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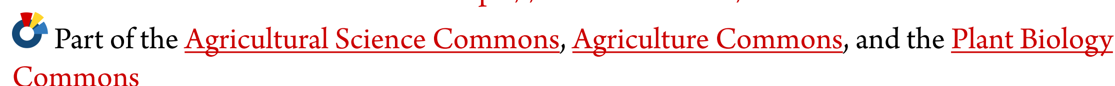

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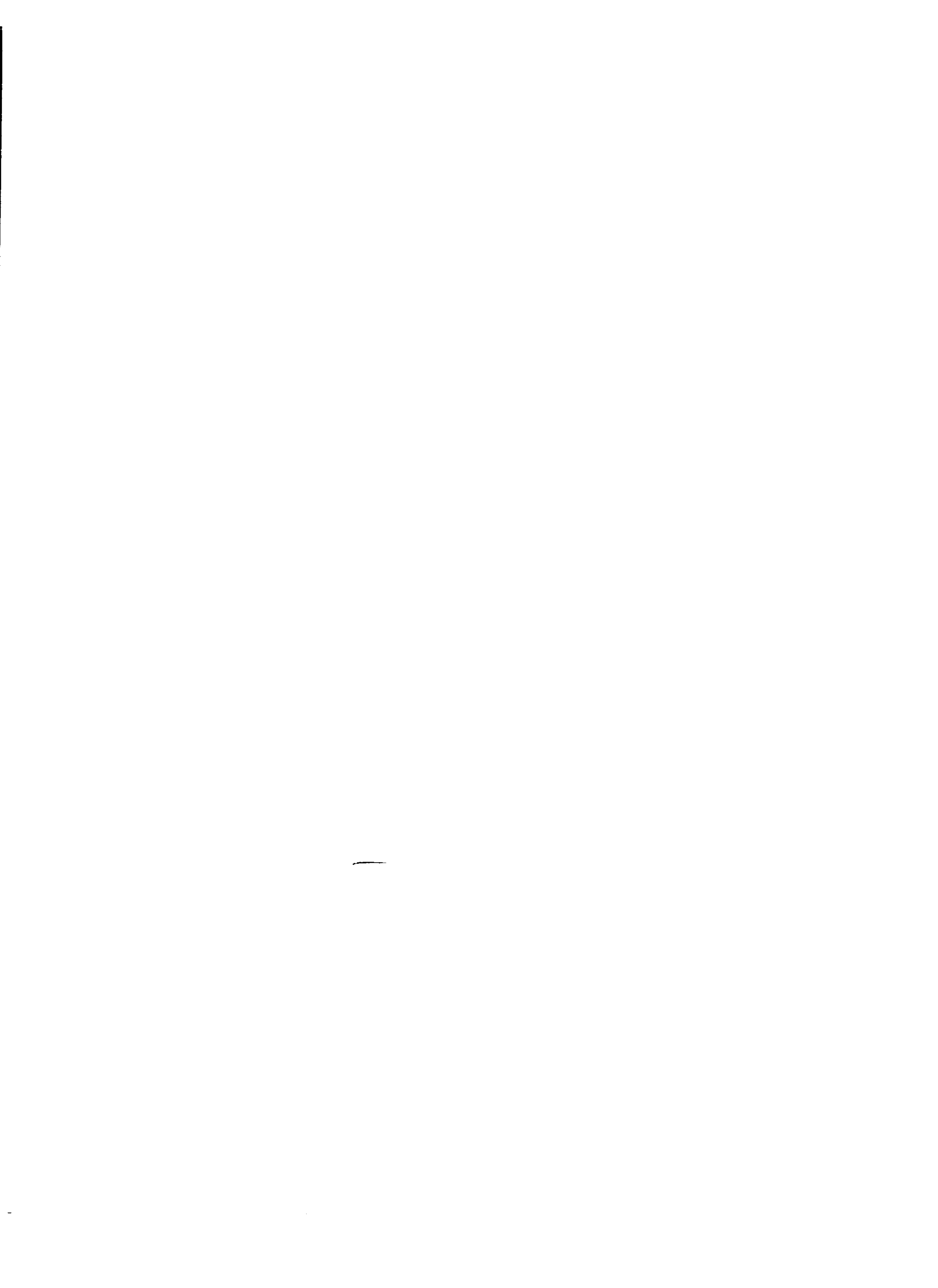

Characterization of the alternative respiratory pathway in soybean

by

David Michael Obenland

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY

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Major: Plant Physiology

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1989

TABLE OF CONTENTS

Page

GENERAL INTRODUCTION.. 1

Explanation of Dissertation Format.................... 2

LITERATURE REVIEW... 4

Introduction.. 4

Structure of the Alternative Pathway.................. 5

Energy Conservation................................... 10

Measurement of Activity and Capacity.................. 11

Control of Activity and Capacity...................... 16

Potential Physiological Roles......................... 20

Summary... 25

PAPER 1. OCCURRENCE OF ALTERNATIVE RESPIRATORY

CAPACITY IN SOYBEAN AND PEA........................... 27

Abstract.. 27

Introduction.. 27

Materials and Methods................................. 29

Results and Discussion................................ 33

Literature Cited...................................... 41

PAPER 2. EFFECT OF RESPIRATORY INHIBITORS ON CO_2

EVOLUTION IN SOYBEAN MEASURED IN THE DARK............. 43

Abstract.. 43

Introduction.. 44

Materials and Methods................................. 46

Results... 47

Discussion.. 50

Conclusion.. 57

Literature Cited.. 57

PAPER 3. RELATIONSHIP OF ALTERNATIVE RESPIRATORY CAPACITY AND ALTERNATIVE OXIDASE AMOUNT DURING GERMINATION AND SEEDLING DEVELOPMENT OF SOYBEAN....... 59

Abstract.. 59

Introduction.. 60

Materials and Methods.. 63

Results and Discussion.. 67

Acknowledgements.. 75

Literature Cited.. 76

SUMMARY.. 78

ADDITIONAL LITERATURE CITED.. 80

APPENDIX.. 87

GENERAL INTRODUCTION

It is important that cyanide-resistant respiration in higher plants be better understood. Lambers (1985) has estimated that 30 to 60% of the photosynthate produced in a day is respired. Some research (Wilson, 1975; Wilson, 1982) indicates that genotypes with high rates of respiration have slower growth rates than genotypes with lower rates of respiration. This suggests that there is a wasteful component of respiration that is more prevalent in genotypes with high respiration rates. As will be discussed in more detail later in this review, one of the most important characteristics of the cyanide-resistant (alternative) respiratory pathway is that it is non-energy-conserving. In other words, electrons that flow through the alternative pathway may be simply wasted. The alternative pathway, then, could represent at least a part of the wasteful component of total respiration just mentioned. Better understanding of the alternative pathway is, therefore, important to the goal of increasing the productivity of crop plants. This may be especially true in soybean (Glycine max [L.] Merr.), an important crop plant that appears to have an unusually large capacity for the alternative pathway in its tissues.

Study of the alternative pathway is also important because its role in plant metabolism is, in almost all

cases, unknown. The widespread distribution of the alternative pathway in the plant kingdom may indicate that the alternative pathway is more than a potentially wasteful process. Instead, the common occurrence of the alternative pathway in plant tissues may indicate that it plays an important, but just not understood, role in plant metabolism.

The objective of this study was to characterize the alternative pathway in soybean and to gain a better understanding of the importance of this respiratory pathway to soybean productivity.

Explanation of Dissertation Format

In the course of my dissertation, I conducted research on three very different topics concerning the characteristics of the alternative pathway in soybean. Because of the diversity of research topics, I have chosen to present my results in the form of three separate papers.

Paper 1 deals with determining if the alternative pathway is present in certain soybean and pea genotypes. All of the mitochondrial work for this paper was conducted by Carrie Hiser of Michigan State University. With the assistance of her advisor, Dr. Lee McIntosh, she prepared both Table 4 and the portion of the "Materials and Methods" titled "Sources of pea mitochondria". The whole tissue

measurements, analysis of the results, and writing the paper were done by me. Assistance and advice in writing the paper were provided by Drs. Richard Shibles and Cecil Stewart. The paper has been published in Plant Physiology (Obenland et al., 1988). All of the parties mentioned as being involved with the paper were listed as co-authors.

Paper 2 reports the results of an investigation into the causes of KCN stimulation of O_2 uptake in soybean leaf tissue. I did all of the experimentation, data analysis, and writing of the paper.

Paper 3 presents results from an experiment that investigated the relationship between alternative oxidase amount and alternative pathway capacity in soybean. Dr. Regina Diethelm, a co-author of the paper, performed the mitochondrial measurements used to construct Table 3. All of the remaining experimental work and the writing of the paper were done by me. I was assisted in data interpretation and writing of the paper by Drs. Cecil Stewart and Richard Shibles, both of whom were co-authors.

LITERATURE REVIEW

Introduction

Cyanide is a potent inhibitor of the mitochondrial, cytochrome electron transport chain. Addition of cyanide to animal tissues generally results in a reduction of the respiratory rate to 1% or less of normal (Douce, 1985). In 1929, Genevois observed that respiration of aged seedlings of sweet pea (Lathyrus odorata) was resistant to cyanide. This observation, as well as research by Van Herk and Badenhuisen (1934) and Van Herk (1937 a,b,c) on the cyanide resistance of Sauromatum guttatum spadix tissue, pioneered the study of cyanide resistance in plant tissues. Later work established that cyanide-resistance was mitochondrial in nature (James and Eliot, 1955) and due to a respiratory pathway separate from the cytochrome pathway (Yokum and Hackett, 1957). The discovery by Schonbaum et al. (1971) of specific inhibitors of the cyanide-resistant pathway, completed the laying of the foundation for further study of the cyanide resistant, or alternative, pathway as it is also known today.

Cyanide-resistance is very widespread in the plant kingdom, and very few tissues are known to be totally sensitive to cyanide (see Henry and Nyns, 1975 for an extensive review). Although not found in animals, cyanide

resistance has also been reported in a number of fungi, yeasts, and microorganisms (Henry and Nyns, 1975).

Structure of the Alternative Pathway

Bendall and Bonner (1971) demonstrated that both the alternative and cytochrome pathways use the same dehydrogenases. They also noted that electrons that flow through the alternative pathway travel to an oxidase separate from the cytochrome oxidase, and in doing so bypass the cytochrome portion of the cytochrome pathway (Figure 1). The insensitivity of the alternative pathway to antimycin, an inhibitor of cytochrome b oxidation, places the branchpoint of the two pathways on the substrate side of cytochrome b (Day et al., 1980). Location of the branchpoint was further clarified by experiments that showed that succinate, a substrate that donates electrons at complex II (Figure 1), could donate electrons to the alternative pathway in the presence of KCN (Passam and Palmer, 1972). All of the previously mentioned results suggest a common pathway for electron flow for both pathways through complexes I and II, with the alternative pathway branching off between complex II and cytochrome b (Figure 1). Experiments have not supported the viewpoint that there may be two parallel pathways (Wilson, 1978).

Storey (1976) noted that under conditions of cytochrome

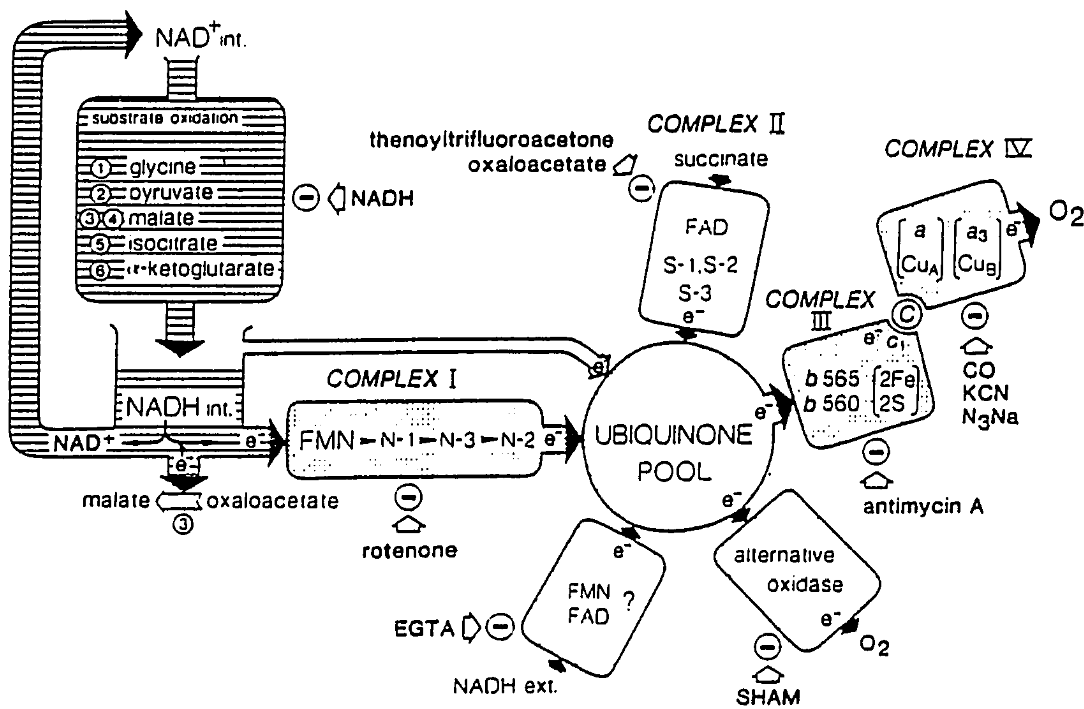

Figure 1. Diagram of the components of the higher plant respiratory chain (Douce, 1985)

pathway inhibition, a pulse of oxygen given to anaerobic mitochondria caused the rapid oxidation of ubiquinone. The rapid oxidation was prevented by salicylhydroxamic acid (SHAM), an inhibitor of the alternative pathway. This experiment gave evidence that ubiquinone, a small hydrophobic molecule, was the branch point between the two pathways. Further experiments, using pentane to deplete the mitochondria of quinones and inhibit both pathways, also supported this view (Lance et al., 1985).

It is commonly observed in higher plant mitochondria that resistance to cyanide varies, depending on the substrate used. Succinate oxidation, for example, is much more cyanide resistant than NADH oxidation (Day et al., 1988). This may indicate that substrates have different accessibilities to the alternative pathway. Lance et al. (1985) has suggested that the reason for this differential accessibility is that there are multiple pools of ubiquinone. These pools may be separated by physical means or by differences in reactivity (Gutman, 1977).

After the alternative pathway branches away from the cytochrome pathway at ubiquinone, it is believed by most researchers that electrons then flow directly to a terminal oxidase. There has been no good experimental support for the presence of intermediates between the branch point and terminal oxidase. Great difficulties have been encountered

in determining the nature of the terminal (alternative) oxidase. One problem is that the alternative oxidase is indistinct in characterization both by electron paramagnetic resonance and by spectrophotometric means (Rich et al., 1977). Also, isolating the alternative oxidase proved troublesome due to its extreme lability (Douce and Neuberger, 1989). A great advance was made when it was discovered that quinols are able to donate electrons at or very close to the alternative oxidase (Huq and Palmer, 1978). Not only did this show that the alternative oxidase was a quinol oxidase, but it also gave researchers a means for assaying directly for alternative oxidase activity. This discovery was to prove vital for later isolation experiments.

A partial purification of the alternative oxidase was achieved by Bonner et al. (1986), and analysis of the preparation indicated that iron seemed to be an important component of the alternative oxidase. By using a different solubilization and purification protocol, Elthon and McIntosh (1987) were able to achieve a 166-fold purification of the alternative oxidase from Sauromatum guttatum. Three main proteins at 35, 36 and 37 kD co-purified with quinol oxidase activity. Immunoblots, made using antibodies to these proteins, indicated that the amount of 35 and 36 kD proteins increased concomitantly with increasing alternative

pathway capacity. This correspondence between amount of protein and alternative pathway capacity was strong evidence that supported the involvement of these proteins with the determination of alternative pathway capacity. Similar proteins appear to be present in other species (Elthon et al., 1989; Paper 3 in this dissertation).

With the exception of a few, such as Rustin et al. (1983), who ascribe to alternate theories, there is little doubt remaining among researchers that the alternative oxidase is a protein. Now that the alternative oxidase has been isolated, work can proceed on further characterization of its structure and control. Progress has been recently reported in cloning the gene(s) that code for the alternative oxidase (Rhoads et al., 1989).

As with the cytochrome pathway, the location of the alternative pathway is the inner mitochondrial membrane. It was found that in aged potato mitochondria, removal of the outer mitochondrial membrane did not affect the alternative pathway (Lance et al., 1985). There has, however, been debate about which side of the inner membrane the alternative oxidase is on. Schonbaum et al. (1971) noted that while hydroxamic acids inhibit alternative pathway function, oxidative phosphorylation, which is known to occur on the inside of the inner membrane, is not affected. This they took to indicate that the alternative pathway is more

accessible to inhibition than is phosphorylation, and, therefore, the oxidase must be on the outside of the membrane. In support of this, Dizengremel et al. (1983) discovered that Triton X-100 can be added at a concentration that affects the alternative pathway, but not the cytochrome pathway. They proposed that the alternative oxidase was on the outside of the membrane, and more accessible to the Triton X-100. On the other hand, Moore et al. (1976) stated that, due to evidence supporting a close linkage between the alternative pathway and succinate dehydrogenase (on the inner side), the alternative oxidase is likely to be on the inner side of the inner membrane.

Energy Conservation

A very important characteristic of the alternative pathway is that passage of electrons through the pathway results in no energy conservation. This is supported by the observation that ADP/O ratios are lower when measured in tissues that are cyanide-resistant, as compared to tissues that are more cyanide-sensitive (Lance et al., 1985). Passam (1974) demonstrated that addition of a cytochrome respiratory inhibitor acted to decrease ADP/O ratios, whereas the addition of an alternative pathway inhibitor increased the ADP/O ratio. Moore et al. (1978) confirmed that the alternative pathway conserves no energy by

demonstrating that no membrane potential generation occurs due to cyanide-insensitive respiration.

Measurement of Activity and Capacity

Assays of alternative pathway activity and capacity (potential size) are generally made using an O_2 electrode to monitor the O_2 uptake rate. Differences in response of O_2 uptake to inhibitors of both the cytochrome and alternative pathways are the basis of the assays.

When cyanide (usually KCN) is added to the reaction mixture, there is a blockage in the cytochrome pathway, and assuming that the alternative pathway is not already saturated with electrons, this blockage causes a diversion of electrons from the cytochrome to the alternative pathway. Generally, in the presence of cyanide, the alternative pathway will fill to capacity with electrons. Sometimes, however, a respiratory uncoupler will be added to ensure that there is no adenylate restriction to the electron flow. The O_2 uptake in the presence of KCN, however, cannot be taken as the capacity of the alternative pathway. This is due to the fact that, even after addition of an inhibitor of the alternative pathway, there will remain a residual O_2 uptake. Although part of the residual rate may be due to incomplete inhibition by the inhibitors used, not all of the residual rate can be so accounted for (Lance et al., 1985).

other oxidases, such as peroxidase and lipoxygenase, are thought to be responsible for much of the residual O_2 uptake. The residual rate can be substantial in whole tissues, but is much less significant in isolated mitochondria.

In order to determine the capacity of the alternative pathway one must first determine the O_2 uptake in the presence of KCN and then subtract from this the O_2 uptake in the presence of KCN plus an inhibitor of the alternative pathway (i.e., the residual rate). In order to obtain the activity (actual flow through the pathway at the time of measurement), what is generally done is to add an inhibitor of the alternative pathway, such as SHAM. Subtraction of the rate in the presence of inhibitor from the rate in the absence of inhibitor gives an estimate of the activity. However, acceptance of this estimate as real requires the assumption that the inhibitor will not cause a diversion of electrons from the alternative to the cytochrome pathway. Although this assumption has been proved correct in some cases (Theologis and Laties, 1978), it may not hold in all situations (Wilson, 1988). Upon obtaining both the activity and capacity by means discussed above, the ratio of activity divided by capacity is often calculated. This ratio gives a measure of the degree of engagement of the alternative pathway.

More elaborate means, involving inhibitor titrations, have also been devised to estimate alternative pathway parameters (Bahr and Bonner, 1973a,b). These techniques, although more time consuming, offer the possible advantage of being able to detect non-specific inhibitor effects.

A major breakthrough in the study of the alternative pathway was the discovery by Schonbaum et al. (1971) that hydroxamic acid derivatives were able to inhibit the alternative pathway without inhibiting the cytochrome pathway. These are now the inhibitors of choice of most researchers. Salicylhydroxamic acid (SHAM) is the most often used, mainly due to availability. The means by which hydroxamic acids act on the alternative pathway is unknown, although Rich et al. (1978) have suggested that the inhibitory effects may be due to competition with the reducing substrate (ubiquinone).

One of the great difficulties in assaying the alternative pathway has been the lack of assurance that SHAM affects only the alternative pathway. While cyanide can also influence other metabolic processes in the plant (Hackett, 1960), for the most part respiratory inhibition by cyanide is almost totally due to inhibition of the cytochrome pathway (Lambers, 1985). SHAM, however, is known to be far less specific in its effects (Lambers, 1985). Other hydroxamic acids present a similar problem, but

because of the more prevalent use of SHAM, the following discussion will be restricted to effects of SHAM.

An example of inhibitor non-specificity is the inhibition of lipoxygenase by SHAM. Lipoxygenase, an enzyme that catalyzes the addition of molecular O_2 across a double bond in polyunsaturated fatty acids, is also insensitive to KCN. This gives a potential for mistaking lipoxygenase activity for both alternative pathway activity and capacity. Goldstein et al. (1980) claimed that by further purifying mitochondria by use of Percoll density gradient centrifugation, both lipoxygenase and the alternative pathway could be eliminated. They showed that what earlier had been taken to be the alternative pathway may have been a non-specific inhibitor interaction. This idea has since been refuted by experiments using disulfiram, an inhibitor that affects the alternative pathway but not lipoxygenase (Miller and Obendorf, 1981). Also, it has been shown that, in all but the one case mentioned (Goldstein et al., 1980), purification of mitochondria to remove lipoxygenase does not significantly reduce the amount of alternative pathway. Unfortunately, since disulfiram cannot penetrate into whole tissues, it is not possible to assess by this means how much (if any) overestimation is occurring in whole tissue alternative pathway measurements due to lipoxygenase. Lipoxygenase, however, is not thought to contribute

significantly to whole tissue respiration under most circumstances.

SHAM can also act to stimulate O_2 uptake. In potato (Solanum tuberosum L.) tuber callus (van der Plas et al., 1987) and in the intact roots of several species (Spreen-Brouwer et al., 1986), the stimulation is a result of a SHAM-stimulated peroxidase. Stimulation by SHAM of O_2 uptake in plasmalemma vesicles of wheat (Triticum aestivum L.) is thought to be due to a plasmalemma-bound redox chain (Moller and Bérczi, 1986). Also, SHAM is an inhibitor of photosynthesis, and even in room light its use can result in an apparent stimulation of O_2 uptake (Diethelm et al., in press), which actually is due to decreased O_2 evolution by photosynthesis.

As can be seen, SHAM affects many other cellular processes that determine net O_2 uptake. One must be extremely cautious in doing experiments and analyzing data when using SHAM, especially when working with whole tissue. In a recent review, Moller et al. (1988) gave possible guidelines in overcoming whole-tissue measurement problems.

There has been a finding recently that may offer researchers a new, non-invasive way to measure alternative pathway activity. Guy et al. (1989) reported that descrimination against ^{18}O is different between the alternative and cytochrome pathways. Use of this technique

could give an estimate of activity without the use of inhibitors.

Control of Activity and Capacity

Experiments done by Bahr and Bonner (1973a) indicated that the amount of electron flux through the alternative pathway was determined by the activity of the cytochrome pathway. The capacity of the cytochrome pathway was always fully utilized whenever the alternative pathway was engaged. Many researchers believe that the alternative pathway is engaged upon the overflow of electrons from the cytochrome pathway into the alternative pathway (Lambers, 1982).

The control point of electron flux is thought to be at the branch point between the pathways, where the electrons are partitioned according to the rate constant of the reaction between ubiquinone and the alternative oxidase, and between ubiquinone and complex III (Douce and Neuberger, 1989). In some cases, it has been found that the alternative pathway can be engaged without electron saturation of the cytochrome pathway (Day et al., 1988). Experiments that have used a quinone-sensitive electrode to measure the redox state of the quinone pool have found that alternative pathway engagement is dependent more on redox state of the quinone pool than on saturation of the cytochrome pathway with electrons (Douce and Neuberger,

1989). It appears that, although amount of electron flow through the cytochrome pathway is an important determinant of alternative pathway activity, shunting of electrons into the alternative pathway is more complex than just a simple spilling over (overflow) of electrons from the cytochrome pathway.

There is a strong relationship between alternative pathway activity and level of substrates _in vivo_. Steingröver (1981) noted that, when storage in the tap root of _Daucus carota_ began, carbohydrates available for respiration decreased, and the contribution of the alternative pathway to total respiration decreased correspondingly. Lambers et al. (1981) observed a decrease in alternative pathway activity when roots of _Plantago coronopus_ were transferred to a saline solution. The saline was found to have induced the synthesis of sorbitol, thus reducing the level of respirable carbohydrate. Other examples supporting the relationship between increased substrate levels and increased alternative pathway engagement _in vivo_ are given in a subsequent section of this literature review. In short, substrate levels appear to be a limiting factor in some cases to alternative pathway engagement.

In assays with isolated mitochondria, saturating levels of substrate are used. However, the type of substrate used

during these assays has been found very important to quantity of alternative pathway activity. Generally, malate oxidation provides the most cyanide resistance, followed by succinate, and then by NADH (Lance et al., 1985). Sometimes succinate can exceed malate in the degree of cyanide resistance (Day et al., 1988). One hypothesis to explain why different substrates provide differing levels of cyanide-resistance is that the substrates have differing amounts of accessibility to the alternative oxidase due to the existence of multiple ubiquinone pools (Lance et al., 1985). An alternate explanation may involve the differential activities of the individual dehydrogenases of each substrate.

Under high substrate levels, adenylates may control the activity of the alternative pathway (Saglio and Pradet, 1980). Extensive flux through the cytochrome pathway, due to high substrate levels, can potentially deplete ADP levels in the cell, leading to an inhibition of electron flow through the cytochrome pathway. Constriction of the cytochrome pathway can lead to increased activity of the alternative pathway, as electrons that cannot travel through the cytochrome pathway are "forced" through the non-phosphorylating, and thus not adenylate restricted, alternative pathway (Lambers, 1985). Adenylates may also influence the alternative pathway indirectly via alterations

in glycolytic rates (Saglio and Pradet, 1980).

One problem with the hypothesis that the alternative pathway functions under adenylate restriction of the cytochrome pathway is that the alternative pathway should still be under adenylate control at Complex I. It has been suggested that electrons can override this control by using the non-phosphorylating, rotenone-insensitive bypass of Complex I (see Figure 1). This may be correct, especially since it has been observed that both the rotenone insensitive bypass and the alternative pathway develop simultaneously in aging potato slices (Lambers, 1985).

The factors that control the development of alternative pathway capacity (potential size) have not been identified. This problem is the topic of Paper 3 of this dissertation. About all that is known for certain is that cytoplasmic protein synthesis appears to be necessary for development of capacity (Morohashi and Matsushima, 1983; Burguillo and Nicolás, 1977). Generally, young tissue has less capacity than does older tissue (Azcon-Bieto et al., 1983; Brown and Possingham, 1957; Sesay et al., 1986). Exposure to cold temperatures (Rychter et al., 1988), aging of potato slices (Dizengremel and Lance, 1976), and the initiation of the respiratory climax in *Arum* spadix (Meeuse, 1975) are all situations that lead to increased alternative pathway capacity.

One factor that appears to be universally associated with development of alternative pathway capacity is that pathway development occurs either immediately after or at the same time as an increase in the total respiration rate. A related observation is that some research has shown there to be a high degree of correlation between total respiration rate of a genotype and its corresponding alternative pathway capacity (Tom Christensen, Iowa State University, personal communication). Positive relationships between rate of respiration and alternative pathway capacity may simply indicate that the alternative pathway does indeed have an important role in plant metabolism, and when total respiration is increased (or is genotypically greater), then alternative pathway capacity must increase (or be greater) to perform this role (Dr. Cecil Stewart, Iowa State University, personal communication).

Potential Physiological Roles

Thermogenesis

The best understood role of the alternative pathway is that of enabling the production of heat in thermogenic tissues (Meeuse, 1975). These tissues have exceptionally large alternative pathways and, due to the non-phosphorylating nature of the alternative pathway, substantial amounts of heat can be generated as the tissue

respires. In <u>Arum maculatum</u> tissue, an increase of 15° C over ambient air temperature can occur (Meeuse, 1975). In the case of the family Araceae, the function of the temperature increase seems to be to volatilize insect attractants in the inflorescence during the time of pollination.

The induction of thermogenesis in <u>Sauromatum</u> is triggered by the release of salicylic acid from the male flower primordia (Raskin et al., 1987). Salicylic acid is able to induce increased production of alternative oxidase protein, leading to enhanced alternative pathway capacity (Elthon and McIntosh, 1987).

<u>Cold tolerance</u>

A number of researchers have investigated the possibility that alternative pathway function in some non-thermogenic tissues is involved in cold temperature tolerance (McNulty and Cummins, 1987; Rychter et al., 1988; Stewart et al., submitted; Van de Venter, 1985). In general, these studies have found that low temperatures act to enhance alternative pathway capacity. The mechanism by which the enhanced capacity of the alternative pathway would confer cold tolerance is unknown. Although heat is generated by the alternative pathway, the amount is very small (McNulty and Cummins, 1987) and is likely to play little or no role in protecting tissues from the cold.

In support of a positive relationship between cold tolerance and alternative pathway capacity, McCaig and Hill (1977) found that winter wheat had greater capacity for the alternative pathway than did spring wheat. Also, cold tolerant corn inbreds grown at cold temperatures had higher alternative pathway capacities than did non-tolerant inbreds (Van de Venter, 1985). However, Stewart et al. (in press) found no consistent relationship between amount of alternative pathway and sensitivity of corn inbreds to growth at cold temperatures.

Energy overflow

Lambers (1982) has proposed that under conditions of excess carbohydrate supply, the alternative pathway may function as an energy overflow acting to rid the cell of excess carbohydrate. This idea has been supported by a number of different studies that have examined alternative pathway activity in tissues differing in cellular carbohydrate levels. For example, comparisons made between young roots of *Hypochaeris radicata* that were unable to store carbohydrates and older, carbohydrate-storing roots, indicated a greater alternative pathway activity in the younger roots (Lambers, 1980). Similarly, roots of *Pisum sativum* grown on NH_4^+ (and needing less energy to utilize the N) had greater amounts of alternative pathway activity than those roots grown on NO_3^- or in *Rhizobium* innoculated

soil (Lambers, 1980). Additional experiments using time of day (Azcon-Bieto et al., 1983) and different light levels (Lambers, 1980) to manipulate carbohydrate levels, have also indicated that greater carbohydrate availability corresponds with increased alternative pathway activity. Although a number of experiments support the idea of the alternative pathway acting as a means to dispose of excess carbohydrate, too little is known about how the activity of the alternative pathway is controlled to draw any conclusions about the validity of this hypothesis.

Maintenance of TCA cycle function

Mitochondria function as more than just the means to convert donated electrons from various substrates to ATP. Mitochondria also function in a biosynthetic mode, in the provision of organic acids from the tricarboxylic acid (TCA) cycle to serve as intermediates for other cellular reactions. Under conditions of high cellular energy charge (high ATP/ADP ratio) and restricted mitochondrial electron transport, the ability of the mitochondria to supply these intermediates could be diminished. A number of researchers (Bahr and Bonner, 1973a; Palmer, 1976) have proposed that a possible role of the alternative pathway is to maintain electron flow and TCA cycle function under these conditions. They base this proposal on the fact that the alternative pathway is non-phosphorylating and electrons could flow

unimpeded through it under conditions of high energy charge.

Cyanide tolerance

Cyanide can occur naturally in plant tissues in the form of cyanogenic glycosides. Under the proper conditions cyanide can be released from these compounds, conceivably then being inhibitory to cytochrome electron transport. One way for the plant to cope with the presence of cyanide in its tissues would be to have an alternate route for electron flow that is insensitive to cyanide. Since the alternative pathway is cyanide insensitive, it could potentially be suited for such a role.

The idea of the alternative pathway being a means for the plant to tolerate cyanide was supported in a study by Passam (1976) in which the respiratory characteristics of *Manihot esculenta* tubers, a tissue containing cyanogenic glycosides, was investigated. In this study it was found that mitochondria isolated from tubers containing greater amounts of cyanide had greater capacities for the alternative pathway. However, although tolerance of cyanide may be one role of the alternative pathway in some tissues, it should be noted that those plant tissues with the largest alternative pathway capacities have no cyanogenic glycosides (Goodwin and Mercer, 1985).

Conclusion

With the exception of its role in generating heat in thermogenic tissues, the purpose of the alternative pathway in plant tissues remains obscure. It has not been possible to substantiate fully other theories regarding the role of the alternative pathway. Perhaps, in the evolutionary history of plants the alternative pathway played an important role, but the reason for this role has since been lost. In other words, the alternative pathway may now be, in most cases, only a useless relic of the past. However, the alternative pathway is so prevalent in plant tissues that it does seem very likely that it plays a significant, but just not yet understood, role in plant metabolism.

Summary

The alternative respiratory pathway is a non-energy-conserving branch off of the main cytochrome pathway in the inner mitochondrial membrane. The terminal (alternative) oxidase is the only component of the pathway that has been identified. The presence of the alternative pathway is widespread in the plant kingdom. Partitioning of electrons between the two pathways is a little understood process that seems to involve differences in rate constants between components at the branch point of the two pathways. The

factors that control capacity (potential size) of the alternative pathway are unknown. With the exception of its role in thermogenesis, the physiological role of the alternative pathway is unknown.

PAPER 1. OCCURRENCE OF ALTERNATIVE RESPIRATORY CAPACITY IN SOYBEAN AND PEA

Abstract

Capacity for the alternative respiratory pathway was assessed in leaf and root tissue of male-sterile and fertile soybean (Glycine max [L.] Merr.) plants and in leaf, embryonic axis, and epicotyl tissue as well as isolated mitochondria of pea (Pisum sativum L.) by measurement of oxygen uptake in the presence and absence of KCN and salicylhydroxamic acid. Male-sterile and fertile soybean tissues showed similar responses to the inhibitors, and both possessed a capacity for alternative respiration. We also found that tissue and isolated mitochondria from 'Progress No. 9' pea possessed alternative respiratory capacity similar to that of 'Alaska' pea.

Introduction

Widespread existence of the alternative respiratory pathway has been well established in a multitude of plant types and tissues (Henry and Nyns, 1975). The question of significance of the alternative pathway to overall plant metabolism, however, remains unanswered. One way to address such a question would be to compare selected physiological parameters of genotypes that differ in alternative pathway

activity. To do this, it is necessary to have plants that exhibit genotypic variability for the alternative pathway. Few studies have identified such variability.

McCaig and Hill (1977), using isolated mitochondria from etiolated coleoptiles, found differences among four cultivars of wheat (Triticum aestivum L.) for alternative pathway capacity that ranged from 8 to 35% of state 3 respiration rate. Day et al. (1985) compared alternative pathway activity and capacity in both leaf and root tissue of two populations of Lolium perenne (L.) and found the capacity between the two populations to differ only in roots. Leaf tissue of the dwarf pea (Pisum sativum L.) cultivar 'Progress No. 9' was reported to lack the alternative pathway, whereas in 'Alaska' pea the pathway was present (Musgrave and Siedow, 1985). In a later paper (Musgrave et al., 1986b), other dwarf pea cultivars were identified as lacking the alternative pathway. Musgrave et al. (1986a) also compared cyanide-insensitive respiration of seven male-sterile lines from four species, one of which was soybean, with that of corresponding fertile lines and reported that, in all but one case, the fertile lines exhibited cyanide-insensitive, SHAM-sensitive O_2 uptake, but the sterile lines did not.

Initially, it was our goal in this study to determine whether there were important differences among soybean

genotypes for the alternative pathway. We surveyed leaf tissue of a number of diverse genetic lines representing both *Glycine max* and *Glycine gracilis* for alternative pathway capacity. Differences in capacity and response to SHAM were found, but they were not great (Appendix Tables A1 and A2). Because of the availability of plant material and the previously mentioned results of Musgrave et al. (1986a), we decided to examine tissues of eight lines from four different soybean genetic male-sterile/fertile groups. Finding no differences between sterile and fertile tissues within any of the four groups, we were led to reevaluate the alternative pathway capacity of Alaska and Progress No. 9 peas. Results from work with both the soybean male-sterile/fertile groups and the pea cultivars are presented.

Materials and Methods

Whole tissue plant material

Soybean (*Glycine max* [L.] Merr.) leaf tissue was obtained from field-grown plants provided by Drs. Reid G. Palmer and Halina Skorupska, USDA-ARS/Iowa State University. At flowering, pollen was examined from rows of plants segregating for sterility/fertility in order to classify the plants by type. Sterility was conditioned by genetic constitution at one of four different loci: *ms1 ms1* (strain T266H), *ms2 ms2* (strain T259H), *ms3 ms3* (strain T273H), *ms4*

ms4 (strain T274H); fertiles either were homozygous dominant or heterozygous. Two-week-old leaves were harvested and taken to the laboratory with petioles immersed in water. The leaves were washed in distilled water containing a small amount of Tween 80. Twenty 0.3-cm^2 discs from leaves of two or more plants of each type were used for oxygen uptake measurements.

Soybean roots were taken from 11- to 13-week-old, greenhouse-grown plants. Seeds of the aforementioned genotypes were sown in 25-cm plastic pots filled with sand. Sterility was determined by pollen examination or, for later stages, noting the presence or absence of pods. After thorough cleaning, lateral roots were removed and cut into 5-mm segments. Approximately 300 mg (fresh weight) was used for each measurement.

Pea (Pisum sativum L.) seeds were obtained from W. Atlee Burpee Company, Warminster, PA, for 'Alaska' and from Mary Musgrave, Duke University, Durham, NC, for 'Progress No. 9'. Plants were grown in a greenhouse in 25-cm plastic pots containing a 1:1:1 mixture of soil, peat and perlite. Recently expanded leaves were taken from 2- to 3-week-old plants and washed in 1 % (v/v) ethanol. For each respiratory measurement, 15 0.3-cm^2 leaf discs were cut from several leaves.

Pea embryonic axes were obtained by soaking seeds in

aerated, distilled water for 2 h, then placing the seeds on moistened filter paper inside petri plates for an additional 18 h. The axis tissue was then carefully cut from the rest of the seed. Six axes were used per measurement.

Pea epicotyl tissue was obtained from seeds germinated on moistened paper towels for 1 week in darkness. Approximately 200 mg (fresh weight) of tissue from several seeds, which was cut into 5-mm segments, was used for each measurement.

Whole tissue measurements

Oxygen uptake measurements were performed at 25° C by use of a Clark-type electrode (Yellow Springs Instruments model 5300) on tissue immersed either in a potassium phosphate buffer (0.1 M, pH 6.3) for pea tissue measurements or distilled water for soybean measurements. The volume used was 5 mL, with the exception of soybean roots, which were measured in 7 mL.

Tissue was allowed to equilibrate 10 to 15 min in the cuvette, after which a control O_2 uptake rate was determined. KCN was then added, and a measurement taken when the inhibitor effect had reached maximum. Then SHAM was added, allowed to act, and a SHAM + KCN rate determined. This method of sequential addition of inhibitors was followed for all tissues except pea epicotyl, for which a separate cuvette was used for each inhibitor and the

control. Media were reoxygenated before each measurement on all tissues except for pea leaf and embryonic axis. For the latter, the short measurement time made reoxygenation unnecessary. Distilled water was used as solvent for KCN, and either DMSO or 2-methoxyethanol for SHAM.

Sources of pea mitochondria

Pea seeds of cvs Alaska and Progress No. 9 were obtained from the W. Atlee Burpee Company, Warminster, PA, for isolation of mitochondria by method A and from James N. Siedow, Duke University, Durham, NC, for isolation of mitochondria by method B. Seeds were soaked overnight in distilled water, planted in vermiculite, watered once with Hoagland solution, and grown in a dark cabinet at room temperature for 7 to 10 d before harvesting.

Isolation of pea mitochondria (method A) Etiolated pea seedlings were cut into small pieces and ground in a blender with a twofold volume of isolation buffer (400 mM sorbitol, 30 mM MOPS [pH 7.6], 1 mM EDTA, 4 mM cysteine, 0.2% BSA, 0.6% PVPP). After filtration through four layers of sterile Miracloth, the homogenate was centrifuged at 1,400*g* for 15 min. The resulting supernatant was centrifuged at 16,300*g* for 15 min to pellet the mitochondria. The pellets were resuspended in wash buffer (350 mM sucrose, 30 mM MOPS [pH 7.2], 1 mM EDTA, 0.2% BSA) and the suspension was centrifuged at 4,300*g* for 2 min. The

supernatant was centrifuged at 12,000g for 5 min to pellet the mitochondria, which were resuspended in assay buffer (250 mM sucrose, 30 mM TES [pH 7.2]). Oxygen uptake by the washed mitochondria was measured in a Rank Brothers electrode apparatus (digital oxygen system model 10) at 25° C in a total volume of 1.0 mL of assay buffer plus sample. Protein concentrations were determined by a modified Lowry method (Larson et al., 1986).

Isolation of pea mitochondria (method B) Washed mitochondria were obtained following the procedures of Musgrave et al. (1986b), except for slight variations in the isolation buffer (400 mM mannitol, 20 mM Hepes [pH 7.2], 1 mM EDTA, 0.5 mM $Na_2S_2O_3$, 0.5% BSA, 0.1% PVPP) and the wash buffer (400 mM mannitol, 20 mM Hepes [pH 7.2], 0.1 mM EDTA, 0.1% BSA). The mitochondria were further purified by sucrose density gradient centrifugation (Douce et al., 1972). Oxygen uptake by the purified mitochondria was measured as described in method A in the assay buffer (Musgrave et al., 1986b) with the addition of 1 mg/mL BSA.

Results and Discussion

Tables 1 and 2 present the oxygen uptake data for soybean leaf and root tissues, respectively. As reported previously (Sesay et al., 1986), oxygen uptake by soybean leaf tissue is stimulated by KCN, but is inhibited in the

Table 1. Oxygen uptake rates of control and inhibitor-treated leaf tissue of fertile (F) and sterile (S) soybean genotypes and percentage of control rate. Mean ± SE, n = 4

	MS1[a]		MS2[a]	
	F	S	F	S
	μmol O_2 (g dry weight)$^{-1}$ (min)$^{-1}$			
Control	1.95±0.10	1.97±0.02	1.73±0.06	1.73±0.07
+ 1 mM KCN	2.53±0.12	2.45±0.06	2.28±0.04	2.28±0.06
	129.7%	124.4%	131.8%	131.8%
+ 1 mM KCN & 10 mM SHAM	0.85±0.04	0.83±0.06	0.83±0.06	0.92±0.09
	43.6%	42.1%	48.0%	53.2%

[a]Refers to the different loci conditioning male sterility/fertility.

MS3[a]		MS4[a]	
F	S	F	S
---------------μmol O_2 (g dry weight)$^{-1}$ (min)$^{-1}$------------			
1.97±0.10	1.87±0.24	1.94±0.19	1.38±0.01
2.45±0.12	2.33±0.14	2.19±0.26	1.52±0.08
124.4%	124.6%	112.9%	110.1%
0.94±0.08	0.79±0.07	0.93±0.01	0.70±0.01
47.7%	42.2%	48.0%	50.7%

Table 2. Oxygen uptake rates of control and inhibitor-treated root tissue of fertile (F) and sterile (S) soybean genotypes and percentage of control rate. Mean ± SE, n = 4

	MS1[a]		MS2[a]	
	F	S	F	S
	------μmol O_2 (g dry weight)$^{-1}$ (min)$^{-1}$-------			
Control	1.04±0.07	1.09±0.08	1.06±0.11	1.17±0.05
+ 2 mM KCN	0.72±0.02	0.76±0.06	0.77±0.06	0.72±0.05
	69.2%	69.7%	72.6%	61.5%
+ 2 mM KCN & 1 mM SHAM	0.39±0.01	0.38±0.02	0.35±0.06	0.39±0.03
	37.5%	34.9%	33.0%	33.3%

[a]Refers to the different loci conditioning sterility/fertility.

MS3[a]	
F	S
---------μmol O_2 (g dry weight)$^{-1}$ (min)$^{-1}$-----------------	
1.04±0.07	1.18±0.07
0.64±0.07	0.76±0.05
61.5%	64.4%
0.28±0.03	0.34±0.03
26.9%	28.8%

presence of both KCN and SHAM. Root respiration, on the other hand, is inhibited 30 to 40% by KCN and an additional 35% by the addition of SHAM in the presence of KCN. As indicated by inhibition by SHAM in the presence of KCN, the alternative respiratory pathway was present in every case. In both leaf and root, response to the inhibitors was nearly equal in male steriles and fertiles of each genetic group, indicating a similar capacity for the alternative pathway in each. Musgrave et al. (1986a) reported that root tissue of the soybean <u>ms1</u> <u>ms1</u> sterile lacks the alternative respiratory pathway. The presence of sterility had no influence on alternative pathway capacity in our study.

Among genetic groups, there seem to be differences in alternative respiratory capacity, but this may be a consequence of developmental differences. Because it was necessary for plants to flower before steriles could be distinguished from fertiles, sampling of the different genetic pairs necessarily occurred at different plant ages.

Respiratory rates for leaf, embryonic axis and epicotyl tissues of Progress No. 9 and Alaska peas are presented in Table 3. These results clearly show that both cultivars have a sizable capacity for the alternative pathway in all tissues examined.

Respiratory rates for mitochondria isolated from etiolated shoots of Alaska and Progress No. 9 peas are

Table 3. Respiratory rates of control and inhibitor-treated tissues, percentage of control, and percentage capacity of alternative pathway (Valt) for Progress No. 9 and Alaska peas. Capacity of alternative pathway is the difference between rate in presence of KCN and rate in presence of KCN + SHAM, expressed as a percentage of the total rate. Mean ± SE, n = 4 to 6

		leaf	embryonic axis	epicotyl
		μmol O_2 (g dry weight)$^{-1}$ (min)$^{-1}$		
Progress No. 9	control	2.20±0.05	2.06±0.23	2.86±0.13
	+ 1 mM KCN	1.66±0.04 (75.4%)	1.22±0.15 (59.2%)	1.73±0.12 (60.5%)
	+ 1 mM KCN & 1 mM SHAM	0.60±0.04 (27.3%)	0.57±0.05 (27.7%)	0.86±0.11 (30.1%)
	Valt	48%	31%	30%
Alaska	control	2.90±0.24	2.06±0.32	2.50±0.15
	+ 1 mM KCN	1.59±0.09 (54.8%)	0.97±0.12 (47.1%)	0.94±0.12 (37.6%)
	+ 1 mM KCN & 1 mM SHAM	0.67±0.13 (23.1%)	0.50±0.02 (24.3%)	0.41±0.07 (16.4%)
	Valt	32%	23%	21%

Table 4. Rates of oxygen uptake by isolated mitochondria and percentage capacity of the alternative pathway. Control rate was measured in the presence of added substrate, and the uncoupled rate was measured after the addition of 0.5 μM FCCP. Alternative respiration was calculated as the rate in 1 mM KCN minus the rate in KCN plus 1 mM SHAM

substrate	control	uncoupled	alternative
	natoms consumed $(min)^{-1}$ (mg protein)$^{-1}$		
Part A:[a]			
Alaska			
1 mM NADH	21.36±3.32	59.76±9.88	4.90±3.36 (8%)
Progress No. 9			
1 mM NADH	20.96±5.16	51.14±8.50	4.52±2.60 (9%)
Part B:[b]			
Alaska			
10 mM succinate + 150 μM ADP	92.95±16.32	145.31±20.73	17.20±5.91 (12%)
Progress No. 9			
10 mM succinate + 150 μM ADP	88.24±13.86	143.99±9.18	10.94±2.28 (8%)

[a]Washed mitochondria isolated by Method A (see Materials and Methods). Values are the mean ± SE of five experiments with three replicate runs per experiment. The mitochondrial samples in each run contained 0.5 to 2.0 mg protein.

[b]Purified mitochondria isolated by method B (see Materials and Methods). Values are the mean ± SE of three experiments with three replicate runs per experiment. The mitochondrial samples in each run contained 0.5 to 1.5 mg protein.

compared in Table 4. Comparison of the respiratory rates using either washed (Part A) or purified (Part B) mitochondria indicates that the capacity for the alternative pathway is measurable in both cultivars. This contrasts with the findings of Musgrave et al. (1986b) who found no alternative respiratory capacity in washed mitochondria from epicotyls of Progress No. 9.

From these respiration studies using whole tissues and isolated mitochondria, we conclude that male-sterile soybean plant tissues and the dwarf pea cultivar, Progress No. 9, possess the alternative respiratory pathway.

Note: We have discussed our results with J. N. Siedow (Duke University). He also has recently observed no difference between Progress No. 9 and Alaska in the levels of SHAM-sensitive, cyanide-resistant oxygen uptake during measurements of intact tissue (epicotyl or leaf) respiration (J. N. Siedow, personal communication). His (and our) efforts to clarify the reason(s) for the previous results (Musgrave et al., 1986b; Musgrave and Siedow, 1985) have been unsuccessful.

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PAPER 2. EFFECT OF RESPIRATORY INHIBITORS ON CO_2 EVOLUTION IN SOYBEAN MEASURED IN THE DARK

Abstract

The effects of the respiratory inhibitors, KCN and salicylhydroxamic acid (SHAM), on dark CO_2 evolution in soybean (Glycine max [L.] Merr.) leaves were determined to test the hypothesis that KCN stimulation of O_2 uptake in this tissue is due to decontrol of glycolysis, caused by a diversion of electrons into the alternative pathway. Both KCN and SHAM, when used individually, caused a 30% increase in CO_2 evolution. When both KCN and SHAM were present together, the resulting rate was approximately equal to the control (no inhibitors present). The stimulatory effect of KCN was taken as evidence that KCN acted to increase the glycolytic rate. The prevention of this increase by the inclusion of SHAM, an inhibitor of the alternative pathway, would have been evidence for the involvement of the alternative pathway in the glycolytic increase. However, because both KCN and SHAM stimulated CO_2 evolution, it was not possible to determine if the KCN stimulation was prevented by SHAM. Therefore, the true cause of KCN stimulation of O_2 uptake in this tissue could not be ascertained. The stimulation of CO_2 evolution by SHAM was unexpected and believed to be due to a non-specific

reaction. There was little effect of leaf age on the action of KCN; at all leaf ages KCN stimulated CO_2 evolution by a similar amount.

Introduction

Cyanide is a potent inhibitor of the cytochrome respiratory pathway. Its addition to plant tissues generally results in a substantial reduction in respiratory rate as measured by oxygen uptake. There are tissues, however, whose respiration is stimulated rather than inhibited by cyanide. The conventional explanation for the occurrence of this stimulation is that it results from decontrol of glycolysis as a consequence of diversion of electrons from the phosphorylating cytochrome pathway to the non-phosphorylating, but cyanide insensitive, alternative pathway. This explanation requires that the unused capacity of the alternative pathway be greater than the flow through the cytochrome pathway. The alternative pathway must be able to accomodate the electrons diverted from the cytochrome pathway, as well as additional electron flow from an increased glycolytic rate, for stimulation to occur.

The occurrence of cyanide respiratory stimulation, as a result of increased electron flow through the alternative pathway, is supported by experiments using salicylhydroxamic acid (SHAM) which inhibits the alternative pathway and the

cyanide stimulation (Sesay et al., 1986). However, the link between increased glycolytic rate, that is known to occur as a result of the addition of cyanide (Solomos and Laties, 1975; 1976), and increased electron flow through the alternative pathway has never been satisfactorily demonstrated. It has only been assumed that such a relationship exists.

The respiration rate of fully expanded soybean leaves, as determined by O_2 uptake, is strongly stimulated by KCN (Sesay et al., 1986). I decided to address the question of how the stimulation occurs by monitoring the amount of CO_2 given off in the dark in response to KCN and SHAM. Since glycolysis and tricarboxylic acid (TCA) cycle function result in the production of CO_2, the rate of CO_2 evolution should give an estimate of the rate of glycolysis, and tissues that show cyanide stimulation of oxygen uptake should also show increased CO_2 evolution.

Cyanide previously has been reported to stimulate CO_2 evolution and the activities of key glycolytic enzymes in several tissues (Solomos and Laties, 1975; 1976). There are no reports, however, of CO_2 evolution being monitored in the presence of both KCN and an inhibitor of the alternative pathway.

In this paper, the connection between glycolysis and KCN stimulation of mitochondrial electron transport will be

further explored by the use of both KCN and SHAM. And, as KCN stimulation of O_2 uptake was reported to occur only in fully expanded soybean leaves (Sesay et al., 1986), the effect of KCN on the CO_2 evolution of different leaf ages will also be examined.

Materials and Methods

The soybean cultivar 'Corsoy 79' was grown in 25 cm plastic pots in the greenhouse. When leaves were 14 days old (day 0 = leaf just unfolding) leaves were harvested by severing the petiole with a razor blade. The cutting was done while the petiole was immersed in water to prevent air bubbles from entering the xylem. Harvesting was done at midday to prevent possible time of day effects. The leaves were transported to the lab in beakers of water.

Individual leaflets were then cut from the leaves with petiolules submerged in water. The cut end of the petiolule was placed into a petri dish containing either water or an inhibitor. The respiratory uncoupler, FCCP, was also included as a treatment to test the effect of a respiratory uncoupler versus the effect of KCN. The level of the fluid in the petri dish was at the bottom of the leaflet, the petiolule being immersed.

The leaflets were allowed to take up the inhibitor for different lengths of time, depending on the inhibitor used.

For KCN and FCCP 30 minutes was required, and for SHAM 60 minutes. These times were selected to give maximal inhibitor effects. Leaflets were placed under a light source during this incubation time to enhance fluid uptake into the leaf. After the allotted uptake time had passed, the leaflet was removed from the water or inhibitor and placed into a LI-6200 Portable Photosynthesis System to monitor CO_2 evolution. Measurements were made in the dark.

To determine the effect of KCN on different leaf ages, measurements were made on leaves from different nodes on the plants. Node 1 corresponded to the upper part of the plant with numbering proceeding down the stem. In this way a series of leaf ages was obtained. The leaf at node 1 was approximately 3 days old, and the next leaf down was nearly fully expanded (7 days old). The concentration of KCN used for all leaf ages was 5 mM.

Results

The maximal effect of KCN was to stimulate CO_2 evolution by 30% (Figure 1). The rate of CO_2 evolution was greatly increased up to 5 mM, after which no further increase occurred. Unexpectedly, SHAM also stimulated CO_2 evolution by a similar percentage (Figure 2). Although only 4 points were included in the SHAM concentration curve, it can be seen that the effect of SHAM was maximal and unchanged at

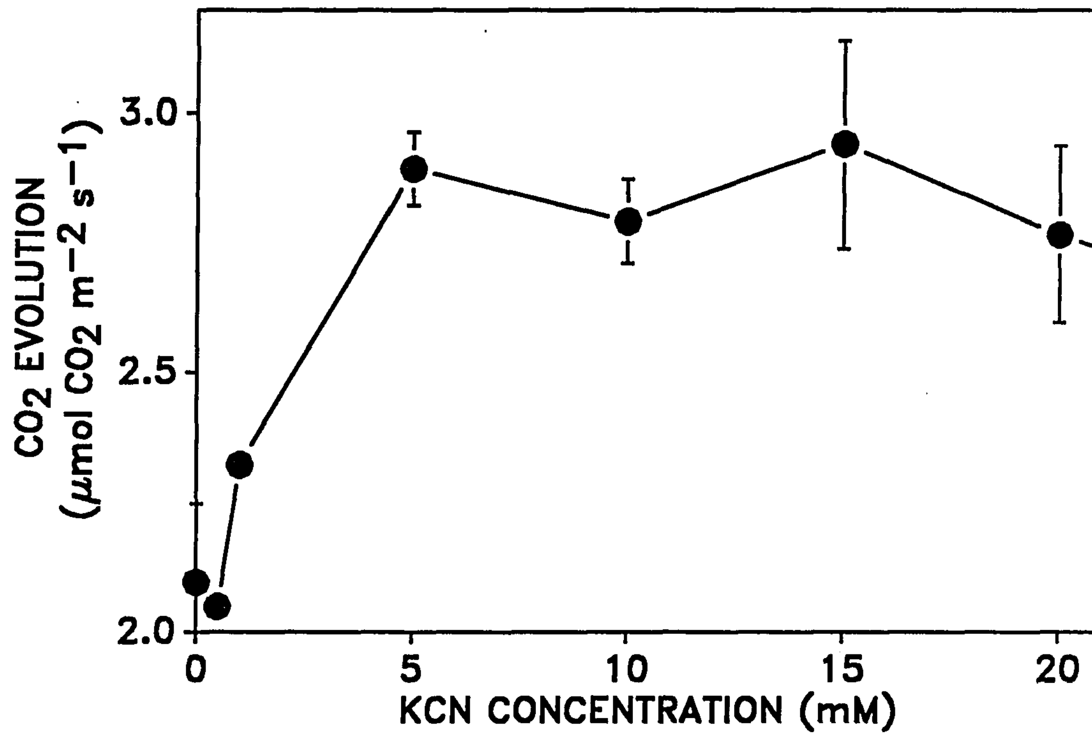

Figure 1. Effect of different KCN concentrations on CO_2 evolution of 14-day-old soybean leaves measured in the dark and expressed on a leaf area basis. The bars indicate ± SE

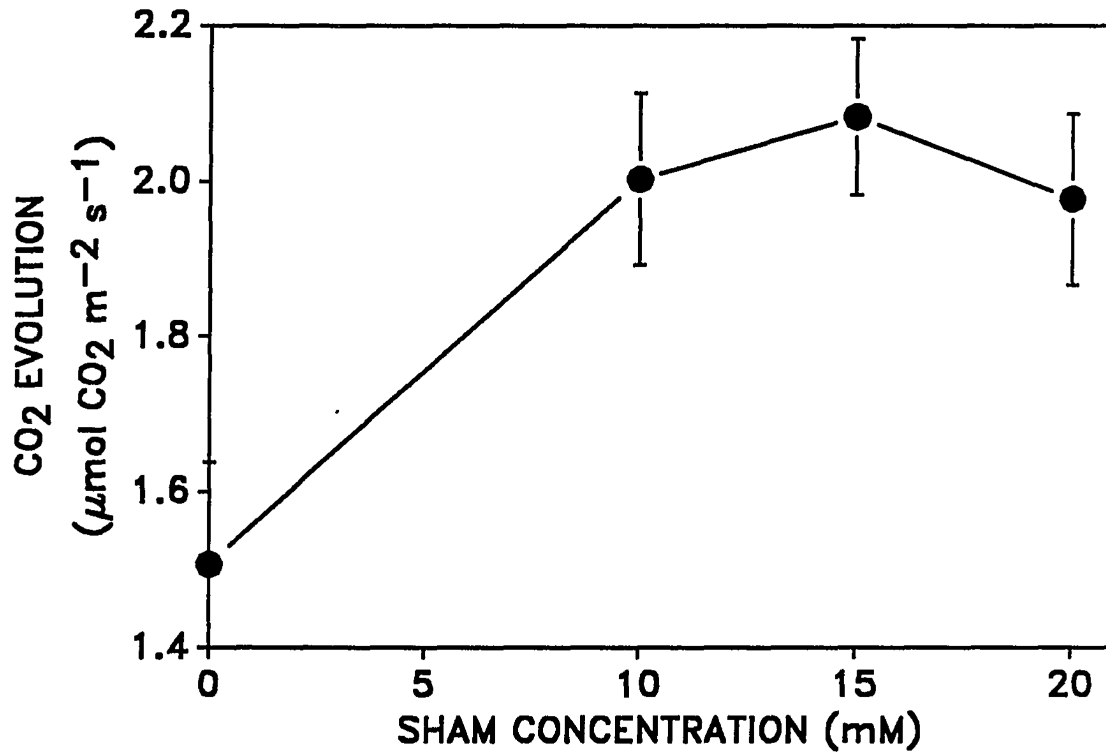

Figure 2. Effect of different SHAM concentrations on CO_2 evolution of 14-day-old soybean leaves measured in the dark and expressed on a leaf area basis. The bars indicate ± SE

concentrations of 10 to 20 mM. Figure 3 shows the effect of increasing KCN concentration on the respiratory rate of tissue given 25 mM SHAM. The effect of KCN was to eliminate the stimulatory effect of SHAM. Both inhibitors together produced a respiratory rate approximately equal to that of the control (no inhibitors).

The effect of the uncoupler, FCCP, was to stimulate CO_2 evolution by approximately 15% (Figure 4). The stimulatory effect was maximal at 6 μM.

The effect of KCN on CO_2 evolution of aging leaves is shown in Figure 5. The decline in control rate from 3 days to 7 days was dramatic, as the rate of 3 day old leaves was more than double that of 7 day old leaves. Thereafter, the decline in control rate was very gradual. At each leaf age, the rate of CO_2 evolution was stimulated by KCN. The difference between the control and the KCN rate was quite similar at all leaf ages.

Discussion

The stimulatory effect of KCN on CO_2 evolution in soybean leaf tissue agrees with previous work done with tissue slices (Solomos and Laties, 1975; 1976); and is consistent with the idea of an increase in glycolysis due to KCN. Greater rates of glycolysis should result in more CO_2 being given off because more pyruvate is produced and

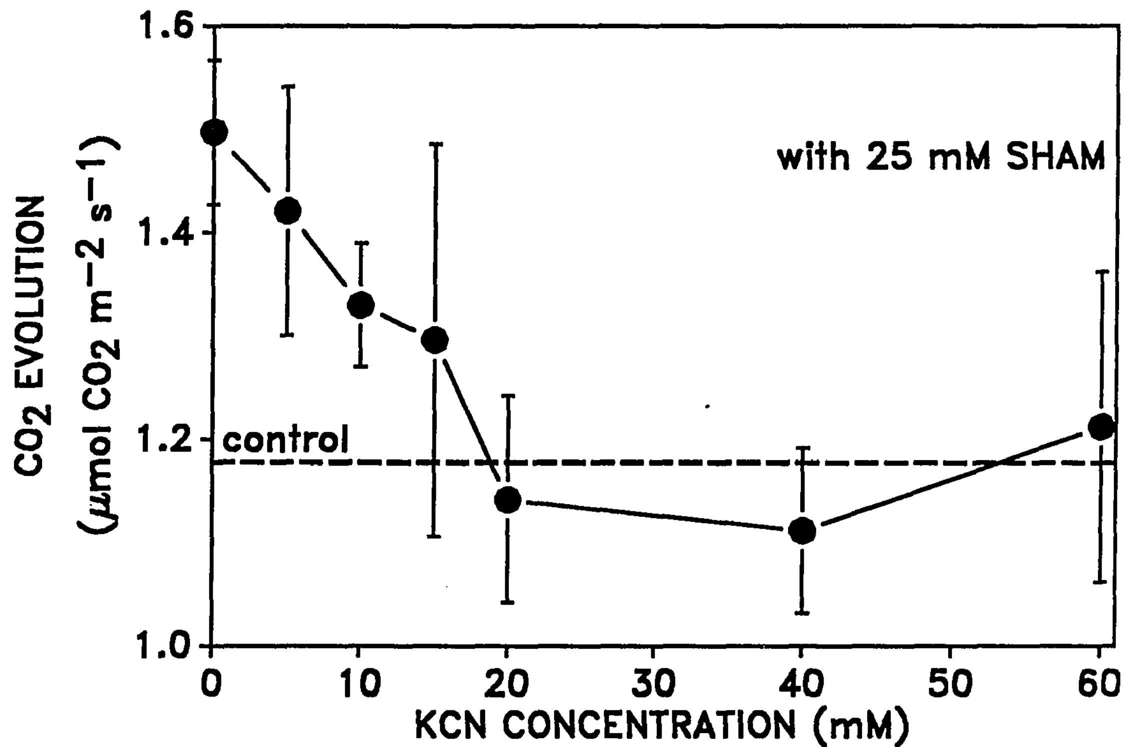

Figure 3. Effect of 25 mM SHAM with different KCN concentrations on CO_2 evolution of 14-day-old soybean leaves measured in the dark and expressed on a leaf area basis. The bars indicate ± SE

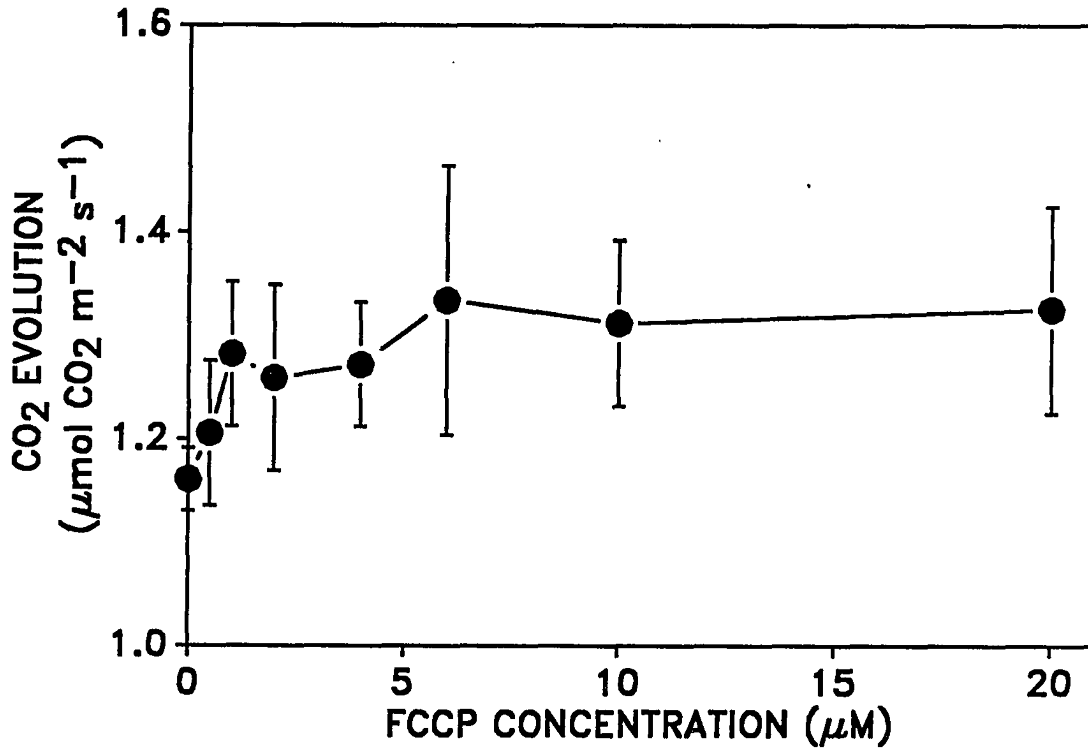

Figure 4. Effect of different FCCP concentrations on CO_2 evolution of 14-day-old soybean leaves measured in the dark and expressed on a leaf area basis. The bars indicate ± SE

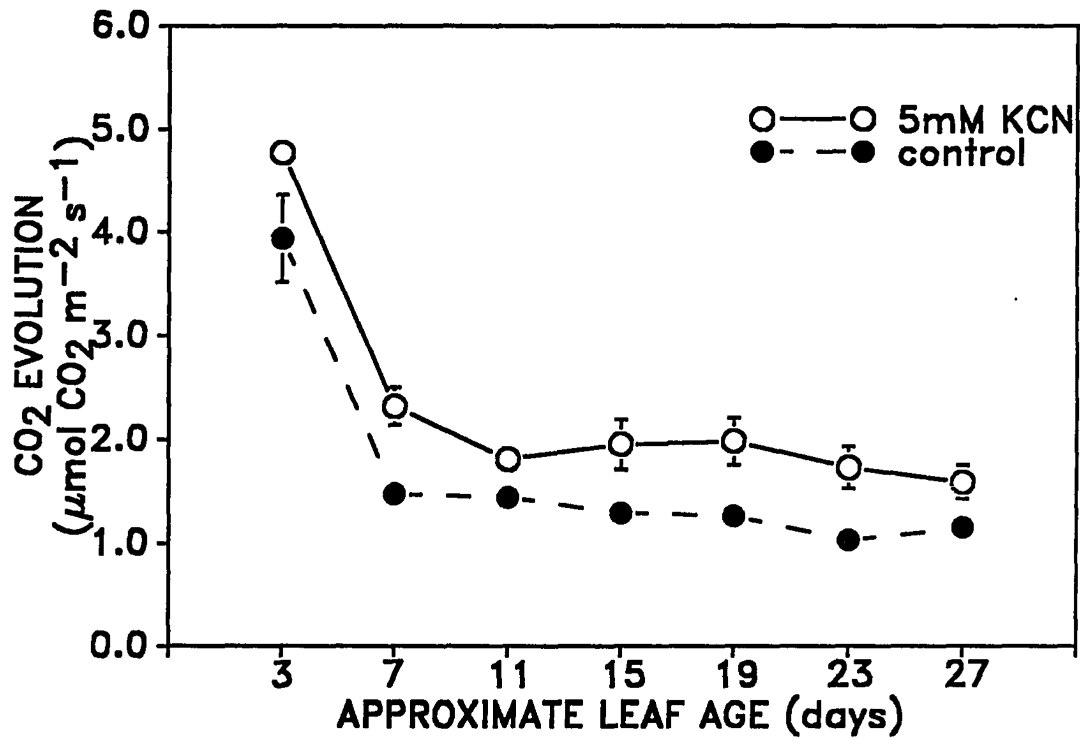

Figure 5. Effect of 5 mM KCN on CO_2 evolution of soybean leaves of different ages measured in the dark and expressed on a leaf area basis. The bars indicate ± SE

metabolized.

The stimulatory action of FCCP on CO_2 evolution shows the linkage between elimination of the proton gradient across the mitochondrial membrane and increased glycolysis. Although it is sometimes inappropriate to compare treatments with different control values, in this case KCN clearly had a greater stimulatory effect on CO_2 evolution than did FCCP. Even though both treatments uncoupled electron transport, FCCP should have been the better uncoupler because all coupling sites were disabled, whereas with KCN coupling site 1 still should have been functional. One might expect that FCCP would stimulate CO_2 evolution to a greater extent than KCN, if reduction in the proton gradient was the only controlling mechanism.

Solomos and Laties (1976) found that, in cherimoya (*Annona cherimola*) fruits, a highly cyanide insensitive tissue, cyanide stimulated glycolysis and concomitantly increased ATP levels. This is evidence that the glycolytic stimulation in some tissues may be mediated by more than just cellular ATP levels as influenced by the proton gradient. Instead, activation of the alternative pathway by cyanide may have a direct effect upon glycolytic rate. This may explain, to some degree, the greater effect of KCN compared with FCCP in this experiment. However, the possibility that FCCP did not achieve its full effect, due

to problems in penetrating the tissue, cannot be ruled out.

The stimulation of CO_2 evolution by KCN is evidence that KCN causes an increase in glycolysis. However, evidence that the stimulation occurs as a result of diversion of electrons into the alternative pathway requires that one show that, with the alternative pathway blocked by SHAM, KCN no longer stimulates CO_2 evolution. Since the presence of both KCN and SHAM together act to restrict electron flow severely, one would predict that the rate of CO_2 evolution would be considerably reduced from that of the control. However, I observed that, in the presence of both KCN and SHAM, CO_2 evolution was approximately equal to the control rate. The seemingly ineffective action of the two inhibitors together is likely explained by the stimulatory effect of SHAM on CO_2 evolution. Propyll gallate, another inhibitor of the alternative pathway, also stimulated CO_2 evolution (data not shown). The observed rate in the presence of both KCN and SHAM is probably the combined effect of the inhibition of cytochrome electron transport by KCN, the inhibition of alternative electron transport by SHAM, and the stimulation by SHAM of some unidentified CO_2 evolving reaction. Unfortunately, because of the stimulatory effect of SHAM on CO_2 evolution, it cannot be determined with any certainty that the KCN stimulation of CO_2 evolution (via glycolysis) is actually inhibited by

SHAM.

The stimulation of CO_2 evolution due to SHAM is almost certainly due to a non-specific action of this inhibitor. Since O_2 uptake is not stimulated by SHAM in soybean leaf tissue of the same age (14 days) measured in the dark (Appendix Figure A1), it is difficult to imagine how SHAM stimulation of CO_2 evolution could occur in anything but a non-specific manner. SHAM has been implicated in the cause of many non-specific side effects, such as the stimulation of O_2 uptake in some intact tissues (Bingham and Farrar, 1987; Spreen-Brouwer et al., 1987; Van der Plas et al., 1987; Diethelm et al., in press) and the inhibition of a number of enzymes and other physiological parameters (Lambers, 1985).

There appears to be little effect of leaf age on the amount of KCN stimulation. At all leaf ages examined, the incremental amount of stimulation of CO_2 evolution was nearly the same. This contrasts with previous O_2 uptake data (Appendix Figure A2) where stimulation occurred only after 3 days of age. Therefore, in a 3-day-old leaf, KCN acts to inhibit O_2 uptake and at the same time to stimulate dark CO_2 evolution. I have no explanation for this lack of correspondence between O_2 uptake and CO_2 evolution.

Conclusion

As a result of the stimulation of CO_2 evolution by SHAM, it is not possible to draw any conclusions about the nature of KCN stimulation of O_2 uptake in soybean leaf tissue. Although KCN stimulates CO_2 evolution, there is no direct evidence to confirm that this stimulation involves the alternative respiratory pathway.

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PAPER 3. RELATIONSHIP OF ALTERNATIVE RESPIRATORY CAPACITY AND ALTERNATIVE OXIDASE AMOUNT DURING GERMINATION AND SEEDLING DEVELOPMENT OF SOYBEAN

Abstract

In soybean (Glycine max [L.] Merr.) axis tissue and light- and dark-grown cotyledons 3 to 9 days of age, increasing amount of alternative oxidase, as determined by immunoblots probed with alternative oxidase antibodies, corresponded with the increasing alternative pathway capacity of isolated mitochondria from these tissues. In older cotyledons, however, little or no change in amount of alternative oxidase occurred even though there were substantial changes in capacity. This suggests that, whereas alternative oxidase content is likely to be important in determining the capacity of the alternative pathway in young cotyledons, other factors regulate capacity in older cotyledons. It is also possible, however, that the alternative oxidase antibodies were unable to detect all of the proteins that form the alternative oxidase. For all cotyledon ages examined, two proteins believed to constitute the alternative oxidase were present at molecular weights of 34 and 39 kD, though only one of the proteins varied significantly during development. In the light-grown cotyledons the amount of 39 kD protein changed, whereas in

the dark-grown cotyledons it was the 34 kD protein that changed. The presence or absence of light, therefore, played a regulatory role. Tissue type (cotyledon or axis) also influenced the protein pattern. Axis tissue possessed only the 39 kD protein.

Introduction

The alternative respiratory pathway is a non-phosphorylating branch off of the cytochrome pathway through which electrons flow in the mitochondrial membrane. Alternative respiration is thought to be nearly ubiquitous in higher plants. With the exception of the genus Arum, in which the alternative pathway has a role in thermogenesis, the function of the pathway in plant metabolism is unclear. The widespread nature of the alternative pathway in higher plants and its non-phosphorylating, and thus potentially wasteful, nature make study and better understanding of it important.

An important step in understanding the alternative pathway is in understanding the long-term pattern of its development and determining what factors control this development. Generally, alternative pathway capacity is less in younger than in older tissues, increases with tissue maturation, and eventually declines as aging proceeds. With whole leaf tissue peak capacity occurs about the time of

full leaf expansion (Azcon-Bieto et al., 1983; Sesay et al., 1986). Changes in capacity of isolated mitochondria from cotyledons of different ages from black gram (*Vigna mungo* L.), pea (*Pisum sativum* L.) and soybean (*Glycine max* [L.] Merr.) were compared in a review by Dizengremel and Turquet (1984). In all three, the pattern of development showed a period of increasing capacity in younger tissue, then decreasing as the cotyledons aged. The time span during which this occurred differed widely for the different species.

Regulation of alternative pathway expression has been best characterized in *Neurospora* where alternative pathway development is thought to proceed via *de novo* cytoplasmic protein synthesis under the control of a mitochondrially synthesized repressor protein (Edwards and Rosenberg, 1976; Lambowitz and Zannoni, 1978). Evidence also supports a requirement for RNA transcription for pathway development in *Neurospora* (Edwards et al., 1974). In higher plants, alternative pathway appearance has also been attributed to the action of nuclear genes (Burguillo and Nicolás, 1977; Dizengremel and Lance, 1976; Morohashi and Matsushima, 1983). In some instances, functioning of the pathway may be determined by the presence or absence of an "engaging factor" (Stegink and Siedow, 1986). Although mitochondrial gene involvement has not been demonstrated in higher plant

alternative pathway appearance, the need for both cytoplasmic and mitochondrial protein synthesis has been described for the disappearance of pathway in black gram cotyledons (Morohashi and Matsushima, 1983). The requirement for RNA transcription in alternative pathway development has not been well studied in higher plants, but in the case of germinating chick pea (*Cicer arietinum* L.) at least RNA transcription was not needed (Burguillo and Nicolás, 1977).

Although valuable knowledge has been gained about the regulation of alternative pathway capacity in the studies previously described, a more complete understanding awaited the purification and characterization of the component(s) comprising the alternative pathway. Recently, the terminal (alternative) oxidase of the alternative pathway has been purified and antibodies have been raised to it (Elthon and McIntosh, 1987). The amount of alternative oxidase protein present could be an important factor in the control of alternative pathway capacity. Our objective in this study was to use the newly developed antibodies to the alternative oxidase to determine the relationship of amount of immunologically reactive protein with alternative pathway capacity, as measured in isolated mitochondria from light- and dark-grown soybean cotyledons and from soybean axis tissue. Using various tissue types and growth conditions

allowed us to determine if the relationship is the same in different tissues of soybean as well as to determine if growth under different environmental conditions (light versus dark) affect this relationship.

Materials and Methods

Plant material

To obtain etiolated cotyledons, soybean seeds were first surface sterilized by soaking for 10 minutes in 5% NaOCl and then rinsed with distilled water. Seeds were then soaked in aerated distilled water for 1.5 h before being rolled into moistened paper towels. Rolled towels were placed upright in vegetable crispers, covered with a plastic bag, and placed in a dark cabinet at approximately 23° C. Axis tissue was obtained by rolling dry seeds into germination paper and placing the rolls upright in covered, water containing beakers inside a dark cabinet. For green cotyledons, seeds were planted in a greenhouse in flats containing a 1:1:1 mixture of soil, peat and perlite. Day 0 for all tissue types was when the seeds were rolled or planted. Emergence in the greenhouse occurred in 2 to 3 days; 3-day-old cotyledons were only partially green at the time of sampling.

Isolation of mitochondria

The method of Day and Hanson (1977) was used for isolation of mitochondria from non-green tissue. Modifications in the procedure were: To the grinding buffer we added 5% (w/v) PVP-40 and 4 mM cysteine; the concentration of BSA in the grinding buffer was increased to 2% (w/v); 50 mM Tes replaced the 50 mM KH_2PO_4 in all solutions; duration of the final centrifugation was reduced to 10 minutes.

Green cotyledons (50 to 100g) were homogenized in two volumes of cold grinding buffer (0.3 M sucrose, 50 mM TES (pH 7.6), 10 mM KH_2PO_4, 2 mM EDTA, 2 mM $MgCl_2$, 30 mM ascorbate, 1% (w/v) PVP-40 and 1% (w/v) BSA) by using a polytron at setting 7 for 3 s. The homogenate was filtered through six layers of cheesecloth and centrifuged at 1,000g for 10 minutes. The supernatant was carefully decanted after removing the lipid layer (if present) and centrifuged at 12,000g for 20 minutes. The resulting pellet was then resuspended in wash buffer (0.3 M sucrose, 10 mM TES (pH 7.2) and 0.1% (w/v) BSA) and the total volume made up to 40 mL in a single centrifuge tube. Centrifugation at 1,000g for 10 minutes then followed. The supernatant was removed by pipette and the mitochondria pelleted by centrifuging the supernatant for 10 minutes at 12,000g. The pellet was resuspended in 2 mL of wash buffer and layered onto a discontinuous gradient formed by use of Percoll and wash

buffer consisting of 8 mL of 55%, 16 mL of 27% and 12 mL of 15% Percoll. The gradient was centrifuged at 7,700*g* for 30 minutes. The white layer at the interface of the 27 and 55% layers was collected using a bent tip Pasteur pipette. The volume of this mitochondrial suspension was then made up to 40 mL with wash buffer and centrifuged for 15 minutes at 12,000*g* to pellet the mitochondria. The resulting mitochondrial pellet was resuspended and centrifuged again as in the preceding step. The final pellet was generally resuspended in 1 mL of wash buffer.

Measurement of mitochondrial respiration

Oxygen uptake was measured by the use of a Clark-type oxygen electrode (Yellow Springs Instruments). Measurements were performed at 25°C in a total volume made to 3 mL with measuring buffer (.25 M sucrose, 10 mM TES (pH 7.2), 5 mM $MgCl_2$, 5 mM KH_2PO_4 and 0.1% BSA) using 10 mM succinate as substrate. ATP at a concentration of 85 μM was always included. State 3 was induced by 120 nmol ADP in the first cycle of ADP addition, and by 180 nmol in the second cycle. Two mM KCN and 2 mM SHAM were added at the attainment of state 4 after the second cycle of ADP addition. Subtraction of the residual rate, estimated in the presence of both KCN and SHAM, from the KCN-insensitive rate was used to calculate the capacity of the alternative pathway (Valt).

Protein and chlorophyll determination

Protein was determined by the method of Bradford (3) using BSA (fraction V) as standard. Chlorophyll was extracted from the mitochondrial sample using 96% ethanol, and the amount of chlorophyll determined using the method of Wintermans and deMots (1965). Mitochondrial protein values were corrected for contamination from chloroplast fragments by using the chlorophyll concentration and assuming a protein:chlorophll ratio of 7 to 1 in the contaminating chloroplast fragments (Lilley et al., 1975).

Electrophoresis and immunoblotting

Samples with 100 to 200 μg of protein were mixed with a sample buffer containing 125 mM THAM (pH 6.8), 20% (w/v) SDS, 30% (v/v) glycerol and .04% (w/v) bromophenol blue tracking dye in a sample to buffer ratio of 2:1. The samples were then heated to 95°C for 2 minutes. After heating, 2 μl of 1 M DTT was added to each sample. Gel electrophoresis was performed as described by Laemmli (1970) with a 5% (w/v) stacking and 12% (w/v) resolving gel. Bio-Rad pre-stained low molecular weight markers were used to estimate molecular weight and the efficiency of the electrotransfer. The procedure of Blake et al. (1984) as modified by Elthon and McIntosh (1987) was used for transfer of the proteins to nitrocellulose and for probing with antibodies to the alternative oxidase.

Results and Discussion

Measurements of O_2 uptake in isolated mitochondria from soybean cotyledons are shown in Figure 1. In both green and etiolated tissues the capacity of the alternative pathway increased with increasing cotyledon age up to 9 days. From 9 to 12 days, however, the capacity in the light-grown cotyledons continued to increase whereas that of the dark-grown declined. In both, alternative pathway capacity, as a percentage of the state 4 rate, changed very little during development, averaging 71% for the dark-grown tissue and 75% for the light-grown.

Figure 2 shows immunoblots which were run using aliquots of the mitochondrial samples used to obtain the data in Figure 1. The blots were probed with antibodies to the alternative oxidase. Two bands are evident, at 34 and 39 kD. These molecular weights are similar to those reported earlier for bands labeled by alternative oxidase antibodies in Sauromatum (Elthon and McIntosh, 1987). In the blot run from non-green samples (Figure 2A) the lower (34 kD) band shows increasing intensity from 3 to 9 days. With the exception of a very slight increase in intensity from 3 to 4 days, the upper (39 kD) band did not change during this period. Previous experiments using Sauromatum (Elthon and McIntosh, 1987) have also reported an alternative oxidase protein that was relatively stable in

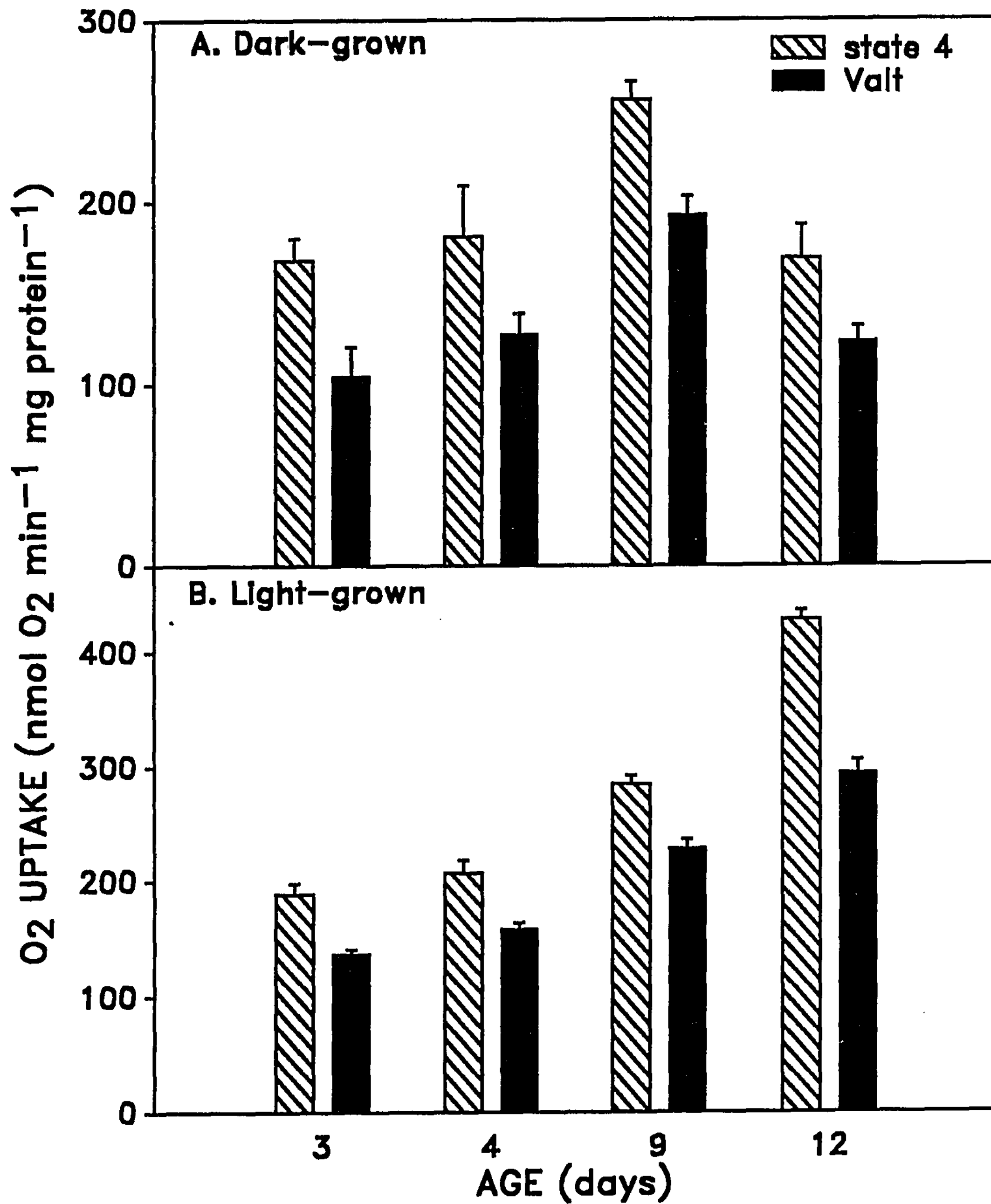

Figure 1. Alternative pathway capacities and rate of state 4 respiration of mitochondria isolated from different ages of soybean cotyledons using succinate as substrate. Data represents the mean of three different preparations ± SE

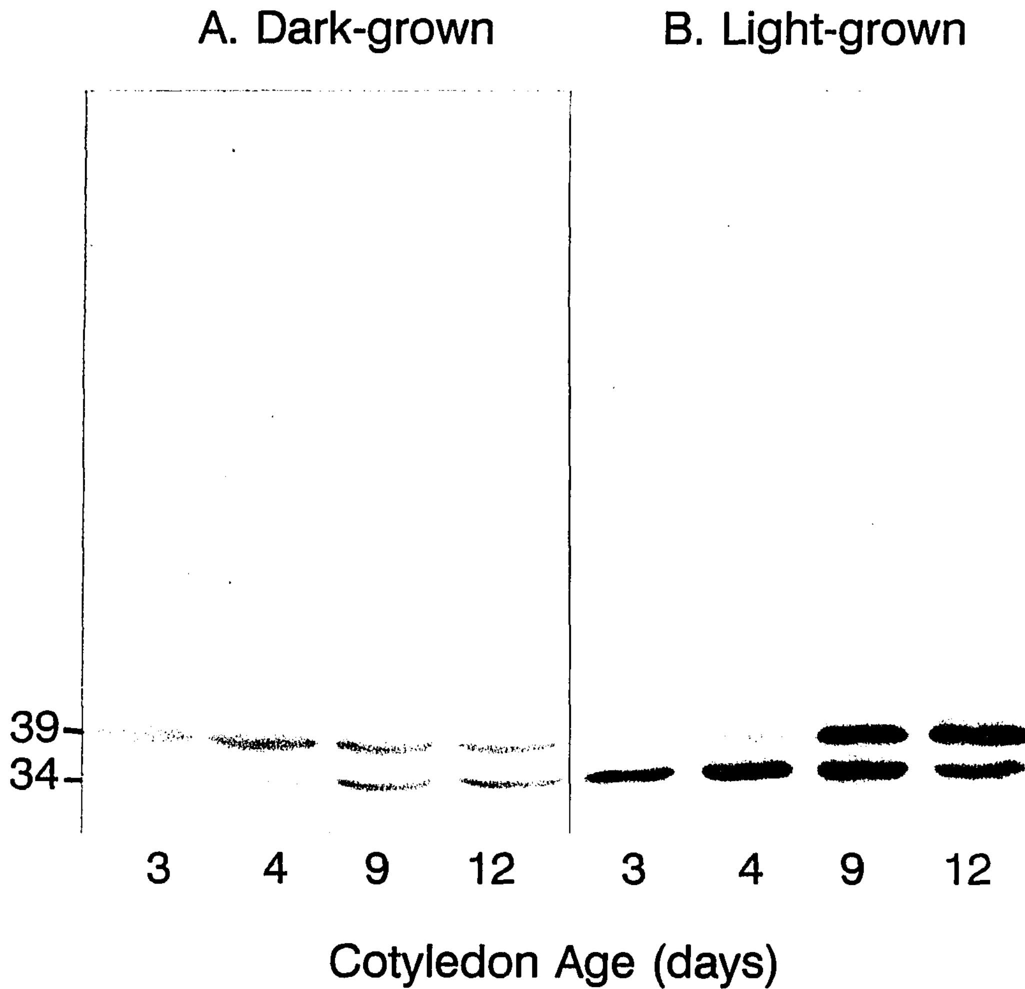

Figure 2. Immunoblots of mitochondrial proteins from light and dark-grown soybean cotyledons of different ages probed with alternative oxidase antibodies. The amount of mitochondrial protein added per lane was 100 μg for light-grown and 200 μg for dark-grown cotyledons. Molecular masses in kD are listed to the left

amount in mitochondrial preparations from tissues having different alternative pathway capacities. Interestingly, the change in band intensity from 3 to 9 days seen in the blot of the dark-grown cotyledons is the reverse of that seen in the light-grown cotyledons (Figure 2B). In the case of the light-grown tissue, it is the lower bands (34 kD) that are nearly unchanged whereas the intensity of the upper bands (39 kD) increased greatly. It seems that light may have a direct or indirect regulatory effect in controlling the distribution of protein in the two bands. In both tissue types, little or no visible change in intensity of either band occurs after 9 days.

The increase in alternative oxidase amount, as evidenced in the immunoblots of both light and dark-grown tissues (Figure 2), corresponds to the increase in alternative pathway capacity from 3 to 9 days (Figure 1). The increasing amount of oxidase may be responsible for the increased capacity. However, there are sizeable changes in capacity from 9 to 12 days in the two tissue types that seem unrelated to change in alternative oxidase amount. This is especially evident in the results from dark-grown cotyledons; in which by 12 days the alternative pathway capacity had fallen to a value similar to that present at 4 days (Figure 1A), but the amount of 34 kD protein clearly had not diminished much. It seems that alternative oxidase

amount is likely an important factor in the control of capacity during development of the cotyledons, from 3 to 9 days, but later (9 to 12 days) other factor(s) predominate as regulator(s). Another possible explanation for the lack of correlation after 9 days is that there may be alternative oxidase protein(s) present that the antibodies do not recognize. These proteins could have changed in amount after 9 days and changed alternative pathway capacity. Mitochondrial protein blots probed with alternative oxidase antibodies have been previously made using a number of different species (Elthon et al., 1989), and from 1 to 4 protein bands have been observed. It is unknown whether this species difference represents a true difference in the number of alternative oxidase proteins present or an inability of the antibody to recognize some of the proteins in certain species.

Figure 3 shows O_2 uptake of etiolated soybean axis mitochondria. Alternative pathway capacity increased from 18 to 66 h after seed imbibition, with little change from 66 to 90 h. Alternative pathway capacity, as a percentage of the state 4 rate, is less than that for cotyledon mitochondria; it ranges from 33% of the state 4 rate in 18-h axis to 53% in 90-h axis mitochondria.

Immunoblots of the axis mitochondrial samples (Figure 4), with the exception of the 18-h lane, show only one band.

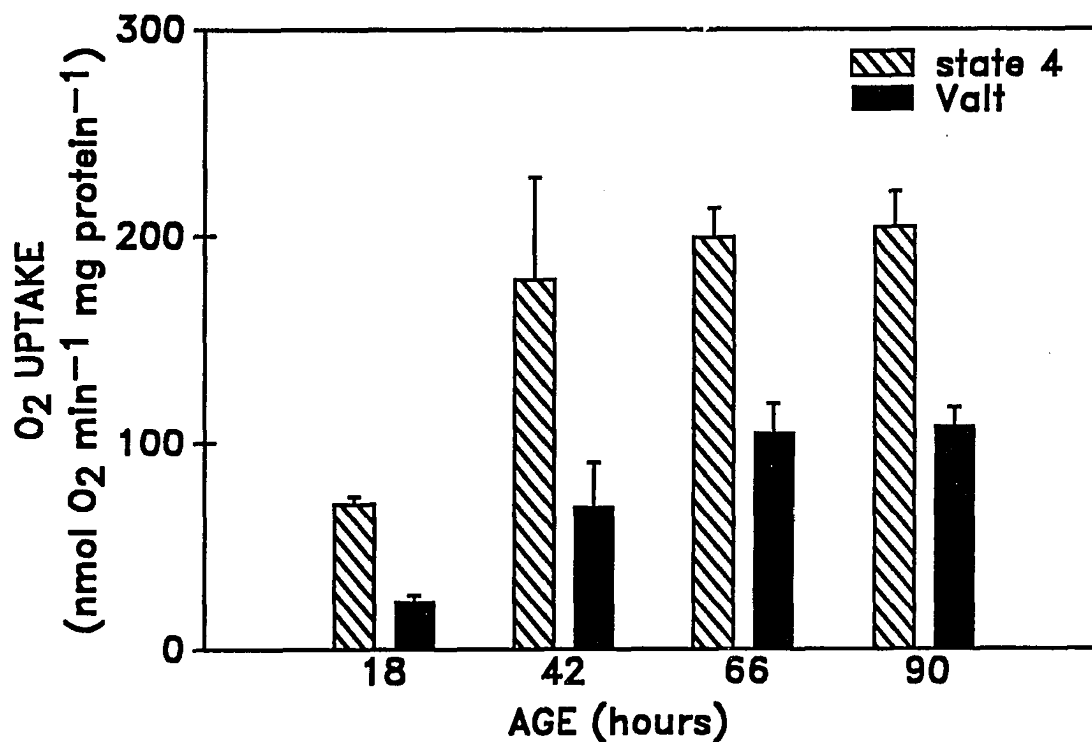

Figure 3. Alternative pathway capacities and rate of state 4 respiration of mitochondria isolated from etiolated soybean axis tissue using succinate as substrate. Data represents the mean of three to five different preparations ± SE

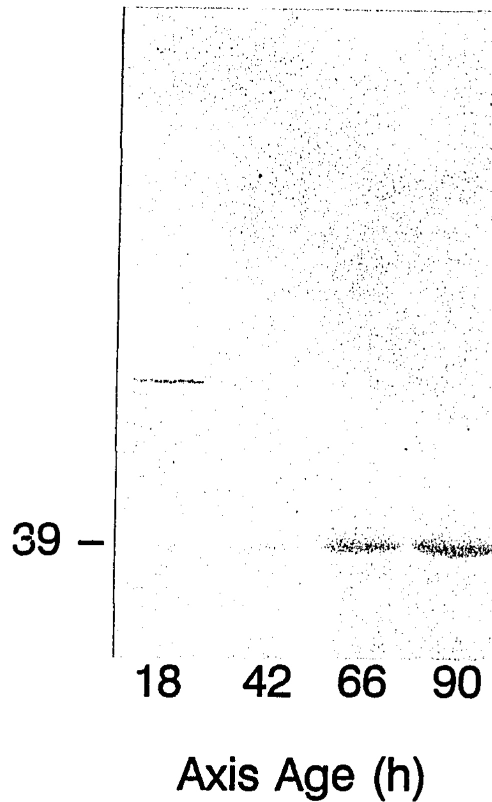

Figure 4. Immunoblots of mitochondrial proteins from soybean axis tissue of different ages probed with alternative oxidase antibodies. The amount of mitochondrial protein added per lane was 200 μg. Molecular masses in kD are listed to the left

This band corresponds to the upper (39 kD) band of the cotyledon tissues. In the 18-h lane there is an additional band present at a higher molecular weight. This band was not always present in immunoblots from other 18-h axis mitochondrial samples, and may be a result of non-specific binding by the alternative oxidase antibodies. Similar to the 39 kD band of the light-grown cotyledon mitochondria, the amount of alternative oxidase in the axis mitochondria increases from 18 to 66 h as seen from increasing band intensity. This increase parallels the alternative pathway capacity increase. From 66 to 90 h there is no discernable darkening of the band, and no detectable change in capacity (Figure 3). As with the younger soybean cotyledons, the amount of oxidase present may be an important factor that determines capacity in axis tissue.

In conclusion, the data indicate that alternative oxidase amount may be important in the determination of alternative pathway capacity in young soybean cotyledons and in axis tissue, but not in older cotyledons. The lack of correlation between alternative oxidase amount and alternative pathway capacity in older cotyledons could be due to the involvement of other controlling factors or to the inability of our antibodies to detect all of the proteins that make up the alternative oxidase.

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SUMMARY

When work for this dissertation was begun, my initial goal was to determine if there were large differences among soybean genotypes for the alternative respiratory pathway. Genotypes exhibiting large differences in the pathway would have provided the basis for further research. Unfortunately, although I did determine that genotypic variability for the alternative pathway existed among the soybean lines tested (Appendix Tables A1 and A2), none of the differences among the genotypes were large enough to merit further study.

The work presented in Paper 1 was an attempt to confirm published reports (Musgrave et al., 1986a,b; Musgrave and Siedow, 1985) indicating that male-sterile lines of many species (including soybean), as well as the pea cultivar Progress No. 9, lack the alternative pathway. We, however, found both the male-sterile soybean genotype cited and the pea cultivar Progress No. 9 to possess substantial alternative pathway capacity. Paper 1 was published (Obenland et al., 1988) to notify other researchers of these inaccuracies in the literature.

It had always been assumed that KCN stimulation of O_2 uptake was due to decontrol of glycolysis as a result of electrons being diverted into the alternative pathway. The question of whether or not this hypothesis is true has

important implications regarding the validity of O_2 uptake measurements in those tissues, including soybean leaves, in which KCN stimulation of O_2 uptake occurs. Unfortunately, my attempt to answer this question, as presented in Paper 2, failed due to an unexpected, non-specific effect of SHAM on the measurements. Nevertheless, in the course of doing the experiments, a lot of documentation about the effects of KCN, SHAM and FCCP on CO_2 evolution of soybean leaf tissue was accomplished. This information may be of value to future researchers.

Prior to the experiments reported in Paper 3, the only consensus concerning the control of alternative pathway capacity in non-thermogenic higher plant tissues was that protein synthesis was required for pathway appearance. My work in Paper 3 demonstrated that in soybean cotyledon and axis tissue, the amount of alternative oxidase is likely an important determinant of alternative pathway capacity. These results represent a beginning toward the eventual goal of understanding all of the factors that control alternative pathway capacity throughout plant development.

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APPENDIX

Table A1. Oxygen uptake rates of control and inhibitor-treated 17- to 18-day-old soybean leaf tissues, percentage of control, and percentage capacity of the alternative pathway (Valt.). Capacity of alternative pathway is the difference between rate in presence of KCN and rate in presence of KCN + SHAM. Method of measurement is as in "Materials and Methods" in Paper 1. Values are the mean of four replications

	Control	1 mM KCN	(%)	1 mM KCN + 10 mM SHAM	(%)	Valt.
	--------μmol O_2 (g dry weight)$^{-1}$ (min)$^{-1}$-------					
Virginia	1.95	2.45	(125.6%)	0.55	(28.2%)	1.90
PI326580	1.86	2.48	(133.3%)	0.60	(32.3%)	1.88
Peking	2.18	2.69	(123.4%)	0.85	(39.0%)	1.84
Med. Green	2.67	3.10	(116.1%)	1.29	(48.3%)	1.81
PI424078	2.36	2.58	(114.0%)	0.80	(33.9%)	1.78
Clark 63	2.54	2.80	(110.2%)	1.08	(42.5%)	1.72
PI153292	1.86	2.41	(129.6%)	0.80	(57.0%)	1.61
PI79593	1.73	2.30	(124.8%)	0.78	(45.1%)	1.52
PI65388	1.88	2.49	(132.4%)	1.02	(54.3%)	1.47
Illini	2.28	2.42	(106.1%)	1.10	(48.2%)	1.32
Mandarin	2.64	2.62	(99.2%)	1.42	(53.8%)	1.20
Harosoy 63	2.58	2.67	(103.5%)	1.51	(58.5%)	1.16

Table A2. Oxygen uptake rates of control and inhibitor-treated 14-day-old soybean leaf tissues, and percentage of control. Method of measurement as in "Materials and Methods" in paper 1. Four replications were performed for each value

	Control	10 mM SHAM	(%)	1 mM KCN + 10 mM SHAM	(%)
	--------μmol O_2 (g dry weight)$^{-1}$ (min)$^{-1}$--------				
Virginia	1.90	2.10	(110.5%)	0.59	(31.0%)
PI326580	2.08	2.21	(106.3%)	0.63	(30.3%)
Peking	2.14	2.04	(95.3%)	0.75	(35.0%)
Med. Green	2.65	2.45	(92.5%)	0.75	(28.3%)
PI424078	2.33	2.35	(100.9%)	0.46	(19.7%)
Clark 63	2.22	2.07	(93.2%)	0.57	(25.7%)
PI153292	2.15	2.20	(102.3%)	0.68	(31.6%)
PI79593	1.79	1.90	(106.1%)	0.84	(46.9%)
PI65388	1.77	2.06	(116.4%)	0.79	(44.6%)
Illini	2.44	2.14	(87.7%)	0.81	(33.2%)
Mandarin	1.99	1.86	(93.5%)	0.74	(37.2%)
Harosoy 63	2.75	2.53	(92.0%)	0.87	(31.6%)

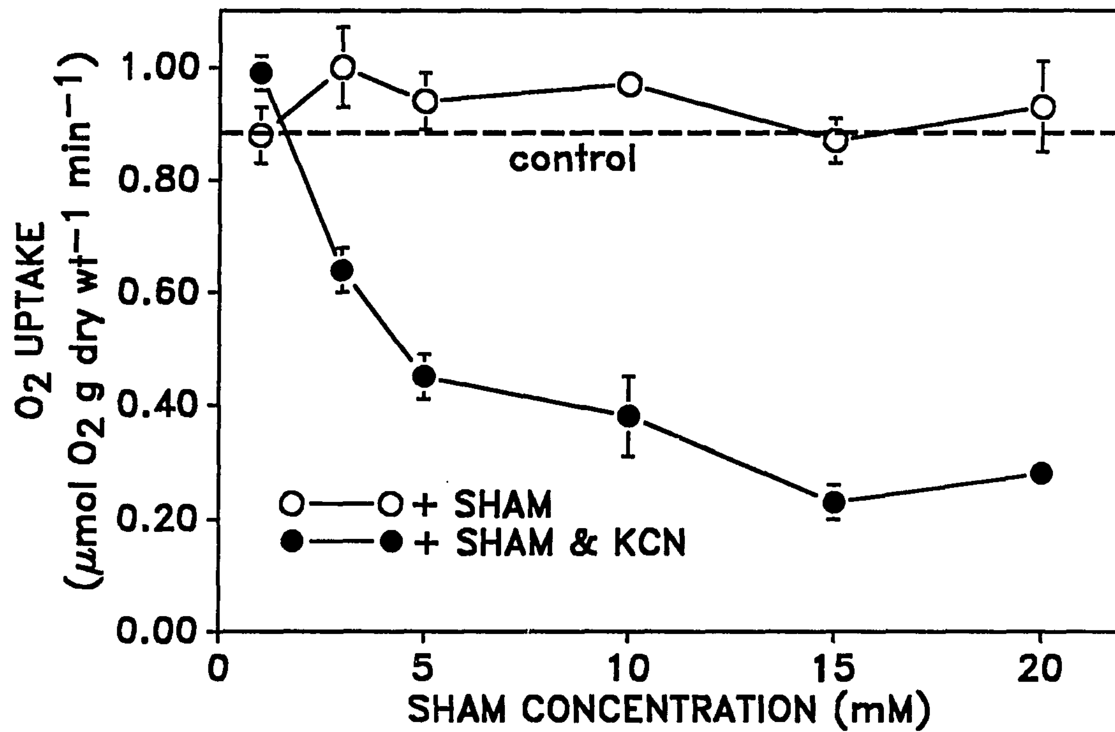

Figure A1. Effect of different SHAM concentrations in the presence or absence of 1 mM KCN on O_2 uptake of 14-day-old soybean leaves. The bars indicate ± SE

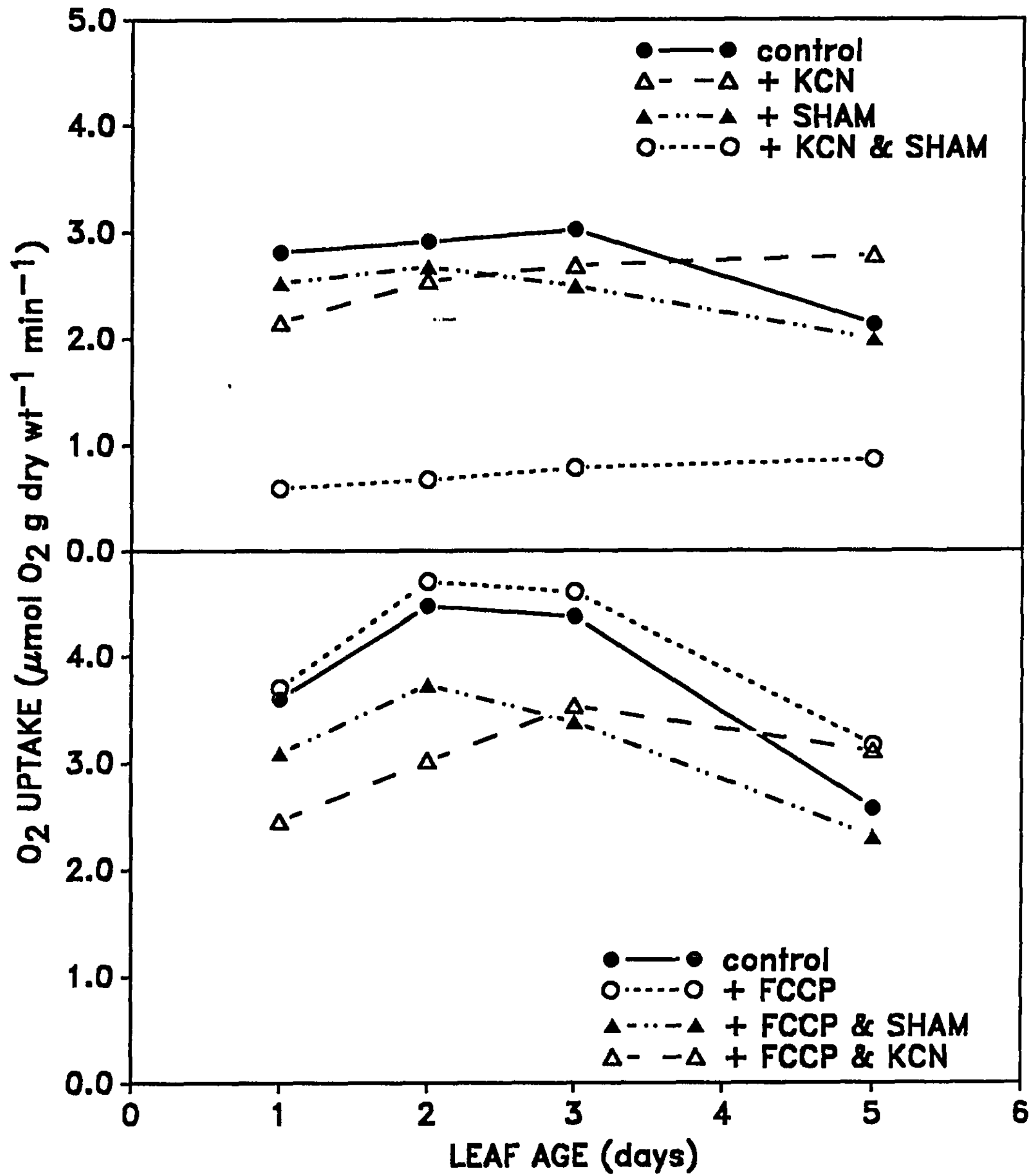

Figure A2. Effect of 1 mM KCN, 10 mM SHAM, and 10 μM FCCP on the O_2 uptake of soybean leaves of different ages