

THE EFFECT OF H₂O₂ ON THE PHOTOSYNTHETIC
BIOCHEMISTRY OF *PISUM SATIVUM*

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PREFACE

The study presented in this thesis was carried out at the Department of Biological Sciences, University of Natal, Durban, under the supervision of Professor N. W. Pammenter and Dr. A. M. Amory.

The experimental studies represented here are original and have not been submitted in any form to another University.

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ABSTRACT

To study the short-term effect of hydrogen peroxide on plant metabolism aminotriazole (2 mM), a catalase inhibitor was applied through the transpiration stream of pea seedlings and the gas exchange characteristics, ascorbate peroxidase, glutathione reductase and catalase activities, and the levels of hydrogen peroxide, glutathione and formate were determined. CO₂ assimilation rates were inhibited after the addition of aminotriazole: photorespiratory conditions (high O₂ and low CO₂ concentrations, high light intensities) exacerbated the inhibition. CO₂ response curves showed that aminotriazole reduced both the RuBP regeneration rate and the efficiency of the carboxylation reaction of Rubisco. The inhibition of the CO₂ assimilation rate during the first 100 minutes after the addition of aminotriazole was alleviated by feeding PGA through the transpiration stream. Catalase activity was completely inhibited within 200 minutes of aminotriazole application, but there was no concomitant increase in the hydrogen peroxide concentration, suggesting that hydrogen peroxide is metabolised by an alternative mechanism. The treatment had no effect on ascorbate peroxidase and glutathione reductase activities or total glutathione pool size, but greatly increased formate levels. These results suggest that hydrogen peroxide is metabolised by reacting with glyoxylate to form formate and CO₂. The increased production of formate may reduce the flow of carbon through the normal photorespiratory pathway and may be used anapleurally as a precursor of products of 1-C metabolism other than serine. This would prevent the return of photorespiratory carbon to the Benson-Calvin cycle in the form of 3-PGA. It is proposed that when RuBP regeneration levels are low high formate levels may be an effector of Rubisco, and competitively inhibit the binding of CO₂ and O₂. These results suggest that under photorespiratory conditions the uninterrupted flow of carbon through the photorespiratory pathway is fundamental to the functioning of photosynthetic metabolism.

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LIST OF ABBREVIATIONS IN FIGURES

ASC - ascorbate

DHA - dehydroascorbate

E - inactive enzyme

E* - active enzyme

E*-C-M - enzyme-CO₂-Mg⁺⁺

E*-C-M-R - enzyme-CO₂-Mg⁺⁺-RuBP

E-C* - enzyme-CO₂

F - effector

Fd_{red} - reduced ferredoxin

Fd_{ox} - oxidized ferredoxin

GLL - glycolate

GLR - glycerate

GLX - glyoxylate

GLY- glycine

GSH - reduced glutathione

GSSG - oxidised glutathione

H₂O₂ - hydrogen peroxide

MDH - monodehydroascorbate

NADP⁺ - oxidised nicotinamide adenine diphosphate

NADPH - reduced nicotinamide adenine diphosphate

O₂⁻ - superoxide

OH· - hydroxyl radical

OH-PYR - hydroxypyruvate

PGA - phosphoglycerate

PGL - phosphoglycolate

PSI - photosystem I

SER - serine

THF - tetrahydrofolate

CHAPTER ONE

INTRODUCTION

Aerobic organisms are presently exposed to an atmosphere that contains at least 20 per cent dioxygen (O_2). Although molecular O_2 is not toxic, it can produce by-products which are potentially damaging. Ground state O_2 contains two unpaired electrons with parallel spin (Greenwood and Earnshaw, 1985) and the reaction of O_2 with a substrate that donates pairs of electrons will require a spin inversion in order to conform to the Pauli Exclusion Principle. Because of the time required to invert the spin of an electron, O_2 has a much greater tendency to react with radical species and unpaired electrons. The univalent reduction of O_2 produces the reactive intermediates, superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH\cdot$) (Salin, 1987). Figure 1 depicts the pathways of univalent O_2 reduction and the formation of active oxygen intermediates. H_2O_2 is therefore a product of the divalent reduction of O_2 or the univalent reduction of O_2^- .

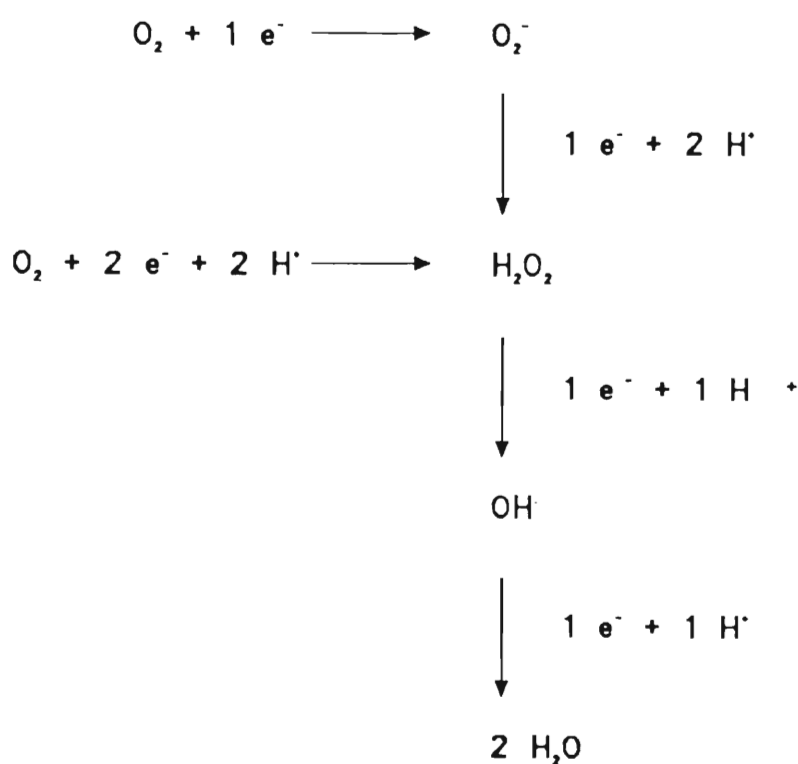


Figure 1. The univalent reduction products of O_2 .

Leaf cells of higher plants are particularly vulnerable to oxidative stress because the internal O_2 concentration during photosynthetic reactions is several times higher than in the surrounding atmosphere (Halliwell, 1987).

This review will discuss the production of the active oxygen species, O_2^- and H_2O_2 , by a number of processes during photosynthesis by higher plants; the metabolism of H_2O_2 , which has been produced during the dismutation of O_2^- or the direct reduction of O_2 ; and the response of these scavenging mechanisms and the photosynthetic metabolic pathways to conditions which enhance O_2^- and H_2O_2 production *in vitro* and *in vivo*.

1.0 PRODUCTION OF SUPEROXIDE AND HYDROGEN PEROXIDE IN LEAF CELLS

H_2O_2 is produced either directly or through the disproportionation of O_2^- by a number of processes during photosynthesis in higher plants. During photorespiration, glycolate is oxidized to glyoxylate, in a reaction catalysed by glycolate oxidase, with the concomitant production of H_2O_2 (Zelitch, 1972). The concentration of H_2O_2 produced by this reaction will be dependent on the rate of glycolate formation and will increase under photorespiratory conditions.

The photoreduction of O_2 in chloroplasts was first discovered by Mehler (1951). It has been difficult to examine the Mehler reaction *in vivo* because O_2 photoreduction occurs simultaneously with mitochondrial and photorespiratory O_2 consumption (Ogren, 1984; Hrubec, Robinson and Donaldson, 1985). This difficulty has been minimized by the *in vivo* discrimination between $^{16}O_2$ photoevolution and $^{18}O_2$ photoconsumption with the use of the mass spectrophotometer (Robinson, 1988). The light-dependent uptake of $^{18}O_2$ has been observed in algal cells (Radmer, Kok and Ollinger, 1978; Radmer and Ollinger, 1980), intact leaves of C_3 plants (Canvin *et al.*, 1980), intact chloroplasts (Egneus, Heber, Matthieson and Kirk, 1975) and isolated bundle sheath strands (Furbank and Badger, 1983).

The primary photoproduct of dioxygen in the chloroplast is O_2^- and almost all of the chloroplastic H_2O_2 is the disproportionation product of O_2^- . Two major pathways of O_2^- -generation exist in the chloroplasts (Figure 2).

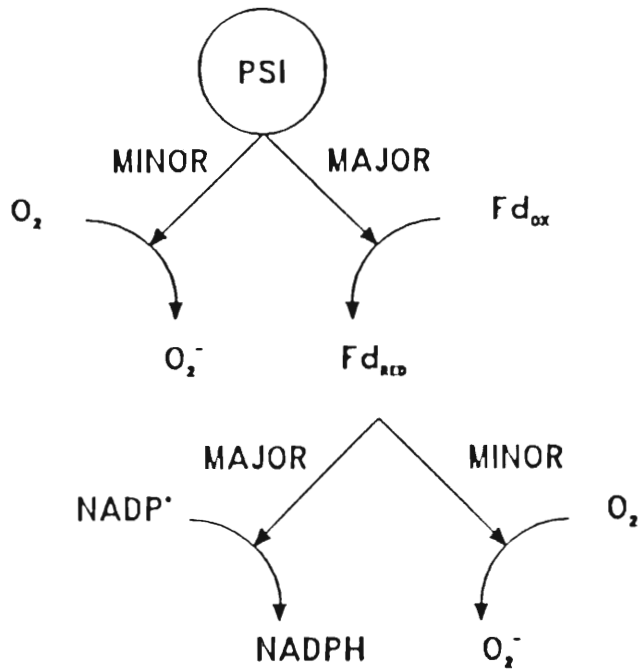
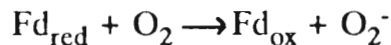
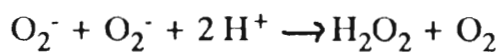


Figure 2. The photoreduction of molecular O₂ by photosystem I (PSI), (after Halliwell, 1987).

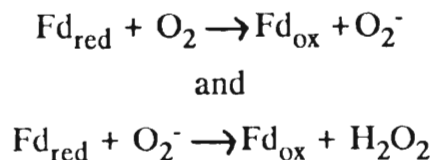
The first mechanism involves the direct interaction of O₂ with a component of the electron transport chain (Badger, 1985; Halliwell, 1987), probably P-430, the primary electron acceptor of PSI (Asada, Kiso and Yoshikawa, 1974). The second and major route of O₂⁻-generation occurs via autoxidation of ferredoxin (Fd). Fd reacts in a one electron transfer reaction with O₂ to form O₂⁻ (Misra and Fridovich, 1971).



The superoxide is in turn dismutated to H₂O₂ and ground state O₂ by superoxide dismutase (Halliwell, 1974b; Salin, 1987). The rate of H₂O₂ production from O₂⁻ is estimated to be about 120 μM s⁻¹ (Asada and Takahashi, 1987).



Under conditions when low concentrations of superoxide dismutase are present, a two-step O₂ reduction forming O₂⁻ and H₂O₂ in sequence, may also occur (Allen, 1975; Elstner, 1982).



Fd_{red} normally passes most of its electrons onto oxidized nicotinamide adenine diphosphate (NADP^+) via Fd-NADP^+ -oxidoreductase (Figure 2). Studies with isolated spinach chloroplast lamellae indicated that O_2 and NADP^+ compete for reducing equivalents (Robinson and Gibbs, 1982). The amount of O_2^- generated will therefore to some extent depend on the amount of NADP^+ available, which in turn depends on the rate of photosynthetic electron transport, the rate of CO_2 reduction and the supply of CO_2 (Halliwell, 1987). However, O_2 has a strong affinity for reducing sites and the O_2 concentration in chloroplasts of illuminated leaves is saturating with respect to the O_2 reduction sites (approximately $250 \mu\text{M}$) (Robinson and Gibbs, 1982). This suggests that the potential for O_2 reduction exists even when NADP^+ is saturating.

The photoreduction of molecular O_2 and the concomitant production of O_2^- and H_2O_2 appears to be physiologically important. The CO_2 assimilation rate of illuminated intact chloroplasts is inhibited upon exposure to anaerobic conditions as a result of an ATP-deficiency. This was alleviated by the addition of trace amounts of O_2 (Ziem-Hanck and Heber, 1980). This suggests that the additional production of ATP by the Mehler reaction is necessary to maintain a sufficiently high ATP/NADPH ratio for the operation of the Benson-Calvin cycle. This appears to be particularly important under conditions where light is limiting (Egneus, Heber, Matthieson and Kirk, 1975; Hosler and Yocum, 1987). Even under conditions where sufficient ATP is provided by cyclic and non-cyclic electron transport, O_2 photoreduction is necessary to prevent the over-reduction of the intermediates of the electron transport chain (Robinson and Gibbs, 1982).

In addition to the control of the ATP/NADPH ratio, the photoreduction of O_2 is considered to be an essential energy dissipating mechanism under conditions where CO_2 assimilation is limiting (Marsho, Behrens and Radmer, 1976). Low rates of CO_2 assimilation will result in a depletion of the NADP^+ pool and a concomitant rise in the photoreduction of O_2 (Figure 2) (Egneus *et al.*, 1975). Significant endogenous rates of O_2 reduction occur during the induction of photosynthesis (Radmer and Kok, 1976), during stomatal closure (Gamble and Burke, 1984) and during the development of greening leaves

when the Benson-Calvin cycle is not fully operational (Gillham and Dodge, 1987).

The production of O_2^- and the concomitant production of H_2O_2 is a necessary component of photosynthetic metabolism and higher plants are continually exposed to varying concentrations of these metabolites.

2.0 IS HYDROGEN PEROXIDE TOXIC?

H_2O_2 is the least reactive of the active oxygen species. It has a completely filled outer orbital and does not exhibit radical properties (Halliwell and Gutteridge, 1985). It is a weak oxidizing agent ($E^0 = 1.77V$) (Salin, 1987) that can oxidize essential thiol groups of active enzymes (Halliwell and Gutteridge, 1985). It readily diffuses into cells where it can react with Fe (III) in the Fenton reaction to form the hydroxyl radical which is a strong oxidizing agent ($E^0 = 2.0V$) that is capable of oxidizing enzymes, nucleic acids and certain small metabolites (Elstner, 1982; Halliwell and Gutteridge, 1985; Salin, 1987). This may be the origin of its most toxic effects.

3.0 HYDROGEN PEROXIDE-SCAVENGING MECHANISMS IN LEAF CELLS

Although H_2O_2 is not particularly cytotoxic it is necessary for plant cells to possess H_2O_2 -scavenging mechanisms to reduce the possibility of production of more injurious oxy-intermediates. Three major mechanisms of H_2O_2 metabolism exist in the plant cell. Peroxisomal H_2O_2 is either enzymatically metabolised by catalase, or it is non-enzymatically or enzymatically removed by reacting with glyoxylate to form formate and CO_2 . In the chloroplast an elaborate sequence of oxidation reduction reactions - the anti-oxidant system - scavenges H_2O_2 .

3.1 Peroxisomes

The enzymatic breakdown of H_2O_2 by catalase is the best-known H_2O_2 -scavenging mechanism (Figure 3). A catalase- H_2O_2 complex, compound I, possesses both catalytic and peroxidatic properties. Compound I is reduced back to the original enzyme either in a

reaction with a second molecule of H_2O_2 which is catalytically broken down to H_2O and O_2 , or during the oxidation of a secondary electron donor (eg. formate). At constant rates of H_2O_2 generation, compound I accumulates to a steady state level in dynamic equilibrium with free catalase and a low concentration of H_2O_2 (Halliwell, 1974b). This mechanism of catalase action has been postulated to prevent the destruction of approximately ten per cent of H_2O_2 produced during glycolate oxidation (Grodzinski and Butt, 1976). It has been proposed that the residual H_2O_2 reacts non-enzymatically with glyoxylate to form formate and CO_2 (Zelitch, 1972; Halliwell and Butt, 1974), (Figure 4).

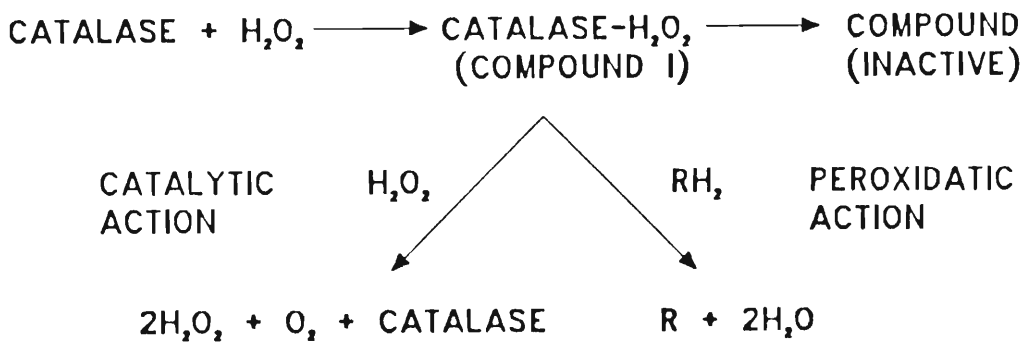


Figure 3. The catalytic and peroxidatic reactions of catalase.

There is controversy about the existence and metabolism of H_2O_2 which has escaped catalase action. It has generally been assumed that sufficient catalase is present in the peroxisomes to destroy all the H_2O_2 generated during glycolate oxidation (Tolbert, 1971). This assumption was supported by Yokota *et al.* (1985) who simulated the glycolate pathway of C_3 plants and showed that the enzymatic decomposition of H_2O_2 by catalase is 10^3 - 10^4 -fold greater than the non-enzymatic decomposition by reaction with glyoxylate. Even if catalase was 98 per cent inhibited it would still be capable of decomposing 95-99 per cent of H_2O_2 generated. However, Grodzinski and Butt (1976) showed that $^{14}CO_2$ was released from ^{14}C -labelled glyoxylate in isolated peroxisomes in the presence of a 200-fold excess of catalase. They proposed that peroxisomal catalase activity was sufficient to hold H_2O_2 at non-toxic levels and the remaining H_2O_2 was non-enzymatically removed by reaction with glyoxylate to form formate and CO_2 . A similar reaction has been reported in soybean mesophyll cells (Oliver, 1979), in isolated corn and sorghum bundle sheath cells (Cossins *et al.*, 1988) and is associated with the chloroplasts of *Euglena* (Yokota *et al.*, 1983).

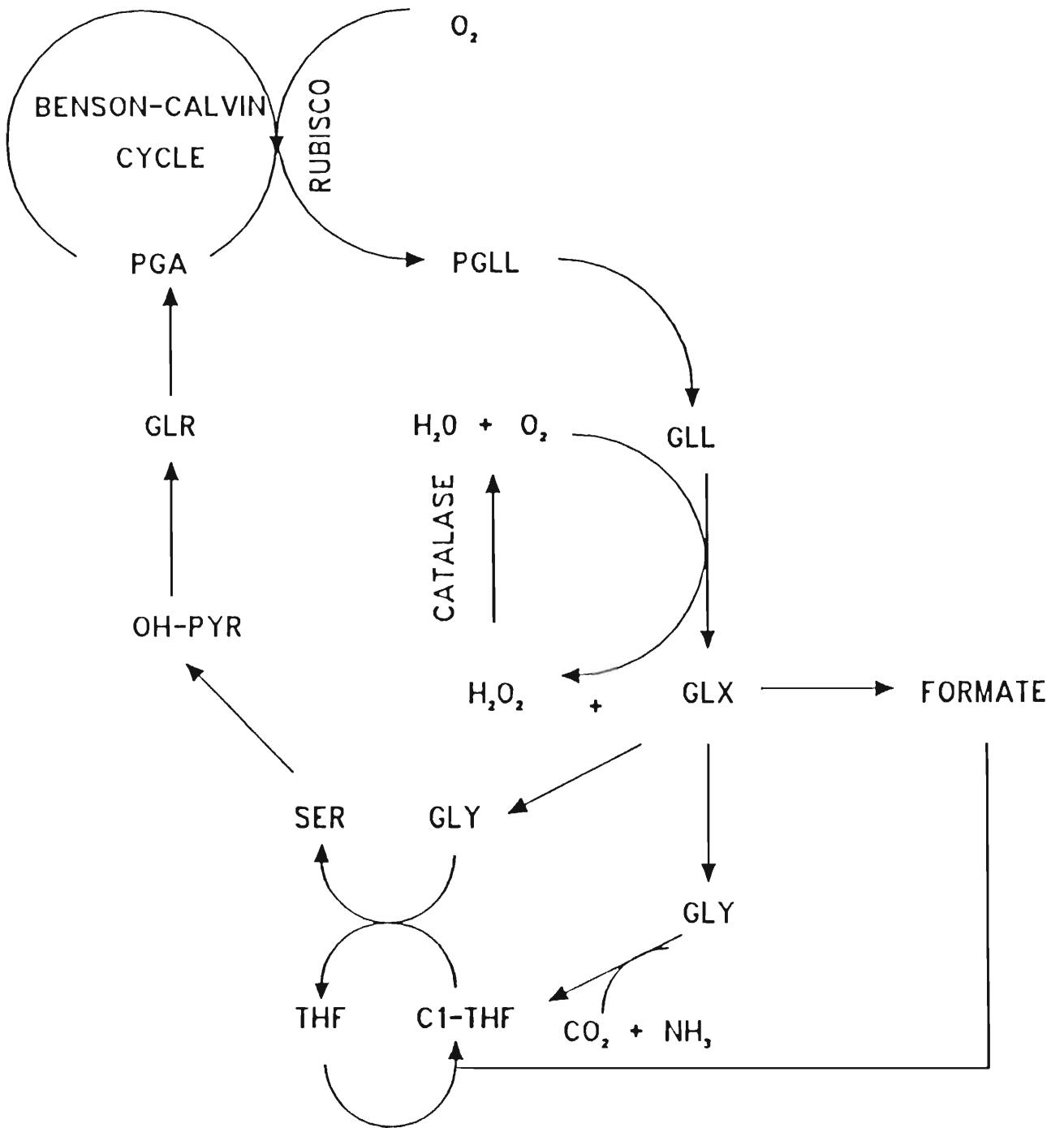


Figure 4. The photorespiratory pathway.

The decarboxylation of glyoxylate by H_2O_2 has been suggested to contribute to photorespiratory CO_2 release (Grodzinski, 1978). In the Tolbert photorespiratory pathway CO_2 is the product of a complex mitochondrial reaction in which glycine is converted to stoichiometric amounts of CO_2 , NH_3 and C1-tetrahydrofolate (THF) (Figure 4). The importance of the glyoxylate- H_2O_2 reaction as an alternative source of photorespiratory CO_2 release has been questioned by Artus, Somerville and Somerville (1986) who noted that *in vivo*, glyoxylate would tend to be converted to glycine rather than to formate and CO_2 . Somerville and Ogren (1981) suggested that the alternative pathway of CO_2 production would be significant only upon depletion of amino donors for the transamination of glyoxylate to glycine. The amino donors serine, alanine and glutamine have been shown to determine the extent of glyoxylate transamination in spinach leaf peroxisomes (Yu, Liang and Huang, 1984). Oliver (1979) suggested that a competition for NADP exists between the enzymes of dark respiration and glycine decarboxylation within the mitochondria. Under conditions where NADP becomes limiting this competition may force substantial amounts of glycolate to be decarboxylated via the alternative photorespiratory pathway (Figure 4). Chemical energy is also required for the reassimilation of NH_3 released in the Tolbert photorespiratory pathway. The alternative pathway would be favourable when nitrogen-donors are scarce and chemical energy becomes limiting (Amory and Cresswell, 1986).

The accumulation of glycolate, following the chemical inhibition of glycolate oxidase by butyl 2-hydroxy-3-butyrate (BHB), was approximately 50 per cent greater than the accumulation of glycine after the glycine-serine conversion was inhibited by isonicotinic acid hydrazide (INH) (Servaites and Ogren, 1977). This indicated that a branch point exists in the glycolate pathway between glycolate and glycine (Figure 4). The rate of CO_2 release from glycine was inhibited over 90 per cent in the presence of INH, while the rate of CO_2 release from glycolate was inhibited by only 50 per cent (Oliver, 1979). These results suggest that some CO_2 can be released from glycolate at an alternative site. The alternative photorespiratory pathway appears to be operational when the Tolbert photorespiratory pathway is blocked by chemical inhibitors.

Studies with spinach chloroplast preparations showed that enhanced levels of glyoxylate increased the flow of carbon through the alternative pathway to a greater extent than through the Tolbert photorespiratory pathway (Zelitch, 1972). The addition of a certain amount of [^{14}C]glyoxylate to leaf peroxisomes resulted in the release of similar quantities

of $^{14}\text{CO}_2$ during the decarboxylation reaction with H_2O_2 (Oliver, 1979). This suggests that under favourable photorespiratory conditions, high light and low CO_2 (Waidyanantha, Keys and Whittingham, 1975; Badger, 1985), when high glyoxylate and H_2O_2 concentrations occur, there is increased flow through the alternative pathway.

These results indicate that the non-enzymic removal of H_2O_2 , by reaction with glyoxylate to form formate and release photorespiratory CO_2 , increases when nitrogen and chemical energy are limiting, in the presence of high H_2O_2 or glyoxylate concentrations, or a reduction in catalase or serine hydroxymethyl-transferase activity. However, under normal physiological conditions the majority of the H_2O_2 produced in the peroxisomes is catalytically removed. This view was supported in studies with leaf peroxisomes, the site of glyoxylate to glycine conversion, where less than 10% of the glyoxylate formed reacted with H_2O_2 to form formate and CO_2 under normal physiological conditions (Shingles, Woodrow and Grodzinski, 1984).

Formate has been shown to inhibit linear electron transport from Q, the primary electron acceptor of photosystem II, to plastoquinone in isolated chloroplasts in the absence of CO_2 (Snel and van Rensen, 1984). Bicarbonate can alleviate this inhibition probably by competitively displacing the formate from two pools of high affinity binding sites in the thylakoid membranes (Stemler, 1977). Snel and van Rensen (1984) proposed a physiological role for formate in the regulation of photosynthetic electron flow. As has already been mentioned, under conditions where CO_2 is limiting carbon is diverted through the glycolate pathway and more formate will be produced. The formate diffuses into the chloroplast and inhibits linear electron flow. Such a mechanism could be regarded as a negative feed-back mechanism which would be advantageous in preventing the over-reduction of electron carriers under conditions where CO_2 assimilation is limiting.

The metabolism of formate generated during photorespiration is uncertain. Evidence has been obtained indicating that formate can be decarboxylated to CO_2 through the peroxidative action of catalase in the peroxisomes and by an NAD^+ -dependent formate dehydrogenase in the mitochondria (Leek, Halliwell and Butt, 1972; Halliwell, 1974a). The oxidation of [^{14}C]formate to $^{14}\text{CO}_2$ by the peroxidative action of catalase was slow relative to the rate of formate production (Grodzinski, 1979). The amount of NAD^+ -formate dehydrogenase and the capacity for formate-dependent O_2 uptake varied greatly among species (Oliver, 1981), but the activity of NAD^+ -formate dehydrogenase is usually much

lower than that of formyl tetrahydrofolate synthetase (Grodzinski, 1979). This suggests that most of the formate is reduced by formyl tetrahydrofolate synthetase to OH-methyl tetrahydrofolate (one-carbon) which participates in serine synthesis by the serine hydroxymethyl transferase reaction (Shingles, Woodrow and Grodzinski, 1984). Formate may also be a precursor of other products of one-carbon metabolism (Cossins, 1980). The decarboxylation reactions of formate were only considered to play an important role in formate metabolism when high rates of formate synthesis occurred (Halliwell, 1974a).

3.2 Chloroplasts

Catalase exists predominantly in the peroxisomes (Whitehouse, Ludwig and Walker, 1971; Gillham and Dodge, 1986) and the chloroplasts require an alternative means of detoxifying H_2O_2 . Vacuum-infiltrated H_2O_2 caused significantly greater ultrastructural damage to mitochondria than to chloroplasts in the light, suggesting that chloroplasts are protected from this oxidizing agent in the light (Amory, 1985). Chloroplasts contain an ascorbate-specific peroxidase (Nakano and Asada, 1981; Groden and Beck, 1979) with a very low K_m for H_2O_2 (Asada and Takahashi, 1987). Evidence for the peroxidative scavenging of H_2O_2 was provided when isolated spinach chloroplasts evolved $^{16}O_2$ from $H_2^{18}O_2$, instead of $^{18}O_2$ as would have been expected from catalytic metabolism (Asada and Badger, 1984). Foyer and Halliwell (1976) proposed an elaborate sequence of oxidation-reduction reactions, initiated by ascorbate peroxidase, to scavenge chloroplastic H_2O_2 (Figure 5). Ascorbate peroxidase catalyses ascorbate-dependent reduction of H_2O_2 to H_2O (Groden and Beck, 1979; Nakano and Asada, 1981). Dehydroascorbate, the primary oxidation product of ascorbate, is then reduced to ascorbate by glutathione-dependent dehydroascorbate reductase (Jablonski and Anderson, 1978; Hossain and Asada, 1984). It has recently been discovered that ascorbate peroxidation may produce monodehydroascorbate which can dismutate non-enzymatically to form ascorbate and dehydroascorbate, or be reduced to ascorbate by NADH-dependent monodehydroascorbate reductase (Hossain, Nakano and Asada, 1984). NADPH-dependent glutathione reductase regenerates reduced glutathione (Law, Charles and Halliwell, 1983). The final electron donor is reduced ferredoxin, photogenerated in the thylakoid, which reduces NADP in a reaction catalysed by Fd:NADP⁺ oxidoreductase (Asada and Takahashi, 1987).

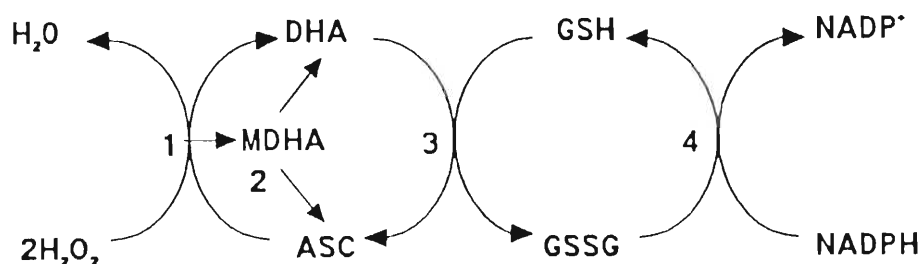


Figure 5. The ascorbate-glutathione H_2O_2 -scavenging system. The enzymes involved are: ascorbate peroxidase (1), monodehydroascorbate reductase (2), dehydroascorbate reductase (3), glutathione reductase (4).

The operation of the ascorbate-glutathione H_2O_2 -scavenging system has been clearly demonstrated in isolated photosynthesizing chloroplasts (Law, Charles and Halliwell, 1983; Asada and Badger, 1984). It is further supported by the localization of the participating enzymes and metabolites in chloroplasts (Anderson, Foyer and Walker, 1983; Gillham and Dodge, 1986) at concentrations in excess of the K_m values for their binding sites (Robinson, 1988). Chloroplasts contain high percentages of leaf ascorbate peroxidase, dehydroascorbate reductase, glutathione reductase (Gillham and Dodge, 1986) and 11 and 30 per cent of the total plant glutathione and ascorbate, respectively (Bielawski and Joy, 1986). The activity of the ascorbate-glutathione scavenging system in ruptured chloroplasts is about a third of that exhibited by intact chloroplasts, which suggests that the activity of the anti-oxidant enzymes is sensitive to the pool sizes of the intermediates (Nakano and Asada, 1981). The minimum concentration of ascorbate required to stabilize ascorbate peroxidase is $20\mu\text{M}$ (Nakano and Asada, 1987). The oxidation of the ascorbate pool below this concentration results in the irreversible deactivation of ascorbate peroxidase (Hossain and Asada, 1984). The level of reduced glutathione (GSH) appears to regulate the activity of glutathione reductase and the enzyme is inactivated at very high GSH concentrations (Jablonski and Anderson, 1978; Wise and Naylor, 1987).

The levels of the intermediates and therefore the activities of the enzymes of the anti-oxidant system respond to changes in the H_2O_2 concentration. The photoreduction of O_2 and the concomitant production of H_2O_2 is expected to be maximal in high light and at low CO_2 concentrations (Asada and Takahashi, 1987). Studies on a variety of field-grown species showed that the ascorbate concentration doubled in summer when plants are exposed to higher light intensities (Foyer, Rowell and Walker, 1983). Gillham and Dodge (1987) attributed increased protection against paraquat-induced damage in high light-grown plants to enhanced chloroplast levels of ascorbate, ascorbate peroxidase, glutathione

reductase and dehydroascorbate reductase. The midday closure of stomata in response to water stress reduces the availability of CO_2 thereby reducing the rate of CO_2 fixation (Boyer, Armond and Sharp, 1987). Under these conditions O_2 may function as an alternate electron acceptor with subsequent production of H_2O_2 (Radmer and Kok, 1976). Gamble and Burke (1984) reported a 2.2-fold increase in the level of glutathione reductase in response to water stress. Even though there was a decrease in protein content the specific activities of all enzymes, except ascorbate peroxidase, were higher in water-stressed leaves compared to non-water stressed leaves (Smirnoff and Colombé, 1988). Increased atmospheric O_2 induces glutathione reductase activity in maize and cotton (Foster and Hess, 1980; 1982). Enhanced levels of anti-oxidant intermediates and enzyme activities may serve to protect chloroplasts against damaging O_2 radicals under conditions of enhanced H_2O_2 production (Gamble and Burke, 1984; Gillham and Dodge, 1987).

4.0 THE EFFECT OF ENHANCED HYDROGEN PEROXIDE CONCENTRATIONS ON PHOTOSYNTHETIC METABOLISM *IN VITRO*

The existence of the anti-oxidant system in the chloroplast and its ability to respond to endogenous increases in the H_2O_2 concentration, implies that the chloroplast should be able to cope with the addition of low concentrations of H_2O_2 . However, exogenously applied H_2O_2 has been shown to be both ultrastructurally and metabolically damaging to isolated chloroplasts. Pea leaf chloroplasts showed extensive ultrastructural damage following the addition of H_2O_2 (Parker and Lea, 1983). Kaiser (1976) showed that $5 \mu\text{M}$ H_2O_2 caused a significant inhibition of CO_2 fixation which was accompanied by a lower level of ribulose biphosphate (RuBP), a higher level of fructose biphosphate (FBP) and sedoheptulose biphosphate (SBP) and higher ATP/ADP ratios (Kaiser, 1979). These responses were all reversed by addition of either catalase or dithiothreitol (DTT) (Kaiser, 1976, 1979). It was proposed that catalase would metabolise the H_2O_2 which had escaped the action of the anti-oxidant system and DTT would mimick the ferredoxin-thioredoxin (Fd-Thd) system *in vitro* (Anderson, Avron and Yoon Park, 1974) and reduce enzymes which had been oxidized by the unmetabolized H_2O_2 . It was therefore suggested that the 'free' H_2O_2 oxidizes light-generated sulphhydryl groups of essential enzymes of the Benson-Calvin cycle, fructose and sedoheptulose biphosphotase and phosphoribulokinase. This would account for the observed decreased rates of RuBP regeneration which would result in decreased CO_2 assimilation rates (Farquhar and Sharkey, 1982). Charles and Halliwell

(1980) showed that fructose biphosphotase was completely inhibited five minutes after the addition of 1 mM H_2O_2 . The activity was completely restored following the addition of DTT or by raising the FBP concentration. This supports the hypothesis that the major effect of H_2O_2 is an oxidation of essential thiol groups which causes a reversion of the enzymes to a form whose substrate affinity is low. These results suggest that the ability of the anti-oxidant system to scavenge H_2O_2 is limited. The Fd-Thd system, which participates in the reduction of the thiol groups, therefore exists *in vivo* to prevent the inhibition of reduced forms of essential Benson-Calvin cycle enzymes by any light-generated H_2O_2 which has not been scavenged by the anti-oxidant system (Anderson and Avron, 1976; Charles and Halliwell, 1981).

These investigations were all conducted with isolated chloroplasts which have an incomplete photorespiratory pathway. The carbon lost to the glycolate pathway as a result of the oxygenation of RuBP would be unable to return to the Benson-Calvin cycle as 3-PGA (Figure 4). Robinson, Smith and Gibbs (1980) showed that catalase did not contribute to a complete recovery of ^{14}C -photoassimilation in isolated spinach chloroplasts exposed to ideal photorespiratory conditions (PH 8.3 or 100 per cent O_2). These results suggest that the depletion of the intermediates of the Benson-Calvin cycle, because of the incomplete photorespiratory pathway, contributed to the observed inhibition of the CO_2 assimilation rate in isolated chloroplasts in the presence of H_2O_2 . Lower CO_2 assimilation rates result in the decreased utilization of ATP and NADPH. An ADP deficiency limits noncyclic electron transport (Rosa and Whatley, 1981) and insufficient Fd is therefore available to activate enzymes via the Fd-Thd system. Photoproduced electrons are diverted to O_2 when $NADP^+$ is limiting (Figure 2, Halliwell, 1987). The increased production of H_2O_2 would then be an indirect consequence of the decreased CO_2 assimilation rate. This situation, where the production of endogenous H_2O_2 has been increased and the operation of the Fd-Thd system is limited, is aggravated by the addition of exogenous H_2O_2 (Rosa and Whatley, 1981). The addition of PGA to isolated chloroplasts prevented the depletion of the intermediates of the Benson-calvin cycle, thereby enhancing the CO_2 assimilation rate and greatly reducing the inhibition of CO_2 assimilation by H_2O_2 (van Leeuwen, 1987). These studies support the hypothesis that photorespiration and the return of carbon potentially lost to the photorespiratory pathway, is essential to prevent depletion of the intermediates of the Benson-Calvin cycle and thereby prevent photooxidative damage (Kirk and Heber, 1976).

5.0 THE EFFECT OF ENHANCED HYDROGEN PEROXIDE CONCENTRATIONS ON PHOTOSYNTHETIC METABOLISM *IN VIVO*

Catalase-deficient mutants or plants which have been treated with the herbicide 3-amino-1,2,4-triazole (aminotriazole) have been used to investigate the effect of enhanced H_2O_2 *in vivo* on metabolism. Aminotriazole, which readily diffuses into cells (Singer and McDaniel, 1982) inactivates catalase by binding directly to its protein moiety (Margoliash, Novogradsky and Schejter, 1960), but does not inhibit other photosynthetic or photorespiratory enzymes (Feierabend and Kemmerich, 1983). The loss of catalase activity is expected to be accompanied by accumulation of H_2O_2 (Feierabend and Schubert, 1978). Studies with aminotriazole-treated suspension-cultured pear fruit cells showed that the H_2O_2 concentration did not increase even in the presence of methyl viologen, a source of free radical production (Ferguson and Dunning, 1986). Reduction of catalase activity by exposure to low temperature was accompanied by a decreased H_2O_2 concentration (Macrae and Ferguson, 1985). These results imply that the other H_2O_2 -scavenging reactions compensate for the lack of catalase and adequately remove endogenously produced H_2O_2 .

The exposure of catalase-deficient barley mutants and aminotriazole-treated wild-type barley to photorespiratory conditions, resulted in a two- to three-fold increase in the total glutathione pool size (Smith *et al.*, 1984; Smith, 1985). Almost all the increase was due to the accumulation of oxidized glutathione (GSSG) and the GSH:GSSG ratio decreased from 0.86 in wild-type plants to 0.42 in plants lacking catalase activity. This effect was dependent on light intensity which suggests that the generation of H_2O_2 directly or indirectly stimulated glutathione synthesis (Smith *et al.*, 1984). It was proposed that the H_2O_2 produced in the peroxisomes, which is usually catalytically removed, diffuses into the chloroplasts (Kendall *et al.*, 1983). The activity of the ascorbate-glutathione scavenging system increased to cope with the higher chloroplastic H_2O_2 levels and there was a concomitant increase in the oxidation of glutathione and decrease in the GSH:GSSG ratio. Reduced glutathione is a feedback inhibitor of the glutathione biosynthetic enzyme and the lower GSH pool would result in additional synthesis of glutathione (Smith *et al.*, 1984, 1985). Once the biosynthesis of glutathione becomes limited by the availability of sulphate the CO_2 assimilation rate is inhibited and other events which eventually lead to the death of the plant are initiated (Smith *et al.*, 1984, 1985). The ascorbate pool was not depleted in

protoplasts when the endogenous production of the H_2O_2 was enhanced in the presence of aminotriazole or at low CO_2 concentrations (Foyer, Rowell and Walker, 1983). The implication is that, provided there is sufficient glutathione and ascorbate, the anti-oxidant system is capable of preventing the concentration of H_2O_2 reaching physiologically damaging levels, in the short-term.

The rate of non-enzymic removal of peroxisomal H_2O_2 by reaction with glyoxylate to form formate and CO_2 increases in the absence of catalase (Grodzinski, 1979). The increased rate of formate production and decarboxylation to CO_2 would prevent the return of carbon lost to the photorespiratory cycle thereby depleting the intermediates of the Benson-Calvin cycle and inhibiting the CO_2 assimilation rate (Somerville and Ogren, 1981; van Leeuwen, 1987). Sivak *et al.* (1987) proposed that the initial events upon exposure of catalase-deficient barley mutants to photorespiratory conditions involve depletion of Benson-Calvin cycle intermediates, the rate of depletion varying with light intensity and O_2 concentration.

Plants lacking catalase activity exhibited enhanced activity of other H_2O_2 -scavenging mechanisms. This indicates that the plant tissue is able to cope with localized increases in the H_2O_2 concentration, in the short term. However, the increase in the activity of the anti-oxidant system is eventually limited by the availability of sulphate (Smith, 1985), and the enhanced production of formate may cause decreased CO_2 assimilation rates. This situation is similar to that which exists in isolated chloroplasts (see Section 5.0). Therefore the long-term inhibition of the catalase activity and enhanced H_2O_2 production *in vivo* will culminate in photooxidative damage including the inhibition of the CO_2 assimilation rate (Smith, 1985), inhibition of carotenoid biosynthesis (Halliwell, 1987) and chlorophyll bleaching (Feierabend and Kemmerich, 1983). Several investigators have suggested that the inactivation of peroxisomal enzymes and the concomitant inhibition of the CO_2 assimilation rate are a secondary consequence of herbicide-induced photooxidative events (Halliwell, 1987). Aminotriazole has been shown to belong to group I herbicides which induce only weak photodestructive damage which, in the case of aminotriazole, follows the inhibition of catalase (Feierabend and Kemmerich, 1983). Plants grown in the presence of aminotriazole showed far less photodestruction of chlorophyll than aminotriazole-treated isolated chloroplasts (Feierabend and Winkelhüsener, 1982), which have been shown to potentially enhance photooxidative damage (see Section 5).

6.0 THE PRESENT STUDY

The existence of several H_2O_2 -scavenging mechanisms which have been reported to be sensitive to the H_2O_2 level suggests that the plant is able to cope with enhanced levels of H_2O_2 , in the short-term. The aim of the present study was to determine the physiological and biochemical response of excised seedlings of *Pisum sativum* to short-term increases in the H_2O_2 concentration. To avoid complications arising from an incomplete photorespiratory pathway, aminotriazole was applied through the transpiration stream of excised pea seedlings to enhance the endogenous H_2O_2 concentration. The concentration of endogenous H_2O_2 produced was varied by exposing seedlings to various light intensities and CO_2 and O_2 concentrations which alter the rate of carbon flow through the photorespiratory pathway. The catalase activity, H_2O_2 concentration and photosynthetic CO_2 uptake rates were monitored throughout the experimental period to determine whether toxic levels of H_2O_2 would accumulate and inhibit CO_2 assimilation. CO_2 response curves were used as a tool to determine the effect of H_2O_2 on the CO_2 uptake mechanism. The photosynthetic intermediate, PGA, was applied through the transpiration stream to determine the extent to which the inhibition of a photorespiratory enzyme reduces the return of carbon to the Benson-Calvin cycle. The activities of ascorbate peroxidase and glutathione reductase and the glutathione pool size were monitored at various CO_2 concentrations to assess the ability of the ascorbate-glutathione cycle to compensate for the lack of catalase. The formate levels were investigated to determine the contribution of the H_2O_2 and glyoxylate reaction to the scavenging of H_2O_2 which is normally removed by catalase .

CHAPTER TWO

MATERIALS AND METHODS

1.0 PLANT MATERIAL AND GROWTH CONDITIONS

Pea seedlings (*Pisum sativum* L. var. Dark Skin Perfection) were grown on vermiculite in a growth chamber with day/night temperatures of 25/22 °C and a 12 hour day of light intensity $250 \mu\text{mol m}^{-2} \text{s}^{-1}$. Plants were watered every second day with a 0.1 % Benlate solution. All experiments were conducted on two week-old seedlings.

2.0 GAS EXCHANGE ANALYSIS

Gas exchange measurements were performed on excised pea seedlings enclosed in a water-jacketed glass cuvette at 25°C. Ambient air (20 per cent O₂, 350 $\mu\text{mol mol}^{-1}$ CO₂) drawn from a buffer tank, or the appropriate gas mixture supplied from a cylinder, were humidified and passed through a dew point controller at 18°C. The leaf-to-air water vapour concentration gradient was approximately 1 kPa. Cylinders containing various CO₂-O₂ combinations were mixed from CO₂, O₂, N₂ and CO₂-free air cylinders manually or by means of mass flow controllers (Hastings, Hi-Tec F-100, U.S.A.). CO₂ and O₂ concentrations of the gas mixtures were measured using an infra red gas analyser (ADC-225-MK3, ADC, Hoddesdon, U. K.) in the absolute mode, and a gas-O₂ meter (Beckman OM-14, U.S.A.), respectively. The gas flow rate (1.2 l min^{-1}) was controlled by a rotameter fitted with a needle valve (Fischer and Porter, 10A3200, Workington, England). The light source was provided by a metal halogen lamp (Wotan Star, HQ1-T 400W, Lascon). CO₂ and water vapour exchange were measured by infra red gas analysis (ADC-225-MK3, ADC, Hoddesdon, U. K.).

Light response of photosynthesis was obtained by step-wise decreases in the light intensity, once the excised pea seedling had reached steady-state CO₂ assimilation at a relatively high photon flux density. The light intensity was then slowly increased until light saturation was achieved. Steady-state assimilation rate was determined at each step. Similarly CO₂ response curves were obtained, once gas exchange had equilibrated at 350 $\mu\text{mol mol}^{-1}$

CO₂, by decreasing the CO₂ concentration below the CO₂ compensation point and then increasing it until CO₂ saturation.

2.1 Inhibitor studies

The catalase activity of suspension-cultured pear fruit cells was 50 per cent-inhibited four hours after the addition of 1 mM 3-amino-1,2,4-triazole (Ferguson and Dunning, 1986). Concentrations of aminotriazole greater than 1 mM were required to completely inhibit all three catalase isozymes from *Zea mays* (Chandlee, Tsafaris and Scandalios, 1983). A concentration of 2 mM aminotriazole was therefore chosen to completely inhibit the catalase activity in the experimental period (360 minutes). Aminotriazole (2 mM) was applied through the transpiration stream of pea seedlings from which the roots had been excised. The gas exchange characteristics of aminotriazole-inhibited seedlings were investigated for 360 minutes after the addition of the inhibitor, at various light intensities, CO₂ concentrations, CO₂-O₂ combinations or in the presence of phosphoglycerate (PGA). The steady-state CO₂ assimilation rate was determined under ambient conditions (350 μmol mol⁻¹ CO₂, 21 per cent O₂ and saturating light intensity) and then under the particular experimental conditions to be investigated, prior to the addition of aminotriazole.

The effect of aminotriazole on the CO₂ assimilation rate was investigated at 350 μmol mol⁻¹ CO₂, 21 per cent O₂ and photon fluxes of 105, 250, 510, 780 and 900 μmol m⁻² s⁻¹. These represent light intensities where the CO₂ assimilation rate is both light-limited and light-saturated.

The gas exchange characteristics of aminotriazole-treated pea seedlings were investigated at various CO₂ and O₂ concentrations and a saturating light intensity of 500 μmol m⁻² s⁻¹. This investigation was conducted at 88, 170, 350, 620, 800 and 1000 μmol mol⁻¹ CO₂ and 21 per cent O₂, or at 800 μmol mol⁻¹ CO₂, 48 per cent O₂. The CO₂:O₂ ratio of the high CO₂, high O₂ combination was the same as that which occurs under ambient conditions (350 μmol mol⁻¹ CO₂:21 per cent O₂).

PGA (5mM), a Benson-Calvin cycle intermediate, and aminotriazole (2 mM) were applied through the transpiration stream simultaneously to determine the effect of this Benson-

Calvin cycle metabolite on the CO₂ assimilation of aminotriazole-treated peas. This investigation was conducted under ambient conditions (350 μmol mol⁻¹ CO₂, 21 per cent O₂ and a photon flux of 500 μmol m⁻² s⁻¹).

Results were replicated three to six times in all the investigations. Different symbols were used in the figures to represent replicates and hand-drawn lines were used to depict trends. Gas exchange parameters were calculated according to von Caemmerer and Farquhar (1981). Between plant variation was reduced by normalizing the results relative to steady-state CO₂ assimilation rates prior to the commencement of the treatment.

2.2 CO₂ response curves

CO₂ response curves were constructed to determine the effect of aminotriazole on the CO₂ uptake mechanism. The slope is an indicator of the efficiency of the carboxylation reaction and the saturation portion of the curve reflects the RuBP regeneration rate (Farquhar and Sharkey, 1982). The CO₂ response of a single aminotriazole-treated plant could not be obtained because the CO₂ assimilation rate is progressively inhibited. The data obtained during the investigation of the effect of various CO₂ concentrations on the gas exchange mechanism of aminotriazole-treated seedlings, were interpolated to enable the construction of CO₂ response curves. A line was fitted to the results obtained for individual replicates at a particular CO₂ concentration and the CO₂ assimilation rates corresponding to 100, 200 and 300 minutes following the addition of aminotriazole were determined. This data was used to construct CO₂ response curves before and 100, 200 and 300 minutes after the commencement of the treatment. The data was normalized to steady state assimilation rates obtained under ambient conditions. The non-linear regression technique of POSSIM (Amory, 1989; University of Natal) was used to fit CO₂ response curves to the data and to analyse the initial linear portion of the CO₂ response curves to determine the Michaelis-Menton constants of the oxygenation and carboxylation reactions.

3.0 FLUORESCENCE

Chlorophyll a fluorescence was measured at 25°C using a pulse-amplitude modulation fluorometer (Model PAM 101; H. Waltz, Effeltrich, F. R. G.). An excised pea seedling was enclosed in a water-jacketed brass cuvette and the second expanded leaf was positioned on

a fine metal grid below a fitting for the fibre optic probe in the perspex lid. This ensured that the leaf and fibre optics were maintained at a constant distance during all the experiments. All fluorescent measurements were preceded by five minutes of complete darkness, followed by a measuring beam of weak light from a light-emitting diode to obtain F_0 . F_0 designates the fluorescence level when all the PSII reaction centres are open (Butler, 1978). F_m , the maximum fluorescence yield (Butler, 1978), was determined by application of a one second pulse of saturating light (Schott KI 1500) which completely reduces the PSI and PSII electron acceptors. The light intensity of the weak and saturating beams and the gain of the measuring system were kept constant throughout the experiments, to allow absolute replication and comparison between control and experimental samples. F_v , the variable fluorescence was obtained from the expression $F_v = F_m - F_0$ (Björkman, 1987). The results are represented as F_v/F_m ratios which corresponds to the photochemical efficiency of PSII (Björkman, 1987). F_v/F_m ratios were determined at intervals during a 360 minute period in control plants and aminotriazole-treated plants.

4.0 BIOCHEMICAL ANALYSES

The effect of aminotriazole on enzyme activities and metabolite pool sizes was investigated at various CO_2 concentrations and times following the commencement of the treatment. Twelve excised pea seedlings were enclosed in a perspex cuvette which was immersed in a waterbath controlled at 25°C. The leaf-air water vapour deficit was maintained at approximately 1 kPa and the light intensity was held constant at $500 \mu\text{mol m}^{-2} \text{s}^{-1}$. The pea seedlings were equilibrated for 60 minutes under the appropriate experimental conditions, prior to the application of aminotriazole. The seedlings were removed and frozen in liquid nitrogen upon completion of the experiment. Between plant variation in enzyme activity and metabolite pool size was reduced by removing the material to be used for the replicates from a random mixture of the frozen leaves of twelve pea seedlings. This procedure was repeated two or three times under each experimental condition.

4.1 Standardization of hydrogen peroxide

As H_2O_2 was used in the assay of ascorbate peroxidase, catalase and for the H_2O_2 standard curve and is readily converted to H_2O , it was important to standardize H_2O_2 at intervals

during the biochemical analysis. H_2O_2 (10 mM) in phosphate buffer (50 mM, pH 7.0) was standardized by its absorbance at 240 nm. The molar absorptivity of hydrogen peroxide is $40 \text{ mol}^{-1} \text{ cm}^{-1}$ at 240 nm (pH 7.0) (Patterson, Macrae and Ferguson, 1984).

4.2 Enzyme activities

The activities of catalase, ascorbate peroxidase and glutathione reductase were investigated at 80, 150 and 350 $\mu\text{mol mol}^{-1} \text{ CO}_2$, before and 100, 200 and 300 minutes following the addition of aminotriazole. Enzyme activities were expressed on a fresh mass basis.

4.2.1 Catalase

Catalase was assayed according to the method of Lück (1965). Pea leaves (1 g) were extracted with 5 ml of sodium phosphate buffer (100 mM, pH 6.5) at 4°C and centrifuged (Beckman Model J2-21 centrifuge, JA 20-1 rotor, U.S.A.) at $10\,000g$ (4°C) for 30 minutes. The reaction mixture contained 1.95 ml potassium phosphate buffer (60 mM, pH 7.0), 1 ml H_2O_2 (10 mM) and 50 μl plant extract. The change in absorbance due to the metabolism of H_2O_2 (a molar absorptivity of $40 \text{ mol}^{-1} \text{ cm}^{-1}$) by catalase was measured in a dual beam spectrophotometer (Varian DMS 80) at 240 nm against a blank containing potassium phosphate buffer and plant extract.

4.2.2 Ascorbate peroxidase

Ascorbate peroxidase was assayed from the decrease in absorbance at 290 nm as ascorbate was oxidized (Nakano and Asada, 1981). The absorbance coefficient of ascorbate at 290 nm was taken to be $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$. The tissue was ground at 4°C in a potassium phosphate buffer (50 mM, pH 7.0) containing MgCl_2 (1 mM), MnCl_2 (1 mM) and ethylenediaminetetraacetate (EDTA, 2 mM). Ascorbate (0.5 mM) and 20 per cent sorbitol were included to stabilise the enzyme (Nakano and Asada, 1987). The brei was centrifuged at $10,000 g$ (4°C) for 15 minutes. The reaction mixture contained 1.5 ml potassium phosphate buffer (50 mM, pH 7.0) containing ascorbate (0.5 mM) and EDTA (0.1 mM) and 0.9 ml H_2O_2 (0.1 mM). The reaction was started by adding 0.1 ml of extract and the decrease in the absorbance relative to a blank, in which H_2O_2 was absent, was monitored for 30 seconds.

4.2.3 Glutathione reductase

Glutathione reductase was assayed spectrophotometrically according to the method of

Jablonski and Anderson (1978). The glutathione reductase extraction buffer was similar to that used for ascorbate peroxidase but ascorbate and sorbitol were excluded. The plant extract was filtered through muslin and centrifuged at 10,000 g (4°C) for 15 minutes. The reaction mixture contained 1.2 ml potassium phosphate buffer (350 mM, pH 8.0) containing EDTA (0.325 mM), 0.5 ml NADPH (0.2 mM), 0.5 ml GSSG (0.2 mM) and 0.1 ml extract. The rate of NADPH oxidation (an absorbance coefficient of 6300 mM⁻¹ cm⁻¹) by GSSG-dependent glutathione reductase was followed at 340 nm against a blank which did not contain GSSG and NADPH.

4.3 Metabolite pool sizes

The pool sizes of H₂O₂, glutathione and formate were determined for seedlings which had been exposed to 80, 350 and 800 μmol mol⁻¹ CO₂, before and 100 and 300 minutes after the addition of aminotriazole. Metabolite pool sizes were expressed on a fresh weight basis.

4.3.1 Hydrogen peroxide

H₂O₂ was assayed by a slight modification of the method of Patterson, Macrae and Ferguson (1984), which was based on the reaction of H₂O₂ with Ti(IV) in the form of its complex pyridylazo-resorcinol (PAR). Pea leaves (2.0 g) were ground to a powder in liquid nitrogen together with 1.0 ml of frozen trichloroacetic acid (TCA, 5 per cent) and activated charcoal (0.7 g). TCA (5 per cent, 7.0 ml) was then added to the frozen powder, while stirring with a glass rod and gently warming in a water bath at 30°C. The extract was filtered through muslin and centrifuged at 18,000g (4°C) (Beckman Model J2-21 centrifuge, JA 20-1 rotor, U.S.A.) for 15 minutes. The excess charcoal was removed by filtering through a 100 μm nylon mesh under pressure from a syringe. Extract (2 ml) was added to the colourimetric reagent, titanium-pyridylazoresorcinol (TiPAR) (2 ml) and adjusted to pH 8.4 with 17 M ammonia solution. The TiPAR reagent was made daily by mixing equal volumes of pyridylazoresorcinol (0.6 mM) and potassium titanium oxalate (0.6 mM). Replicate samples were incubated with catalase (1 μg) at 20°C for 10 minutes, prior to adding the TiPAR reagent and adjusting the pH, to provide reagent blanks. A standard curve was constructed every time the assay was conducted and standards were within the range 20-200 nmol ml⁻¹ H₂O₂. The assay mixture, blanks and the standards were incubated at 45 °C for 60 minutes and then at room temperature for 30 minutes, before the absorbance at 508 nm was measured.

4.3.2 Glutathione

The total glutathione content was determined by a slight modification of the method of Griffith (1980). Glutathione disulphide (GSSG) was selectively determined by derivitization of reduced glutathione (GSH) (Smith, 1985). Pea leaves (1 g) were homogenized in sulphosalicylic acid (5 per cent, 10 ml) with acid-washed sand and centrifuged at 5,000g for 15 minutes. A 1 ml aliquot of the brei was added to 1.5 ml of potassium phosphate (0.5 M, pH 7.5) and used for the assay of total glutathione. 2-Vinylpyridine (0.2 ml) was added to another 1 ml aliquot of the extract and the tube mixed until an emulsion formed. This tube was used for the assay of GSSG. The tubes were incubated at 25°C for 1 hour after which they were extracted twice with diethyl ether to remove the 2-vinylpyridine. However, this did not appear to remove all the vinylpyridine which inhibited glutathione reductase and resulted in an underestimation of the GSSG content. Recently, an assay for GSSG using a recycling reaction followed by spectrophotometric detection in a flow injection analysis system has been proposed (Redegeld *et al.*, 1988). As yet, this revised method has not been carried out in our laboratory.

The extract (0.1 ml) or GSH standard (0.1 ml) were incubated with 0.5 ml sodium phosphate (0.1 M, pH 7.5) containing EDTA (5 mM), 0.1 ml NADPH (2 mM) and one unit of yeast glutathione reductase type III (Sigma Chemical Co.) for 20 minutes to convert all the glutathione to the reduced form. The colourimetric compound 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) was then added and the formation of the thiolate anion, which has a significant absorbance at 412 nm, was followed for 650 s at 25°C. A standard curve was constructed every time the assay was conducted with GSH standards in the range 0 to 8.7 nmol ml⁻¹.

4.3.3 Formate

Formate was assayed by the method provided by the Sigma Chemical Company. The extraction of formate (after Woodburn, 1988) involved grinding leaf material (1 g) to a fine powder in liquid nitrogen. Water (3 ml) was added and the homogenate was left on ice until the temperature reached 4°C. The homogenate was filtered through 4 layers of muslin and chloroform was added to the filtrate and centrifuged (Beckman GP centrifuge, U. K.) at 1,000g. The aqueous layer was removed and centrifuged at 45,000g (4°C) for 15 minutes.

The reaction mixture contained 0.75 ml sodium phosphate buffer (0.2 M, pH 7.0), 1.85 ml water, 0.3 ml β -NAD (7mg ml^{-1} , Sigma Chemical Co.) and 0.1 ml of a formate dehydrogenase (0.5 units ml^{-1} , Sigma Chemical Co.)- β -NAD (1 mg ml^{-1}) solution. The increase in the absorbance was measured at 340 nm. A standard curve was constructed in the range $20\text{-}100\text{ nm ml}^{-1}$ sodium formate every time the assay was conducted.

5.0 STATISTICAL ANALYSIS

The Kolmogorov-Smirnov two-sample test was used to determine the approximate significance level between two distributions obtained during gas exchange analysis. The significance of the difference between gas exchange results of aminotriazole-treated pea seedlings obtained for each of the experimental conditions investigated, and those obtained under ambient conditions ($350\ \mu\text{mol mol}^{-1}$, 21 per cent O₂ and a photon flux of $500\ \mu\text{mol m}^{-2}\text{ s}^{-1}$), in the absence of PGA, was determined. The significance level was also determined between the CO₂ response curve obtained before and those obtained 100, 200 and 300 minutes after the addition of aminotriazole.

A one-way analysis of variance with time was conducted on the biochemical results obtained at each CO₂ concentration. The significance level of the difference between results obtained at different CO₂ concentrations was also investigated using a one-way analysis of variance.

CHAPTER THREE

RESULTS

1.0 THE PHOTOSYNTHETIC CHARACTERISTICS OF EXCISED PEA SEEDLINGS

Light (Figure 6) and CO₂ (Figure 7) response curves of *Pisum sativum* seedlings were constructed in order to define the experimental conditions under which the inhibitor studies were to be conducted. The CO₂ assimilation rate of pea seedlings was found to saturate at a photon flux density of approximately 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Figure 6) and at an internal CO₂ concentration of about 450 $\mu\text{mol mol}^{-1}$ (Figure 7). Both the light and the CO₂ response curves are typical for a C₃ photosynthetic plant (Ogren, 1984).

From these results the following experimental conditions were defined:

Light-limiting: 80 and 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$,

Light-saturating: 780 and 900 $\mu\text{mol m}^{-2} \text{s}^{-1}$

CO₂-limiting: 80 and 150 $\mu\text{mol mol}^{-1}$ - external CO₂ (c_a)

CO₂-saturating: 620, 800 and 1000 $\mu\text{mol mol}^{-1}$ (c_a)

Standard: 350 $\mu\text{mol mol}^{-1}$ CO₂ (c_a) and a photon flux of 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$

Under standard conditions excised pea seedlings assimilated at a rate of 8-10 $\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$; this did not fluctuate by more than two per cent for the following 360 minutes, the experimental period (results not shown).

2.0 THE EFFECT OF AMINOTRIAZOLE ON THE BIOCHEMISTRY OF *PISUM SATIVUM*

Aminotriazole, applied through the transpiration stream of photosynthesizing pea seedlings should inhibit catalase activity, alter the H₂O₂ concentration, and affect the photosynthetic processes. To quantify these effects the catalase activity, H₂O₂ concentration and gas exchange and fluorescence characteristics were determined at different CO₂ concentrations.

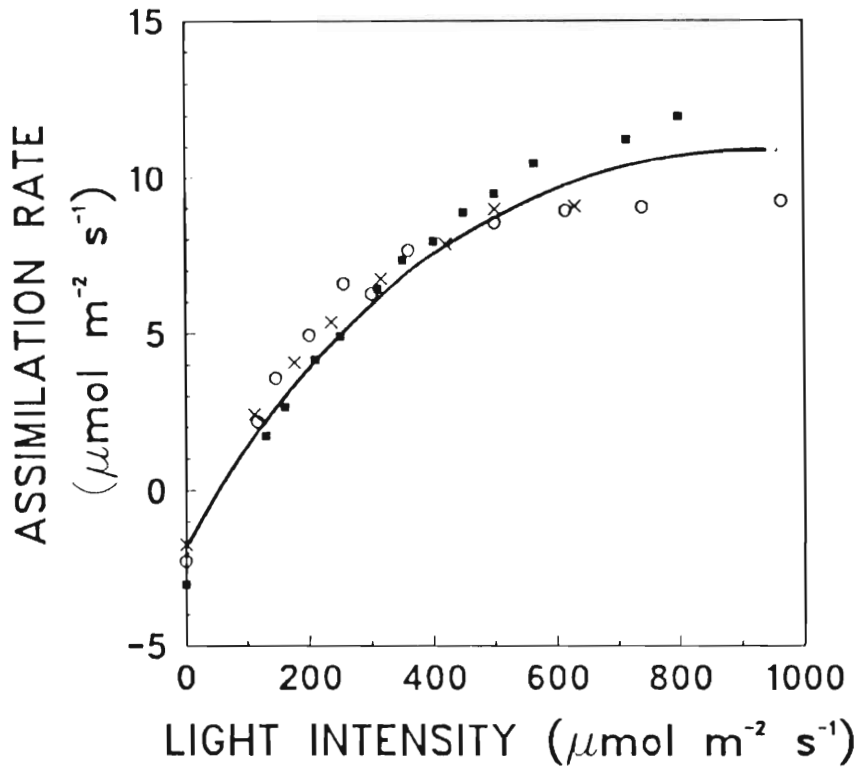


Figure 6. Light response curves of *Pisum sativum* in air ($350 \mu\text{mol mol}^{-1} \text{CO}_2$, 21 per cent O_2) ($n = 3$).

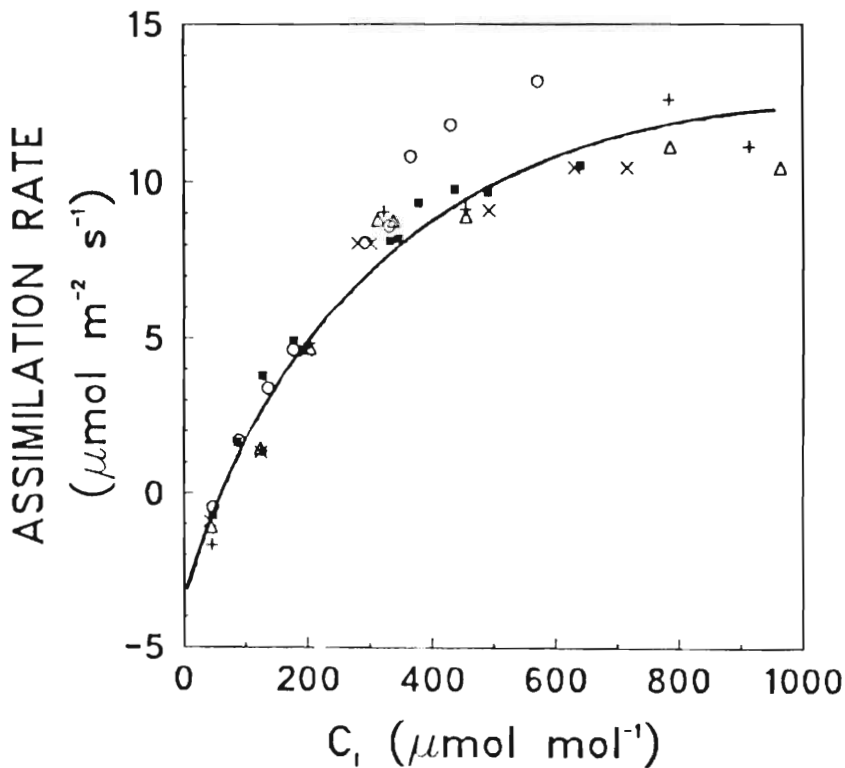


Figure 7. $A:c_i$ curve of *Pisum sativum* at a photon flux of $500 \mu\text{mol m}^{-2} \text{s}^{-1}$ ($n = 5$).

2.1 Catalase activity

The catalase activity of control pea seedlings and the rate of catalase inhibition by aminotriazole, were affected by the CO₂ concentration (Figure 8). The catalase activity prior to inhibition at 80 and 150 μmol mol⁻¹ CO₂ was significantly different from that at 350 μmol mol⁻¹ CO₂. Complete inhibition of catalase activity was achieved after 200 minutes at 80 μmol mol⁻¹ CO₂, and after 300 minutes at 150 and 350 μmol mol⁻¹ CO₂.

2.2 Hydrogen peroxide concentration

The addition of aminotriazole had little effect on the H₂O₂ concentration at any of the CO₂ concentrations investigated (Figure 9). The H₂O₂ concentrations were significantly higher at low c_a (80 μmol mol⁻¹ CO₂), most probably as a result of increased photorespiratory activity and enhanced rates of O₂ photoreduction at this low CO₂ concentration. There appears to be no relationship between catalase activity and H₂O₂ concentration.

2.3 Gas exchange characteristics

The CO₂ assimilation rate was reduced from 9.2 to 4.2 μmol m⁻² s⁻¹ approximately 100 minutes following the addition of aminotriazole at 350 μmol mol⁻¹ CO₂ and a photon flux density of 500 μmol m⁻² s⁻¹ (standard conditions) (Figure 10). Since stomatal conductance was unaffected at this stage (Figure 10), the inhibition of CO₂ assimilation appears to be independent of stomatal effects. However, in order to gain carbon most economically with respect to water loss, stomata usually function to maintain the water cost of assimilating CO₂ constant (Farquhar and Sharkey, 1982), and the stomatal conductance eventually decreased in response to the lower CO₂ assimilation rate. After 360 minutes both the CO₂ assimilation rate and stomatal conductance were reduced by approximately 80 per cent.

Since the initial CO₂ assimilation rates and the CO₂ assimilation response to aminotriazole varied amongst plants, the results of the studies with aminotriazole were normalized against the steady-state CO₂ assimilation rate prior to the commencement of the treatment. This enabled comparison between experiments conducted under different conditions. The addition of aminotriazole under standard conditions caused a 50 per cent reduction in the normalized CO₂ assimilation rate after 100 minutes and an 80 per cent reduction after

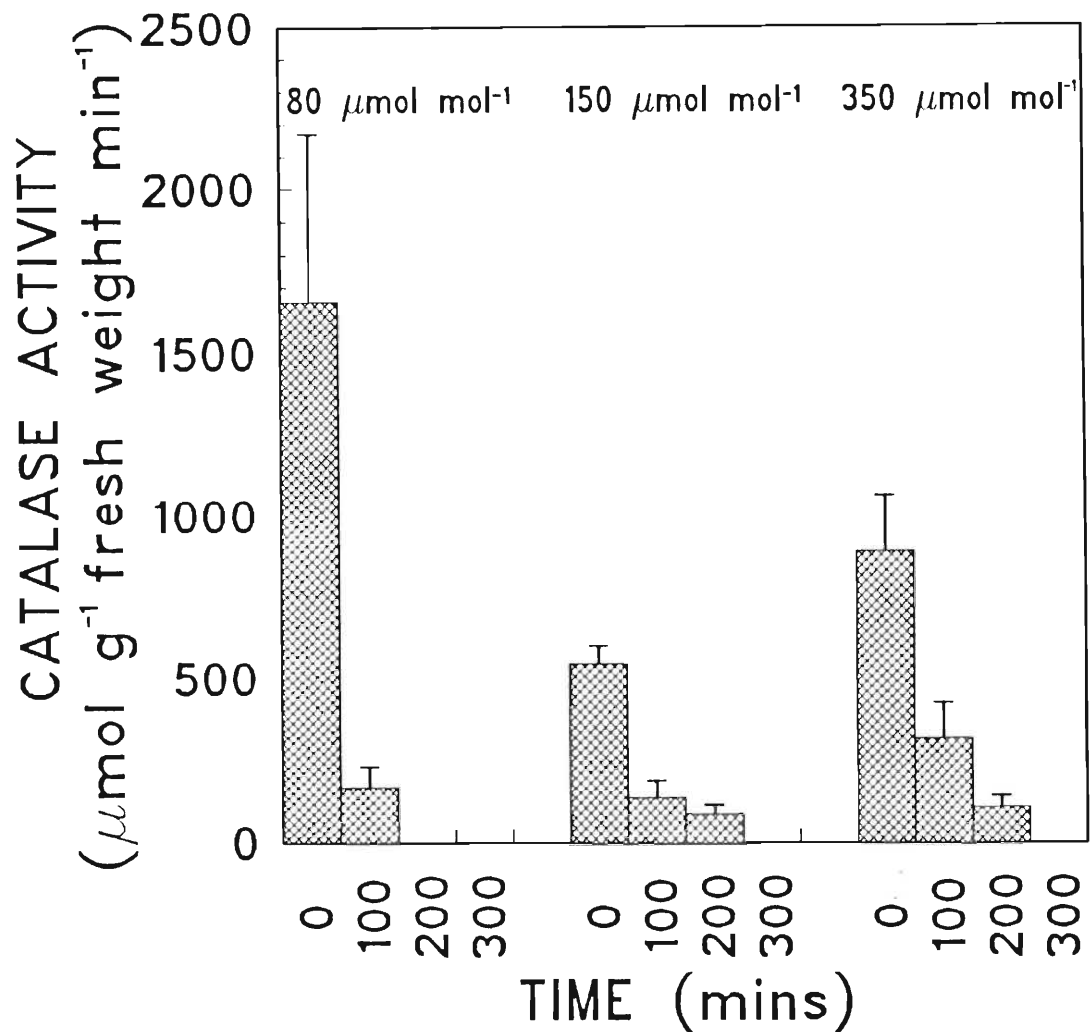


Figure 8. Catalase activity of pea seedlings exposed to 80, 150 or 350 $\mu\text{mol mol}^{-1}$ CO_2 before ($t = 0$) and 100, 200 and 300 minutes following the addition of 2 mM aminotriazole (bars represent mean \pm standard deviation, $n = 6$). (Significant difference in activity prior to the addition of aminotriazole at 80 and 150 $\mu\text{mol mol}^{-1}$ CO_2 from standard conditions ($P = 0.05$), significant difference with time at all the CO_2 concentrations investigated ($P < 0.001$))

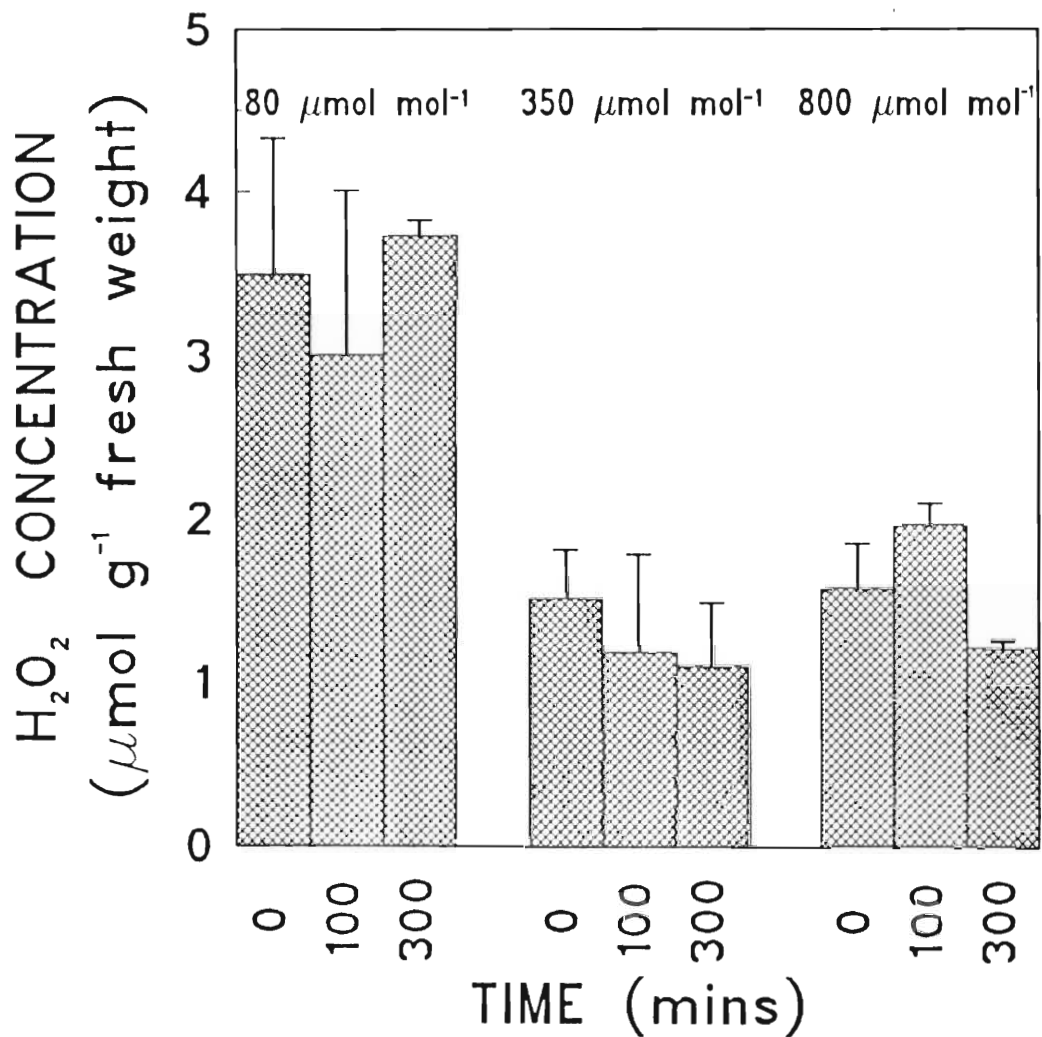


Figure 9. H₂O₂ content prior to the addition of aminotriazole (t = 0), and at 100 and 300 minutes after the addition of aminotriazole at 80, 350 and 800 μmol mol⁻¹ CO₂, 21 per cent O₂ (bars represent mean ± standard deviation, n = 6). (Significant difference with CO₂ concentration at 80 μmol mol⁻¹ CO₂, P = 0.0001).

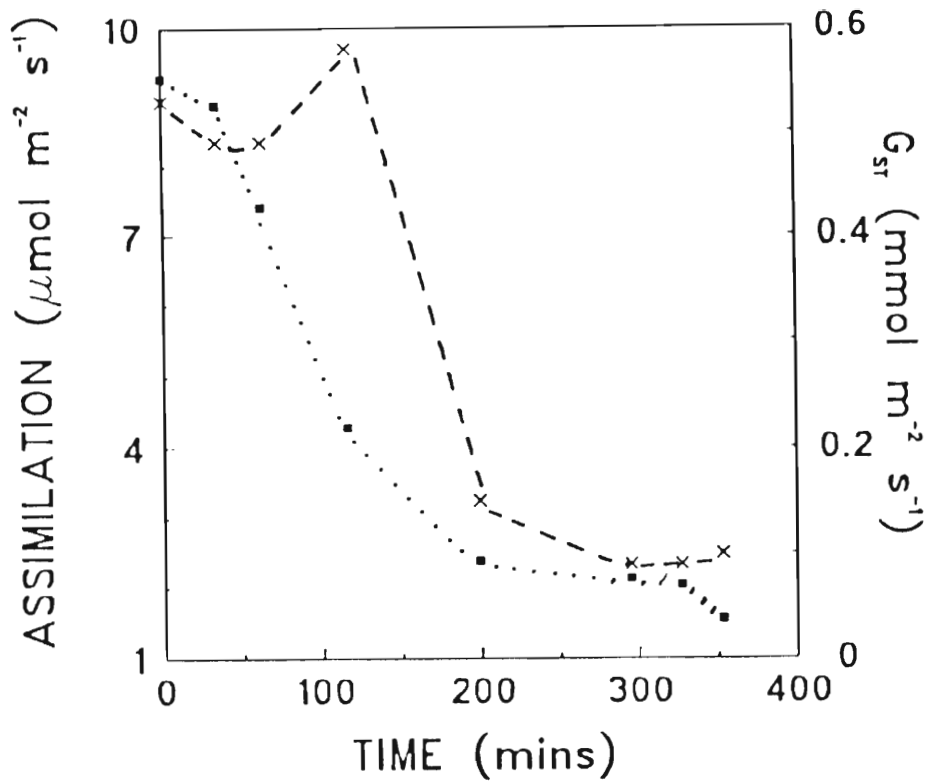


Figure 10. A typical response of CO_2 assimilation (\blacksquare) and stomatal conductance to CO_2 (\times) following the addition of aminotriazole at a photon flux density of $500 \mu\text{mol m}^{-2} \text{s}^{-1}$ in air.

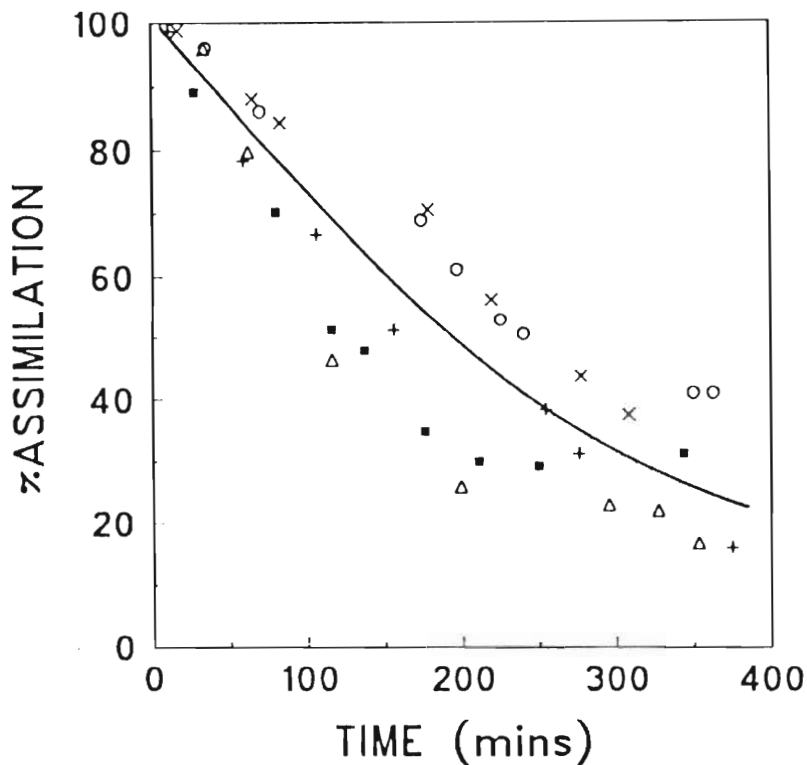


Figure 11. The effect of aminotriazole on the normalized CO_2 assimilation rates of pea seedlings under standard conditions. Steady-state CO_2 assimilation prior to the addition of aminotriazole was $9.0 \mu\text{mol m}^{-2} \text{s}^{-1}$.

approximately 200 minutes (Figure 11). The effect of aminotriazole on the CO₂ assimilation rate under various other CO₂, O₂ and light conditions was compared to that exhibited under standard conditions.

2.4 Fluorescence characteristics

It has been suggested that the primary effect of aminotriazole is to block the biosynthesis of the photosynthetic pigments β -carotene and xanthophyll (Halliwell, 1987). Chlorophyll is assumed to be secondarily bleached in the absence of protecting carotenoids (Feierabend and Schubert, 1978) which would affect F_0 , F_M (the fluorescence emitted from the PSII chlorophyll molecules when Q_A is completely reduced), and hence the F_V/F_M ratio (a measure of the photochemical efficiency of photosystem II) (Björkmann, 1987). The addition of aminotriazole had no effect on the F_0 and F_M values (Table 1) or the F_V/F_M ratios (Figure 12 A, B). F_0 was in the range 0.31 to 0.38, F_M 1.1 to 1.4 and F_V/F_M 0.68 to 0.77 during the experimental period (360 minutes). Aminotriazole does not appear to inhibit carotenoid biosynthesis, induce chlorophyll photobleaching or incur lesions in the electron transport chain of photosystem II, in the short-term.

TREATMENT	TIME (mins)	F_0	F_M
control	0	0.32	1.31
aminotriazole	0	0.36	1.28
control	360	0.37	1.4
aminotriazole	360	0.37	1.16

Table 1. The F_0 and F_M values of control pea seedlings at steady state photosynthesis and after 360 minutes in the presence and absence (control) of aminotriazole.

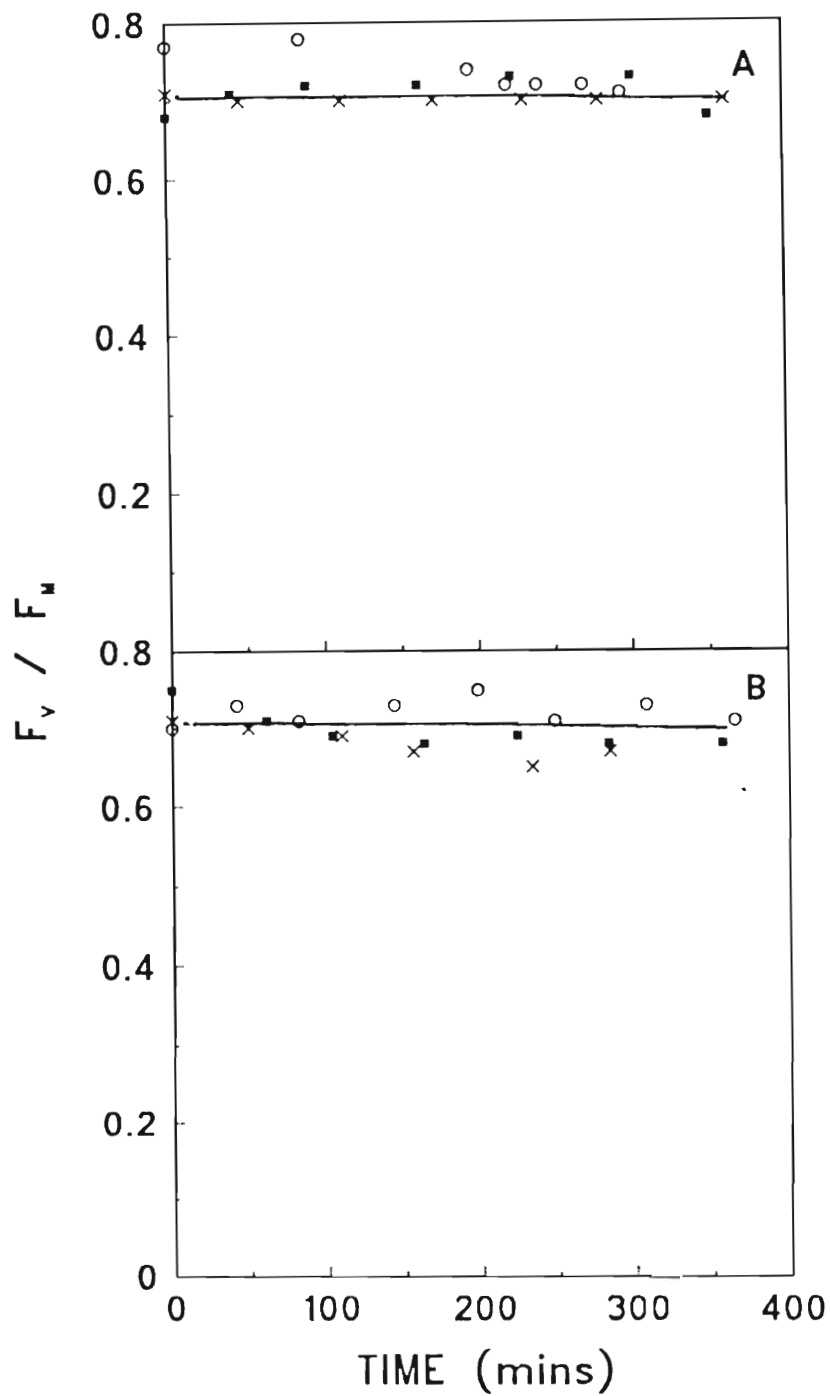


Figure 12. The F_v / F_m ratios of control pea seedlings (A) and aminotriazole-treated pea seedlings (B) during a 360 minute period under standard conditions ($n = 3$).

These results show that the addition of aminotriazole through the transpiration stream of pea seedlings inhibited catalase activity and lowered the CO₂ assimilation rate, but had no apparent effect on the H₂O₂ concentration or the F_V and F_M values. This suggests that the inhibition of CO₂ assimilation is not a result of an increase in the H₂O₂ concentration to toxic levels or a decrease in the efficiency of photosystem II. A number of techniques and experimental procedures were therefore used in an attempt to clearly quantify the effect of aminotriazole on the biochemistry of pea seedling leaves. Results of these experiments are reported in the following section.

3.0 THE MECHANISM OF THE INHIBITION OF CO₂ ASSIMILATION

3.1 CO₂ response curves

TIME (mins)	K _c	K _o	V _{MAXC}	r ²
0	285	348	270	0.99
100	793	238	323	0.99
200	1283	1236	314	0.98
300	2713	2005	380	0.95

Table 2. V_{cm_{ax}}, K_o, K_c, and r values determined by non-linear regression using the linear portion of the CO₂ response curve for control pea seedlings and 100, 200 and 300 minutes after the addition of aminotriazole to pea seedlings.

CO₂ response curves convey fundamental information regarding the biochemistry of photosynthesis (Sharkey, 1985) and were therefore used as a tool to investigate the effect of aminotriazole on the photosynthetic biochemistry of *Pisum sativum*. It has recently been demonstrated that certain treatments such as mild water stress cause heterogeneous stomatal closure (Sharkey and Seeman, 1989) which invalidates the concept of an average c_i. It was assumed that a 'patchy' stomatal response did not occur during these investigations to enable interpretation of the A:c_i curves. The reduction in the CO₂ assimilation rate following the addition of aminotriazole results from a decrease in both the RuBP regeneration rate (the saturated portion of the curve) and the efficiency of the

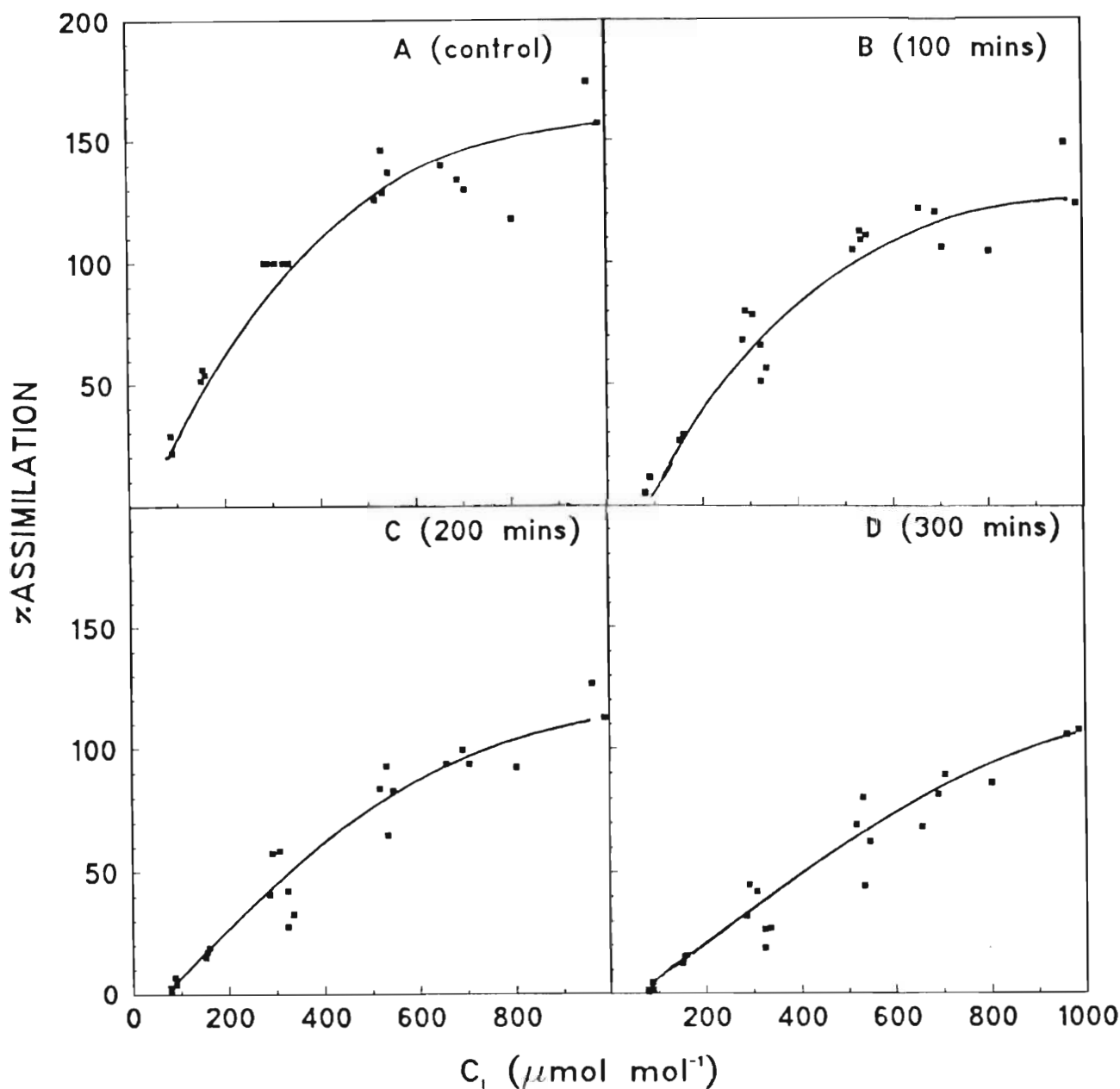


Figure 13. CO₂ response curves were interpolated before (A), and 100 (B), 200 (C) and 300 (D) minutes after the addition of aminotriazole to pea seedlings at a light intensity of 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$; 100 per cent assimilation - approximately 9 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at ambient CO₂. (Each point on the graph represents an individual replicate. B, C and D differ from A at the 99 per cent significance level).

carboxylation reaction (the initial linear portion of the curve) (Figure 13). These effects are apparent after 100 minutes (Figure 13 B) and are maximal 200 minutes after the commencement of the treatment (Figure 13 C). The CO₂ response curves constructed 200 and 300 minutes after the addition of the inhibitor are not significantly different (Figure 13 C, D).

Analysis of the initial linear portion of the CO₂ response curves showed that the affinity of Rubisco for CO₂ (K_c) and O₂ (K_o) was reduced with the addition of aminotriazole (Table 2). There was almost a ten-fold increase in the Michaelis-Menton constants of both the carboxylation (K_c) and oxygenation reactions (K_o), 300 minutes after the addition of aminotriazole. However, the potential rate of the carboxylation reaction (V_{maxc}) was unaffected. This suggests that Rubisco is not directly inhibited by aminotriazole or H₂O₂.

3.2 PGA effects

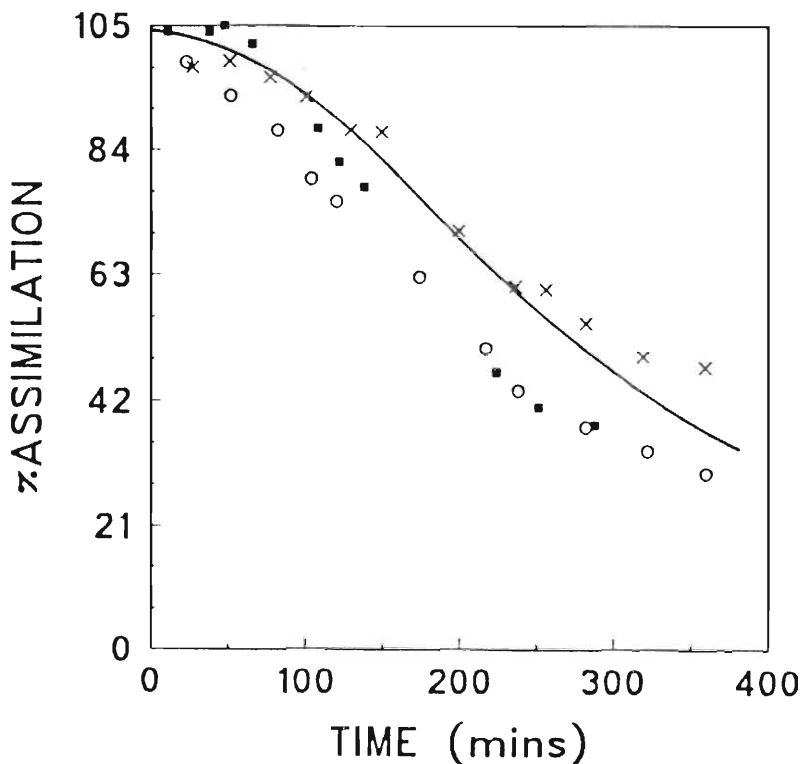


Figure 14. Effect of PGA on the CO₂ assimilation rate of aminotriazole-treated pea seedlings under standard conditions ($n = 3$). Results were normalized relative to a steady-state CO₂ assimilation rate of $8.5 \mu\text{mol m}^{-2} \text{s}^{-1}$ in the presence of PGA. (significant difference during the first 100 minutes between aminotriazole-treated seedlings supplied with PGA (Figure 14) and those not supplied with PGA (Figure 11), $P < 0.05$).

The effect of aminotriazole on the RuBP regeneration rate of pea seedlings may be due to a perturbation of the photorespiratory pathway which affects the rate of carbon return to the Benson-Calvin cycle, in the form of PGA. To test this hypothesis PGA was applied through the transpiration stream of excised pea seedlings. The response of CO₂ assimilation 100 minutes after the addition of aminotriazole in the presence of PGA (Figure 14) was significantly different from that which occurs under identical CO₂ and light conditions in the absence of PGA (Figure 11). The addition of PGA to aminotriazole-treated pea seedlings appears to initially alleviate the inhibition of CO₂ assimilation. It appears that the return of carbon, temporarily lost to the glycolate pathway as a result of the oxygenation reaction of Rubisco, to the Benson-Calvin cycle, is the first process to be affected in the short-term. A depletion of the intermediates of the Benson-Calvin cycle will affect the rate of RuBP regeneration.

3.3 CO₂ and O₂ effects

The lack of catalase activity in barley mutants or aminotriazole-treated barley seedlings was accompanied by reduced CO₂ assimilation rates under photorespiratory conditions, but CO₂ assimilation was unaffected under non-photorespiratory conditions (Smith *et al.*, 1984). The relationship between the rate of inhibition of CO₂ assimilation and the photorespiratory activity of aminotriazole-treated pea seedlings was investigated by exposing plants to different CO₂ and O₂ concentrations.

The rate of inhibition of CO₂ assimilation of aminotriazole-treated pea seedlings was greatest at CO₂ concentrations below ambient (Figures 15 A, B) and became reduced at high CO₂ concentrations (Figures 15 D, E, F). The CO₂ assimilation rate was reduced linearly with time until approximately 200 minutes after the start of the treatment, when it appeared to reach a minimum, under CO₂-limiting conditions at 80 μmol mol⁻¹, 170 μmol mol⁻¹ and 350 μmol mol⁻¹ CO₂ (Figures 15 A, B; Figure 11, respectively). The inhibition of the CO₂ assimilation rate increased almost linearly with time at the CO₂-saturating concentrations of 620, 800 and 1000 μmol mol⁻¹ CO₂ (Figures 15 C, D, E). The CO₂ assimilation rate was reduced to approximately eight per cent of the steady-state level at 80 μmol mol⁻¹ CO₂, while the CO₂ assimilation rate was only 40 per cent inhibited at 800 and 1000 μmol mol⁻¹ CO₂, 360 minutes following the commencement of the treatment. It appears that the extent of inhibition at the completion of the experiment decreased with increasing CO₂ concentrations until 800 and 1000 μmol mol⁻¹ CO₂.

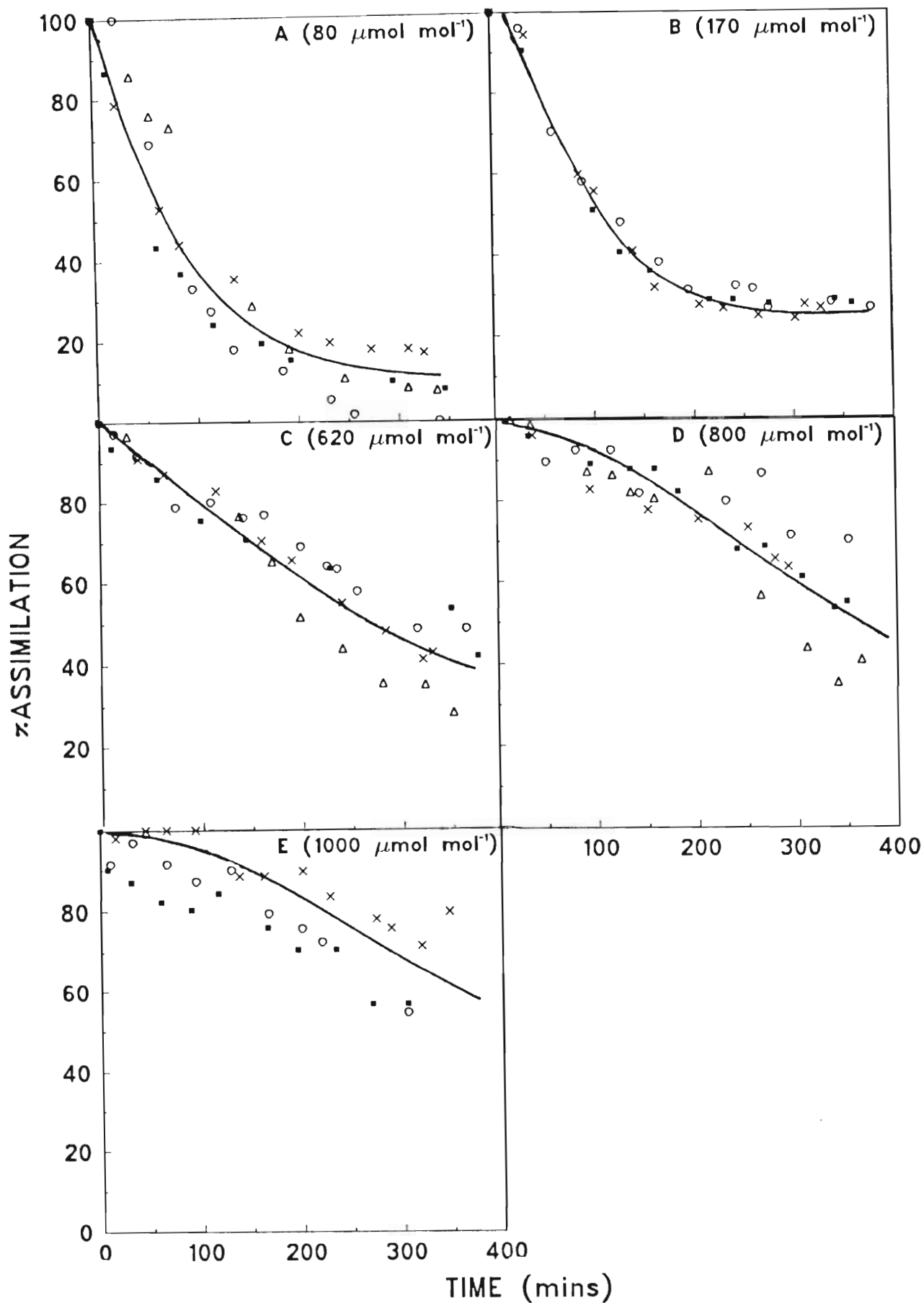


Figure 15. The effect of aminotriazole on the CO₂ assimilation rate of pea seedlings at 80 (A), 150 (B), 620 (D), 800 (D) and 1000 (E) μmol mol⁻¹ CO₂, 21 per cent O₂ and a photon flux density of 500 μmol m⁻² s⁻¹. (Between 3-7 replicates were conducted at each CO₂ concentration and time interval.)

(Figure 16), where the rate of photorespiration would be reduced to a minimum (Canvin *et al.*, 1980).

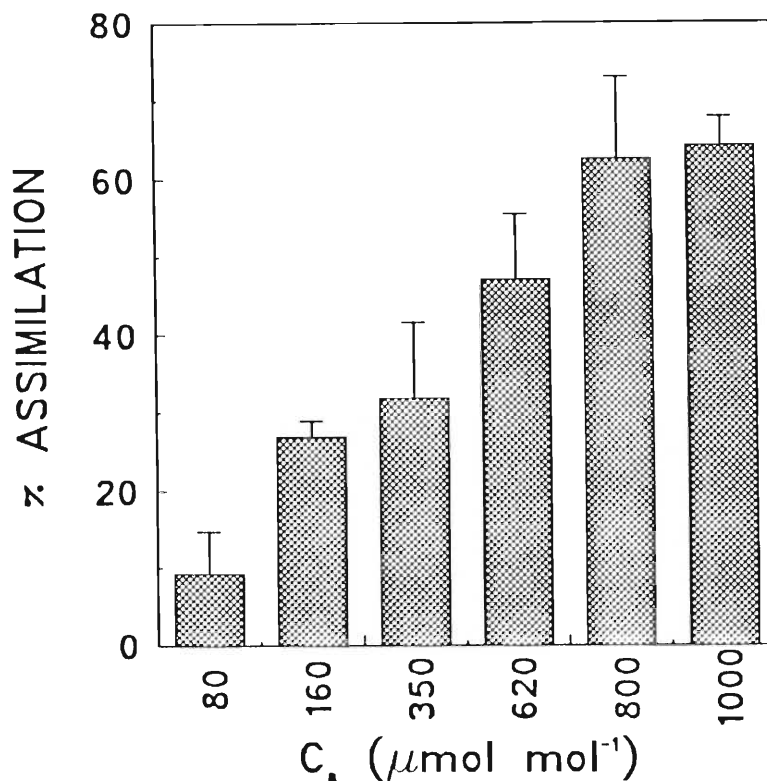


Figure 16. A summary of the effect of c_a on the assimilation of CO_2 , 300 minutes after the addition of aminotriazole ($n = 3-7$).

The exposure of pea seedlings to $800 \mu\text{mol mol}^{-1} \text{CO}_2$ and 45 per cent O_2 resulted in a slight inhibition of the CO_2 assimilation rate during the experimental period (Figure 17 A). O_2 uptake, during photorespiration and the Mehler reaction, saturates at about 30 per cent O_2 (Canvin *et al.*, 1980) and higher O_2 concentrations may induce oxidative damage. The inhibition of the assimilation rate of aminotriazole-treated pea seedlings at $800 \mu\text{mol mol}^{-1} \text{CO}_2$, 45 per cent O_2 (Figure 17 B) was not significantly different to that exhibited at $350 \mu\text{mol mol}^{-1} \text{CO}_2$, 21 per cent O_2 (Figure 11), but was almost double that at $800 \mu\text{mol mol}^{-1} \text{CO}_2$, 21 per cent O_2 (Figure 15 D). The degree of inhibition of CO_2 assimilation exhibited by aminotriazole-treated pea seedlings appears to be dependent on the rate of carbon flow through the photorespiratory pathway, which is controlled by the $\text{CO}_2:\text{O}_2$ ratio.

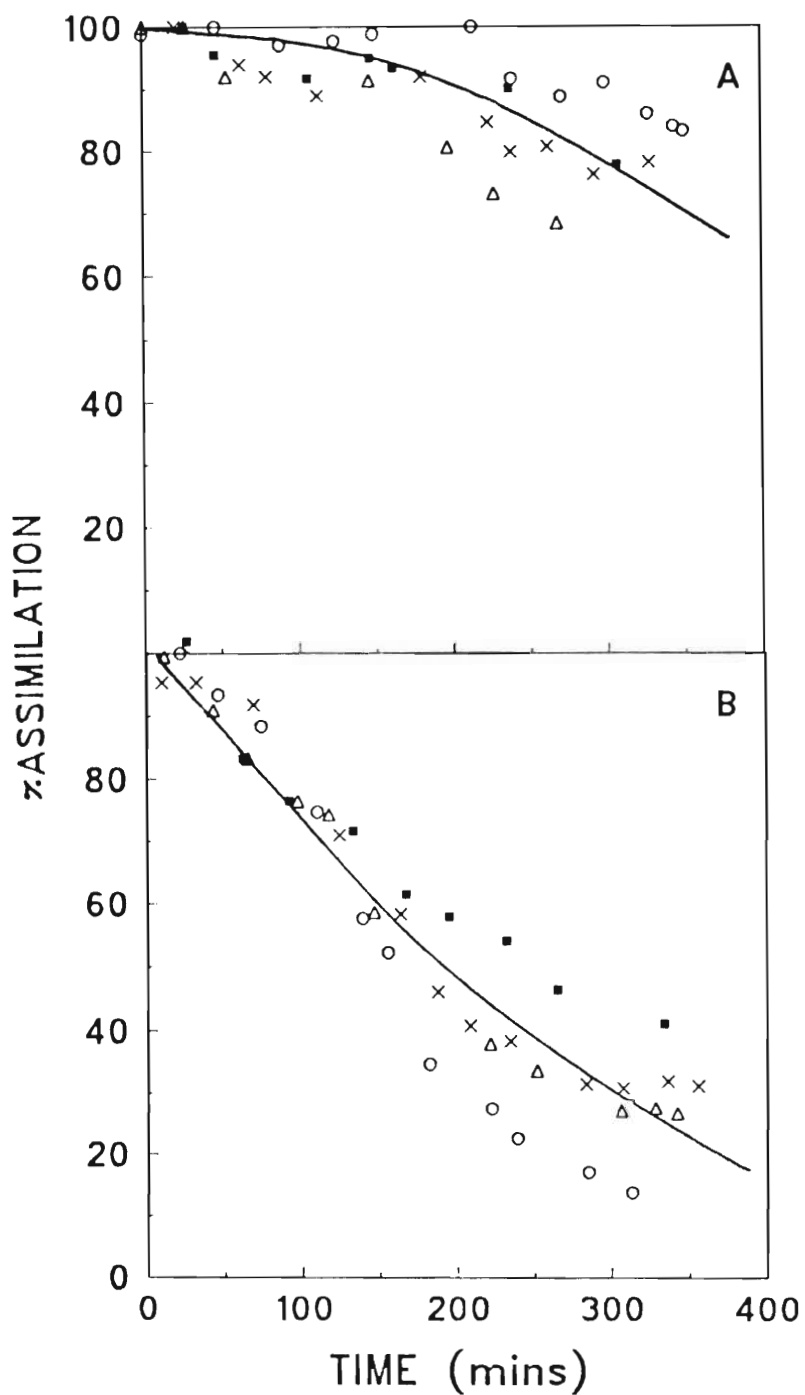


Figure 17. The effect of 45 per cent O_2 , $800 \mu\text{mol mol}^{-1} \text{CO}_2$ on control (A) and aminotriazole-treated pea seedlings (B). The steady-state CO_2 assimilation rates were $9.0 \mu\text{mol m}^{-2} \text{s}^{-1}$ (control) and $9.4 \mu\text{mol m}^{-2} \text{s}^{-1}$ (aminotriazole-treated). (Significant difference from effect at $800 \mu\text{mol mol}^{-1} \text{CO}_2$, 21 per cent O_2 (Figure 15 D), $P < 0.001$).

3.4 The hydrogen peroxide-scavenging mechanisms

The results from CO₂ response curves suggested that the mechanism of the inhibition of CO₂ assimilation of aminotriazole-treated pea seedlings may involve a reduction in the regeneration of the CO₂ acceptor molecule, RuBP. RuBP-regeneration requires light-derived chemical energy and the return of carbon from the photorespiratory pathway, in the form of 3-PGA, lost due to the oxygenation reaction of Rubisco (Sharkey, 1985). The H₂O₂ concentration of aminotriazole-treated pea seedlings was found not to increase to toxic levels. The reduction in the RuBP-regeneration rate would, therefore, not be a result of the direct oxidation of essential Benson-Calvin cycle enzymes by H₂O₂, as previously suggested (Kaiser, 1976; 1979). However, the compensatory removal of H₂O₂ by metabolic systems other than those involving catalase may reduce the availability of reducing power for the operation of the Benson-Calvin cycle (Asada and Takahashi, 1987) or prevent the return of carbon from the glycolate pathway in the form of PGA. Some of the enzyme activities and metabolite pool sizes of these alternative H₂O₂-scavenging mechanisms were therefore investigated.

3.4.1. The ascorbate-glutathione hydrogen peroxide scavenging system

The removal of H₂O₂ by the ascorbate-glutathione system requires photogenerated light energy (Law, Charles and Halliwell, 1983; Asada and Takahashi, 1987). An increase in the activity of the ascorbate-glutathione scavenging system to enable the compensatory removal of H₂O₂, normally metabolised by catalase, may create a competition for reductant between the ascorbate-glutathione system and the Benson-Calvin cycle. Under light-limiting conditions insufficient chemical energy may be available to support the potential H₂O₂-scavenging and RuBP-regeneration rates, causing a reduction in CO₂ assimilation. Contrary to expectation, the rate of inhibition of CO₂ assimilation was greater at the light-saturating photon fluxes of 500, 780 and 900 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Figure 11; Figure 18 C, D) than at 100 and 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ where the rate of photoreductant generation limits CO₂ uptake (Figure 18 A, B). The rate of inhibition was lower at a light intensity of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ than 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and the extent of inhibition after 300 minutes was correspondingly less (Figure 19). However, there was no significant difference in the rate or the extent of inhibition (Figure 19) exhibited under light-saturating conditions. Under light saturating conditions the activity of the photorespiratory pathway reaches a maximum, being limited by the RuBP-regeneration rate (Badger, 1985). The degree of inhibition of the CO₂ assimilation rate of aminotriazole-treated pea seedlings

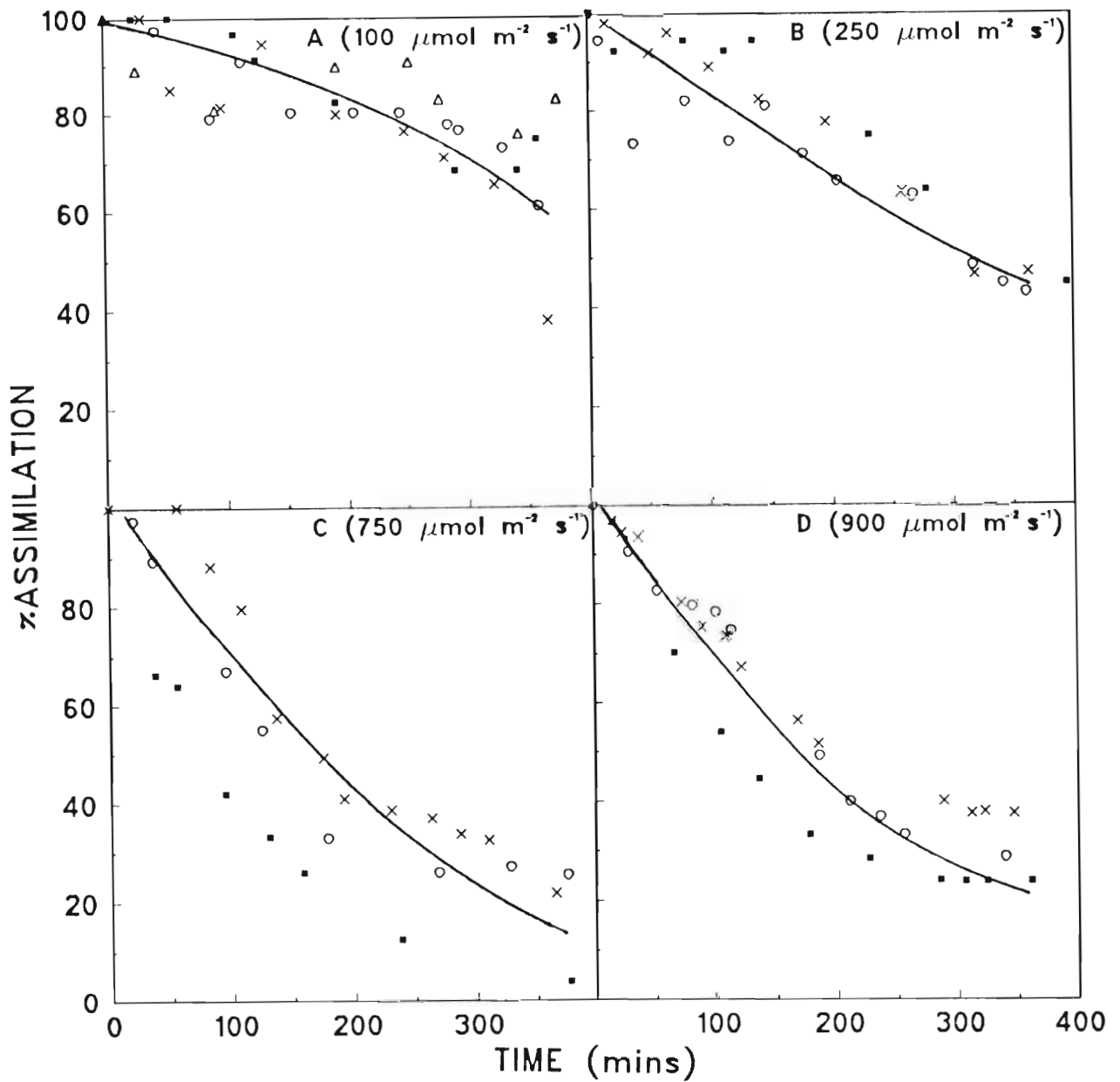


Figure 18. The CO_2 assimilation response of aminotriazole-treated pea seedlings to light intensities of 100 (A), 250 (B), 780 (C) and 900 (D) $\mu\text{mol m}^{-2} \text{s}^{-1}$ in air (investigations were replicated 3-7 times at each light intensity). (A and B significantly different from standard conditions (Figure 11), $P < 0.01$).

appears to be directly proportional to the rate of carbon flow through the photorespiratory pathway.

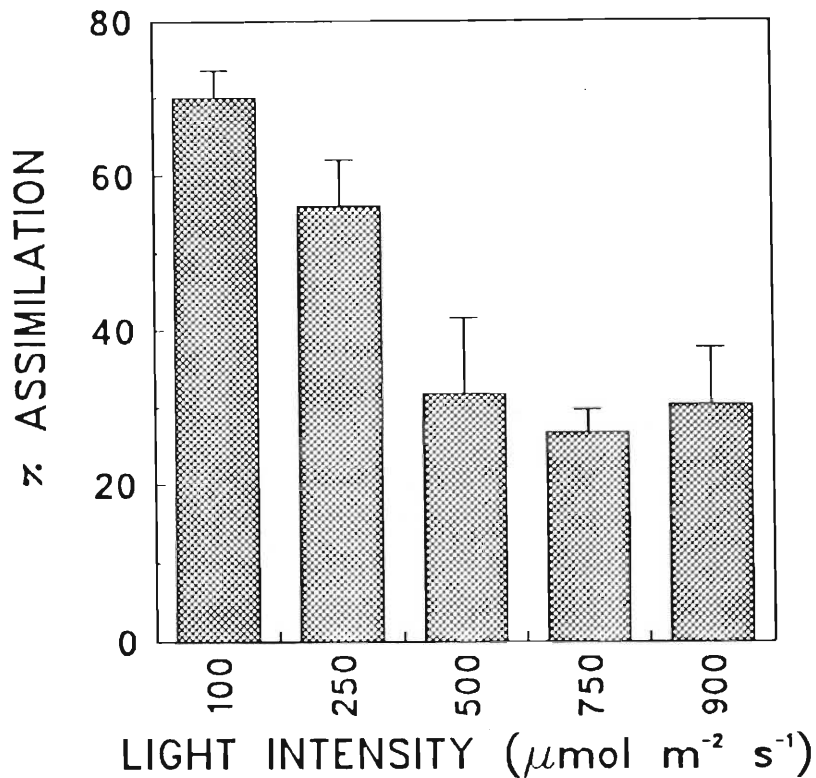


Figure 19. A summary of the effect of light intensity on the CO_2 assimilation rate of aminotriazole-treated seedlings 300 minutes after the commencement of the treatment (n = 3-7).

An increase in the activity of the ascorbate-glutathione H_2O_2 -scavenging mechanism has been regarded as an adaptation of the photosynthetic apparatus to oxidative stress (Gillham and Dodge, 1984; 1987). The effect of aminotriazole on the activity of the enzymes, ascorbate peroxidase and glutathione reductase and the total glutathione and oxidised glutathione (GSSG) pool sizes was therefore investigated.

Aminotriazole did not induce an increase in the ascorbate peroxidase activity of pea seedlings at any of the CO_2 concentrations investigated (Figure 20). The ascorbate peroxidase activity of the control pea seedlings was significantly greater at $80 \mu\text{mol mol}^{-1} \text{CO}_2$ than at the higher CO_2 concentrations investigated and was reduced to the level exhibited at the higher CO_2 concentrations 200 minutes after commencement of the treatment.

Apart from a slight increase in the glutathione reductase activity 300 minutes after the addition of aminotriazole at $150 \mu\text{mol mol}^{-1} \text{CO}_2$, the activity of this anti-oxidant system

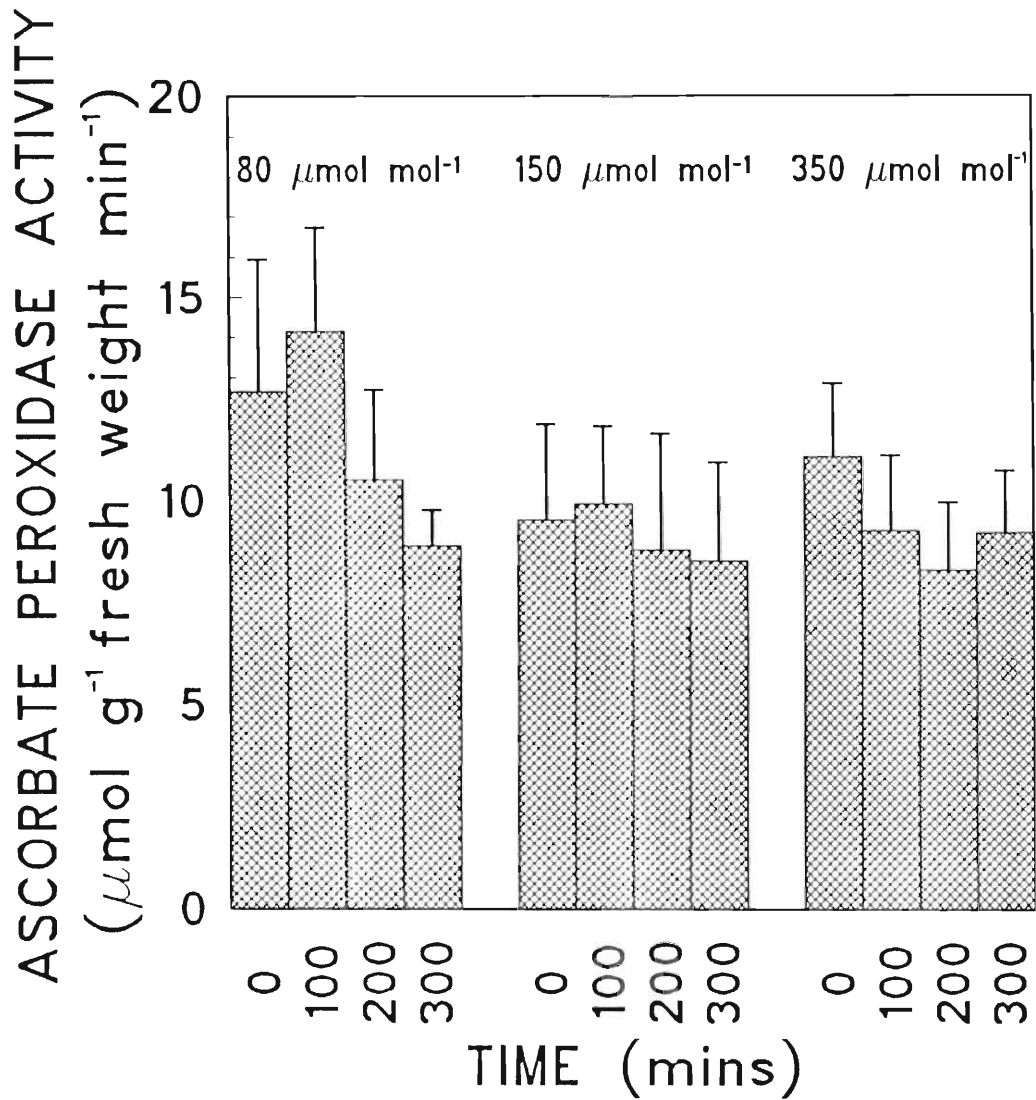


Figure 20. Ascorbate peroxidase activity before ($t = 0$), and at 100, 200, and 300 minutes after the addition of aminotriazole at 80, 150 and 350 $\mu\text{mol mol}^{-1} \text{CO}_2$, 21 per cent O_2 and a photon flux density of $500 \mu\text{mol m}^{-2} \text{s}^{-1}$ (bars represent mean \pm standard deviation, $n = 10$). (Significant difference with time at 80 $\mu\text{mol mol}^{-1} \text{CO}_2$, $P < 0.01$, and a significantly greater control activity at 80 $\mu\text{mol mol}^{-1} \text{CO}_2$, $P < 0.01$).

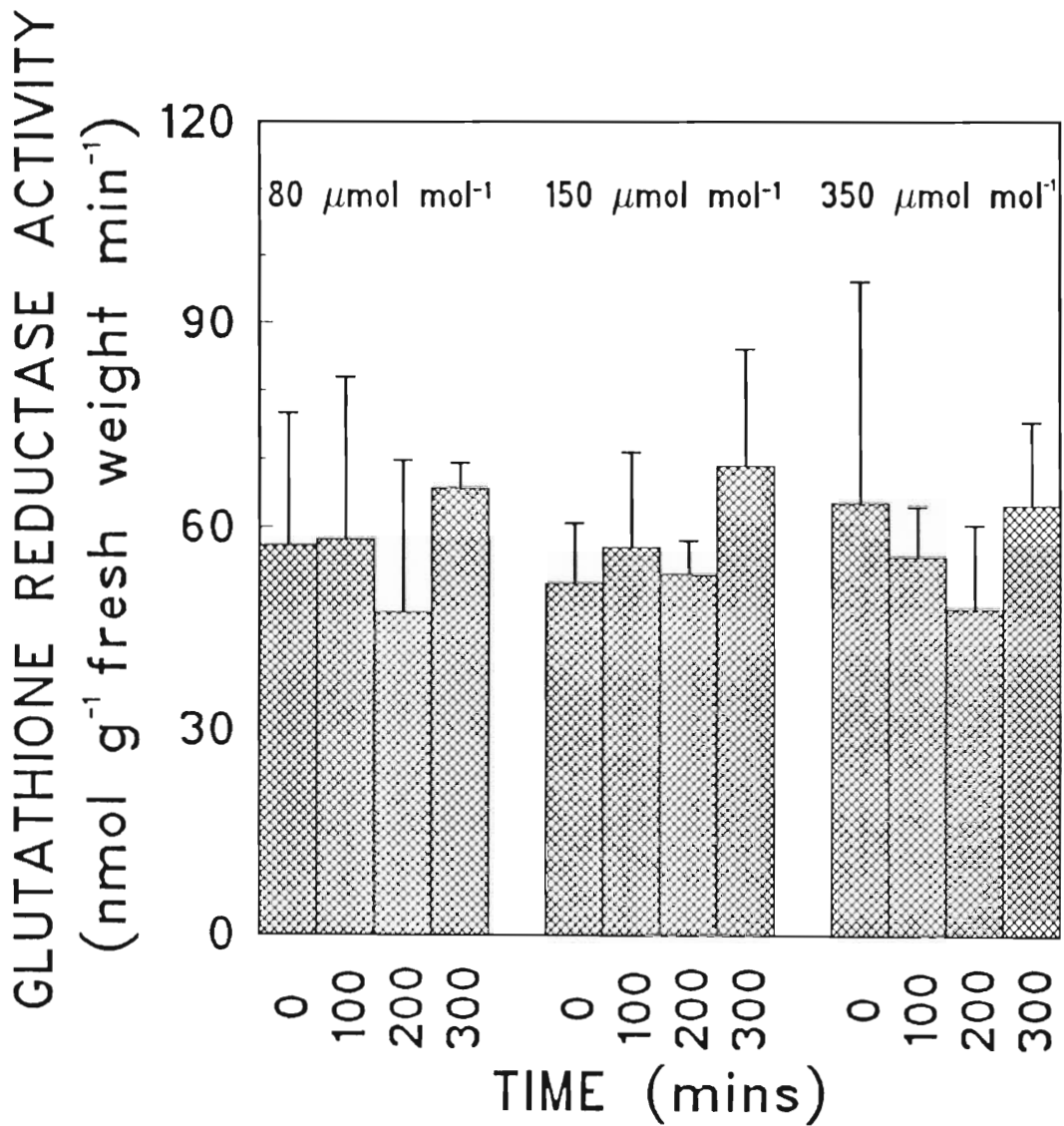


Figure 21. The effect of aminotriazole on glutathione reductase activity before ($t = 0$) after 100, 200 and 300 minutes at 80, 150 and 350 $\mu\text{mol mol}^{-1}$ CO_2 ($n = 10$, vertical lines represent standard deviation). (Significant difference 300 minutes after the addition of aminotriazole at 150 $\mu\text{mol mol}^{-1}$ CO_2 , $P < 0.01$).

enzyme was not significantly effected by treatment with aminotriazole at any of the CO₂ concentrations investigated (Figure 21). Glutathione reductase activity of pea seedlings appeared to be insensitive to changes in the background CO₂ concentration and remained relatively constant at 80, 150 and 350 μmol mol⁻¹ CO₂ (Figure 21).

The enhanced H₂O₂ levels exhibited by pea seedlings at 80 μmol mol⁻¹ CO₂ were accompanied by high total glutathione levels, which decreased following treatment with aminotriazole (Figure 22). The total glutathione pool sizes of control pea seedlings exposed to 350 and 800 μmol mol⁻¹ CO₂ were approximately 50 per cent lower than at 80 μmol mol⁻¹ CO₂ and were not affected by treatment with aminotriazole (Figure 22). The measured GSSG levels were always unrealistically low, possibly as a result of vinylpyridine contaminating the assay medium and inhibiting glutathione reductase. The results were therefore considered to be unreliable.

The activity of the ascorbate-glutathione pathway does not appear to increase with the addition of aminotriazole. Either the activity of the anti-oxidant system of the control pea seedlings is sufficient to enable compensatory removal of H₂O₂ normally metabolised by catalase, or the H₂O₂ is removed by an alternative mechanism, possibly by reaction with glyoxylate to form formate and CO₂.

3.4.2 The alternative photorespiratory pathway

The inhibition of catalase by aminotriazole may have been accompanied by a compensatory removal of peroxisomal H₂O₂ by reaction with glyoxylate to form formate and CO₂. (Servaites and Ogren, 1977). The concentration of formate was almost double the control level 100 minutes after the addition of aminotriazole at 350 and 800 μmol mol⁻¹ CO₂ (Figure 23). However, the pool size returned to the level of the control pea seedlings 300 minutes following the commencement of the aminotriazole-treatment. At 80 μmol mol⁻¹ CO₂ the formate concentration of control pea seedlings was significantly higher than at 350 or 800 μmol mol⁻¹ CO₂. The addition of aminotriazole, at this low CO₂ concentration did not significantly enhance the formate levels after 100 minutes, but instead decreased the level to below that exhibited by the control after 300 minutes (Figure 23). It appears that the removal of peroxisomal H₂O₂ by reaction with glyoxylate to form formate and CO₂ increases in the presence of enhanced peroxisomal H₂O₂ concentrations which occur at low c_a and initially following the inhibition of catalase by aminotriazole.

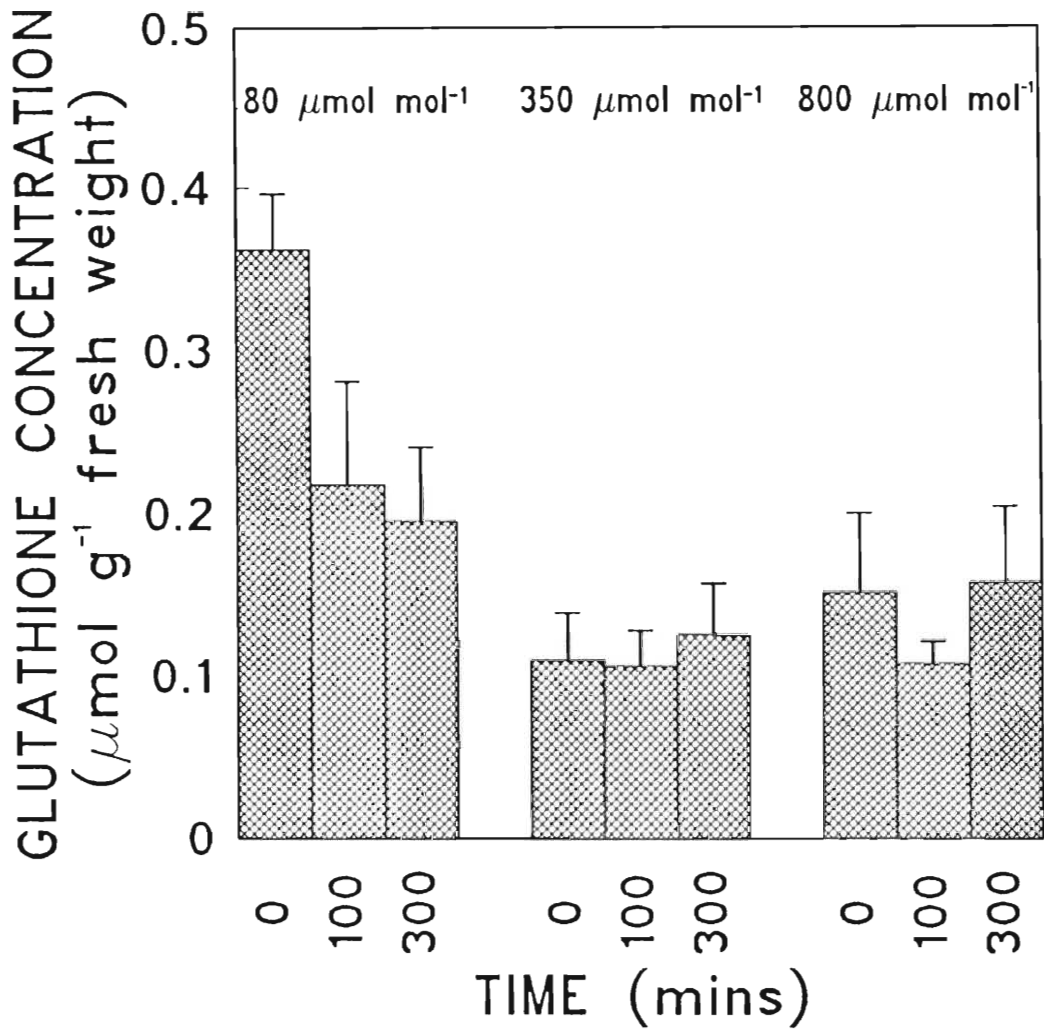


Figure 22. The total glutathione pool size of control pea seedlings and 100 and 300 minutes following the commencement of the treatment at 80, 350, and 800 $\mu\text{mol mol}^{-1}$ CO_2 , 21 per cent O_2 and a photon flux density of 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ ($n = 9$). (Significant difference with time and from the other CO_2 concentrations at 80 $\mu\text{mol mol}^{-1}$ CO_2 , $P < 0.001$).

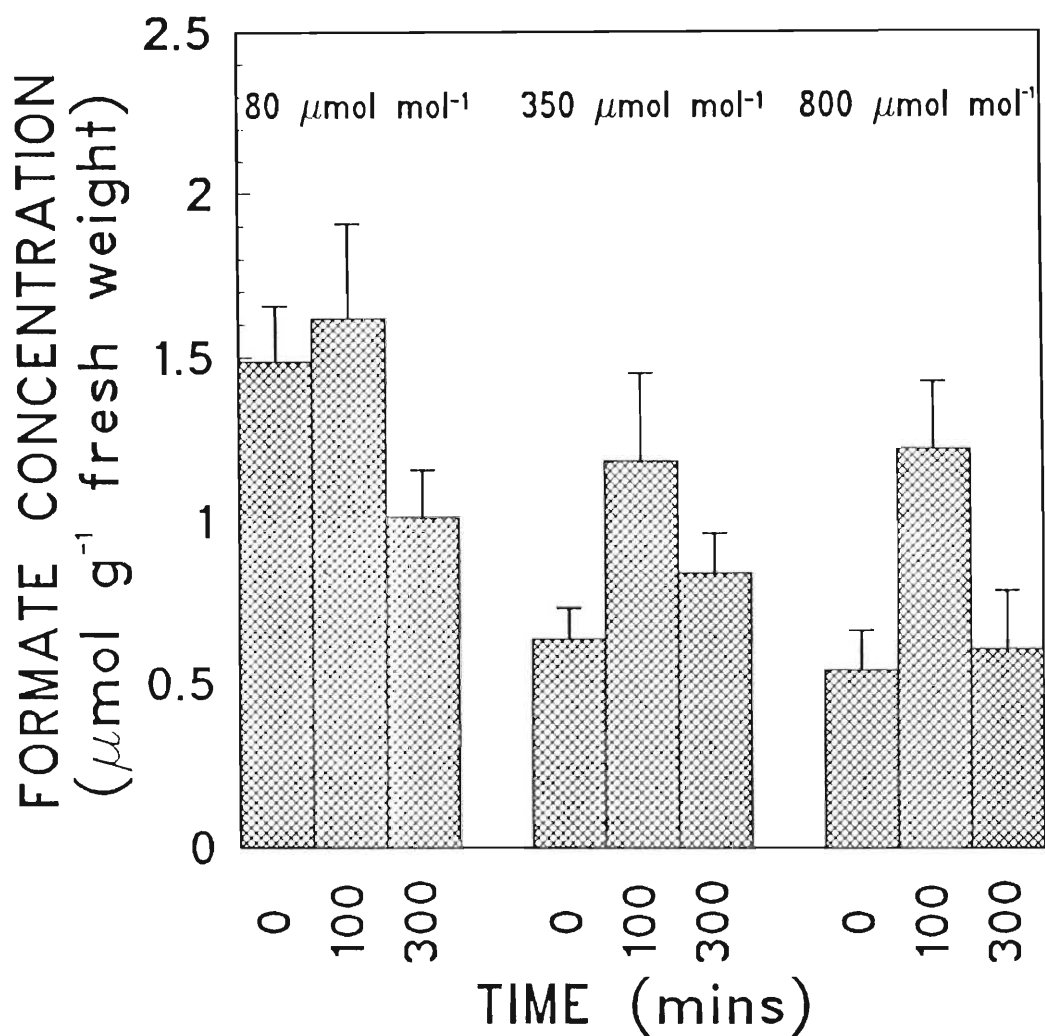


Figure 23. The formate pool sizes of control and aminotriazole-treated seedlings exposed to 80, 350 and 800 $\mu\text{mol mol}^{-1}$ CO_2 , 21 per cent O_2 and a photon flux density of 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (Vertical lines represent standard deviation, $n = 9$). (Significant difference 100 minutes after the addition of aminotriazole at 350 and 800 $\mu\text{mol mol}^{-1}$ CO_2 , $P < 0.001$, and after 300 minutes at 80 $\mu\text{mol mol}^{-1}$ CO_2 , $P = 0.0001$).

The increased production of formate in aminotriazole-treated peas may reduce the photorespiratory carbon flow through the Tolbert photorespiratory pathway (van Leeuwen, 1987). The formate produced may be completely decarboxylated to CO₂ (Leek, Halliwell and Butt, 1972; Halliwell, 1974a) or act as a precursor of products of one-carbon metabolism, other than serine (Cossins, 1980). Under these conditions considerably less of the photorespiratory carbon would be returned to the Benson-Calvin cycle via both the Tolbert and the alternative photorespiratory pathways and the rate of RuBP regeneration and the CO₂ assimilation rate would be reduced. If this was the primary cause of the inhibition of the CO₂ assimilation rate following the addition of aminotriazole, then the addition of isonicotinic acid hydrazide (INH), a specific inhibitor of serine transhydroxymethylase activity (Servaites and Ogren, 1977), to aminotriazole-treated seedlings should not enhance the inhibition of CO₂ assimilation. INH has been shown to decrease the incorporation of ¹⁴C from ¹⁴C-labelled glycolate into the serine and phosphoglycerate pools, but increase the incorporation into glycine and formate (Servaites and Ogren, 1977; Grodzinski, 1979). The addition of INH (5 mM) to pea seedlings caused a 60 per cent inhibition of the CO₂ assimilation rate 300 minutes after treatment (Figure 3.18 A). This is presumably a result of decreased activity of the Tolbert photorespiratory pathway. The simultaneous addition of INH and aminotriazole to excised pea seedlings did not increase the inhibition of the CO₂ assimilation above that exhibited by aminotriazole-treated pea seedlings (Figure 3.18 B), which suggests that the mechanism of the inhibition of CO₂ assimilation by INH and aminotriazole may be similar.

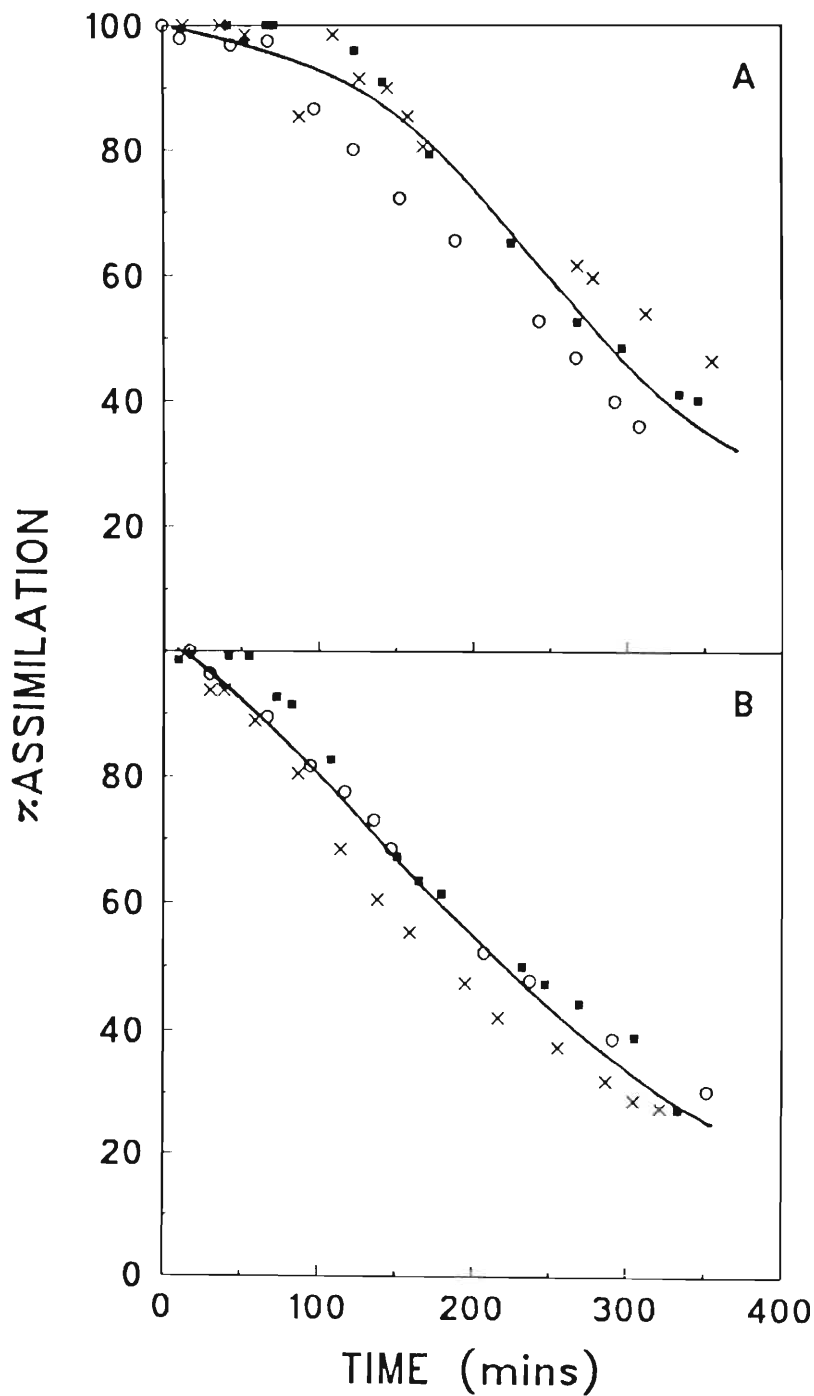


Figure 24. The effect of INH on the CO₂ assimilation rate in the presence (B) and absence of aminotriazole (A), ambient air and a light intensity of 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$. (No significant difference between A and B, $P = 0.05$).

CHAPTER FOUR

DISCUSSION

The present study showed that there was no concomitant increase in H_2O_2 concentration with the inhibition of catalase activity by aminotriazole. The CO_2 assimilation rate was, however, inhibited. These results suggest that H_2O_2 is compensatorily removed by an H_2O_2 -scavenging mechanism other than catalase. The increased activity of these mechanisms may contribute to the decreased photosynthetic CO_2 uptake rate by depleting the reductant and/or carbon supply to the Benson-Calvin cycle.

1.0 THE EFFECT OF AMINOTRIAZOLE ON THE PHOTOSYNTHETIC METABOLISM OF *PISUM SATIVUM*

Aminotriazole (2 mM), applied through the transpiration stream of excised pea seedlings, completely inhibited catalase and severely decreased CO_2 assimilation rates, but did not enhance the *in vivo* H_2O_2 concentration .

The catalase activity was maximally inhibited approximately 300 minutes after the addition of aminotriazole at all the CO_2 concentrations investigated (Figure 8), but the initial rate of inhibition was greatest at $80 \mu\text{mol mol}^{-1} \text{CO}_2$. Since the inhibitory action of aminotriazole is exerted on the catalase- H_2O_2 complex, Compound I (Halliwell and Gutteridge, 1985), the enhanced H_2O_2 levels exhibited at this low CO_2 concentration (Figure 9) may have facilitated the formation of Compound I, thereby increasing the rate of inhibition.

With the inhibition of catalase, the concentration of H_2O_2 may increase to toxic levels; this could contribute to photooxidative damage. However, in agreement with observations of Macrae and Ferguson (1985) with cold-treated tissue, Volk and Feierabend (1989) with high light-treated tissue, and Ferguson and Dunning (1986) with aminotriazole-treated cells, the inhibition of catalase was not accompanied by an increase in the H_2O_2 levels of pea seedlings at any of the CO_2 concentrations in these investigations (Figure 9). This suggests that with catalase inhibition, in the short-term, there are alternative H_2O_2 -

scavenging mechanisms which prevent the accumulation of H_2O_2 . The high H_2O_2 concentrations associated with $80 \mu\text{mol mol}^{-1} \text{CO}_2$ are probably a result of enhanced photorespiratory activity (Canvin *et al.*, 1980) and enhanced photoreduction of molecular O_2 (Egneus *et al.*, 1975).

Aminotriazole (2 mM) had an almost immediate and increasingly severe effect on the CO_2 assimilation rate of pea seedlings under the standard conditions (Figure 10, 11). It has been proposed that unmetabolized H_2O_2 oxidises sulphhydryl groups of essential Benson-Calvin cycle enzymes, thereby inhibiting CO_2 assimilation (Kaiser, 1976; 1979; Charles and Halliwell, 1980). It is also conceivable that enhanced H_2O_2 concentrations may affect electron transport and reduce the availability of reducing power for the operation of the Benson-Calvin cycle. However, the absence of H_2O_2 accumulation after the addition of aminotriazole to pea seedlings suggests that there is no direct relationship between this metabolite and the inhibition of photosynthetic CO_2 assimilation. The inhibition of the CO_2 assimilation rate also appears to be independent of stomatal effects, although the stomatal conductance did eventually decrease in response to an increasing inhibition of CO_2 assimilation, probably to reduce the water cost of assimilating CO_2 (Sharkey, 1984).

Several chlorosis-inducing herbicides, such as aminotriazole, have been shown to initiate photooxidative damage in the chloroplast by directly inhibiting carotenoid biosynthesis, thereby preventing the quenching of excited triplet chlorophyll molecules (Halliwell, 1987). The concomitant photodestruction of chlorophyll may affect the photochemistry and reduce the availability of reducing power for the assimilation of CO_2 . These effects may therefore be manifested as an inhibition of the CO_2 assimilating mechanism of aminotriazole-treated pea seedlings in this investigation. However, chlorophyll fluorescence measurements showed no evidence of chlorophyll photodestruction or uncoupling of electron transport by aminotriazole (Table 1; Figure 12 A, B). It therefore appears that aminotriazole, in the short-term, does not directly induce photobleaching of chlorophyll. This is in agreement with the observations of Feierabend and Schubert (1978) and Feierabend and Winkelhüsener (1980) who proposed that aminotriazole is a group I herbicide which inhibits catalase specifically and exhibits only weak chlorophyll photodestruction. It is probable that in the long-term H_2O_2 may accumulate to levels that the plant is unable to detoxify completely and this may induce photooxidative events such as chlorophyll photobleaching.

The present observations suggest that the inhibition of catalase by aminotriazole significantly alters the photosynthetic biochemistry of pea seedlings. The lack of H_2O_2 accumulation suggests that the activity of the alternative H_2O_2 -scavenging mechanisms may have increased to enable the compensatory removal of peroxisomal H_2O_2 . Aminotriazole-treated peas also exhibited decreased levels of CO_2 assimilation which does not appear to be a result of a direct inhibition of Benson-Calvin cycle enzymes by H_2O_2 or of carotenoid biosynthesis by aminotriazole. The inhibition of photorespiratory enzymes, such as catalase, therefore appears to have far-reaching consequences on the integrated operation of the biochemical pathways and represents a far more complex situation than previously envisaged.

2.0 THE MECHANISM OF THE INHIBITION OF THE CO_2 ASSIMILATION RATE.

The reduced CO_2 assimilation rate exhibited by aminotriazole-treated pea seedlings appears to be a combined effect of reduced RuBP regeneration rates and decreased efficiency of the carboxylation reaction of Rubisco (Figure 13). Factors which may affect the RuBP regeneration rates and carboxylation efficiencies of Rubisco will be discussed below.

2.1 RuBP regeneration rate

The most obvious explanation for the decreased RuBP-regeneration rates is the direct inhibition of Benson-Calvin cycle enzymes, fructose and sedoheptulose biphosphatases, by unmetabolized H_2O_2 (Kaiser, 1976; 1979). However, the lack of an increase in H_2O_2 following the addition of aminotriazole suggests that H_2O_2 is not directly responsible for the reduction in CO_2 assimilation.

RuBP-regeneration rates are reduced by a limiting supply of reducing power (Sharkey, 1985), or when the return of carbon lost to the photorespiratory pathway is deterred. Increased activity of the H_2O_2 -scavenging systems to prevent the accumulation of H_2O_2 to toxic levels, after catalase inhibition, may generate these effects. Since the ascorbate-glutathione H_2O_2 -scavenging mechanism utilizes reducing power for the regeneration of reduced ascorbate from monodehydroascorbate and of reduced glutathione (Robinson and Gibbs, 1982; Asada and Takahashi, 1987), it is conceivable that a large increase in the

activity of the ascorbate-glutathione cycle may result in competition with the Benson-Calvin cycle for available reducing power. The operation of the ascorbate-glutathione cycle may take precedence over the Benson-Calvin cycle to prevent the accumulation of H_2O_2 to toxic levels, which may result in decreased turnover of the Benson-Calvin cycle and decreased RuBP regeneration rates. H_2O_2 may be removed by reaction with glyoxylate to form formate and CO_2 (Grodzinski and Butt, 1976). The incorporation of the formate produced into products of carbon metabolism, other than serine, (Cossins *et al.*, 1988) or the decarboxylation of formate (Leek, Halliwell and Butt, 1972; Grodzinski, 1979) would prevent the return of photorespiratory carbon to the Benson-Calvin cycle, thereby depleting its intermediates.

It has been postulated that the most probable mechanism of compensatory removal of H_2O_2 , usually metabolised by catalase, is via the chloroplastic ascorbate-glutathione cycle (Macrae and Ferguson, 1985; Ferguson and Dunning, 1986). The activities of the enzymes and the pool sizes of the metabolites of this system have been shown to increase under conditions of enhanced H_2O_2 production. Increased ascorbate peroxidase and glutathione reductase activities are associated with enhanced H_2O_2 concentrations produced at high light intensities (Gillham and Dodge, 1987) and at high O_2 concentrations (Foster and Hess, 1982). Enhanced levels of ascorbate and a two- to three-fold increase in total glutathione have been observed in catalase-deficient barley mutants (Smith *et al.*, 1984; 1985), aminotriazole-treated barley seedlings (Smith, 1985) and high light-treated tissue deficient in catalase (Volk and Feierabend, 1989). However, the activities of ascorbate peroxidase and glutathione reductase, and the total glutathione pool size were unaffected by aminotriazole in these investigations (Figure 20, 21, 22, respectively). The lack of an increase in the activity of the ascorbate-glutathione cycle suggests that the turnover rate of the cycle is sufficient to prevent the accumulation of H_2O_2 , in the short-term, following the inhibition of catalase. It is also possible that the peroxisomal H_2O_2 , usually catalytically removed by catalase, is metabolised by a system other than the ascorbate-glutathione cycle. The exposure of pea seedlings to low CO_2 concentrations ($80 \mu\text{mol mol}^{-1} \text{CO}_2$), which enhance O_2 photoreduction and increase the chloroplastic H_2O_2 concentration (Canvin *et al.*, 1980), stimulated the ascorbate peroxidase activity and increased the total glutathione pool size (Figure 20, 22). It is therefore conceivable that in the long-term the H_2O_2 may accumulate to greater levels which would be accompanied by an increase in the activity of the ascorbate-glutathione cycle.

In the present investigation the formate concentration approximately doubled following the addition of aminotriazole at 350 and 800 $\mu\text{mol mol}^{-1}$ CO_2 and increased to a lesser extent at 80 $\mu\text{mol mol}^{-1}$ CO_2 (Figure 23), suggesting that the peroxisomal H_2O_2 is being compensatorily removed by reaction with glyoxylate to form formate and CO_2 (Grodzinski and Butt, 1976). This supports the reports that inhibition of catalase by aminotriazole is accompanied by an increased reaction of glyoxylate with H_2O_2 (Oliver, 1979) and caused increased labelling of the formate pool (Cossins *et al.*, 1988). The high formate concentration present in the tissue exposed to 80 $\mu\text{mol mol}^{-1}$ CO_2 reported here, suggests that the rate of formate production is dependent on the rate of carbon flow through the photorespiratory cycle. The decreased formate concentration after 300 minutes at all CO_2 concentrations investigated may therefore be a result of decreased carbon flow through the photosynthetic and photorespiratory pathways or increased metabolism of formate.

The alleviation of the inhibition of the CO_2 assimilation rate by PGA, during the first 100 minutes following the addition of aminotriazole, was coincident with the increased production of formate (Figure 14). The formate produced by the reaction of H_2O_2 and glyoxylate may be metabolised to various products other than serine, which do not re-enter the photorespiratory cycle via the methyltetrahydrofolate pool (Cossins, 1980; Shingles, Woodrow and Grodzinski, 1984), and thus the carbon returned to the Benson-Calvin cycle may be reduced, thereby reducing the RuBP regeneration potential and CO_2 assimilation rate. Formate may also be decarboxylated to CO_2 by an NAD^+ -dependent formate dehydrogenase (Leek, Halliwell and Butt, 1972) or by the peroxidative action of catalase (Grodzinski, 1979). Since the peroxidative action of catalase is inhibited by aminotriazole and the activity of formate dehydrogenase is usually much lower than that of formyl tetrahydrofolate synthetase (Grodzinski, 1979), it is unlikely that the decarboxylation of formate was significant in this investigation. Formate has been reported to inhibit linear electron transport (Snel and van Rensen, 1984) which would reduce the CO_2 assimilation rate. However, chlorophyll fluorescence measurements gave no indication of a reduction of efficiency of PSII.

An inhibitor of the glycine-serine conversion, INH, did not enhance the inhibition of the CO_2 assimilation exhibited by aminotriazole-treated seedlings (Figure 24) which suggests that the mechanism of inhibition of CO_2 uptake by INH and aminotriazole are similar. INH has been reported to divert the flow of photorespiratory carbon to formate (Oliver, 1979) with a concomitant inhibition of CO_2 assimilation as a result of a depletion of

Benson-Calvin cycle intermediates (Somerville and Ogren, 1977).

Pea seedlings therefore appeared, in the short-term, to be able to prevent the accumulation of H_2O_2 to toxic levels following the inhibition of catalase. Peroxisomal H_2O_2 may be removed by reaction with glyoxylate to form formate, and not by the ascorbate-glutathione cycle as previously suggested (Macrae and Ferguson, 1985; Ferguson and Dunning, 1986), thereby reducing the carbon flow through the Tolbert photorespiratory pathway. It has been demonstrated by the inhibition of photorespiratory enzymes (Servaites and Ogren, 1977), and in mutants deficient in certain photorespiratory enzymes (Somerville and Ogren, 1981), that the diversion of photorespiratory carbon flow away from the Tolbert pathway reduces the return of carbon to the Benson-Calvin cycle. Furthermore, the formate produced during the compensatory removal of H_2O_2 following catalase inhibition may have been metabolised to products of one-carbon metabolism other than photorespiratory intermediates, thus reducing the return of carbon to the Benson-Calvin cycle and the RuBP-regeneration and CO_2 assimilation rate. Thus the compensatory removal of H_2O_2 , following the inhibition of catalase, initially affects the CO_2 assimilation rate by reducing the rate of RuBP regeneration. Since the addition of exogenous PGA, which would maintain RuBP regeneration rates, did not prevent the inhibition of the CO_2 assimilation rate after 100 minutes with aminotriazole the decreased efficiency of the carboxylation reaction appears to be the limiting factor in the longer term.

2.2 Carboxylation efficiency

Despite an almost 10-fold increase in the estimated K_c 300 minutes after the addition of aminotriazole, the calculated V_{maxc} remained constant (Table 2). The decreased efficiency of the carboxylation reaction of Rubisco therefore appears to be predominantly due to an increase in the Michaelis-Menton constant for CO_2 and not a direct inhibition of the enzyme by H_2O_2 . The interpretation of the kinetic analysis requires consideration of the activation-deactivation mechanism of Rubisco.

Rubisco has been shown to exist in two distinct kinetic forms, an inactive high K_c form (200 μM) and an active low K_c form (20 μM) (Andrews, Badger and Lorimer, 1975). It has been proposed that a soluble chloroplast enzyme, Rubisco activase catalyses the first step in the activation-deactivation transition (Portis, Salvucci and Ogren, 1986). However, recently it has been demonstrated that the activation step does not necessarily require an

activase, but involves the binding of CO_2 to the active site to form a carbomyl compound (Butz and Sharkey, 1989). Various activated enzyme-activator intermediates are formed during the binding of Mg^{2+} ions and then RuBP (Figure 25) to the activated form of the enzyme (the carbomyl compound), followed by the binding of either CO_2 or O_2 at the catalytic site of the fully activated enzyme to produce phosphoglycerate and phosphoglycolate, respectively (Perchorowitz and Jensen, 1983). Various positive and negative effectors can bind to the different intermediates. Negative effectors are metabolites which preferentially bind to the deactivated enzyme and positive effectors bind and stabilize the activated enzyme-activator complexes, but halt further activation and catalysis.

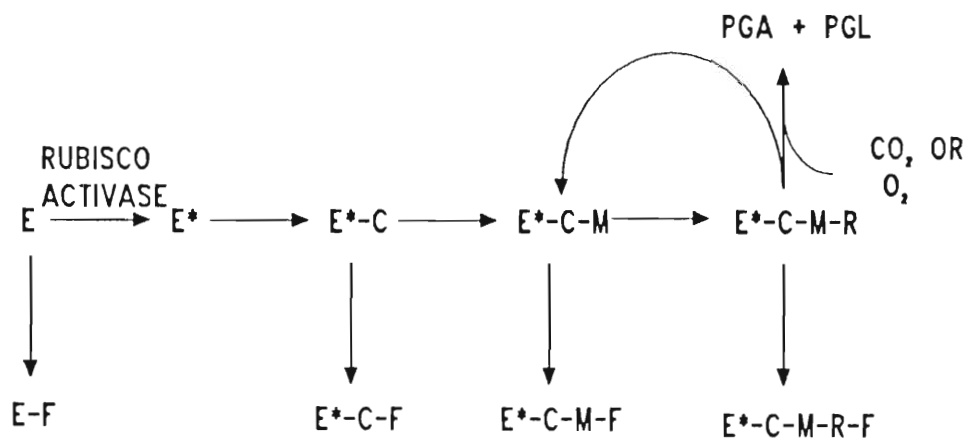


Figure 25. The activation-deactivation mechanism of Rubisco.

At RuBP concentrations above the binding site concentration on the carboxylase enzyme the active enzyme predominantly exists as the $\text{E}^*-\text{C}-\text{M}-\text{R}$ form. However, a drop in the RuBP concentration below the binding site concentration facilitates the binding of various positive effectors at the active site of the enzyme (Perchorowitz and Jensen, 1983). The transition state analogue, carboxypentitol bisphosphate, phosphogluconate (Pierce, Tolbert and Barker, 1980), fructose and sedoheptulose bisphosphate and PGA (Foyer, Furbank and Walker, 1987) exhibit competitive inhibition with respect to RuBP. Since most of these intermediates have low carboxylase binding affinities compared to RuBP, this mechanism of competitive inhibition of the carboxylation reaction requires either high concentrations of these intermediates or very low concentrations of RuBP. The reduction in the level of RuBP regeneration, following the addition of aminotriazole in this investigation, suggests that the level of RuBP available to occupy the carboxylase binding

sites may decrease. However, the decreased RuBP regeneration rates would probably be accompanied by a reduction in the pool sizes of most of the above-mentioned positive effectors to a level where they are unlikely to prevent the formation of the E^* -C-M-R complex. Physiological concentrations of PGA are capable of competitively inhibiting the binding of RuBP to the carboxylase enzyme and have been demonstrated to illicit a response similar to that exhibited in the present investigations, at low RuBP levels. PGA was reported to severely decrease the efficiency of the carboxylation reaction of Rubisco by inducing a 10-fold increase in the K_c of the carboxylase enzyme, while the V_{maxC} remained relatively constant (Foyer, Furbank and Walker, 1987). This was accompanied by an 85 per cent inhibition of the $^{14}CO_2$ assimilation rate. Since PGA (5 mM) initially alleviated the inhibition of the CO_2 assimilation rate of aminotriazole-treated pea seedlings (Figure 14), it is unlikely that PGA competitively inhibits the binding of RuBP to the carboxylase enzyme in these investigations. Despite the possible decrease in the RuBP pool size of aminotriazole-treated seedlings, the decreased efficiency of the carboxylation reaction does not appear to be an inhibition of the binding of RuBP to the carboxylase enzyme.

Badger *et. al* (1980) observed that H_2O_2 competitively inhibits the carboxylation and oxygenation reaction by reacting with the E^* -C-M-R intermediate. It has already been established that the H_2O_2 concentration did not reach toxic levels during this investigation. However, formate, which is a linear molecule and structurally similar to CO_2 and O_2 , was found to increase initially. The possibility therefore exists that formate exhibits competitive inhibition with respect to CO_2 and O_2 which would decrease the efficiency of the carboxylation reaction of Rubisco. Inhibition of photosynthesis and decreased efficiency of the carboxylation reaction of Rubisco have been demonstrated in several mutants of *Arabidopsis thaliana* deficient in glycine decarboxylase, serine transhydroxymethylase, serine-glyoxylate aminotransferase, but not glycolate-P phosphatase (Chastain and Ogren, (1985). It was proposed that a metabolite associated with the photorespiratory pathway, probably glyoxylate or a product of glyoxylate metabolism (such as formate), is a positive effector of Rubisco and accumulated to a sufficiently high concentration in these mutants to exhibit competitive inhibition with respect to metabolites involved in the further activation or catalysis of Rubisco. The binding of formate to the E^* -C-M-R complex would be consistent with the kinetic data obtained as it would result in an increase in both K_c and K_o . Although formate binding would reduce catalysis, it would not deactivate the enzyme and the V_{maxC} may therefore remain unaffected. Furthermore, an inhibition of the oxygenation reaction of Rubisco would reduce the rate of carbon flow through the

photorespiratory pathway, which might explain the reduction in the level of formate 300 minutes after the addition of aminotriazole.

The inhibition of the CO₂ assimilation rate following the addition of aminotriazole appears to be divided into two stages. The compensatory removal of H₂O₂ by reaction with glyoxylate to produce formate resulted in a decrease of carbon returned to the Benson-Calvin cycle. This depleted the Benson-Calvin cycle intermediates, thereby reducing the RuBP regeneration rates and inhibiting the CO₂ assimilation rate. Formate appeared to accumulate to a concentration which could competitively inhibit the oxygenation and carboxylation reactions of Rubisco decreasing the efficiency of the carboxylation reaction of Rubisco. Although the pea seedlings were able to metabolize the H₂O₂ in the absence of catalase, the inhibition of this photorespiratory enzyme appears to upset the equilibrium which exists between the integrated photosynthetic and photorespiratory pathways.

3.0 THE EFFECT OF PHOTORESPIRATORY ACTIVITY ON THE PHOTOSYNTHESIS OF AMINOTRIAZOLE-TREATED SEEDLINGS

The degree of inhibition of the photosynthetic CO₂ uptake rate exhibited by aminotriazole-treated pea seedlings was proportional to the rate of carbon flow through the photorespiratory pathway. Conditions which enhance photorespiratory activity; high light, low CO₂ and high O₂ concentrations (Canvin *et al.*, 1980) were associated with a proportionally greater inhibition of the CO₂ assimilation rate following the addition of aminotriazole (Figure 16, 17, 19, respectively). A similar dependence of the inhibition of photosynthetic CO₂ uptake on the activity of the photorespiratory cycle was observed with mutants of *Arabidopsis thaliana* deficient in certain photorespiratory enzymes (Somerville and Ogren, 1981), following the inhibition of photorespiratory enzymes of isolated soybean leaf cells with INH and BHB (Servaites and Ogren, 1977) and in catalase-deficient barley mutants and aminotriazole-treated barley seedlings (Smith *et al.*, 1984).

Conditions which promote photorespiratory activity, such as low CO₂ concentrations, are associated with increased production of H₂O₂ (Figure 9) and it was originally thought that the inhibition of catalase under these conditions would enhance the rate of accumulation of H₂O₂ to toxic levels which would inhibit photosynthesis. However, H₂O₂ did not accumulate following the addition of aminotriazole at any of the CO₂ concentrations investigated. But at 80 μmol mol⁻¹ CO₂ the concentration of formate 100 minutes after the

addition of aminotriazole was greater than at the higher CO₂ concentrations (Figure 23). These results suggest that the addition of aminotriazole under conditions of enhanced photorespiratory activity would increase the rate of compensatory removal of H₂O₂ to form formate. The accumulation of formate and therefore the accumulation of Benson-Calvin cycle intermediates, and the reduction in the RuBP regeneration rates and the efficiency of the carboxylation reaction of Rubisco would be greater than following the addition of aminotriazole under conditions associated with low photorespiratory activity. This is in agreement with the observations of Sivak *et al.* (1987) who proposed that the exposure of catalase-deficient barley mutants initially resulted in a depletion of Benson-Calvin cycle intermediates: this effect was proportional to the rate of carbon flow through the photorespiratory pathway.

It appears that the photorespiratory cycle functions to return glycolate carbon back to the Benson-Calvin cycle. The inhibition of a photorespiratory enzyme, such as catalase appears to upset glycolate metabolism and there is a resultant inhibition of the photosynthetic CO₂ uptake rate. In the long-term this may increase the level of available reducing power, which would increase the photoreduction of O₂ and therefore the production of H₂O₂ and may eventually lead to photooxidative damage. The Benson-Calvin cycle and photorespiratory cycle appear to be closely associated and interdependent on one another to maintain the efficient functioning of photosynthetic metabolism.

4.0 CONCLUSION

The inhibition of catalase by aminotriazole (2 mM) was not accompanied by an accumulation of H₂O₂. The peroxisomal H₂O₂, normally metabolised by catalase, was probably compensatorily removed by reaction with glyoxylate to form formate and CO₂ and not by the ascorbate-glutathione cycle. The diversion of photorespiratory carbon flow to formate has a two-fold effect on the CO₂ assimilation mechanism. The accumulation of formate prevents the return of photorespiratory carbon to the Benson-Calvin cycle which reduces the RuBP regeneration rate and competitively inhibits the carboxylation and oxygenation reactions of Rubisco by binding to the E^{*}-C-M-R complex (Figure 25). Although the plant is capable of preventing the accumulation of H₂O₂ to toxic levels in the short term the inhibition of a photorespiratory enzyme appears to destroy the equilibrium which exists between the Benson-Calvin and the photorespiratory cycles, particularly under conditions associated with high photorespiratory activity, when more carbon flows through

the photorespiratory cycle and thus more H_2O_2 (or formate) is produced. The reduced operation of the photorespiratory and Benson-Calvin cycles may result in photooxidative damage as a result of an accumulation of reducing power, in the longer term. Alteration of the photorespiratory pathway therefore appears to have far-reaching consequences. The photosynthetic biochemistry of *Pisum sativum* did not appear to be effected by enhanced H_2O_2 levels in the short-term, but the inhibition of normal glycolate metabolism had a direct and severe effect on the photosynthetic CO_2 uptake mechanism.

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