ORGANELLE FUNCTION IN PHOTORESPIRATORY GLYCINE METABOLISM

A thesis submitted to the University of Adelaide

as a requirement for the degree of

DOCTOR OF PHILOSOPHY

by

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January, 1984

CONTENTS

SUMMARY

(1) The state 3 respiratory rates of isolated pea leaf and cauliflower mitochondria were found to be sensitive to changes in the external ATP/ADP ratio. Increases in the ratio led to decreases in the state 3 respiratory rates. The sensitivity of the control, however, was found to be markedly influenced by the total concentration of adenylates (ATP + ADP) present, indicating that respiration by plant mitochondria is regulated, not by the value of the external ATP/ADP ratio, but by the absolute concentration of ADP available for uptake into the mitochondria. For the range of adenylate levels examined (based on published levels in leaf tissue), little effect on respiration was observed unless the value of the ATP/ADP ratio exceeded approximately 20. Decreasing the concentration of both magnesium and phosphate 10-fold had little effect on the respiratory response of mitochondria to changes in the ratio. When these results are compared to measurements of *in vivo* ratios in light-treated leaf tissue it is concluded that if mitochondrial respiration is inhibited in the light, some mechanism other than changes in the ATP/ADP ratios must be responsible.

(2) Glycine oxidation by pea leaf mitochondria under both state 3 and state 4 conditions, as measured with an ammonia electrode, was unaffected by the presence of other respiratory substrates which compete at either

the NAD level (e.g. malate, 2-OG) or respiratory chain level (e.g. succinate, NADH). The measured rate of O_2 uptake under these conditions was lower than would be predicted on the basis of a summation of the individual substrate rates, indicating that the oxidation of these other respiratory substrates had been inhibited. External NADH oxidation was found to be particularly sensitive to competition from not only glycine, but also malate, 2-oxoglutarate and succinate. Re-oxidation of glycine NADH could also be achieved through the reversal of malate dehydrogenase in the presence of OAA, however, this capacity could be demonstrated equally well for all NAD-linked substrate dehydrogenases present in the mitochondria, with no apparent preference for the NADH generated by glycine oxidation. The results demonstrate that a mechanism exists in pea leaf mitochondria for the preferential oxidation of glycine via the respiratory chain. Tissue measurements suggest that the rate of glycine oxidation via the respiratory chain *in vivo* (20-30 µmol CO_2 evolved mg^{-1}Chl h^{-1}) is barely adequate to account for estimated rates of photorespiratory flux (25-35 µmol CO_2 evolved mg^{-1} Chl h^{-1}), however, it appears that the limitation lies at the absolute level of glycine decarboxylase activity and not with the oxidative capacity of the respiratory chain.

(3) The addition of glycine to pea leaf mitochondria oxidizing malate in state 3, led to a marked increase in the rate

of O_2 uptake. Other NAD-linked substrates (citrate, 2-OG) however, had little effect on the rate of O_2 uptake in the presence of malate. Further investigation revealed that citrate and 2-OG were inhibiting malate oxidation directly, most probably through the exchange of malate out of the mitochondria during their uptake. Glycine was also found to stimulate the state 4 rate of O_2 uptake in the presence of malate. Similarly, malate could be shown to stimulate the state 3 and state 4 rates in the presence of glycine. The second-substrate mediated increases in the state 4 rates were not due to increases in the rate of O_2 uptake via the alternate pathway.

The rotenone-insensitive state 3 rates of malate and glycine oxidation by freshly isolated mitochondria could be stimulated by the addition of NAD. However, these NAD-limited rotenone-insensitive rates could also be stimulated by the addition of a second NAD-linked substrate. Experiments with NAD-depleted mitochondria showed access of glycine decarboxylase to the rotenone-insensitive bypass to be much more sensitive to NAD loss than the malate enzymes, malate dehydrogenase and malic enzyme. The results presented strongly contradict previously accepted models of a uniform distribution of enzymes within mitochondria, with access to a common NAD pool, and suggest that glycine decarboxylase and the malate oxidizing enzymes may have differential access, both in terms of size and location, to NAD within pea leaf mitochondria.

(4) Intact isolated chloroplasts from pea leaves carried out light dependent (NH_3, 2-OG) and (glutamine, 2-OG)-dependent O_2 evolution at rates of 3.3. ± 0.7 (n = 7) and 6.0 ± 0.4 (n = 5) μmol mg^{-1} Chl h^{-1} respectively. Malate stimulated the rate of (NH_3, 2-OG)-dependent O_2 evolution 2.1 ± 0.5 (n = 7) fold in the absence of glutamine, and 3.3 ± 0.4 (n = 11) fold in the presence of glutamine. Malate also stimulated (glutamine, 2-OG)-dependent O_2 evolution in the presence of a high concentration of glutamine. The affinity ($K\frac{1}{2}$) of (NH_3, glutamine, 2-OG)-dependent O_2 evolution for 2-OG was estimated at 200-250 μM in the absence of malate and 50-80 μM when malate (0.5 mM) was present. In contrast to malate and various other dicarboxylates, aspartate, glutarate and glutamate did not stimulate (NH_3, glutamine, 2-OG)-dependent O_2 evolution by isolated pea chloroplasts. Similar results to those above were also obtained with spinach chloroplasts except that, in contrast to pea chloroplasts, glutamine had no effect on the degree of stimulation, by malate, of (NH_3, 2-OG)-dependent O_2 evolution. Using both *in vitro* assays and reconstituted chloroplast systems, malate was shown to have no direct effect on the activities of either glutamine synthetase or glutamate synthase. It is proposed that malate stimulates O_2 evolution by increasing the rate of 2-OG uptake into the chloroplast.

The concentration of malate required for maximal stimulation of O_2 evolution was dependent on the concentration of 2-OG present. However, the small extent of the competition between malate and 2-OG for uptake, was not consistent with that predicted by the current 'single carrier' model proposed for the uptake of dicarboxylates into chloroplasts, and may indicate the existence of two separate dicarboxylate carriers in the chloroplast envelope.

(5) A reconstituted chloroplast system was developed for the measurement of *in vitro* glutamate synthase (GOGAT) activity. GOGAT-dependent O_2 evolution in the reconstituted system required added ferredoxin (Km 0.9 μM and was inhibited by DCMU and azaserine. No O_2 evolution was observed in the absence of either glutamine or 2-oxoglutarate. The system demonstrated a broad pH optimum with a peak at pH 7.6 and also displayed an absolute requirement for the presence of divalent cations, in particular Mg^{2+}. This requirement could not be attributed to the photosynthetic electron transport chain as shown by the high rates of NADP-dependent O_2 evolution in the absence of these cations. Maximal rates of activity were only observed following pre-incubation with 2-OG, with the time required for full activation being dependent on the concentration of 2-OG added.

(6) Up to 80% of total cellular hexokinase activity in pea leaves was found to be associated with particulate fractions. Fractionation on sucrose density gradients showed this particulate activity to be associated exclusively with mitochondria. In the presence of glucose and ATP, the bound mitochondrial hexokinase could support rates of O_2 uptake of up to 30% of normal ADP-stimulated rates. This stimulation of O_2 uptake by hexokinase was completely sensitive to oligomycin, indicating that it resulted from an increase in the supply of ADP for mitochondrial oxidative phosphorylation. Spectrophotometric measurements of the mitochondrial hexokinase activity showed that ADP could support rapid rates of activity provided oxidizable substrates were also present to support the conversion of ADP to ATP in oxidative phosphorylation. Mitochondrial hexokinase demonstrated a slightly higher affinity for, and V_{max} with, ATP produced via oidative phosphorylation in comparison to added or external ATP. Carboxyatractyloside, an inhibitor of mitochondrial adenine nucleotide uptake, inhibited this ADP-supported activity, but had no effect on hexokinase activity in the presence of added ATP, demonstrating that the hexokinase enzyme was located external to the inner mitochondrial membrane. Glucose (Km 53 μM) was the preferred substrate of pea leaf mitochondrial hexokinase compared to fructose (Km 5.1 mM). Hexokinase was not solubilized in the presence of glucose-6-phosphate.

DECLARATION

The investigations described in this thesis were performed in the Botany Department, University of Adelaide from March, 1981 to November, 1983. The following papers were written during the period of study:

(1) Role of the external Adenosine Triphosphate/Adenosine Diphosphate Ratio in the Control of Plant Mitochondrial Respiration, by I.B. Dry and J.T. Wiskich, Arch. Biochem. Biophys. (1982) **217**: 72-79.

(2) Characterization of Dicarboxylate Stimulation of Ammonia, Glutamine and 2-Oxoglutarate-Dependent O_2 Evolution in Isolated Pea Chloroplasts, by I.B. Dry and J.T. Wiskich, Plant Physiol. (1983) **72**: 291-296.

(3) Measurement of *in vitro* glutamate synthase activity using an oxygen-evolving reconstituted chloroplast system. Evidence for enzyme activation by Mg^{2+} and 2-oxoglutarate, by I.B. Dry and J.T. Wiskich, FEBS Lett. (1983) **151**: 31-35.

(4) The mitochondrial localization of hexokinase in pea leaves, by I.B. Dry, D. Nash and J.T. Wiskich, Planta (1983) **158**: 152-156.

(5) Preferential oxidation of glycine by the respiratory chain of pea leaf mitochondria, by I.B. Dry, D.A. Day and J.T. Wiskich, FEBS Lett. (1982) **158**: 154-158.

A sixth paper entitled 'Contrasting characteristics of glycine and malate oxidation by pea leaf mitochondria. Evidence of differential access to NAD and respiratory chains' is in preparation.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university. To the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text. I consent to this thesis being made available for photocopying and loan.

IAN B. DRY

ACKNOWLEDGEMENTS

I would sincerely like to thank Dr Joseph T. Wiskich whose unique form of supervision has enabled me to derive the maximum benefit from the past 3 years.

I would also like to thank Richard Norrish and Brian Rowland for their help in building and maintaining critical pieces of equipment; Cecelia Marcelline for typing this thesis; and Drs S.P. Robinson, D. Nash and J.H. Bryce for helpful discussions.

This work would not have been possible had it not been for the continued support of my parents and my talented, loving wife, Helen.

I am grateful to the University of Adelaide for the financial support of the George Fraser Scholarship.

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ABBREVIATIONS

ADP	adenosine diphosphate
AdN	adenine nucleotide
ATP	adenosine triphosphate
Asp	aspartate
Aza	azaserine
BSA	bovine serum albumen
CAM	crassulacean acid metabolism
C_3	photosynthetic carbon reduction cycle
C_4	photosynthetic dicarboxylic acid cycle
Chl	chlorophyll
DCMU	3-(3,4-dichlorophenyl)-1, 1-dimethylurea
DTT	dithiothretol
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol (β aminoethylether) N, N^1 tetraacetic acid
FAD	flavin adenine dinucleotide
FCCP	p-trifluromethoxy (carbonylcyanide) phenyl hydrazone
FH_4	tetrahydrofolic acid
G6P	glucose-6-phosphate
GGAT	glutamate-glyoxylate aminotransferase
Gln	glutamine
Glu	glutamate
GOGAT	glutamate synthase
GS	glutamine synthetase
Hepes	N-2-hydroxyethylpiperazine-N^1-2-ethanesulphonic acid
Mal	malate
MDH	malate dehydrogenase

ME	malic enzyme
Mes	2-(N-morpholino)-ethanesulphonic acid
Mp	mitochondria (purified)
MSO	methionine sulphoximine
Mw	mitochondria (washed)
NAD(H)	nicotinamide adenine dinucleotide (reduced)
NADP(H)	nicotinamide adenine dinucleotide phosphate (reduced)
OAA	oxaloacetate
2-OG	2-oxoglutarate
OH-PYR	hydroxypyruvate
PCO	photosynthetic carbon oxidation
PCR	photosynthetic carbon reduction
pmf	proton-motive force
PVP	polyvinyl pyrrolidone
RCR	respiratory control ratio
RuBP	ribulose-1,5-bisphosphate
SHAM	salicylic hydroxamic acid
TCA	tricarboxylic acid
Tes	N-tris (hydroxymethyl)-methyl-2-aminoethanesulphonic acid
TPP	thiamine pyrophosphate
Tris	Tris (hydroxymethyl) aminomethane

CHAPTER I

GENERAL INTRODUCTION

1.1. *Photorespiration: definition and significance*

Photorespiration in its simplest form refers to the light dependent evolution of CO_2 and uptake of O_2 in photosynthetic cells, which results from the operation of a metabolic pathway separate from mitochondrial or 'dark' respiration. This pathway is, in fact, a cycle of enzymic reactions involving chloroplasts, peroxisomes and mitochondria. The photorespiratory cycle is obligatorily linked to the photosynthetic carbon reduction cycle (Lorimer and Andrews, 1981) through the common initial substrate, ribulose-1,5-bisphosphate (RuBP) as illustrated in Fig. 1.1. RuBP carboxylase can react with CO_2 in the normal photosynthetic process to form 2 molecules of 3-phosphoglycerate or alternatively with O_2, to form 1 molecule each of 3-phosphoglycerate and the photorespiratory substrate, phosphoglycolate. The remaining reactions of the photorespiratory cycle then act to recycle the carbon trapped in phosphoglycolate, back into the PCR cycle.

Photorespiration is of greatest significance in C_3 plants and may attain rates of 15-50% of net photosynthesis, depending on the species and the method of measurement (Zelitch, 1975, 1979; Canvin 1979; Somerville and Somerville, 1983). It is likely that C_4 plants also photorespire, but at a much reduced rate due to the special anatomical features of C_4 leaves (Edwards and Huber, 1981).

At present there is much conjecture as to the role of photorespiration in plants (Lorimer and Andrews, 1981). Osmond and Bjorkman (1972) proposed that photorespiration may provide a mechanism for the orderly dissipation of excess photochemical energy absorbed by the light-harvesting apparatus, under conditions of limiting CO_2 e.g. following stomatal closure during water stress. Alternatively, it has been suggested that photorespiration is an unavoidable consequence of the active site of RuBP carboxylase which will bind both CO_2 and O_2 (Lorimer and Andrews, 1973).

Irrespective of whether the oxygenase reaction of RuBP carboxylase is essential or unavoidable, the photorespiratory pathway has important ramifications, not only for photosynthetic metabolism, but also for respiration and nitrogen assimilation. During photorespiratory glycine metabolism, NADH and NH_3 may be produced at rates of up to 3 (Ludwig and Canvin, 1971) and 10 fold (Miflin and Lea, 1980; Lorimer and Andrews, 1981) respectively that normally arising from 'dark' respiration or primary nitrogen assimilation. It is therefore of considerable importance to understand how the subsequent metabolism of these two photorespiratory products is integrated with the primary metabolic processes of the cell.

1.2. *The biochemistry of the photorespiratory cycle*

Numerous reviews have appeared on this topic (Zelitch, 1975; Schnarrenberger and Fock, 1976; Chollet, 1977; Tolbert 1979, 1980; Keys, 1980; Lorimer and Andrews, 1981; Lorimer, 1981; Osmond, 1981; Wallsgrove *et al*. 1983). What follows is an overview of the

current information on the biochemical basis of the cycle. Separate sections will be devoted to those aspects of the cycle which have particular relevance to my studies. A general outline of the photorespiratory carbon cycle is given in Fig. 1.2. It can be seen that of the 4 carbons which leave the chloroplast (as two glycolate molecules), three are returned in the form of a glycerate molecule and one is lost as CO_2 during glycine decarboxylation in the mitochondria.

1.2.1. *The initial oxygenase reaction*

The initial step of the photorespiratory pathway involves the addition of O_2 to RuBP by the enzyme RuBP carboxylase. This causes a cleavage between carbons 2 and 3 of RuBP (Pierce *et al.* 1980) producing a molecule of phosphoglycolate and a molecule of 3-phosphoglycerate (Bowes *et al.* 1971; Lorimer *et al.* 1973).

Both CO_2 and O_2 bind at the same active site on the RuBP carboxylase enzyme (Lorimer and Andrews, 1981) and therefore, the relative rates of carboxylation or oxygenation will be regulated by the concentrations of each species around the active site. The enzyme exhibits a much higher affinity for CO_2 i.e. Km of 10-20 μM (Jensen and Bahr, 1977) than for O_2 i.e. Km of 400-500 μM (Tolbert, 1980). Even so, the high O_2/CO_2 ratio which is present at the active site (Ku and Edwards, 1977a) results in phosphoglycolate production. The competition between CO_2 and O_2 is further exacerbated at higher temperatures due to a larger decrease in CO_2 solubility relative to O_2 (Ku and Edwards, 1977a) and to alterations in the kinetic properties of RuBP carboxylase favouring oxygenation more so than carboxy-

lation (Badger and Andrews, 1974; Laing *et al*. 1974). Consequently, photorespiratory activity in C_3 plants is increased at higher temperatures (Ku and Edwards, 1977b).

This is not the case for C_4 species however, which grow most efficiently at these elevated temperatures. Photorespiration is significantly reduced in C_4 plants because the CO_2 concentration at the active site of RuBP carboxylase, is 5-6 times that achieved by equilibrium with the atmosphere (Rathnam, 1978). Carbon dioxide is fixed initially in the mesophyll cells by phosphoenolpyruvate carboxylase and then transported, in the form of a dicarboxylic acid, to the bundle sheath cells where it is released and subsequently refixed by RuBP carboxylase (Edwards and Huber, 1981). This CO_2-concentrating mechanism, combined with an apparently high diffusive resistence of bundle sheath cell walls to O_2 (Edwards and Huber, 1981) maintains a high CO_2/O_2 ratio at the active site and minimizes the oxygenase reaction.

The next step in the photorespiratory cycle involves the conversion of phosphoglycolate to glycolate, within the chloroplast (Douce *et al*. 1973), by the enzyme phosphoglycolate phosphatase. The role of this enzyme in photorespiratory metabolism is well demonstrated by the inability of *Arabidopsis* mutants, deficient in phosphoglycolate phosphatase, to grow in normal air. These mutants could only survive under CO_2-enriched conditions (Somerville and Ogren, 1979). This enzyme demonstrates similar regulatory characteristics to some of the PCR cycle enzymes (Robinson and Walker, 1981), in that it requires Mg^{2+} for activation (Christeller and Tolbert,

1978). Light-dark regulation may be involved as the Mg^{2+} concentration of the stroma changes during light-dark transitions (Barber, 1976).

1.2.2. *Glycolate metabolism*

The movement of glycolate from the chloroplast to the peroxisome is still the subject of some debate. Takabe and Akazawa (1981) have suggested that glycolate movement across the chloroplast envelope is via diffusion, while the data of Howitz and McCarty (1982, 1983) indicate the existence, within the outer membrane of a specific glycolate transporter which is competitively inhibited by glycerate and glyoxylate. If a specific glycolate carrier is present in the chloroplast membrane, it is unlikely to catalyse a glycolate/glycerate exchange *in vivo* based on (a) the recent findings of Robinson (1982a,b) of the existence of a specific glycerate transporter and (b) because the stoichiometry of the exchange requires that 2 molecules of glycolate be exchanged for every 1 glycerate molecule taken up by the chloroplast.

Once in the peroxisome, glycolate is oxidised to glyoxylate by glycolate oxidase. This reaction leads to the uptake of O_2 and the production of H_2O_2 which is then further broken down to H_2O and O_2 by the action of peroxisomal catalase. Glycolate oxidase, purified from pumpkin cotyledons, demonstrated a Km (glycolate) of 0.33 mM and a Km (O_2) of 76 µM (Nishimura et al. 1983). Inhibition of glycolate oxidase in leaf tissue with 2-hydroxy-3-

butynoate led to an accumulation of glycolate and an inhibition of photosynthesis (Kumarsinghe *et al.* 1977; Servaites and Ogren, 1977; Jenkins *et al.* 1982). In contrast, Oliver and Zelitch (1977) reported that the addition of potassium glyoxylate to leaf tissues led to a marked stimulation of photosynthesis through an inhibition of glycolate synthesis and proposed this as a method for the metabolic regulation of photorespiration. It has subsequently been shown however, that this stimulation could be duplicated with KCl and that the effect of glyoxylate was not related to photorespiratory metabolism but was due to a salt mediated increase in the availability of CO_2 through decreased stomatal resistance (Madore and Grodzinski, 1983).

Glyoxylate has also been reported to inhibit CO_2 fixation by isolated chloroplasts (Oliver and Zelitch, 1977). This inhibition has been attributed to a number of factors including (a) an inhibition of ribulose-1,5-P_2 carboxylase activity (Cook and Tolbert, 1982) (b) NADPH depletion through the operation of chloroplastic NADP-glyoxylate reductase (Mulligen *et al.* 1983; Lawyer *et al.* 1983) or (c) glyoxylate acting as a weak acid and causing acidification of the stromal compartment (Mulligen *et al.*, 1983; Lawyer *et al.* 1983). It is questionable however, whether such a regulatory system would operate *in vivo* as glyoxylate is not found to accumulate appreciably during photosynthesis (Lawyer and Zelitch, 1979; Somerville and Ogren 1981; Peterson 1982a).

Various pathways have been proposed for the further metabolism of glyoxylate in leaf tissue. Glyoxylate can be oxidized to formate and CO_2 in a non-enzymic reaction with H_2O_2, in either the

peroxisome or the chloroplast (see Fig. 1.2; Zelitch, 1972; Grodzinski and Butt, 1976; Grodzinski 1978, 1979; Oliver, 1979) and it has been suggested that this form of CO_2 release may account for a significant proportion of the CO_2 evolved in the photorespiratory process (Oliver, 1979). Recent work with *Arabidopsis* mutants (Somerville and Ogren, 1981), soybean mesophyll cells (Oliver, 1981) and isolated spinach and pea protoplasts (Chang and Huang, 1981; Walton and Woolhouse, 1983) however, suggest that this source of CO_2 will be of little significance *in vivo* (i.e. < 2.5% of the total photorespiratory CO_2 evolution) unless there is a shortage of amino donors required for the major pathway of glyoxylate metabolism in peroxisomes.

This major pathway, as shown in Fig. 1.2, involves the amination of glyoxylate to glycine and involves the operation of 2 separate aminotransferase enzymes: glutamate-glyoxylate aminotransferase (GGAT) and serine - glyoxylate aminotransferase (SGAT). The involvement of these enzymes is confirmed by the inhibitory effects of aminotransferase inhibitors on photorespiratory metabolism (Jenkins *et al.* 1983). Each aminotransferase is highly specific for glyoxylate and the respective amino donor, and the reactions are physiologically irreversible in the direction of glycine formation (Nakamura and Tolbert, 1983). The peroxisomal activity of both SGAT and GGAT are also found to be light-inducible (Noguchi and Fujiwara, 1982).

It can be seen in Fig. 1.2 that one amino group is effectively recycled within the cycle itself from serine → glyoxylate → glycine → serine and back to glyoxylate again, while the other amino group is lost as NH_3 during glycine decarboxylation in the mitochondria.

This NH_3 must be re-assimilated to form glutamate as it is required for the transamination of the second of the glyoxylate molecules. It is necessary to aminate two glyoxylate molecules at this step as two glycine molecules are required for the formation of one serine molecule.

It is clear from the evidence of Walton and Butt (1981) that serine is the preferred amino donor to glyoxylate in the aminotransferase reaction. They showed that (a) SGAT activity in spinach beet peroxisomes was greater than GGAT activity, (b) at low glyoxylate concentrations, SGAT was saturated at much lower serine concentrations than the concentration of glutamate required to saturate GGAT and (c) GGAT was competitively inhibited by serine, but glutamate had little effect on SGAT activity. Such a system would ensure that carbon flow through the photorespiratory cycle was not held up at the serine step, through an over utilization of glutamate as the amino donor in preference to serine. In this,way the role of glutamate would be as a supplementary amino donor only (Walton and Butt, 1981).

Glutamate-glyoxylate aminotransferase is also found to catalyse alanine-glyoxylate aminotransferase activity (Rehfeld and Tolbert, 1972; Noguchi and Hayashi, 1981; Nakamura and Tolbert, 1983). Indeed, rates of glyoxylate amination by this aminotransferase enzyme may be higher in the presence of alanine than in the presence of glutamate (Noguchi and Fujiwara, 1982; Walton, 1983). Alanine is present in large quantities in leaf tissue (Chapman and Leech, 1979) and,as such,may represent an alternative donor to glutamate in this step (Betsche, 1983a; Nakamura and Tolbert, 1983).

1.2.3 *Glycine decarboxylation*

Glycine is oxidatively decarboxylated to serine in the mitochondria in a two enzyme step (shown below) involving glycine decarboxylase (step 1) and serine hydroxymethyl transferase (step 2) (Bird *et al.* 1972; Woo and Osmond, 1976; Moore *et al.* 1977; Woo, 1979). The stoichiometry of the reaction is such that 2 molecules of glycine are metabolised to form 1 molecule of serine. Mutants of *Arabidopsis*, lacking mitochondrial serine hydroxymethyl transferase, are unable to metabolise photorespiratory glycine (Somerville and Ogren, 1981).

(1) glycine + NAD^+ + FH_4 $\rightleftharpoons$ CO_2 + NH_3 + methylene-FH_4 + NADH + H^+

(2) glycine + methylene-FH_4 + H_2O $\rightleftharpoons$ serine + FH_4

Net: 2 glycine + NAD^+ + H_2O $\rightleftharpoons$ serine + CO_2 + NH_3 + NADH + H^+

The mechanism of glycine uptake into the mitochondrion is most probably via diffusion (Arron and Edwards, 1980a; Day and Wiskich 1980; Proudlove and Moore, 1982; Moore *et al.* 1983) despite some reports that uptake is carrier-mediated (Dench *et. al.* 1978; Walker *et al.* 1982a). Non-photosynthetic tissues show little capacity to oxidize glycine (Gardeström *et al.* 1980). Arron and Edwards (1980b) observed a light-induced development of mitochondrial glycine oxidative capacity in sunflower cotyledons, which paralleled increases in the activity of glycolate oxidase. Mitochondria isolated from bundle sheath cells of some C_4 species oxidized glycine, but at a reduced rate compared to C_3 species, while mitochondria of C_4 mesophyll cells showed little or no glycine oxidative capacity (Woo and Osmond, 1977; Rathnam, 1979).

Glycine decarboxylase was inhibited by isonicotinyl hydrazine, glycine hydroxamate, and aminoacetonitrile (Lawyer and Zelitch,

1979; Usuda *et al.* 1980). It has also been reported that the activities of glycine decarboxylase and serine hydroxymethyl transferase are inhibited by glyoxylate (Peterson, 1982b) however, the concentrations of glyoxylate required for significant inhibitions are unlikely to be reached *in vivo*.

Studies with glycine decarboxylase from both animal tissue and bacteria (Kikuchi, 1973) have shown it to be a multienzyme complex comprised of four catalytic proteins, together with the cofactors pyridoxal phosphate,FAD and tetrahydrofolate. Investigations into the structure of plant glycine decarboxylase have been made difficult by the sensitivity of this enzyme to mitochondrial membrane breakage (Woo and Osmond, 1976; Moore *et al.* 1977). Recently, an active preparation of glycine decarboxylase has been obtained from pea leaf mitochondria (Sarojini and Oliver, 1983). The cofactor requirements of the glycine decarboxylase enzyme indicated that the plant system may involve a reaction sequence or complex of enzymes similiar to that of the animal or bacterial systems. The enzyme exhibited an apparent Km (glycine) of 1.7 mM which compares with the measurements of 1-2 mM made for glycine oxidation with isolated intact mitochondria (Moore *et al.* 1977; Day and Wiskich, 1981).

Measurement of tissue rates of glycine decarboxylase activity, based on mitochondrial activities corrected for breakage during isolation, yield rates of 25-46 µmol CO_2 evolved mg^{-1} Chl h^{-1} (Woo and Osmond, 1976; Day and Wiskich, 1981). These rates are only just sufficient to account for the most widely accepted estimates of photorespiratory CO_2 release i.e. 25-35 µmol CO_2 mg^{-1} Chl h^{-1} (Ludwig and Canvin, 1971; D'Aoust and Canvin, 1973; Mahon *et al.* 1974) and

it has been suggested that these rates may represent overestimations of the true photorespiratory flux through the glycine decarboxylase step (Day and Wiskich, 1981). Conversely, it has also been proposed that these values are, in fact, underestimations of the true photorespiratory flux (Zelitch, 1979) since they do not account for the potential refixation of photorespired CO_2 within the leaf, which may be of the order of 30% or higher of that produced (Fock *et al.* 1979; Somerville and Somerville, 1983).

1.2.4. *The conversion of serine to phosphoglycerate*

Serine is deaminated to hydroxypyruvate by SGAT in the peroxisome as discussed in section 1.2.2. Hydroxypyruvate is then reduced to glycerate in the peroxisome by the action of NAD-linked hydroxypyruvate reductase. This enzyme has been purified from cucumber cotyledons and found to have Km for hydroxypyruvate and NADH of 62 and 5 μM respectively (Titus *et al.* 1983). Hydroxypyruvate reductase also catalysed the NAD-linked reduction of glyoxylate to glycolate, however, the Km (glyoxylate) of 5.7 mM suggests this enzyme would not be utilised for glyoxylate reduction *in vivo* (Titus *et al.* 1983).

The source of NADH for hydroxypyruvate reduction is still unresolved. Peroxisomes contain large quantities of NAD-malate dehydrogenase (Yamazaki and Tolbert, 1970; Schmitt and Edwards, 1983a) which could generate the NADH for this reaction. However, both mitochondria (Woo and Osmond, 1976; Journet *et al.* 1981) and chloroplasts (Tolbert, 1980) have been implicated as potential sources of malate for this reaction. The movement of malate between

the respective organelles would presumably be mediated via a malate/OAA shuttle (Woo and Osmond, 1976) or alternatively via a malate/aspartate shuttle (Journet *et al.* 1981; Tolbert, 1980) as shown in Fig. 1.3. Schmitt and Edwards (1983a) have pointed out that a malate/aspartate shuttle may not be viable, based on the low activities of glutamate-oxaloacetate aminotransferase (GOT) present in peroxisomes (see also Rehfeld and Tolbert, 1972; Huang *et al.* 1976). The feasibility of these shuttles, with particular reference to mitochondrial shuttles, will be considered further in section 1.3.1.

Glycerate is phosphorylated to 3-phosphoglycerate by glycerate kinase. This enzyme is located entirely within the chloroplast (Usuda and Edwards, 1980; Schmitt and Edwards, 1983b) thereby necessitating the transport of glycerate across the chloroplast envelope. Robinson (1982a, 1982b) has recently demonstrated the presence of a light-activated glycerate transporter in the outer membrane of spinach chloroplasts which catalyses the uptake of glycerate at rates of approximately 40 μmol mg^{-1} Chl h^{-1}. Data from a range of C_3 plants (Schmitt and Edwards, 1983b; Kleczkowski and Randall, 1983) indicates that, in contrast to the results of Heber *et al.* (1974), glycerate kinase is present in sufficiently high activity to ensure that the photorespiratory cycle is not limited at this step. The activity of this enzyme was also found to be essentially insensitive to changes in adenylate charge and pH similar to those which occur in the chloroplast stroma during dark-light transitions. Thus, the activity of this enzyme appears to be regulated solely by substrate supply (Schmitt and Edwards 1983b) which would ensure the rapid re-incorporation of glycerate back into the PCR cycle.

It is interesting to note that while the bundle sheath cells of C_4 plants appear to contain all of the photorespiratory enzymes necessary for the conversion of phosphoglycolate to glycerate (Edwards and Huber, 1981), the enzyme glycerate kinase is apparently absent and appears to be localised in the mesophyll cells (Usuda and Edwards, 1980).

1.3. *The fate of products of glycine metabolism*

The conversion of glycine to serine within the mitochondria leads to the production of serine, CO_2, NADH and NH_3 (Fig. 1.2). Serine is then further metabolised via the photorespiratory carbon cycle, while CO_2 is released into the atmosphere or refixed within the cell by RuBP carboxylase (Zelitch, 1979). I would now like to consider the metabolic fates of the other two products of the reaction, namely NADH and NH_3, and consider the potential influences of other metabolites and cellular processes on the subsequent metabolism of these products.

1.3.1. *NADH: the influence of 'dark' respiration*

It is clear that in order for the turnover of the photorespiratory cycle to be maintained, an efficient mechanism for the re-oxidation of NADH must be available to glycine decarboxylase. Two such mechanisms have been demonstrated *in vitro*. The first is via the mitochondrial electron transport chain and is coupled to three phosphorylation sites (Woo and Osmond, 1976; Moore *et al.* 1977; Douce *et al.* 1977 ; Day and Wiskich, 1981). The ratio of O_2 uptake to CO_2 release was found to be 1:2 (Arron and Edwards, 1979; Day and Wiskich, 1981) in agreement with the production of equal quantities of CO_2

and NADH (the subsequent oxidation of which via the respiratory chain results in the uptake of a $\frac{1}{2}$ O_2). Mitochondrial oxygen uptake, in the presence of glycine, was inhibited by the electron transport chain inhibitors rotenone, antimycin-A and KCN (Woo and Osmond, 1976; Moore *et al*. 1977), although recent evidence suggests that KCN may also act as a direct inhibitor of serine hydroxymethyl transferase (Gardeström *et al*. 1980).

The second mechanism of NADH re-oxidation which has been demonstrated *in vitro*, proceeds via a reversal of malate dehydrogenase in the presence of OAA (Woo and Osmond 1976; Moore *et al*. 1977; Day and Wiskich 1981). In the presence of OAA, re-oxidation occurs independently of and at rates slightly superior to the electron transport chain (Woo and Osmond, 1976; Moore *et al*. 1977). It has been proposed that such a system could operate *in vivo* to supply the reducing power required for hydroxypyruvate reduction, via the operation of a malate/OAA shuttle (Fig. 1.3) between mitochondria and peroxisomes (Woo and Osmond, 1976). Rates of glycine NADH re-oxidation by a reconstituted malate /OAA shuttle, using isolated pea leaf mitochondria, were less than in the presence of excess OAA, indicating that the activity of such a shuttle may be limited by the rate of malate export from the mitochondria (Day and Wiskich, 1981). Furthermore,when rates where calculated on a tissue basis, the malate/ OAA shuttle could only support rates of NADH re-oxidation of 10-22 µmol mg^{-1} Chl h^{-1}, in comparison to rates of re-oxidation via the respiratory chain of 26-46 µmol mg^{-1} Chl h^{-1} (Day and Wiskich, 1981).

A further question regarding the operation of such a shuttle is the feasibility of moving OAA from the peroxisomes to the mitochondria across the cytoplasmic compartment, as this also contains large quantities of malate dehydrogenase (Rocha and Ting, 1971) which may reduce the OAA to malate before it reaches the mitochondria. Journet *et al.* (1981) have proposed that this problem may be avoided through the operation of a malate/aspartate shuttle (Fig. 1.3), similar to that invoked for the movement of NADH between glyoxysomes and mitochondria during the operation of the glyoxylate cycle (Wiskich, 1980), and originally proposed as a method of movement of reducing equivalents across animal mitochondrial membranes (Chappell, 1968). Aspartate and 2-oxoglutarate were shown to enter isolated spinach mitochondria at sufficiently high rates to support such a shuttle (Journet *et al.* 1981).However, as mentioned in section 1.2.4, the operation of such a shuttle *in vivo* would appear doubtful, based on the low activities of GOT found in leaf peroxisomes (Schmitt and Edwards, 1983a).

Of key importance in determining which of the two re-oxidation systems are most likely to operate *in vivo* is a considerat-ion of the potential influence of the process of 'dark' respiration. The rate of 'dark' respiration in the light i.e. during the period of photorespiratory activity, may influence the degree of access of glycine decarboxylase to the NADH re-oxidizing capacity of the mito-chondrial respiratory chain. For example, the TCA cycle involves four NAD-linked dehydrogenases, all of which may theoretically compete with glycine decarboxylase for access to both NAD and NADH re-oxidation capacity (in the form of the NADH dehydrogenase enzymes linked to the mitochondrial respiratory chain). Succinate dehydro-

genase could also compete with glycine decarboxylase at the electron transport chain level, through competition of the NADH and succinate dehydrogenases for access to the electron chain carrier, ubiquinone.

In a recent review on the topic of the effects of light on 'dark' respiration, Graham (1980) concluded that the evidence from both green algae and the leaves of higher plants, strongly indicated the continued operation of the TCA cycle in the light at rates approaching those in the dark. Indeed, such a situation would appear to be necessary if the carbon skeleton requirements of light-dependent synthetic reactions, such as amino acid and porphyrin synthesis, are to be satisfied (Graham, 1980). Thus, the question arises as to what degree of influence this TCA cycle turnover, in the light, will have on the re-oxidation of glycine NADH by the mitochondrial respiratory chain. Before this question can be considered, however, one must first ascertain whether the mitochondrial respiratory chain is in fact operating in the light.

There have been a number of reports of a light-dependent inhibition of respiratory O_2 uptake in micro-organisms (Hoch *et al.* 1963; Ried, 1969; Healy and Myers, 1971). More recently, Canvin *et al.* (1980) reported that O_2 uptake via 'dark' respiration in C_3 leaves may also be suppressed in the light. In contrast, Gerbaud and Andre (1980) using similar gas exchange techniques, came to the opposite conclusion i.e. that respiratory O_2 uptake did persist during photosynthesis. This is further supported by evidence of succinate metabolism in leaf tissue in the light. Succinate oxidation in the TCA cycle, leads to the production of FADH which can only be re-oxidised via the mitochondrial respiratory chain.

Consequently, an inhibition of the chain would lead to a depletion of FAD and an inhibition of succinate oxidation. The reports of Stepanova and Baranova (1972),Chapman and Graham (1974) and Jordan and Givan (1979) however, suggest that succinate metabolism does continue in the light.

An important part of the development of the theory of a light-mediated inhibition of the mitochondrial respiratory chain has been the proposed metabolic basis of the inhibition. This was orginally proposed by Heber and Santarius (1970) and has since gained wide acceptance; so much so that it has been incorporated into models devised to explain the metabolic regulation or operation of both the photorespiratory (Journet *et al*. 1981) and nitrate assimilatory (Sawhney *et al*. 1978; Woo *et al*. 1980) pathways.

Heber and Santarius (1970) observed an increase in the extrachloroplastic ATP/ADP ratio in leaf cells of *Elodea densa* following dark to light transitions and suggested that such an increase may be expected to inhibit mitochondrial oxidative phosphorylation and thus coupled electron flow to O_2, through its effect on the adenylate translocator. The increase in the extrachloroplastic ratio was thought to result from the operation of a triose phosphate/ 3-phosphoglycerate shuttle, operating across the chloroplast envelope, which could export both NAD(P)H and ATP from the chloroplast to the cytoplasm in the light (Heber, 1974). Similar increases in the extrachloroplastic ATP/ADP ratio following dark to light transitions have been observed in tobacco (Keys and Whittingham, 1969) and wheat leaves (Sellami, 1976). Recent work by Stitt *et al*. (1980) however, suggests that changes in the extrachloroplastic ATP/ADP ratio may

not be as significant as first thought. Using protoplasts and improved sampling techniques, they estimated that the extra-chloroplastic ATP/ADP ratio may be as low as 1-2. Furthermore, their results indicated that any increase in the ATP/ADP ratio which was observed in the light, was in fact only transient and that the ratio returned to its original 'dark' value following 4-5 min in the light.

Clearly, if the mitochondrial respiratory chain is inhibited in the light by increases in the ATP/ADP ratio, the NADH generated during photorespiratory glycine metabolism must be re-oxidised via another mechanism, and it is on this basis that the various shuttle systems have been proposed (Woo and Osmond, 1976; Journet *et al.* 1981). One advantage of such a system would be that glycine oxidation could be utilised as a source of NADH for hydroxypyruvate reduction. However, if the respiratory chain is not inhibited by increases in the cytoplasmic ATP/ADP ratio and continues to operate in the light, two important questions arise: (a) what influence will TCA cycle turnover in the light, have on the re-oxidation of glycine NADH via the respiratory chain and (b) what is the source of NADH for hydroxypyruvate reduction.

1.3.2. *NH_3: the potential role of malate in refixation*

There is now little doubt that primary NH_3 assimilation in higher plants occurs via the glutamine synthetase (GS)/glutamate synthase (GOGAT) pathway (Miflin and Lea, 1980). The kinetic and regulatory properties of these two enzymes have been reviewed by Stewart *et al.* (1980). It is also now firmly established that the

re-assimilation of NH_3 released during the operation of the photorespiratory cycle, is also carried out via this pathway as depicted in Fig. 1.4 (Wallsgrove *et al.* 1983). Labelling studies involving the infiltration of spinach leaf tissue with ^{15}N-glycine, demonstrated rapid incorporation of the label into glutamine, indicating that the released $^{15}NH_3$ had been re-assimilated via GS (Woo *et al.* 1982). Similarly, the inhibition of GS with methionine sulphoximine, led to a significant increase in the amount of $^{15}NH_3$ evolved from wheat leaves in the presence of ^{15}N-glycine (Keys *et al.* 1978). This accumulation of NH_3 could be reduced by up to 90% by incubating the tissue under non-photorespiratory conditions, showing that the NH_3 refixed by GS under normal conditions is predominately photorespiratory in origin (Frantz *et al.* 1982). The key role of the GOGAT enzyme in photorespiratory NH_3 re-assimilation was clearly demonstrated by Somerville and Ogren (1980) with a GOGAT-deficient mutant of *Arabidopsis*, which was found to accumulate NH_3 to toxic levels under photorespiratory conditions.

Mitochondria have been proposed as the initial site of photorespiratory NH_3 refixation by GS. Jackson *et al.* (1979) found sufficient GS activity associated with isolated spinach leaf mitochondria, to cope with estimates of photorespiratory NH_3 release. Subsequent investigations however, have revealed that GS is not associated with the mitochondrial fraction (Wallsgrove *et al.* 1979; Wallsgrove *et al.* 1980; Bergman *et al.* 1981; Nishimura *et al.* 1982a). Alternatively, mitochondrial glutamate dehydrogenase has been suggested as a potential site for NH_3 re-fixation (Tolbert, 1980). Isolated mitochondria supplied with 2-oxoglutarate in the presence of

glycine however, did not refix NH_3 into glutamate (Hartmann and Ehmke, 1980; Day and Wiskich, 1981). This is not surprising based on the particularly high Km(NH_3) of glutamate dehydrogenase enzymes from plant tissues (Stewart *et al*. 1980).

It is now clear that initial refixation of photorespiratory NH_3, by GS, may take place in either the cytoplasm or the chloroplast. Higher plants contain two isozymes of glutamine synthetase; one located in the cytoplasm (GS_1) and one located in the chloroplast (GS_2) (Hirel and Gadal, 1981; Hirel *et al*. 1982a; Hirel and Gadal 1982). The relative amounts of each of these isozymes varies between tissues. McNally *et al*. (1983) have identified four groups of plants on the basis of the relative distributions of GS between these two compartments. Two groups contain either cytoplasmic (e.g. achlorophyllous parasitic species) or chloroplastic (e.g. spinach, tobacco) GS only. The other two groups include plants containing both isozymes and are separated according to whether the major component of the GS activity is located in the chloroplast (C_3 grasses and temperate legumes such as peas, beans) or the cytoplasm (C_4 and CAM species).

Based on this distribution data, it would appear that in those plants with significant rates of photorespiration i.e. C_3 plants a chloroplastic location for GS is favoured, and that the initial refixation is most likely to occur in the chloroplast compartment. This is in contrast to the model proposed by Keys *et al*. (1978) which envoked refixation by cytoplasmic GS. Obviously, in plants such as spinach, chloroplastic refixation is the only option. In C_3 plants containing both isozymes however, the role of chloroplastic

GS in photorespiratory metabolism is supported by the observation that it is this isozyme, and not the cytoplasmic form, which is absent from etiolated tissue and synthesised in response to light (Nishimura *et al*. 1982b; Hirel *et al*. 1982b). As mentioned previously, this is also a characteristic common to a number of other photorespiratory enzymes e.g. glycolate oxidase, SGAT and glycine decarboxylase. Similarly, the activity of the ferredoxin-linked GOGAT enzyme, which is located wholly in the chloroplast, is also found to be light-inducible (Matoh and Takahashi, 1981, 1982; Wallsgrove *et al*. 1982).

Isolated chloroplasts have been shown to carry out both (NH_3, 2-oxoglutarate) and (glutamine, 2-oxoglutarate)-dependent O_2 evolution, which is sensitive to inhibitors of GS and GOGAT and which demonstrates ratios of moles of O_2 evolved per mole of substrate added of approximately 0.5, as predicted from the stoichiometry of the ferrodoxin linked-GOGAT reaction (Anderson and Done 1977a, 1977b; Woo and Osmond, 1982). Rates of O_2 evolution of NH_3 refixation in each of these studies were low however, (e.g. maximal rates of 8-10 µmol O_2 mg^{-1}Chl h^{-1} or 16-20 µmol NH_3 assimilated mg^{-1} Chl h^{-1}) in comparison with the minimum predicted rates of photorespiratory NH_3 release of 25-35 µmol mg^{-1} Chl h^{-1} (see section 1.2.3).

The results of Woo and Osmond (1982) indicated that the rate of (NH_3, 2-OG)-dependent O_2 evolution, by isolated spinach chloroplasts, may be limited by the apparent poor affinity of the system for 2-OG. Furthermore, they showed that the affinity of the chloroplast system for 2-OG, could be dramatically improved by the addition of various dicarboxylates including malate, succinate

and fumarate. Rates of (NH_3, 2-OG)-dependent O_2 evolution were stimulated 2-4 fold in the presence of malate, and thus under these conditions could be shown to account for the predicted rates of photorespiratory NH_3 release. Woo and Osmond (1982) proposed that the dicarboxylate-mediated stimulation of O_2 evolution may be explained, in part, by a stimulation of 2-OG uptake into the chloroplast. However, rates of 2-OG uptake into isolated chloroplasts, have been estimated at 26-32 μmol mg^{-1} Chl h^{-1} (Lehner and Heldt, 1978; Proudlove and Thurman, 1981) and thus,theoretically, should not have been a limiting factor in the O_2 evolving system.

Clearly, this observation requires further investigation to ascertain firstly, the mechanism by which dicarboxylate molecules, such as malate, stimulate (NH_3, 2-OG)-dependent O_2 evolution in isolated chloroplasts and secondly, whether such a mechanism may have a role in the operation of the photorespiratory nitrogen cycle *in vivo*.

1.4. *The present study*

This investigation initially set out to examine three main questions relating to the metabolic rate of products of photorespiratory glycine metabolism:

(1) Is the electron transport chain of plant mitochondria regulated by the external ATP/ADP ratio and, if so, are the ratios measured in the extrachloroplastic fractions of leaf tissue, sufficiently high to inhibit leaf mito-

chondrial respiration *in vivo*.

(2) What influence will TCA cycle turnover, in the light, have on the potential re-oxidation of glycine NADH via the mitochondrial respiratory chain and, thus, what is the most likely metabolic fate of NADH generated during photorespiratory glycine metabolism.

(3) What is the mechanism of dicarboxylate stimulation of photorespiratory NH_3 refixation in isolated chloroplasts and is this likely to have a role in the operation of the photorespiratory nitrogen cycle *in vivo*.

The results of investigations into these questions are covered in the Chapters 3-6.

As part of the investigation into question (3), it was necessary to develop a sensitive assay system for GOGAT activity, which closely approximated conditions within the intact chloroplast. Thus, an O_2 evolving reconstituted chloroplast system for the assay of *in vitro* GOGAT activity was developed, and the characteristics of this assay system are presented in Chapter 7. Furthermore, during investigations into question (1), evidence was obtained of endogenous hexokinase activity associated with pea leaf mitochondria. While there have been a number of reports of particulate hexokinase in non-photosynthetic tissues, little is known of the distribution of this enzyme in photosynthetic tissues. Therefore, the results of such a study, with particular emphasis on the hexokinase activity associated with the mitochondrial fraction, are presented in Chapter 8.

Figure 1.1. The integration of the photosynthetic carbon reduction (PCR) and photorespiratory carbon oxidation (PCO) cycles. (Lorimer and Andrews, 1981).

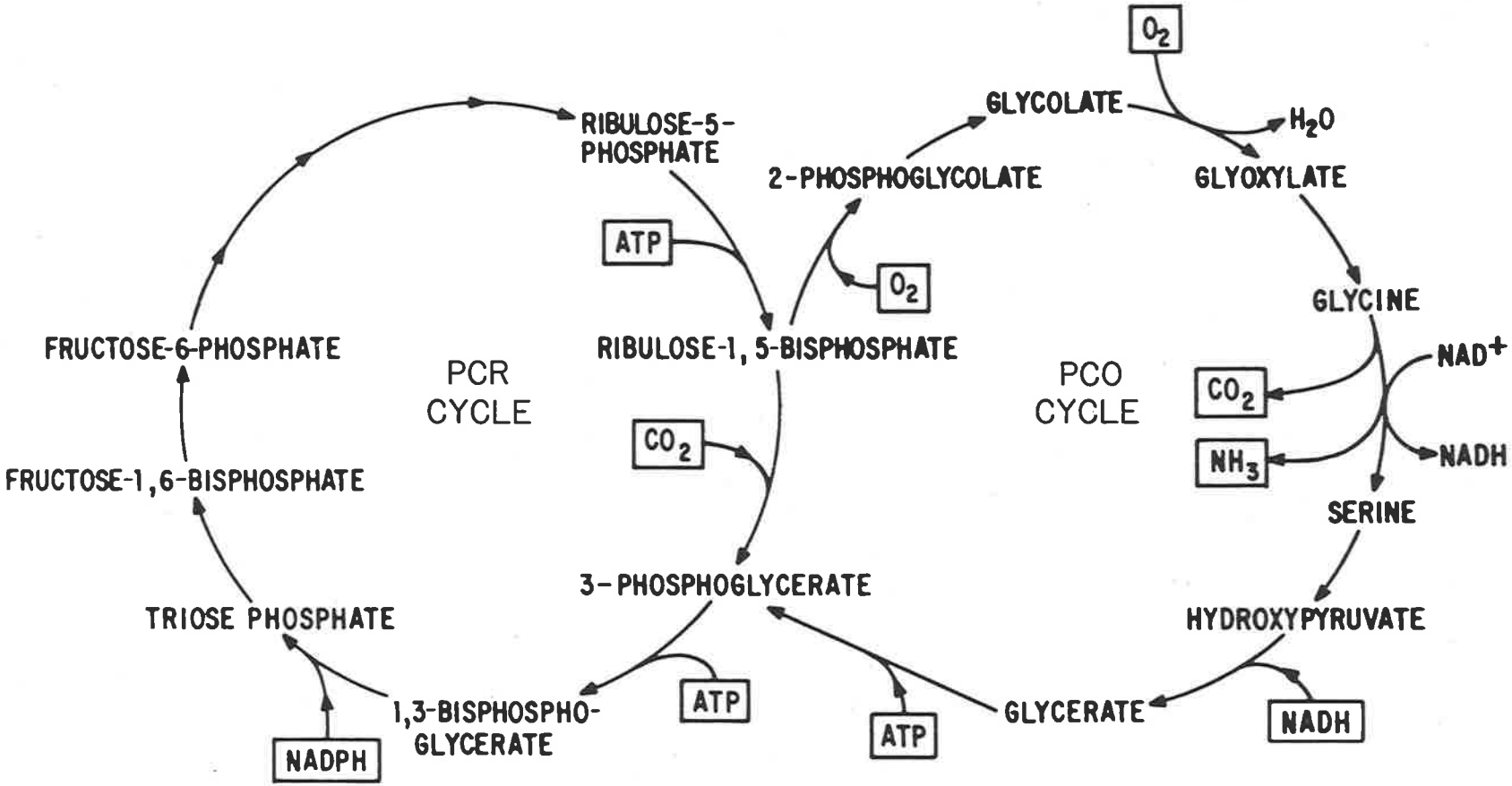

RIBULOSE-5-PHOSPHATE
ATP
RIBULOSE-1, 5-BISPHOSPHATE
CO_2
3-PHOSPHOGLYCERATE
ATP
1,3-BISPHOSPHO-GLYCERATE
NADPH
TRIOSE PHOSPHATE
FRUCTOSE-1,6-BISPHOSPHATE
FRUCTOSE-6-PHOSPHATE
PCR CYCLE
O_2
2-PHOSPHOGLYCOLATE
GLYCOLATE
H_2O
GLYOXYLATE
GLYCINE
CO_2
NH_3
NAD+
NADH
SERINE
HYDROXYPYRUVATE
NADH
GLYCERATE
ATP
PCO CYCLE

Figure 1.2. The photorespiratory carbon cycle (adapted from Chollet, 1977).

Enzymes:

(1) RuBP carboxylase - oxygenase

(2) phosphoglycolate phosphatase

(3) glycolate oxidase

(4) serine-glyoxylate aminotransferase

(5) glutamate - glyoxylate aminotransferase

(6) glycine decarboxylase

(7) serine hydroxymethyl transferase

(8) hydroxypyruvate reductase

(9) glycerate kinase

(10) catalase.

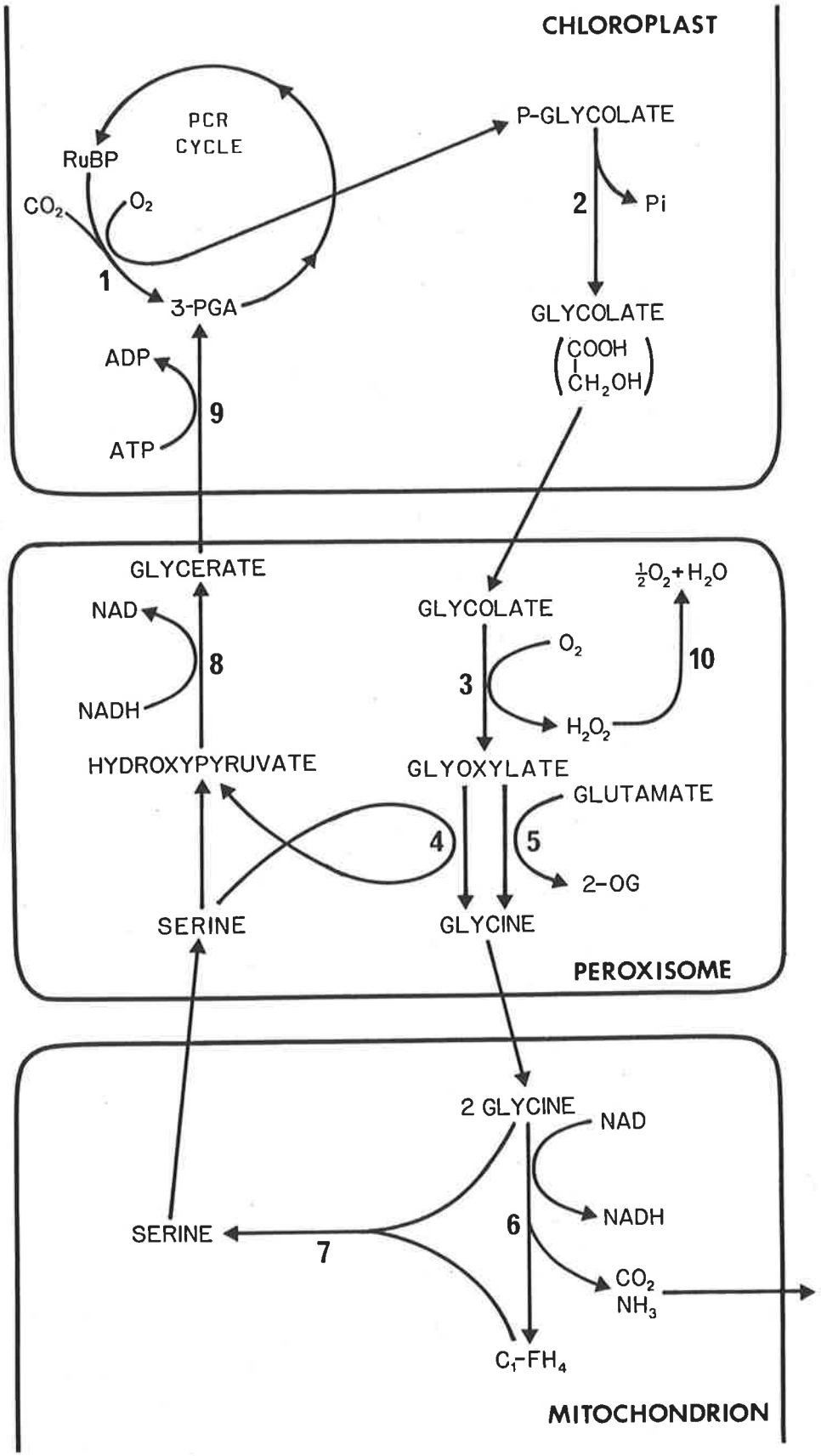

CHLOROPLAST
PCR CYCLE
RuBP
CO_2
O_2
1
3-PGA
P-GLYCOLATE
2
Pi
GLYCOLATE
COOH
CH_2OH
ADP
ATP
9
GLYCERATE
NAD
NADH
8
HYDROXYPYRUVATE
GLYCOLATE
O_2
3
H_2O_2
$\frac{1}{2}O_2+H_2O$
10
GLYOXYLATE
GLUTAMATE
4
5
2-OG
SERINE
GLYCINE
PEROXISOME
2 GLYCINE
NAD
NADH
SERINE
7
6
CO_2
NH_3
C_1-FH_4
MITOCHONDRION

Figure 1.3. Metabolite movement during the operation of a malate/aspartate shuttle between mitochondria and peroxisomes for the supply of NADH for hydroxypyruvate reduction.

This shuttle can be adapted for the movement of NADH between the chloroplast and the peroxisome by linking OAA reduction via chloroplastic NAD(P)-malate dehydrogenase to the photosynthetic electron transport chain (Anderson and House, 1979).

Enzymes:

(1) NAD-malate dehydrogenase

(2) glutamate-oxaloacetate aminotransferase

(3) glycine decarboxylase/serine hydroxymethyl transferase

(4) serine-glyoxylate aminotransferase

(5) NAD-hydroxypyruvate reductase

Symbols:

malate/OAA shuttle (▪▪▪▪▪▪▪)

malate/aspartate shuttle (———).

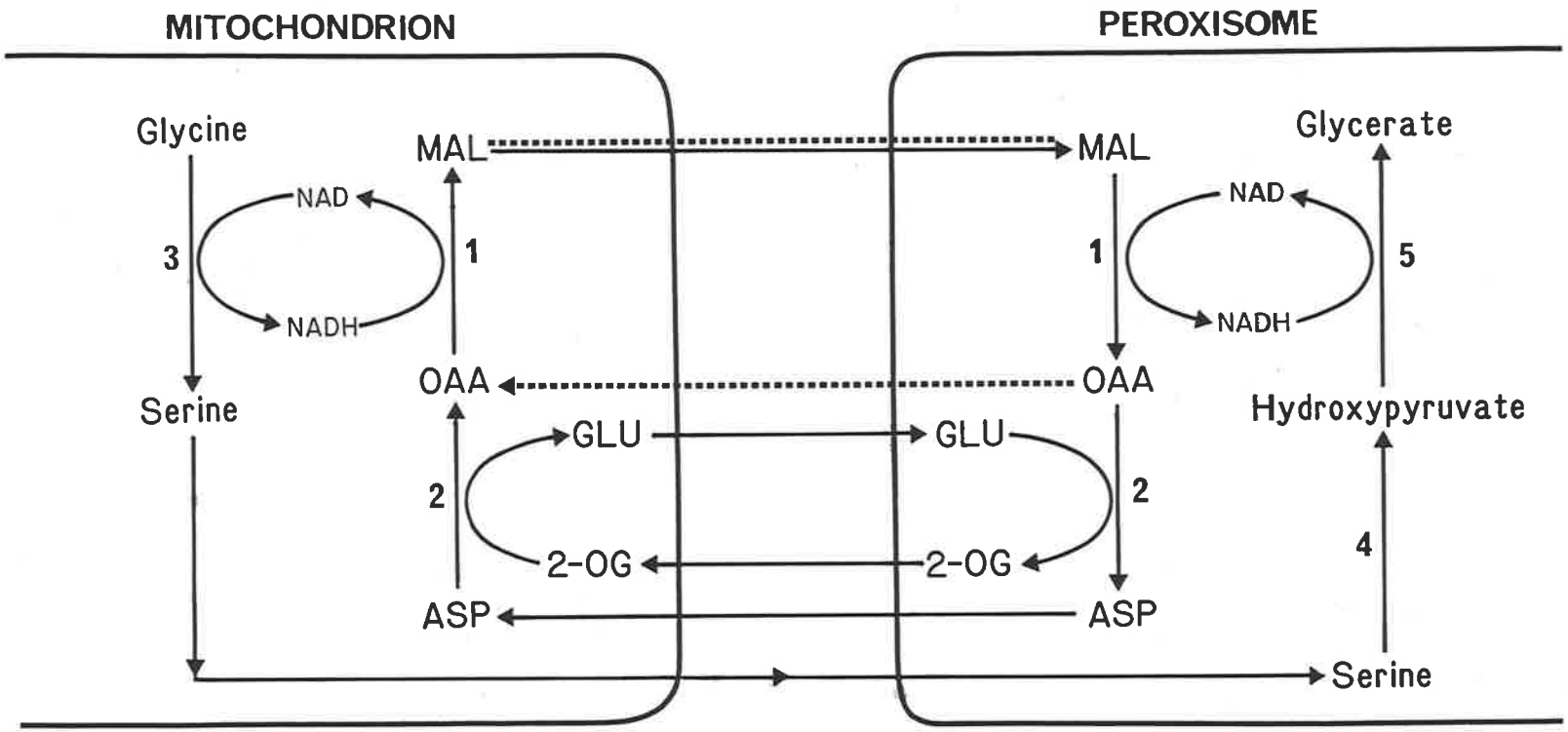
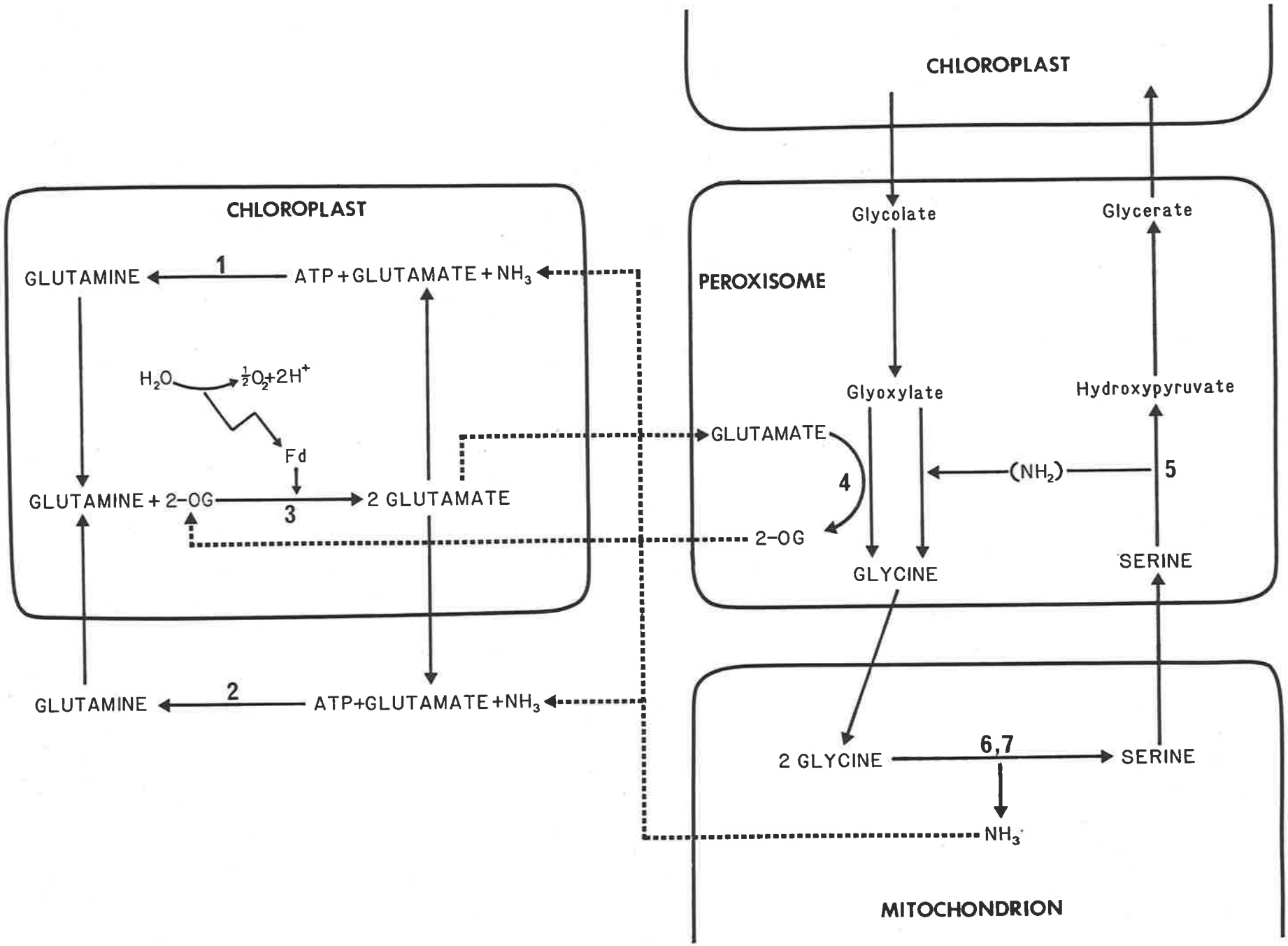

MITOCHONDRION
PEROXISOME
Glycine
MAL
MAL
Glycerate
NAD
NAD
3
1
1
5
NADH
NADH
OAA
OAA
Serine
Hydroxypyruvate
GLU
GLU
2
2
4
2-OG
2-OG
ASP
ASP
Serine

Figure 1.4. The photorespiratory nitrogen cycle.

Enzymes:

1. glutamine synthetase (chloroplastic)
2. glutamine synthetase (cytoplasmic)
3. glutamate synthase
4. glutamate - glyoxylate aminotransferase
5. serine - glyoxylate aminotransferase
6. glycine decarboxylase
7. serine hydroxymethyl transferase


CHLOROPLAST
CHLOROPLAST
Glycolate
Glycerate
PEROXISOME
GLUTAMINE
1
ATP+GLUTAMATE+NH3
H2O
½O2+2H+
Fd
Glyoxylate
Hydroxypyruvate
GLUTAMATE
4
(NH2)
5
GLUTAMINE + 2-OG
3
2 GLUTAMATE
2-OG
GLYCINE
SERINE
GLUTAMINE
2
ATP+GLUTAMATE+NH3
2 GLYCINE
6,7
SERINE
NH3
MITOCHONDRION

CHAPTER II

MATERIALS AND METHODS

2.1. *Materials*

Pea seeds (*Pisum sativum* cv. Massey Gem, Greenfeast), spinach seeds (*Spinacia oleracea* cv. Hybrid 102) and cauliflower (*Brassica oleracea*) were purchased locally.

Peas were grown in vermiculite in a glasshouse for 10-15 days. Spinach was grown in pots filled with gravel for 4-5 weeks in a glasshouse and watered with $\frac{1}{2}$ strength Hoaglands solution (Hoagland and Arnon, 1950) Cauliflower was used fresh.

All chemicals and enzymes were analytical reagent grade. Sucrose was obtained from B.D.H. Ltd. (Poole, U.K.), DE52 from Whatman Ltd. (Kent, U.K.), Percoll and PD-10 Sephadex columns from Pharmacia Fine Chemicals (Uppsala, Sweden) and ^{14}C-glutamate from Amersham Aust. Pty. Ltd. (N.S.W., Aust.)

2.2. *Isolation of washed mitochondria*

(a) *Pea leaf*

Forty g of leaves (chilled for 30 min) were disrupted with a Polytron PTA-35 probe for 2-3 s in 200 ml of ice-cold medium containing 0.3 M sorbitol, 50 mM Tes, 1 mM EDTA, 1 mM $MgCl_2$, 10 mM isoascorbate, 1% (w/v) PVP-40 and 0.4% (w/v) BSA, adjusted to pH 7.5. The homogenate was filtered through two layers of Miracloth (Calbiochem, Cal., U.S.A.) and centrifuged at

2,000 *g* for 5 min. The supernatant was centrifuged at 10,000 *g* for 20 min and the pellets washed by resuspending in 60 ml of 0.3 M sorbitol containing 20 mM Tes and 0.1% (w/v) BSA (adjusted to pH 7.5) and recentrifuged at 10,000 *g* for 20 min. Final resuspension was in 2-3 ml of wash medium. All isolation procedures were carried out at 1-2°C.

(b) *Cauliflower*

Young florets (200 g) were chilled for 30 min and disrupted using a Moulinex liquidizer in 200 ml of medium containing 0.3 M sorbitol, 50 mM Tes, 3 mM EDTA and 0.5% (w/v) BSA, all adjusted to pH 7.2. The homogenate was filtered through two layers of Miracloth and centrifuged at 1,500 *g* for 10 min. The supernatant was centrifuged at 12,000 *g* for 15 min. The mitochondrial pellet was washed by resuspending in 60 ml of 0.3 M sorbitol and recentrifuged at 12,000 *g* for 15 min. Final resuspension was in 2-3 ml of wash medium with 0.5% (w/v) BSA added.

2.3. *Isolation of purified mitochondria*

Following the first centrifugation (2,000 *g* for 10 min) 75 ml of the resulting supernatant were carefully layered onto a sucrose density gradient and centrifuged for 90 min, at 21,000 rpm in a Model L-2 ultracentrifuge, using a SW25.2 rotor (Beckman Instruments, Cal., U.S.A.). Each tube consisted of a 5 ml "cushion" of 54% (w/w) sucrose underneath a 30-ml continuous gradient of 30.6%

to 41.5% (w/w) sucrose and 25 ml of supernatant. All sucrose solutions contained 1 mM EDTA, 1 mM Tes (pH 7.5) and 0.1% (w/v) BSA. The mitochondrial fraction [located just below the green band of chloroplast fragments - see Nash and Wiskich (1983)] was slowly diluted at 0°C over a period of 30-40 min with 100 ml of 0.3 M sorbitol, 20 mM Tes and 0.1% (w/v) BSA (pH 7.5). The suspension was centrifuged at 10,000 *g* for 20 min in a fixed-angle rotor and the mitochondrial pellet resuspended in 1-2 ml of the dilution medium. For fractionation studies the gradients were removed with an ISCO density gradient fractionator (model 640) by pushing the gradient up from the bottom.

2.4. *Isolation of chloroplasts*

(a) Pea

Pea shoots (60 g) were disrupted with a Polytron probe for 2-3 s in 200 ml of ice-cold medium containing 0.33 M sorbitol, 50 mM Mes, 20 mM NaCl, 2 mM EDTA, 2 mM $NaNO_3$, 2 mM isoascorbate, 1 mM $MgCl_2$, 0.5 mM NaH_2PO_4 and 0.4% (w/v) BSA, adjusted to pH 6.2. The brei was squeezed through two layers of Miracloth containing a layer of cotton wool and the filtrate centrifuged at 1,500 *g* for 30 s in a M.S.E. Super Minor centrifuge. The chloroplast pellet was rinsed with 5-10 ml of medium containing 0.33 M sorbitol, 50 mM Hepes, 20 mM NaCl, 2 mM EDTA, 2 mM $NaNO_3$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5 mM NaH_2PO_4 and 0.4% (w/v) BSA, adjusted to pH 6.7, and resuspended in 1-2 ml of the same medium using a cotton bud.

For percoll-purified chloroplasts, the chloroplast pellet was resuspended in 6-8 ml of the above medium, underlaid with 4 ml of resuspension medium containing 40% (v/v) percoll and centrifuged at 1,500 *g* for 1 min. The resulting chloroplast pellet was resuspended by gently swirling in 1-2 ml of resuspension medium. All procedures were carried out at 1-2°C.

(b) *Spinach*

Isolated chloroplasts were prepared from deribbed spinach leaves, as for pea leaves, except for the isolation medium (0.33 M sorbitol, 10 mM sodium pyrophosphate, 5 mM $MgCl_2$, 2 mM isoascorbate and 0.1% (w/v) BSA, adjusted to pH 6.5 with HCl) and the resuspension medium (0.33 M sorbitol, 50 mM Hepes, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 0.2% (w/v) BSA, adjusted to pH 7.6).

2.5. *Preparation of reconstituted chloroplast components*

The chloroplast extract and envelope-free fractions were prepared by resuspending the isolated chloroplast pellet in 5.5 ml of a solution containing 1:25 dilution of standard chloroplast reaction medium (see section 2.8) and 5 mM dithiothretol, all adjusted to pH 7.6. After stirring for 2 min at 2°C, the suspension was centrifuged at 14,000 *g* for 10 min. The supernatant (chloroplast) extract) was collected and the pellet resuspended in 40 ml of the same medium and recentrifuged at 14,000 *g* for 10 min. The pellet was resuspended in 2-3 ml of full strength chloroplast reaction medium to form the envelope-free chloroplast fraction.

2.6. *NAD-depletion treatment*

Mitochondria were resuspended to a concentration of approx. 0.2 mg mitochondrial protein ml^{-1}, in a large volume of mitochondrial reaction medium (section 2.8) which also contained 1 mM EGTA. The mitochondria were then incubated with gentle stirring, at 4°C (unless otherwise stated), in the presence of 2 mM glycine or malate for up to 5 hr.

2.7. *Sampling of extramitochondrial ATP/ADP ratios*

A 1 ml aliquot was removed from the reaction vessel and rapidly filtered through a 0.45 µm cellulose nitrate membrane filter (Sartorius, Gottingen,Ger.), into a test tube containing 46 µl of 11.5 N perchloric acid (on ice). The filtrate was rapidly vortexed, left on ice for 5 min and then centrifuged for 4 min in a Beckman Model 152 microfuge (Beckman Instruments, Cal., U.S.A.) at 2°C. A 700 µl sample was neutralized to pH 7.0 with 250 µl of neutralizing solution (2M KOH, 1M Hepes) and immediately frozen.

2.8. *Oxygen uptake and evolution*

Oxygen uptake or evolution was measured polarographically with a Rank oxygen electrode (Rank Bros., Cambridge, U.K.) connected to a Rikadenki recorder (Rikadenki Kogyo Co., Ltd., Tokyo, Japan). For all measurements the temperature of the reaction vessel was maintained at 25°C with a circulating water bath. Oxygen concentration of air-saturated medium was taken as 240 µM. Respiratory control and ADP/O ratios were calculated as described by Estabrook (1967).

(a) Mitochondria

Pea leaf mitochondria were assayed in 2.0 to 2.5 ml of medium containing 0.3 M sorbitol, 10 mM Tes, 10 mM KH_2PO_4, 2 mM $MgCl_2$, and 0.1% (w/v) BSA, adjusted to pH 7.2. Cauliflower mitochondria were assayed in a medium containing 0.25 M sucrose, 10 mM Tes, 10 mM KH_2PO_4 and 5 mM $MgCl_2$ at pH 7.4.

(b) Chloroplasts

Pea chloroplasts were illuminated in a 2 ml reaction medium containing 0.33 M sorbitol, 50 mM Hepes, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, adjusted to pH 7.6. Spinach chloroplasts were assayed in the same medium, but included 0.2% (w/v) BSA and 600 units of catalase. The light source was a 150-w tungsten halogen projector lamp, giving a light intensity of 550 $\mu E\ m^{-2}\ s^{-1}$ at the centre of the vessel.

For CO_2-dependent O_2 evolution, 4.5 mM sodium pyrophosphate, 0.9 mM ATP, 0.18 mM NaH_2PO_4 and 4.5 mM $NaHCO_3$ were added. Glyceraldehyde (10 mM) was present during (NH_3, 2-OG)-, (NH_3, glutamine, 2-OG)-, (glutamine, 2-OG)-and (Asp, 2-OG)-dependent O_2 evolution, to inhibit endogenous CO_2-dependent O_2 evolution (Stokes and Walker 1972).

Chloroplast intactness was measured with ferricyanide according to the method of Lilley *et al.* (1975).

Chloroplasts (15-20 µg Chl) were illuminated in 2.0 ml of reaction medium (0.4 or 0.1 M sucrose, 50 mM Hepes, 10 mM KH_2PO_4, 5 mM $MgCl_2$, pH 7.6), 2.4 mM $K_3Fe(CN)_6$ and 7 mM NH_4Cl (to uncouple photosynthetic electron transport). The percentage intactness was calculated as:

$$\frac{\text{uncoupled rate (0.1 M)} - \text{uncoupled rate (0.4 M)}}{\text{uncoupled rate (0.1 M)}} \times 100$$

(c) Reconstituted chloroplasts

Oxygen evolution by a reconstituted chloroplast system was measured in 2 ml of an assay mixture containing 0.33 M sorbitol, 50 mM Hepes, 10 mM $MgCl_2$, 2 mM EDTA, 1 mM $MnCl_2$, 1 mM NH_4Cl, envelope-free chloroplasts (200 µg Chl), chloroplast extract (50-200 µl), 200 µg of spinach ferredoxin and 600 units of catalase at pH 7.6.

(d) Whole tissue : leaf slices

Leaves (0.2 g fwt) were cut into slices (approximately 1 mm wide) under a solution containing 50 mM Hepes and 0.2 mM $CaCl_2$ (pH 6.6), using a razor blade. These slices were placed into a Rank O_2 electrode, containing 4 ml of the above medium, for O_2 uptake measurements.

2.9. *Simultaneous measurement of NH_3 release and O_2 uptake*

Ammonia release was measured simultaneously with O_2 uptake in a sealed perspex vessel into which were fitted an Orion ammonia electrode (Orion Res. Inc., M.A., U.S.A.) connected to a Beckman pH

meter (Beckman Instruments, Cal., U.S.A.) and a Clark-type O_2 electrode (Yellow Springs, Oh., U.S.A.). The signals from both electrodes were monitored with a twin-channel Rikadenki recorder. The capacity of the vessel was 9 ml. Mitochondria (1.5-2.0 mg protein) were added to 8.5 of reaction medium (section 2.8). Ammonia release was calibrated by injecting known amounts of NH_4Cl.

2.10. *Enzyme assays*

Spectrophotometric measurements were made with an Amino Dw 2a spectrophotometer (American Instrument Co., Maryland, U.S.A.) at room temperature.

(a) Hexokinase

Hexokinase was assayed by following NADP reduction at 340 nm, in the presence of excess G6P dehydrogenase. Assays involving intact mitochondria were carried out in the presence of 2.5 ml mitochondrial reaction medium (with $MgCl_2$ adjusted to 5 mM), 2 mM glucose, 0.3 mM NADP and 0.3 unit of G6P dehydrogenase. The reaction was initiated with 2 mM ATP. In the case of cell-fractionation studies and the fractionation of sucrose gradients, the mitochondrial reaction medium was replaced with 0.1 M Tris-HCl buffer (pH 8.0) containing 5 mM $MgCl_2$. Where fructose was utilized as a substrate, 1 unit of phosphoglucose isomerase was also included in the assay mixture.

(b) *NAD-isocitrate dehydrogenase*

The method of Cox (1969) was followed. A 0.1 ml sample was placed into a volume of 3.1 ml of 44 mM Hepes-KOH (pH 7.6), 0.04% (v/v) Triton X-100, 1 mM $MnSO_4$ and 0.6 mM NAD. The reaction was initiated with 3.9 mM D, L-isocitrate.

(c) *Hydroxypyruvate reductase*

Hydroxypyruvate reductase was measured as described by Tolbert (1971), using a 0.1 ml sample in a total volume of 3.0 ml containing 5 mM KH_2PO_4/K_2HPO_4 buffer (pH 6.3), 0.04% (v/v) Triton X-100, 0.2 mM NADH and 1 mM hydroxypyruvate to initiate the reaction.

(d) *Glutamine synthetase*

Glutamine synthetase was assayed according to the method of O'Neal and Joy (1973), by following the oxidation of NADH at 340 nm in the presence of pyruvate kinase and lactate dehydrogenase. The standard reaction mixture contained 0.1 M Hepes-KOH (pH 7.8), 60 mM KCl, 20 mM $MgSO_4$, 18 mM glutamate, 7 mM ATP, 1 mM phosphoenolpyruvate, 0.2 mM NADH, 10 units of lactate dehydrogenase and 10 units of pyruvate kinase. The reaction was initiated with 5.5 mM NH_4Cl.

(e) *Fumarase (Fumarate hydratase)*

Fumarase activity was measured in a coupled assay with NADP-malic enzyme, according to the method of Hatch (1978). The reaction mixture contained 25 mM Hepes-KOH (pH 7.5), 0.04% (v/v) Triton X-100, 5 mM KH_2PO_4, 4 mM $MgCl_2$, 0.4 mM NADP and 0.2 unit of NADP-malic enzyme in a total of 1 ml. The reaction was initiated with 10 mM fumarate.

Chicken liver malic enzyme was prepared for use in the assay by diluting 0.5 ml of enzyme, suspended in $(NH_4)_2SO_4$, with 2.0 ml of 20 mM Hepes-KOH (pH 7.5) containing 2 mM dithiothretol. This solution was desalted by passing it through a 9 ml Sephadex column (PD-10) loaded with G-25, that had previously been equilibrated with the above resuspending medium. The desalted malic enzyme remained stable for several days at -15°C.

(f) *NADH cytochrome c-reductase*

Antimycin-A-insensitive NADH cytochrome c-reductase activity was measured by following the reduction of cytochrome c at 550 nm. The reaction mixture contained 2.5 ml of mitochondrial reaction medium (section 2.8), 10 mM KCN, 50 µM cytochrome c, 5 µM antimycin-A and 50 µl membrane suspension. The reaction was initiated by adding 0.5 mM NADH.

2.11. *Metabolite assays*

(a) ATP

The method of Bergmeyer (1974) was followed. The assay mixture contained 50 mM Hepes-KOH (pH 7.4), 10 mM $MgCl_2$, 5 mM EDTA, 4 mM glucose, 70 µM NADP, 0.7 unit of GGP dehydrogenase and sample containing 0-70 nmol ATP in a total volume of 2.6 ml. The reaction was initiated with 0.5 unit of hexokinase and the increase in absorbance at 340 nm measured.

(b) ADP

The method of Bergmeyer (1974) was followed. The assay mixture contained 75 mM Hepes-KOH (pH 7.5), 5 mM $MgCl_2$, 0.4 mM phosphoenolpyruvate, 13 µM NADH, 0.5 unit of lactate dehydrogenase and sample containing 0-5 nmol ADP in a total volume of 2.5 ml. The reaction was initiated with 2 units of pyruvate kinase and the decrease in absorbance at 340 nm measured.

(c) 2-oxoglutarate

The measurement of 2-OG was achieved using a coupled assay system involving GOT and MDH. The assay mixture contained 50 mM Tes-KOH (pH 7.4), 10 mM $MgSO_4$, 5 mM EDTA, 7 mM aspartate, 0.2 mM NADH, 15 units of MDH and 150-200 nmol of 2-OG. The reaction was initiated with 1 unit of GOT and the decrease in absorbance at 340 nm measured.

(d) *NAD*

NAD was assayed according to the method of Klingenberg (1974). Immediately prior to the extraction of NAD from intact mitochondria, it is important to ensure the total conversion of intramitochondrial NADH to NAD (Tobin *et al.* 1980), due to the instability of NADH in acid extracts (Klingenberg, 1974). This was achieved by incubating mitochondria (in the absence of substrates) with 0.15 mM ATP and 4 µM FCCP in a test tube for 2 min, while vigorously vortexing the tube to maintain the incubation mixture in an aerobic state. The mitochondria were extracted in 0.3 N perchloric acid for 5 min on ice, and then neutralized with 5 N KOH to pH 7-8.

The assay mixture contained 90 mM pyrophosphate buffer, pH 8.9 (using a stock buffer solution of 0.15 M produced by dissolving 9.0 g sodium pyrophosphate and 1.0 g semicarbazide hydrochloride in 132 ml of double distilled water and adjusting to pH to 8.9 with KOH), 90 mM ethanol and 0.5 nmol NAD in a total volume of 1.1 ml. The reaction was initiated with 5 units of alcohol dehydrogenase and the increase in absorbance at 340 nm measured.

2.12 *Glutamate transport*

Back exchange of ^{14}C-glutamate was measured with the silicone oil technique. Chloroplasts were first loaded with ^{14}C-glutamate by incubation for 20 min in the dark at 0°C, in

9.5 ml of resuspension medium (section 2.4) containing 10 mM ^{14}C-glutamate (10 µCi ml^{-1}) and chlorophyll at a concentration of 2 mg ml^{-1}. The incubation mixture was diluted with 3 ml of resuspension medium and underlaid with 2 ml of resuspension medium containing 40% (v/v) percoll. This was centrifuged at 1,500 *g* for 1 min and the pellet resuspended in the above medium.

Polypropylene tubes (0.4 ml) were loaded in the following order: 20 µl 1N perchloric acid, 75 µl silicone oil (density 1.04 g ml^{-1}, viscosity 120 centistokes; type AR from the Wacker Chemical Co., Munich, Ger.) and 200 µl chloroplast sample. Chloroplasts (70 µg Chl ml^{-1}) were incubated in the dark at 2°C, in a medium containing 0.33 M sorbitol, 50 mM Hepes, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, pH 7.6. After 1 min, various dicarboxylates were added (1 mM) and back exchange allowed to proceed for 30 s, after which time samples (200 µl)were removed for centrifugation using a Beckman microfuge. Release of ^{14}C-glutamate was determined from the amount of ^{14}C-label in the supernatant.

2.13. *Estimation of respiratory rates on a tissue basis*

Pea seeds (*Pisum sativum* cv Massey Gem) were sown in vermiculite on day 1 and shoots emerged on day 7. Seedlings were watered with a soluble fertilizer 'Aquasol' (Hortico Ltd., Adel., Aust.)

Leaf tissue samples (10 g) were disrupted in 100 ml of isolation medium according to the method for mitochondrial preparation (section 2.2), except that the isolation medium also contained 2 mM

dithiothretol. The homogenate was passed through a double layer of Miracloth and the total volume measured. A 1 ml sample was removed for chlorophyll estimation and another 10 ml removed for total fumarase activity estimation. The remaining volume was utilised for the preparation of the mitochondrial fraction as described in section 2.2.

The method used for the estimation of total fumarase activity in crude tissue extracts was based on Hatch (1978). The 10 ml homogenate sample was first incubated with 0.1% deoxycholate for 10 min at 4°C. This treatment was found to solubilize up to 96-98% of the total mitochondrial fumarase activity. The detergent treatment was followed by centrifugation at 12,000 *g* for 10 min. The supernatant was removed and 2 ml passed through a 9 ml Sephadex column (PD-10) loaded with G-25, which had previously been equilibrated with 20 mM Hepes-KOH (7.6) containing 2 mM dithiothretol. The fumarase activity was collected in a total volume of 3.5 ml and assayed according to the method in section 2.10.

Mitochondrial activities were assayed with a Rank O_2 electrode as described in section 2.8, while total mitochondrial fumarase activity was assayed spectrophotometrically (section 2.10). A comparison of the total homogenate fumarase activity with the fumarase activity recovered in the mitochondrial fraction, allowed for an estimation of the % recovery of intact mitochondria. The average mitochondrial recovery was found to be 34.2 ± 2.9% (n=7). This figure was then used to give an estimate of the total oxidative activities in the homogenate.

The amount of tissue which had been disrupted and which contributed to the total homogenate activity, was estimated by comparing the total chlorophyll yield in the homogenate, with measurements of chlorophyll concentrations of the leaf tissue on a g fwt basis. Tissue chlorophyll levels were measured in 80% acetone according to the method of Strain *et al.* (1971). The method of Wintermans and DeMots (1965) involving the use of 96% ethanol, yielded similar results. Up to 0.5 g of leaf tissue was ground for 5 min in 15 ml of 80% (v/v) acetone with an Ultraturax (Janke and Kunkel, Staufen, Ger.). The brei was centrifuged for 10 min at 15,000 *g* and the absorbance of the supernatant at 665 nm and 649 nm measured. Total chlorophyll (μg ml^{-1}) was calculated using the equation: 6.45 (A_{665}) + 17.72 (A_{649}). Tissue chlorophyll estimates for mature pea leaves were found to be of the order of 2.18 ± 0.06 mg^{-1} Chl g^{-1} fwt (n=7). Chlorophyll levels in the homogenate were also estimated using the 80% (v/v) acetone method.

2.14. *Preparation of ferredoxin*

Ferredoxin was prepared from 4 kg of spinach leaves by the method of Rao *et al.* (1971). All procedures were carried out at 4°C. The spinach was deribbed and disrupted in 20 mM KH_2PO_4, pH 7.5 (1 l kg^{-1}) with a homogeniser. The brei was filtered through a layer of muslin between two layers of Miracloth and centrifuged at 16,000 *g* for 10 min. NaCl (6 g l^{-1}) was added to the supernatant and the pH adjusted to pH 7.5 with NaOH. DE 52 slurry (40 ml l^{-1}) was added while stirring and the suspension left to stand in the cold for ½ hr. The DE 52 had previously been equilibrated as follows: 125 g at DE 52 was washed with 5 l of distilled water

followed by 1 l of 1 M KH_2PO_4, pH 7.5, and finally 10 l of 20 mM KH_2PO_4, pH 7.5.

The supernatant was decanted and the DE 52 slurry packed into a 3 cm (wide) column. The column was washed overnight with 20 mM KH_2PO_4, 0.15 M NaCl (pH 7.5) until the eluate was clear. The ferredoxin (red-brown band) was eluted with high salt buffer (20 mM KH_2PO_4, 0.8 M NaCl, pH 7.5). Ammonium sulphate (0.5 g ml^{-1}) was slowly added to the ferredoxin eluate, the pH adjusted to 7.5 with NaOH and the suspension left to stand for 30 min.

The solution was then centrifuged at 10,000 *g* for 30 min and the supernatant (approximately 40 ml), dialysed overnight against 4l of 20 mM KH_2PO_4, pH 7.5. The dialysate was loaded onto a small column packed with DE 52 (equilibrated as before) and washed for 4 h with 20 mM KH_2PO_4, 0.15 M NaCl (pH 7.5). The ferredoxin was eluted with 20 mM KH_2PO_4, 0.8 M NaCl (pH 7.5) and stored, under nitrogen, at - 15°C. Immediately before use, the ferredoxin was desalted by passing it through a 9 ml Sephadex column (PD-10) loaded with G-25, in 20 mM KH_2PO_4 (pH 7.5).

2.15. *Chemical assays*

Protein content was measured by the method of Lowry *et al.* (1951), with $CuSO_4$ in 1% citrate (rather than tartrate) and BSA (fraction V) was used as a standard. Except for section 2.13, chlorophyll was estimated from acetone extracts according to the method of Arnon (1949). The protein content of washed leaf mitochondrial suspensions was corrected for the contribution by broken thylakoids by assuming a thylakoid protein : chlorophyll ratio of 7:1 (Nash and Wiskich, 1982).

Refractivity of sucrose gradient solutions was measured with an Abbe refractometer Model 10460 (American Optical Corp., Buffalo, U.S.A.).

CHAPTER III

ROLE OF THE EXTERNAL ATP/ADP RATIO IN THE CONTROL OF MITOCHONDRIAL RESPIRATION

3.1. INTRODUCTION

It has been proposed (see section 1.3.1) that a light-mediated increase in the cytoplasmic ATP/ADP ratio of leaf cells, leads to an inhibition of the mitochondrial electron transport chain (Heber, 1974). Numerous studies with animal mitochondria (Davis and Lumeng, 1975; Küster, *et al*. 1976; Davis and Davis - van Thienen, 1978; Kunz *et al*. 1981) have shown that coupled respiration, as measured by oxygen consumption, may indeed be regulated or controlled by the external ATP/ADP ratio, through competition between ADP and ATP for access to the AdN translocator (Pfaff and Klingenberg, 1968; Souverijn *et al*. 1973). Vignais and Lauquin (1979) however, have pointed out that despite these findings, a role for the AdN translocator in *in vivo* regulation of oxidative phosphorylation is unlikely, since the ATP/ADP ratios measured in animal tissues are well below those needed to cause significant inhibitions of mitochondrial respiration *in vitro*.

To test the hypothesis that ATP/ADP ratios control mitochondrial respiration in photosynthetic tissue, it was decided to examine the effects of changes in the external ATP/ADP ratio on the respiratory rate of isolated plant mitochondria and in particular of those isolated from leaf tissue. Any regulatory effects of the external ATP/ADP ratio on respiration could then be compared with the measured valves of ATP/ADP ratios in leaf tissue, to determine the potential regulatory influence of these ratios on *in vivo* respiration.

Following completion of the work for this chapter, a number of relevant references regarding ATP/ADP ratios in leaf cell compartments were published (Goller *et al.* 1982; Stitt *et al.* 1982; Hampp *et al.* 1982) and the implications of their results will be considered in the discussion section.

3.2. RESULTS

Isolated pea leaf and cauliflower mitochondria demonstrated good respiratory control and ADP/O ratios of 2.1-2.2 and 2.4-2.5 respectively, with malate as substrate.

3.2.1. *ADP-generating system*

The addition of creatine kinase to cauliflower and pea leaf mitochondria, in the presence of creatine and ATP (either added or generated by oxidative phosphorylation) led to a stimulation of O_2 uptake (Fig. 3.1; A,C). No stimulation was observed in the absence of creatine (Fig. 3.1; B). Furthermore, the O_2 uptake induced by creatine kinase was sensitive to both oligomycin and carboxyatractyloside (Fig. 3.1; A,C) showing firstly, that it was due to a stimulation of oxidative phosphorylation through the production of ADP and, secondly, that all ADP entry into the mitochondria was via the AdN translocator. Hexokinase has also been used successfully as an extramitochondrial ADP-generating system with animal mitochondria (Küster, *et al.* 1976). However, it was not suitable for use with pea leaf mitochondria, due to the presence of an inherent hexokinase activity associated with the mitochondrial fraction. This mitochondrial hexokinase activity was investigated further in Chapter 8.

Figure 3.2 illustrates the use of creatine kinase, in the presence of creatine and ATP, for the generation of various respiratory rates between state 2 (designated here as O_2 uptake in the presence of substrate only with no added ADP) and state 3 (as determined by the addition of excess ADP in the absence of ATP). The stimulation of O_2 uptake was linear with creatine kinase activity until state 3 was approached, at which point ADP supply from the ADP-generating system was no longer limiting oxidative phosphorylation.

The creatine kinase system has been criticized (Brawand *et al*. 1980) on the basis that the concentration of inorganic phosphate (Pi) is not constant and decreases during the incubation period through the formation of phosphocreatine,as shown below:

creatine + ATP $\rightleftharpoons$ phosphocreatine + ADP

At the most only 7-10% of the Pi initially present in my system (10 mM) would be utilized during the incubation period, prior to sampling. Data will be presented later in the chapter, to show that decreases in the Pi level of this magnitude have no effect on the respiratory rate observed in the presence of a particular ATP/ADP ratio.

3.2.2. *Effect of ATP on ADP-dependent respiration*

Figure 3.3 illustrates the response of respiratory O_2 uptake,by pea leaf mitochondria,to increasing ADP concentration in the presence of various levels of ATP. Steady state ADP concentrations and respiratory rates were generated with the creatine kinase

system. Rates of electron flow via the three phosphorylation sites of the respiratory chain were maximized by the addition of both malate and glycine as substrates (see section 4.2.1). The ATP or adenylate levels chosen for examination approximated the range of published values of extrachloroplastic adenylate levels in leaf tissue (Keys and Whittingham, 1969; Sellami, 1976; Stitt *et al*. 1980). It should be noted that while adenylate refers to (ATP + ADP), in most cases due to the much higher concentrations of ATP than ADP, the terms ATP and adenylate are interchangeable.

The results indicate that ATP does have an inhibitory effect on the rate of ADP-stimulated oxygen uptake, but that the extent of the inhibition is both small and very much influenced by the absolute ADP concentration. For example, at the low ADP concentration of 8.5 µM, increasing the level of ATP from 240 to 1600 µM resulted in a 23% decrease in the respiratory rate. This inhibitory effect was decreased at 30 µM ADP however, with a similar increase in ATP concentration causing only a 10% decrease in the rate of O_2 uptake. Thus, as the amount of ADP present increased, so the inhibitory effect of ATP on the respiratory rate was decreased; suggestive of a competitive type inhibition of ATP on ADP uptake. The apparent $K\frac{1}{2}$ for ADP of oxidative phosphorylation, at the lowest adenylate concentration,was estimated at between 6-8 µM which compares with the value of 13 µM measured by Davis and Lumeng (1975) for rat liver mitochondria.

Similar results were obtained with mitochondria isolated from non-green tissue (cauliflower) as shown in Fig. 3.4. As with

pea leaf mitochondria, the concentration of ADP required to obtain a particular rate of state 3 respiration, increased as a function of the total adenylate or ATP concentration.

3.2.3. *Effect of ATP/ADP ratios on respiration*

The effect of the steady-state extramitochondrial ATP/ADP ratio on the rate of O_2 uptake is shown in Fig. 3.5. As reported previously for animal mitochondria, increases in the external ATP/ADP ratio led to a decrease in the state 3 respiratory rate of pea leaf mitochondria. However, as observed by Davis and Lumeng (1975) this control is not absolute; since for a given ATP/ADP ratio, the inhibitory effect on respiration varies with changes in the adenylate concentration. Thus, while an ATP/ADP ratio of 60 produces a 50% inhibition of respiratory O_2 uptake at an adenylate concentration of 0.24 mM, there is little effect of this ratio at 1.6 mM. Clearly then, other factors are important in determining the sensitivity of mitochondrial respiration to the external ATP/ADP ratios. Even so, it can be seen that at all adenylate levels examined, marked inhibition of respiration was only found to occur at values of the ATP/ADP ratio of approximately 20 or greater; ratios below this value having little or no effect on the respiratory rate.

While fewer samples were taken at the higher range of respiratory rates for cauliflower mitochondria, the similarity in the influence of the ATP/ADP ratio on respiration (Fig. 3.6) between these two types of mitochondria is apparent, with the sensitivity to the external ATP/ADP ratio increasing as the adenylate level decreased.

3.2.4. *Effect of Mg^{2+}*

It is known that the AdN translocator specifically transports the uncomplexed (with respect to Mg^{2+} ions) forms of ADP and ATP (Pfaff *et al.* 1969). Furthermore, ATP complexes Mg^{2+} more strongly than does ADP (Burton, 1959) such that at saturating concentrations of Mg^{2+}, most of the ATP would be present in the Mg ATP form, unable to compete with uncomplexed ADP for access to the translocator. As data for the previous figures were collected from mitochondria incubated in the presence of excess levels of Mg^{2+} (5 mM), it was necessary to test the effect of lower Mg^{2+} concentrations on the inhibitory influence of ATP/ADP ratios on the respiratory rates. Figure 3.7 illustrates the effect of decreasing the Mg^{2+} concentration in the reaction medium from 5 to 0.5 mM in the presence of a constant adenylate concentration. Considering that maximal state 3 rates were, on average, 7% lower for mitochondria incubated with 0.5 mM Mg^{2+} than they were with 1 or 5 mM Mg^{2+}, it can be seen that decreasing the Mg^{2+} concentration in the medium has had little effect on the sensitivity of the respiratory rate to changes in the ATP/ADP ratio. More significant effects may have been observed had treatments been included in which the Mg/ATP ratio was less than unity. It is unlikely, however, from present knowledge of the relative concentration of both Mg^{2+} (1-4 mM; Wyn-Jones *et al.* 1979) and ATP (Stitt *et al.* 1980) *in vivo*, that the ratio would normally fall below unity in the cytoplasm of leaf cells and, consequently, such treatments would have limited physiological significance.

3.2.5. *Effect of Pi*

It has been suggested by various groups working with animal mitochondria (Owen and Wilson, 1974; Holian *et al*. 1977; Erecinska *et al*. 1977) that oxidative phosphorylation or respiration rate is controlled, not by the extramitochondrial ATP/ADP ratio, but by the phosphorylation potential or ATP/ADP.Pi ratio. Thus, phosphate concentration may also influence the extent of the inhibitory effect of ATP/ADP ratios on the respiratory rate.

Figure 3.8 illustrates the Pi requirements of isolated pea leaf mitochondria for maximal state 3 rates of oxidative phosphorylation. Maximal state 3 rates were almost maximal in the presence of 1 mM Pi, but were markedly inhibited at 0.5 mM Pi. Therefore any effects of low phosphate (1 mM) on the extent of the inhibitory effect of ATP/ADP ratios on respiration, could be attributed to changes in the phosphorylation potential and not to direct phosphate limitations of oxidative phosphorylation. Figure 3.9 compares the respiratory response of pea leaf mitochondria to varying ATP/ADP ratios, in the presence of 10 mM and 1 mM Pi. The results indicate that reducing the phosphate concentration 10 fold, had little effect on the general relationship between the ATP/ADP ratio and the respiratory rate.

3.3. DISCUSSION

A unique feature of the operation of the AdN translocator in the inner mitochondrial membrane is its regulation by the membrane potential (Klingenberg, 1979). The protein itself demonstrates no

intrinsic preference for either ADP or ATP as the transportable substrate, as is well illustrated by the symmetrical exchange of these nucleotides across the inner membrane of 'uncoupled' mitochondria. In normal energized mitochondria however, the exchange becomes highly asymmetric: ADP^{3-} in for ATP^{4-} out. This particular electrogenic exchange results in the effective movement of one negative charge from the matrix to the extramitochondrial space, which is the highly favoured exchange reaction under the influence of mitochondrial membrane potential.

Souverijn *et al.* (1973) have shown that the affinity of the AdN translocator for ADP and ATP is dependent on the energy state of the mitochondria. Thus, while the Km of the translocator for $ADP_{(in)}$ (1-3 μM) remains constant, the Km for $ATP_{(in)}$ increases dramatically (from 1 μM to 150 μM) upon energization of the mitochondria. This effect may be mediated through changes in the conformation of the AdN translocator protein (Souverijn *et al.* 1973; Vignais and Lauquin, 1979). However, ATP may still compete with ADP for access to the carrier, under these conditions, provided a sufficiently large extramitochondrial ATP/ADP ratio is imposed (Davis and Lumeng, 1975; Küster *et al.* 1976; Kunz *et al.* 1981).

Similarly, the results presented here show that ATP may compete with ADP for uptake into plant mitochondria. Figure 3.3 illustrates that the rate of respiration is dependent, not only on the availability of ADP, but also on the external concentration of ATP. Conversely, the extent of the influence of ATP on ADP uptake or ADP-stimulated respiration will be a function of the ADP

concentration as shown by the decreasing inhibition of O_2 uptake by ATP with increases in the level of ADP (Fig. 3.3). Consequently, the influence of the ATP/ADP ratio on respiration (Fig. 3.5) will be the results of the interaction of a number of different factors affecting ADP uptake. Firstly, the numerical value of the ratio, in concert with the total adenylate concentration, will determine the absolute concentration of ADP available for uptake by the AdN translocator. Secondly, the value of the ATP/ADP ratio will, through the competition between ADP and ATP for access to the translocator, regulate what proportion of this ADP may actually be transported into the mitochondria for use in the oxidative phosphorylation reaction.

From a consideration of these factors, one can propose explanations for the apparent differences in sensitivity of the respiratory rate, to changes in the ATP/ADP ratio at different adenylate levels (Figs 3.5, 3.6). At low adenylate concentrations, the limiting amounts of ADP present will mean that small increases in the ATP/ADP ratio, while only causing small changes in the absolute concentration of ADP, will result in large alterations of the respiratory rate as shown in Fig. 3.3. At the high adenylate levels however, the presence of a much larger ADP pool (for the same ATP/ADP ratio) will mean that increases in the ratio, which result in decreases in the ADP concentration, will have significantly less effect on the ADP-stimulated respiratory rate (Fig. 3.3.). Therefore, at these levels, inhibition of respiration by increased ATP/ADP ratios is, in constrast to the lower adenylate levels, due to increased competition for uptake between these two adenylate species.

In summary, it can be seen that plant mitochondrial respiration is not controlled by the extramitochondrial ATP/ADP ratio, but as for animal mitochondria, is a function of the rate of transport of ADP into the mitochondrial matrix (Jacobus *et al.* 1982) as mediated by (a) the kinetic properties of the adenine nucleotide translocator and (b) the availability of ADP.

The results of Fig. 3.5 show a close parallel to the results obtained with animal mitochondria in that respiration was only inhibited at ATP/ADP ratios exceeding approximately 20; independent of the level of adenylates present. Thus, the ratios required for significant inhibition of respiration *in vitro*, are far in excess of those *in vivo* ratios (between 2 and 8) measured in the extrachloroplastic fractions of light-treated leaf tissue (Heber and Santarius, 1970; Keys and Whittingham, 1969; Sellami, 1976; Stitt *et al.* 1980). This indicates that competitive inhibition of ADP uptake by ATP, under our experimental conditions, does not appear to be sufficiently effective to cause an inhibition of mitochondrial respiration in intact leaf tissue. This also appears to be the case in the presence of very low phosphate concentrations (Fig. 3.9), which suggests that even at concentrations as low as 1 mM, sufficient phosphate is transported into the mitochondrial matrix, via the phosphate translocator, to maintain an internal phosphorylation potential favourable for ATP synthesis. Indeed, recent work by Rebeille *et al.* (1983) suggests that mechanisms may exist, within cells, to maintain the cytoplasmic Pi concentration at a reasonably constant level, despite large changes in total

cellular Pi. Using ^{31}P-NMR techniques, they estimated the cytoplasmic Pi concentration of sycamore cells to be of the order of 6 mM which compares to the measurement of 9 mM made by Ullrich *et al.* (1965) for the cytoplasmic compartment of spinach leaves.

It could be argued that to date, methods employed in the estimation of *in vivo* cytoplasmic ATP/ADP ratios have been not only too slow, but also too imprecise,with the combining of the cytoplasmic and mitochondrial pools into one extrachloroplastic fraction. Recently, techniques have been developed which allow for the rapid separation of chloroplasts, mitochondria and the cytoplasmic compartment from protoplasts (Lilley *et al.* 1982; Hampp *et al.* 1982). Estimations of the cytoplasmic ATP/ADP ratio during dark-light transitions with these new techniques however, do not support the inhibition hypothesis. For example, Hampp *et al.* (1982) demonstrated that the cytoplasmic ATP/ADP ratio in oat protoplasts rose from 1.5. to 8.3 in the first 60 s following exposure to light, but that this increase was only transient,with the ratio returning to a value of 2.4 after 5 min illumination. Furthermore, Stitt *et al.* (1982) found the cytosolic ATP/ADP ratio of wheat protoplasts to actually be higher in the dark (9.2) than in the light (6.5). A later publication (Stitt *et al.* 1983),using the same technique confirmed the value of the cytoplasmic ratio to be of the order of 7-8, but found it to be virtually unaffected by light - dark transitions. Clearly the observations above, together with the data presented in this chapter, refute the hypothesis that the mitochondrial electron transport chain in photosynthetic tissue is inhibited in the light through increases in the cytoplasmic ATP/ADP ratio.

If respiration is switched off in leaf tissue, in the light, the question remains as to what the possible mechanism of inhibition could be. Based on the estimates of Stitt *et al.* (1983) of cytoplasmic ADP levels of 5-10 nmol/mg Chl and cytosolic volumes of 10-12 µl/mg Chl (R. Douce cited as a personal communication in Stitt *et al.* 1983) the concentration of ADP in the cytoplasm would be of the order of 0.4-1.0 mM. A comparison of these figures with the ADP requirements of isolated mitochondria (Figs 3.3 and 3.4) suggests that the mitochondrial respiratory chain is unlikely to be limited *in vivo* by ADP supply. Indeed, for the ATP/ADP ratios measured (< 10), this concentration of ADP in the cytoplasm would mean that in the absence of a light mediated inhibitory mechanism, the mitochondrial respiratory chain could operate *in vivo* at maximal state 3 rates, provided substrate was available.

Stitt *et al.* (1982) suggested that their measurements of the extramitochondrial and intramitochondrial ATP/ADP ratios, in wheat protoplasts, indicated a light-mediated de-energisation of the mitochondrial membrane potential. It should be noted, however, that mechanisms for the inhibition of mitochondrial respiration in the light which lead to a dissipation of the membrane potential (for example through an inhibition of electron flow or an inhibition of the supply of reducing equivalent to the chain) will, in so doing, stimulate the breakdown of cytoplasmic ATP by the mitochondrial ATPase enzyme. Consequently, unless mitochondria have a specific role in the leaf cell in the light, to act as ATP-energy 'sinks', it is necessary that the inhibitory mechanism act in such a way as to allow the membrane potential to be maintained, or alternatively, cause a direct inhibition of the mitochondrial ATPase.

It is important to remember that there is still no definitive evidence to show that the mitochondrial respiratory chain in photosynthetic tissue is inhibited in the light. The work of Goller *et al.* (1982), for example, indicates that light has no direct effect on the mitochondrial respiratory chain and that the rate of antimycin-sensitive electron transport is potentially equal under light and dark conditions. If the mitochondrial respiratory chain does operate in the light, it is likely that NADH generated within the mitochondrial matrix will be re-oxidized via the chain and not be available for transport to, and subsequent utilization in, reduction reactions outside of the mitochondria. Thus, questions are raised as to possible alternative sources of NADH for nitrate reduction (see Reed and Canvin, 1982) and photorespiratory hydroxypyruvate reduction. Also of importance is the possible influence of TCA cycle turnover in the light (Graham, 1980) on the potential re-oxidation, by the mitochondrial respiratory chain, of NADH generated during photorespiratory glycine oxidation. This question will be considered in the next chapter.

Figure 3.1. Stimulation of respiratory O_2 consumption by creatine kinase.

Cauliflower (A,B) and pea leaf (C) mitochondria (0.5 mg protein) were added to 2.5 ml of reaction medium (section 2.8) containing 5 mM $MgCl_2$, 7 mM malate, 7 mM glutamate and 0.1 mM TPP. Additions as indicated: 30 mM creatine, 0.24 mM ATP, 0.18 mM ADP, 2 units creatine kinase (CK), 20 µM carboxyatractyloside (CAT) and 5 µg of oligomycin. Rates shown represent nmol O_2 consumed mg^{-1} protein min^{-1}.

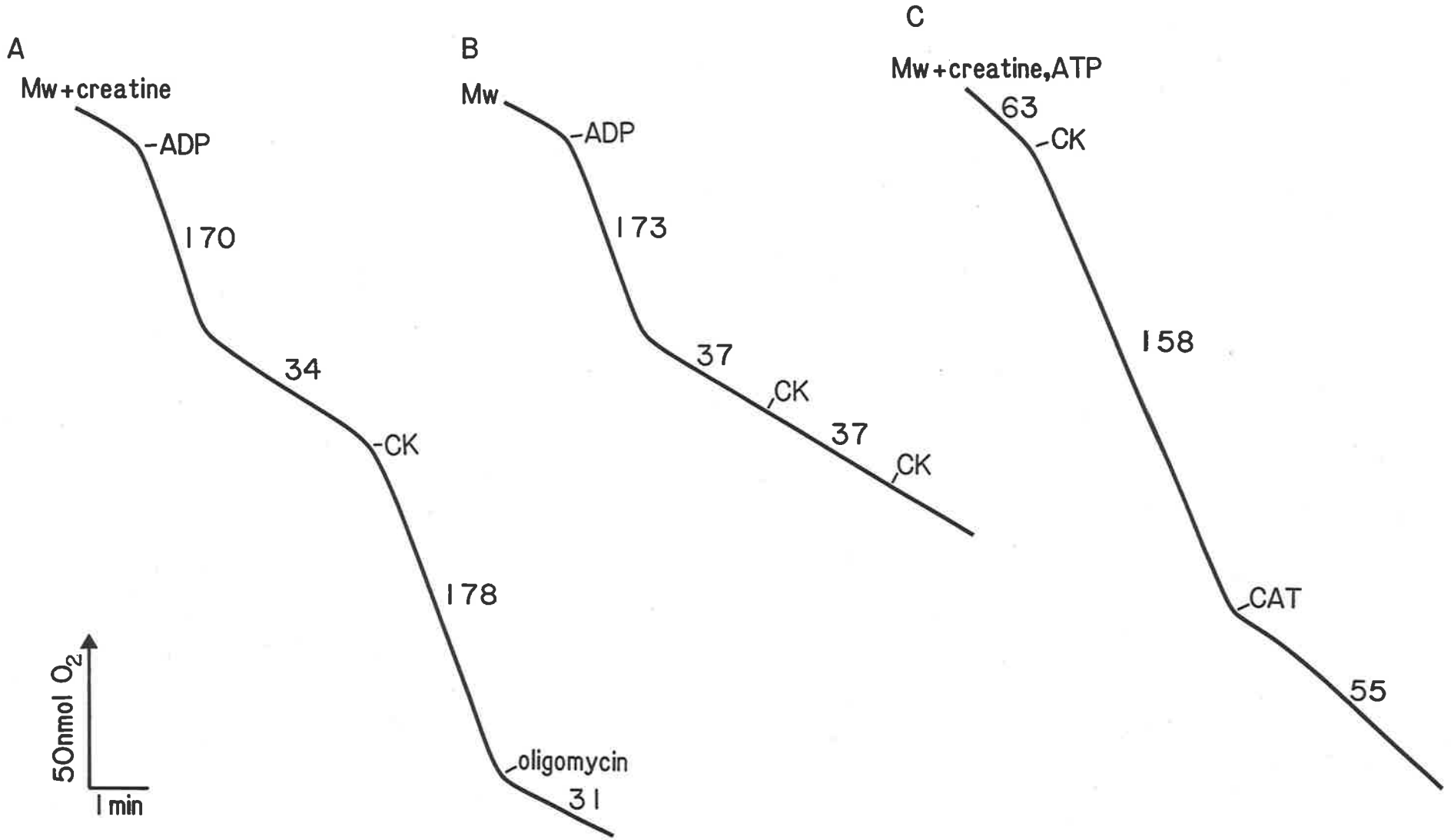

A
Mw+creatine
-ADP
170
34
-CK
178
oligomycin
31
50nmol O_2
1 min
B
Mw
-ADP
173
37
CK
37
CK
C
Mw+creatine,ATP
63
CK
158
CAT
55

Figure 3.2. Generation of various respiratory rates by creatine kinase.

Cauliflower mitochondria (0.5 mg protein) were incubated for 2-3 min with 7 mM malate, 7 mM glutamate, 0.1 mM TPP, 30 mM creatine and 2 mM ATP. Various intermediate respiratory rates were initiated by the addition of creatine kinase (40 units/ml).

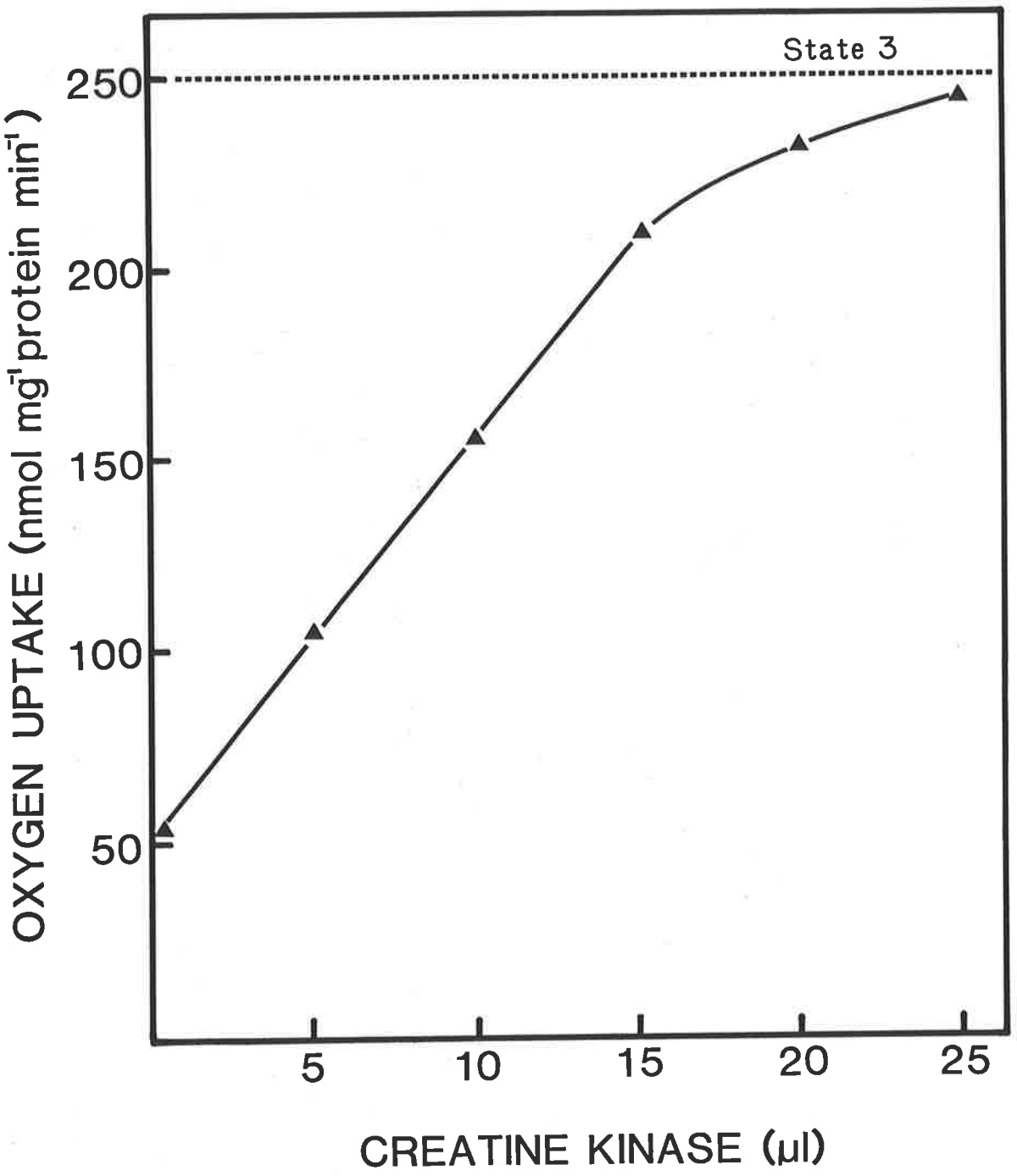

State 3
250
200
150
100
50
OXYGEN UPTAKE (nmol mg⁻¹ protein min⁻¹)
5
10
15
20
25
CREATINE KINASE (µl)

Figure 3.3. Effect of ATP concentration on the rate of ADP-dependent respiration in pea leaf mitochondria.

Mitochondria (0.8 mg protein) were incubated in 2.5 ml reaction medium (section 2.8) containing 5 mM $MgCl_2$, 10 mM malate, 10 mM glutamate, 10 mM glycine, 0.1 mM TPP and 30 mM creatine to which varying amounts of ATP were added: 0.24 mM (●), 0.8 mM (▲), 1.6 mM (○). Different respiratory rates were initiated with creatine kinase as shown in Fig. 3.2, and samples of the extra-mitochondrial solution removed and analysed as described in sections 2.7 and 2.11. The state 3 rate was 160 nmol O_2 consumed mg^{-1} protein min^{-1}.

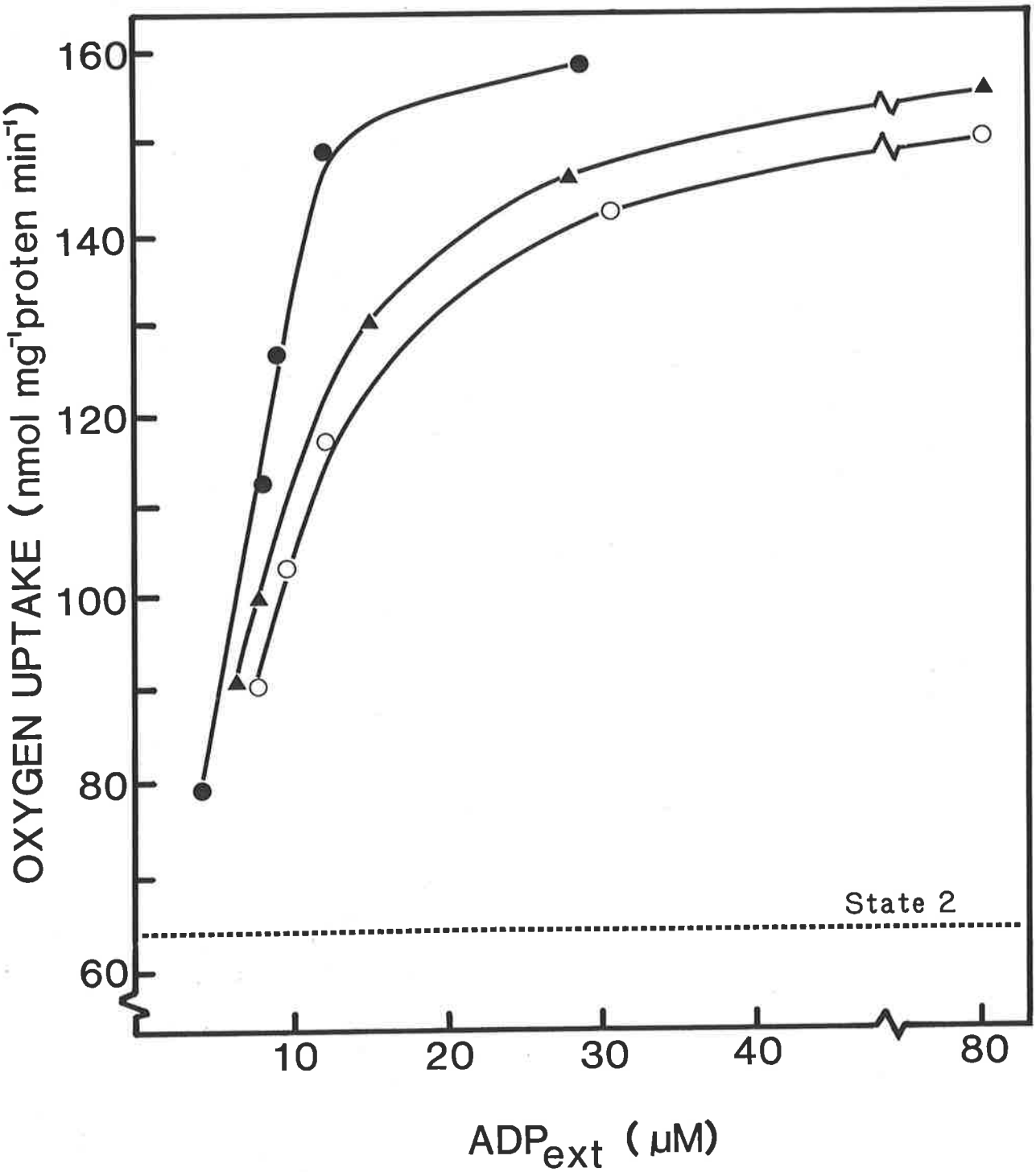

160
140
120
100
80
60
OXYGEN UPTAKE (nmol mg⁻¹proten min⁻¹)
State 2
10
20
30
40
80
ADPext (µM)

Figure 3.4. Effect of ATP concentration on the rate of ADP-dependent respiration in cauliflower mitochondria.

Mitochondria (0.5 mg protein) were incubated in 2.5 ml reaction medium (section 2.8) containing 5 mM $MgCl_2$, 10 mM malate, 10 mM glutamate, 0.1 mM TPP and 30 mM creatine to which varying amounts of ATP were added: 0.27 mM (▲), 1.6 mM (△), 2.4 mM (■). Other experimental procedures were followed as described in Fig. 3.2. The state 3 rate was 247 nmol O_2 consumed mg^{-1} protein min^{-1}.

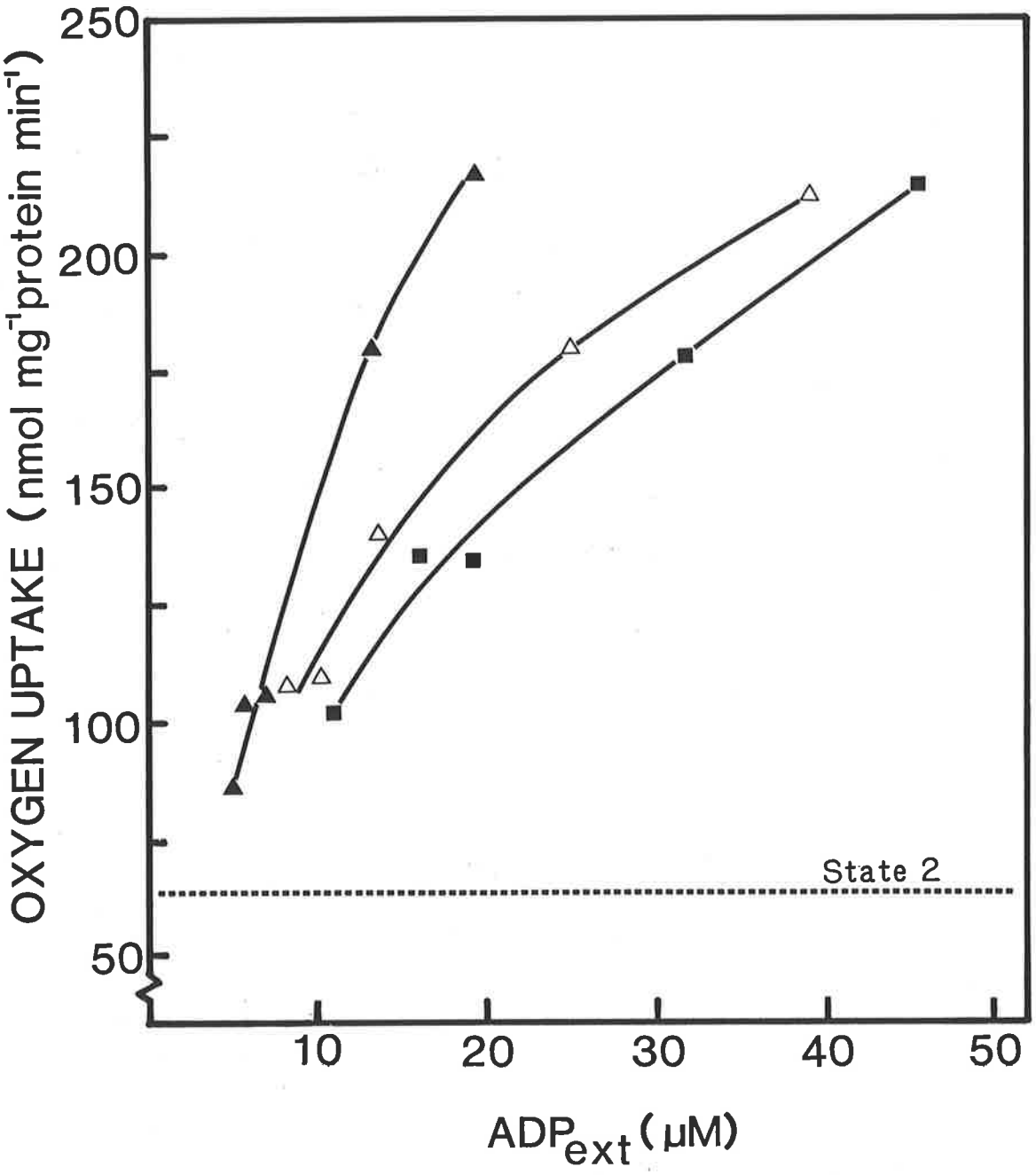

250
200
150
100
50
OXYGEN UPTAKE (nmol mg-1protein min-1)
State 2
10
20
30
40
50
ADPext (μM)

Figure 3.5. Effect of the extramitochondrial ATP/ADP ratio on the respiratory rate of pea leaf mitochondria.

Experimental procedures as given in Fig. 3.3. Data taken from 3 separate experiments with varying total adenylate concentrations: (a) 0.24 mM(●); 0.8 mM (▲); 1.6 mM (○); (b) 0.45 mM (■); (c) 0.60 mM (△). Oxygen uptake rate is expressed as a percentage of the state 3 rate.

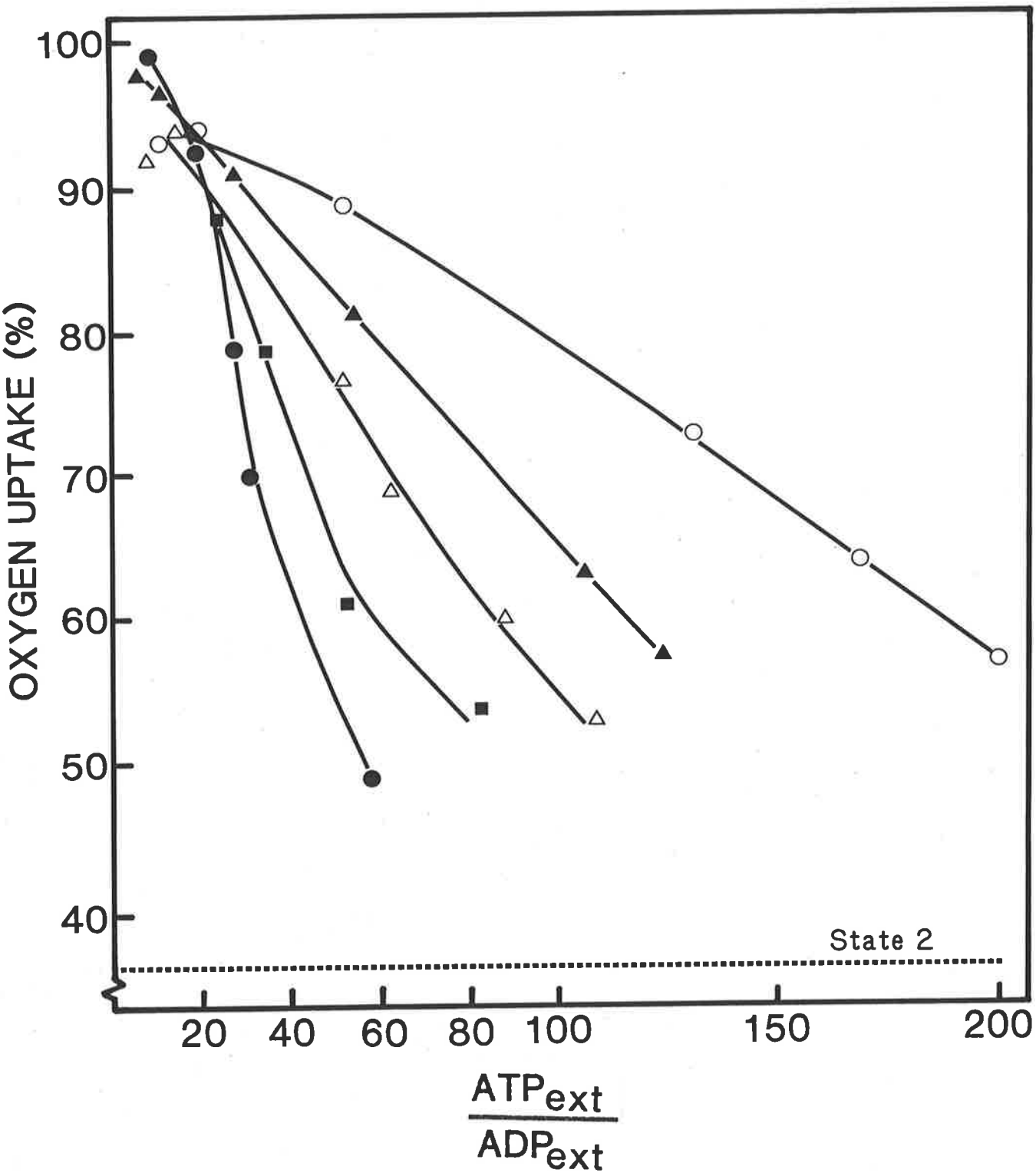

100
90
80
70
60
50
40
OXYGEN UPTAKE (%)
State 2
20
40
60
80
100
150
200
ATPext / ADPext

Figure 3.6. Effect of the extramitochondrial ATP/ADP ratio on the respiratory rate of cauliflower mitochondria.

Experimental procedures as given in Fig. 3.4. Total adenylate concentrations: 0.27 mM (▲); 1.6 mM (△); 2.4 mM (■). Oxygen uptake rate expressed as a percentage of the state 3 rate.

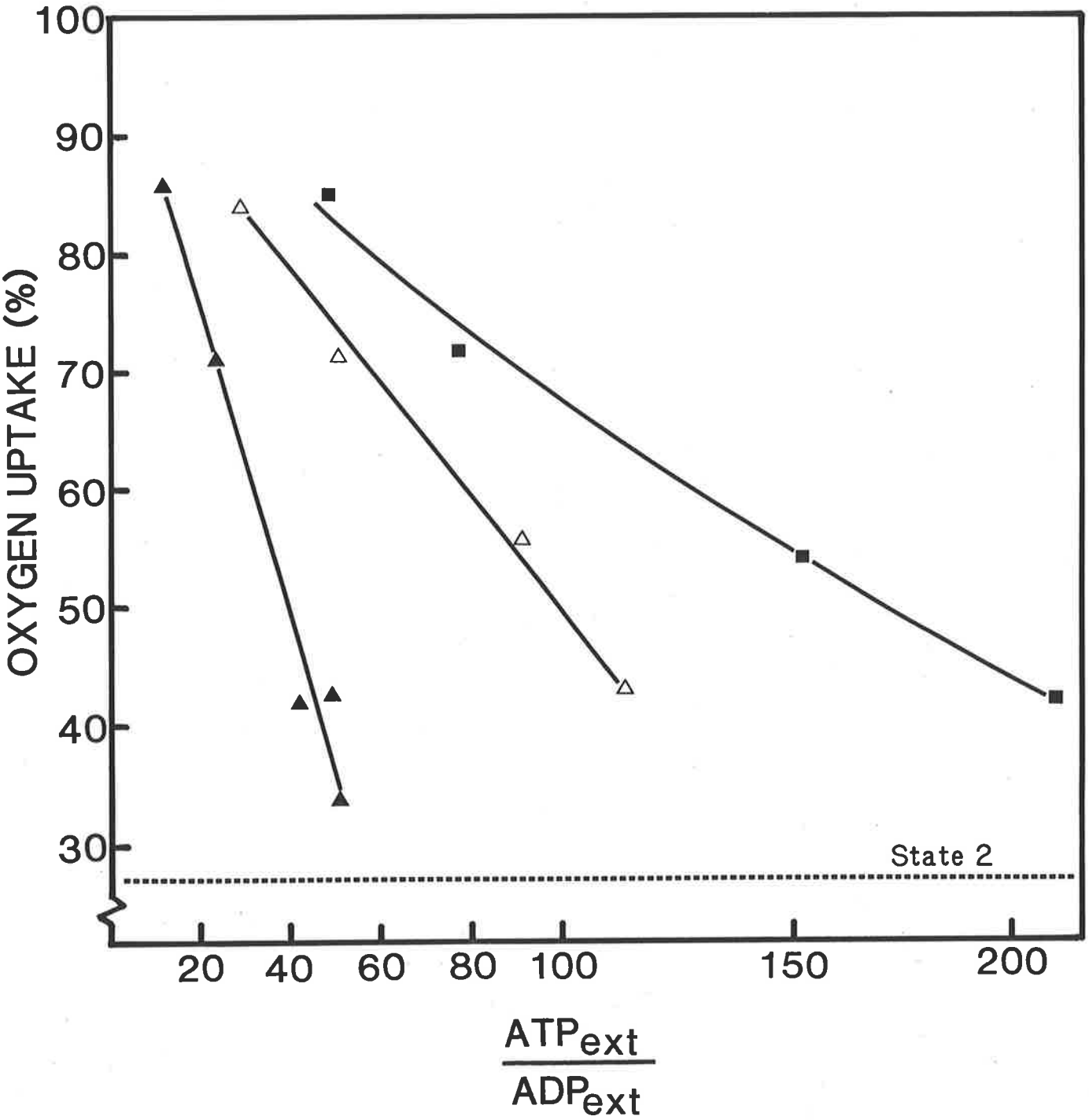

100
90
80
70
60
50
40
30
OXYGEN UPTAKE (%)
State 2
20
40
60
80
100
150
200
ATPext / ADPext

Figure 3.7. Effect of Mg^{2+} concentration on the respiratory rate of pea leaf mitochondria in the presence of various ATP/ADP ratios.

Experimental procedures as described in Fig. 3.3 except that the concentration of ATP in all treatments was 0.45 mM, while the Mg^{2+} concentration of the reaction medium was varied: 0.5 mM (○), 1.0 mM (▲); 5.0 mM (●). State 3 rate was 119 nmol O_2 consumed mg^{-1} protein min^{-1}.

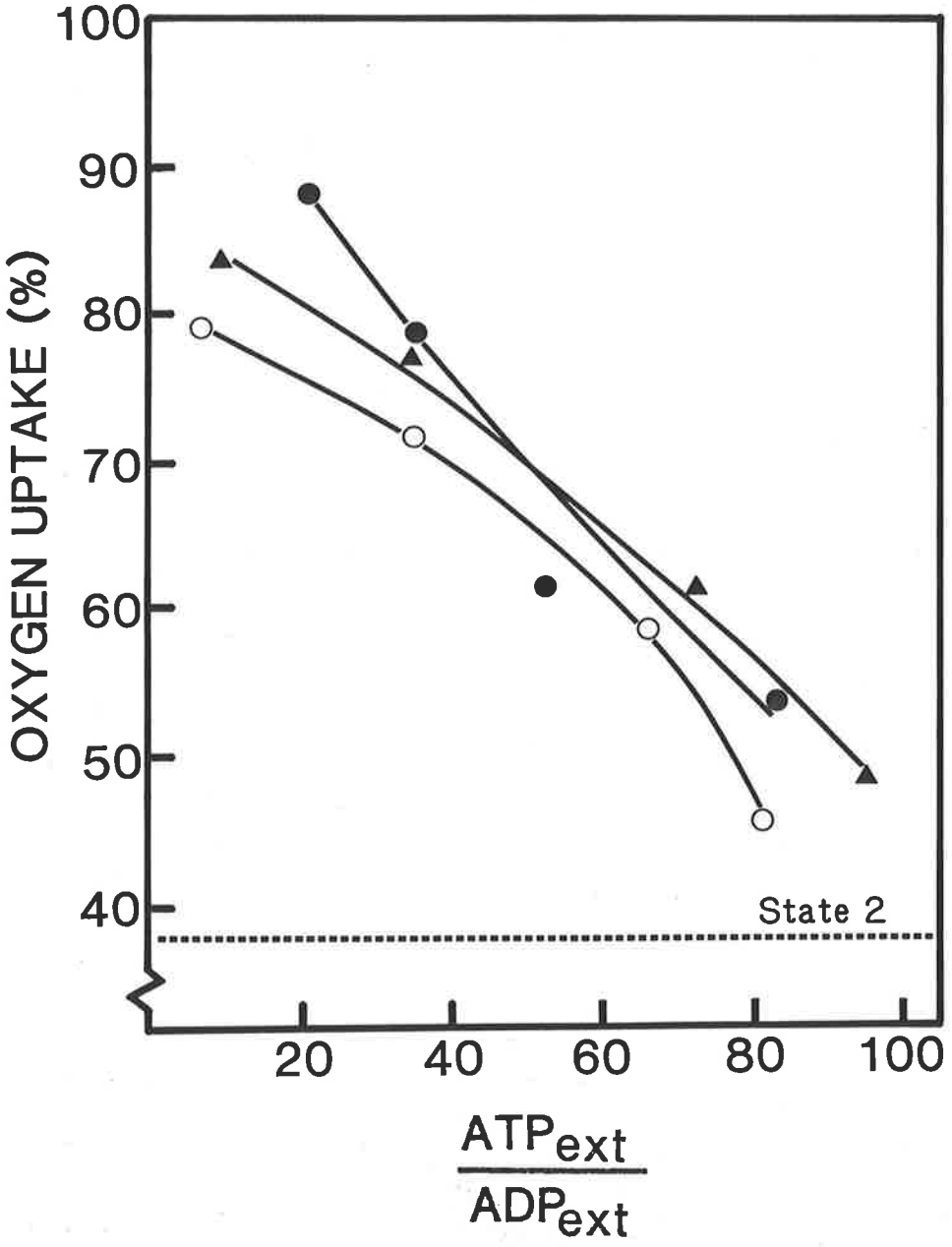

100
90
80
70
60
50
40
OXYGEN UPTAKE (%)
State 2
20
40
60
80
100
ATPext
ADPext

Figure 3.8. Effect of Pi concentration on the maximal state 3 respiratory rates of pea leaf mitochondria.

Mitochondria (0.4 mg protein) were added to 2.6 ml of reaction medium (section 2.8) containing 5 mM $MgCl_2$, 10 mM malate, 10 mM glutamate, 0.1 mM TPP and varying levels of Pi. Total buffer concentration was maintained at 20 mM by the addition of variable amounts of Tes buffer. Additions as indicated: 0.20 mM ADP. Rates shown represent nmol O_2 consumed mg^{-1} protein min^{-1}.

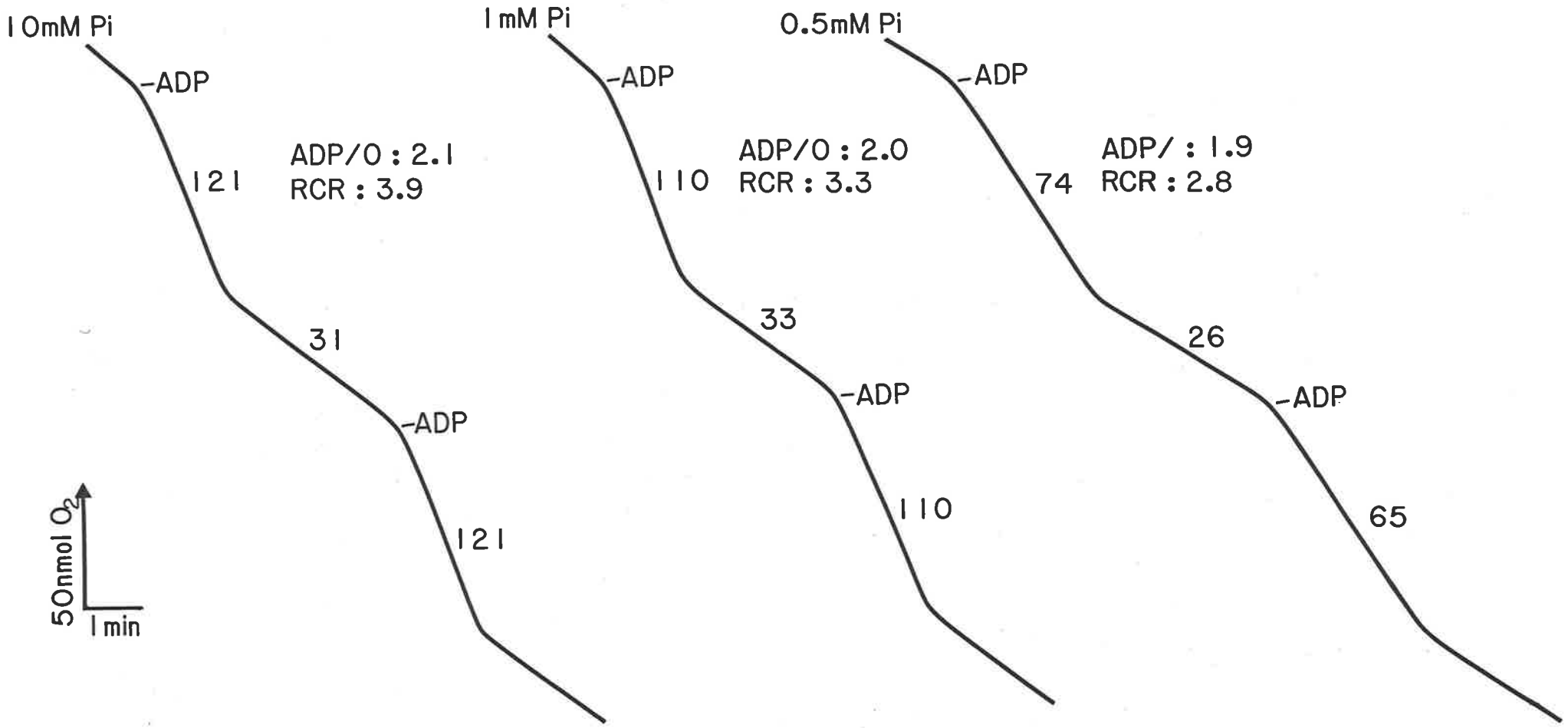
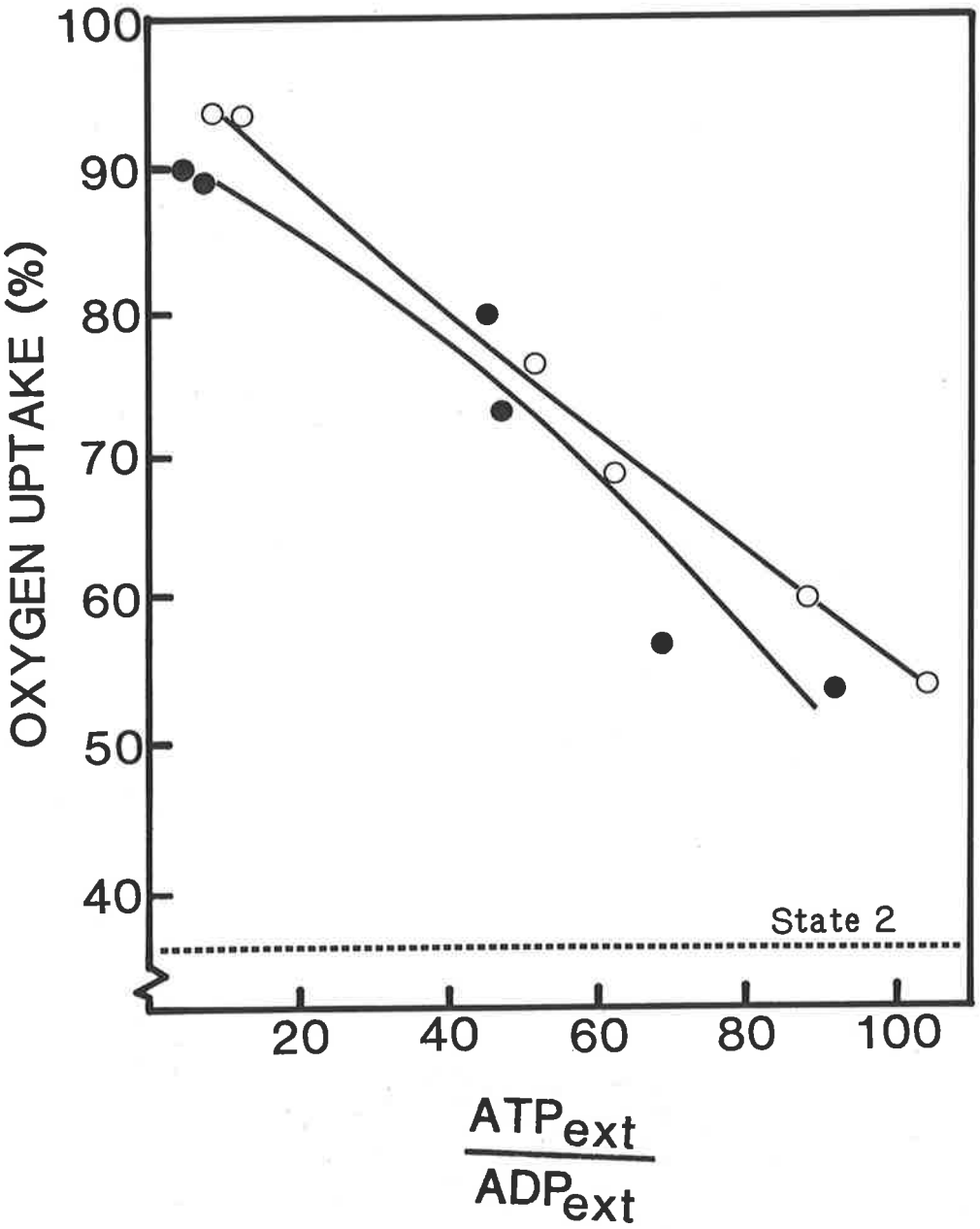

10mM Pi
-ADP
121
ADP/O : 2.1
RCR : 3.9
31
-ADP
121
50nmol O2
1 min
1mM Pi
-ADP
110
ADP/O : 2.0
RCR : 3.3
33
-ADP
110
0.5mM Pi
-ADP
74
ADP/ : 1.9
RCR : 2.8
26
-ADP
65

Figure 3.9. Effect of Pi concentration on the respiratory rate of pea leaf mitochondria in the presence of various ATP/ADP ratios.

Experimental procedures were followed as described in Fig. 3.3 except that the ATP concentration in all treatments was 0.6 mM while the Pi concentration of the reaction medium was varied: 1 mM (●), 10 mM (○). Total buffer concentration was maintained at 20 mM as described in Fig. 3.8. State 3 rate was 149 nmol O_2 consumed mg^{-1} protein min^{-1}.


100
90
80
70
60
50
40
OXYGEN UPTAKE (%)
State 2
20
40
60
80
100
ATPext
ADPext

CHAPTER IV

PREFERENTIAL OXIDATION OF GLYCINE BY THE RESPIRATORY CHAIN

4.1. INTRODUCTION

In this chapter, the potential competitive effects of TCA cycle substrate oxidation on glycine oxidation by the respiratory chain of pea leaf mitochondria are investigated. Also, estimates of tissue rates of glycine decarboxylase activity (which could be supported by the mitochondrial respiratory chain of pea leaves), are compared to current estimates of *in vivo* photorespiratory cycle activity.

4.2. RESULTS

Mitochondria used in these experiments demonstrated ADP/O ratios of 2.0-2.2 with glycine and 2.2-2.5 with malate as substrates. With the exception of experiments detailed in section 4.2.4., alternate pathway activity did not exceed 15% of the total oxidative capacity of the mitochondria used.

4.2.1. *Effect of respiratory substrates on glycine oxidation under state 3 conditions*

Figure 4.1 demonstrates the extent of the potential competition which could arise between substrate oxidations linked to the respiratory chain. As indicated by the non-additive increase in O_2 uptake following substrate addition (based on the summation of the individual substrate rates), competition to glycine

oxidation via the respiratory chain could arise at two levels. Firstly, at the NADH dehydrogenase level, between glycine decarboxylase and the NAD-linked dehydrogenases of the TCA cycle and secondly, at the chain level, from succinate dehydrogenase and external NADH dehydrogenase. The competition which exists between NAD-linked substrates specifically will also be considered in Chapter 5.

The effect of these respiratory substrates on glycine oxidation (via the respiratory chain) can be measured through the use of an NH_3 electrode to follow glycine oxidation specifically. The use of the NH_3 electrode is demonstrated in Table 4.1, which shows state 3 - state 4 transitions in O_2 uptake and NH_3 release during glycine oxidation by pea leaf mitochondria. Ratios of O_2 uptake to NH_3 release were of the order of 1:2 as predicted from theoretical considerations (see section 1.2.3.)

The effects of various respiratory substrates on glycine oxidation in state 3, are shown in Table 4.2. The 'predicted' rate of O_2 uptake represents the summation of O_2 uptake rates as measured with the individual substrates. The use of an ADP-generating system (glucose + hexokinase) allowed for the maintenance of the state 3 condition over extended periods of time (e.g. 15-20 min), thus ensuring that the respiratory substrates attained their maximum rates of oxidation following their addition to the reaction medium. It can be seen that glycine oxidation in state 3 was unaffected by the presence of other respiratory substrates. Oxygen uptake, however, was markedly stimulated, demonstrating that concurrent oxidation of substrates did occur.

The discrepancy between the predicted rate and the observed rate of O_2 uptake indicates the extent of inhibition of oxidation of the other respiratory substrates in the presence of glycine (in no case was NH_3 release from glycine inhibited). The rate of malate oxidation, for example, was inhibited 30% in the presence of glycine. Similarly, external NADH oxidation was inhibited approximately 27% in the presence of glycine (Table 4.3), although concurrent oxidation of succinate, malate or 2-OG, also inhibited external NADH oxidation. The presence of $CaCl_2$, which has been observed to activate external NADH oxidation by pea leaf mitochondria (Nash and Wiskich, 1983), had no effect on the relative degree of inhibition of external NADH oxidation caused by these substrates (Table 4.4). In the presence of multiple substrates, not all of the difference between the predicted and observed rates of O_2 uptake could be accounted for by decreases in external NADH oxidation alone. Thus, the oxidation of other substrates e.g. succinate, malate was also inhibited (Table 4.3).

4.2.2. *Effect of respiratory substrates on glycine oxidation under state 4 conditions*

Wiskich (1980) has suggested that it is unlikely that cellular respiration would operate under state 3 conditions. Rather, it is probable that normal *in vivo* respiratory rates would be somewhere between the state 3 and state 4 rates, as limited by ADP supply. I therefore examined the effect of some respiratory substrates on glycine oxidation under state 4 conditions. The results in Table 4.5, clearly show that, even under these restricted respiratory conditions, glycine is preferentially oxidized by the electron trans-

port chain at the expense of the other respiratory substrates present. State 4 malate oxidation (as in state 3) was inhibited 30% upon the addition of glycine. The glycine state 4 rate, however, as judged by NH_3 release, was unaffected by the presence of other respiratory substrates.

4.2.3. *Re-oxidation of NADH by malate dehydrogenase*

It is well established that the addition of OAA to leaf mitochondria oxidizing glycine leads to a dramatic inhibition of respiratory O_2 uptake, while maintaining glycine oxidation at a similar or even increased rate (Woo and Osmond, 1976; Day and Wiskich, 1981; Journet *et al*. 1981). This is attributed to the rapid re-oxidation of NADH by MDH and re-cycling of the NAD to glycine decarboxylase. If such a system were to operate *in vivo*, one might expect there to be a specialized degree of interaction between the two enzyme systems involved (i.e. glycine decarboxylase and MDH), to ensure the efficient removal of reducing equivalents in this way. The results of Table 4.6, however, indicate that this does not appear to be the case, since OAA-dependent re-oxidation of NADH occurs with a range of mitochondrial substrates. This demonstrates that MDH has equal access to all sources of intramitochondrial NADH, regardless of which enzyme reduces the NAD. Thus, there appears to be a ubiquitous distribution of MDH within the matrix of pea leaf mitochondria.

4.2.4. *Tissue rates of glycine oxidation via the respiratory chain*

Part of the question regarding the possible coupling of glycine oxidation to the mitochondrial respiratory chain *in vivo*, is whether sufficient rates of oxidation could be maintained to account for the estimated rates of photorespiratory turnover. As pointed out in section 1.2.3., current estimates of respiratory-linked glycine oxidation, on a tissue basis, are only just sufficient to account for whole leaf photorespiratory rates (Woo and Osmond, 1976; Day and Wiskich, 1981). It has also recently been suggested that alternate pathway activity associated with the mitochondrial respiratory chain (Day *et al*. 1980) may have a role in glycine oxidation *in vivo* (Azcón-Bieto *et al*. 1983), particularly under conditions of ADP-limitation of the cytochrome chain (Wiskich, 1980).

It was therefore decided to re-examine the tissue estimates of respiratory chain-linked glycine decarboxylase activity in pea leaves, and investigate the potential contribution of the alternate pathway to photorespiratory glycine oxidation *in vivo*. Measurements were made on leaf tissue sampled from plants ranging from young emergent shoots (day 8; leaves not unfolded, only 20% of total chlorophyll differentiated) to mature plants (day 21; leaves fully developed and expanded), as this enabled a more reliable estimate of pea leaf glycine oxidative capacity to be made. Mitochondria were isolated from the leaf tissue and measurements of state 3, state 4 and alternate pathway activity in the presence of various respiratory substrates (including glycine) were made. These measurements were then extrapolated to tissue rates (see section 2.13) based on: (a) estimates of mitochondrial recovery

through comparisons of fumarase activity in the leaf homogenate and the mitochondrial pellet and (b) chlorophyll content of the leaf homogenate relative to the chlorophyll content of the original leaf tissue.

Figure 4.2 shows that the total respiratory capacity of pea leaf tissue decreased with age. The rates of malate (Fig. 4.2.), succinate (54 down to 14 µmol O_2 g^{-1} fwt h^{-1}) and external NADH oxidation (69 down to 28 µmol O_2 uptake g^{-1} fwt h^{-1}) were all found to decrease with tissue development. In contrast, the rates of alternate pathway activity, with these substrates, were found to increase initially and then remain constant over the period examined. Thus, the decrease in respiratory activity can be attributed to decreases in the total cytochrome pathway activity per g.fwt of tissue.

Glycine oxidative capacity was low in the young emergent tissue (day 8), which had a low chlorophyll concentration, and increased, in concert with chlorophyll development (Fig. 4.3), to a maximum tissue rate of 48 µmol O_2 uptake g^{-1} fwt h^{-1} at around day 11. Subsequently, the rate was maintained at a reasonably constant level of 30 µmol O_2 uptake g^{-1} fwt h^{-1} (Fig. 4.2). A similar pattern is observed for glycine activity if expressed on a µmol CO_2 evolved mg^{-1} Chl h^{-1} basis (i.e. 2 x CO_2 evolved per 1 x O_2 taken up). The initially high rate of activity measured at day 8, when the results are expressed on a chlorophyll basis, indicates the close relationship which exists between the development of chlorophyll or photosynthetic activity and glycine decarboxylase activity (compare Figs 4.2 & 4.3) in pea leaf tissue.

Apart from the peak in activity at around day 11 (which was a reproducible feature of these leaf development experiments), rates of glycine oxidation via the respiratory chain (under state 3 conditions) were found to be in the region of 20-30 μmol CO_2 mg^{-1} Chl h^{-1}. Indeed, samples from five separate groups of pea plants between the ages of 14-16 days gave a mean activity of 27.4 ± 2.7 μmol CO_2 mg^{-1} Chl h^{-1}. A similar assay of glycine decarboxylase activity in spinach leaf tissue gave a rate of 28.9 μmol CO_2 mg^{-1} Chl h^{-1}.

The maximal rates of glycine oxidation which could be supported by alternate pathway activity in pea leaf tissue were of the order of 5-8 μmol O_2 uptake g^{-1} fwt h^{-1} (Fig. 4.2). Malate, however, was oxidized via the alternate pathway at rates 30-50% higher than that achieved in the presence of glycine, indicating that glycine decarboxylase did not have access to all of the alternate pathway activity present in this tissue.

4.3. DISCUSSION

4.3.1. *Preferential oxidation of glycine via the respiratory chain*

Day and Wiskich (1981) originally reported that the addition of certain TCA cycle intermediates to mitochondria oxidizing glycine had little effect on the rate of glycine decarboxylation. The data presented in this chapter confirms this original observation and shows that pea leaf mitochondria preferentially oxidize glycine when confronted with a mixture of glycine and TCA cycle substrates (Table 4.2). Even under state 4 (ADP-limited) conditions, when competition between substrates for electron

transport could be expected to be most severe, the oxidation of glycine was not diminished (Table 4.5). Therefore, it appears that the electron transport chain of pea leaf mitochondria has an absolute preference for the NADH generated from glycine oxidation.

This preference also extends to the competition between external NADH and glycine (Tables 4.3, 4.4). It is known that external NADH oxidation is inhibited by intramitochondrial oxidation reactions in cauliflower and Jerusalem artichoke mitochondria (Day and Wiskich, 1977a; Cowley and Palmer 1980). The sensitivity of external NADH oxidation to intramitochondrial NADH oxidation may be linked to differences in the redox potential drop between the respective NADH dehydrogenases and ubiquinone, such that electron transport from the internal NADH dehydrogenase to ubiquinone is more highly favoured in a competitive situation.

The inhibitory effect of glycine on malate oxidation in pea leaf mitochondria (Table 4.2) has also been observed by Walker *et al.* (1982). They observed a 37% decrease in malate oxidation, under state 3 conditions, on the addition of glycine. However, in contrast to the results presented here (see Table 4.5), they did not find malate oxidation to be inhibited by glycine under state 4 conditions. There is no obvious explanation for the difference in these results, but it may be related to the fact that Walker *et al.* (1982b) measured the effect of glycine on malate oxidation via ME only, whereas the experiments carried out here involved malate oxidation via both MDH and ME.

Recently, Ericson *et al.* (1983) have provided evidence to suggest that a similar system of preferential glycine

oxidation is also present in spinach mitochondria. Their results indicated that glycine oxidation had preferred access to the mitochondrial electron transport chain in comparison to malate, succinate and external NADH and, thus, suggested that the oxidation of glycine must be more tightly coupled to the respiratory chain than these other substrates. The actual mechanism by which glycine is preferentially oxidized via the respiratory chain is yet to be elucidated and will be considered further in Chapter 5. However, it may be related to the close association of glycine decarboxylase with the respiratory chain,on the inner mitochondrial membrane.

It is essential that glycine oxidation be maintained for the continued operation of the photorespiratory cycle. In the face of competition from the TCA cycle dehydrogenases, it might be expected that the mechanism responsible for the continued oxidation of glycine *in vivo*, would show some preference for this photorespiratory intermediate. The results in Table 4.6 indicate that MDH, in the presence of OAA, represents a very general mechanism for the re-oxidation of intramitochondrial NADH, without any apparent preference for a particular substrate. On the other hand, the respiratory chain demonstrates a clear preference for the NADH generated during glycine oxidation. No similiar system of preferential glycine oxidation is present in animal mitochondria. Indeed, glycine oxidation by rat-liver mitochondria was severely restricted in the presence of respiratory substrates such as malate, succinate and 2-OG (Hampson *et al*. 1983).

It is therefore tempting to speculate that the system found in plant mitochondria has a specific role in photorespiratory metabolism and that oxidative decarboxylation of glycine *in vivo* is linked to the mitochondrial respiratory chain, rather than to a metabolic shuttle involving OAA and MDH. This proposal is supported by the recent findings of Oliver (1983) who showed that the light-dependent release of CO_2 by glycine decarboxylase in pea leaf protoplasts, was inhibited by the respriatory chain inhibitor, rotenone. This indicates that glycine oxidation *in vivo* was linked to the mitochondrial respiratory chain. Of course, a further implication of the above proposal, which is also supported by Oliver's findings, is that the mitochondrial electron transport chain is operating in the light.

4.3.2. *Photorespiratory glycine metabolism in vivo*

While the discussion to this point would appear to strongly support the concept of photorespiratory glycine oxidation via the mitochondrial respiratory chain, there are still important questions to be answered regarding the potential rates of glycine oxidation which can be handled by this pathway *in vivo*. The data presented in section 4.2.4 indicates that tissue rates of respiratory-linked glycine decarboxylation, in both pea and spinach leaf tissue, are of the order of 20-30 μmol CO_2 mg^{-1} Chl h^{-1}. These rates were measured under state 3 conditions and are only just sufficient to account for current estimates of photorespiratory cycle turnover (see section 1.2.3.). Furthermore, if respiratory chain activity is limited *in vivo* by ADP availability, the maximal rate of glycine oxidation via the respiratory chain may

be even further reduced below that required to account for *in vivo* photorespiratory rates.

It is also clear that, for the pea leaf tissue examined here, alternate pathway activity would not play a significant role in photorespiratory glycine oxidation *in vivo*. Indeed, glycine decarboxylase appeared to have a relatively poor access to the alternate pathway in comparison to the malate oxidizing enzymes (Fig. 4.2). A possible explanation for this observation may be found in Chapter 5.

Perhaps a more important question regarding these tissue estimates of glycine decarboxylase activity pertains to the validity of the current photorespiratory flux measurements. It can be seen in Fig. 4.2 that under state 3 conditions glycine oxidation was limited, not by the capacity of the cytochrome chain (since total respiratory capacity always exceeded the rate of glycine oxidation), but by absolute glycine decarboxylase activity. Consequently, it would appear that even in the absence of a limitation of glycine oxidation by the respiratory chain, maximal rates of glycine decarboxylase activity would be barely sufficient to cope with photorespiratory cycle turnover. It has been proposed that some glycine decarboxylase activity may be lost during mitochondrial isolation (Woo and Osmond 1976; Day and Wiskich, 1981), due to the particularly labile nature of this enzyme. Walker *et al*. (1982b) found that isolated pea leaf mitochondria rapidly lost the capacity to metabolise glycine (compared to other substrate oxidation capacities) unless stored in the presence of a low concentration of this substrate. The

inclusion of glycine in the isolation media used in section 4.2.4, however, did not increase the recovery of glycine decarboxylase activity.

Thus, doubts must be raised as to the accuracy of published rates of photorespiratory flux through the glycine decarboxylase step in leaf tissue. This issue, together with the questionable capacity of the respiratory chain to cope with glycine oxidation *in vivo*, will be considered further in Chapter 9.

Table 4.1. Simultaneous measurement of O_2 uptake and NH_3 release during the oxidation of glycine by pea leaf mitochondria.

Experimental procedures as given in section 2.9. Assays contained 11 mM glycine and 0.17 mM ADP.

	NH_3 Release	O_2 uptake
	nmol mg^{-1} protein min^{-1}	
Glycine + ADP (state 3)	156	81
state 4	99	49
+ ADP	163	78
state 4	92	46

Table 4.2. Effect of various respiratory substrates on glycine oxidation by pea leaf mitohcondria in state 3.

Experimental procedures as given in section 2.9. Assays contained 11 mM glycine, 11 mM glucose and 4 units of hexokinase. State 3 was initiated by the addition of 0.28 mM ADP. Following the establishment of a steady state rate of glycine oxidation, additions were made to the following final concentrations: 11 mM malate, 11 mM succinate, 11 mM 2-OG, 0.8 mM NADH. Both glutamate (11 mM) and TPP (0.1 mM) were also included in the assay medium on the addition of malate, while TPP (0.1 mM) and malonate (5 mM) were included with 2-OG. Oxidation rates for succinate and NADH were 83 and 130 nmol O_2 consumed mg^{-1} protein min^{-1}.

	NH_3 release	O_2 uptake	Predicted O_2 uptake
	nmol mg^{-1} protein min^{-1}		
Glycine	150	71	
+ malate	150	142	171
Glycine	120	64	
+ succinate, NADH	120	192	277
Glycine	100	51	
+ 2-OG	100	118	120
Glycine	125	63	
+ malate, succinate NADH	130	247	376

Table 4.3. Effect of glycine, succinate, malate and 2-OG oxidation on NADH oxidation by pea leaf mitochondria.

Substrates were incubated with NADH for 3 min prior to initiation of state 3 with ADP. Substrate concentrations as given in Table 4.2. Pthalonate (5 mM) was also present in assays containing malate to inhibit external malate dehydrogenase mediated NADH oxidation resulting from the export of any OAA during malate oxidation. Note that the rate of NADH oxidation is halved to make it equivalent to O_2 consumption.

	O_2 uptake			NADH Oxidation ($\frac{1}{2}$)	
	measured	predicted	deficit	measured	control-measured
	nmol mg^{-1} protein min^{-1}				
Exp. 1					
NADH	212	-	-	216	0
NADH + glycine	252	308	56	158	58
NADH + succinate	242	335	93	158	58
NADH + succinate, glycine	267	427	160	97	119
Exp. 2					
NADH	175	-	-	175	0
NADH + malate	201	249	48	132	43
NADH + malate, glycine	225	326	101	96	79
Exp. 3					
NADH	175	-	-	175	-
NADH + 2-OG	192	233	41	134	41
NADH + 2-OG, glycine	216	294	78	101	74

Table 4.4. Effect of succinate and glycine on external NADH oxidation by pea leaf mitochondria in the presence of $CaCl_2$.

Experimental conditions as given in Table 4.3 except that $CaCl_2$ (1 mM) was also present in the reaction medium.

	O_2 uptake			NADH oxidation ($\frac{1}{2}$)	
	measured	predicted	deficit	measured	control-measured
	nmol mg^{-1} protein min^{-1}				
NADH	210	-	-	229	-
NADH + succinate	221	291	70	176	53
NADH + glycine	243	291	48	176	53
NADH + succinate, glycine	243	372	129	124	105

Table 4.5. Effect of malate and 2-OG on glycine oxidation by pea leaf mitochondria in state 4.

Concentration of substrates as given in Table 4.2 except that the initial ADP addition was to 0.17 mM.

	NH_3 release	O_2 uptake	Predicted O_2 uptake
		nmol mg^{-1} protein min^{-1}	
Glycine + ADP	138	68	-
state 4	86	43	-
Malate + ADP	-	102	-
state 4	-	47	-
+ glycine	86	76	90
2-OG + ADP	-	71	-
state 4	-	32	-
+ glycine	88	65	75

Table 4.6. Accessibility of various NADH-generating enzymes in pea leaf mitochondria to OAA-dependent re-oxidation by malate dehydrogenase.

Assays were carried out in 2 ml of reaction medium (section 2.8) and contained mitochondria (0.5 mg protein) and either 11 mM glycine, 11 mM 2-OG, 11 mM citrate, 11 mM glutamate or 11 mM malate and 2 mM arsenite. Malonate (5 mM) and TPP (0.1 mM) were also included with 2-OG. State 3 was initiated by the addition of 0.7 mM ADP followed by the addition of 0.7 mM OAA.

Substrate	Assumed enzyme	O_2 uptake	
		State 3	+OAA
		nmol mg^{-1} protein min^{-1}	
Glycine	Glycine decarboxylase	114	10
2-OG	2-OG dehydrogenase	45	12
Citrate	Isocitrate dehydrogenase	28	6
Glutamate	Glutamate dehydrogenase	27	0
Mal, arsenite	*Malic enzyme	40	10

*Possibly some malate dehydrogenase also

Figure 4.1. Concurrent oxidation of respiratory substrates by pea leaf mitochondria.

Mitochondria (0.5 mg protein) were placed in 2 ml of reaction medium (section 2.8) containing 11 mM glycine. Additions as indicated: 1 mM ADP, 11 mM malate (including 11 mM glutamate and 0.1 mM TPP), 11 mM succinate, 0.8 mM NADH. Rates shown represent nmol O_2 consumed mg^{-1} protein min^{-1}. Numbers in the brackets represent the total predicted rate of O_2 consumption based on a summation of the individual substrate rates.

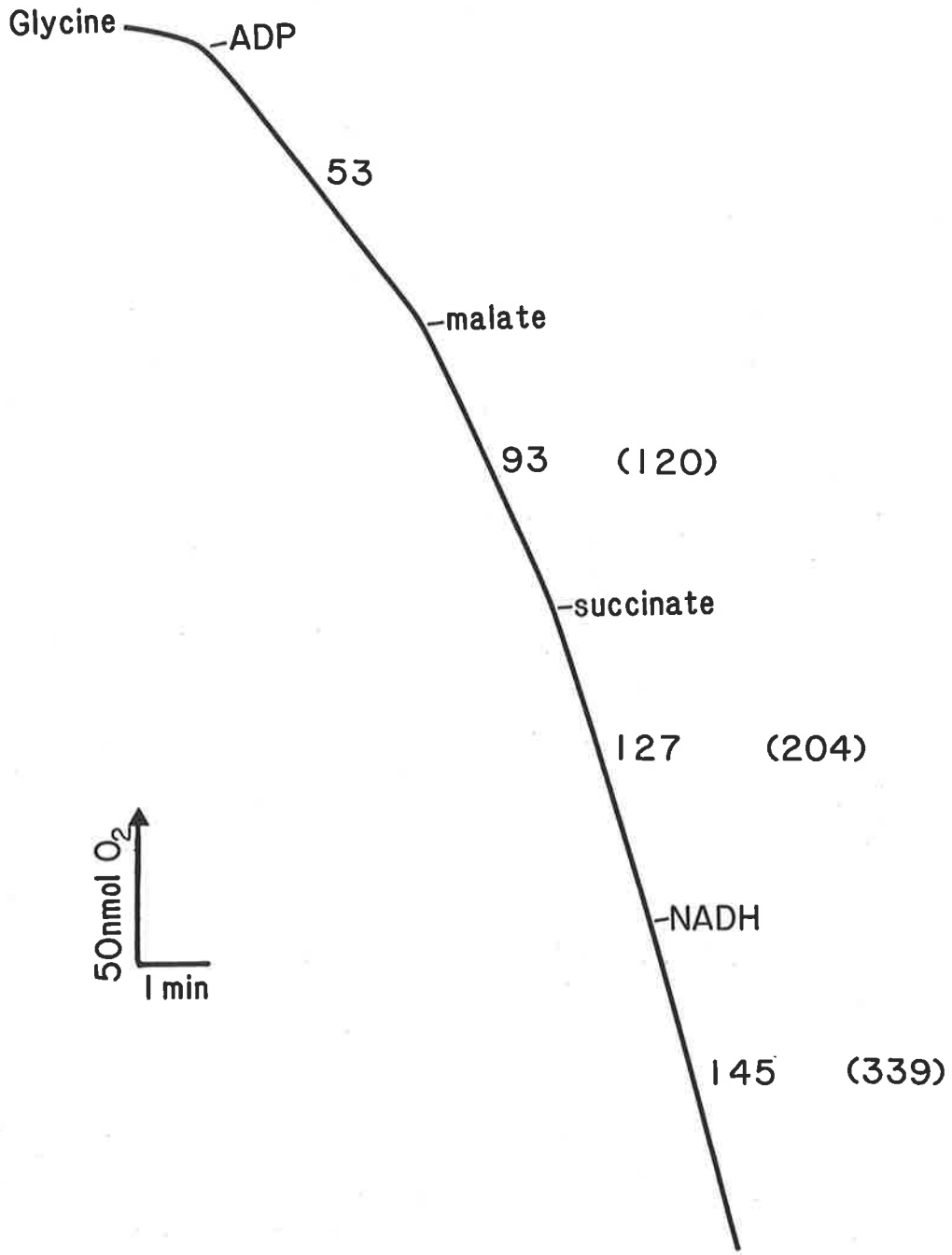

Glycine
-ADP
53
-malate
93 (120)
-succinate
127 (204)
-NADH
145 (339)
50nmol O_2
1 min

Figure 4.2. Changes in tissue rates of respiratory-linked oxidations during the development of pea leaves.

Experimental procedures as described in section 2.13. Substrate oxidation rates were measured under state 3 conditions in the presence of excess ADP. Total oxidative capacity was measured in the presence of glycine, malate, succinate and NADH. Alternate pathway activity was calculated from the rate of disulfiram-sensitive, antimycin-A-insensitive O_2 uptake. Concentrations used: 11 mM malate, 11 mM glycine, 11 mM succinate, 0.8 mM NADH, 150 µM disulfiram, 5 µM antimycin-A. Glutamate (11 mM) and TPP (0.1 mM) were also present when malate was a substrate. Calculation of rates of glycine decarboxylation (CO_2 release) were based on the ratio of 2 x CO_2 evolved per 1 x O_2 consumed by the respiratory chain.

Symbols:

(a) state 3 O_2 uptake - total (■)
malate (▲)
glycine (●)

(b) alternate pathway O_2 uptake - total (□)
malate (△)
glycine (○)

(c) state 3 glycine decarboxylation - (●).

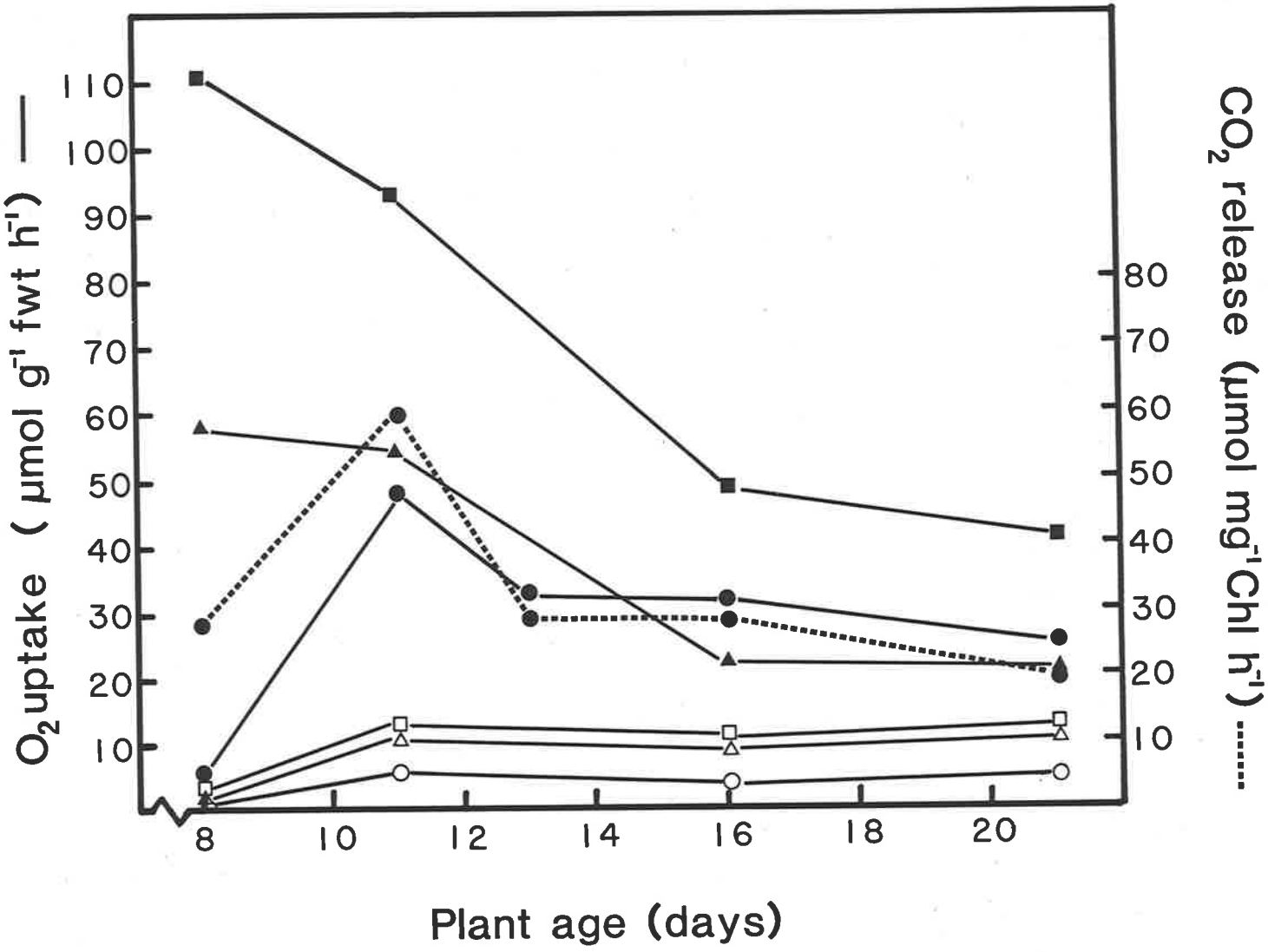
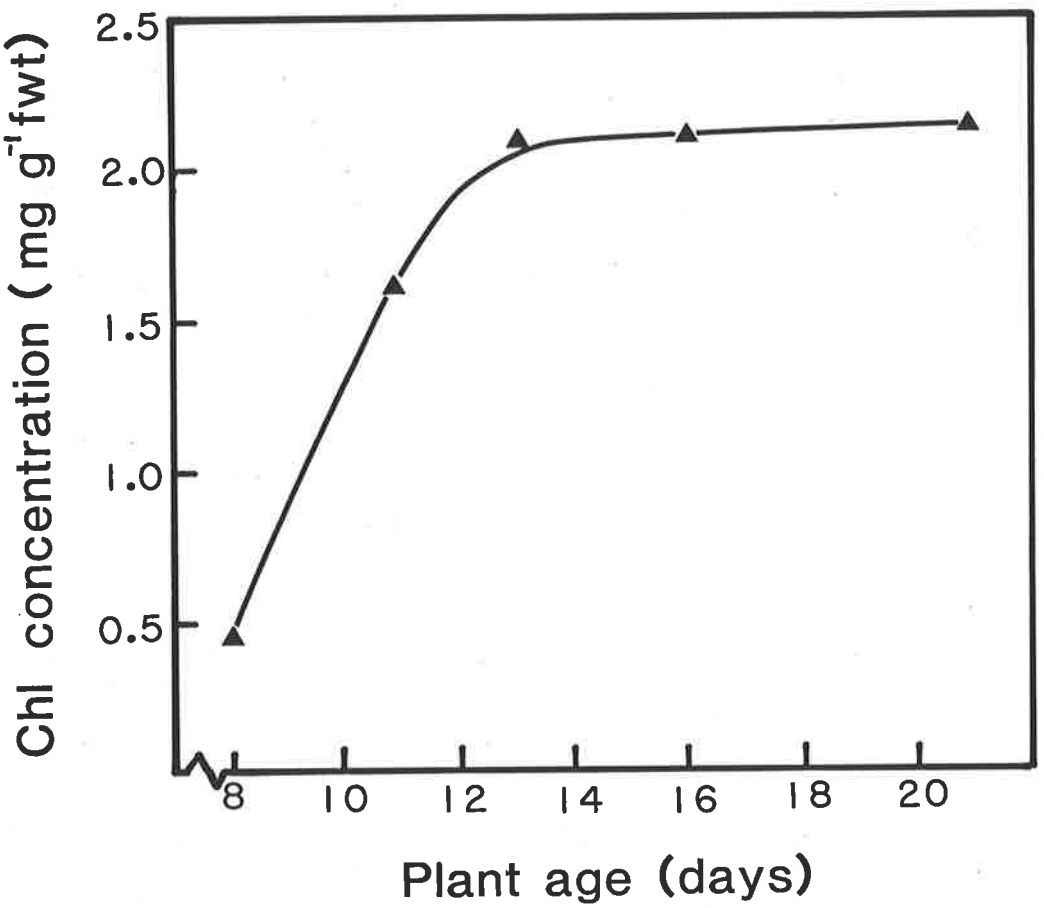

O2 uptake (µmol g-1 fwt h-1) ——
CO2 release (µmol mg-1Chl h-1) .....
110
100
90
80
70
60
50
40
30
20
10
80
70
60
50
40
30
20
10
8
10
12
14
16
18
20
Plant age (days)

Figure 4.3. Chlorophyll development in pea leaves.

Experimental procedures as described in section 2.13.


Chl concentration (mg g⁻¹ fwt)
2.5
2.0
1.5
1.0
0.5
8
10
12
14
16
18
20
Plant age (days)

CHAPTER V

DIFFERENTIAL ACCESS OF ENZYMES INVOLVED IN GLYCINE AND MALATE OXIDATION TO NAD AND RESPIRATORY CHAINS IN PEA LEAF MITOCHONDRIA

5.1. INTRODUCTION

In the previous chapter I presented evidence to suggest that a mechanism may exist in pea leaf mitochondria for the preferential oxidation of glycine via the mitochondrial respiratory chain. The basis of the mechanism is unknown, but may indicate the existence of some form of compartmentation of enzymes within the mitochondrial matrix. This would allow for the differential access of certain enzymes to separate NAD pools and respiratory chains.

Such a model has been proposed previously by Rustin *et al.* (1980) to account for apparent differences in the rotenone and cyanide sensitivity of malate oxidation via ME and MDH. They proposed that a functional connection existed between ME and the alternate pathway, through the rotenone-insensitive NADH dehydrogenase (see also Rustin and Moreau, 1979; Moreau and Romani, 1982). However, these claims have since been questioned by Wiskich and Day (1982), who concluded that ME and MDH have equal access to the rotenone- and cyanide-resistant pathways.

Glycine decarboxylase appears to be membrane-bound (Sarojini and Oliver, 1983) and, as such, may behave differently from matrix soluble NAD-linked enzymes such as MDH. The results of Chapter 4 certainly suggest that a unique association exists between glycine decarboxylase and the respiratory chain in pea

leaf mitochondria. Thus, the aim of this chapter is to examine glycine oxidation in greater detail and, in particular, compare its behaviour to that of other NAD-linked substrates, in order to provide information on the possible compartmentation of glycine decarboxylase with specific NAD pools and respiratory chains within pea leaf mitochondria

5.2. RESULTS and DISCUSSION

5.2.1. *Concurrent oxidation of NAD-linked substrates by pea leaf mitochondria under state 3 conditions*

The addition of glycine to pea leaf mitochondria oxidizing malate led to a marked stimulation of the state 3 rate of O_2 uptake (Table 5.1, Fig. 5.1; A). The average stimulation of the malate state 3 rate by glycine was 1.43 ± 0.14 fold (n = 19), which contrasts with the results of Walker *et al.* (1982b), who found that glycine had a negligible effect on the state 3 rate of malate oxidation by pea leaf mitochondria. Conversely, the addition of other NAD-linked substrates (e.g. 2-OG and citrate) had little effect on the rate of O_2 uptake in the presence of malate (Table 5.1). These same substrates, however, like malate (Fig. 5.1; B), could be shown to markedly stimulate the respiratory rate in the presence of glycine (Table 5.1).

Wiskich and Day (1982) have suggested that the observed lack of stimulation of malate-dependent O_2 evolution by 2-OG and citrate in cauliflower mitochondria may be due to competition between the respective dehydrogenase enzymes for access to matrix

NAD and/or NADH dehydrogenase capacity. However, as seen from the results in Table 5.2, an alternative explanation may be that both 2-OG and citrate inhibit malate oxidation directly, such that their addition has little effect on the net rate of O_2 uptake or, in the case of citrate, produces a slight inhibition. For example, the very slight stimulatory effect of 2-OG on O_2 uptake in the presence of malate is actually reduced if the concentration of 2-OG is increased (Table 5.2). More significantly, if 2-OG is added in the presence of arsenite (an inhibitor of 2-OG dehydrogenase), a marked inhibition of malate-dependent O_2 uptake is observed, which can be reversed by the further addition of malate. Similarly, the citrate inhibition can also be reversed by an increase in the malate concentration (Table 5.2)

The effects of 2-OG and citrate on malate oxidation may be mediated through substrate transport effects, rather than competition for enzymes or enzyme co-factors. Both 2-OG and citrate are transported into mitochondria on specific carriers, in exchange for internal dicarboxylate molecules, such as malate (Wiskich, 1977). Thus, the addition of 2-OG or citrate to mitochondria oxidizing malate may effectively remove malate from the matrix which could otherwise be utilised by MDH or ME. If the incoming substrate molecule is oxidized itself at a rate similar to malate oxidation, there should be no net effect on the rate of O_2 uptake, as is observed with 2-OG addition (Table 5.1). This will not be the case with citrate, however, because of the unfavourable equilibrium of aconitase (Lehninger, 1975), which will restrict the rate of citrate oxidation and,

consequently, produce a net inhibition of O_2 uptake upon citrate addition (Table 5.2).

There is also the possibility that 2-OG inhibition of malate oxidation may be mediated through a reduction in the rate of removal of OAA by GOT. In the presence of 2-OG and aspartate (which would have been synthesized by GOT in the presence of glutamate), the equilibrium of the GOT reaction would no longer be as favourable for OAA removal, causing an accumulation of OAA and an inhibition of MDH activity.

If 2-OG is added to pea leaf mitochondria oxidizing malate under conditions favouring ME (at pH 6.8, in the absence of glutamate and in the presence of arsenite), an inhibition of malate oxidation is still observed (Table 5.3). However, the extent of inhibition is reduced in comparison to the effect on malate oxidation in the presence of glutamate (at pH 7.2) which favours MDH activity. If part of the 2-OG effect is mediated via a reversal of GOT, it should be possible to demonstrate a similar effect with aspartate. The results in Table 5.3 demonstrate that, like 2-OG, aspartate does indeed inhibit malate oxidation in the presence of glutamate. However, also like 2-OG, part of this inhibition can be attributed to effects other than a reversal of GOT (i.e. transport effects), since some inhibition of malate oxidation, by aspartate, is observed in the absence of glutamate (Table 5.3).

Overall these results indicate that NAD-linked substrate may interact not only at the enzyme or NAD level, but also at the transport level through the existence of carriers

in the mitochondrial membrane which catalyse exchange reactions between the different NAD-linked substrates. These results also raise the interesting possibility that aspartate uptake into mitochondria may also involve a carrier which catalyses an aspartate/malate exchange. Proudlove and Moore (1982) could find no evidence for carrier mediated uptake of aspartate or glutamate into pea leaf mitochondria and suggested that uptake was via diffusion only. Day and Wiskich (1977b), on the other hand, demonstrated that glutamate uptake into cauliflower and beetroot mitochondria did require the presence of phosphate and malate, indicating that a carrier may exist which mediates a glutamate/malate exchange across the inner mitochondrial membrane. Aspartate may also be taken up by this carrier in exchange for internal malate.

From these results, it is clear that no valid conclusions can be drawn regarding the comparative access of TCA cycle dehydrogenases and glycine decarboxylase to NAD and/or NADH dehydrogenase capacity within the mitochondria. Glycine uptake is via diffusion (section 1.2.3) and, consequently, O_2 uptake rates in the presence of glycine would not be subject to the same potentially inhibitory effects of second-substrate addition as malate-dependent respiratory rates appear to be. It was therefore decided that all further experiments should involve comparisons between glycine and malate oxidation only, to avoid the obvious complications associated with the concurrent oxidation of NAD-linked TCA cycle intermediates.

5.2.2. *Effect of substrate addition under state 4 conditions*

Second substrate additions also led to a marked stimulation of the state 4 respiration rates (Fig. 5.1; C,D). The state 4 rates were measured for up to 4 min prior to the addition of the second substrate to avoid any biphasic behaviour of the malate state 4 rate (Palmer *et al.* 1982). Walker *et al.* (1982b) reported that glycine stimulated the state 4 rate of O_2 uptake with malate, but found that this stimulation was, under certain conditions, mediated through an increase in O_2 uptake via the alternate pathway. In contrast to their results, the alternate pathway inhibitor disulfiram (Fig. 5.1; C,D) had little or no effect on the state 4 stimulations observed in my preparations, nor was any stimulation of O_2 uptake (by second-substrate addition) observed in the presence of antimycin-A (Fig. 5.1; E).

Both SHAM and disulfiram were found to inhibit glycine oxidation, by pea leaf mitochondria, to a much greater extent than predicted from the amount of alternate pathway activity measured with antimycin-A (Table 5.4). It should be noted that antimycin-A was used, instead of KCN, to estimate alternate pathway activity with malate and glycine because of the inhibitory effects of KCN on glycine decarboxylase (Gardeström *et al.* 1981) and GOT (Wiskich and Day, 1982). The inhibition of glycine oxidation by SHAM and disulfiram increased gradually with time, with a 40-50% inhibition of state 3 oxidation via the cytochrome

chain observed after 5 min in their presence (Table 5.4). The significance of this result is that the glycine stimulation of the malate state 4 rate (as shown in Fig. 5.1) was often found to be reduced by these alternate pathway inhibitors, 3-4 min after their addition. This reduction, however, was not due to an inhibition of O_2 uptake via the alternate pathway, but due to the direct effects of SHAM and disulfiram on glycine oxidation via the cytochrome chain. It is assumed that the inhibition of glycine oxidation by these compounds results from an effect on the glycine decarboxylase enzyme directly, since similar magnitudes of inhibition of state 3 malate oxidation via the cytochrome pathway, were not observed (Table 5.4)

These second-substrate-mediated increases in the state 4 rate of O_2 uptake (Fig. 5.1) would appear to be in contradiction to our current understanding of the state 4 condition. Day *et al.* (1980) have stated that the rate of O_2 uptake, under state 4 condtions, is restricted by the proton-motive force (pmf) across the inner mitochondrial membrane. The stimulation of the malate state 4 rate by glycine and vice versa (Fig. 5.1), however, suggests that the pmf may not be limiting the state 4 rates of respiration with these substrates. The pmf may be partly dissipated by metabolite ion transport (Day *et al.* 1980) and led to an increase in the rate of O_2 uptake upon substrate addition. While a transient stimulation of the glycine state 4 rate by malate may possibly be explained in these terms, the opposite result (i.e. the stimulation of the malate state 4 rate by glycine;Fig. 5.1) cannot, since glycine uptake occurs via diffusion (see section 1.2.3). It was there-

fore necessary to determine what factors limit the state 4 rates of oxidation of these substrates before an explanation for the observed stimulation of state 4 respiration by substrate addition, could be formulated.

The results in Table 5.5 indicate that the control exerted by the pmf during state 4, on that part of the electron transport chain between ubiquinone and O_2, does not limit the individual rates of oxidation of glycine or malate. Succinate or external NADH oxidation, which link directly into ubiquinone (Day *et al.* 1980), were found to support much higher state 4 rates than those of the individual NAD-linked substrates (Table 5.5). Indeed, even in the presence of malate and glycine together, the maximal state 4 rate of electron transport between ubiquinone and cytochrome oxidase was not achieved, as demonstrated by the higher rates of O_2 uptake in the presence of glycine + succinate or glycine + NADH (Table 5.5).

The limitation of the pmf under state 4 conditions must therefore be exerted at the first phosphorylation site or at NADH dehydrogenase. Plant mitochondria possess two distinct NADH dehydrogenases on the matrix side of the inner membrane; a rotenone-sensitive dehydrogenase which is coupled to the first phosphorylation site and a rotenone-insensitive dehydrogenase which bypasses the first phosphorylation site (Møller and Palmer, 1982). The rotenone-insensitive dehydrogenase exhibits a much lower affinity for NADH than does the rotenone-sensitive dehydrogenase, i.e. Km of 80 µM and 8 µM respectively (Møller and Palmer, 1982). Thus, high matrix NADH concentrations must

be established, (as under state 4 conditions or in the presence of rotenone), before this bypass pathway will be activated (Palmer *et al*. 1982). The results in Table 5.6 indicate that the rotenone-insensitive bypass could account for a significant proportion of glycine and malate oxidation in pea leaf mitochondria under state 4 conditions.

If state 4 rates are limited by the affinity of the rotenone-insensitive dehydrogenase for NADH, it is difficult to understand how second-substrate stimulations can occur, based on the hypothesis that all NAD-linked enzymes have access to a common NAD pool. For example, it would appear that when glycine is added to pea leaf mitochondria already oxidizing malate in state 4 (Fig. 5.1) an even higher NADH/NAD ratio is generated (as indicated by the higher state 4 rate) than if glycine were the only substrate present (see also Table 5.5). Given that the glycine state 4 rate is not limited by absolute enzyme activity (see the higher rate ofglycine oxidation in state 3) and also given that glycine decarboxylase had access to all NAD, the question arises as to why the glycine decarboxylase enzyme was not able to produce this supposedly elevated NADH/NAD ratio or NADH concentration (and thus the higher state 4 rate) when operating on its own One possible explanation may be that glycine decarboxylase does not have access to the total NAD pool within the mitochondria. This proposal is further investigated in the next section.

5.2.3. *Effect of substrate addition under rotenone-inhibited state 3 conditions*

Unlike state 4, we can be reasonably certain that the rate of rotenone-insensitive state 3 O_2 uptake is limited by the affinity of the rotenone-insensitive dehydrogenase for NADH, and that the rate of electron flow via this bypass pathway will be dependent on the intramitochondrial concentration of NADH. The addition of NAD to freshly isolated pea leaf mitochondria led to marked increases in the rotenone-insensitive state 3 rates of glycine and malate oxidation (Fig. 5.2; B,D). All experiments involving the addition of NAD were carried out in the presence of EGTA to inhibit the oxidation of any external NADH which may be generated by soluble malate dehydrogenase released from broken mitochondria (Palmer *et al.* 1982). Indeed, external NADH oxidation accounted for up to 50% of the total stimulation of rotenone-insensitive state 3 malate oxidation by NAD in pea leaf mitochondria in the absence of EGTA (Table 5.7).

As shown previously by Tobin *et al.* (1980) the stimulation of respiratory O_2 uptake by NAD results from an increase in the size of the mitochondrial NAD pool, following NAD uptake. This allows for the generation of higher NADH concentrations within the matrix and, thus, increases the rate of electron flow via the rotenone-insensitive bypass (Palmer *et al.* 1982). What is shown by the results in Fig. 5.2, however, is that these NAD-limited, rotenone-insensitive state 3 rates of malate and glycine oxidation could also be markedly stimulated by the addition of a second NAD-limited substrate (Fig. 5.2; A,C). These stimulations

could not be elicited through increases in the concentration of the original substrate being oxidised (Fig. 5.2 A,C). It should also be noted that the malate stimulation of the rotenone-insensitive glycine rate could be achieved on the addition of malate only, i.e. in the absence of glutamate and TPP, although the extent of the stimulation was less than in their presence.

As with the second-substrate stimulations of state 4 rates, these observations clearly contradict current thinking regarding the distribution of enzymes within mitochondria. If all enzymes had equal access to a common NAD pool, an NAD limitation of the rotenone-insensitive rate for one enzyme, as shown in Fig. 5.2, should represent an NAD limitation for all other enzymes with access to that same NAD pool. These results (Fig. 5.2) cannot be explained in terms of one enzyme, e.g. MDH, possessing a higher affinity for NAD or a more favourable equilibrium than the other enzyme, e.g. glycine decarboxylase. If this were the case, it should be possible to demonstrate the same rate of rotenone-insensitive O_2 utpake in the presence of malate only, as is achieved on its addition to mitochondria already oxidizing glycine. Clearly, this is not so, as shown in Fig. 5.2, since the addition of malate as the second substrate resulted in rates of rotenone-insensitive O_2 uptake well above that achieved with malate only. The same argument also applies with glycine as the second substrate.

These results, therefore, also support the theory that glycine decarboxylase and the malate oxidizing enzymes have access to NAD, within pea leaf mitochondria, which is not readily accessible to the other respective enzyme/s. This would explain how the

addition of an NAD-linked substrate, like malate, could produce a stimulation of the rate of O_2 uptake when the glycine rate was already limited by NAD. In order to test the hypothesis that these respective enzymes had access to separate NAD pools within pea leaf mitochondria, experiments were designed in which mitochondria were depleted of NAD and the relative effects on glycine and malate oxidation examined. If the enzymes shared a common NAD pool it would be expected that glycine and malate oxidation should respond in a similar manner. If, however, the enzymes have access to separate NAD pools, it may be possible to demonstrate a difference in the response of glycine and malate oxidation to the NAD-depletion treatments, if the NAD pools differ in size or rate of depletion.

5.2.4. *Comparative effects of NAD-depletion treatments on glycine and malate oxidation*

Mitochondria were incubated in large volumes of reaction medium as described in section 2.6, to deplete them of NAD. Figure 5.3 demonstrates the effect of this treatment on the NAD content of pea leaf mitochondria. The levels of NAD in the freshly-isolated pea leaf mitochondria were similar to the NAD content of mung bean hypocotyls reported by Tobin *et al.* (1980).

The rates of malate and glycine oxidation during the incubation period were measured in the presence or absence of NAD. It can be seen (Fig. 5.4), that the state 3 rate of glycine oxidation, in the absence of NAD, decreased during the incubation period

whereas the malate rate remained reasonably constant. Part of the fall in the glycine rate could be accounted for by a loss in total glycine decarboxylase activity (as measured under state 3 conditions, in the presence of NAD). The complication arising from losses in total enzymic capacity during the incubation period can be removed by plotting the data in terms of the rate observed in the absence of NAD, as a percentage of the comparable rate in the presence of added NAD. Figure 5.5 presents the comparative state 3 data in this form. The results indicate that a small decrease in the state 3 glycine rate was observed upon the loss of NAD in comparison to malate.

Much more significant effects of NAD loss were observed if substrate oxidation was carried out under state 4 conditions. Figure 5.4 shows that the glycine state 4 rate decreased markedly (in the absence of NAD), while the malate rate was virtually unaffected. It should be noted that despite the fall in total glycine oxidation capacity there was always sufficient activity to account for the glycine state 4 rate, as shown by the constant rate of glycine oxidation in the presence of added NAD. This difference in response of glycine and malate oxidation to NAD loss under state 4 conditions is also illustrated in Fig. 5.6.

The increased sensitivity of glycine oxidation to NAD loss under these conditions, in comparison to the effect under state 3 conditions (Fig. 5.5.), may reflect the involvement, in state 4, of the rotenone-insensitive NADH dehydrogenase. The poor affinity of this enzyme for NADH, relative to the rotenone-sensitive dehydrogenase (which should predominate under state 3 conditions), would make this enzyme much more sensitive to decreases in the intramitochondrial NAD(NADH) concentration. One

should therefore expect rotenone-insensitive oxidation to be particularly sensitive to NAD loss. A similar experiment was carried out to test the effect of NAD loss on the rates of rotenone-insensitive malate and glycine oxidation. The results shown in Fig. 5.7 illustrate that the rotenone-insensitive state 3 oxidation rates were indeed highly sensitive to NAD loss. Furthermore, it was glycine oxidation that again was much more sensitive to NAD loss than the comparable malate oxidation rate. This is also apparent from a comparison of the ratios of the -NAD/+NAD rotenone-insensitive state 3 rates as shown in Fig. 5.8 which takes into account the decreases observed in the rotenone-insensitive state 3 rates in the presence of added NAD i.e. due to factors other than NAD loss.

The results presented above indicate that NAD loss is having its primary effect on the access of glycine decarboxylase to the rotenone-insensitive bypass. These observations cannot be explained in terms of differential effects of NAD loss on enzyme activity directly e.g. Km effects, since if this were the case, similar inhibitions of oxidation rates should be observed under all respiratory conditions. In fact, if NAD loss were affecting enzyme activity directly, the most dramatic inhibitory effects should be observed under state 3 conditions when enzyme activity is not subject to other limitations which are present under state 4 or rotenone-inhibited conditions. However, the opposite result is observed with the rotenone-insensitive state 3 glycine rate decreasing to a greater extent in response to NAD loss than the un-inhibited state 3 rate, as shown by the reduction in the percent rotenone-insensitivity of glycine oxidation (Fig. 5.9). Furthermore, as the results in Fig. 5.4 and 5.7 clearly show,

state 3 rates in NAD-depleted mitochondria were always much higher than the state 4 or rotenone-insensitive state 3 rates, thereby demonstrating that sufficient NAD was present, in terms of the affinity of the glycine decarboxylase enzyme for this co-factor, to support rates far in excess of the state 4 or rotenone-insensitive state 3 rate observed. Therefore,the affinity of glycine decarboxylase for NAD was not limiting the state 4 or rotenone-insensitive rates in NAD-depleted mitochondria.

Much larger decreases in the rotenone-insensitive malate rate (relative to the rate in the presence of added NAD) were observed if mitochondria were incubated or depleted at a higher temperature (Fig. 5.10). This most probably reflects an increased rate of NAD diffusion out of the mitochondria at this elevated temperature. Glycine oxidation was still much more sensitive to the depletion treatment than malate, reaching a value of 20% for the ratio of -NAD/+NAD rotenone-insensitive rates after only 100 min incubation period (Fig. 5.10), compared to 200 min at 4°C (Fig. 5.8).

The dependence of NAD-depleted or deficient pea leaf mitochondria on added NAD is further illustrated in Fig. 5.11. Trace A represents freshly isolated mitochondria (which themselves are already somewhat NAD-deficient; see Fig. 5.2; C,D) and can be compared to mitochondria which have been experimentally depleted for 2 hr at 4°C (traces B,C). The addition of NAD led to marked increases in the rate of rotenone-insensitive state 3 glycine oxidation (Trace B). However, what is more significant is that the addition of malate could also be shown to produce large increases in the rotenone-insensitive rate of O_2 uptake under these severely NAD-limited conditions.

5.3. CONCLUSIONS

The results demonstrate that glycine decarboxylase and the malate oxidizing enzymes have differential access, both in terms of size and location, to NAD within pea leaf mitochondria. Furthermore, it is proposed that access of glycine decarboxylase to NADH dehydrogenase or electron transport chains is also restricted in comparison to the malate enzymes. The observed effects of second-substrate additions under both state 4 and rotenone-inhibited state 3 conditons clearly indicated that the glycine and malate enzymes did not have access to the same common NAD pool. This was later confirmed by NAD-depletion experiments which demonstrated a clear difference in the response of these respective enzymes to NAD loss. The differential sensitivity of these enzymes to NAD-depletion could not be explained on the basis of potential differences in the affinities of these enzymes for NAD, as the observed effects of NAD loss on glycine decarboxylase in these particular experiments, were mediated primarily through effects on the access of this enzyme to the rotenone-insensitive NADH dehydrogenase.

These results may arise as a consequence of the difference in the distribution of these enzymes within the mitochondrial matrix as depicted in Fig. 5.13. Glycine decarboxylase being membrane bound (Sarojini and Oliver, 1983), would only have access to the NAD and the electron transport chains within its immediate environment. The malate-oxidizing enzymes on the other hand (and in particular MDH)could have a much wider distribution within the matrix and, as such, would have access to more

NAD and respiratory chains than are available to glycine decarboxylase. This may also explain the findings (Chapter 4) that glycine decarboxylase appeared to have a restricted access to the alternate pathway in comparison to the malate enzymes.

Compartmentation-type models have been proposed to explain certain aspects of acetyl-CoA and glutamate metabolism in animal mitochondria through the existence of separate pools of substrate within the mitochondrial matrix (von Glutz and Walter, 1975; Schoolwerth and LaNoue, 1980). However, unlike these models I am not proposing that it is the NAD which is necessarily compartmented into discrete pools, but rather the enzymes which may be distributed or compartmented in such a way as to restrict the access of certain of them(e.g. membrane bound enzymes such as glycine decarboxylase) to less than the total pool of NAD and respiratory chains within the mitochondria. Therefore, during NAD-depletion treatments, while NAD is being lost from the total pool and thus from all NAD-linked enzymes within the mitochondria, the most dramatic effects are observed on an enzyme (such as glycine decarboxylase) which already has a restricted access to the total NAD pool. Not surprisingly, the effects of NAD loss on glycine decarboxylase are reflected most clearly in the inability of this enzyme to donate electrons to the rotenone-insensitive bypass, because of the poor affinity of this bypass pathway for NADH.

The lack of correlation between the initial rates of NAD loss from the mitochondria (Fig. 5.3) and the rapid decrease in the rotenone-insensitive state 3 rate of glycine oxidation

(see Figs 5.7, 5.8) can also be explained in terms of restricted access to the total NAD pool. Thus, while the initial NAD loss (Fig. 5.3) may only represent a small fraction of the total intramitochondrial NAD pool, it may constitute a large proportion of the NAD required by glycine decarboxylase for access to the rotenone-insensitive bypass. As a result, this apparently small loss of NAD leads to marked decreases in the rate of glycine oxidation via the rotenone-insensitive pathway (Fig. 5.7).

As mentioned in the introduction, it has been postulated that ME is compartmented with specific NAD pools within plant mitochondria, which are linked specifically to the rotenone-insensitive NADH dehydrogenases (Rustin *et al*. 1980). If this were the case, one might expect ME to show a similar response to NAD loss as glycine decarboxylase. Figure 5.12 illustrates the response of malate oxidation via ME to NAD-depletion. It can be seen that the response is virtually identical to the response observed in the presence of both MDH and ME (Figs 5.6, 5.8) and does not display the sensitivity demonstrated by glycine decarboxylase to NAD loss. This data would therefore not support the hypothesis (Rustin *et al*. 1980) that the access of ME to NAD or respiratory chains, within the mitochondria, was in any way restricted or specialised. Further work is required to investigate the response of other NAD-linked mitochondrial enzymes to NAD-depletion treatments. Of particular interest would be the response of other multienzyme complexes such as 2-OG dehydrogenase and pyruvate dehydrogenase which, like glycine dcarboxylase,may be attached to the inner mitochondrial membrane.

One question which is yet to be resolved regarding the results of the NAD-depletion experiments is why NAD stimulates malate oxidation as much as glycine oxidation in freshly prepared mitochondria (Figs 5.2, 5.4), yet is clearly much less sensitive than glycine oxidation, to the further loss of NAD during the depletion treatments. It may be possible that part of this apparent NAD stimulation of malate oxidation, in fresh mitochondria, is associated with EGTA-insensitive oxidation of NADH (generated by external MDH in the presence of NAD; see section 5.2.3), via the internal NADH dehydrogenase enzymes of broken mitochondria within the preparation. The maximum rate of EGTA-insensitive NADH oxidation was found to be of the order of 10-15 nmol O_2 consumed mg^{-1} protein min^{-1} in these washed mitochondrial preparations. This problem could be eliminated through the use of purified mitochondria. However, there are problems associated with the use of purified mitochondria for this type of experiment due to: (a) the potential loss of intramitochondrial NAD during the purification procedure and (b) obtaining a sufficiently large yield of highly active (particularly with respect to glycine decarboxylase) mitochondria to carry out these long term NAD-depletion experiments.

Recently, Laties (1983) has presented evidence to suggest that isocitrate dehydrogenase may be distributed in two separate compartments within potato mitochondria. One form of the enzyme is thought to be located in the matrix and operates with matrix NAD, while the other form is found to be associated with the inner surface of the inner mitochondrial membrane, with its

activity appearing to be dependent on external (cytosolic) NAD (Laties, 1983; Tezuka and Laties, 1983). This latter form shows distinct similarities to the glycine decarboxylase enzyme found in pea leaf mitochondria in that they are both associated with the inner membrane, but more significantly, are both particularly sensitive to NAD loss or to the addition of exogenous NAD under conditions of NAD-deficiency. While the NAD response of membrane bound isocitrate dehydrogenase was observed by Laties (1983) with freshly isolated potato mitochondria, it is well established that mitochondria isolated from such tissue are already very NAD deficient i.e. 0.8-1.1 nmol mg^{-1} protein (Neuburger and Douce, 1980; Tobin *et al.* 1980) in comparison to mitochondria isolated from pea leaf tissue (5-6 nmol NAD mg^{-1} protein) or mung bean hypocotyls (5-6 nmol NAD mg^{-1} protein; Tobin *et al.* 1980). Consequently, it would not have been necessary to artificially deplete these potato mitochondria of NAD (as done here) in order to see the observed response of this enzyme to external or added NAD.

A further question which arises form these observations of NAD stimulations of membrane-bound enzymes is whether the added NAD penetrates the inner mitochondrial membrane and increases the concentration of NAD in the matrix (Tobin *et al.* 1980; Palmer *et al.* 1982) or whether the NAD is bound within the inner membrane (Moore and Cottingham, 1983) and thus stimulates the access of certain membrane bound enzymes to respiratory chains. It is clear that the addition of NAD to NAD-depleted mitochondria isolated from potato (Tobin *et al.* 1980) and Jerusalem-artichoke

tubers (Palmer *et al.* 1982) does stimulate malate oxidation and that this result is consistent with NAD entry into the matrix space (Tobin *et al.* 1980; Palmer *et al.* 1982). However, the initial loss of NAD from pea leaf mitochondria which produced the rapid decrease in the rotenone-insensitive state 3 rate of glycine oxidation had little effect on malate oxidation under similar conditions (Fig. 5.7). Taken together these results may indicate two potential sites of action of NAD in mitochondria: (a) within the inner mitochondrial membrane from which NAD is easily lost to and taken up from the external medium and which controls the access of membrane-bound enzymes like glycine decarboxylase to respiratory chains and, in particular, the rotenone-insensitive bypass and (b) the mitochondrial matrix from which NAD is lost more slowly and the level of which regulates the activity of soluble NAD-linked enzymes such as MDH and ME.

Despite the compartmentation of the enzymes within the mitochondria, the malate enzymes do appear to have access to the NAD (be it bound or otherwise) and respiratory chains utilised by glycine decarboxylase. For example, the NADH generated during glycine oxidation is rapidly re-oxidised by MDH in the presence of OAA (Table 4.6; see also Woo and Osmond, 1976; Day and Wiskich, 1981). Furthermore, the addition of both glycine and malate to pea leaf mitochondria does not lead to an additive increase in O_2 uptake, in terms of the respective rates obtained with the individual substrates (Chapter 4). However, it is the rate of glycine oxidation which is maintained under such conditions, at the expense of malate oxidation. This may indicate the existence

of a special interaction between glycine decarboxylase and the NADH dehydrogenase enzymes on the inner mitochondrial membrane which ensures that glycine is preferentially oxidized in a competitive situation involving glycine decarboxylase and the malate enzymes.

Table 5.1. Concurrent oxidation of NAD-linked substrates by pea leaf mitochondria.

Mitochondria (0.6 mg protein) were incubated for 2-3 min in 2 ml of reaction medium (section 2.8) containing 10 mM glucose and 0.20 mM ADP and the initial substrate as stated. Hexokinase (1.4 units) was then added to initiate state 3 oxidation. The pre-incubation was designed to ensure that the initial substrate had reached its maximal state 3 rate of oxidation, prior to the addition of the other NAD-linked substrates. Concentrations used: 12 mM malate, glycine, 2-OG and citrate. Glutamate (11 mM) and TPP (0.1 mM) were always present with malate, while malonate (5 mM) and TPP (0.1 mM) were added with 2-OG. The results of 2 separate experiments are shown.

	Sequential additions	O_2 uptake
		nmol mg^{-1} protein min^{-1}
Exp. 1	Malate	92
	+ glycine	146
	Malate	86
	+ 2-OG	95
	+ citrate	79
	+ glycine	130
	Malate	88
	+ citrate	71
	+ 2-OG	85
	+ glycine	140
	2-OG	41
	+ malate	98
	+ citrate	69
	+ glycine	125
	Citrate	21
	+ 2-OG	55
	+ malate	77
	+ glycine	124
Exp. 2	Glycine	64
	+ 2-OG	106
	+ malate	122
	Glycine	66
	+ citrate	79
	+ 2-OG	100
	+ malate	106

Table 5.2. Effect of 2-OG and citrate on malate oxidation by pea leaf mitochondria in the presence and absence of arsenite.

Experimental procedures as given in Table 5.1. Initial concentrations and additions of substrates were at 12 mM. Arsenite was present at 1 mM.

Sequential additions	O_2 uptake
	nmol mg^{-1} protein min^{-1}
Malate	100
+ 2-OG	118
+ 2-OG	109
+ citrate	92
Malate, arsenite	73
+ 2-OG	41
+ malate	68
Malate	108
+ citrate	84
+ malate	105

Table 5.3. Effect of 2-OG and aspartate on malate oxidation by pea leaf mitochondria in the presence and absence of glutamate.

Mitochondria (1-2 mg protein) were incubated for 2-3 min in 2 ml of reaction medium (section 2.8) at pH 7.2 or 6.8, containing 1 mM arsenite and 11 mM malate (± 11 mM glutamate) and 0.20 mM ADP. State 3 malate oxidation was initiated by the addition of 1.0 mM ADP, and after the establishment of a constant rate further substrates were added (each addition represents 11 mM). Also shown is the percent inhibition of the initial rate of malate oxidation. The results of 3 separate experiments are shown.

Sequential additions	O_2 uptake	
	nmol mg^{-1} protein min^{-1}	*% inhibition*
Exp. 1 Malate, glutamate (pH 7.2)	82	-
+ 2-OG	44	46
+ 2-OG	30	63
+ malate	39	52
Exp. 2 Malate, glutamate (pH 7.2)	55	-
+ aspartate	37	33
+ aspartate	28	49
+ malate	35	36
Exp. 3 Malate (pH 6.8)	40	-
+ 2-OG	31	23
+ 2-OG	23	43
+ malate	37	8
Malate (pH 6.8)	39	-
+ aspartate	34	13
+ aspartate	31	21
+ malate	45	-

Table 5.4. The effect of disulfiram and SHAM on glycine and malate oxidation by pea leaf mitochondria.

Mitochondria (0.6 mg protein) were placed in 2 ml of reaction medium (section 2.8) containing either 11 mM glycine or 11 mM malate, 11 mM glutamate and 0.1 mM TPP. State 3 oxidation was initiated by the addition of 1.0 mM ADP. Following establishment of a constant rate, antimycin-A, SHAM or disulfiram were added and the resultant rate of O_2 uptake after 5 min measured. SHAM was dissolved in methoxy-ethanol. The numbers in the brackets represent the percent inhibition of O_2 uptake via the cytochrome chain i.e. that decrease in the rate of O_2 uptake which cannot be accounted for by the inhibition of the alternate pathway by SHAM and disulfiram (as estimated by the rate of O_2 uptake in the presence of antimycin-A).

Additions	O_2 uptake	
	Glycine	Malate
	nmol mg^{-1} protein min^{-1}	
State 3 (control)	81	101
+ antimycin-A (5 µM)	16	18
+ SHAM (1.0 mM)	57 (12)	89 (0)
+ SHAM (2.0 mM)	33 (49)	77 (7)
+ disulfiram (150 µM)	37 (43)	69 (17)

Table 5.5. Oxidative properties of pea leaf mitochondria.

Mitochondria (0.5 mg protein) were placed in 2 ml of reaction medium (section 2.8). Subtrate concentrations: 11 mM malate, glycine, succinate, 0.9 mM NADH. Glutamate (11 mM) and TPP (0.1 mM) were included with malate. The state 3 and state 4 rates given were those measured following one state 3/state 4 transition. Glycine (11 mM) was added after the establishment of a constant state 4 rate. The glycine-stimulated rate represents the rate achieved in the presence of 150 µM disulfiram.

	O_2 uptake			
Substrates	state 3	state 4	ADP/O	RCR
	nmol mg^{-1} protein min^{-1}			
Malate	116	51	2.4	2.3
+ glycine	-	76	-	-
Glycine	110	53	2.2	2.1
Succinate	117	76	1.4	1.5
+ glycine	-	92	-	-
NADH	161	69	1.5	2.3
+ glycine	-	84	-	-

Table 5.6. Effect of rotenone on state 4 malate and glycine oxidation by pea leaf mitochondria.

Experimental conditions as given in Table 5.5. Concentrations used were 0.14 mM ADP and 20 µM. Rotenone was dissolved in methoxy-ethanol.

Sequential Additions	O_2 uptake
	nmol mg^{-1} protein min^{-1}
Malate + ADP	150
state 4	59
+ rotenone	47
Glycine + ADP	106
state 4	56
+ rotenone	59

Table 5.7. Effect of EGTA on the degree of stimulation of malate oxidation by NAD in pea leaf mitochndria.

Mitochondria (0.5 mg protein) were placed in 2 ml of reaction medium (section 2.8) containing 11 mM malate, 11 mM glutamate and 0.1 mM TPP. O_2 uptake rates were measured in the presence or absence of 1 mM EGTA and 1 mM NAD. Additions: 0.18 mM ADP, 20 µM rotenone.

Sequential Additions	O_2 uptake			
	− EGTA		+ EGTA	
	− NAD	+ NAD	− NAD	+ NAD
	nmol mg^{-1} protein min^{-1}			
Malate + ADP	111	127	108	122
state 4	61	76	61	72
+ ADP	119	152	118	140
+ rotenone	87	119	86	102

Figure 5.1. Effect of substrate addition on state 3 and state 4 oxidation rates by pea leaf mitochondria.

Mitochondria (0.5 mg protein) were placed in 2 ml of reaction medium (section 2.8). Additions as indicated were: 11 mM malate and glycine, 150 µM disulfiram, 5 µM antimycin-A (Anti-A), 0.14 mM ADP (first addition) and 0.7 mM ADP (second addition). Glutamate (11 mM) and TPP (0.1 mM) were included with all malate additions. Note that the mitochondria had already gone through one state 3/state 4 transition prior to the first addition of ADP shown. Traces (A,B), (C,D) and E came from different experiments. Rates shown are expressed as nmol O_2 consumed mg^{-1} protein min^{-1}.

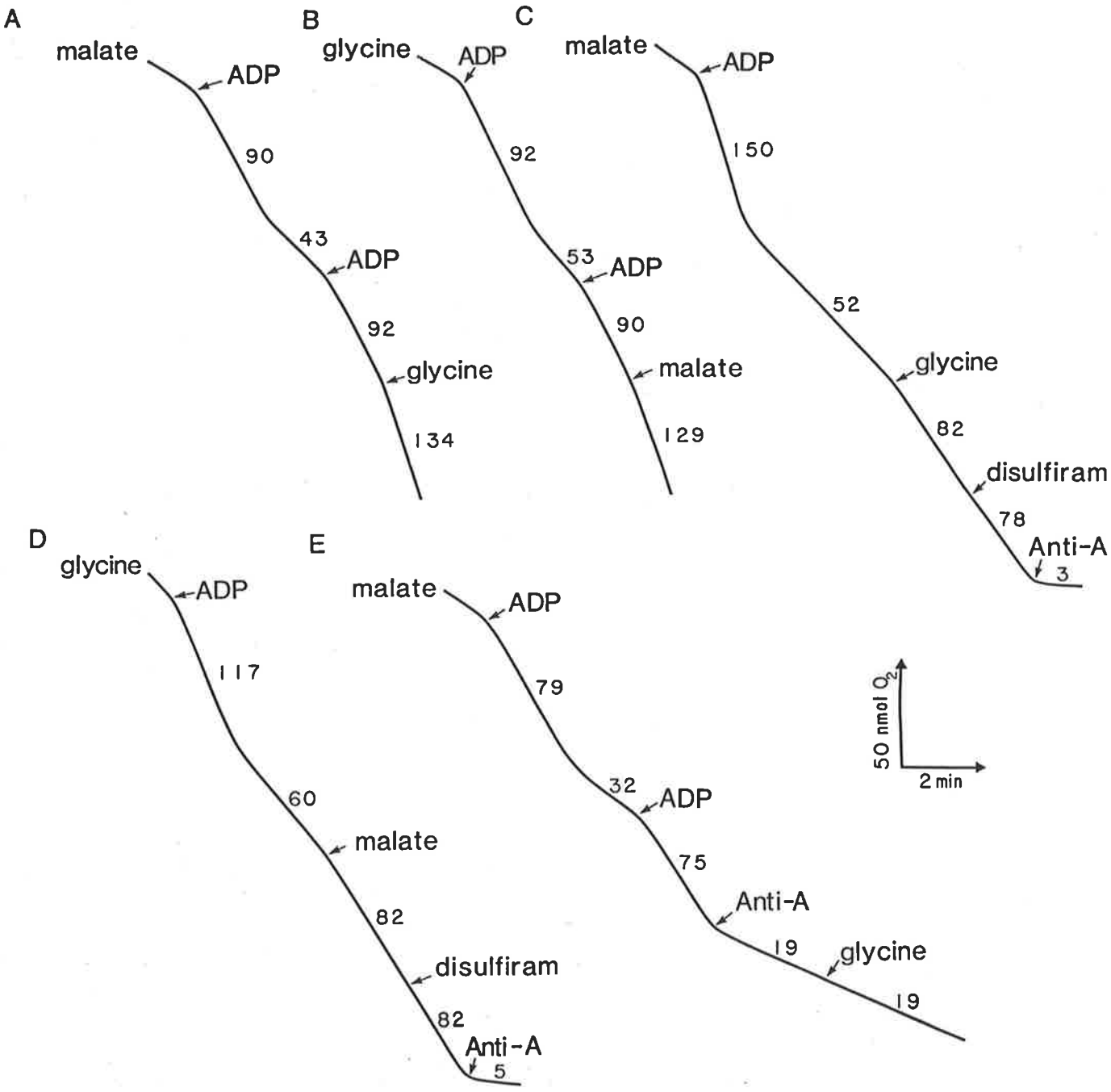

A
malate
ADP
90
43
ADP
92
glycine
134
B
glycine
ADP
92
53
ADP
90
malate
129
C
malate
ADP
150
52
glycine
82
disulfiram
78
Anti-A
3
D
glycine
ADP
117
60
malate
82
disulfiram
82
Anti-A
5
E
malate
ADP
79
32
ADP
75
Anti-A
19
glycine
19
50 nmol O2
2 min

Figure 5.2. Effect of NAD and substrate addition on rotenone-insensitive state 3 rates of malate and glycine oxidation by pea leaf mitochondria.

Experimental conditions as given in Fig. 5.1 except that assays also contained 1 mM EGTA. Concentrations used: 22 µM rotenone, 1 mM NAD.

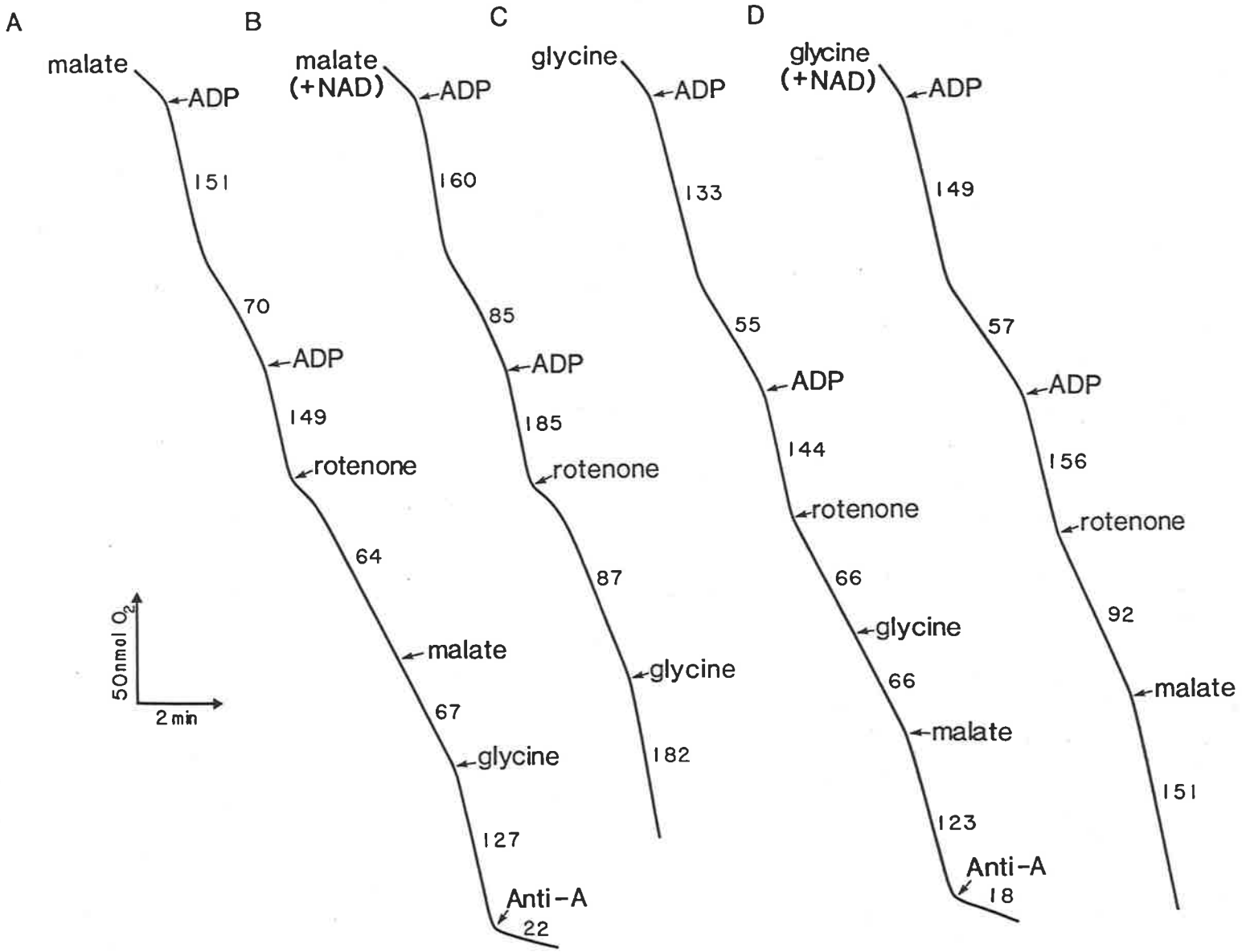

A
malate
ADP
151
70
ADP
149
rotenone
64
malate
67
glycine
127
Anti-A
22
50nmol O2
2 min
B
malate
(+NAD)
ADP
160
85
ADP
185
rotenone
87
glycine
182
C
glycine
ADP
133
55
ADP
144
rotenone
66
glycine
66
malate
123
Anti-A
18
D
glycine
(+NAD)
ADP
149
57
ADP
156
rotenone
92
malate
151

Figure 5.3. NAD-depletion of pea leaf mitochondria.

Mitochondria were incubated in reaction medium as described in section 2.6, except that there were no substrates present. Samples (17 ml) were removed at regular intervals and centrifuged at 10,000 *g* through a 10 ml pad of 0.6 M sucrose, to separate the mitochondrial pellet from any NAD in the incubation medium. The pellet was resuspended in 1 ml of resuspension medium (section 2.2) without BSA. The NAD content of the resuspended mitochondrial pellet was determined as described in section 2.11.

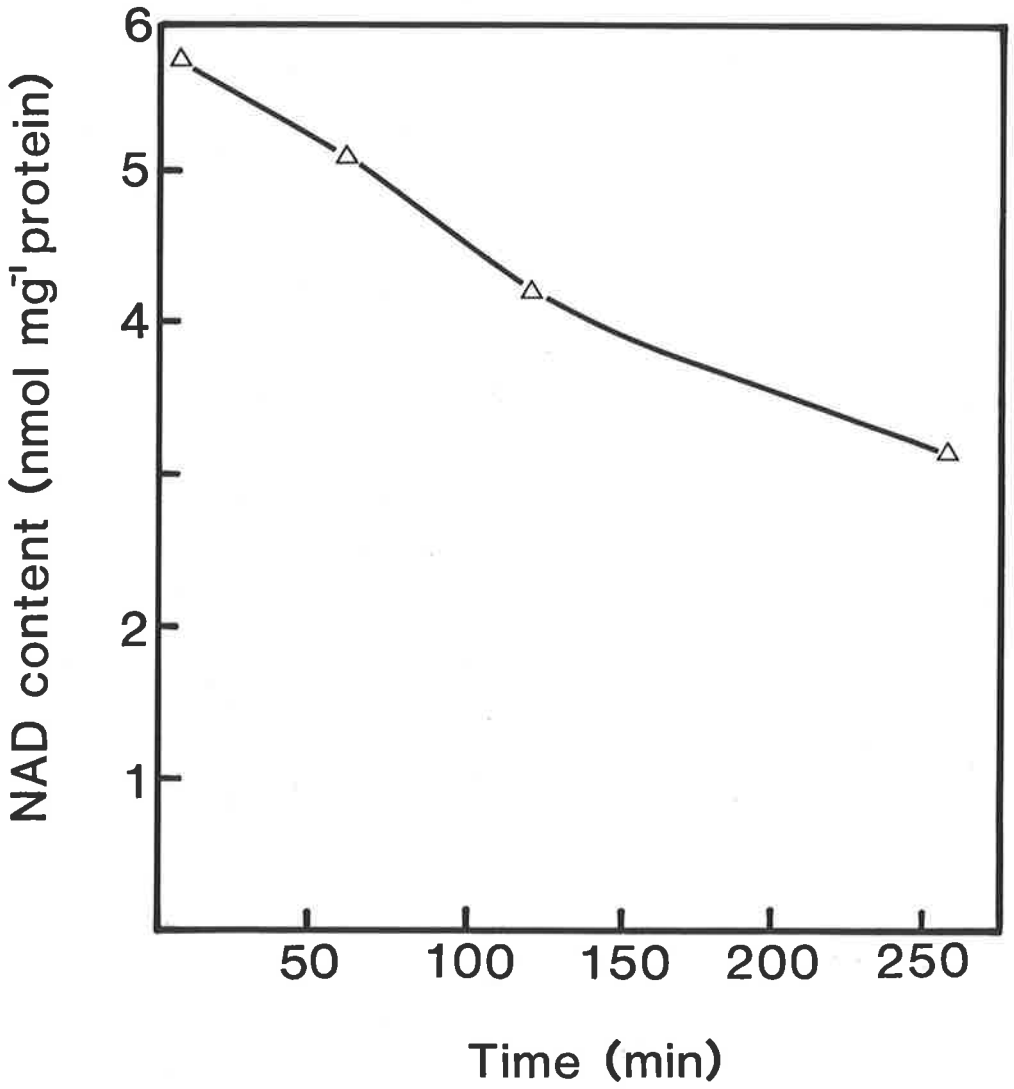

NAD content (nmol mg-1 protein)
6
5
4
2
1
50
100
150
200
250
Time (min)

Figure 5.4. Effect of NAD-depletion on state 3 and state 4 rates of malate and glycine oxidation by pea leaf mitochondria.

Experimental procedures as given in section 2.6. Samples of mitochondria (2 ml) were withdrawn at regular intervals and placed in an unsealed O_2 electrode and incubated with or without 1 mM NAD for 4 min at 25° C. The electrode was then sealed and either 12 mM glycine or 12 mM malate, glutamate and 0.1 mM TPP added to initiate O_2 uptake. The state 3 and state 4 rates shown represent the rates achieved following two state 3/state 4 transitions.

Symbols:

state 3 + NAD (●——●)

state 3 - NAD (●·····●)

state 4 + NAD (○——○)

state 4 - NAD (○·····○)

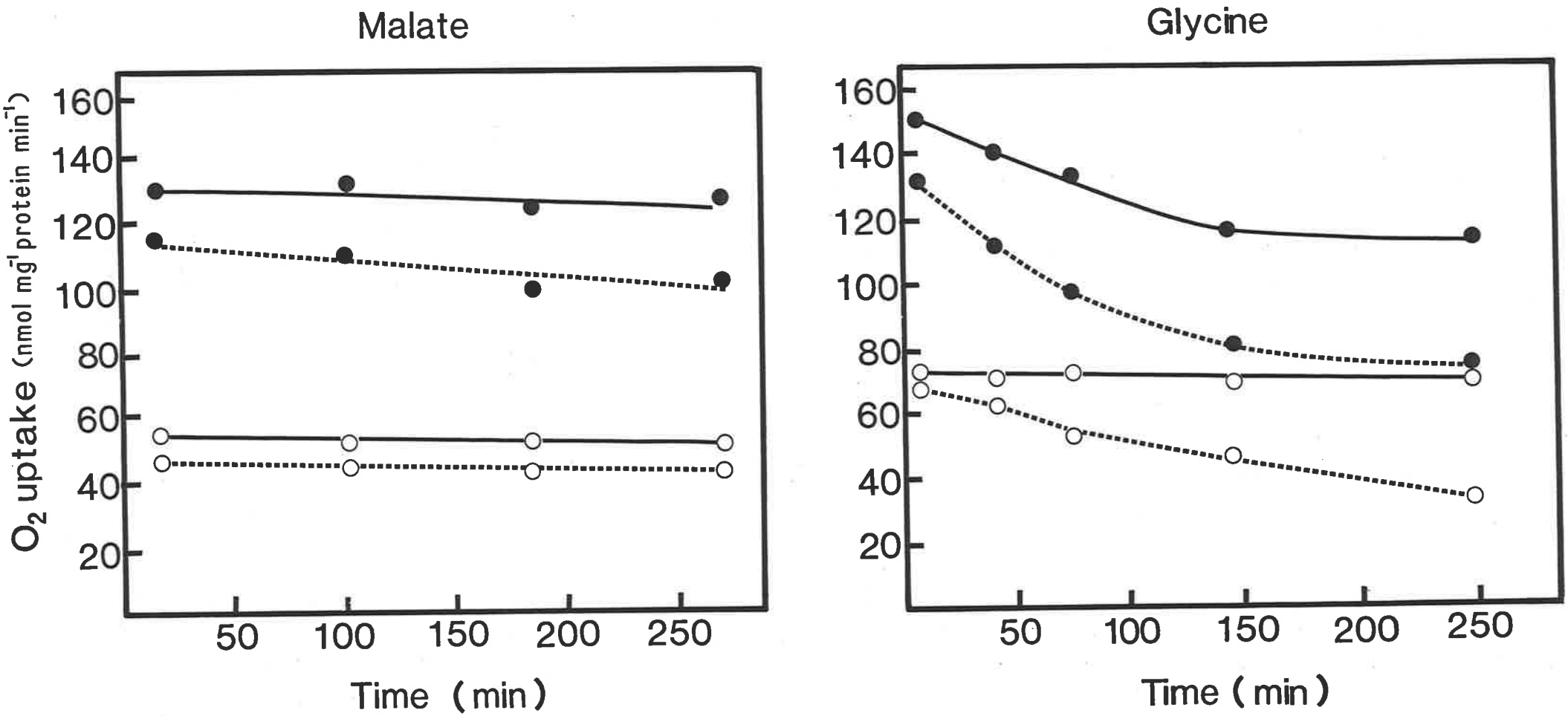

Malate
Glycine
O_2 uptake (nmol mg^{-1} protein min^{-1})
160
140
120
100
80
60
40
20
50
100
150
200
250
Time (min)

Figure 5.5. Comparative effects of NAD-depletion on state 3 malate and glycine oxidation by pea leaf mitochondria.

Data from Fig. 5.4 have been replotted in terms of the state 3 rates in the absence of added NAD, as a percentage of the state 3 rates measured in the presence of added NAD.

Symbols:

malate (▲)

glycine (△)

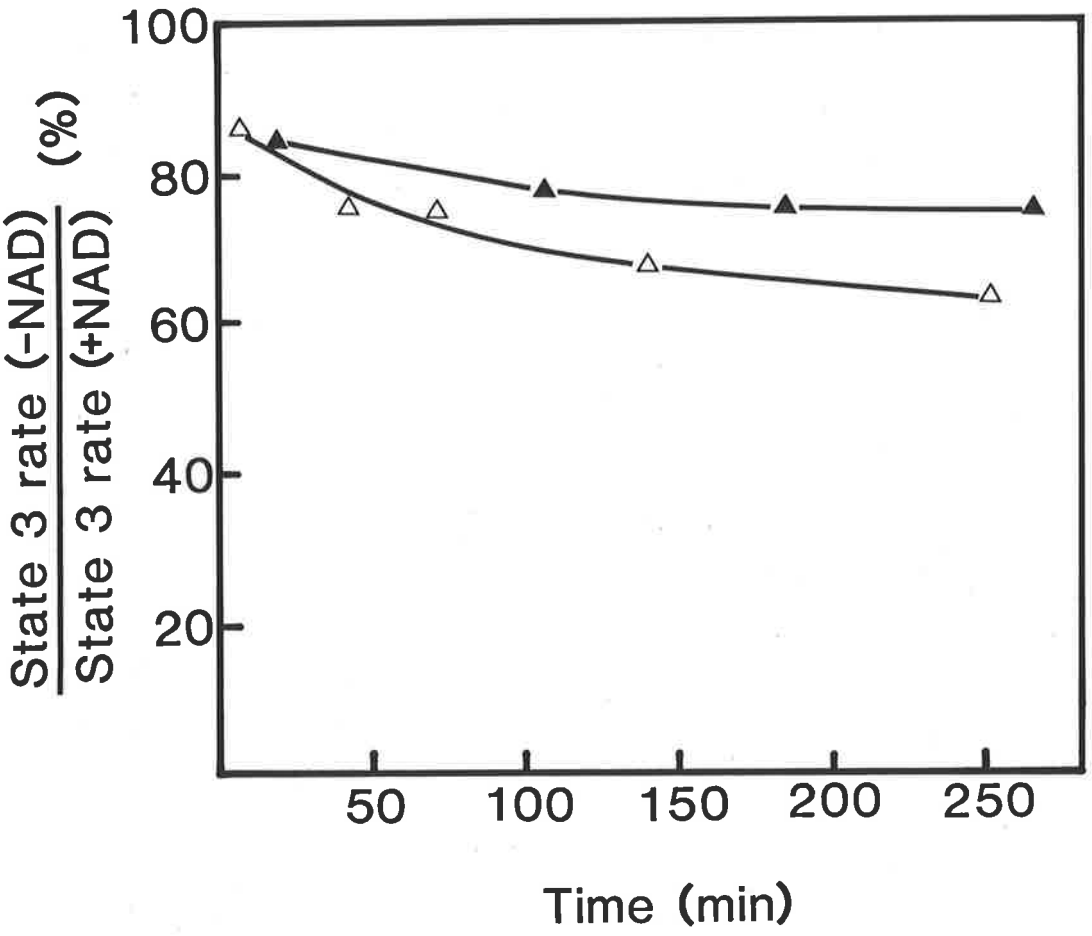

100
80
60
40
20
State 3 rate (-NAD) / State 3 rate (+NAD) (%)
50
100
150
200
250
Time (min)

Figure 5.6. Comparative effects of NAD-depletion on state 4 malate and glycine oxidation by pea leaf mitochondria.

Data from Fig. 5.4 have been replotted in terms of the state 4 oxidation rates in the absence of added NAD, as a percentage of the state 4 rates measured in the presence of added NAD.

Symbols:

malate (●)

glycine (○)

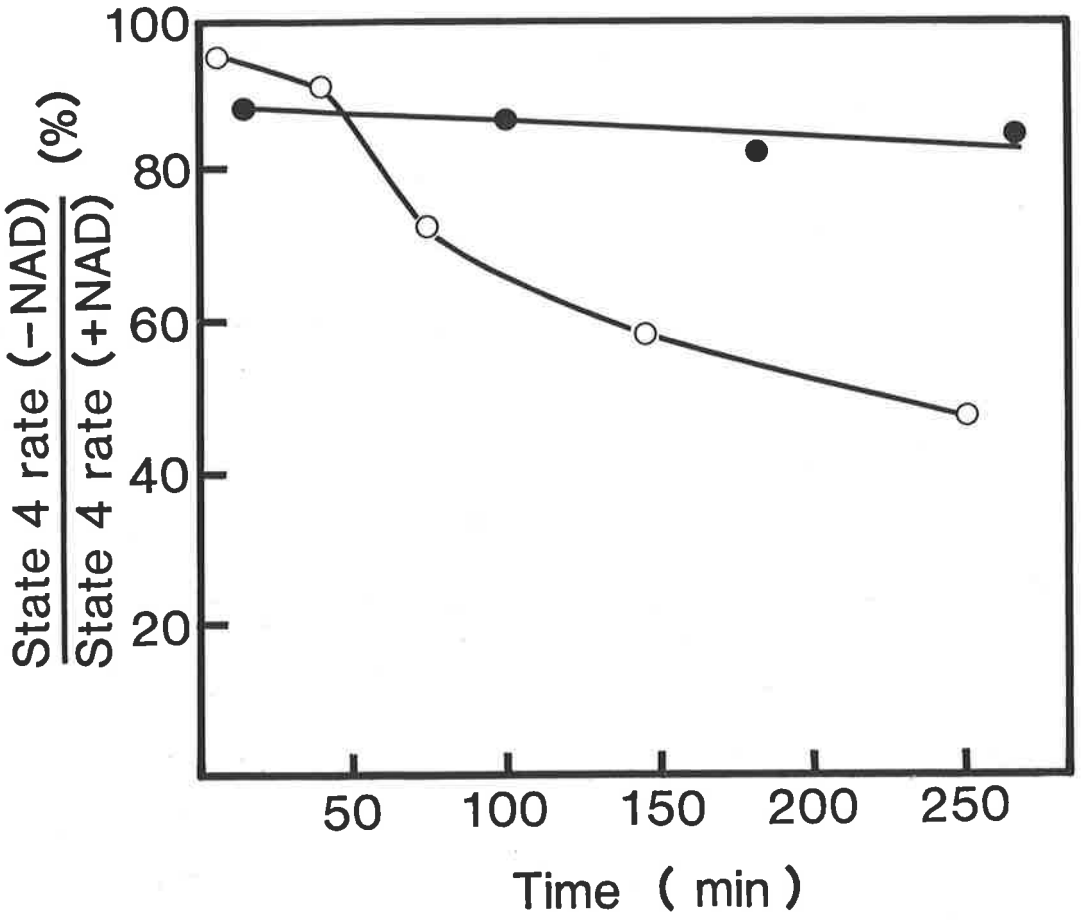

100
80
60
40
20
State 4 rate (−NAD) / State 4 rate (+NAD) (%)
50
100
150
200
250
Time (min)

Figure 5.7. Effect of NAD-depletion on the rotenone-insensitive state 3 rates of malate and glycine oxidation by pea leaf mitochondria.

Experimental procedures as given in Fig. 5.4. Rotenone (22 µM) was added during state 3 oxidation and the rate measured after 3-4 min. The numbers in the brackets represent the uninhibited state 3 rate of glycine oxidation by NAD-depleted (-NAD) mitochondria at that time.

Symbols:

glycine - NAD (○ ····· ○)

glycine + NAD (○ —— ○)

malate - NAD (● ····· ●)

malate + NAD (● —— ●)

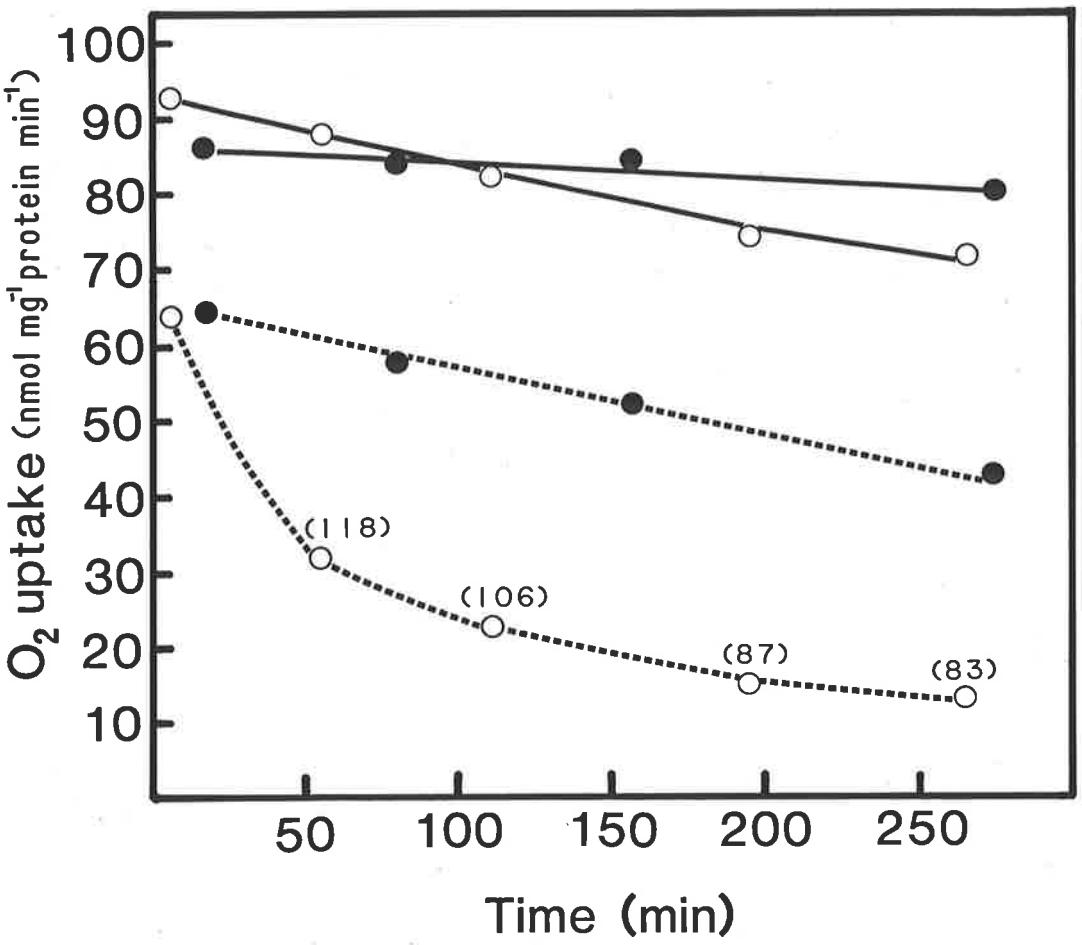

100
90
80
70
60
50
40
30
20
10
O_2 uptake (nmol mg^{-1} protein min^{-1})
(118)
(106)
(87)
(83)
50
100
150
200
250
Time (min)

Figure 5.8. Comparative effects of NAD-depletion on rotenone-insensitive state 3 rates of malate and glycine oxidation by pea leaf mitochondria.

Data from Fig. 5.7 have been replotted in terms of the rotenone-insensitive (R-I) state 3 oxidation rates in the absence of added NAD, as a percentage of the R-I rate in the presence of added NAD.

Symbols:

malate (●)

glycine (○)

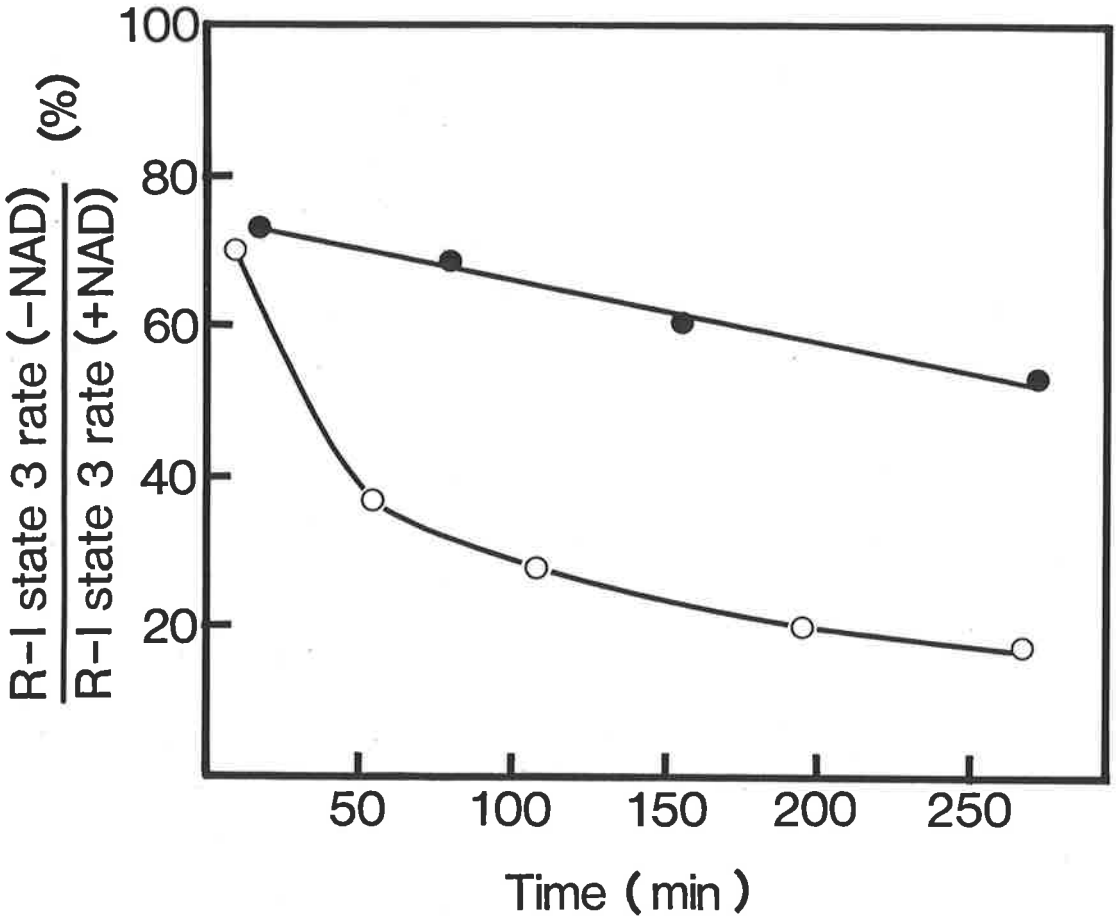

100
80
60
40
20
R-I state 3 rate (-NAD) / R-I state 3 rate (+NAD) (%)
50
100
150
200
250
Time (min)

Figure 5.9. Comparative effects of NAD-depletion on the degree of rotenone-insensitivity of the state 3 rates of malate and glycine oxidation by pea leaf mitochondria.

Experimental procedures as given in Fig. 5.7. Data plotted in terms of the rotenone-insensitive (R-I) state 3 rate as a percentage of the state 3 rate for both NAD-depleted (-NAD) and NAD-supplemented (+NAD) mitochondria.

Symbols:

glycine + NAD (○ —— ○)

glycine - NAD (○ ····· ○)

malate + NAD (● —— ●)

malate - NAD (● ····· ●)

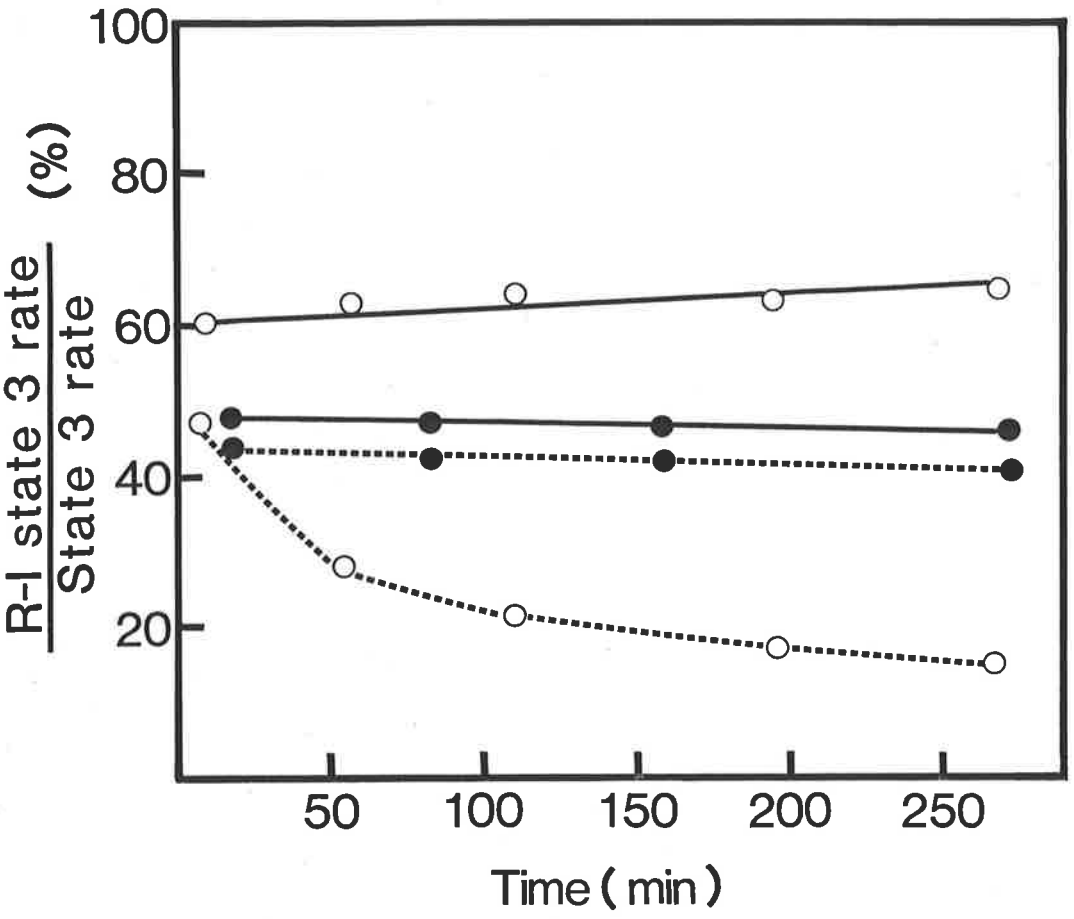

100
80
60
40
20
R-I state 3 rate / State 3 rate (%)
50
100
150
200
250
Time (min)

Figure 5.10. Comparative effects of NAD-depletion at 13°C on the rotenone-insensitive (R-I) state 3 rates of malate and glycine oxidation by pea leaf mitochondria.

Experimental procedures as given in Fig. 5.7 except that the incubation temperature was 13°C. Data plotted according to Fig. 5.8.

Symbols:

malate (▲)

glycine (Δ)

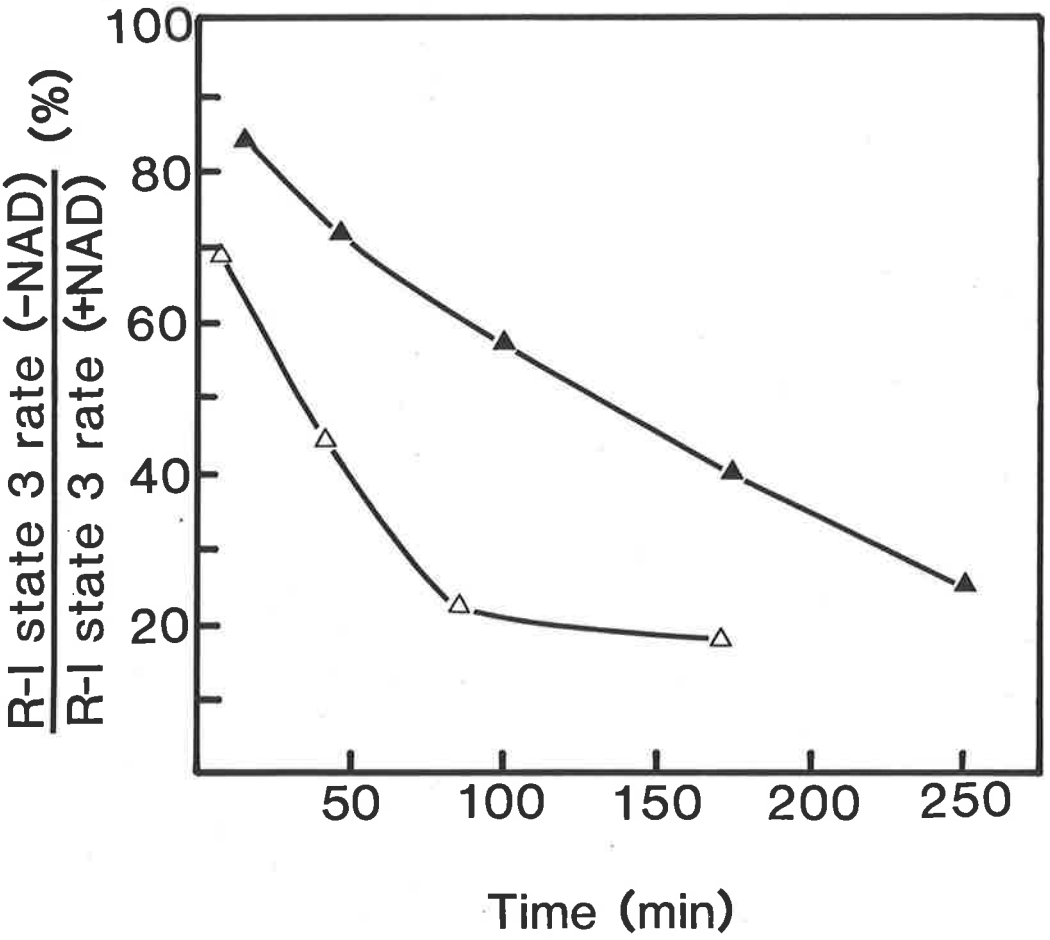

R-I state 3 rate (-NAD) / R-I state 3 rate (+NAD) (%)
100
80
60
40
20
50
100
150
200
250
Time (min)

Figure 5.11. Effect of NAD and substrate addition on rotenone-insensitive state 3 glycine oxidation by fresh and NAD-depleted pea leaf mitochondria.

Experimental procedures as given in Fig. 5.4. Trace A: fresh mitochondria, traces B and C: same mitochondria following 2 hr of NAD-depletion at 4° C.

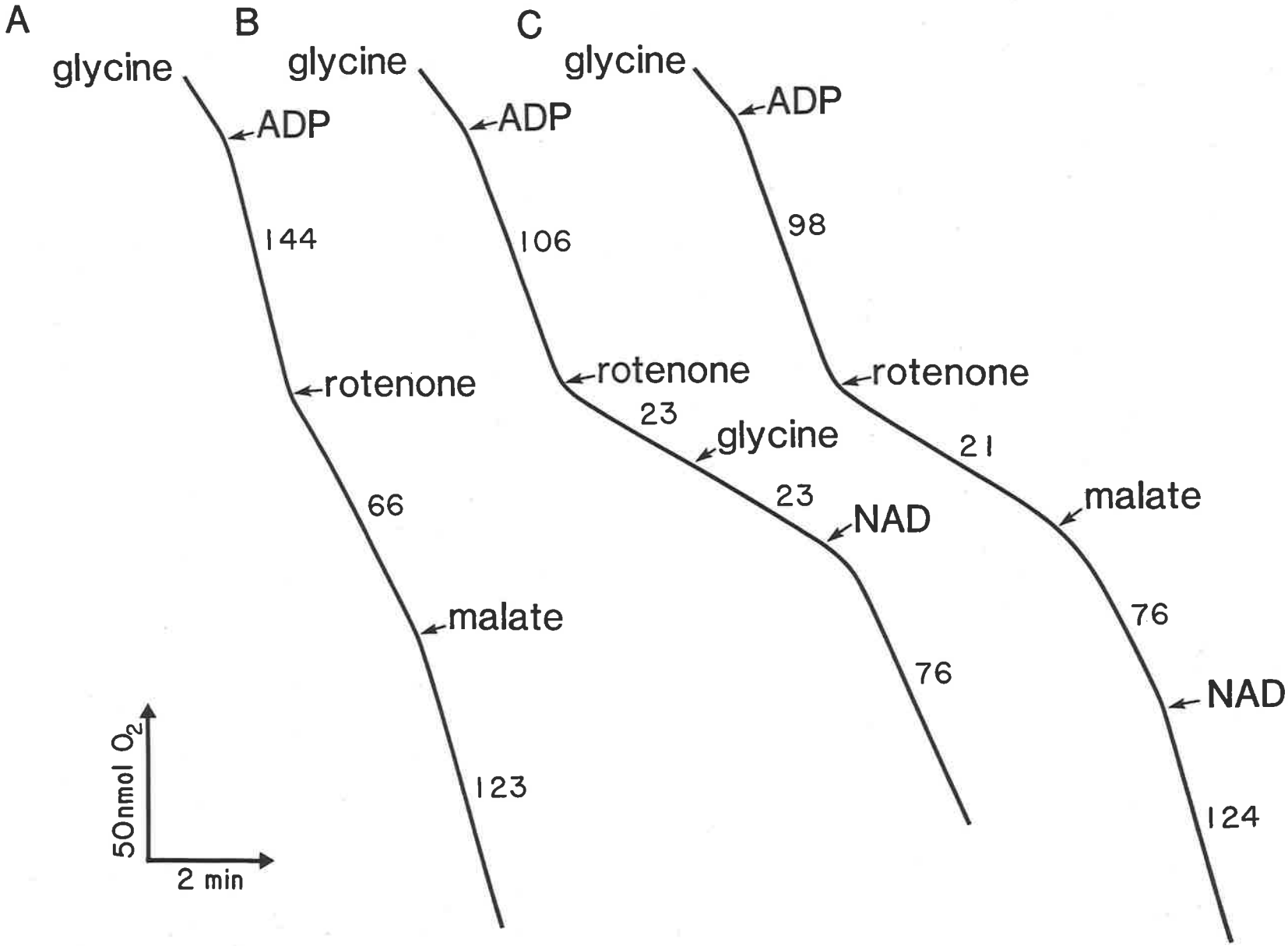

A
glycine
ADP
144
rotenone
66
malate
123
B
glycine
ADP
106
rotenone
23
glycine
23
NAD
76
C
glycine
ADP
98
rotenone
21
malate
76
NAD
124
50nmol O_2
2 min

Figure 5.12. Effect of NAD-depletion on the state 4 and rotenone-insensitive (R-I) state 3 rates of malate oxidation at pH 6.8.

Experimental procedures as given in Fig. 5.4, except that malate oxidation was assayed at pH 6.8 in the absence of glutamate and the presence of arsenite (1 mM) to favour oxidation via malic enzyme (Neuburger and Douce, 1980). Data plotted according to Figs 5.6 and 5.8.

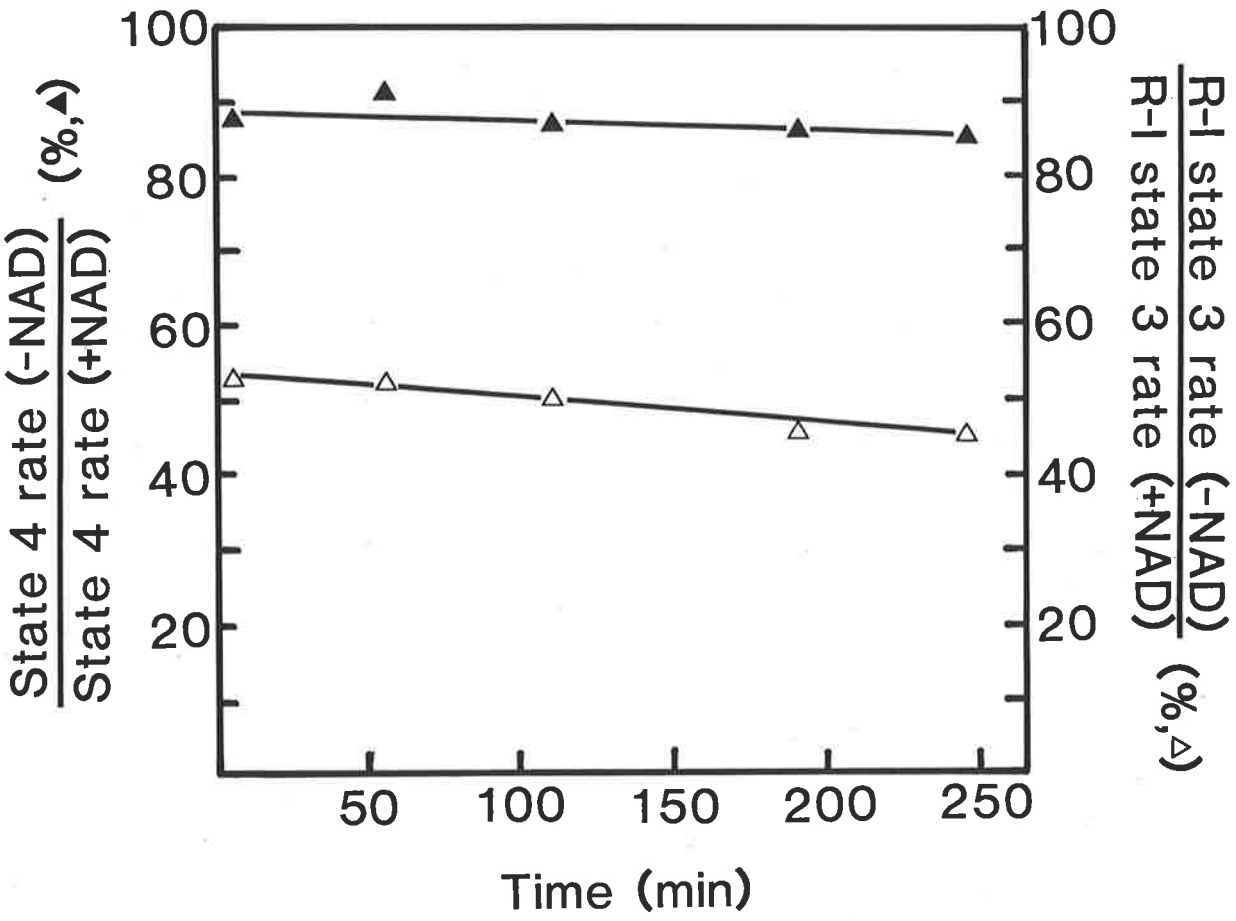

State 4 rate (-NAD) / State 4 rate (+NAD) (%,▲)
R-I state 3 rate (-NAD) / R-I state 3 rate (+NAD) (%,△)
100
80
60
40
20
50
100
150
200
250
Time (min)

Figure 5.13. Differential distribution of glycine decarboxylase and malate dehydrogenase within pea leaf mitochondria.

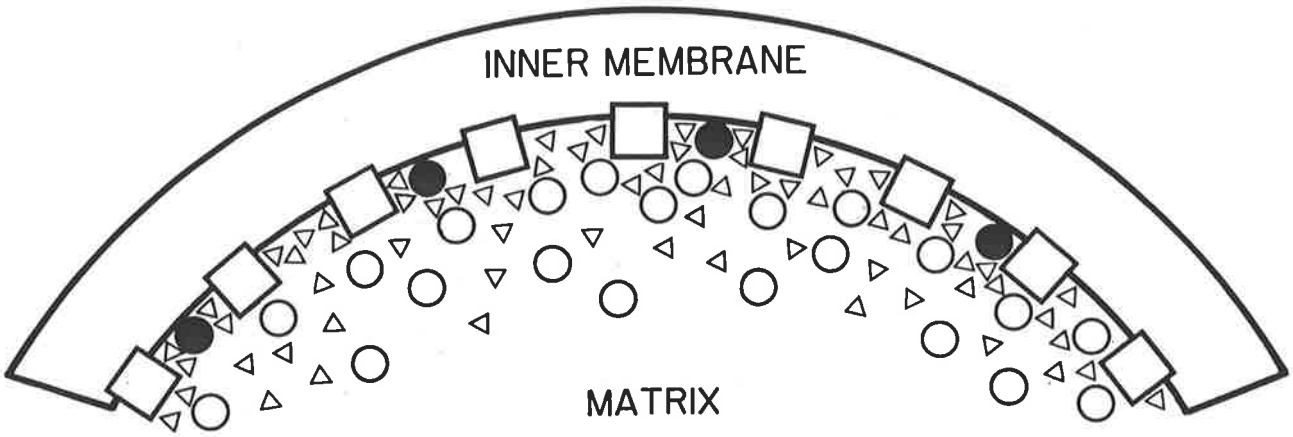
Symbols:

NADH dehydrogenase □

glycine decarboxylase ●

malate dehydrogenase ○

NAD △


INNER MEMBRANE
MATRIX

CHAPTER VI

DICARBOXYLATE STIMULATION OF PHOTORESPIRATORY NH_3 REFIXATION IN ISOLATED CHLOROPLASTS

6.1. INTRODUCTION

It has been suggested that a major factor limiting the rate of (NH_3, 2-OG)-dependent O_2 evolution by isolated chloroplasts is 2-OG uptake and that this uptake, and consequently the rate of O_2 evolution, could be enhanced by the addition of certain dicarboxylates (e.g. malate, succinate, fumarate) to the assay system (Woo and Osmond, 1982). This suggestion, however, would appear to be in contradiction of our current understanding of the mechanism of dicarboxylate uptake across chloroplast membranes. Firstly, work on isolated spinach (Lehner and Heldt, 1978) and pea (Proudlove and Thurman, 1981) chloroplasts demonstrates the dicarboxylate carrier to have both a high affinity and a high V_{max} for 2-OG transport and thus would not be expected to limit (NH_3, 2-OG)-dependent O_2 evolution. Secondly and more importantly, however, these uptake studies reveal that the dicarboxylate carrier is broadly specific and mediates the uptake of a number of different dicarboxylate molecules such 2-OG, malate, succinate and fumarate all with approximately equal affinity (Lehner and Heldt, 1978). Thus, if 2-OG uptake is the limiting factor to (NH_3, 2-OG)-dependent O_2 evolution, one might predict that the addition of a dicarboxylate such as malate should result, not in a stimulation as observed by Woo and Osmond (1982), but in an inhibition of O_2 evolution as both malate and 2-OG would compete for access to the dicarboxylate carrier, resulting in a further reduction in the rate of 2-OG entry.

The aim of this chapter therefore, was to determine the mechanism by which these dicarboxylate molecules stimulate O_2 evolution associated with the photorespiratory NH_3 re-assimilatory pathway in isolated chloroplasts, and to interpret these findings in terms of our current understanding of the operation of (a) the chloroplast dicarboxylate carrier and (b) the photorespiratory nitrogen cycle.

6.2. RESULTS

6.2.1. *Effect of malate on (NH_3, 2-OG)- and (glutamine, 2-OG)-dependent O_2 evolution*

Isolated pea chloroplasts carried out light-dependent O_2 evolution in the presence of NH_4Cl and 2-OG or glutamine and 2-OG, which was sensitive to the photosynthetic electron transport chain inhibitor DCMU (Fig. 6.1. A,B). Oxygen evolution was carried out in the absence of PPi, ADP and high concentrations of Mg^{2+}, all suggested previously as essential for (NH_3, 2-OG)-dependent O_2 evolution by isolated pea chloroplasts (Anderson and Done, 1977a).

The addition of malate to the assay system stimulated (NH_3, 2-OG)-dependent O_2 evolution by isolated pea chloroplasts, the extent of which was found to be dependent on the presence or absence of added glutamine (Fig. 6.1; C,D). Thus, while malate was found to stimulate (NH_3, 2-OG)-dependent O_2 evolution in the absence of glutamine (Fig. 6.1, C), the full extent of the stimulation was only observed when glutamine was also present

(Fig. 6.1; C,D,E). It should be noted that glutamine addition, in the absence of malate, caused only a minor increase in the rate of O_2 uptake (Fig. 6.1; A).

Methionine sulphoximine and azaserine, inhibitors of GS and GOGAT respectively (Ronzio *et al.* 1969; Wallsgrove *et al.* 1977), inhibited (NH_3, 2-OG)-dependent O_2 evolution, both in the presence and absence of malate (Fig. 6.2), indicating that malate-stimulated O_2 evolution resulted from the operation of the GS/GOGAT pathway and not from the metabolism of malate within the chloroplast. This is further demonstrated in Fig. 6.3 in which limiting amounts of either NH_4Cl or 2-OG were added to stimulate O_2 evolution in the presence of excess amounts of the second substrate(s). It can be seen that oxygen evolution ceased when all of the added substrate had been utilised, even in the presence of added malate, and was only re-initiated after the addition of more substrate. Examination of the molar ratios of O_2 evolved per substrate (NH_4Cl, 2-OG) addition from such traces (Table 6.1), showed the ratios to be in reasonable agreement with the theoretical value of 0.5 and previous measurements of such ratios (Anderson and Done, 1977a; Woo and Osmond, 1982). Malate did not appear to significantly alter the value of this ratio, although ratios measured with limiting amounts of 2-OG were consistently closer to the theoretical value (0.5) when measured in the presence of malate.

Malate could also be shown to stimulate (glutamine, 2-OG) dependent O_2 evolution (in the absence of NH_4Cl), but this was only observed at high glutamine concentrations (Fig. 6.4).

This may be due to the limited rate of uptake of glutamine into isolated pea chloroplasts, as noted by Barber and Thurman (1978).

The pH dependence of malate stimulation of (NH_3, glutamine, 2-OG)-dependent O_2 evolution is shown in Fig. 6.5. It can be seen that the optimum pH range for O_2 evolution both with and without added malate is in the range 7.2. to 7.6.

6.2.2. *Effect of malate on in vitro GS and GOGAT activity*

The possibility that malate stimulated O_2 evolution by activating either the GS or GOGAT enzymes directly was investigated using *in vitro* assay systems. Glutamine synthetase was readily assayed in a broken chloroplast system (Fig. 6.6), with the NH_4Cl-dependent activity being completely sensitive to MSO (Fig. 6.6; B). Malate was found to have no effect on this *in vitro* GS activity over the range of substrate concentrations tested (Fig. 6.6; A,C,D).

As GOGAT is linked *in vivo* to ferredoxin and, thus, the photosynthetic electron transport chain, it was decided that the closest *in vitro* approximation to *in vivo* conditions would be a reconstituted assay system. The development of such an O_2 evolving reconstituted chloroplast system for the assay of *in vitro* GOGAT activity is discussed in Chapter 7. Figure 6.7 shows that O_2 evolution in this system was light-dependent and completely sensitive to azaserine, but was not stimulated by the addition of malate. The stimulation of O_2 evolution upon the addition of more chloroplast extract (CE, containing the GOGAT enzyme)demonstrates that *in vitro* GOGAT activity, in the presence of malate, was not

limited by the supply of reducing equivalents from the photosynthetic electron transport chain.

Pre-incubation of isolated intact chloroplasts with malate (1 mM) for periods of up to 1 h was also found to have no effect on the rate of (NH_3, glutamine, 2-OG)-dependent O_2 evolution, when assayed in the absence of malate.

6.2.3. *Effect of malate on the affinity of the chloroplast system for 2-OG*

The dependence of (NH_3, 2-OG)-dependent O_2 evolution by isolated pea chloroplasts on 2-OG concentration, both in the presence and absence of glutamine and of malate, is shown in Fig. 6.8. Again it can be seen that malate stimulated (NH_3, 2-OG)-dependent O_2 evolution, both with and without added glutamine, over the range of 2-OG concentrations tested. The $K\frac{1}{2}$ for 2-OG in the absence of malate was estimated at 200-250 µM. Glutamine addition had no effect on this value, while the presence of malate reduced the $K\frac{1}{2}$ estimate to 50-80 µM. Similar affinities for 2-OG were measured with isolated spinach chloroplasts (Fig. 6.9). Malate stimulated (NH_3, 2-OG)-dependent O_2 evolution by spinach chloroplasts, but had little effect on the affinity of the system for 2-OG which, as for peas, was of the order of 100-200 µM. In contrast to pea chloroplasts, however, glutamine had no effect on the degree of stimulation, by malate, of (NH_3 2-OG)-dependent O_2 evolution by isolated spinach chloroplasts. This result was also observed by Woo and Osmond (1982).

6.2.4. *Competitive interactions between 2-OG and malate*

The relationship between malate concentration and the degree of stimulation of (NH_3, glutamine, 2-OG)-dependent O_2 evolution is illustrated in Fig. 6.10. In the presence of 1 mM 2-OG, half maximal stimulation of O_2 evolution was achieved on addition of malate to a concentration of 150 µM, and maximal stimulation reached when the value of the ratio of concentrations of malate to 2-OG was in the range 1-2. However, when malate was added in the presence of an increased concentration of 2-OG (10 mM), stimulation at each malate concentration (below 2 mM) was found to be reduced and the $K_{\frac{1}{2}}$ for malate increased to approximately 700 µM.

These results indicated that while malate could significantly stimulate (NH_3, glutamine, 2-OG)-dependent O_2 evolution, the degree of stimulation produced by a particular malate concentration was dependent on the concentration of 2-OG also present. This is further illustrated in Fig. 6.11. Here the malate concentration was kept constant (0.5 mM) while the 2-OG concentration was varied between 0 and 20 mM. The resultant stimulation of (NH_3, glutamine, 2-OG)-dependent O_2 evolution was compared to that produced by malate, when present at the same concentration as 2-OG i.e. a malate : 2-OG concentration ratio of 1. As the results show, the degree of stimulation of (NH_3, glutamine, 2-OG)-dependent O_2 evolution produced by a particular malate concentration, was dependent on the concentration of 2-OG present.

Conversely, increasing the amount of malate added in the presence of a constant 2-OG concentration, was also found to lead to a decrease in the rate of malate-stimulated (NH_3, glutamine, 2-OG)-dependent O_2 evolution (Fig. 6.12).

6.2.5. *Specificity of the dicarboxylate stimulation*

The effects of a range of dicarboxylates on (NH_3, glutamine, 2-OG)-dependent O_2 evolution by isolated pea chloroplasts are shown in Table 6.2. Malate, succinate, fumarate, L-tartarate and thiomalate were all found to markedly stimulate O_2 evolution. In contrast, aspartate and glutarate produced only very minor increases, while glutamate addition resulted in an inhibition of (NH_3, glutamine, 2-oxoglutarate)-dependent O_2 evolution. Malonate which is not thought to be transported into the chloroplast (Lehner and Heldt, 1978) had no effect.

Spinach chloroplasts gave similar results to pea chloroplasts with respect to the percent stimulation of various dicarboxylates, relative to malate. For example, the addition of L-tartrate and thiomalate at 2 mM, produced stimulations of 58 and 33% respectively (compare to Table 6.2). However, in contrast to pea chloroplasts, glutarate was found to have no effect, while aspartate and glutamate were both very inhibitory to (NH_3, 2-OG)-dependent O_2 evolution by isolated spinach chloroplasts.

6.2.6. *Comparative rates of NH_3 refixation by the different chloroplast systems*

Table 6.3 summarizes the mean values of the various types of O_2 evolving systems associated with the photorespiratory NH_3 re-assimilatory pathway, which have been investigated in this chapter. The rates of O_2 evolution have been corrected for chloroplast breakage during isolation to allow for the estimate of the maximal potential rates of NH_3 refixation which can be achieved under the various conditions, based on the theoretical ratio of 2 x NH_3 refixed per 1 x O_2 released in the ferredoxin-linked GOGAT reaction. It should be noted that plants grown under high nitrate status (i.e. 10 mM KNO_3) showed similar rates of (NH_3, 2-OG)-dependent O_2 evolution to those given in Table 6.3. Furthermore, percoll purified chloroplasts showed similar rates of O_2 evolution and stimulatory effects by malate to those of unpurified chloroplasts.

The most variable parameter listed in Table 6.3 was found to be the ratio D : E i.e. the rate of malate-stimulated (NH_3, 2-OG)-dependent O_2 evolution in the absence of glutamine, compared to the maximal rate of malate-stimulated O_2 evolution in the presence of glutamine. This was not only true for chloroplast preparations within the variety *Massey Gem*, but also between pea varieties, with the variety *Green feast* consistently showing a much smaller value for this ratio (e.g. 0.20-0.25) than *Massey Gem*. This most probably reflects differences in the levels of endogenous intermediates of the GS/GOGAT pathway, within chloroplasts isolated from these different pea varieties. Differences

were also evident between chloroplasts isolated from different plant species, as glutamine had no effect on the degree of stimulation, by malate, of (NH_3, 2-OG)-dependent O_2 evolution by spinach chloroplasts (i.e. a D : E ratio of 1.0).

6.3. DISCUSSION

6.3.1. *The mechanism of dicarboxylate stimulation of O_2 evolution*

The results demonstrate that isolated pea chloroplasts are capable of supporting (NH_3, 2-OG)-dependent O_2 evolution in the absence of PPi, ADP or excessively high concentrations of $MgCl_2$ (10 mM) as used previously (Anderson and Done 1977a). This is especially significant with respect to $MgCl_2$, in that recent reports (Huber and Maury, 1980; Maury *et al.* 1981) have shown low concentrations (4 mM) of $MgCl_2$ to cause significant acidification of chloroplast stroma and inhibit CO_2-dependent O_2 evolution.

In contrast to the observations of Woo and Osmond (1982), malate was found to stimulate (NH_3, 2-OG)-dependent O_2 evolution by isolated pea chloroplasts, in the absence of added glutamine (Fig. 6.1). However, the full stimulatory effect of malate was only seen when glutamine was also present, with NH_4Cl. This requirement for glutamine most probably results from a deficiency of glutamate for GS, due to the unusually high K_m of this enzyme for this substrate e.g. 3.5 to 13 mM (O'Neal and Joy, 1974) and the presence, in isolated pea chloroplasts, of endogenous glutamate at concentrations of only 5 to 6 mM (Mills and Joy, 1980). This is in contrast to spinach chloroplasts,

which did not require glutamine for maximal stimulation of (NH_3, 2-OG)-dependent O_2 evolution by malate (see also Woo and Osmond, 1982) and which have been found to contain glutamate at concentrations of up to 15 mM (Lehner and Heldt, 1978).

The demonstration of malate-stimulated (glutamine, 2-OG)-dependent O_2 evolution (Fig. 6.4) indicates that the effect of malate is on GOGAT-dependent O_2 evolution. It is also clear that the extent of the stimulation is dependent on the relative availability of glutamine to the GOGAT enzyme. When the only source of glutamine is via uptake (e.g. Fig. 6.4) malate may be seen to have little or no effect, due to the slow transport of this amino acid across the chloroplast membrane (Barber and Thurman, 1978). When isolated pea chloroplasts were incubated with both glutamine and NH_4Cl, however, the availability of glutamine within the chloroplast was markedly increased through the operation of GS, thereby allowing the maximal stimulatory effect of malate on GOGAT-dependent O_2 evolution to be observed (Figs 6.1, 6.8).

No evidence was found to suggest that the observed stimulation of(NH_3, glutamine, 2-OG)-dependent O_2 evolution by malate resulted from the metabolism of malate within the chloroplast, or that the addition of malate led to any activation of the pathway enzymes. Furthermore, since malate can be shown to stimulate both (NH_3, 2-OG) and (glutamine, 2-OG)-dependent O_2 evolution it is unlikely that the malate effect is mediated through direct effects on glutamine or NH_3 uptake. More probably, malate increases the availability to GOGAT of its other substrate

(2-OG) either by stimulating 2-OG uptake or increasing the affinity of the GOGAT enzyme for this substrate. Woo and Osmond (1982) observed a dramatic decrease in the $K\frac{1}{2}$ for 2-OG in (NH_3, 2-OG)-dependent O_2 evolution from 6 mM to 73 µM on the addition of malate to spinach chloroplasts. My measurements with isolated pea and spinach chloroplasts, however, showed the $K\frac{1}{2}$ estimates for 2-OG, both in the presence and absence of malate (50-250 µM; see also Anderson and Done, 1977b) to be in the range expected from previous studies on chloroplast transporters (i.e. 190 µM; Lehner and Heldt,1978) and the glutamate synthase enzyme (i.e. 150 µM; Wallsgrove *et al*. 1977). Thus, the stimulatory effect of malate on (NH_3, glutamine, 2-OG)-dependent O_2 evolution probably results from an increase in the rate of 2-OG uptake, via exchange reactions with malate across the chloroplast membrane.

Isolated chloroplasts would not contain sufficient endogenous dicarboxylates (Lehner and Heldt, 1978) to maintain this type of rapid exchange for any length of time following the addition of 2-OG, and, once exchanged out of the chloroplast into the surrounding medium, would be too dilute to facilitate their rapid re-uptake for use in further exchange reactions. This may explain the apparent anomaly between the estimated rates of 2-OG uptake, as measured in ^{14}C - uptake experiments over 10-20 s i.e. 26-32 µmol mg^{-1} Chl h^{-1} (Lehner and Heldt, 1978; Proudlove and Thurman 1981)and those rates approximated from polarographic measurements, as done here, of between 8-12 µmol mg^{-1} Chl h^{-1} (Table 6.3), which represent steady state rates calculated over 5-10 minutes.

The obvious contradiction to the above theory is that glutamate, a dicarboxylate known to exchange across the chloroplast membrane (Lehner and Heldt, 1978), would be produced during (NH_3, glutamine, 2-OG)-dependent O_2 evolution and, indeed, would normally be expected to be exchange for 2-OG *in vivo* in order to maintain the stoichiometric balance of the photorespiratory cycle (see Fig. 1.4). However, it would appear from the limited rate of (NH_3, glutamine, 2-OG)-dependent O_2 evolution, in the absence of malate, that glutamate (in contrast to malate) may not be the optimal substrate for exchange reactions involving 2-OG, and that a more complex shuttle involving the net exchange of 2-OG and glutamate on separate carriers may occur *in vivo*.

6.3.2. *Evidence for separate dicarboxylate carriers in the chloroplast membrane*

The demonstration of malate stimulated 2-OG uptake provides strong evidence for the existence of separate carriers in the chloroplast membrane. If, for example, the chloroplast membrane contained only one type of dicarboxylate carrier, the addition of malate during (NH_3, glutamine, 2-OG)-dependent O_2 evolution should result in a competition between malate and 2-OG for uptake and, consequently, an inhibition of O_2 evolution. However, for the opposite result to occur, as is indeed the case, at least two separate carriers must be present. The first carrier must exhibit a much higher affinity for malate than 2-OG to allow for the rapid uptake of malate in the presence of 2-OG. This is well illustrated in Figs 6.10 and 6.11 which show that while an

inhibition of stimulation by malate is observed in the presence of very large 2-OG: malate concentration ratios, the inhibitory effect of 2-OG on malate uptake is much lower than would be predicted from inhibitor kinetics equations (Webb, 1963). This is based on the assumption that the Ki for 2-OG, with respect to malate uptake, should be similar to the $K_{\frac{1}{2}}$ or K_m for 2-OG uptake, if both malate and 2-OG were bound at the same carrier site. For example, Lehner and Heldt (1978) showed that the uptake of malate into spinach chloroplasts was inhibited approximately 60% by the presence of a 3 fold higher concentration of succinate or fumarate. My results, however, show that the presence of a 3 fold higher concentration of 2-OG had virtually no effect on the stimulation of (NH_3, glutamine, 2-OG)-dependent O_2 evolution by malate (Fig. 6.11)

Conversely, the second carrier must exhibit the reverse characteristics or affinities to the first (i.e. a much higher affinity for 2-OG than malate) to ensure that the added malate does not readily compete with 2-OG for uptake into the chloroplast, in exchange for the internal malate. Figure 6.12 demonstrates that the carrier responsible for 2-OG uptake does indeed have only a low affinity for malate, since the presence of very high malate : 2-OG concentration ratios have only a small effect on the maximum rate of (NH_3, glutamine, 2-OG)-dependent O_2 evolution. Clearly, these results support the theory that 2-OG and malate are taken up on separate carriers with a small degree of overlapping specificity.

Further information regarding the specificity of this '2-OG' carrier, may also be obtained from the results given in Table 6.2. The stimulatory effect of malate on (NH_3, glutamine, 2-OG)-dependent O_2 evolution could be reproduced by a number of other dicarboxylates. Aspartate, however, had little effect on the rate of O_2evolution while the addition of glutamate was inhibitory. The affinity of the so-called dicarboxylate carrier for the uptake of the various dicarboxylate species into the chloroplast is thought to be as follows: malate > aspartate > glutamate > L-tartrate and thiomalate (Lehner and Heldt, 1978). Furthermore, aspartate uptake in the presence of 2-OG must be rapid, since rates of (Asp, 2-OG)-dependent O_2 evolution of approximately 20 µmol mg^{-1} Chl h^{-1}(or 40 µmol 2-OG uptake mg^{-1} Chl h^{-1}) are observed in the presence of 1 mM concentrations of each substrate. Thus, the inability of aspartate and glutamate to stimulate (NH_3, glutamine, 2-OG)-dependent O_2 evolution, in contrast to the other dicarboxylates (Table 6.2), may reflect the specificity of the '2-OG' carrier on the stromal side of the chloroplast membrane. It would appear that in contrast to it's specificity for 2-OG on the outside of the membrane, the stromal side of this carrier may exhibit a high degre of specificity for C_4 type dicarboxylates (e.g. malate, succinate, L-tartrate), and that the addition of an amino group (e.g. aspartate), an extra carbon (e.g. glutarate), or both (e.g. glutamate), significantly reduces the binding of such dicarboxylate molecules to this carrier, for use in exchange reactions with 2-OG.

Anderson and Done (1977b) have previously reported that glutamate inhibits (glutamine, 2-OG)-dependent O_2 evolution by pea chloroplasts through product inhibition of GOGAT. Certainly, the results of Table 6.4 indicate that the glutamate inhibition of either (glutamine, 2-OG) or (NH_3, glutamine, 2-OG)-dependent O_2 evolution (see also Table 6.2) is associated with competitive interactions between 2-OG and glutamate. However, the glutamate inhibition could be reversed, not only by 2-OG, but also by malate (Table 6.4). This suggests that the glutamate inhibition of (glutamine, 2-OG)- and (NH_3, glutamine, 2-OG)-dependent O_2 evolution is mediated through an inhibition of 2-OG uptake by glutamate, and that in the presence of malate, 2-OG uptake occurs on a separate carrier which has a much lower affinity for glutamate. Woo (1983) has also recently reported that both glutamate and aspartate inhibit (NH_3, 2-OG)-dependent O_2 evolution by spinach chloroplasts (as seen here) and that this inhibition could be reversed by various dicarboxylates. Consequently, he too has proposed the possible existence of a separate dicarboxylate carriers in the chloroplast membrane.

To summarize, the results suggest the existence of two separate carriers in the outer chloroplast membrane, of similar specificity to those found in the inner mitochondrial membrane: (a) a dicarboxylate exchange carrier (I in Fig. 6.13) responsible for the rapid uptake/exchange of a wide range of dicarboxylates (excluding 2-OG) and(b) a 2-OG/dicarboxylate exchange carrier (II in Fig. 6.13) which mediates the rapid uptake of 2-OG in exchange for specific dicarboxylates e.g. malate. As illustrated in Fig. 6.13 (broken lines), the stimulation of 2-OG uptake by

malate in (NH_3, glutamine, 2-OG)-dependent O_2 evolution, in reality represents a 'short circuit' in the reaction and transport sequence which normally supports rapid rates of 2-OG uptake during the operation of a malate/aspartate shuttle, with malate being taken up directly into the chloroplast instead of being produced internally.

The predicted rapid uptake of malate, in exchange for glutamate (Fig. 6.13) is not, however, supported by ^{14}C- uptake data. The results in Table 6.5 show that the rate of efflux of ^{14}C- glutamate from isolated spinach chloroplasts was almost 3 fold lower in the presence of malate, than with 2-OG. Similar results have also been reported by Woo (1983). One possible interpretation of these results is, of course, that malate uptake in not mediated via an exchange reaction with glutamate as proposed in Fig. 6.13. However, with the clear discrepancies which appear to exist between the results predicted on the basis of ^{14}C-uptake studies and those observed with the O_2 electrode, I feel the results of Table 6.5 cannot be considered conclusive in ruling out the possibility of a malate/glutamate exchange under the incubation conditions used for the O_2 electrode studies. This raises the question of the validity of uptake experiments carried out over short periods of time (i.e. 15-30 s), in the absence of light, and their applicability to steady state exchange reactions occurring across chloroplast membranes in the light as measured with the O_2 electrode. This is particularly relevant to light-mediated changes in the pH of chloroplast stroma, which may affect the uptake of dicarboxylate molecules into the chloroplast.

Finally, whilst the findings regarding the potential existence of separate carriers in the chloroplast membrane are of obvious importance, the physiological significance of malate-stimulated (NH_3, glutamine 2-OG)-dependent O_2 evolution to the operation of the photorespiratory cycle *in vivo* is yet to be justified. If photorespiratory NH_3 recycling does indeed occur at rates of approximately 30 µmol mg^{-1} Chl h^{-1}, then such a mechanism for the uptake of 2-OG would appear, from our studies with isolated pea chloroplasts, to be required to operate *in vivo*. If, however, this is an over-estimation of the true NH_3 flux through the chloroplast, then the rates of NH_3 refixation in the absence of such a mechanism, as measured here e.g. 12-18 µmol NH_3 mg^{-1} Chl h^{-1} (Table 6.3, corrected for chloroplast breakage), may be sufficient.

Table 6.1. Molar ratios of O_2 evolution per substrate addition during (NH_3, 2-OG) and (NH_3, glutamine, 2-OG)-dependent O_2 evolution by pea chloroplasts.

Data taken from the results shown in Fig. 6.3.

Non-limiting substrate(s)	Limiting substrate	Molar ratio: moles O_2 evolved per mole substrate added		
		Exp. 1	*Exp. 2*	*Exp. 3*
		.48	.46	-
2-OG, 10 mM	NH_4Cl	.49	.48	-
		-	.48	-
NH_4Cl, 1 mM		.41	.39	.41
glutamine, 1 mM	2-OG	.42	.41	-
malate, 0.5 mM		.42	.41	-
		.36	.34	.34
NH_4Cl, 1 mM	2-OG	.36	.36	.37
glutamine, 1 mM		.34	-	-

Table 6.2. Specificity of the dicarboxylate stimulation of (NH_3–glutamine, 2-OG)-dependent O_2 evolution by pea chloroplasts.

Chloroplasts (40 µg Chl ml^{-1}) were added to 1.8 ml of reaction medium (section 2.8) containing 1 mM NH_4Cl and 1 mM glutamine. O_2 evolution was initiated by the addition of 1 mM 2-OG together with the dicarboxylate specified. Amino-oxyacetic acid (5 mM) was used to inhibit (Asp, 2-OG)-dependent O_2 evolution in assays involving aspartate. There was no effect of amino-oxyacetic acid on the rate of (NH_3, glutamine, 2-OG)-dependent O_2 evolution. The control rate represents the rate of O_2 evolution in the absence of added dicarboxylate. Intactness: 72, 69%. CO_2 - dependent O_2 evolution: 92, 93 µmol mg^{-1} Chl h^{-1}.

DICARBOXYLATE	Experiment 1		Experiment 2	
	O_2 evolution	% of stimulation by malate	O_2 evolution	% of stimulation by malate
	$\mu mol\ mg^{-1}\ Chl\ h^{-1}$		$\mu mol\ mg^{-1}\ Chl\ h^{-1}$	
Control	4.6	-	5.2	-
Malate, 2 mM	16.6	100	15.0	100
Succinate, 2 mM	16.3	98	-	-
Fumarate, 2 mM	16.6	100	-	-
L-Tartarate, 2 mM	12.8	68	11.1	60
L-Tartarate, 5 mM	13.4	73	12.3	72
Aspartate, 2 mM	5.4	6	6.0	8
5 mM	6.1	12	6.5	13
Glutarate, 2 mM	6.4	15	-	-
5 mM	7.1	21	6.5	13
Glutamate, 2 mM	2.0	-	-	-
5 mM	1.0	-	-	-
Thiomalate, 2 mM	-	-	8.9	38
5 mM	-	-	11.4	63
Malonate, 2 mM	4.6	0	-	-
5 mM	4.1	-	-	-

Table 6.3. Summary of mean values for various forms of O_2 evolution by pea chloroplasts.

Assays A,B,D and E involved 1 mM concentrations of all substrates. Assay C involved 2 mM glutamine and 1 mM 2-OG. Rates corrected for chloroplast breakage during preparation (C.F.B.) are also given (mean intactness: 68%) together with maximal rates of NH_3 re-assimilation which can be achieved with each system.

Type of O_2 evolution		n	mean	s.d.	C.F.B.	NH_3 re-assimilatory capacity
			$\mu mol\ O_2\ mg^{-1}\ Chl\ h^{-1}$			$\mu mol\ NH_3\ mg^{-1}\ Chl\ h^{-1}$
NH_3, 2-OG	(A)	7	3.3	0.7	4.8	9.6
NH_3, glutamine, 2-OG	(B)	12	4.4	0.9	6.4	12.8
glutamine, 2-OG	(C)	5	6.0	0.4	8.8	17.6
NH_3, 2-OG, malate	(D)	7	6.9	1.6	10.0	20.0
NH_3, glutamine, 2-OG, malate	(E)	11	14.6	2.2	21.5	43.0
(D) / (E)		7	0.5	0.1	-	-
(E) / (B)		11	3.3	0.4	-	-

Table 6.4. Effect of glutamate on various forms of O_2 evolution by pea chloroplasts.

Chloroplasts (44 µg Chl ml^{-1}) were added to 1.8 ml of reaction medium (section 2.8). Assay concentrations were all 1 mM except for glutamine in the (glutamine, 2-OG) system which was 2mM. The numbers shown in brackets represent final concentrations in the assay medium. Intactness: 71% CO_2-dependent O_2 evolution: 94 µmol mg^{-1} Chl h^{-1}.

Sequential additions	Type of O_2 evolution		
	(glutamine, 2-OG)	(NH_3, glutamine, 2-OG)	(NH_3, glutamine, 2-OG, malate)
		µmol mg^{-1} Chl h^{-1}	
Control	6.1	5.0	16.3
+ glutamate (5 mM)	3.2	1.8	-
+ glutamate (10 mM)	2.2	1.1	12.3
+ glutamine (6 mM)	2.2	-	-
+ 2-OG (5 mM)	5.6	5.5	13.5
+ malate (2 mM)	-	-	16.3
Control	6.0	5.2	15.9
+ glutamate (5 mM)	3.1	1.6	-
+ glutamate (10 mM)	-	-	11.9
+ malate (2 mM)	6.0	13.0	13.6
+ 2-OG (5 mM)	-	-	15.6

Table 6.5. Rate of ^{14}C-glutamate efflux from spinach chloroplasts in the presence of various dicarboxylates.

Chloroplasts were percoll-purified as described in section 2.4 and the rate of back-exchange of ^{14}C-glutamate measured according to the method in section 2.12. Intactness: 97%.

Dicarboxylate added	^{14}C-glutamate efflux
	nmol mg^{-1} Chl.30 s
None	3.0
Glutamate	16.5
Malate	13.5
2-OG	31.0

Figure 6.1. Effect of glutamine, DCMU and malate on (NH_3, 2-OG) and (glutamine, 2-OG)-dependent O_2 evolution by pea chloroplasts.

Chloroplasts (47 µg Chl ml^{-1}) were added to 1.8 ml of reaction medium (section 2.8). Assay concentrations: 1 mM 2-OG, 1 mM NH_4Cl and 1 mM glutamine (Gln). Additions: 1 mM 2-OG, 1 mM malate, 1 mM NH_4Cl, 1 mM glutamine and 50 µM DCMU. Chloroplast intactness: 69%. CO_2-dependent O_2 evolution: 92 µmol mg^{-1} Chl h^{-1}. Values beside the curves represent µmol O_2 evolved mg^{-1} Chl h^{-1}.

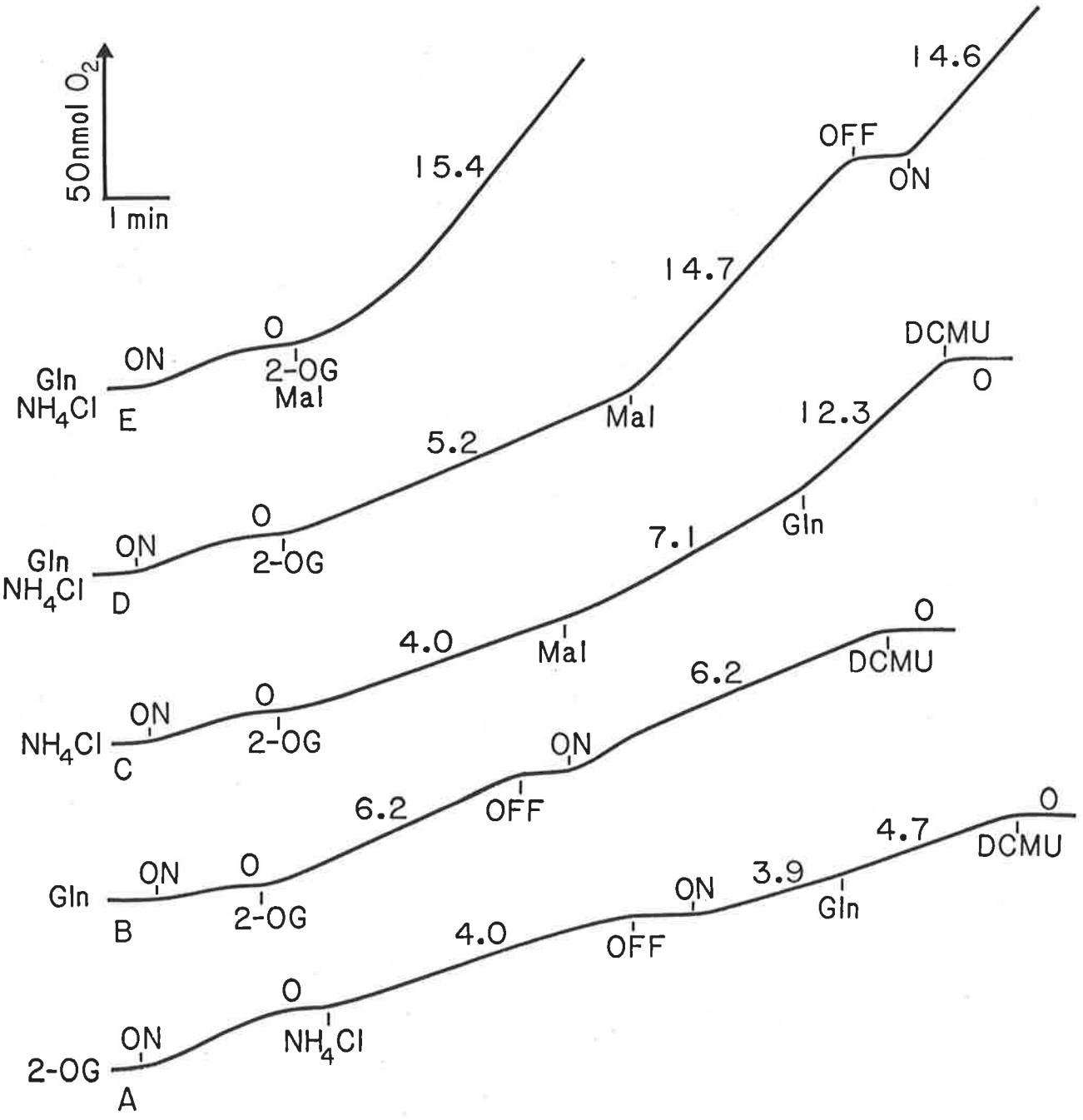

50nmol O_2
1 min
15.4
14.7
14.6
OFF
ON
O
ON
2-OG
Mal
Gln
NH_4Cl
E
Mal
DCMU
O
12.3
5.2
O
ON
2-OG
Gln
NH_4Cl
D
Gln
7.1
4.0
Mal
O
DCMU
6.2
ON
2-OG
NH_4Cl
C
6.2
OFF
ON
O
4.7
DCMU
3.9
ON
Gln
Gln
B
2-OG
4.0
OFF
O
ON
NH_4Cl
2-OG
A

Figure 6.2. Effect of methionine sulphoximine and azaserine on (NH_3, glutamine, 2-OG)-dependent O_2 evolution by pea chloroplasts.

Chloroplasts (42 µg Chl mg^{-1}) were added to 1.8 ml of reaction medium (section 2.8). Assay concentrations: 1 mM 2-OG, 1 mM NH_4 Cl, 1 mM glutamine and 2.5 mM methi nine sulphoximine (MSO). Additions: 1 mM NH_4Cl, 1 mM glutamine, 1 mM 2-OG, 1 mM malate, 1 mM OAA, 1 mM aspartate and 0.25 mM azaserine (Aza). Intactness: 69%. CO_2-dependent O_2-evolution: 73 µmol mg^{-1} Chl h^{-1}. Values beside the curves represent µmol O_2 evolved mg^{-1} Chl h^{-1}.

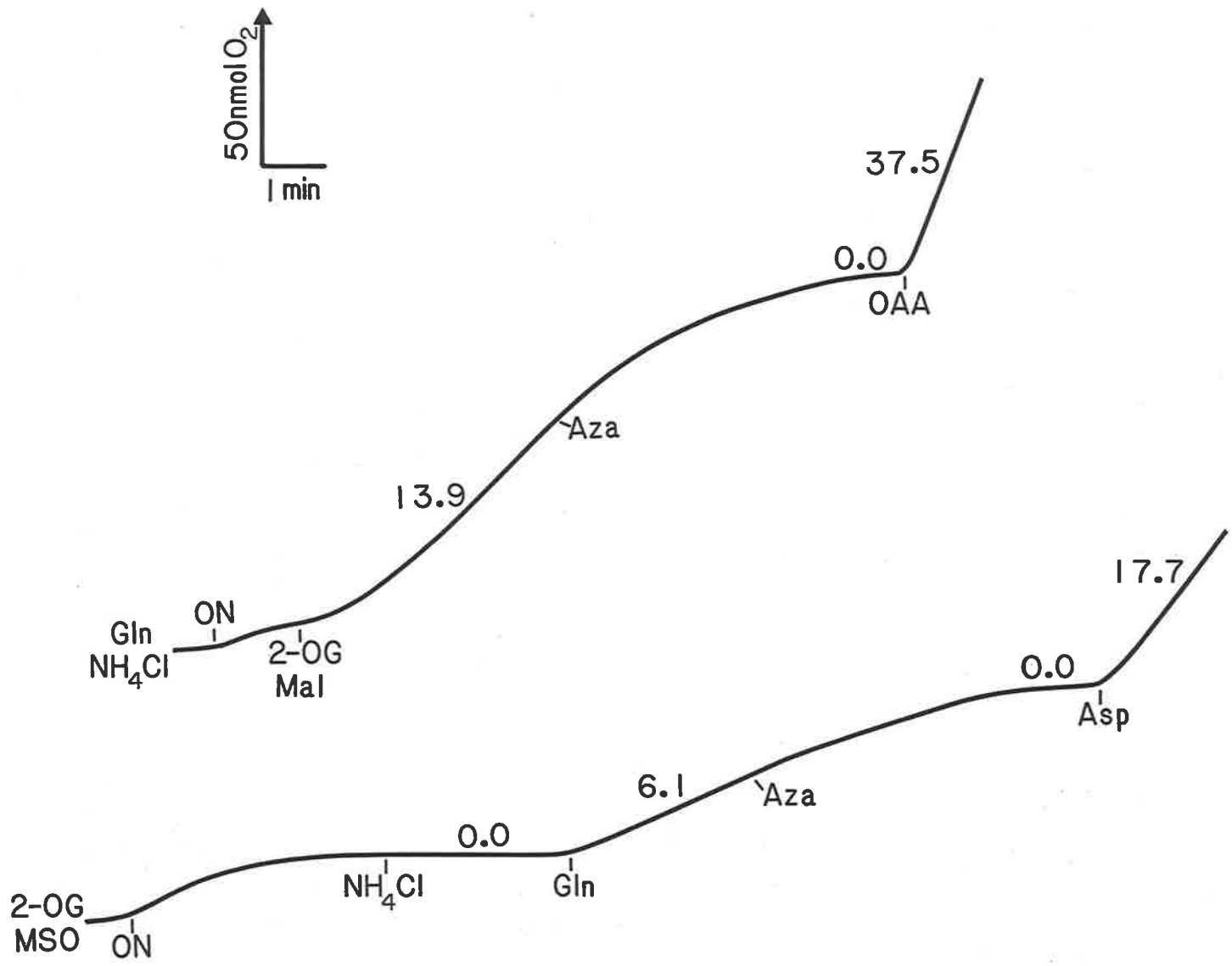

50nmol O_2
1 min
37.5
0.0
OAA
Aza
13.9
ON
Gln
NH_4Cl
2-OG
Mal
17.7
0.0
Asp
Aza
6.1
0.0
NH_4Cl
Gln
2-OG
MSO
ON

Figure 6.3. Oxygen evolution by pea chloroplasts in the presence of limiting amounts of 2-OG and NH_4Cl.

Chloroplasts (42 µg Chl ml^{-1}) were added to 1.8 ml of reaction medium (section 2.8). Assay concentrations: trace A, 1 mM NH_4Cl, 1 mM glutamine; trace B, 1 mM NH_4Cl, 1 mM glutamine, 0.5 mM malate; trace C, 10 mM 2-OG. Additions: 180 nmol 2-OG and 150 nmol NH_4Cl. Intactness: 69%. CO_2-dependent O_2 evolution: 77 µmol mg^{-1} Chl h^{-1}. Values beside the curves represent µmol O_2 evolved mg^{-1} Chl h^{-1}.

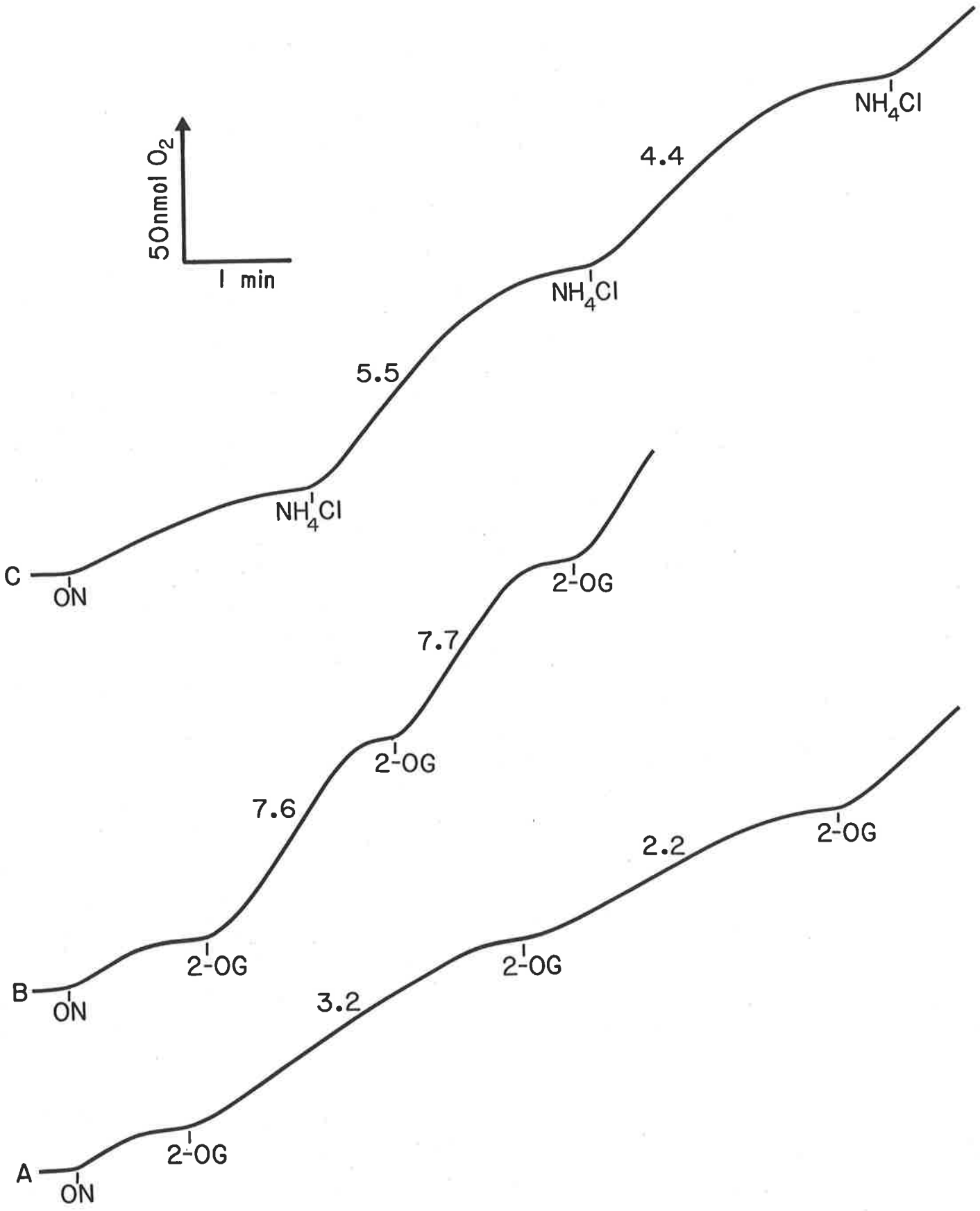

50nmol O_2
1 min
A
B
C
ON
2-OG
NH_4Cl
3.2
7.6
7.7
5.5
2.2
4.4

Figure 6.4. Effect of malate on (glutamine, 2-OG)-dependent O_2 evolution by pea chloroplasts.

Chloroplasts (50 µg Chl ml^{-1}) were added to 1-8 ml of reaction medium (section 2.8) containing 1 mM 2-OG. O_2 evolution was initiated by the addition of glutamine. Malate was added following the establishment of a steady state rate. Intactness: 66%. CO_2-dependent O_2 evolution: 64 µmol mg^{-1} Chl h^{-1}.

Symbols:

no malate (●)

0.1 mM malate (○)

1.0 mM malate (△)

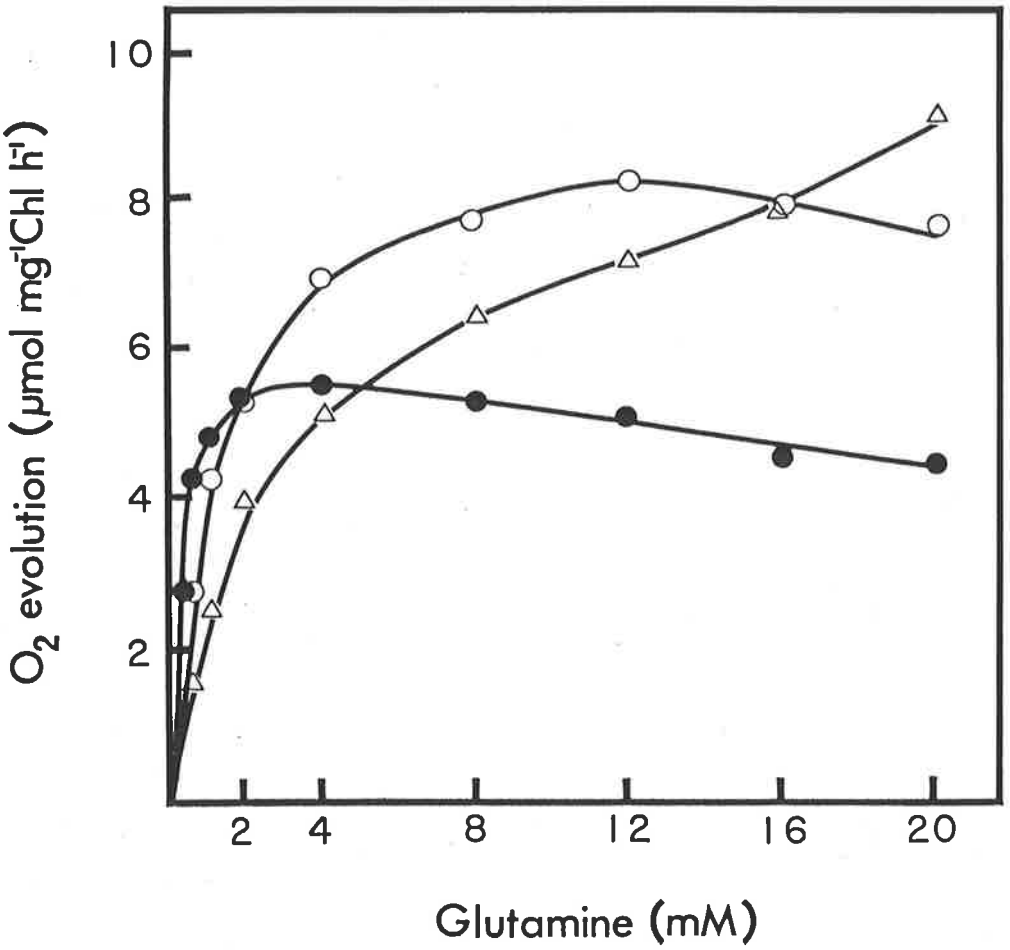

10
8
6
4
2
O_2 evolution (μmol mg^{-1}Chl h^{-1})
2
4
8
12
16
20
Glutamine (mM)

Figure 6.5. Effect of pH on (NH_3, glutamine, 2-OG)-dependent O_2 evolution by pea chloroplasts.

The standard assay contained: 44 µg Chl ml^{-1} chloroplasts, 1 mM 2-OG, 1 mM NH_4Cl, 1 mM glutamine and ± 1 mM malate. Intactness: 78%. CO_2-dependent O_2 evolution: 75 µmol mg^{-1} Chl h^{-1}.

Symbols:

- malate (●)

+ malate (○)

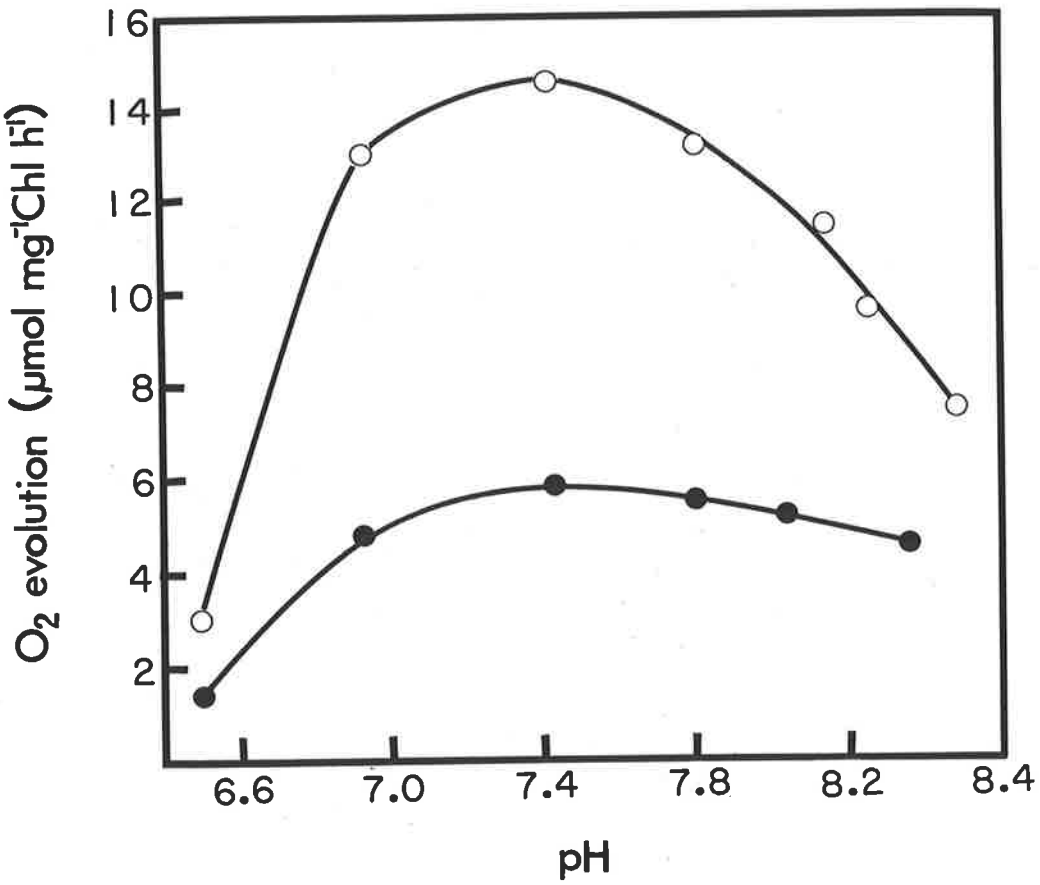

16
14
12
10
8
6
4
2
O2 evolution (μmol mg-1Chl h-1)
6.6
7.0
7.4
7.8
8.2
8.4
pH

Figure 6.6. Effect of malate on in vitro glutamine synthetase activity.

Chloroplastic GS was assayed according to the method described in section 2.10. Assay concentrations: trace A, 18 mM glutamate, 7 mM ATP; trace B, 18 mM glutamate, 7 mM ATP, 5 mM MSO; trace C, 3.6 mM glutamate, 7 mM ATP; trace D, 18 mM glutamate, 1.8 mM ATP. All reactions were initiated with 5.5 mM NH_4Cl. Malate was added in each case to a concentration of 2 mM. Rates beside the curves represent µmol NADH oxidized mg^{-1} Chl h^{-1}.

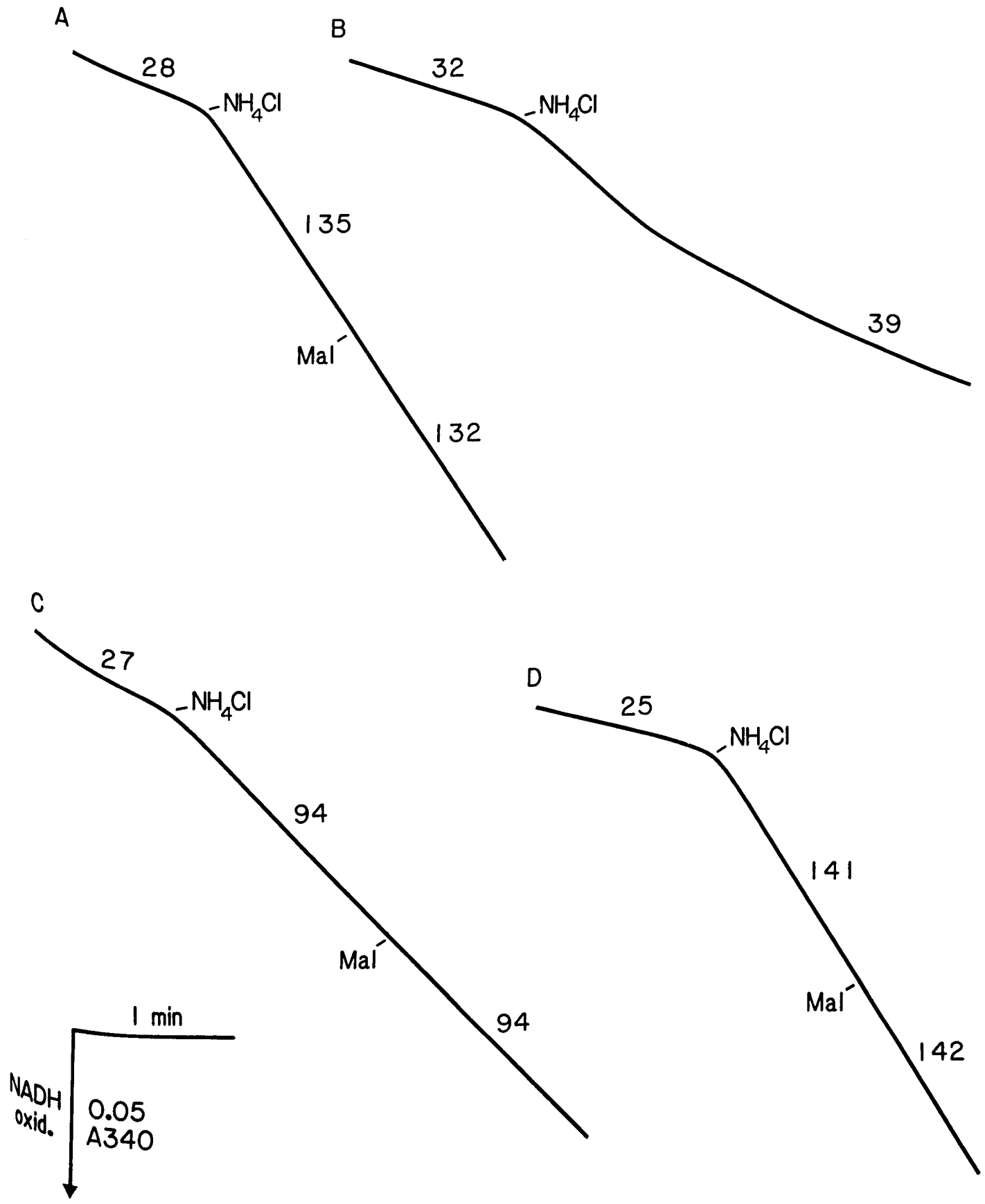

A
28
NH4Cl
135
Mal
132
B
32
NH4Cl
39
C
27
NH4Cl
94
Mal
94
1 min
NADH oxid.
0.05 A340
D
25
NH4Cl
141
Mal
142

Figure 6.7. Effect of malate on in vitro glutamate synthase activity.

GOGAT activity was measured with a reconstituted chloroplast system as described in section 2.8. The assays contained 115 µg Chl ml^{-1}, 30 µl chloroplast extract and 5 mM glutamine. Additions as indicated: trace A, 0.25 mM 2-OG, 1 mM malate, 4 mM malate, 0.25 mM 2-OG, 2 mM 2-OG,150 µl of chloroplast extract (CE); trace B, 1 mM 2-OG, 1 mM malate, 4 mM malate, 150 µl of chloroplast extract, 0.5 mM azaserine. Rates beside the curves represent µmol O_2 evolved mg^{-1} Chl h^{-1}.

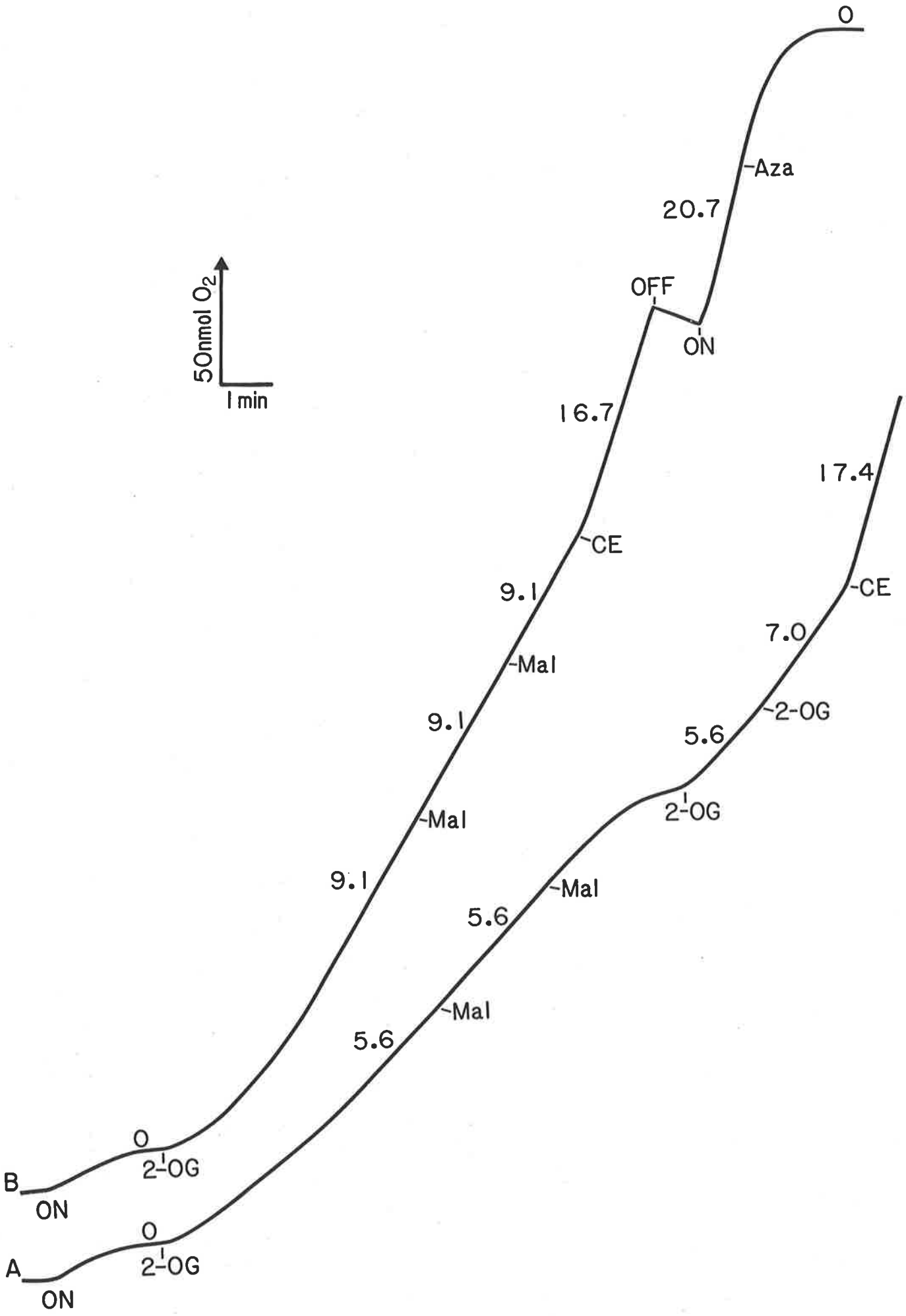

50nmol O_2
1 min
B
ON
0
2-OG
9.1
-Mal
9.1
-Mal
9.1
-CE
16.7
OFF
ON
20.7
-Aza
0
A
ON
0
2-OG
5.6
-Mal
5.6
-Mal
5.6
-CE
7.0
-2-OG
17.4

Figure 6.8. Effect of 2-OG concentration on (NH_3, 2-OG)-dependent O_2 evolution by pea chloroplasts.

Chloroplasts (45 µg Chl ml^{-1}) were added to 1.8 ml of reaction medium (section 2.8) containing 1 mM NH_4Cl and ± 1 mM glutamine. O_2 evolution was initiated by the addition 2-OG (± 1 mM malate). The recorded rates represent the steady state rates of O_2 evolution after 5 min. Intactness: 75%. CO_2-dependent O_2 evolution: 70 µmol mg^{-1} Chl h^{-1}.

Symbols:

NH_3, 2-OG (●)

NH_3, glutamine, 2-OG (○)

NH_3, 2-OG, malate (▲)

NH_3, glutamine, 2-OG, malate (△)

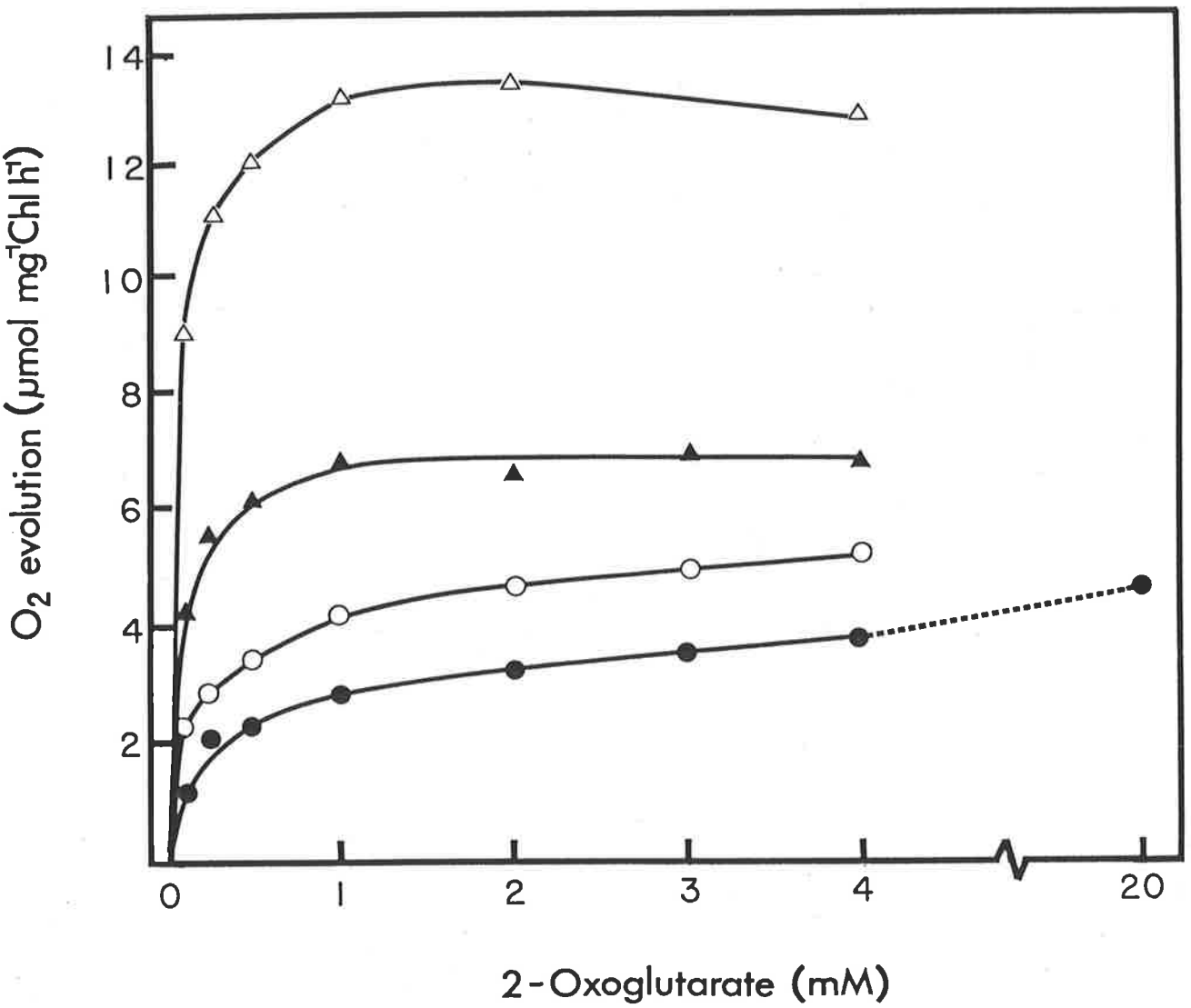

14
12
10
8
6
4
2
0
1
2
3
4
20
O2 evolution (µmol mg-1Chl h-1)
2-Oxoglutarate (mM)

Figure 6.9. Effect of 2-OG concentration on (NH_3, 2-OG)-dependent O_2 evolution by spinach chloroplasts.

Experimental procedures as given in Fig. 6.8, except that glutamine was absent. Intactness: 50%. CO_2-dependent O_2 evolution: 81 µmol mg^{-1} Chl h^{-1}.

Symbols:

NH_3, 2-OG (▲)

NH_3, 2-OG, malate (△)

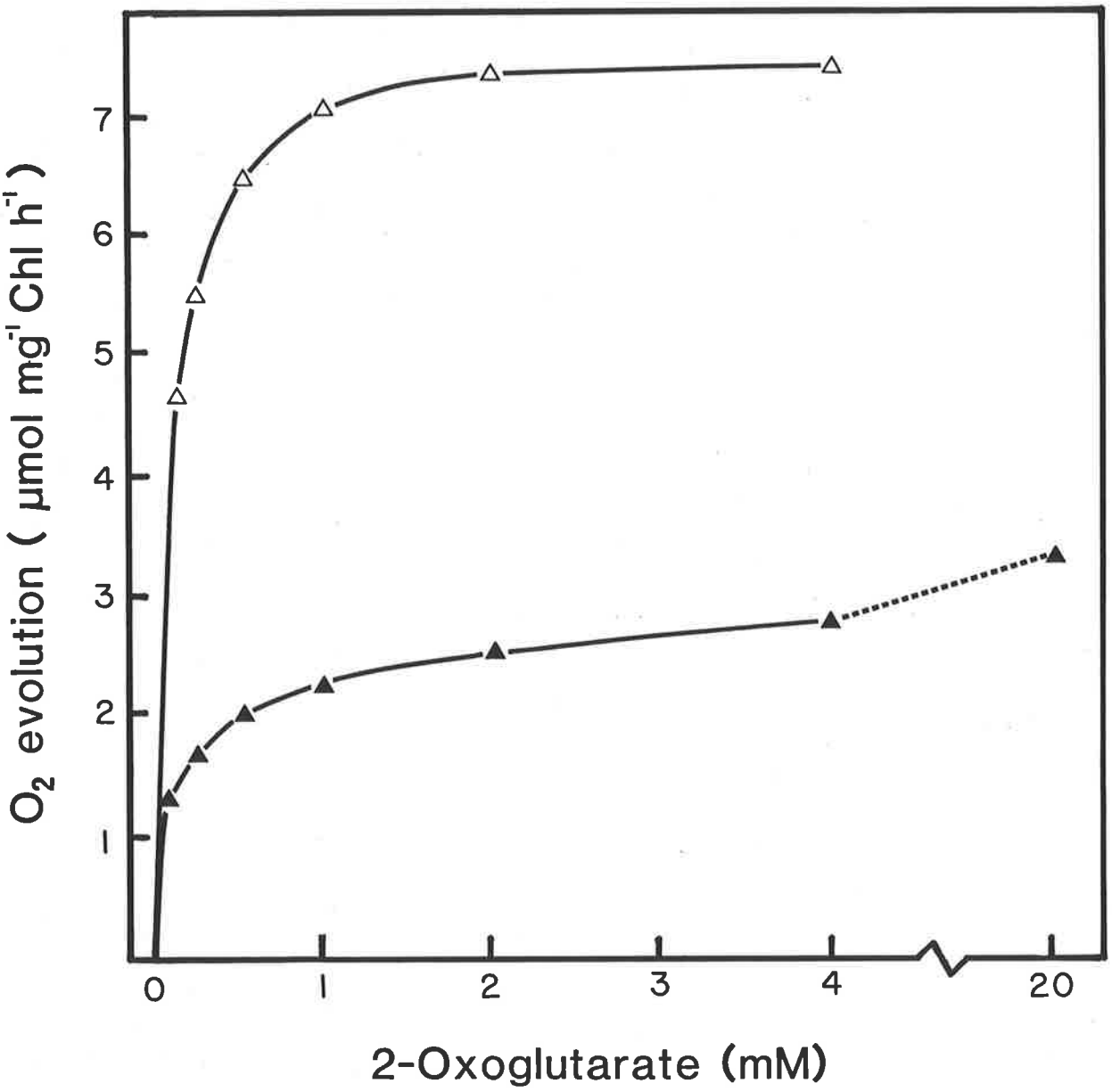

O2 evolution (µmol mg-1 Chl h-1)
7
6
5
4
3
2
1
0
1
2
3
4
20
2-Oxoglutarate (mM)

Figure 6.10. Effect of malate concentration on (NH_3, glutamine, 2-OG)-dependent O_2 evolution by pea chloroplasts.

Chloroplasts (40 µg Chl ml^{-1}) were added to 1.8 ml reaction medium (section 2.8) containing 1 mM NH_4Cl and 1 mM glutamine. Malate was added with 2-OG which was used to initiate O_2 evolution. Intactness: 70%. CO_2-dependent O_2 evolution: 85 µmol mg^{-1} Chl h^{-1}.

Symbols:

1 mM 2-OG (●)

10 mM 2-OG (○)

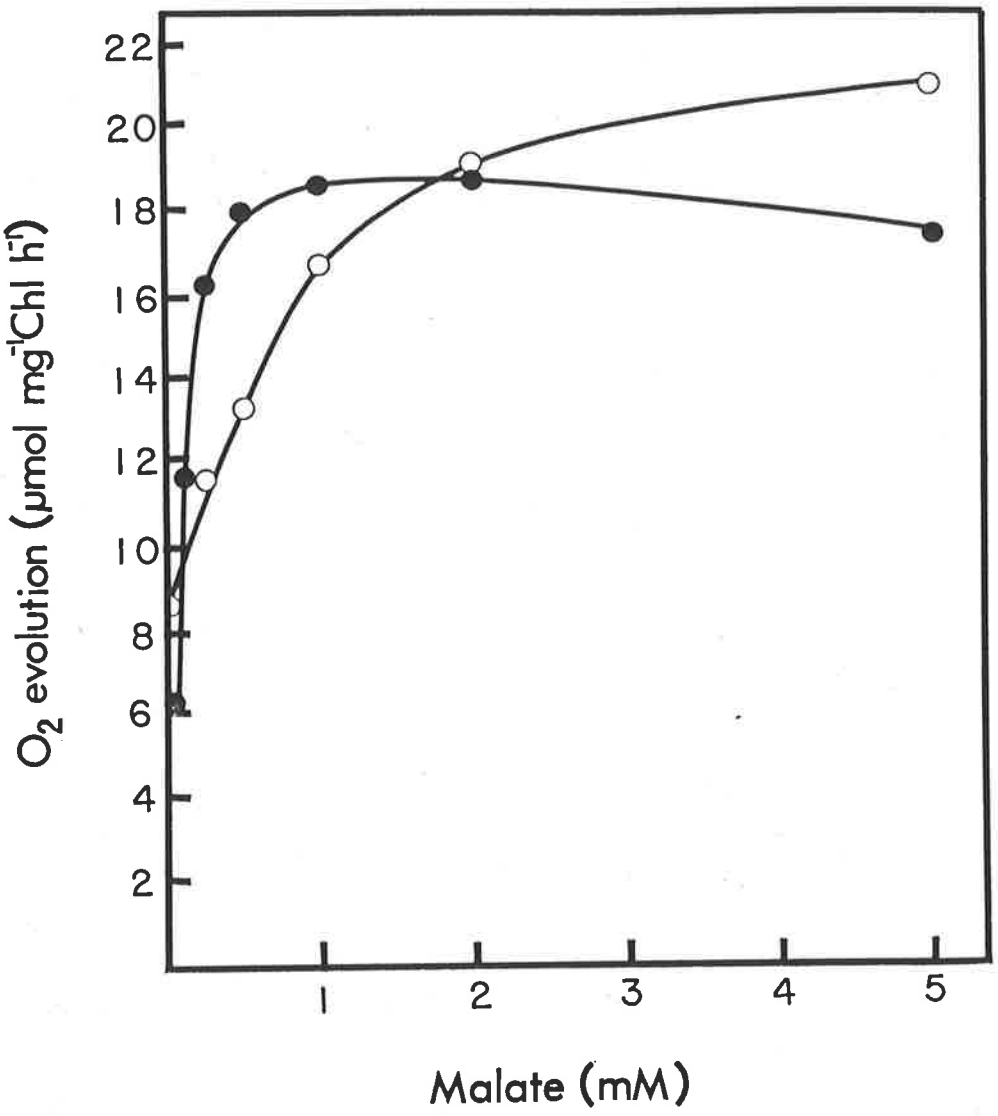

22
20
18
16
14
12
10
8
6
4
2
1
2
3
4
5
O2 evolution (μmol mg-1Chl h-1)
Malate (mM)

Figure 6.11. Effect of 2-OG concentration on the malate stimulation of (NH_3, glutamine, 2-OG)-dependent O_2 evolution by pea chloroplasts.

Chloroplasts (38 µg Chl ml^{-1}) were added to 1.8 ml of reaction medium (section 2.8) containing 1 mM NH_4Cl and 1 mM glutamine. O_2 evolution was initiated by 2-OG addition (●). Where present, malate addition was made with 2-OG to a concentration of 0.5 mM (○) or to the same concentration as 2-OG (▲). Intactness: 72%. CO_2-dependent O_2 evolution: 98 µmol mg^{-1} Chl h^{-1}.

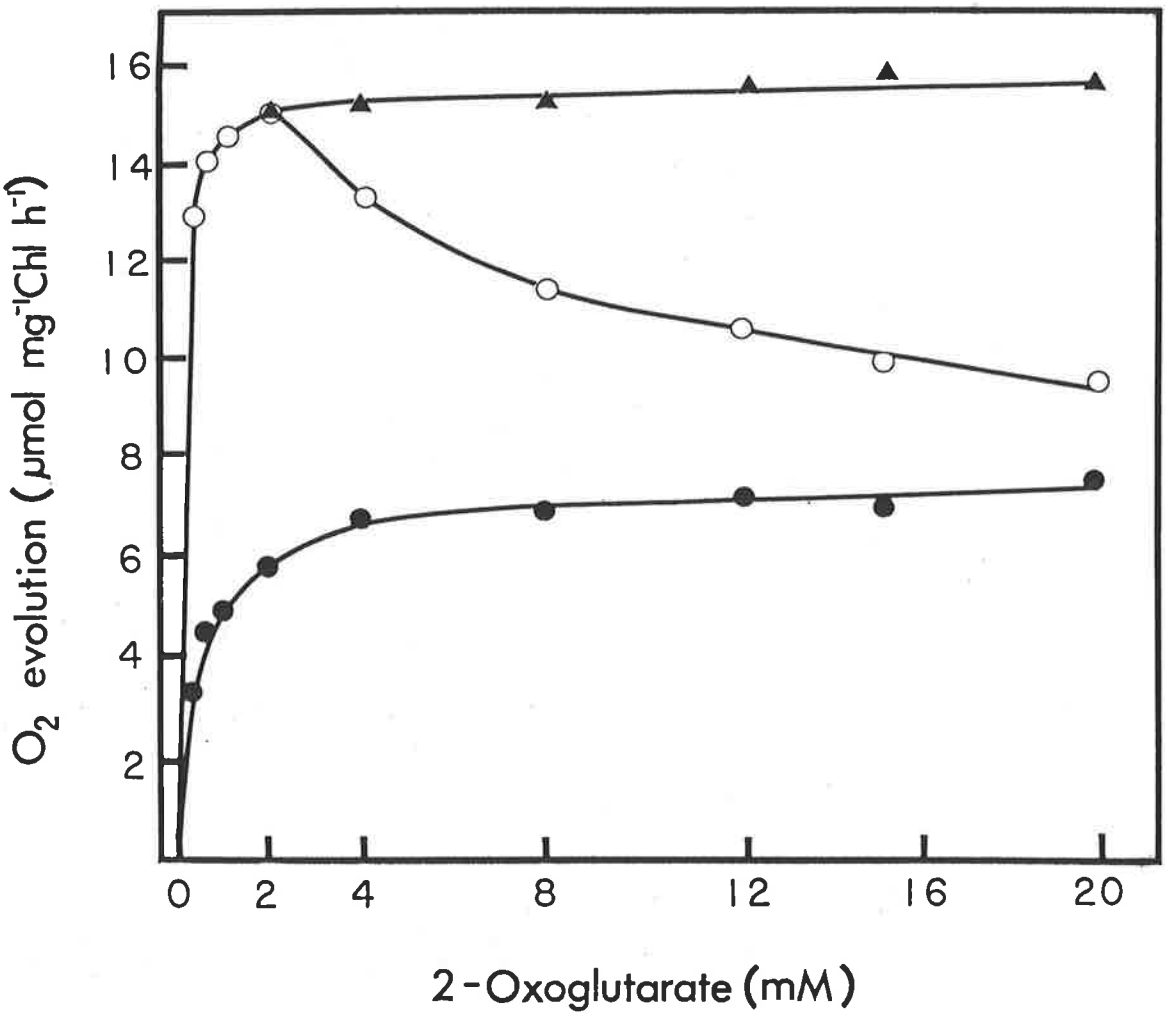

16
14
12
10
8
6
4
2
0
2
4
8
12
16
20
O2 evolution (μmol mg-1Chl h-1)
2-Oxoglutarate (mM)

Figure 6.12. Effect of increasing the malate concentration on its stimulatory effect on (NH_3, glutamine, 2-OG)-dependent O_2 evolution by pea chloroplasts.

Chloroplasts (43 µg Chl ml^{-1}) were added to 1.8 ml reaction medium (section 2.8) containing 1 mM NH_4Cl and 1 mM glutamine. O_2 evolution was initiated by the addition of 0.5 mM 2-OG together with malate at varying concentrations. The dotted line represents the rate of O_2 evolution in the absence of added malate. Intactness: 71%. CO_2-dependent O_2 evolution: 100 µmol mg^{-1} Chl h^{-1}.

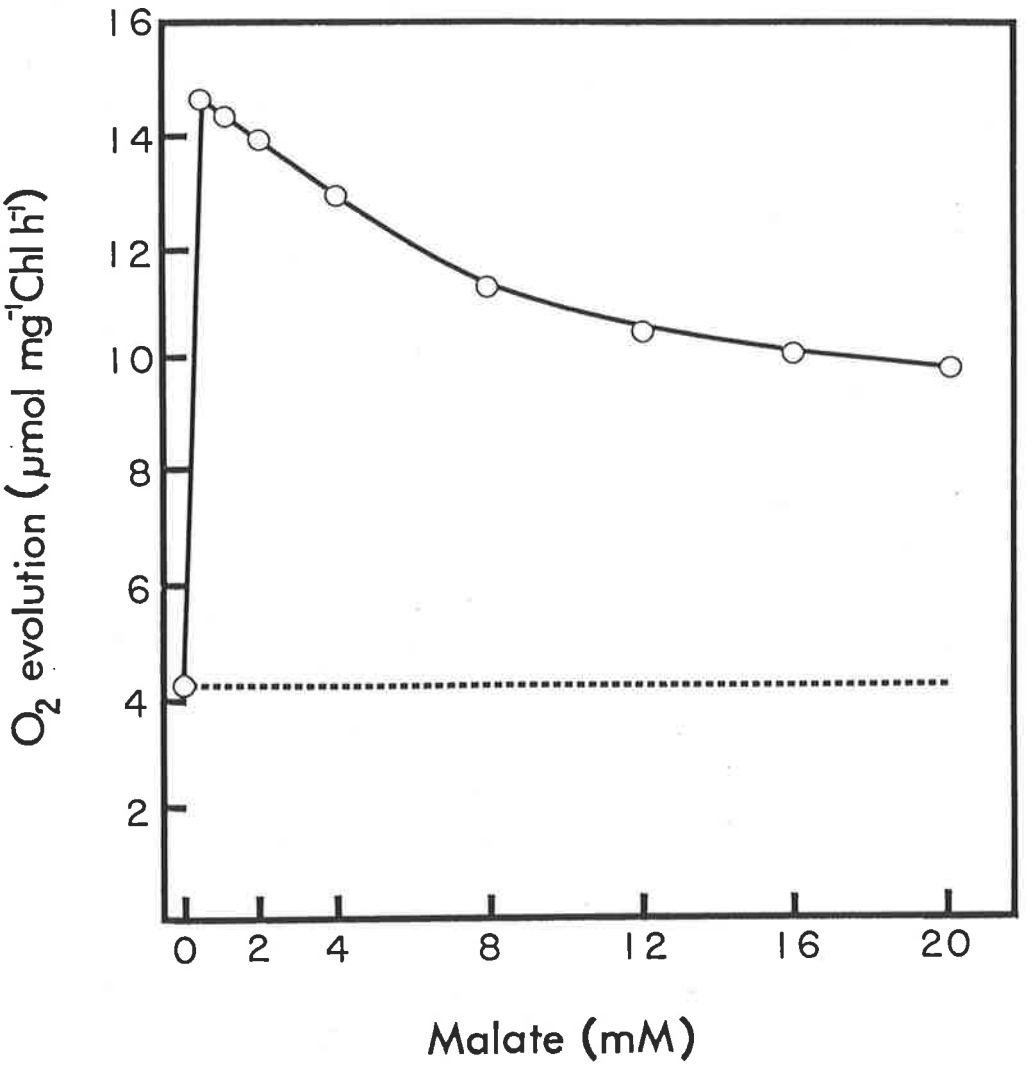
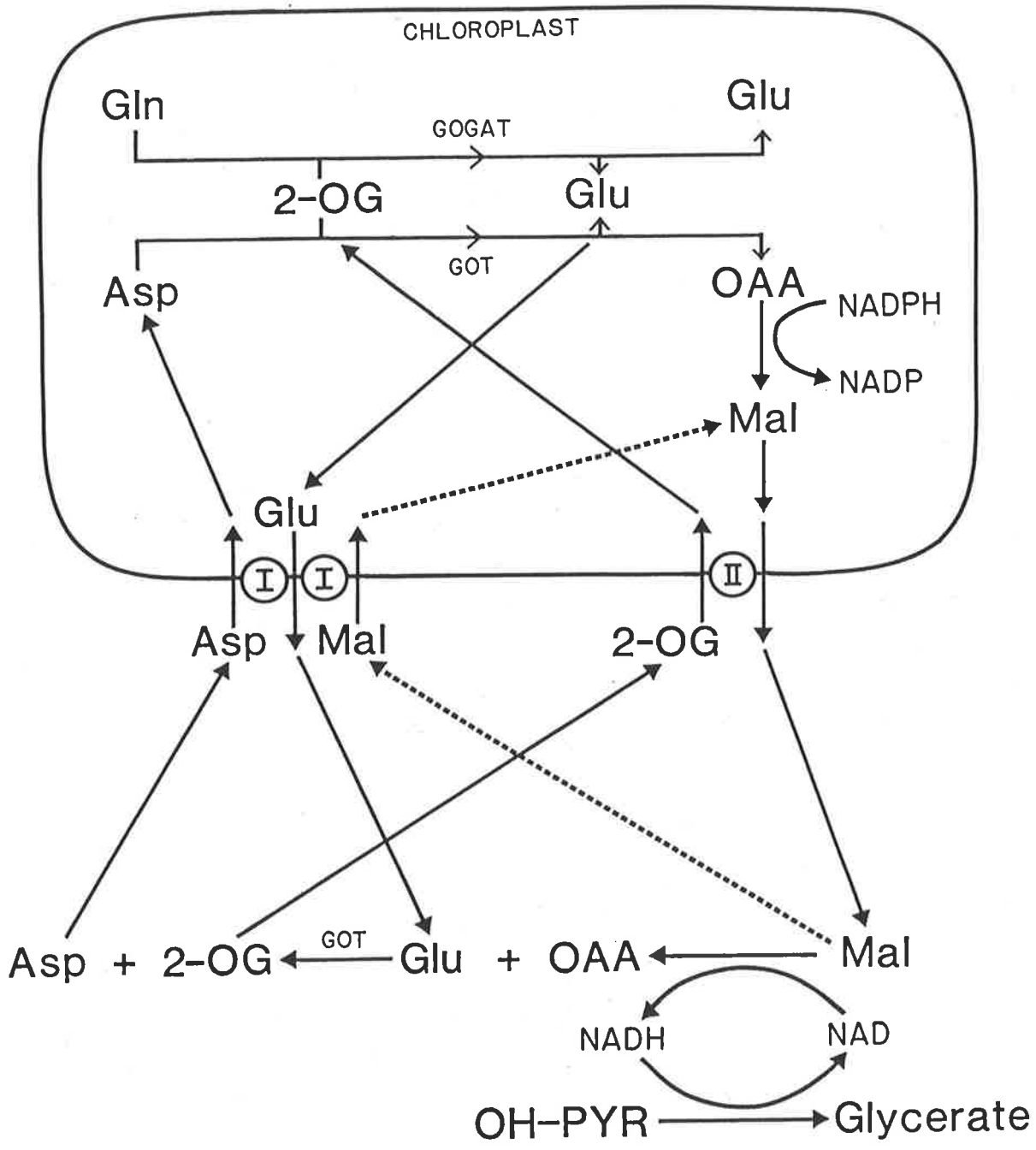

16
14
12
10
8
6
4
2
O_2 evolution (μmol mg^{-1}Chl h^{-1})
0
2
4
8
12
16
20
Malate (mM)

Figure 6.13. Movement of dicarboxylate molecules across the chloroplast envelope.

This diagram illustrates the possible movement of dicarboxylate molecules during (a) the operation of a malate/aspartate shuttle (solid lines) and (b) malate-stimulated uptake of 2-OG for GOGAT-catalysed O_2 evolution (broken lines). See text for explanation of symbols I and II.


CHLOROPLAST
Gln
Glu
GOGAT
2-OG
Glu
GOT
Asp
OAA
NADPH
NADP
Mal
Glu
I
I
II
Asp
Mal
2-OG
Asp + 2-OG
GOT
Glu + OAA
Mal
NADH
NAD
OH-PYR
Glycerate

CHAPTER VII

AN OXYGEN-EVOLVING RECONSTITUTED CHLOROPLAST SYSTEM FOR THE ASSAY OF IN VITRO GLUTAMATE SYNTHASE ACTIVITY

7.1. INTRODUCTION

Measurement of *in vitro* ferredoxin-linked GOGAT activity has, in the past, been slow and labourious because assay of the reaction product (glutamate) required the use of various chromatographic methods (Rathnam and Edwards, 1976; Rhodes *et al.* 1976; Wallsgrove *et al.* 1977; Stewart and Rhodes, 1978; Matoh *et al.* 1979; Cullimore and Sims, 1981). These assay systems were also far removed from the *in vivo* situation through the use of the artificial electron donor, dithionite, and in some cases the artificial electron carrier, methyl viologen (Rathnam and Edwards, 1976; Wallsgrove *et al.* 1977; Cullimore and Sims, 1981).

Hucklesby *et al.* (1980) proposed a new assay system involving electrolytically reduced methyl viologen. Such a system would allow GOGAT activity to be measured directly, by following the rate of oxidation of reduced methyl viologen, thereby avoiding the time-consuming glutamate determinations. However, special equipment was reqired to produce the electrolytically reduced methyl viologen. More recently Suzuki *et al.* (1982) demonstrated that partially purified GOGAT could be linked to the light dependent reduction of ferredoxin by thylakoid membranes. This system represented the first demonstration of light dependent GOGAT activity *in vitro*, but utilized thylakoid membranes which could not evolve O_2 and thus was also reliant on chromatographic techniques for the determination of enzyme activity.

In this chapter, I present a method for the measurement of *in vitro* GOGAT activity, using an O_2-evolving reconstituted chloroplast system. This assay system is rapid and highly sensitive and allows for the direct measurement of light-dependent enzyme activity. It also utilizes recently isolated and unmodified GOGAT and assays it under conditions which closely resemble *in vivo* conditions. The use of this assay system has led to the discovery of regulatory characteristics, not previously reported for this enzyme. Following completion of this work, a report was published on the characterisation of a similar O_2-evolving reconstituted system using spinach chloroplasts (Anderson and Walker, 1983).

7.2. RESULTS

A reconstituted chloroplast system prepared according to the method of Lilley and Walker (1974), exhibited light-dependent O_2 evolution in the presence of 2-OG and glutamine (Fig. 7.1). It can be seen that O_2 evolution did not occur in the absence of either substrate and was inhibited by DCMU (Fig. 7.1; B) and the GOGAT-specific inhibitor, azaserine (Wallsgrove *et al.* 1977). Azaserine had no effect on NADP-dependent O_2 evolution by this reconstituted chloroplast system (Fig. 7.1; A).

The rate of O_2 evolution achieved on a per mg chlorophyll basis in the reconstituted system was dependent on the amount of chloroplast extract (containing GOGAT) added (Fig. 7.2). In the majority of experiments, suboptimal levels of chloroplast extract were used to ensure that the supply of reducing equivalents from the chlorophyll fraction, did not limit

the rate of GOGAT-catalysed O_2 evolution, and consequently, the rates given in the other figures do not represent the maximum rates of O_2 evolution which could have been achieved using this reconstituted system.

The dependence of the reconstituted chloroplast system on added ferredoxin is illustrated in Fig. 7.3. The Km for ferredoxin was estimated at 0.9 μM which compares to the values of 2 μM and 1.7 μM for ferredoxin-linked GOGAT purified from field bean (Wallsgrove *et al.* 1977) and corn (Matoh *et al.* 1979) respectively. Maximal rates of GOGAT-dependent O_2 evolution were achieved at 5-6 μM ferredoxin, in agreement with the requirements of NADP-dependent O_2 evolution by reconstituted chloroplast systems (Lilley and Walker, 1979). Some background activity was observed in the absence of added ferredoxin, when larger volumes of chloroplast extract were employed in the reaction mixture. This was probably due to the presence of some ferredoxin removed from the pea chloroplasts, during the preparation of the chloroplast extract.

Glutamate synthase-dependent O_2 evolution by the reconstituted system demonstrated a broad pH optimum with a peak at 7.6 (Fig. 7.4). A similar broad response to pH was observed with the field bean enzyme, although the peak occurred at pH 7.3 (Wallsgrove *et al.* 1977). The system also displayed an absolute requirement for Mg^{2+}, which could not be attributed to a requirement of the photosynthetic electron transport chain for this metal ion, as demonstrated by the high rate of NADP-dependent O_2 evolution in the absence of added $MgCl_2$ (Fig. 7.5). No GOGAT-dependent

O_2 evolution was observed in the absence of added $MgCl_2$. This requirement for Mg^{2+} could be replaced to a certain extent by Ca^{2+} and Mn^{2+}, but not by K^+ or Na^+ (Table 7.1) showing the effect to be specific for a divalent cation.

As shown in Fig. 7.1 (trace A), a lag period is observed between the time of 2-OG addition to the reaction mixture and the achievement of the maximal rate of O_2 evolution. This did not occur, however, if the GOGAT enzyme was pre-incubated with 2-OG prior to the initiation of O_2 evolution with glutamine or light (Fig. 7.1; B,C). As this may indicate some form of allosteric interaction between the GOGAT enzyme and 2-OG, the effect of varying concentrations of this substrate on the rate of O_2 evolution was investigated.

The results shown in Fig. 7.6 do not indicate any obvious sigmoidal or allosteric behaviour of this enzyme with respect to 2-OG. However, it should be noted that the rates plotted are, in fact, the steady state rates achieved following the lag period, and that this lag period varied depending on the concentration of 2-OG present e.g. 4 min at 0.15 mM 2-OG compared to 2 min at 2.0 mM 2-OG. The length of this lag was found to be independent of the glutamine concentration.

Analysis of the data in Fig. 7.6, on a double-reciprocal plot (Fig. 7.7), does reveal a departure from linearity at the low 2-OG concentrations (i.e. < 0.5 mm). A Km estimate taken from the points corresponding to 2-OG concentrations above 0.5 mM gives a value of 0.27 mM. If a further addition of 2-OG

to the same initial concentration) was made following the cessation of O_2 evolution upon full utilization of the first 2-OG addition a marked increase in the steady state rates of O_2 evolution was observed for 2-OG concentrations below 0.5 mM. With the inclusion of these points in Fig. 7.7, a linear relationship is established among the various 2-OG concentrations. Clearly, the GOGAT enzyme was not fully activated by the first addition of the low (< 0.5 mM) 2-OG concentrations. The Km (2-OG) estimate for the fully activated enzyme is 0.23 mM. This is similar to the values measured for GOGAT isolated from field bean (Wallsgrove *et al.* 1977) corn (Matoh *et al.* 1979) and rice leaves (Suzuki and Gadal, 1982).

7.3. DISCUSSION

The results presented demonstrate that ferredoxin-linked GOGAT activity may be assayed *in vitro*, in a rapid and sensitive manner through the use of a standard reconstituted chloroplast system as developed originally by Stokes and Walker (1971). Such systems have been used in the past to study enzymes and enzyme sequences associated with the PCR cycle (Lilley and Walker, 1974; Walker and Slabas, 1976; Slabas and Walker, 1976; Lilley and Walker, 1979), however, this is the first report of GOGAT-dependent O_2 evolution by a reconstituted chloroplast system.

The absolute requirement of the GOGAT enzyme, in this system, for divalent cations and in particular Mg^{2+} is in direct contrast to the results of previous studies which have found these cations to have an inhibitory effect on GOGAT activity

(Wallsgrove *et al.* 1977; Suzuki and Gadal, 1982). This is, however, a characteristic which has been reported for a number of other chloroplast enzymes, including RuBP carboxylase (Lorimer *et al.* 1976) and fructose-1, 6-diphosphatase (Baier and Latzko, 1975), and has been proposed as a mechanism for their regulation *in vivo* through light dependent alterations in the concentration of Mg^{2+} ions in the chloroplast stroma (Robinson and Walker, 1981).

The activation of the GOGAT enzyme by its substrate, 2-OG, as evidenced by the lag in activity if this substrate is not first pre-incubated with the enzyme, also shows a close parallel to the interaction of the RuBP carboxylase enzyme with its substrate, CO_2. Lorimer *et al.* (1976) have shown that RuBP carboxylase forms an active ternary complex (enzyme-CO_2-Mg^{2+}), in which CO_2 first binds to the enzyme in a slow, rate determining step, followed by the rapid binding of Mg^{2+}. Consequently, unless the enzyme is pre-incubated with CO_2, a lag in activity will result, the length of which is found to be dependent on to the concentration of CO_2, but independent of Mg^{2+}. My results show that a similar relationship appears to exist between GOGAT, 2-OG and Mg^{2+}; with the interaction between the enzyme and 2-OG being the slow, rate limiting step, while Mg^{2+} binding was rapid and did not result in a lag in activity on addition (Fig. 7.8).

Suzuki and Gadal (1982) have also found evidence of an allosteric interaction between the GOGAT enzyme and glutamine. However, in contrast to 2-OG, this substrate appeared to act as a negative effector. Further investigation is required to identify

potential allosteric sites on the GOGAT enzyme, to which 2-OG, Mg^{2+} and glutamine may bind, as such sites may represent important points of regulation of the photorespiratory cycle.

Table 7.1. Effect of inorganic salts on the rate of GOGAT-dependent O_2 evolution by a reconstituted chloroplast system.

Experimental procedures as described in sections 2.5 and 2.8. Assays contained 150 µl of chloroplast extract, 4 mM glutamine and 2 mM 2-OG. The various salts were added to a concentration of 10 mM after incubation of the reaction mixture in the light for 1 min.

Salt	O_2 evolution
	µmol mg^{-1} Chl h^{-1}
$MgCl_2$	15.2
$CaCl_2$	12.3
$MnCl_2$	9.9
KCl	0.0
NaCl	0.0

Figure 7.1. GOGAT-dependent O_2 evolution by a reconstituted chloroplast system.

Experimental procedures as in sections 2.5 and 2.8. Assays contained 125 µl of chloroplast extract as well as: trace A, 4 mM glutamine; trace B, 1 mM 2-OG; trace C, 4 mM glutamine, 1 mM 2-OG. Additions: trace A, 1 mM 2-OG,0.5 mM azaserine, 0.16 mM NADP; trace B, 4 mM glutamine, 50 µM DCMU. Rates beside the curves represent µmol O_2 evolved mg^{-1} Chl h^{-1}.

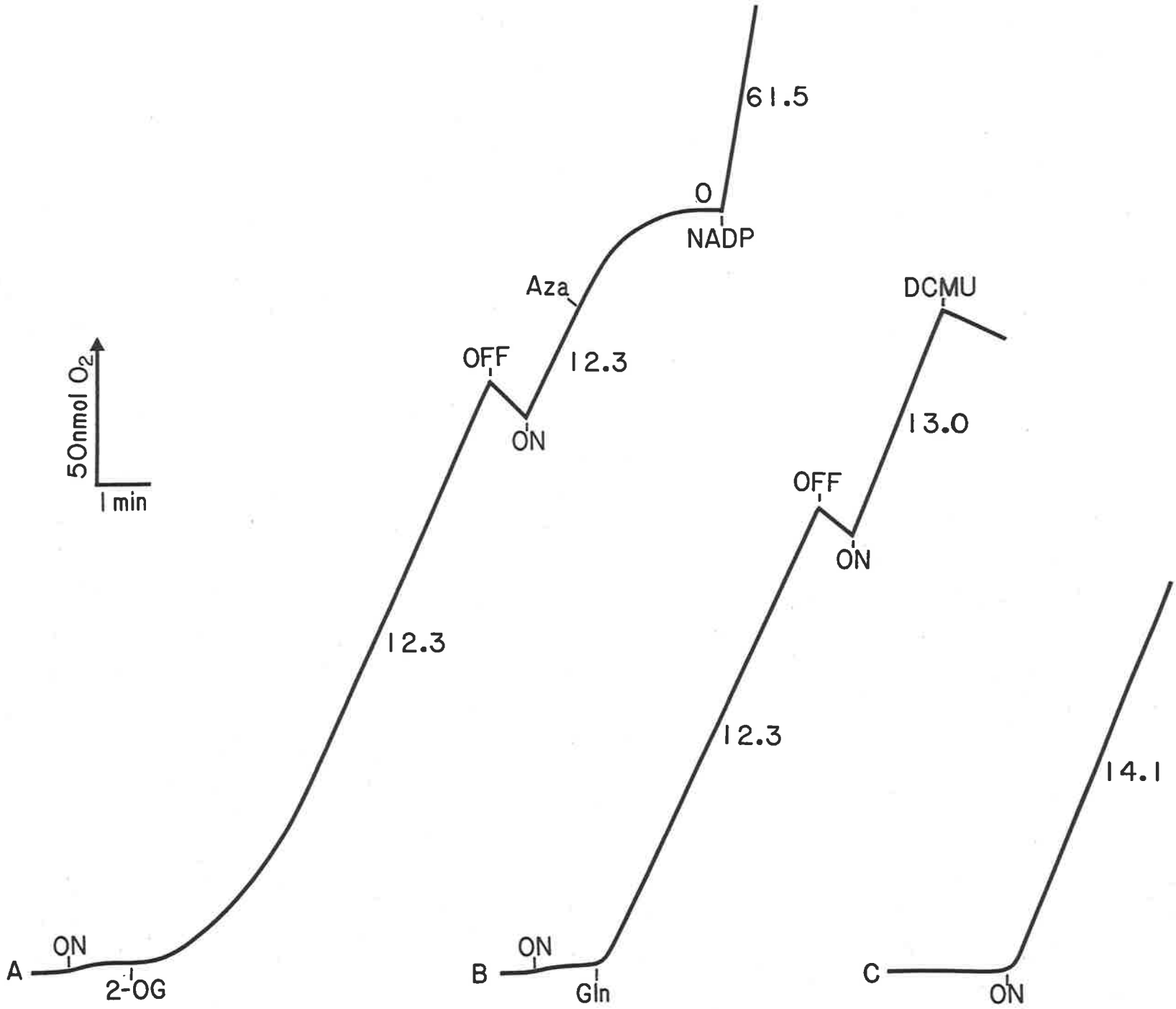

50nmol O_2
1 min
A
ON
2-OG
12.3
OFF
ON
12.3
Aza
O
NADP
61.5
B
ON
Gln
12.3
OFF
ON
13.0
DCMU
C
ON
14.1

Figure 7.2. Effect of the chloroplast extract on the rate of GOGAT-dependent O_2 evolution by a reconstituted chloroplast system.

Experimental procedures as described in sections 2.5 and 2.8. Assay concentrations: 4 mM glutamine, 2 mM 2-OG. Reaction mixtures were pre-incubated with both substrates and chloroplast extract for 1 min in the dark prior to initiating O_2 evolution with light.

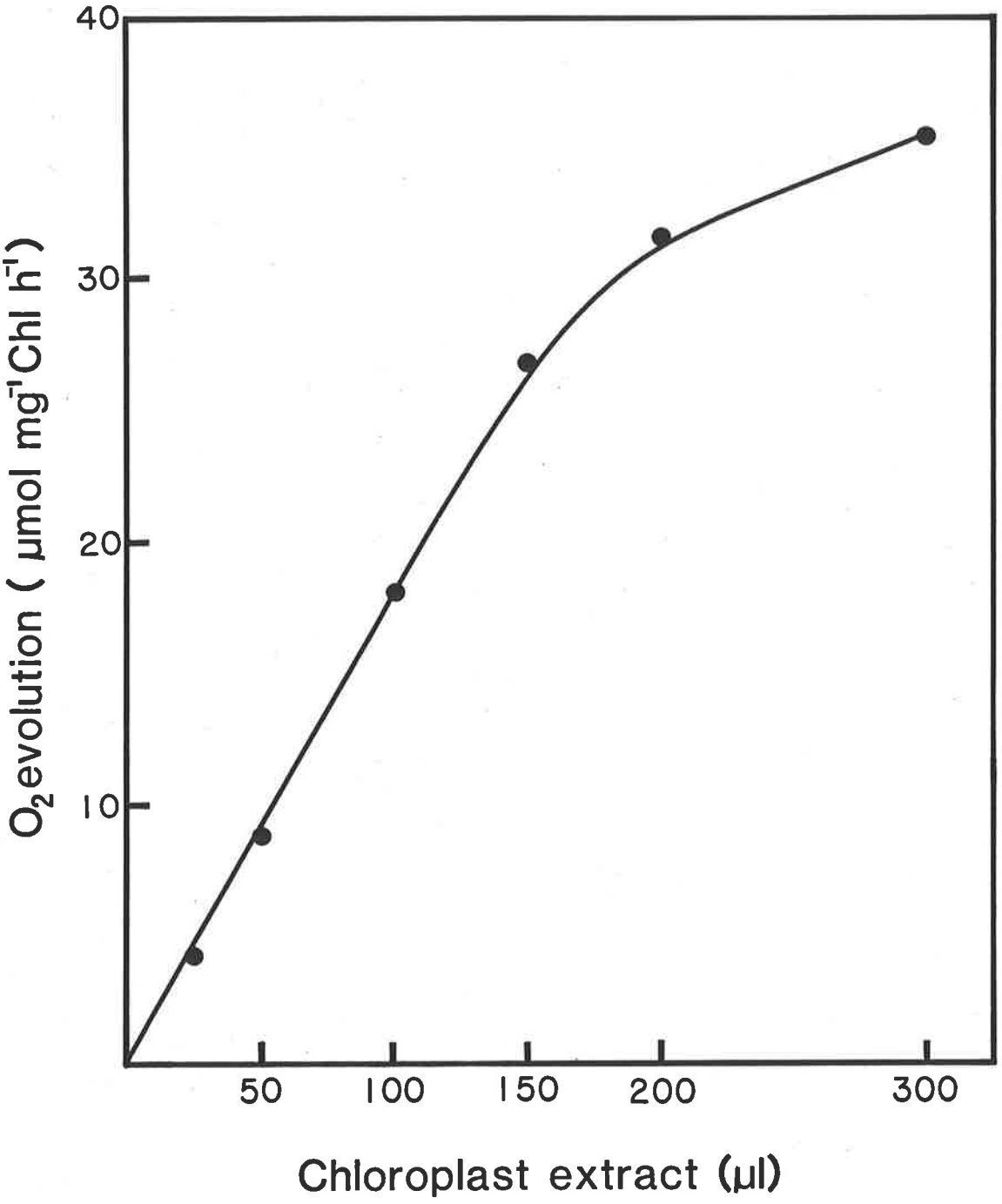

40
30
20
10
O_2 evolution (µmol mg^{-1} Chl h^{-1})
50
100
150
200
300
Chloroplast extract (µl)

Figure 7.3. Effect of spinach ferredoxin on the rate of GOGAT-dependent O_2 evolution by a reconstituted chloroplast system.

Experimental procedures as described in sections 2.5 and 2.8. Assays contained 50 µl of chloroplast extract, 4 mM glutamine and 2 mM 2-OG. The reaction mixtures were pre-incubated for 1 min in the dark prior to initiating O_2 evolution with light.

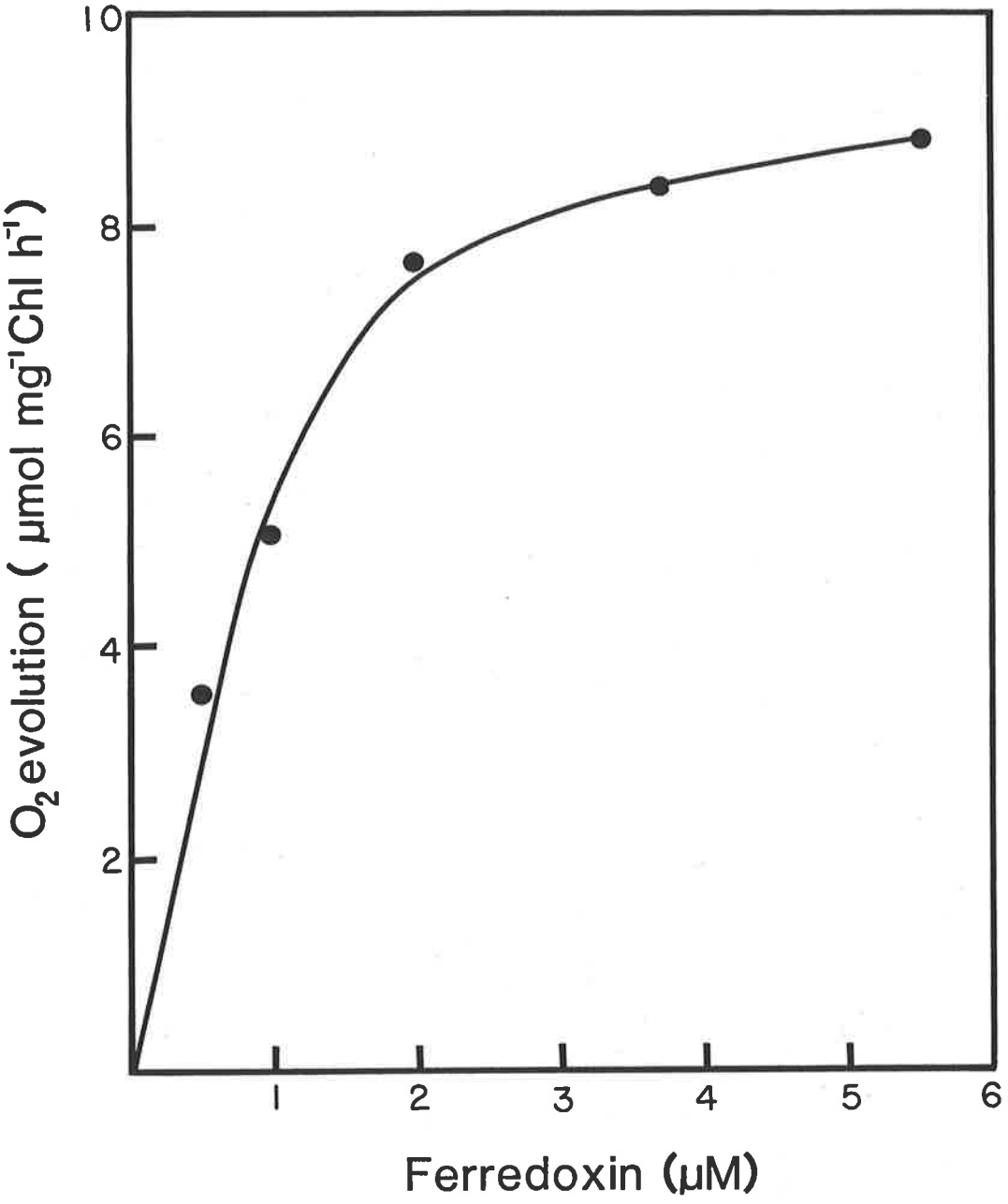

10
8
6
4
2
O_2 evolution (µmol mg^{-1}Chl h^{-1})
1
2
3
4
5
6
Ferredoxin (µM)

Figure 7.4. Effect of pH on the rate of GOGAT-dependent O_2 evolution by a reconstituted chloroplast system.

Experimental procedures as described in sections 2.5 and 2.8. Assays contained 100 µl of chloroplast extract, 5 mM glutamine and 2.5 mM 2-OG. The reaction mixtures were pre-incubated for 1 min in the dark prior to initiated O_2 evolution with light.

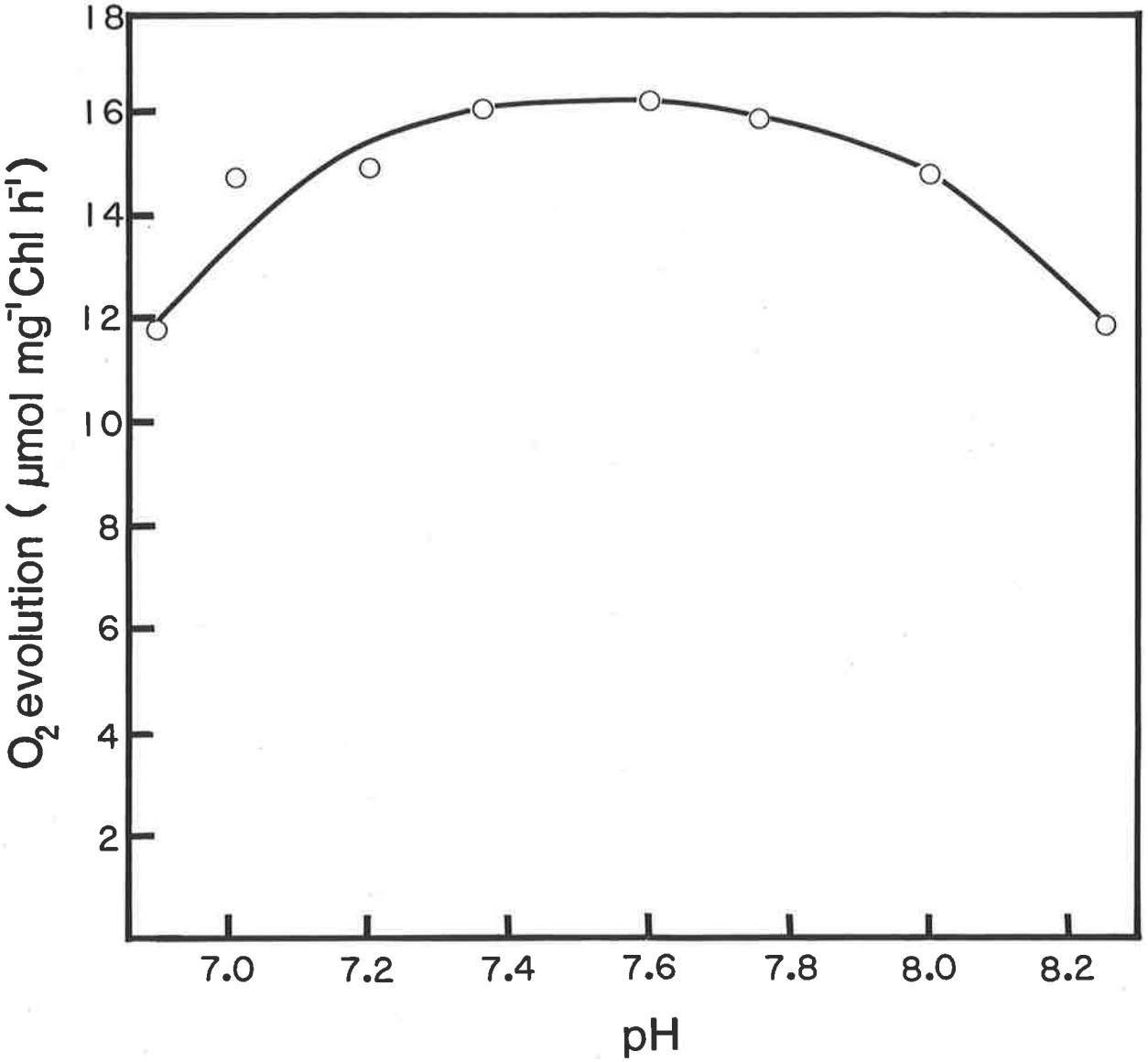

18
16
14
12
10
8
6
4
2
O2 evolution (µmol mg-1Chl h-1)
7.0
7.2
7.4
7.6
7.8
8.0
8.2
pH

Figure 7.5. Effect of $MgCl_2$ concentration on the rate of GOGAT and NADP-dependent O_2 evolution by a reconstituted chloroplast system.

Experimental procedures as described in section 2.5 and 2.8. Assays contained 125 µl of chloroplast extract, 4 mM glutamine and 2 mM 2-OG. Assay of NADP-dependent O_2 evolution as for the GOGAT system, but contained 100 µg chlorophyll, 125 µg spinach ferredoxin and 1.0 mM NADP. Reaction mixtures were pre-incubated for 1 min in the dark prior to initiating O_2 evolution with light.

Symbols:

GOGAT-dependent O_2 evolution (●)

NADP -dependent O_2 evolution (○)

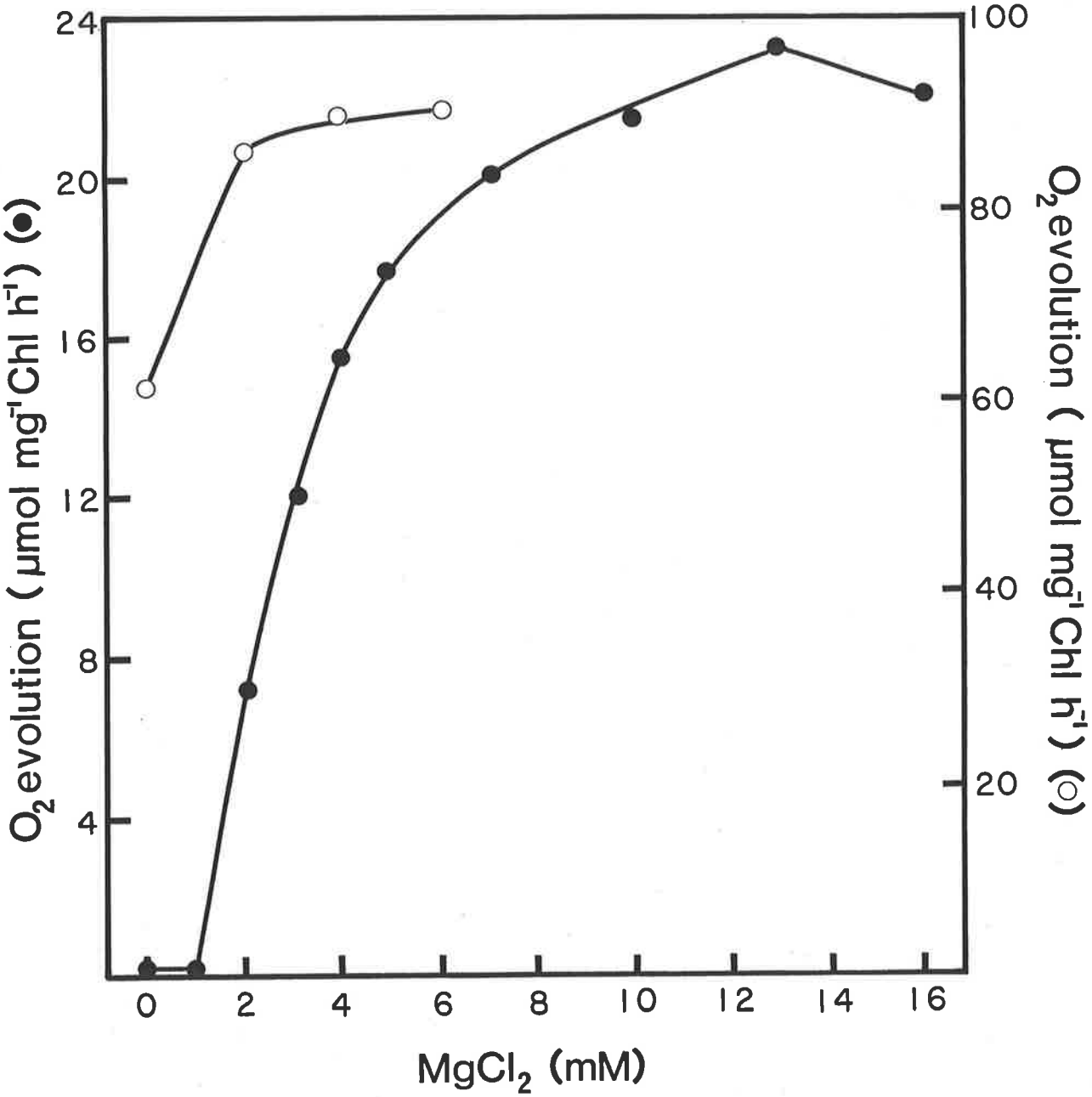

24
20
16
12
8
4
100
80
60
40
20
0
2
4
6
8
10
12
14
16
O2 evolution (µmol mg-1Chl h-1) (●)
O2 evolution (µmol mg-1Chl h-1) (○)
MgCl2 (mM)

Figure 7.6. Effect of 2-OG concentration on the rate of GOGAT-dependent O_2 evolution by a reconstituted chloroplast system.

Experimental procedures as described in sections 2.5 and 2.8. Assays contained 125 µl of chloroplast extract and 4 mM glutamine. The reaction mixtures were pre-incubated for 1 min in the light prior to initiating O_2 evolution with 2-OG.

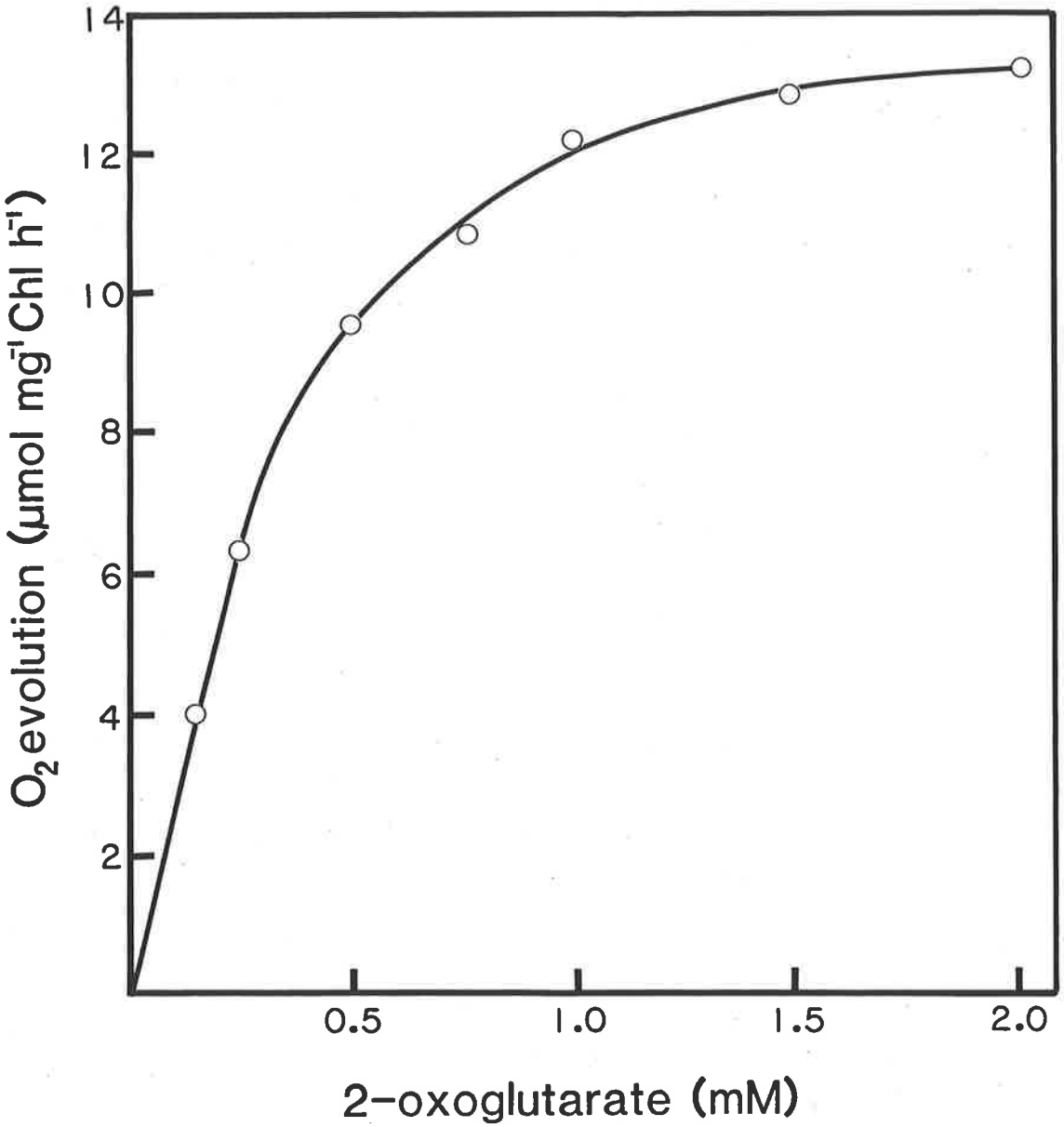

14
12
10
8
6
4
2
O_2 evolution (μmol mg^{-1} Chl h^{-1})
0.5
1.0
1.5
2.0
2-oxoglutarate (mM)

Figure 7.7. Double-reciprocal plot of response of GOGAT activity to varying 2-OG concentrations.

Data shown is taken from Fig. 7.6. (○) and from rates measured following a second addition of 2-OG (●) as described in the text. The dotted line indicates the departure from linearity of the rates achieved with the first addition of the low 2-OG concentrations (i.e. < 0.5 mM) in comparison to those observed with the second 2-OG addition to the same concentration.

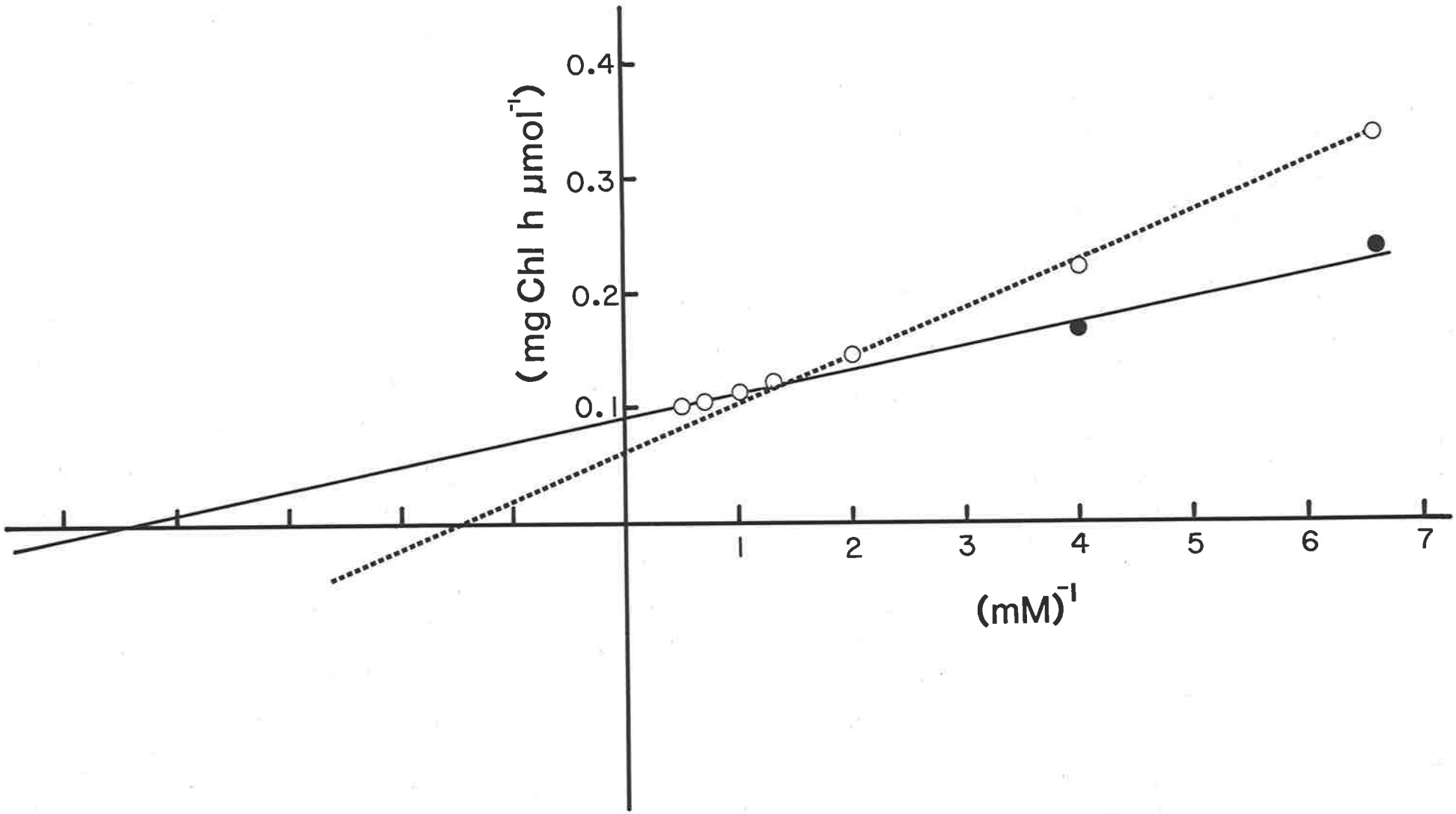
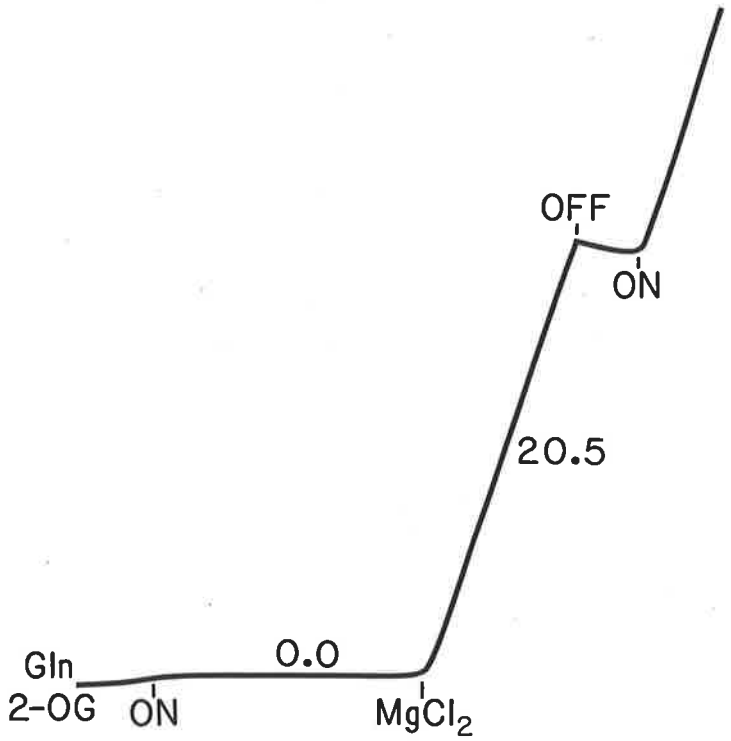

0.4
0.3
0.2
0.1
(mg Chl h µmol⁻¹)
1
2
3
4
5
6
7
(mM)⁻¹

Figure 7.8. Response of GOGAT-dependent O_2 evolution by a reconstituted chloroplast system to $MgCl_2$ addition.

Experimental procedures as described in sections 2.5 and 2.8 except that $MgCl_2$ was absent from the assay medium. Assay contained 125 µl of chloroplast extract, 4 mM glutamine and 2 mM 2-OG. Addition: 10 mM $MgCl_2$. Rates beside the curve represent µmol O_2 evolved mg^{-1} Chl h^{-1}.


OFF
ON
20.5
0.0
Gln
2-OG
ON
MgCl2

CHAPTER VIII

MITOCHONDRIAL HEXOKINASE IN PEA LEAVES

8.1. INTRODUCTION

Investigations into the distribution of hexokinase activity in plant cells have concentrated on non-photosynthetic tissue and shown the activity to be distributed between both the soluble and particulate fractions (Medina and Sols 1953; Marré *et al.* 1968; Kursanov *et al.* 1970; Baxter and Duffus 1973). Some evidence indicates that hexokinase may have a similar distribution in photosynthetic tissues (Saltman 1953; Baldus *et al.* 1981), and that the particulate activity may be associated with the mitochondrial fraction (Baijal and Sanwal 1977).

During experiments discussed in Chapter 3, similar evidence of hexokinase activity associated with pea leaf mitochondria was also obtained. Mitochondrial hexokinase has been demonstrated to be present in a number of different animal tissues (Wilson, 1968; Vallejo *et al.* 1970; Gots and Bessman, 1974; Font *et al.* 1975; Viitanen and Geiger, 1979; Bustamante *et al.* 1981) and found to be particularly important in the metabolic regulation of brain tissue (Wilson, 1980). This chapter details on investigation into the distribution of hexokinase in pea leaf tissue and examines, in particular, the properties of hexokinase activity associated with the mitochondrial fraction. During the course of this investigation I became aware that a similar study was being carried out on pea shoots by another group, the results of which have since been published (Tanner *et al.* 1983).

8.2. RESULTS

Initial investigations with washed mitochondria isolated from pea leaves indicated the presence of endogenous hexokinase activity which, in the presence of both glucose and ATP, could support substantial rates of O_2 uptake e.g. 25-30% of state 3 rates (Fig. 8.1). The stimulation of O_2 uptake by both endogenous and added hexokinase was inhibited by oligomycin and carboxyatractyloside. These inhibitions indicate that hexokinase stimulated O_2 uptake by increasing the external supply of ADP, because oligomycin inhibits oxidative phosphorylation and carboxyatractyloside inhibits the transfer of ADP into the mitochondria.

Using differential centrifugation it could be shown that the distribution of hexokinase activity in the various fractions isolated from pea leaves closely followed the distribution of the mitochondrial enzyme NAD-isocitrate dehydrogenase. Up to 80% of the total cellular hexokinase activity was found to be present in the 2,000 *g* and 15,000 *g* fractions combined (Table 8.1). Further washing of the 15,000 *g* pellet did not reduce the amount of hexokinase found in this fraction.

Pea leaf mitochondria were fractionated on a linear sucrose density gradient and the distribution of hexokinase relative to various markers for chloroplasts (chlorophyll), mitochondria (NAD-isocitrate dehydrogenase) and peroxisomes (hydroxypyruvate reductase) examined. Figure 8.2 illustrates that hexokinase activity was found to be associated exclusively with the mitochondrial band within the gradient. The separate peaks in this mitochondrial band were found to be reproduceable and may

indicate the existence of separate populations of mitochondria in pea leaf cells. The narrow band of hexokinase activity located at the top of the gradient was associated with antimycin-A-insensitive NADH cytochrome c-reductase activity, as found on outer mitochondrial membranes and endoplasmic reticulum. From the relative activity of NAD-isocitrate dehydrogenase in the mitochondrial band it was possible to estimate the amount of hexokinase activity still associated with the mitochondria, after purification, to be approx. 40% of the total cellular activity.

Purified pea leaf mitochondria exhibited stimulations of O_2 uptake in the presence of glucose and ATP similar to those of washed mitochondria and again this stimulation was sensitive to oligomycin (Fig. 8.3).

Hexokinase activity was followed spectrophotometrically by measuring NADP reduction at 340 nm in the presence of G6P dehydrogenase. The results given in Fig. 8.4 illustrate that NADP reduction was immediate on the addition of ATP (trace A), but occurred after a lag period (1-2 min) following the addition of ADP (trace B), provided Krebs cycle intermediates such as malate or succinate were present to support oxidative phosphorylation of the added ADP to ATP by the mitochondria. No ADP- supported NADP reduction was observed in the presence of oligomycin (Fig. 8.4; C) or in the absence of oxidisable substrate (Fig. 8.4; E). Hexokinase activity was found to be very sensitive to the concentration of ADP present, maximal rates only achieved in the presence of large ATP/ADP ratios (Fig. 8.4; B,C). More importantly, however, it could be shown that while ADP-supported NADP reduction

was completely inhibited by carboxyatractyloside, a specific inhibitor of adenine nucleotide uptake into mitochondria, this inhibitor had no effect on the rate of ATP-supported NADP reduction (Fig. 8.4; D), indicating that the hexokinase enzyme was located externally to the inner mitochondrial membrane and not in the mitochndrial matrix.

Pea leaf mitochondrial hexokinase demonstrated a high affinity for glucose (Km 53 μM; Fig. 8.5) and a low affinity for fructose (Km 5.1 mM). The V_{max} of this enzyme with fructose, however, was approx. 1.5 fold higher than with glucose.

Work with animal mitochondrial hexokinase (Gots and Bessman, 1974; Viitanen and Geiger 1979) suggests that newly formed ATP from oxidative phosphorylation may have better access to the hexokinase enzyme than added or external ATP. Indeed, Gots and Bessman (1974) were able to show the affinity of rat liver mitochondrial hexokinase for ATP, produced during oxidative phosphorylation, to be 3-4 fold higher than for ATP added directly to the assay medium. A similar investigation was carried out with pea leaf mitochondrial hexokinase (Fig. 8.6). ADP was added in the presence of malate and succinate to maximize the rate of respiratory electron flow and thus oxidative phosphorylation of the added ADP to ATP. Figure 8.6 illustrates that pea leaf mitochondrial hexokinase activity was higher in the presence of ATP produced via oxidative phosphorylation than with added ATP. A double-reciprocal plot of this data (Fig. 8.7) reveals that the hexokinase enzyme exhibited a slightly higher

affinity for this recently synthesized ATP (and a higher maximal velocity), than with external ATP. However, these differences were smaller than have been observed with animal mitochondrial hexokinase (Gots and Bessman, 1974).

It has been shown for brain mitochondrial hexokinase that incubation with G6P will, under certain conditions, result in the removal of the enzyme from the mitochondrial membrane (Rose and Warms, 1967; Felgner and Wilson, 1977). Incubation of pea leaf mitochondria with 1 mM G6P for 30 min at 25°C resulted in, at best, only 3% of the total hexokinase activity being solubilized from the mitochondrial membranes. This is in agreement with the results of Tanner *et al.* (1983) on the same tissue, but in stark contrast to the ready solubilization, by G6P, of hexokinase associated with mitochondria isolated from the endosperm of *Ricinus communis* (J.A. Miernyk, personal communication).

8.3. DISCUSSION

The results demonstrate that up to 80% of the total cellular hexokinase activity in pea leaf tissue may be associated with the particulate fraction. Tanner *et al.* (1983) also observed that approximately 65% of the total hexokinase activity in pea stem tissue was pelleted at 10,000 g. These figures are much higher than previous estimates of particulate activity in other types of photosynthetic tissue e.g. spinach leaves (Saltman, 1953; Baldus *et al.* 1981) and *Cuscuta reflexa* filaments (Baijal and Sanwal, 1977), but are comparable to the amounts found associated with the particulate fractions of brain tissue

(Wilson, 1980). More importantly, the hexokinase activity in this particulate fraction was found to be associated exclusively with the mitochondria (Fig. 8.2), in agreement with reports involving various plant (Baijal and Sanwal 1977; Tanner *et al*. 1983) and animal tissues (Vallejo *et al*. 1970).

The kinetic properties of pea leaf mitochondrial hexokinase were found to be very similar to those of the particulate hexokinase isolated from spinach leaves (Baldus *et al*. 1981) and the mitochondrial hexokinase isolated from pea shoots by Tanner *et al*. (1983). The high affinity of this enzyme for glucose, relative to fructose, and its sensitivity to inhibition by ADP (Fig. 8.4), shows it to be similar to the hexokinase II enzyme isolated from pea seeds (Turner and Copeland, 1981). Some evidence was also found (Fig. 8.6) to suggest that the binding of hexokinase to pea leaf mitochondria may give it some preferential access to intramitochondrially generated ATP, as originally observed for rat liver mitochondrial hexokinase (Gots and Bessman, 1974).

The hexokinase enzyme is located externally to the inner mitochondrial membrane, as shown by the insensitivity of external ATP-supported hexokinase activity to carboxyatractyloside inhibition (Fig. 8.4; see Vallejo *et al*. 1970), and is most probably bound to the outer membrane of pea leaf mitochondria (Tanner *et al*. 1983). Felgner and Wilson (1977) demonstrated that the binding of hexokinase to the outer membrane of brain mitochondria was electrostatic in nature and it has since been shown that this electrostatic interaction occurs between hexokinase and a specific hexokinase binding protein within the outer membrane (Felgner *et al*. 1979).

The degree of binding of hexokinase to mitochondria in brain tissue is regulated, in the main, by the intracellular levels of G6P (Wilson, 1980). This effect is thought to be mediated through G6P-induced conformational changes in the hexokinase enzyme which reduce the degree of electrostatic interaction between the enzyme and the hexokinase binding protein, thereby resulting in its solubilization. Furthermore, it has been demonstrated that it is the bound form of the enzyme which is most active, due to its decreased susceptibility to product inhibition by G6P and its increased affinity for ATP (Wilson, 1980). Consequently, under conditions of increased glycolytic demand, the decrease in the intracellular level of G6P results in an increase in the proportion of the hexokinase in the bound state, which in turn activates the enzyme to respond to increased demand for G6P. While such a regulatory system may be required to operate in a tissue, such as the brain, which utilizes glucose almost exclusively as an energy source (Sokoloff *et al.* 1977), the requirement or role for a similar system in photosynthetic tissues is not obvious. In fact our results, along with those of Tanner *et al.* (1983), would tend to suggest that a similar system of G6P-mediated binding does not operate with mitochondrial hexokinase in pea leaves.

One possible explanation for the mitochondrial location of this enzyme in plant tissues may be that it serves to maintain a close relationship with the ATP-generating system of oxidative phosphorylation. This would ensure that the hexokinase enzyme could respond rapidly to changes in cellular demand for G6P,

which is known to be a key intermediate in a number of different metabolic pathways, including glycolysis, and the pentose phosphate pathway.

Table 8.1. Distribution of chlorophyll, hexokinase and NAD-isocitrate dehydrogenase in various subcellular fractions of pea leaf tissue.

A pea leaf homogenate was prepared according to the method in section 2.2 and centrifuged at 2,000 *g* for 10 min. The supernatant was then centrifuged at 15,000 *g* for 15 min, the pellet resuspended in 60 ml of isolation medium and re-sedimented by centrifugation at 15,000 *g* for 15 min. The original 15,000 *g* supernatant was centrifuged at 105,000 g for 90 min. The pelleted fractions were resuspended in a small volume of isolation medium using a glass homogeniser. Hexokinase and isocitrate dehydrogenase were assayed as described in section 2.10 and chlorophyll as in section 2.15.

	Chlorophyll		Hexokinase		NAD-isocitrate dehydrogenase	
	mg	%	Activity	%	Activity	%
			nmol min^{-1}		*nmol min^{-1}*	
Homogenate	22.5	100	2600	100	2770	100
2000 *g* pellet	11.0	49	1325	51	1570	57
15,000 *g* pellet	2.8	12	790	30	1130	41
105,000 *g* pellet	0.8	4	150	6	0	0
105,000 *g* supernatant	7.1	32	251	10	0	0

Figure 8.1. Stimulation of O_2 consumption in washed pea leaf mitochondria by endogenous hexokinase activity.

Mitochondria (0.5 mg protein) were placed in 2.5 ml of reaction medium (section 2.8) at pH 7.5, containing 5 mM $MgCl_2$, 10 mM malate, 10 mM glutamate and 0.1 mM TPP. Additions as shown in order were as follows: trace A, 0.18 mM ADP, 0.18 mM ADP, 12 mM glucose, 1.5 mM ATP, 1.3 units hexokinase (HK), 5 µg oligomycin; trace B, 0.2 mM ADP, 2 mM ATP, 10 mM glucose, 0.8 mM ADP, 20 µM carboxyatractyloside (CAT). Rates shown represent nmol O_2 consumed mg^{-1} protein min^{-1}.

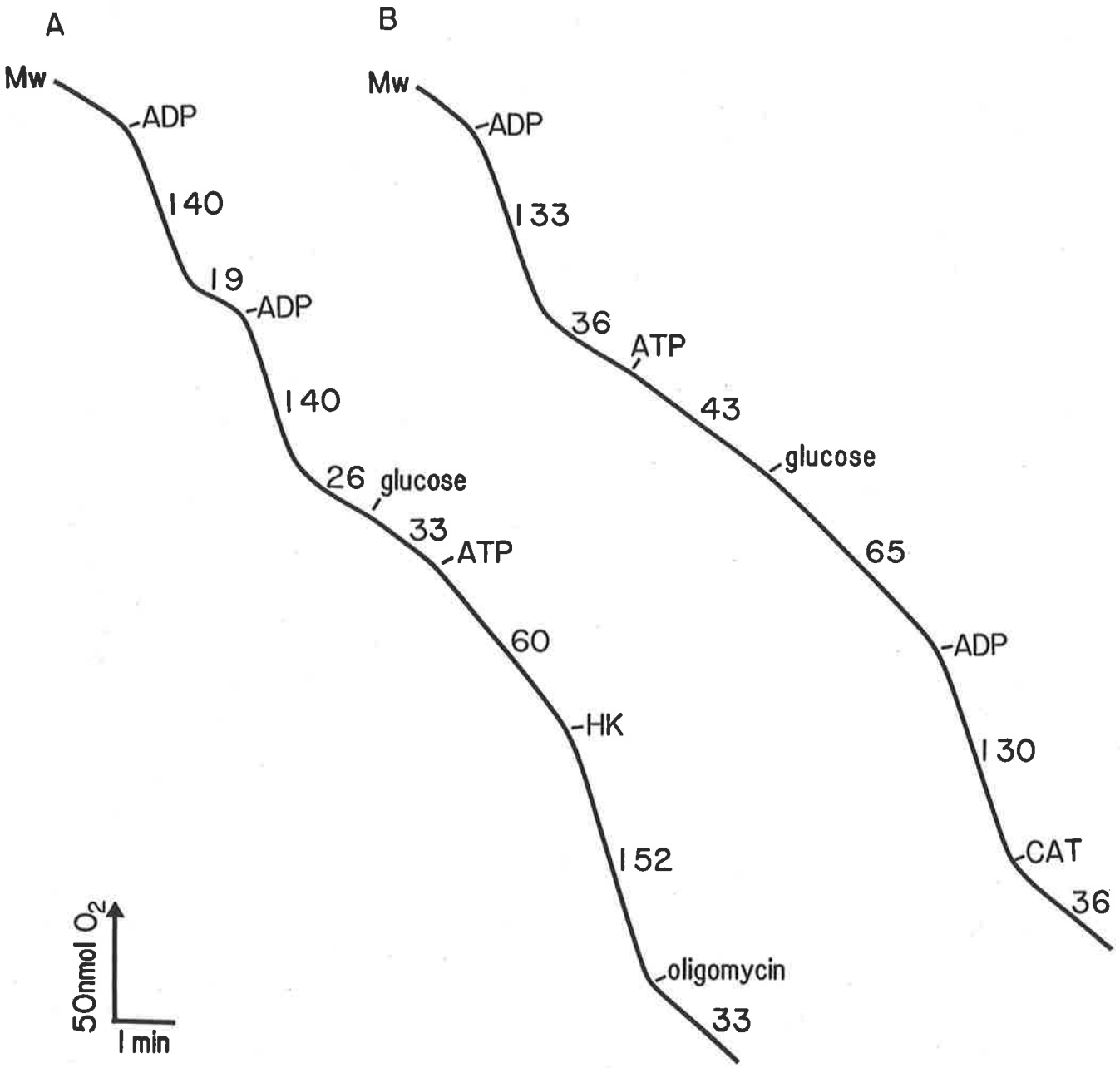

A
Mw
ADP
140
19
ADP
140
26
glucose
33
ATP
60
HK
152
oligomycin
33
B
Mw
ADP
133
36
ATP
43
glucose
65
ADP
130
CAT
36
50nmol O2
1 min

Figure 8.2. Distribution of chlorophyll and enzyme activities in a sucrose density gradient.

Experimental procedures as described in section 2.3. The sucrose gradient was fractionated into 1.2 ml volumes and the chlorophyll and enzyme activities in each fraction measured as described in sections 2.10 and 2.15. Units: sucrose (%, w/v); chlorophyll (μg), NAD-isocitrate dehydrogenase activity (nmol isocitrate oxidized min^{-1}); hexokinase activity (nmol G6P produced min^{-1}); hydroxypyruvate reductase activity (nmol hydroxypyruvate reduced min^{-1}).

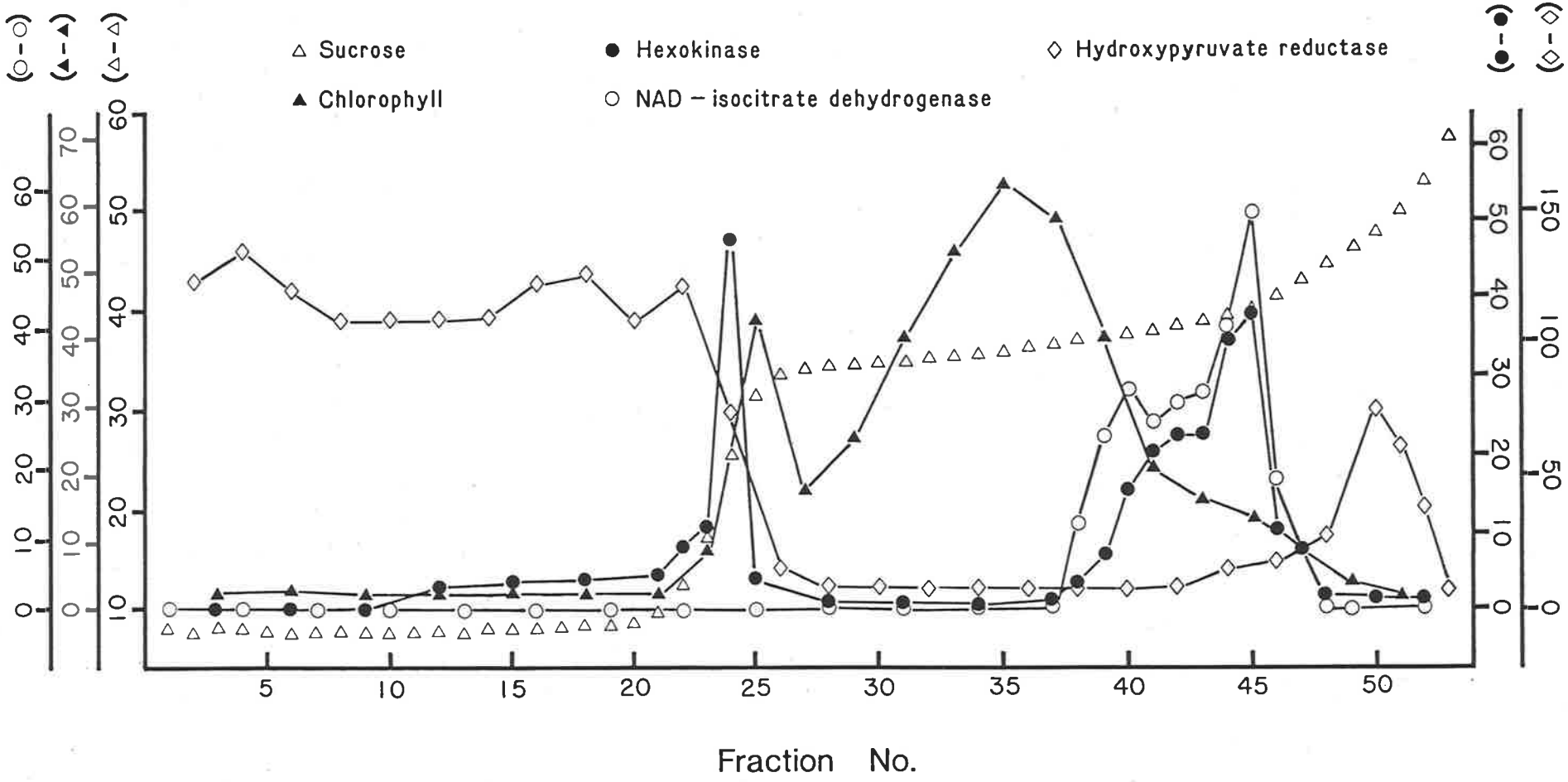

(○-○)
(▲-▲)
(△-△)
(●-●)
(◇-◇)
△ Sucrose
● Hexokinase
◇ Hydroxypyruvate reductase
▲ Chlorophyll
○ NAD – isocitrate dehydrogenase
0
10
20
30
40
50
60
70
0
10
20
30
40
50
60
0
50
100
150
5
10
15
20
25
30
35
40
45
50
Fraction No.

Figure 8.3. Stimulation of O_2 consumption in purified pea leaf mitochondria by endogenous hexokinase.

Mitochondria (0.3 mg protein; purified as described in section 2.3) were placed in 2.0 ml of reaction medium (section 2.8) pH 7.5, containing 5 mM $MgCl_2$, 10 mM malate, 10 mM glutamate and 0.1 mM TPP. Additions in the order indicated: 0.2 mM ADP, 1.0 mM ATP, 10 mM glucose, 0.8 mM ADP and 5 µg oligomycin. Rates shown represent nmol O_2 consumed mg^{-1} protein min^{-1}.

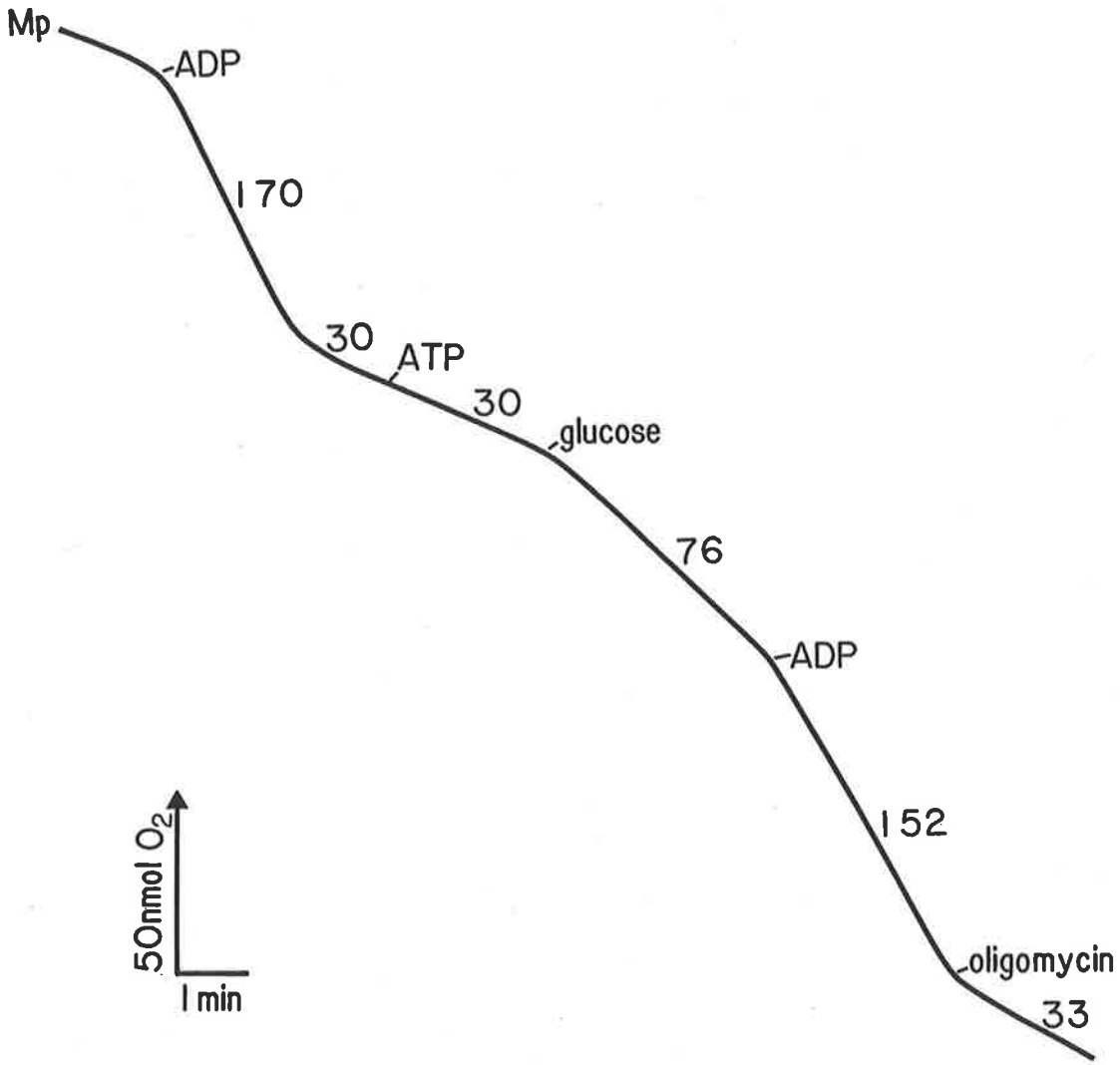

Mp
ADP
170
30
ATP
30
glucose
76
ADP
152
oligomycin
33
50nmol O2
1 min

Figure 8.4. Assay of endogenous hexokinase activity in pea leaf mitochondria via NADP reduction in a coupled enzyme system.

Hexokinase activity of intact pea leaf mitochondria was assayed in reaction medium as described in section 2.10. All assays contained 0.15 mg protein as well as: trace B, 10 mM malate, 10 mM glutamate, 0.1 mM TPP; trace C, 10 mM malate, 10 mM glutamate, 0.1 mM TPP, 5 µg oligomycin; trace D, 10 mM malate, 10 mM glutamate, 0.1 mM TPP, 20 µM carboxyatractyloside; trace E, nil. Additions as indicated: trace A, 1.0 mM ATP, 5 units hexokinase; trace B, 0.1 mM ADP, 0.4 mM ADP, 3.7 mM ATP; trace C, 0.1 mM ADP, 1.0 mM ATP, 3.5 mM ATP; trace D, 0.1 mM ADP, 1.0 mM ATP, 3.5 mM ATP; trace E, 0.1 ADP, 1.0 mM ATP, 10 mM malate and glutamate, 0.1 mM TPP. Rates shown are nmol NADP reduced mg^{-1} protein min^{-1}.

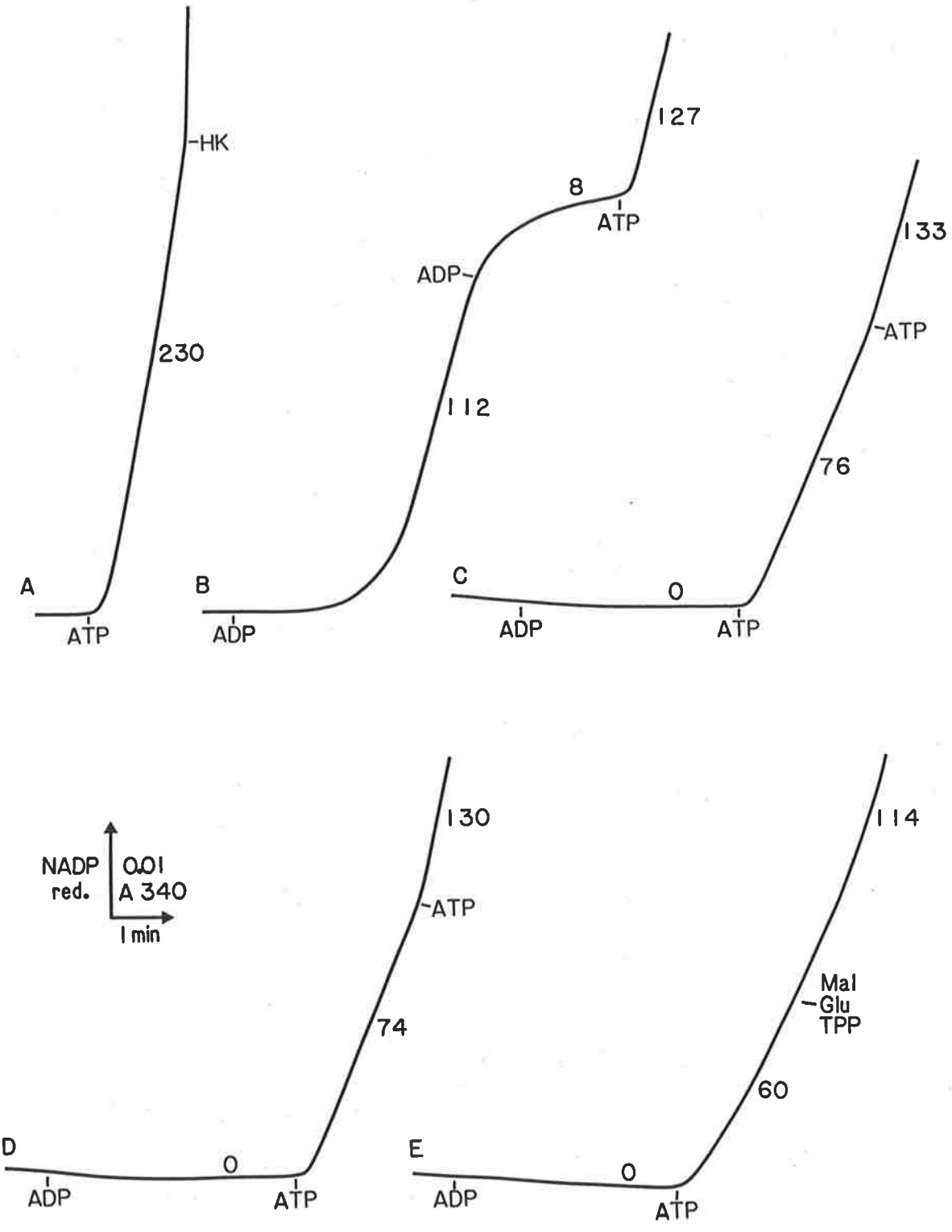

A
ATP
230
HK
B
ADP
112
ADP
8
ATP
127
C
ADP
0
ATP
76
ATP
133
NADP
red.
0.01
A 340
1 min
D
ADP
0
ATP
74
ATP
130
E
ADP
0
ATP
60
Mal
Glu
TPP
114

Figure 8.5. Effect of glucose concentration on the activity of pea leaf mitochondrial hexokinase.

Hexokinase (HK) activity was assayed in Tris-HCl buffer as described in section 2.10. Assays contained 0.4 mg protein.

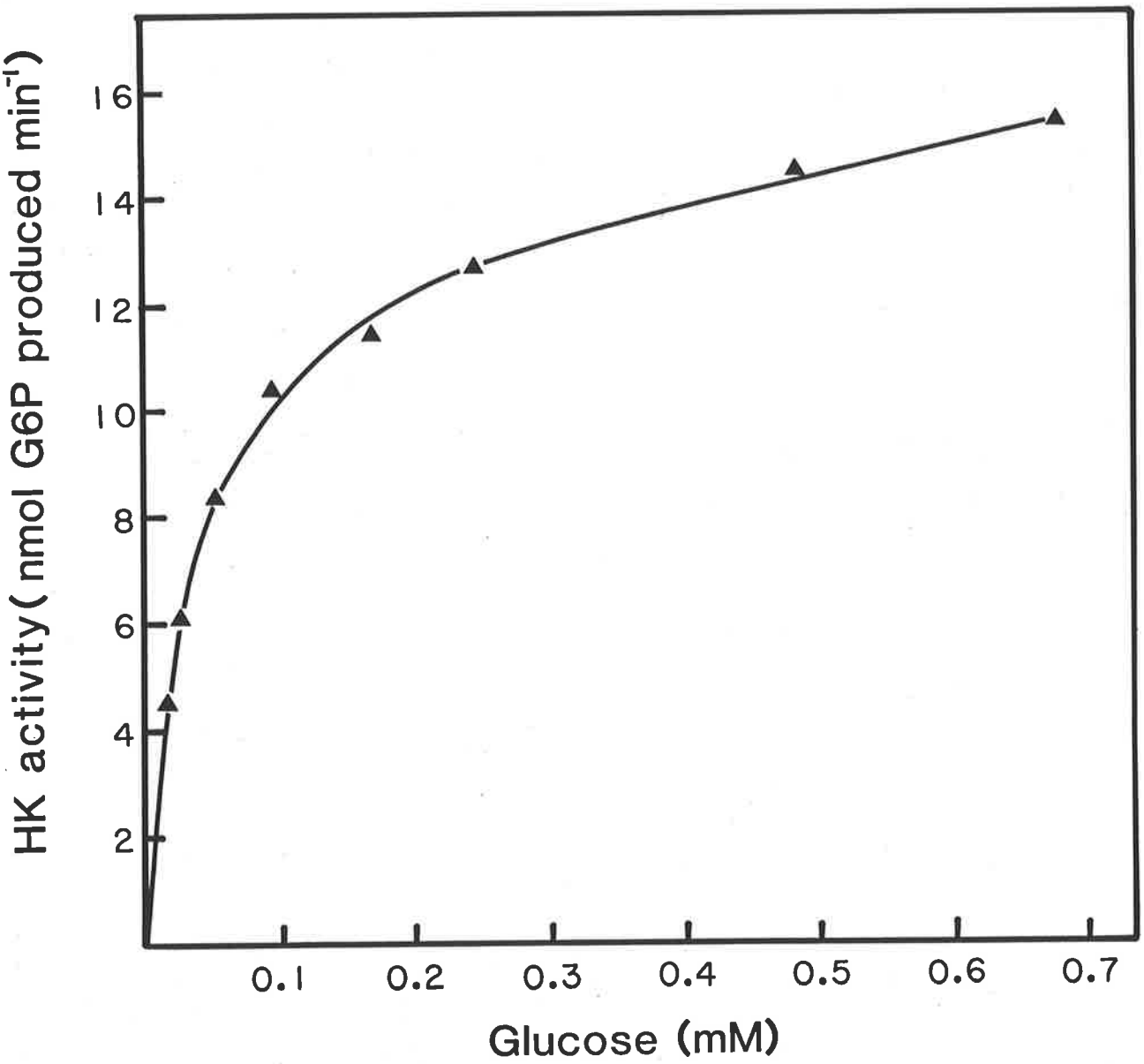

HK activity(nmol G6P produced min-1)
16
14
12
10
8
6
4
2
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Glucose (mM)

Figure 8.6. Effect of ATP concentration on pea leaf mitochondrial hexokinase activity.

Hexokinase (HK) activity of intact pea leaf mitochondria was assayed in reaction medium as described in section 2.10. ATP was added directly or generated via oxidative phosphorylation in the presence of ADP and oxidizable substrates. The ATP-dependent assay contained 10 mM phosphocreatine and 1.4 units of creatine kinase to recycle ADP produced during the hexokinase reaction back to ATP. The ADP-dependent assay contained 10 mM malate, 10 mM glutamate and 10 mM succinate.

Units:

nmol G6P produced min^{-1}

Symbols:

ATP (▲)

ADP (△)

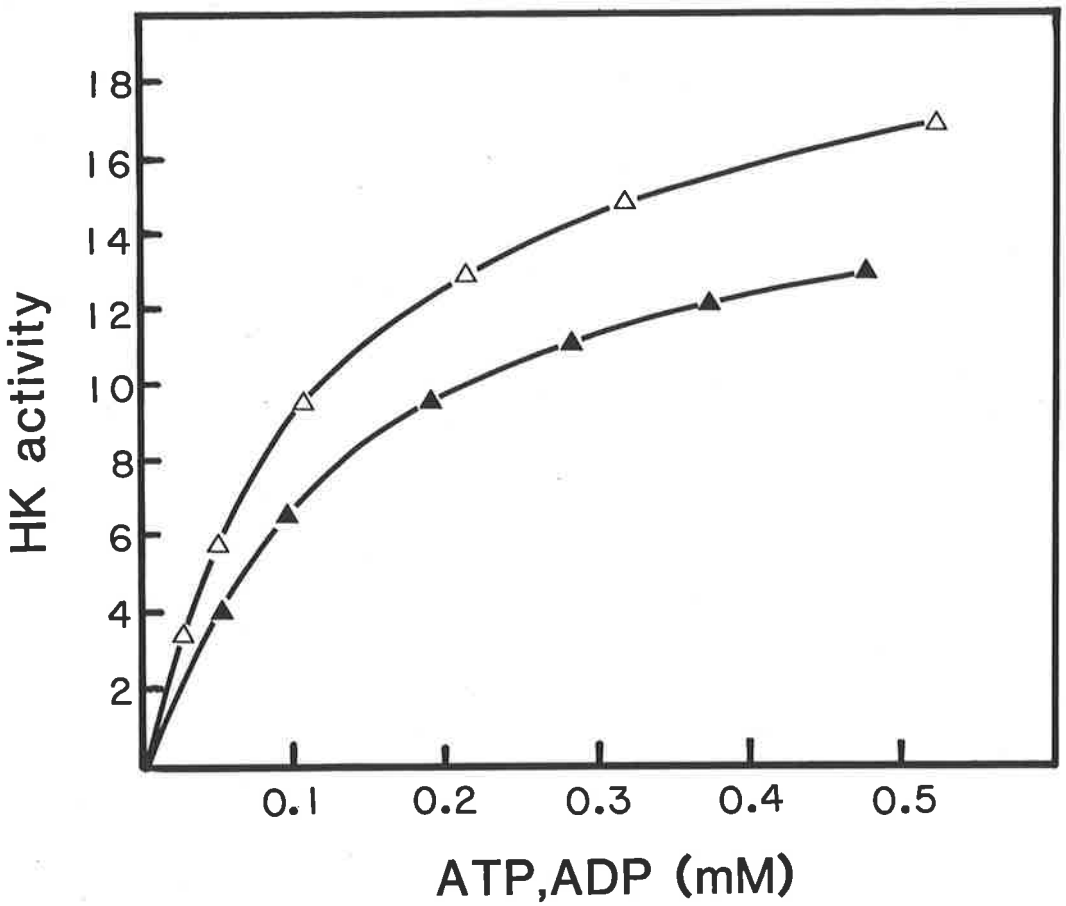

HK activity
18
16
14
12
10
8
6
4
2
0.1
0.2
0.3
0.4
0.5
ATP,ADP (mM)

Figure 8.7. Double-reciprocal plot of response of mitochondrial hexokinase to varying ATP concentration

Data taken from Fig. 8.6.

Symbols:

ATP (▲)

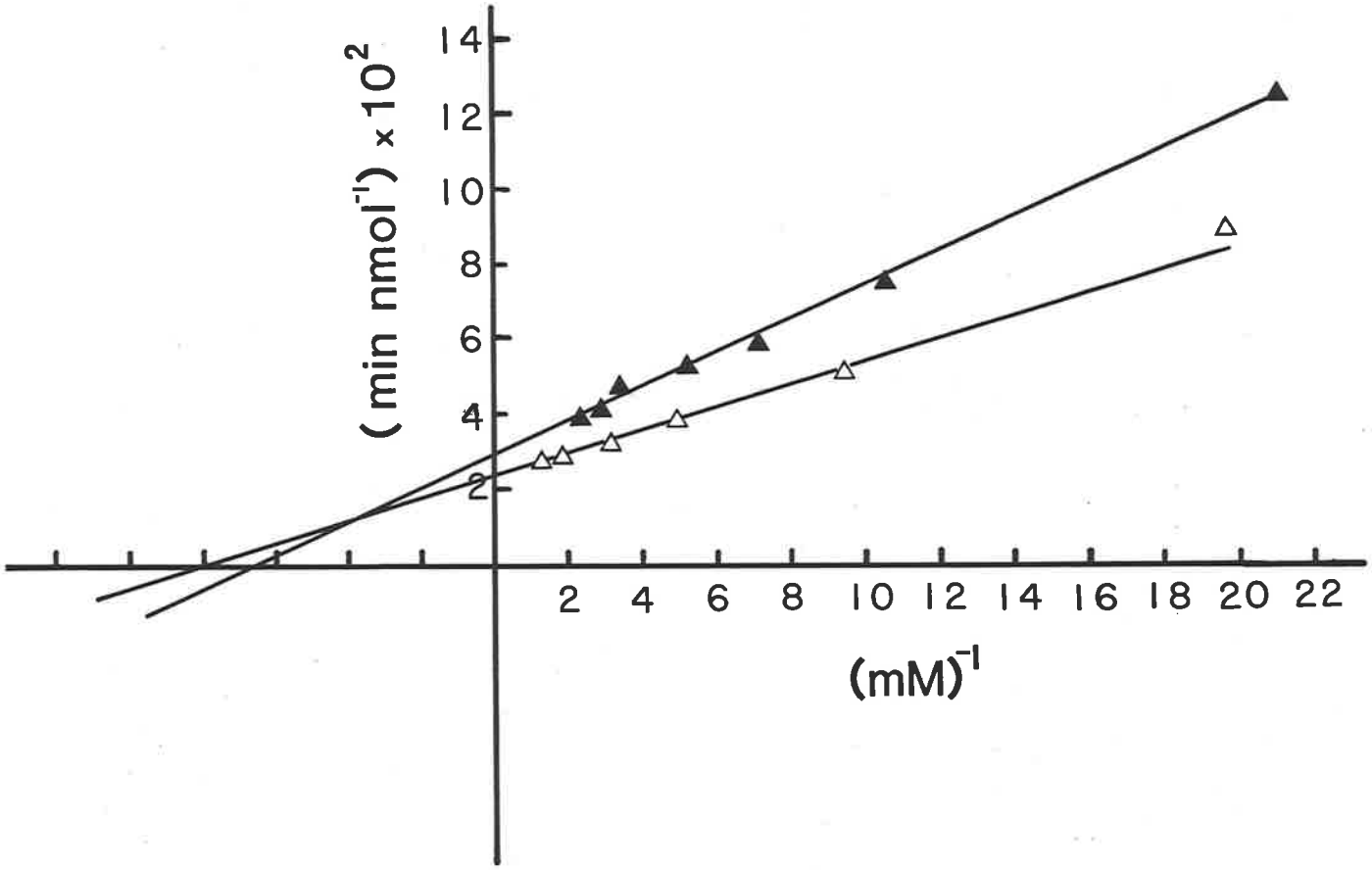
ADP (△)

Km (ADP) - 0.12 mM

Km (ATP) - 0.16 mM

Vmax (ADP) - 43 nmol G6P produced min^{-1}

Vmax (ATP) - 35 nmol G6P produced min^{-1}


(min nmol^{-1}) x 10^2
14
12
10
8
6
4
2
2 4 6 8 10 12 14 16 18 20 22
(mM)$^{-1}$

CHAPTER IX

GENERAL DISCUSSION

9.1. *The role of the mitochondrial respiratory chain in photorespiratory glycine metabolism*

One of the most important questions yet to be resolved regarding photorespiratory metabolism is the role of the mitochondrial respiratory chain. Under normal circumstances one might predict that NADH generated within the mitochondria, during photorespiratory glycine oxidation, would be re-oxidized via the electron transport chain in the same way as NADH generated during the TCA cycle oxidation reactions. However, in the past this potential fate for glycine NADH has been avoided because of the belief of many plant biochemists that the mitochondrial respiratory chain, in photosynthetic tissue, was 'switched off' in the light.

This theory of a light-mediated inhibition of respiration,in reality, had little experimental support, but relied mainly on an elegant biochemical explanation of the phenomenon; an apparent light-mediated increase in the cytoplasmic ATP/ADP ratio which led to an inhibition of mitochondrial oxidative phosphorylation and, thus, coupled electron flow via the respiratory chain to O_2 (Heber and Santarius, 1970). Clearly, the results presented in Chapter 3 demonstrate that on the basis of current estimates of cytoplasmic ATP/ADP ratios in leaf cells, such a regulatory system cannot function. Plant mitochondrial respiration, like that of animal mitochondria, is regulated by ADP supply or uptake into the

mitochondria and not by the external ATP/ADP ratio (Chapter 3). Consequently, it is likely that leaf mitochondrial respiration *in vivo* will be regulated by the availability of ADP.

This leads to the interesting observation made in Chapter 3 that current estimates of the levels of cytoplasmic ADP in leaf cells (Hampp *et al.* 1982; Stitt *et al.* 1982; Stitt *et al.* 1983) are found to be between 4 and 10 fold in excess of the amount of ADP required for maximal state 3 rates of electron flow via the respiratory chain. On the basis of these estimates, respiratory chain activity *in vivo* should never be ADP-limited and would always operate at maximal state 3 rates. This, however, is in contrast to our current concept of respiratory activity *in vivo*, as stated by Wiskich (1980), that the respiratory chain is operating at somewhere between state 3 and state 4 rates, due to a limitation of ADP availability.

Recent investigations into the levels of adenine nucleotides in animal tissues suggest there may be large discrepancies between the total levels of ADP measured in certain cellular compartments and the actual amount of free ADP which is available for, or involved in, metabolic reactions (Veech *et al.* 1979; Wilson *et al.* 1983). These studies involved the measurement of the levels of the reactants of various enzyme reactions within the separate cellular compartments, from which estimates of the concentrations of free ADP and ATP in these compartments could be calculated based on a knowledge of the equilibrium kinetics of these particular enzymic reactions. On the basis of these experiments, Wilson *et al.* (1983) suggested that only 10% of the

total ADP estimated to be present within mitochondria may be in a free form which can interact with matrix enzymes; the rest appearing to be bound and not taking part in metabolic reactions. The use of similar techniques for the measurement of free ADP levels in the cytoplasm of leaf cells may demonstrate the levels of free ADP to be significantly lower than estimated by current techniques, and in the region which will exert a regulatory influence on mitochondrial respiratory activity.

The results of Chapter 4 suggest, albeit indirectly, that the respiratory chain may function in the light to specifically re-oxidise NADH generated during glycine oxidation. Pea leaf mitochondria were shown to preferentially oxidise glycine via the respiratory chain in comparison to TCA cycle intermediates such as malate, 2-OG and succinate and,as mentioned in Chapter 4, similar results have now also been observed with isolated spinach mitochondria (Ericson *et al*. 1983). The mechanism of this preferential oxidation of glycine via the respiratory chain may be linked to the differential distribution or compartmentation of glycine decarboxylase and other TCA cycle dehydrogenase enzymes such as MDH and ME, within the mitochondria (Chapter 5). It is envisaged that the location of glycine decarboxylase on the inner mitochondrial membrane may help to reduce the extent of the competition between glycine decarboxylase and soluble NAD-linked mitochondrial enzymes for access to NAD and NADH dehydrogenase. Whether this compartmentation also extends to the level of NAD (i.e. bound and soluble pools) is not known. However, as discussed in Chapter 5, it is

interesting to note that Laties (1983) has recently reported that isocitrate dehydrogenase may be distributed within separate compartments of potato mitochondria and that these two forms (i.e. membrane-bound and matrix soluble) may operate with separate NAD pools. It is also proposed that the location of glycine decarboxylase on the inner membrane results in a special interaction between this enzyme and the NADH dehydrogenase enzymes of the respiratory chain, which ensures that NADH generated during glycine oxidation will be re-oxidised in preference to that produced by other NAD-linked TCA cycle enzymes.

The absence of a similar system of preferential glycine oxidation in animal mitochondria (Hampson *et al*. 1983) leads one to speculate that this mechanism, and thus the respiratory chain itself, has a specific role in photorespiratory metabolism in leaf tissue. Indeed, the recent findings of Oliver (1983) would support this view (see Chapter 4). Despite this, tissue estimates of *in vivo* glycine oxidation via the mitochondrial respiratory chain (Chapter 4) are only just sufficient to account for the most widely accepted (Somerville and Somerville, 1983) estimates of photorespiration. This limitation cannot be ascribed to the respiratory chain, however, since total chain capacity clearly exceed that utilised during state 3 glycine oxidation (Chapter 4). It appears, therefore, that estimates of *in vivo* rates of glycine oxidation in leaf tissue are limited, not by the affinity of the respiratory chain for glycine NADH, but by the absolute activity of glycine decarboxylase in the tissue.

As noted by Day and Wiskich (1981) the above result is unusual, since enzyme activities in tissue extracts are normally substantially higher than the rates which proceed via that enzyme step *in vivo*. One explanation for this apparent anomaly may be that tissue rate estimations, based on extrapolations from mitochondrial rates (as in Chapter 4), may underestimate the true tissue glycine decarboxylase activity, because inactivation of the enzyme may occur during mitochondrial isolation. Alternatively, we may not yet have discovered the optimal conditions under which to assay this enzyme. Initial studies with RuBP carboxylase from leaf extracts for example, led to a similar conclusion: that the activity of this enzyme was insufficient to account for the *in vivo* rates of photosynthetic CO_2 fixation (Peterkofsky and Racker, 1961). Subsequent investigations, however, revealed that ample activity could be demonstrated to account for *in vivo* rates, provided the enzyme was assayed under optimal conditions (Lilley and Walker, 1975).

A final possibility is that the currently accepted rates of photorespiratory flux overestimate the true *in vivo* rate of glycine decarboxylation. This would appear unlikely, however, based on: (a) a lack of evidence of a significance release of photorespiratory CO_2 release from reactions other than glycine decarboxylation (see section 1.2.2) and (b) the general agreement between workers in this field, that the dual-isotope gas exchange method used for the estimation of the photorespiratory rates quoted here i.e. 25-35 μmol CO_2 mg^{-1} Chl h^{-1} (Ludwig and Canvin, 1971; D'Aoust and Canvin, 1973; Mahon *et al*. 1974; Fock *et al*. 1979) and recognised as the most accurate technique (Zelitch, 1979;

Somerville and Somerville, 1983), may underestimate photorespiratory flux by up to 30-50% (D'Aoust and Canvin, 1974; Fock *et al*. 1979; Somerville and Somerville, 1983). This error stems from problems associated with the estimation of internal re-assimilation of photorespiratory CO_2 (Canvin, 1979).

A final consideration to be taken into account regarding the possibility of *in vivo* glycine oxidation via the respiratory chain, is that this process necessarily leads to the production of ATP, which must be recycled back as ADP at a rate sufficient to account for the potential rate of glycine-dependent oxidative phosphorylation i.e. 75-90 µmole ATP mg^{-1} Chl h^{-1} (based on the tissue rates in Chapter 4 and the production of 3 x ATP per 1 x CO_2 evolved). Rates of 'dark' respiration of leaf slices of 13-14 day old pea plants (measured according to the method in section 2.8) were found to be of the order of 22.5 ± 2.8 µmol O_2 uptake g^{-1} fwt h^{-1} (n = 11) which is equivalent to 62.5 µmol ATP produced mg^{-1} Chl h^{-1}. If the'dark' respiratory rate is ADP-limited, the advent of glycine in the light would result in competition between glycine decarboxylase and the TCA cycle dehydrogenases for access to the respiratory chain; a situation in which glycine would be preferentially oxidised at the expense of the TCA cycle intermediates (Chapter 4). However, current evidence suggests that TCA cycle turnover is maintained in the light at similar rates as in the dark (see section 1.3.1). Furthermore, it can be seen that the rate of respiratory chain activity in the dark would be insufficient to account for the total rate of photorespiratory glycine oxidat-

ion via the chain in the light. Thus, even with preferential glycine oxidation, the rate of ATP turnover or ADP supply to the mitochondria would need to be increased in the light, if the respiratory chain was to be able to support this increased rate of electron flow in the presence of glycine.

In the photorespiratory nitrogen cycle proposed by Keys *et al.* (1978), it was proposed that part of the glycine-mediated increase in ATP production, by the respiration chain, was utilised by cytoplasmic GS in the refixation of NH_3 released during glycine oxidation. This is clearly not possible in species which lack cytoplasmic GS (see section 1.3.2) and also appears unlikely to account for a significant proportion of the ATP produced in C_3 species (e.g. pea) which do contain some cytoplasmic GS. Rates of NH_3 refixation by isolated pea chloroplasts, in the presence of glutamine and 2-OG, were limited by glutamine uptake and could not account for *in vivo* rates of photorespiratory NH_3 refixation (Chapter 6). Thus, a significant proportion, if not all of the NH_3 re-assimilation, most probably occurs within the chloroplast compartment and, consequently, would not utilize the cytosolic ATP produced during glycine oxidation. It should be noted that the operation of ferredoxin-linked GOGAT during the second step of this NH_3 re-assimilatory pathway in chloroplasts would, in fact, result in the production of sufficient ATP via the photosynthetic electron transport chain to support the activity of chloroplastic GS.

It is difficult to reconcile, therefore, how respiratory-linked glycine oxidation could operate *in vivo*, in the absence of

a mechanism for the rapid recycling of cellular ATP under photorespiratory conditions. This problem could, of course, be negated if glycine oxidation was linked to the non-phosphorylating alternate pathway (Azcón-Bieto *et al.* 1983). However, the results of Chapter 4 indicate that for the pea species used in these studies there would be insufficient alternate pathway activity to support *in vivo* rates of photorespiratory glycine oxidation. This conclusion would also apply to spinach tissue, based on the estimates of alternate pathway activity in isolated spinach leaf mitochondria (15-25% of the state 3 glycine oxidation rate; Moore *et al.* 1977; Douce *et al.* 1977) and the tissue rates of glycine oxidation in spinach leaves as measured in Chapter 4. It would appear that, because of the compartmentation of glycine decarboxylase within mitochondria (Chapter 5), this enzyme has only a limited access to the total complement of respiratory chains or alternate pathways which are present. Clearly, this finding does not support the hypothesis that the alternate pathway has a specific role in the oxidation of glycine during the operation of the photorespiratory cycle.

To summarize, the role of the mitochondrial respiratory chain in photorespiratory metabolism is still uncertain. However, the evidence presented in this study indicates that, given the continued operation of the electron transport chain in the light, a major part of the NADH produced during photorespiratory glycine oxidation may be re-oxidised via the respiratory chain. Clearly,

under these conditions, glycine oxidation could not be linked to hydroxypyruvate reduction in the peroxisome (Woo and Osmond, 1976; Osmond, 1981; Journet *et al*. 1981). However, in a competitive oxidation situation involving glycine decarboxylase and the TCA cycle enzymes (in which glycine would be preferentially oxidised via the respiratory chain), the NADH resulting from the operation of the TCA cycle may be transferred to the peroxisome to allow TCA cycle turnover to be maintained in the light.

It could be envisaged that the preferential oxidation of glycine via the respiratory chain, in the presence of TCA cycle turnover, would lead to an increase in the intramitochondrial NADH/NAD ratio. This would tend to inhibit the direct oxidation of malate to OAA within the mitochondria, due to the unfavourable equilibrium of the MDH reaction. The rate of malate oxidation could be maintained if malate was transported to the peroxisome where it could be oxidised by peroxisomal MDH to produce NADH which, in turn, could be used to support hydroxypyruvate reduction. The OAA formed could be transported back to the mitochondria via the shuttle systems illustrated in Fig. 1.3, with the exception that, once in the mitochondrion, the OAA would not be reduced back to malate, as proposed by Woo and Osmond (1976) or Journet *et al*. (1981), but would be utilised in its normal role as an acetyl-CoA acceptor molecule. In this way complete TCA cycle turnover would be maintained, while at the same time transferring reducing equivalents to the peroxisome. Furthermore, glycine oxidation would be linked directly to the respiratory chain and would not be reliant on the efficiency of various shuttle systems for NADH

re-oxidation. Such a system is attractive in terms of the relative priorities associated with maintaining photorespiratory and TCA cycle turnover in the light.

The actual amount of TCA cycle NADH which would be available for hydroxypyruvate reduction would be dependent on the relative rates of respiratory chain activity (in the light) and intramitochondrial NADH production by the TCA cycle and from photorespiratory glycine metabolism. If respiratory chain activity, in the light, was sufficient to account for the production of NADH by both of these processes, an alternative source of reducing power would be required to support hydroxypyruvate reduction. The most likely candidate would be the chloroplast. However, its role as a potential donor of reducing equivalents for this reaction must be considered in terms of the following questions: (a) is the rate of photosynthetic electron transport *in vivo* sufficient to account for the reducing power demands of CO_2 fixation, photorespiratory NH_3 refixation and photorespiratory hydroxypyruvate reduction and (b) how would the ATP production associated with this increased photosynthetic electron transport be utilised or recycled in order to maintain ADP supply for photosynthetic phosphorylation.

9.2. *The current model of photorespiratory NH_3 re-assimilation*

On the basis of the results in Chapter 6 and those published previously (Anderson and Done 1977a,b; Woo and Osmond, 1982) it would appear that maximal rates of NH_3 refixation by isolated chloroplasts are observed when the initial NH_3 refixation step, catalysed by GS, occurs within the chloroplast. It is

likely that glutamine uptake would limit the maximal rate of photorespiratory NH_3 re-assimilation if the GS step occurred outside of the chloroplast, i.e. via cytoplasmic GS. Betsche (1983b) has also recently provided evidence to suggest that the chloroplast is the major site of photorespiratory NH_3 re-assimilation in species containing both the chloroplastic and cytoplasmic forms of GS.

Even so, the results of studies with isolated chloroplasts (Chapter 6) indicate that maximal rates of NH_3 re-assimilation within the chloroplast compartment will only occur at rates sufficient to account for *in vivo* rates of photorespiratory NH_3 release if a dicarboxylate (e.g. malate) is also present. The uptake of 2-OG into isolated chloroplasts, in direct exchange for glutamate, does not appear (despite the findings of ^{14}C- uptake studies) to be rapid enough to support estimated rates of photorespiratory NH_3 release and a more complex shuttle system involving 2-OG exchange with malate appears necessary.

One problem regarding such a shuttle is to determine from where the exchanging malate would originate. Stimulated 2-OG uptake cannot be coupled to a malate/aspartate shuttle, since this is a closed system; aspartate first having to react with 2-OG to produce the malate required for further exchange reactions (see Fig. 6.13). Alternatively, 2-OG uptake could be coupled with a malate/OAA shuttle as proposed by Woo and Osmond (1982) which may operate to supply reducing equivalents to peroxisomal hydroxypyruvate reductase. Indeed, the stoichiometry of the photorespiratory cycle is such that one 2-OG molecule would be available for uptake for every malate (NADH) molecule required for hydroxypyruvate

reduction, thereby making the operation of such a coupled system *in vivo*, theoretically feasible. Woo (1983), however, could find no evidence of an increase in (NH_3, 2-OG)-dependent O_2 evolution by isolated spinach chloroplasts upon the addition of OAA. This may have been due to the design of the experiment, as OAA was added directly to the chloroplast system and thus may have 'drained' reducing power away from the ferredoxin-linked GOGAT reaction during the rapid reduction of OAA to malate via chloroplastic NADP-MDH. Consequently, despite an increase in 2-OG uptake, through exchange with malate, GOGAT activity may not have had access to sufficient reducing power to utilize the increase in 2-OG availability. A more suitable system to test this hypothesis may be to use an external OAA generating system (using external MDH) with which the rate of OAA availability can be controlled (Anderson and House, 1979). Under these conditions, it may be possible to demonstrate the operation of a coupled system, involving malate export for hydroxypyruvate reduction and facilitated 2-OG uptake for photorespiratory NH_3 re-assimilation.

It has been proposed that glutamate may not be the sole amino donor in glyoxylate amination (section 1.2.2) and that alanine may also play an important role in this reaction *in vivo*. If this is the case, it may provide an alternative means of transporting NH_3 from the site of refixation (chloroplast) to the site of amination (peroxisome), without being subject to the apparent limitations associated with 2-OG transport across the chloroplast membrane. In such a system, the glutamate formed in the GOGAT reaction (see Fig. 1.4) would be de-aminated in a reaction with

pyruvate to form 2-OG and alanine. This reaction could be catalysed by chloroplastic glutamate - pyruvate aminotransferase (Kirk and Leech, 1972). The alanine could then be exported to the peroxisome where it could be utilised for glyoxylate amination by GGAT and the pyruvate so formed, returned to the chloroplast to act as the amino acceptor in the reaction with glutamate. The obvious advantage of such a system would be that glutamate and 2-OG are recycled within the chloroplast and thus their transport across the chloroplast membrane (in the absence of malate) would not limit the rates of photorespiratory NH_3 refixation. The operation of such a system must be considered somewhat doubtful, however, based on the following observations: (a) that pyruvate uptake into isolated pea chloroplasts appears to be via diffusion only and occurs at rates of approximately 6.5 μmol mg^{-1} Chl h^{-1} (Proudlove and Thurman, 1981) and (b) a recent report suggesting that glutamate - pyruvate aminotransferase may not, in fact, be associated with the chloroplast fraction (Biekmann and Feieraben, 1983; quoted in Wallsgrove *et al*. 1983).

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