The relative contributions of the cytochrome and the alternative respiratory pathways in the post-chilling respiratory burst in chill-sensitive Cucumis sativus.

Catherine Mary Kiener
University of Massachusetts Amherst
THE RELATIVE CONTRIBUTIONS OF THE CYTOCHROME AND THE ALTERNATIVE RESPIRATORY PATHWAYS IN THE POST-CHILLING RESPIRATORY BURST IN CHILL-SENSITIVE CUCUMIS SATIVA

A Thesis Presented
By
CATHERINE MARY KIENER

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Approved as to style and content by:

William J. Bramlage, Chairperson of Committee

Paul H. Jennings, Member

Bruce S. Jacobson, Member

Allen V. Barker, Department Head, Plant and Soil Science
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The following abbreviations have been used in this text

A/A Antimycin A
ADP Adenosine diphosphate
ATP Adenosine triphosphate
AVG Aminoethoxyvinylglycine
BSA Bovine serum albumin
CCP Carbonyl cyanide m-chlorophenylhydrazone
CLAM Chloro-benzyhydroxamic acid
DNP Dinitrophenol
EDTA Ethylenediamine tetraacetate
KCN Potassium cyanide
NAD(H) Nicotinamide-adenosine dinucleotide (reduced)
PEG Polyethylene glycol
PSI Photosystem I
PSII Photosystem II
RC Respiratory control ratio
SHAM Salicylhydroxamic acid
SMP's Submitochondrial particles
TES N-tris(hydroxymethyl)methyl 2-aminoethane sulfonic acid
TPP Thiamine pyrophosphate
TMPD N'N'N'-N'-Tetramethyl-p-phenylenediamine
ABSTRACT

Chill-sensitive plants that are exposed to low temperatures exhibit a burst of respiration after they are returned to normal temperatures. In this study, this response was analysed in chill-sensitive cucumber hypocotyls using the titration method of Bahr and Bonner (3) to ascertain whether the alternative respiratory pathway contributed to this post-chilling burst of respiration. Cucumber seedlings were grown to 8 days in 27°C, 85% R. H. in the light and then either transferred to 2°C, 95% R. H. in the dark or kept at 27°C but in the dark. The chilled seedlings were returned to 25°C, 85% R. H. to recover.

Although the alternative pathway was not operating to any marked extent before chilling, at 6 hours after chilling it was operating at 40% of its full capacity. This activity, however, only accounted for up to 50% of the increased respiration rate at this point in the post-chilling respiratory burst. By 24 hours, when the seedlings were fully recovered the flux through the alternate pathway had declined to the low level of the controls. Eliminating the post-chilling burst of ethylene with AVG had no effect on this operation of the alternative pathway during the post-chilling burst of respiration. This argued against ethylene having a role in switching on the alternative pathway.

The direct effect of temperature on respiration rates and response to inhibitors of unchilled hypocotyls was investigated. Between 20 and 15°C (which corresponds to the start of the chilling range for
cucumbers) respiration because sensitive to SHAM, with a concomitant loss of sensitivity to KCN. Since unchilled hypocotyls equilibrated to the temperature of the oxygraph water bath within 2 minutes, these observed changes were a rapid response.

It would seem, therefore, that the shift from the cytochrome pathway to the alternative pathway is an immediate response to low temperature. Therefore, the post-chilling burst of respiration may be an after-effect, a consequence of the continued operation of the alternative pathway together with renewed cytochrome pathway activity.

Studies with isolated mitochondria reinforced the assumptions made that the effects of SHAM were indicative of alternative pathway activity. The patterns of respiration and response to inhibitors with different substrates corresponded with the known alternative pathway of plants.

Short-term chilling (0 to 6 hrs) of hypocotyls prior to extraction had marked effects of the properties of the resultant mitochondria. The results suggested the possibility that the isolation procedure per se, which necessarily involves 45 minutes at 6°C, might cause the alternative pathway to switch on. Therefore, caution is needed in extrapolation of results from in vitro mitochondrial activity.
CHAPTER I

INTRODUCTION

Many plant species that originate from tropical or sub-tropical regions exhibit sensitivity to low but non-freezing temperatures. This phenomenon is known as chilling sensitivity and is expressed as a variety of symptoms; prolonged exposure results in injury and eventual death. The chilling temperature range is generally regarded as being up to 15 °C, although this depends on the plant species; some species are sensitive to temperatures as high as 17° C (46).

The extent of injury is inversely proportional to the temperature within the "chilling range" and is a direct function of the time held at that temperature. Up to a point the effects of low temperature are reversible but then a threshold time is reached when irreversible effects develop: these result in chilling injury. Any theory of chilling injury has to take this into account. The extent of injury is modified by a variety of environmental and physiological factors such as relative humidity and the age of the tissue (46, 48).

Since many chill-sensitive plant species are important economic crops, often grown outside their ecological range, a considerable amount of energy has been expended towards the understanding of chilling injury in the hopes of reducing the large economic losses brought about by this phenomenon. A greater understanding of chilling sensitivity could enhance the chances of breeding chill-resistant strains.
The largest losses occur at the germination and young seedling stages, where the crop is being grown outside or at the edge of its natural range, and in the cold storage of harvested crops.

**Physiological Effects**

The more obvious physiological effects of low temperature on chill-sensitive plant tissues include:

1) a rapid cessation of cytoplasmic streaming which occurs within 30 seconds for chill-sensitive plants but not at all in chill-resistant plants.

2) increases in membrane permeability which eventually lead to a flaccid wilted appearance, a common symptom of chilling injury. This leads to a decreased cell water potential which causes stomatal closure. This in turn markedly reduces photosynthesis.

3) chlorosis.

4) increased ethylene production.

5) metabolic imbalance which arises because different enzymes are affected to different degrees by the low temperature.

6) necrosis.

**Cytoplasmic streaming**

Graham and Patterson reported that there were dramatic differences in the cytoplasmic streaming of chill-sensitive and chill-resistant plants as the temperature was varied (65, 66). There are still no detailed studies of this phenomenon even though it is likely
to have marked effects on the metabolism of the cell. Cyclosis ensures the thorough mixing of enzymes and substrates thus preventing local depletions within the cell. It may also affect the binding of regulatory enzymes to membranes or actin (53). Cytoplasmic streaming is thought to be mediated by actin interactions with myosin-type ATPases and; or the plasma membrane (42, 71).

**Solute leakage**

It has been well documented that after chill-sensitive plant tissue has been exposed to low temperature there is an increased loss of solutes, indicative of increases in membrane permeability (13, 46, 48, 61, 63, 67). Direct studies on the changes in membrane permeability have been hampered by the presence of the cell wall. The favored way to measure changes in membrane permeability has been to look at solute leakage, but this has many inherent problems. There is no way of detecting those ions desorbing from the cell wall or those that leak past the membrane but get absorbed to the cell wall. It has also been difficult to interpret measurements, by direct probe, of cell membrane potential.

More recent reports have focused on the timing of chill-induced leakage. Cucumber leaf discs floating on water had to be exposed to 5° C for 4 days before any appreciable leakage was seen (96). It took several hours at 0 °C before chill-sensitive *Cornus* callus tissue showed appreciable changes of plasmalemma or tonoplast permeability (113). Similarly, increased root exudation was delayed (13).
Conversely, other studies have shown increased permeability in cucumber and bean to be a fairly rapid response, occurring within the first 2 hours at low temperature (46, 58).

Some of these differences may be due to differences in relative humidity during chilling. Wright looked in detail at the effect of relative humidity and concluded that although a water deficit or chilling alone had no effect on leakage, a chill-induced water deficit was responsible for the leakage (110). Chilling caused the stomates to open widely which, combined with a decreased water uptake by the roots, caused a water deficit if the plant was in low relative humidity.

To date there have been no studies using protoplasts to study membrane permeability changes directly; these should resolve the timing question.

**Ethylene production**

Several workers have shown that when chill-sensitive plants are returned to a normal growth temperature after chilling they exhibit a burst of ethylene production, the extent of which is proportional to the length of exposure, unless the point of irreversible damage has been reached (60). Ethylene production only occurs after the tissue has been exposed to low temperature so this seems to argue against ethylene having a primary role in the chilling response; it may be just a consequence of injury.
Photosynthesis

Rates of photosynthesis decline in chill-sensitive tissue after they have been held at low temperature (46). Phaseolus plants held at 5°C for one night showed decreased photosynthetic rates when placed back at 20°C. However, the same effect was observed if only the roots were chilled, which suggested that there was an indirect effect of chilling on photosynthesis. It was found that this was in fact due to the effect of reduced water uptake by the roots on stomatal closure (19).

Studies aimed at determining whether low temperature per se has an effect on the photosynthetic pathways have yielded mixed results when the tissue was chilled in the dark (26, 40, 46, 63, 85, 90). Garber compared the effect of low temperature on cucumber (sensitive) and spinach (resistant) leaf discs and thylakoids and found no differences in the various photosynthetic activities that were measured in the discs other than a generally lower activity in cucumber (26). However, cucumber thylakoids were very sensitive to low temperature. In contrast other workers have found altered Hill activity of isolated chloroplasts from tomato, bean and cucumber below 10-12°C, whereas isolated chloroplasts from chill-resistant lettuce, spinach and peas showed no decline in activity (40, 85, 90).

There is agreement over the detrimental effect of chilling sensitive tissue in the light. There is a rapid loss of Hill activity, PSI and PSII (26, 34). Low light intensity partially reduces, and chilling under nitrogen further reduces these effects; this implies
photo-oxidation. Electron flow in chill-sensitive thylakoids is slowed down such that the chlorophyll pigments stay in the triplet state long enough to induce photo-oxidation (34, 97).

Low temperature has also been shown to affect photosynthesis indirectly by reducing chlorophyll synthesis (55, 97, 98).

**Enzyme activity**

Chilling injury has been thought to arise from the differing effects of low temperatures on different enzymes so as to lead to metabolic imbalance, but enzymatic studies have yielded little information about this (46). Bananas have a well known browning response to low temperature; the greatly increased polyphenol oxidase activity that causes this is a consequence of the release of membrane-bound enzyme at low temperature. Graham and co-workers looked at temperature effects on phosphoenolpyruvate carboxylase isolated from C_3 plants. The activity of the enzyme from tomato showed a great increase in activation energy below 10°C, whereas the enzyme from temperate and alpine species all showed a steady linear decline. However, passion fruit (chill-sensitive) phosphoenolpyruvate carboxylase also showed a linear, albeit steep, decline. Kinetic studies on the tomato enzyme suggested that low temperature induced conformational changes (28). Studies on pyruvate phosphate dikinase from corn revealed a dramatic decline in activity below 10°C, but this inactivation could be prevented by pyruvate or phosphoenolpyruvate. This led to the finding that the active form of the enzyme is a tetramer and low
temperature causes it to dissociate into monomers (30).

Respiration

The effects of low temperature on respiration have been well studied (29, 30, 46). Generally, respiration rates decline at low temperatures to a much greater extent with chill-sensitive plants or tissue than with chill-resistant plants. However, when chill-sensitive plants or tissue are returned to a non-chilling environment from a chilling one they exhibit an exaggerated burst of respiration. The extent and length of this burst is related to the time held at the chilling temperature and inversely related to the temperature (21). It has been shown that the onset of visible symptoms of injury when the sensitive tissue is being held at low temperature coincides with the time at which respiration rates become insensitive to the uncoupling agent DNP (17).

It was originally thought that one of the causes of chilling injury could be due to a reduction in ATP due to the effect of low temperature on respiration (46). However several studies have shown that the ATP levels do not usually decline until the onset of visible symptoms of injury (38, 109). It would seem that anabolism is affected to such an extent that the ATP is used very slowly.

Lyons and co-workers studied, in detail, the respiration of mitochondria as a function of temperature (46, 47, 49). Mitochondria from a range of chill-sensitive species showed less swelling and a much greater decrease in oxidative activity at low temperatures than
did mitochondria from chill-resistant species. Furthermore, fatty-acid analysis revealed a correlation in which the mitochondria from chill-resistant species had a higher content of unsaturated fatty-acids (49). If succinate oxidation by isolated mitochondria was plotted against temperature, in the form of an Arrhenius plot, for a wide range of plant species, a linear decrease from 25°C down to 9-12°C was found; below this there was a sharp decrease in slope. This was interpreted as representing a change in $Q_{10}$ from 2.2 to 6.3 for mitochondria from chill-sensitive species whereas mitochondria from chill-resistant species showed a steady $Q_{10}$ throughout the range 1.5 - 25°C. Phosphorylative efficiency, in contrast, was not influenced by temperature in any of the species that were studied. There was no comment on the fact that the procedure for isolation of the mitochondria took 1 hour at 4°C (47). The "soluble" enzymes that were studied did not show this break in the Arrhenius plots.

The Membrane Phase Change Theory

Lyons and co-workers postulated a unifying concept for chilling injury based on their results described above. It was argued that the primary effect of chilling sensitive species is on the cell membranes. This hypothesis was supported by electron spin resonance (ESR) studies using a nitroxide radical as a probe of the membrane structure. The experiments showed that there was a sudden decrease in membrane mobility at the temperature corresponding to the onset of the chilling range for the sensitive species. Mitochondria from resistant species
did not show this change in mobility, in the temperature range studied (45, 72). This was taken to indicate a temperature-dependent phase change that occurred at the onset of the chilling range. This theory overcame the problems of trying to find a mechanism for chilling injury which was previously bedevilled by the heterogeneity of responses of individual proteins to temperature even though the chilling-sensitive plants showed a common chilling range.

Some similar types of studies support this hypothesis. Apple cultivars (6) of different chilling susceptibility all showed breakpoints in the Arrhenius plots on mitochondrial oxidation, ranging from 3.6 - 9.5°C. However there was not a good correlation between the transition temperature of individual cultivars and their relative susceptibility (53). When 3 different organelles, mitochondria, proplastids and glyoxysomes, from germinating castor beans were compared, all 3 intact organelles showed breakpoints in the Arrhenius plots of spin mobility around 10-12°C. Reconstituted vesicles of the phospholipids from the respective organelles showed breakpoints between 8-10°C. This indicates that the phase changes obtained with the intact organelles were in fact due to changes in the lipid bilayer (7). The activities of mitochondrial membrane-bound enzymes also showed breakpoints in the Arrhenius plots of around 10°C. However 2 enzymes of gluconeogenesis, malate synthase and citrate synthase, had linear Arrhenius plots throughout the range 25 - 1.5°C. It was concluded that the phase changes must have no effect on the activity of these two enzymes (108). Seasonal changes in artichoke
mitochondrial activity closely correlated with membrane fluidity as measured by ESR (10). Correlations between the transition temperature of spin motility and membrane-linked activity have also been observed for ion uptake (8), DNA synthesis (14), chlorophyll synthesis (90), chloroplast permeability (63), NADPH reduction by isolated chloroplasts (85, 90) and glutamate, oxaloacetate transaminase (20).

The lipid phase changes of a range of Passiflora species correlated with their known temperature response for growth (67). A relationship between growth and phase-change temperature was also found for Vigna radiata (73). A similar situation also occurs in senescing tissue where a progressive loss of membrane fluidity corresponds with the declining activity of several enzyme systems (54).

Minchin and Simon noted certain qualifications to Lyon's original theory when they found that mitochondrial membranes and tonoplasts from the same tissue differed in their transition temperatures (58).

However, as a counterbalance there are also many negative reports where membrane-related processes do not show breaks in activity; most notably with photosynthetic activities of isolated chloroplasts and thylakoids (6, 25, 26, 56, 64). When chlorophyll a fluorescence is used as an intrinsic probe of membrane fluidity there are no indications of phase-changes in chill-sensitive species (6, 26). It has been noted that, in contrast to other biological membranes, the lipids of chloroplast membranes are mainly glycolipids; most notably monogalactosyldiacylglycerol (MGG) and digalactosyldiacylglycerol (DGG).
These glycolipids show a high degree of unsaturation in their acyl chains whether from chill-sensitive or chill-resistant species (6). Detailed $^{13}$C NMR, ESR and differential scanning calorimetric studies of chloroplasts from a range of sensitive and resistant species reinforced the conclusions that phase-transitions or separations would not be expected to occur above 0 °C. Another study showed that up to 40% of the double bonds in the acyl chains could be hydrogenated without affecting photosynthetic activity (74).

Seed germination is another process that does not fit with Lyon's hypothesis (88, 89). Since imbibing seeds do not have developed functional membrane structures, chilling sensitivity in seeds has been attributed to protein denaturation (89).

These contradictions have resulted in a reappraisal of the original theory (48, 56). It is now appreciated that with a complex lipid mixture, such as that found in biological membranes, a lowering of the temperature generally leads to the lateral migration of the lipids with the highest melting points which then assemble to form rigid gel patches. There may or may not be co-crystallisation of different lipid types. As the temperature is lowered still further these gel patches can grow by accretion of more lipids (44, 103). This leads to zones of phase separation areas.

Phase transition temperatures of individual lipids are highly dependent on the chain length, the degree of unsaturation of the acyl chains and on the type of polar head group. Artificial membrane systems have shown that 2 similar phospholipids will co-crystallize
at a temperature intermediate to the phase transition temperatures of the individual phospholipids (103). Sterols can greatly moderate the structure of lipid bilayers since they have a condensing effect on liquid-crystalline states and a liquefying effect on gel states. Relatively small changes in the lipid composition of membranes can therefore alter the physical properties significantly.

Freeze-fracture studies are consistent with other observations using different physical probes. They reveal the progressive formation of smooth patches, and increasing aggregation of particles (84, 103). This illustrates that an important feature of gel formation would seem to be the exclusion of proteins, at least in the initial gelling stages. A further complication in natural membranes arises from the asymmetry of the lipid bilayer. Proteins have a profound effect on the surrounding lipids. Adjacent lipids form a more rigid layer compared to the fluid bilayer (39). The situation in natural membranes is clearly a complex one.

The interpretation of the ESR results from the original studies on chilling sensitivity have also been reappraised. There was no indication in these studies of which microlipid environment or what depth of the membrane the probe was monitoring. At best it could be taken as a monitor of bulk lipid properties (69). Raison and co-workers have used a more specific probe, trans-parinaric acid, one that is especially sensitive to the first few percent gel formation (111). They concluded that the nitroxide probe had been monitoring the start of a succession of mini phase changes rather than a general phase
transition temperature; this temperature is now called $T_s$. This shift in interpretation can now accommodate criticisms leveled at the use of Arrhenius plots to indicate phase-changes, when the point could equally well have been analysed as a series of break-points or even a curve. The data still indicate that an abrupt change in the order of the cell membranes occurs at the start of the chilling range.

There are several reports of the correlation of bulk unsaturated to saturated fatty-acid ratios, or double bond indices, to the chilling response of different species (46, 50), but there are an equal number of reports where there is no such correlation (46). However, in light of the complexity of biological membranes and their response to temperature it is hardly surprising that these types of studies do not contribute much to a greater understanding of the chilling response, especially in cases where such studies have looked at total membrane complements of the tissue.

Despite these complicating factors, membrane fluidity does seem to be a major factor in low temperature responses (44, 48, 103). Acclimation studies with rats (12), goldfish (16) and Tetrahymena (103) all showed that there was an increased level of unsaturation in the fatty-acyl chains, especially in the phospholipids, when the organisms were grown at a lower temperature. In Tetrahymena this has been shown to be via de novo synthesis which occurs after cells that have been grown at 39.5°C are shifted to 15°C. There is a rapid increase in fatty-acid unsaturation of the phospholipids of microsomes and after a 30 minute lag period there is a similar increase in the
unsaturation of the phospholipids in the plasma membrane (104). Cellular fatty-acid desaturases are localised in microsomal membranes. Several studies confirm that fatty-acyl desaturases from bacteria, animals and plants are all activated by membrane fluidity changes, such that a rigid environment enhances activity (105).

Similar results have been found in studies of acclimation on callus tissue from several plant species (52). Further evidence for the importance of increased fatty-acyl unsaturation in acclimation to low temperature was obtained by the use of inhibitors of the 18:2 to 18:3 conversion. Cotton seedlings which had been sprayed with these inhibitors could no longer be hardened for low temperature tolerance (93).

The fatty-acid complement of rats and E. coli can be manipulated by diet (49, 103). In both cases, lack of polyunsaturated fatty-acids led to a decrease in membrane fluidity and an increase in sensitivity to low temperature. In rats, manipulation of the fatty-acid complement was shown to affect the allosteric properties of several enzymes (23). Rat kidney (Na\(^+\) and K\(^+\)) stimulated ATPase was solubilised and reconstituted with either exogenous phosphoserine or phosphoglycerol. Arrhenius plots of activity showed break-points at temperatures which corresponded to the phase change temperature. When the \( T_s \) was modified by the addition of cholesterol the activity showed a corresponding change (41). Similar results have been shown in the effect of the fluidity of the phospholipids associated with cytochrome oxidase, on its activity (39).
Wolfe has proposed that an important factor in chilling sensitivity is the formation of phase separation zones rather than gel versus fluid states *per se*. He argued that for conformational changes to occur, which are often necessary to produce enzymatic activity, enzymes require lateral compressibility in the adjacent lipid bilayer. Lateral compressibility is greatest in phase separation zones. It has also been shown that these areas correspond to the areas of maximum permeability (111). Although this is undoubtedly an important consideration it is not the complete answer to chilling sensitivity.

Submitochondrial particles that had been fused with varying amounts of exogenous phospholipid vesicles, and separated into different phospholipid: protein classes by sucrose density gradients, and then assayed for NADH, succinate and Cyt$_c$-UQ reductases, NADH dehydrogenase, cytochrome oxidase and ATPase activities all showed decreasing activity with increasing phospholipid content. This reinforced the idea that the respiratory chain components in these mitochondria are freely diffusible yet require close orientation of the respective components for activity and this is upset by the addition of extra phospholipid (82). Inner mitochondrial membranes are found to be relatively lipid-poor so small changes in phospholipid content could have marked effects on the mobility and orientation of mitochondrial membrane proteins (24).

Ultrastructural studies of the effects of low temperature on chill-sensitive tissue have been interpreted both in favor (34) and against (62) the phase-change theory. Changes in membrane structure do occur but Niki and Uritani argue that visible changes in membranous
components do not occur until after several hours in a low temperature. Their work suggests that the onset of chilling injury coincides with the disruption of the tonoplast, one of the last membrane systems to show a visible response to low temperature (62).

Wilson found that *Phaseolus* plants can be either chill or drought hardened. Yet, although the former resulted in a greater level of unsaturated fatty-acids, the latter did not (109). Both types of hardening did induce stomatal closure (110). Experiments comparing the effects of chilling sensitive tissue at 25%, 45% and 100% relative humidity revealed that high humidity prevented chilling injury (110). It has been suggested therefore that chilling injury was a consequence of a chill-induced water deficit rather than a membrane phenomenon *per se*. However, it should be noted that stomatal opening is governed by $K^+$ and malate effluxes in the guard cells, so that rapid chill-induced stomatal opening could be a consequence of altered membrane properties (50).

Other reservations about the main theory have also been made; again leveled at the use of the Arrhenius plots. Silvius and coworkers have shown that the break-points in Arrhenius plots can arise from the temperature dependence of substrate binding affinity, and so need not be a consequence of phase changes at all (87).

Yamonota and Nishimuri have noted that the structural water around membranes undergoes a change at about 8°C and this may in fact have an important role in low temperature responses (112).
Cyanide-Resistant (Alternative) Respiration

Many plant tissues exhibit a phenomenon known as cyanide-resistant or alternative respiration (32, 57, 89). This was first discovered by Van Herk in 1937 in the aroid Sauromatum spadices and its appearance correlated with the heat burst exhibited by these aroid inflorescences (106). This cyanide-resistant oxygen uptake was later shown to be associated with the electron transport chain of the mitochondria (36). It develops on the first day of aroid flowering, the day when respiration rates start to increase rapidly. The mitochondria isolated from spadices at this stage were uncoupled and this uncoupling was shown to be due to extensive use of the alternative pathway (143).

This so called alternative respiration has since been demonstrated in many different plant species and also in some fungal species, although it often only exists at certain stages of the life cycle (32, 89). Interestingly, these stages are often observed when marked metabolic changes are occurring, such as in germinating seeds or in seedlings (37), in ageing tissue (102), after wounding (99), or after infection (15).

Ikuma and Bonner, in their studies on mung bean mitochondria, showed that CN\(^-\) and N\(_3^-\) inhibited state 3 respiration (in the presence of ADP) by 65-80\%, but state 4 respiration (ADP limiting) was much less affected. Antimycin A also inhibited state 3 respiration but had very little effect on state 4, with both malate and succinate as substrates. Rotenone completely inhibited malate oxidation. This suggested
a possible branch point around ubiquinone which bypassed the cyto-
chromes to oxygen. Malate oxidation via the alternative pathway gave
an ADP:O ratio approaching 1.0, compared to the normal ratio of 3.0
for the cytochrome pathway, whereas succinate oxidation by this pathway
was uncoupled, that is there were no phosphorylation sites (33).

Earlier studies using oxygen pulse labeling of anaerobic mito-
chondria in the presence of inhibitors of either the cytochrome path-
way (CN⁻, N₃⁻ or antimycin A) or the alternative pathway (thiocyanate,
α-adipyrridyl or 8-hydroxyquinolate; these inhibitors are not very
specific) supported the idea that ubiquinone was the branch point and
also indicated that none of the cytochromes seemed to be involved in
the alternative pathway (5, 91).

With the discovery of substituted hydroxamic acids as specific
inhibitors of the alternative oxidase, subsequent studies were greatly
enhanced (83).

Bahr and Bonner developed a titration technique with these
inhibitors which enabled them to determine the relative contribution
of each pathway in normal (uninhibited) rates of respiration. One
important finding was that the cytochrome respiration rate was not
affected by the operation of the alternative pathway, at least in the
systems that they studied (3, 4). Their results eliminated the possi-
bilities that the electron flux through the alternative pathway was
controlled by the energy charge, or that the energy state of the cyto-
chromes had any regulatory role (except perhaps that a b-type cytochrome
and its associated flavoprotein may be involved) or that the electron
flux through the 2 pathways was determined by simple competition. The only model that would fit was one with a branch point where 2 components were in equilibrium with each other (4).

Storey measured the rate of oxidation of the different respiratory chain components after oxygen was pulsed through CO - anaerobic skunk cabbage mitochondria. Ubiquinone and part of the flavoprotein complex were oxidised within 1 second but this was inhibited by CLAM. He compared this with potato mitochondria, which do not possess the alternative pathway, and found the same rates of oxidation (very slow) as found in skunk cabbage mitochondria in the presence of CLAM. This provided conclusive proof that ubiquinone was in the region of the branch point and that a flavoprotein with an $E_0$ of 50 mV more negative than ubiquinone linked it to the alternative pathway. It was thought that this was the flavoprotein $Fp_{ma}$ (89, 95). Neither the nature of the alternative oxidase nor its products have been elucidated (32, 57, 89).

All the known inhibitors of the alternative oxidase are metal chelators, which suggests that a non-heme Fe-protein is involved (89). Detailed ESR studies have been done on a 'poky' mutant of Neurospora crassa as a model system. This mutant has a cyanide-resistant respiratory pathway that resembles the plant pathway in all respects. It also shows an ESR signal, around $g = 1.92$, associated with the functioning of the alternative pathway (32, 59, 77, 89). Ferredoxin proteins were excluded as potential candidates but further analysis of this signal (a HiPiP type) did suggest that it was from the S3 center of succinate dehydrogenase. This further suggested that the alternative
oxidase was an auto-oxidizable subunit of succinic dehydrogenase (SDH) (59, 75). However, this did not bear out since submitochondrial particles (SMP) did not need to have any associated SDH to show alternative oxidase activity. The S3 subunit is just the portion of SDH that donates electrons to the alternative pathway (76). Rich and Bonner now propose the following potential candidates:

1) An auto-oxidizable quinol/quinonone system.
2) A copper dimer system that is ESR silent.
3) A non-heme or non-FeS but Fe-containing system, again ESR silent (76).

Mung bean mitochondria seemed to produce more H$_2$O$_2$ in state 3 than state 4 respiration and its production increased in the presence of antimycin A (33, 91). This pointed to the possibility of H$_2$O$_2$ being the primary product of the alternative oxidase; the H$_2$O$_2$ would be rapidly broken down to oxygen by mitochondrial catalases and/or peroxidases. However, other results showed that the mitochondrial peroxidase was an artifact of isolation and this cast doubts on this theory (70). It has also been proposed that superoxide is a primary product of the alternative oxidase since an assay using nitro-blue tetrazolium indicated that more superoxide was formed when the alternative pathway was operating (89). Huq and Palmer showed that neither H$_2$O$_2$ nor superoxide were end products (31). They found that SMP's that possessed the powerful alternative respiratory of Arum did produce both H$_2$O$_2$ and superoxide but in insufficient quantities for the very high respiration rates sustained by the alternative oxidase. Furthermore,
their results indicated that $H_2O_2$ was produced when NADH but not succinate was used as the substrate. Rich and co-workers disagree with these results and still favor a role for $H_2O_2$. They found that mitochondria that had been cleaned up by sucrose density gradient centrifugation, had sufficient catalase and peroxidase activity to use any $H_2O_2$ produced by the alternative pathway. They prepared SMP's and found that in the absence of catalase or peroxidases the rate of $H_2O_2$ production in the presence of antimycin A could easily account for the electron flux through the alternative pathway (78).

To date the most accepted scheme to account for the alternative pathway in relation to the cytochrome pathway is illustrated in Figure 1.

Until 1978 the hydroxamic acids CLAM and SHAM were assumed to be specific inhibitors of the cyanide-resistant pathway and consequently SHAM/CLAM sensitivity was used as a test for the presence of the alternative pathway. However, 3 reports now cast doubts about this specificity. The most comprehensive study, by Rich and co-workers, demonstrated that substituted hydroxamic acids inhibit the activity of a number of redox enzymes by reversibly binding to a site adjacent to, but not directly on, the heme moiety; they are not acting as chelating agents (79). Increased rates of SHAM-sensitive oxygen uptake in soybean particles have been attributed to lipoxygenase activity and it was suggested that a comparison of propyl gallate, a known lipoxygenase inhibitor, and SHAM effects could distinguish between alternative
Figure 1

Plant Respiratory Pathways

NADH (endogenous) ~ Fp,i
NADH-DH (endogenous) (internal)
Rotenone

NADH-DH (external)
NADH-DH ~ Fp,i

KCN
Ascorbate
Cyt b<sub>553</sub>
Cyt b<sub>557</sub>
Cyt c<sub>549</sub>
Cyt a,a<sub>3</sub>

Site I

SDH
Malonate
Succinate

Site II

Antimycin A

Site III

Alternate Oxidase

SHAM (hydroxamic acids)
oxidase and lipoxygenase activity (41). However, a more rigorous study by Siedow and co-workers revealed that not only does SHAM inhibit the lipoxygenase activity but also that propyl gallate could inhibit the alternative oxidase, albeit to a smaller extent than SHAM (86). Clearly, great care has to be taken in interpretations of SHAM effects on intact tissue.

Experimental evidence from numerous plant tissues has illustrated that the alternative pathway is, at most, linked to only one phosphorylation site. Careful monitoring of the development of the cyanide-insensitive pathway in Arum spadices indicated that the respiratory efficiency (ADP: O ratios) dropped drastically as the alternative pathway activity developed, leading to uncoupled mitochondria (36, 43). It is now well established that the operation of the alternative pathway in aroids permits uncoupled respiration which leads to thermogenesis (32, 36, 43, 57, 89).

Thermogenesis does not, however, explain why the alternative pathway is found in so many other plants, often only at clearly defined stages of the life cycle (32, 89). The alternative pathway could be envisaged as a mechanism for controlled uncoupling to allow for turnover of Krebs cycle intermediates for anabolism, without feedback inhibition by ATP which normally occurs when the cytochrome pathway is operating. Such a role has been hard to demonstrate.

Solomos and Laties suggested that there was a link between ethylene stimulation of respiration and cyanide-resistant respiration in those tissues that possessed the resistant pathway (92). Other work has
supported this idea (80).

However, recent work by Theologis and Laties failed to show a role for the alternative pathway in 2 well documented cases of ethylene stimulated respiratory bursts; wound induced respiration and the climacteric respiration of ripening fruits (99, 100, 101, 102). They determined the relative contribution of each respiratory pathway using the titration technique of Bahr and Bonner (3).

Although slicing potato tubers induced the cyanide-resistant pathway, its contribution to the wound induced respiration was found to be nil. Furthermore, uncoupling agents revealed that the cytochrome pathway was not operating at full flux in fresh tissue and so could easily accommodate wound respiration. The alternative pathway was only found to operate in aged slices in the presence of uncouplers which means that electron flux only occurs through the alternative pathway when the cytochrome pathway is saturated. Similar results were obtained with sweet potato tubers which already possess the alternative pathway in intact tubers.

The alternative pathway was also found to contribute little to the climacteric burst of respiration in avocado and banana. Uncoupling agents again demonstrated that this respiratory rise could be accommodated by the unused capacity of the cytochrome pathway found in preclimacteric fruit. Also, the alternative pathway only operated if uncoupling agents were added during the respiratory rise, that is after the cytochrome pathway was saturated.

These results now cast doubt on what had seemed a logical role
Two very recent papers are relevant to this problem; one also relates to the low temperature studies. Chaveau and co-workers studied the response of mitochondria from a number of different plant species to high temperature (90 minutes at 45°C) and found that the alternative pathway activity was specifically reduced; the co-existing cytochrome pathway was relatively resistant to high temperature. In contrast the cytochrome pathway in cyanide-sensitive mitochondria was highly sensitive to temperature. The authors suggested that there was a correlation between the presence of the alternative pathway and a higher degree of resistance to the thermal denaturation of the cytochrome pathway (11). Yoshida and Tagawa looked at low temperature stress on a chill sensitive callus line of Cornus and found that the alternative pathway was present but apparently redundant above 15°C, below this temperature the bulk of the electron flow was diverted from the cytochrome pathway, through the alternative pathway (114). These results raise new possibilities and fresh impetus in the search for the control mechanism behind the function and role of the alternative pathway.
Cucumber seedlings (*Cucumis sativis* L. cv. Marketmore 70) were used throughout this study. The seeds were imbibed in water for 2 hours at room temperature (22°C) and then sown on vermiculite, in trays, at a density of approximately one per square centimeter (1/cm²). They were then grown for 8 days at 27 ± 1°C in continuous light (intensity 160 meins/sec) at a relative humidity of 80 ± 5%. After this time the cotyledons were fully extended. Seedlings to be chilled were transferred to a growth room at 2 ± 1°C for 48 hours, in the dark at a relative humidity of 95 ± 3%. This high humidity was important since it minimised the chill-induced complications of water stress in the seedlings. Sasson (81) has found that 48 hours at 2°C was near the maximum period for which the seedlings could be chilled and still recover. Control plants were kept at 27°C but in the dark, for this 48 hr. period, as a check against dark effects. The rationale of chilling the seedlings in the dark was to eliminate any possibility of photo-oxidation that can occur if chilled in the light.

Seedlings to be treated with aminoethoxyvinylglycine (AVG) were sprayed with a 150 ppm solution that contained 0.1% Triton B1956. to act as a surfactant, when they were 7 days old. This was 24 hours before they were transferred to 2°C.
Measurement of respiration.

Hypocotyl respiration was measured by the following procedure:

1) A (1/2 cm) length was cut from the top centimeter of the hypocotyl of each of 10 seedlings for each sample; these were weighed.

2) Each sample was assayed 20 minutes after cutting, in 1.5 ml TES (N-tris(hydroxymethyl)methyl 2-aminoethane sulfonic acid) buffer, 10 mM, pH 7.0, in a Yellow Springs Clark-type oxygen electrode, or in 2.5 ml TES buffer in a Rank-Brothers Clark-type oxygen electrode, to measure initial rates of oxygen uptake. Unless otherwise noted all assays were run at 28°C.

3) The samples were then incubated in solutions of the test compound(s) and reassayed. The optimal incubation times for maximum effects were found to be 60 minutes for SHAM, 30 minutes for KCN, and 15 minutes for both DNP and CCP. These incubation times are in agreement with those found by Sasson (81) but in this study the required concentrations for SHAM and KCN were found to be $10^{-4}$ M rather than $10^{-5}$ M.

Stock solutions of 1 M KCN, 0.2 M SHAM, 50 mM DNP and 50 mM CCP were made up twice each week. The membrane, used with both types of electrode, was changed at the beginning of each day. Between assays the chamber was rinsed with 90% ethanol followed by distilled water to prevent build-up of SHAM or CCP.

Studies on hypocotyl respiration.

Wounding effect on respiration. The respiration rates of ten 1/2 cm lengths of hypocotyls with cuts at 0, 0.1 and 0.25 cm intervals were measured over 90 minutes and the results compared.
Net effects of respiratory inhibitors and uncoupling agents.

The respiration rates of hypocotyl samples were assayed before and after incubation in one of each of the following: 1) $10^{-4}$ M KCN; 2) $10^{-4}$ M SHAM; 3) $10^{-4}$ M SHAM + $10^{-4}$ M KCN; 4) $10^{-5}$ M DNP; 5) $10^{-6}$ M CCP.

Samples were taken from 8, 9 and 10 day old unchilled seedlings (controls) and from chilled seedlings 3, 6, 9, 12 and 24 hours after transfer back to 25°C.

Direct effect of temperature on respiration. Hypocotyl samples from 8 day old seedlings were assayed at 1, 10, 15, 20, 25, and 30°C before and after incubation in $10^{-4}$ M KCN, $10^{-4}$ M SHAM or $10^{-4}$ M KCN + $10^{-4}$ M SHAM.

Relative contributions of the cytochrome and alternate respiratory pathways. The relative contributions of the cytochrome and alternate pathways to the overall respiration rates were calculated by using the method of Bahr and Bonner (3) (see Appendix I).

Hypocotyls from 8 day old unchilled seedlings, chilled seedlings that had recovered for 6 hours at 25°C and chilled seedlings that had recovered for 24 hours at 25°C were assayed for initial oxygen uptake rates before and after incubation in $10^{-4}$ M KCN together with a given concentration of SHAM in TES buffer, pH 7.0.

Relationship between ethylene production and the alternate pathway. Seedlings which were 7 days old were sprayed with either distilled water (control) or 150 ppm. AVG (in 0.1% Triton B1956). Seedlings that were to be chilled were transferred to 2°C after 24 hours (day 8).
for 48 hours, in the dark. To determine the amount of ethylene produced 2 seedlings were placed in a 15 ml test-tube (10 replicates) for each experiment. Distilled water (0.5 ml) was added to each test-tube and the tubes were sealed with serum caps. After 90 minutes enough ethylene was generated for the samples to be assayed. Gas samples (2 ml) were withdrawn with a syringe and the ethylene concentration measured by gas chromatography.

The time between sealing the test-tubes and assaying the samples was standardised by drawing off each 2 ml aliquot at the same time, and sealing the syringe needles with rubber bungs before they were assayed. Aliquots of ethylene standard and laboratory air were treated in the same way to check leakage from the syringes.

The relative contribution of each respiratory pathway was determined for AVG-treated 8 day old unchilled seedlings, and AVG-treated chilled seedlings 6 and 24 hours after transfer back to 25°C as described earlier.

Mitochondrial studies on the cucumber seedlings.

Isolation procedure. About 150 g of hypocotyls from 8-day-old unchilled seedlings (equivalent to 1.5 trays of seedlings) were used per extraction.

One of the most critical steps in the isolation of intact organelles from plant tissues is the initial disruption of the tissue. It is important to find a balance between a method harsh enough to break through cell wall but not so harsh that it also disrupts the organelles
within the cell. Since a polytron, which acts mainly by shear force rather than by a cutting action, was not available the best compromise was to first roughly chop the hypocotyls with scissors, then to chop them more finely with a hand onion chopper before blending them in a Waring blender. These earlier steps reduced the blending time to 2 1 second low speed bursts.

The isolation procedure that worked best for these hypocotyls is illustrated in the flow diagram (pp. 30). The method was developed by Gardestrom and co-workers (27) so that a greater proportion of intact chloroplasts and membraneous debris was removed than was done by more traditional methods.

The following solutions were used:

Grinding medium: 0.35 sucrose, 10 mM TES, 0.1% Bovine serum albumin (BSA, essentially fatty acid free), 5 mM EDTA and 2 mM cysteine pH 7.6.

Biphasic system: 0.3 M sucrose, 10 mM TES, 6.1% polyethylene glycol (PEG) molecular weight 4,000, 6.1% dextran molecular weight 500,000, 5 mM KCl, pH 7.2.

Wash medium: 0.3 M sucrose, 10 mM TES and 0.1% BSA, pH 7.2.

Reaction medium: 0.25 M sucrose, 10 mM TES, 0.1% BSA, 10 mM KH$_2$PO$_4$, and 5 mM MgCl$_2$, pH 7.2.

For each assay 0.3 to 0.5 ml of mitochondrial suspension was placed in the reaction chamber of the oxygen electrode and the volume made up to a total of 3 ml by the addition of the reaction medium.
MITOCHONDRIAL ISOLATION FLOW DIAGRAM

150g hypocotyl tissue : 300ml grinding medium

1) cut with scissors
2) chop with an onion chopper
3) 2 second in Waring blender, low speed
4) filter through 6 layers of muslin

Filtrate

- centrifuge at 6,000g for 2 minutes
- Pellet
discard
- supernatant

- Pellet
discard
- supernatant

1) resuspend in 4 ml of top phase
   of biphasic system
2) mix with 5 ml of bottom phase
3) centrifuge at 600g for 4 minutes

- Top phase
discard
- Interphase and bottom phase

- Top phase
discard

1) mix with 4 ml of fresh top phase
2) centrifuge at 600g for 2 minutes

- Interphase and bottom phase

1) dilute 7x with wash medium
2) centrifuge at 6,000g for 2 minutes

- supernatant
discard
- Pellet
discard

- supernatant
discard

suspend in 2 ml wash medium
(this is a suspension of the mitochondria)
The ADP: O and respiratory control ratios were measured with each of the following substrates: NADH, succinate, malate + pyruvate + thiamine pyrophosphate (TPP), and ascorbate + N N'N'N'-tetramethyl p-phenylenediamine (TMPD). Stock solutions (0.1 M) were made up and adjusted to pH 7.0. The final concentration of each of the substrates in the reaction chamber was 6.67 mM.

The effects of the following inhibitors were tested with each of the substrates: KCN (5 x 10^{-4} M); SHAM, in 90% ethanol, (5 x 10^{-5} M); anticycin A, in 90% ethanol, (10^{-6} M); rotenone, in 90% ethanol, (10^{-5} M); malonate (6.67 mM); CCP, an uncoupler, (10^{-6} M).

Each state 3 / state 4 respiratory cycle was initiated by the addition of 25 x 10^{-7} moles of ADP (see Appendix II).

Solutions were added to the reaction chamber, with a Hamilton syringe, through the central pore.

For each extraction the protein concentration of the mitochondrial suspension was estimated with coomassie blue. This allowed the data to be standardised into terms of milligrams of mitochondrial protein. The coomassie blue reagent was prepared by the following method:

Coomassie brilliant blue G2 (100g) was dissolved in 50 ml of 95% ethanol, 100 ml of 85% phosphoric acid were then added and the volume made up to 1 l with distilled water. The solution was then filtered, under gravity, through a fluted Whatman No. I filter paper.

To assay the protein 0.01 ml of the sample together with 0.09 ml of TES buffer were added to 1 ml of the coomassie blue reagent; the
mixture was left for 10 minutes at room temperature. The absorbance of the solution at 595 nm was then read on a Gilford spectrophotometer. A calibration curve for the reagent was obtained using BSA in the TES buffer.

All the reagents used in this study were obtained from Sigma Chemical Co., St. Louis, Missouri.
CHAPTER III
RESULTS

Studies of Hypocotyl Respiration

When cucumber seedlings were chilled for 48 hours at 2°C in 95% relative humidity they exhibited injury symptoms of seedling collapse within 2-3 hours of being returned to 25°C, 85% relative humidity (Plate 1). After 12 hours the seedlings showed signs of recovery, and by 24 hours they were fully turgid (Plates 2 and 3).

Wound-induced effects on respiration. When using excised plant tissues for respiratory studies there is always the problem of wound-induced respiration. A comparison of samples of hypocotyl segments with 0, 1 or 2 extra slashes revealed that the rates of oxygen uptake immediately increased after cutting, but then decreased and stabilised by 15 to 30 minutes (0 or 1 slash) or by 45 minutes (2 slashes) and there was no appreciable change over the remainder of the 90 minutes period (Table 1). However, the more cuts there were on the segments the slightly higher the final rate, which indicates that there is a small residual effect of cutting. To take into account these observed phenomena, throughout this study segments were left for 20 minutes in 10 mM TES buffer before they were assayed for the rates of oxygen uptake.

Net effects of respiratory inhibitors and uncoupling agents. As a preliminary, the original work of Sasson (81) was repeated and extended
Plate 1 Comparison of 10 day old unchilled seedlings and seedlings that have been chilled at 2 °C, 95% relative humidity for 48 hr., and then returned to 25 °C for 3 hr.
Plate 2 Comparison of 10 day old unchilled seedlings and seedlings that have been chilled at 2 °C, 95% relative humidity for 48 hours, and then returned to 25 °C for 12 hr.
Plate 3  Comparison of 10 day old unchilled seedlings and seedlings that have been chilled at 2 °C, 95% relative humidity for 48 hr., and then returned to 25 °C for 24 hr.
TABLE 1

Effects of Cutting on Respiration Rates of Cucumber Hypocotyl Segments

Ten, 0.5 cm lengths of hypocotyls were cut with 0, 1 or 2 slashes of a razor blade, and the respiration rates followed over time.

<table>
<thead>
<tr>
<th>Number of cuts</th>
<th>5-10 min</th>
<th>15-30 min</th>
<th>45-60 min</th>
<th>75-90 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>63.4 ± 2.1</td>
<td>57.2 ± 3.2</td>
<td>55.8 ± 3.4</td>
<td>55.4 ± 2.8</td>
</tr>
<tr>
<td>1</td>
<td>77.7 ± 2.3</td>
<td>63.0 ± 2.5</td>
<td>62.8 ± 2.1</td>
<td>62.7 ± 2.2</td>
</tr>
<tr>
<td>2</td>
<td>85.3 ± 3.7</td>
<td>73.3 ± 6.8</td>
<td>65.7 ± 3.8</td>
<td>65.3 ± 3.7</td>
</tr>
</tbody>
</table>
to include the effects of KCN and SHAM together, and of CCP on the rates of oxygen uptake.

Since growth would effectively cease over the two day period of chilling at 2°C, changes in respiration rates and responses with age for unchilled hypocotyls over this period were studied. There was a gradual but significant decrease in rates of oxygen uptake as the seedlings aged (Table 2). Table 3 shows there was also a concomitant decrease in response to SHAM with 10 day old seedlings showing no response to SHAM. However, in the presence of cyanide the respiration rates were always reduced by SHAM. This suggests that the alternative pathway is still present, although it may not be operating by day 10. Sensitivity to cyanide, CCP and DNP remained fairly constant throughout this period. CCP proved to be a more potent uncoupler than did DNP.

The response of 10 day old chilled seedlings to respiratory inhibitors during their recovery at 25°C is illustrated in Figure 2. The rate of oxygen uptake immediately after chilling was too low to be measured meaningfully, but the rate increased sharply up to 3 hours at 25°C (data not shown). Detailed assays of hypocotyl respiration were started at this point. Oxygen uptake declined from the maximum rate exhibited at 3 hours until by 12 hours it had stabilised at a rate equivalent to that of 8 day old unchilled seedlings. Throughout this respiratory burst (between 3 and 12 hours at 25°C) hypocotyl oxygen uptake showed a relatively constant degree of sensitivity to SHAM. However, when the seedlings were fully recovered, after 24 hours at 25°C, the hypocotyls were no longer sensitive to SHAM. The rate in
TABLE 2

Effect of Seedling Age on the Rate of Oxygen Uptake

Samples of 10, 0.5 cm lengths of cucumber hypocotyls were assayed in 10 mM TES buffer, pH 7.0, using a Rank Bros. Clark-type oxygen electrode (n = 8).

<table>
<thead>
<tr>
<th>Seedling Age</th>
<th>Rate of Oxygen Uptake. $(\text{O}_2 \cdot \text{min}^{-1} \cdot \text{g Fresh Wt.}^{-1})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 days</td>
<td>0.173 ± 0.050</td>
</tr>
<tr>
<td>9 days</td>
<td>0.153 ± 0.00</td>
</tr>
<tr>
<td>10 days</td>
<td>0.142 ± 0.026</td>
</tr>
</tbody>
</table>
Figure 2  Samples of 10, 0.5 cm lengths of cucumber hypocotyls were assayed for initial rates of oxygen uptake with a Rank Bros. Clark-type oxygen electrode. They were then reassayed after incubation in the test compound. (n = 8)

- X --- uninhibited rate
- ● --- rate in the presence of SHAM
- -X- --- rate in the presence of SHAM + KCN
- -▲- --- rate in the presence of KCN
- -◇- --- rate in the presence of DNP
- -△- --- rate in the presence of CCP
the presence of cyanide showed no change throughout this period, but
the oxygen uptake of chilled tissue was much less sensitive to cyanide
than was that of unchilled tissue (Table 3). The effect of DNP was
negligible at 3 hours recovery, but steadily increased as seedling
recovery progressed. CCP did uncouple respiration at this peak of
oxygen uptake although the uncoupling effect increased as the burst
subsided. The actual rate of oxygen uptake in the presence of CCP
remained constant throughout this period.

Direct effects of temperature on oxygen uptake. Although this study
was primarily aimed at the post-chilling respiratory burst of oxygen
uptake, it was thought useful to briefly investigate the direct effect
of temperature from 5 to 30°C on hypocotyl segments and on their re¬
response to inhibitors. The results are illustrated in Figure 3. Oxygen
uptake, either with or without SHAM, increased logarithmically with
increase in temperature. In the presence of KCN the curve showed a
two stage response; below 15 to 20°C the rate showed no appreciable
increase and only from 20°C did the rate increase exponentially.

To highlight any changes in $Q_{10}$, the data were plotted in logar¬
ithmic form against the reciprocal of absolute temperature (Figure 4).
There appeared to be an increase in $Q_{10}$ between 15 and 20°C in the
absence of inhibitors. Unfortunately, the equipment could not be
maintained at less than ±1°C, at low temperature, so more data points
could not be obtained. This may have helped to determine whether there
was an actual break-point in this plot. However, the data did reveal
TABLE 3

Effects of Respiratory Inhibitors on the Respiration Rates of 8, 9 and 10-Day-Old Unchilled Seedlings

Hypocotyl samples were assayed before and after incubation in the test compound in 10 mM TES buffer, pH 7.0.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Percent Initial rate of oxygen uptake</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>8 day old</td>
<td>9 day old</td>
<td>10 day old</td>
<td></td>
</tr>
<tr>
<td>$10^{-4}$ M SHAM</td>
<td>$70.7 \pm 11.0$</td>
<td>$81.6 \pm 11.1$</td>
<td>$101.9 \pm 8.2$</td>
<td></td>
</tr>
<tr>
<td>$10^{-4}$ M KCN</td>
<td>$45.3 \pm 5.4$</td>
<td>$43.8 \pm 6.1$</td>
<td>$56.9 \pm 5.9$</td>
<td></td>
</tr>
<tr>
<td>SHAM + KCN</td>
<td>$23.4 \pm 4.2$</td>
<td>$30.2 \pm 3.8$</td>
<td>$22.9 \pm 6.3$</td>
<td></td>
</tr>
<tr>
<td>$10^{-6}$ M DNP</td>
<td>$114.0 \pm 8.1$</td>
<td>$124.7 \pm 7.2$</td>
<td>$116.4 \pm 4.2$</td>
<td></td>
</tr>
<tr>
<td>$10^{-7}$ M CCP</td>
<td>$144.2 \pm 7.9$</td>
<td>$153.0 \pm 11.7$</td>
<td>$142.9 \pm 4.2$</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3  Samples of hypocotyls (10, 0.5 cm lengths/sample) were assayed for oxygen uptake in the presence or absence of $10^{-4}$ M SHAM or $10^{-4}$ M KCN, as a function of temperature.

- **X**  uninhibited rate
- **O**  rate in the presence of $10^{-4}$ M SHAM
- **A**  rate in the presence of $10^{-4}$ M KCN
Figure 4  A plot of the data from Figure 3 expressed as the log, (mean hypocotyl respiration, nmoles O₂/hour . g. fresh weight) against 1/absolute temperature.

- in the absence of inhibitors
- in the presence of 10⁻³ M KCN
- in the presence of 10⁻⁴ M SHAM
a distinct change in response to the inhibitors SHAM and KCN between 15 and 20°C. It appears that the inhibitory effects of SHAM increased at low temperature whereas cyanide lost its inhibitory effects at low temperature.

**Ethylene production after chilling.** Figure 5 illustrates ethylene production by 10 day old cucumber seedlings after 48 hours at 2°C. Seedlings that had not been sprayed with AVG, had the highest ethylene production at the earliest time of measurement, i.e. after 2 to 3 hours of recovery. The rate then declined until, by 24 hours, the rate was back to the level of production in the control unchilled plants. The control seedlings had a steady rate of ethylene production. AVG markedly decreased the level of ethylene production in unchilled seedlings and it also abolished the post-chilling burst of ethylene production.

**Relative contributions of the cytochrome and alternative respiratory pathways before and after chilling.** Bahr and Bonner's method (3) was applied to cucumber hypocotyl respiration to estimate, the proportion of the alternative pathway that is actually operating (see Appendix 1). The plot of $V_T$, (the total respiratory oxygen uptake of the mitochondria) against $g(i)$ (the maximal contribution of the alternative pathway at given concentrations of SHAM) gives (the slope of the resulting line) $\rho$. When $\rho = 0$ the pathway is not operative, when $\rho = 1$ it is fully operative.

These parameters were plotted for unchilled hypocotyls (Figure 6) and hypocotyls 6 hours (Figure 7) and 24 hours (Figure 8) after chilling.
Figure 5. 8 Day old cucumber seedlings were either chilled at 2°C or left at 25°C for 48 hours. The chilled seedlings were then returned to 25°C and assayed for ethylene production (each sample had 2 seedlings) after various time intervals. The total number of samples in each of 6 experiments per treatment was 10.

- - X - - unchilled 10 day old seedlings

- - - X - unchilled 10 day old seedlings sprayed with AVG on day 7.

- - O - - chilled 10 day old seedlings

- - - - chilled 10 day old seedlings sprayed with AVG on day 7.
Figure 6 A plot of $V_T'$ (total mitochondrial oxygen uptake) against $g(i)$ (the maximum contribution by the alternative pathway at a given concentration of SHAM) to estimate (the proportion of the alternative pathway that is actually operating) for unchilled cucumber hypocotyl tissue, ($n = 7$)

- O — non-AVG treated tissue
- X — AVG treated tissue
Figure 8  A plot of $V_T$ against $g(i)$ to estimate of hypocotyl tissue 24 hours after chilling. ($n = 6$)

- O -  non-AVG treated tissue
- X -  AVG treated tissue
with and without AVG treatment. The alternative pathway is apparently not engaged in AVG-treated unchilled tissue, since $p = 0$, and only to a low extent in unchilled tissue that has not been sprayed with AVG (Figure 6). The greater slopes in Figure 7 indicate that the alternative pathway is engaged to a much greater extent 6 hours after chilling. Blocking the post-chilling surge of ethylene biosynthesis with AVG had only a small effect on this use of the alternative pathway during recovery.

After 24 hours the slopes are much less, indicating that the flux through the alternative pathway has diminished. Once again, at 24 hours recovery, blocking the post-chilling ethylene with AVG had no effect on the degree to which the alternative pathway was engaged.

Table 4 summarises the various components of oxygen uptake and the values obtained for $p$. Although the increased use of the alternative pathway did not account for all of the increase in oxygen uptake at 6 hours of recovery, it did account for a sizeable portion of the increase. After 24 hours when the tissue is fully recovered, and the past the post-chilling burst of oxygen uptake, the flux through the alternative pathway had declined to 9% (non-AVG treated tissue) or 8% (AVG treated tissue) of its full capacity.

It was noted that there was a difference in the actual rates of oxygen uptake and sensitivity to SHAM between the data shown in Tables 2 and 3 and that shown in Table 4, although the data within these blocks of experiments were highly consistent. The seedlings used to obtain the first set of data (Tables 2 and 3) were from old seeds (more
The seedlings were chilled at 2°C, in a relative humidity of 95% for 48 hours. They were sprayed with either AVG or distilled water 24 hours before chilling. (n = 6 or 7) If $r = 0.90$, the data gave a good fit to a straight line of slope $r$. Consequently, is a good indication of the actual proportion of the alternative pathway that is operating.

<table>
<thead>
<tr>
<th>Component</th>
<th>Unchilled</th>
<th>6 Hr after chilling</th>
<th>24 hr after chilling</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{res}}$</td>
<td>$16 \pm 1$</td>
<td>$25 \pm 3$</td>
<td>$22 \pm 3$</td>
</tr>
<tr>
<td>$V_{\text{cyt}}$</td>
<td>$78 \pm 8$</td>
<td>$96 \pm 8$</td>
<td>$90 \pm 8$</td>
</tr>
<tr>
<td>$V_{\text{alt}}$</td>
<td>$46 \pm 4$</td>
<td>$70 \pm 5$</td>
<td>$70 \pm 5$</td>
</tr>
<tr>
<td>$V_T'$</td>
<td>$79 \pm 3$</td>
<td>$140 \pm 3$</td>
<td>$138 \pm 5$</td>
</tr>
</tbody>
</table>

$r = 0.13$ (if $r = 0.90$)

<table>
<thead>
<tr>
<th>% of $V_T'$</th>
<th>0.370</th>
<th>0.455</th>
<th>0.13</th>
<th>0.13</th>
</tr>
</thead>
<tbody>
<tr>
<td>by the alt. pathway</td>
<td>$(r = 0.98)$</td>
<td>$(r = 0.97)$</td>
<td>$(r = 0.79)$</td>
<td>$(r = 0.86)$</td>
</tr>
</tbody>
</table>

actual rate through the alt. pathway | 6 | 0 | 27 | 32 | 8 | 7 |
TABLE 5
Effects of Short-Term Chilling Prior to Extraction on Malate Oxidation by Isolated Cucumis Mitochondria

* Rates are expressed as nmoles O₂/mg mitochondrial protein.minute. for 0 and 2 hr. n = 8, for 1, 4 and 6 hr. n = 6.

<table>
<thead>
<tr>
<th>Hr. at 4°C</th>
<th>Respiration rates</th>
<th>ADP : 0</th>
<th>R.C.</th>
<th>% Initial uninhibited</th>
<th>State 3 rate with inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>State 3</td>
<td>State 4</td>
<td></td>
<td></td>
<td>50 μM KCN</td>
</tr>
<tr>
<td>0</td>
<td>1.87</td>
<td>1.22</td>
<td>2.52</td>
<td>1.57</td>
<td>30.71</td>
</tr>
<tr>
<td></td>
<td>± 0.03</td>
<td>± 0.02</td>
<td>± 0.3</td>
<td>± 0.11</td>
<td>± 5.89</td>
</tr>
<tr>
<td>1</td>
<td>0.85</td>
<td>0.67</td>
<td>2.18</td>
<td>1.4</td>
<td>5 .95</td>
</tr>
<tr>
<td></td>
<td>± 0.02</td>
<td>± 0.03</td>
<td>± 0.02</td>
<td>± 0.4</td>
<td>± 0.19</td>
</tr>
<tr>
<td>2</td>
<td>1.01</td>
<td>0.80</td>
<td>1.54</td>
<td>1.24</td>
<td>48.20</td>
</tr>
<tr>
<td></td>
<td>± 0.01</td>
<td>± 0.01</td>
<td>± 0.3</td>
<td>± 0.3</td>
<td>± 0.95</td>
</tr>
<tr>
<td>4</td>
<td>0.51</td>
<td>0.51</td>
<td>1.2</td>
<td>1.13</td>
<td>50.01</td>
</tr>
<tr>
<td></td>
<td>± 0.01</td>
<td>± 0.01</td>
<td>± 0.4</td>
<td>± 0.2</td>
<td>± 14.42</td>
</tr>
<tr>
<td>6</td>
<td>0.1</td>
<td>0.1</td>
<td>0</td>
<td>1</td>
<td>91.50</td>
</tr>
<tr>
<td></td>
<td>± 0.02</td>
<td>± 0.01</td>
<td></td>
<td></td>
<td>± 21.97</td>
</tr>
</tbody>
</table>
It was also noticeable that mitochondria isolated after 6 hours of chilling had lost most of their sensitivity to KCN (Table 5). Further, it was noted that mitochondria isolated after 4 or 6 hours of prior chilling behaved differently to KCN in the following way. On addition of KCN oxygen uptake was not inhibited immediately, as it was in mitochondria from unchilled tissue, but rather there was a momentary increase in apparent oxygen uptake which lasted 1 to 2 minutes before settling down to a slower inhibited rate. This occurred whether or not SHAM was present.

It therefore seems that mitochondria are susceptible to chilling injury, and the effects are progressive with the time held at low temperature. Although the mitochondria from unchilled tissue showed good activity it seems likely that the isolation procedure at low temperature may have some effect on the mitochondrial properties. Therefore, detailed analyses of the relative contributions of the two pathways, using isolated mitochondria, were not done.

The characteristics of the mitochondria from unchilled hypocotyls are shown in Tables 6 and 7. The organelles showed good ADP: 0 ratios with reasonable R.C. with malate and succinate, and were able to oxidise exogenous NADH with an ADP : 0 ratio close to 2 (as would be expected, see schematic in Figure 1). Ascorbate oxidation was extremely rapid. In the absence of substrate, with or without ADP, the rates of oxygen uptake were very low which indicates that most of the activity can be attributed to mitochondria rather than to extraneous oxidative contaminants.
TABLE 6

Properties of Isolated Mitochondria from Unchilled Cucumis Hypocotyls

*Respiration rates are expressed as nmoles O₂/mg protein.minute, n = 10

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Respiration Rates*</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>State 3</td>
<td>State 4</td>
<td>ADP : 0</td>
<td>R. C.</td>
</tr>
<tr>
<td>malate + pyruvate</td>
<td>2.78 ± 0.54</td>
<td>1.9 ± 0.24</td>
<td>2.5 ± 0.03</td>
<td>1.37 ± 0.09</td>
</tr>
<tr>
<td>succinate</td>
<td>3.92 ± 1.10</td>
<td>2.90 ± 0.47</td>
<td>1.91 ± 0.3</td>
<td>1.21 ± 0.07</td>
</tr>
<tr>
<td>ascorbate</td>
<td>28.05 ± 3.7</td>
<td>26.35 ± 3.38</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>NADH</td>
<td>6.7 ± 1.72</td>
<td>4.89 ± 1.3</td>
<td>1.8 ± 0.2</td>
<td>1.18 ± 0.0</td>
</tr>
<tr>
<td>no substrate no ADP</td>
<td>0.09 ± 0.04</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>no substrate + ADP</td>
<td>0.22 ± 0.08</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
TABLE 7
Properties of Isolated Mitochondria from Unchilled *Cucumis* Hypocotyls

The concentrations represent the final concentrations in the reaction chamber of the oxygen electrode.

\( n = 10 \)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Percent of initial uninhibited State 3 respiration rate, in inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 ( \mu M ) CCP</td>
</tr>
<tr>
<td>malate + pyruvate</td>
<td>131.33 ± 1.1</td>
</tr>
<tr>
<td>succinate</td>
<td></td>
</tr>
<tr>
<td></td>
<td>83.43 ± 6.25</td>
</tr>
<tr>
<td>NADH</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.15 ± 11</td>
</tr>
<tr>
<td>ascorbate</td>
<td>100</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Substrate</th>
<th>A/A rotenone rotenone SHAM + A/A + KCN + SHAM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 g/ml 50 M + A/A + SHAM</td>
</tr>
<tr>
<td>malate + pyruvate</td>
<td>50.81 ± 12</td>
</tr>
<tr>
<td>succinate</td>
<td>57.38 ± 8.53</td>
</tr>
<tr>
<td>NADH</td>
<td>52.9 ± 5.3</td>
</tr>
<tr>
<td>ascorbate</td>
<td>100</td>
</tr>
</tbody>
</table>
Oxidation of malate, succinate and NADH were all sensitive to SHAM; that of succinate being the most sensitive. Succinate oxidation was the least sensitive to KCN. The oxidation of all 3 substrates was inhibited by Antimycin (A/A) to the same extent, and the effects of A/A and KCN on malate and NADH oxidation were very similar.

Rotenone was less inhibitory to malate oxidation than were SHAM and KCN and as would be expected it had little effect on the oxidation of succinate and NADH.

The oxidation of ascorbate behaved as was expected; KCN totally blocked its oxidation whilst the other inhibitors had no effect.
It has been well documented that after chill-sensitive tissues are kept at a low temperature, they exhibit a post-chilling respiratory burst of oxygen uptake (21, 46). Cucumber hypocotyls proved to be no exception, displaying an increase of greater than 50% over the unchilled rate during the recovery period. The question that arose was whether the alternative pathway could account for this increase in oxygen uptake.

The data in Table 3 and in Figure 2 demonstrate that during this respiratory burst, the respiration is much more sensitive to SHAM. A more detailed analysis (Figures 6, 7, 8 and Table 1*) revealed that the alternative pathway was operating to a much greater extent during this respiratory burst than it was before or after. Although the flux through this pathway did not account for all of the increase, it did account for a sizeable portion (approximately 50%) of it.

Solomos and Laties have noted that there is a relationship between the effects of ethylene on respiration and the alternative pathway (109, 110). The pathway for ethylene biosynthesis has now been determined and it has been demonstrated that AVG has a specific site of action at a crucial step along this pathway (1, 2). Consequently, AVG was used to block the post-chilling rise of ethylene production; this it did very effectively as can be seen in Figure 5. However, the
suppression of ethylene production had no effect on the increased flux through the alternative pathway, which would argue against ethylene playing a role in the control of the alternative pathway.

The direct effects of temperature on oxygen uptake were found to be very interesting. It would appear that low temperature induces a switch in electron flow from the cytochrome pathway to the alternative pathway. This was exactly what Yoshida and Tagawa described in a recent paper for oxygen uptake by mitochondria from a chill-sensitive Cornus callus culture line (114). Obviously more detailed work needs to be done to determine the exact temperature at which this switch occurs.

Chilling sensitivity has been attributed, to a large extent, to critical changes in the membrane fluidity (45, 46, 48, 105). In order for electron flow down the respiratory chain, the components of the cytochrome respiratory chain have to be in closely associated precise orientation. Schneider and co-workers have demonstrated that with soybean mitochondria increasing the lipid content of the inner membrane dispersed the membrane proteins hence destroying their precise orientation, and causing a large reduction of respiratory oxidation (82). It is well documented that the inner mitochondrial membrane has a very high protein to lipid ratio (24). Thus it might be expected that any phase changes in the inner mitochondrial membrane would have drastic effects on the orientation of the respiratory chain components with a concomitant reduction of electron flow through the cytochrome chain. From the data described in this thesis it would seem that the alternative pathway may become more active and partially compensate for this. The
present evidence indicates that the alternative pathway consists of far fewer components than the cytochrome pathway, consequently it would be expected to be much less sensitive to lipid phase-change disruption (32, 57, 89). The changes in response of hypocotyl oxygen uptake to SHAM and KCN occurred immediately the temperature was lowered, which suggests that the switching of the cytochrome pathway off and the alternative pathway on, occurred immediately. This is in keeping with the membrane phase-change theory. This then raises the possibility that the post-chilling respiratory burst of oxygen uptake might arise because the alternative pathway, turned on during chilling, is still operating during the initial recovery period.

Theologis and Laties have demonstrated by using uncouplers, that for ripening fruits and ageing wounded tissue, the rise in respiration can be largely accounted for by the unused capacity of the cytochrome pathway, and it appeared that the alternative pathway only became active when the cytochrome pathway was operating near full capacity (99, 101, 102). This would not seem to be the case in the respiratory rise seen after chilling cucumber seedlings, since the uncoupler CCP showed that even at the peak of the respiratory rise the 2 pathways were not operating at full capacity. However, the analysis would have to be repeated in the presence of uncoupler to say definitively whether the cytochrome pathway is operating at full capacity during the respiratory burst. An earlier report suggested that respiration became completely uncoupled after chilling (17), but DNP was used as the uncoupler; as shown in this study DNP is much less effective as an
know whether peroxide is being produced to a greater extent the longer the mitochondria have been chilled. This might be the reason why antimycin A inhibited oxygen uptake far more than did KCN, although if KCN was acting solely on the cytochrome oxidase these 2 inhibitors shoudl have the same effect. A greater production of $H_2O_2$ at low temperature could also be envisaged as a possible source of chilling injury.

The patterns of oxidation of malate, NADH succinate and ascorbate and their responses to inhibitors were as expected (from the way that they feed into the respiratory chain), although malate oxidation did not appear to be fully sensitive to rotenone (Tables 6 and 7). From the data it can be tentatively said that electrons from succinate have a greater tendency to flow through the alternative oxidase. Succinate dehydrogenase has been found to be closely associated with the alternative pathway (59, 75).

The results described in this thesis and by Yoshida and Tagawa (114) point to the alternative pathway being switched on in response to low temperature. If this is so it raises the question of whether the reports of alternative oxidase activity in isolated plant mitochondria from various sources might be a consequence of the low temperature during isolation and not necessarily an inherent property of the tissue concerned; such extrapolations must be done with caution.

It is also interesting that the alternative pathway switches on in response to high temperature (11). It may be that the role of the alternative pathway is one of protection against both high and low
temperatures, in reserve for the failure of the more sensitive cytochrome pathway. If the alternative pathway does indeed have a role, three possibilities can be envisaged. Firstly, although electron flow through the alternative pathway bypasses two phosphorylation sites it is still tied to one phosphorylation site (when electrons are being donated via endogenous NADH). So, although not as efficient as the cytochrome pathway, it could still allow for some ATP formation. Secondly, it could be envisaged as a mechanism for allowing the continued turnover of the Krebs cycle. This provides key intermediates for metabolism and it might be that these are needed for repair of temperature-stress-induced injury. A third possibility, referring specifically to low temperature stress, could be that switching on of the alternative pathway as a response to low temperature represents an attempt (albeit futile or no), at thermogenesis; to locally raise the temperature to enable repair of any injury resulting from chilling. Thus, it would be acting a role analogous to that found in avoid spadices. Obviously much more work would have to be done before any role could be assigned to the switching on of the alternative pathway in response to low temperature.

**Concluding Remarks**

It has been suggested that the post-chilling burst of respiratory activity is a result of the build-up of toxic intermediates from respiration (60). There has always been the question of how the plant accommodates this rapid rise in respiratory activity when returned to
normal temperatures. The results from cucumber hypocotyls described in this thesis suggest that the alternative pathway can account for some of this rise, but not all of it. However because it would appear that the alternative pathway switches on in response to low temperature, it may be that this respiratory rise is an after effect of changes in respiratory activity that occurs during chilling, and not solely a response to the build-up of toxic intermediates. The respiratory rise corresponds to the time when the respiratory cytochrome activity has recovered and the alternative pathway is still operating.


Calculation of the Relative Contributions of the Cytochrome and Alternative Respiratory Pathways

Bahr and Bonner (3) described the overall respiration rate \( V_T \) as:

\[
V_T = \rho \ g(i) + V_{cyt} + V_{res}
\]

where \( g(i) \) is the maximum contribution of the alternative pathway at a given concentration of SHAM; \( \rho \) is the proportion of this pathway that is operating; \( V_{cyt} \) is the rate through the cytochrome pathway and \( V_{res} \) is the residual rate of oxygen uptake (non KCN or SHAM sensitive oxygen uptake due to non-mitochondrial oxidation). \( V_{res} \) can be determined and subtracted from \( V_T \) to give \( V_{T'} \):

\[
V_{T'} = \rho \ g(i) + V_{cyt}
\]

If the assumption is made that when electron flow through the alternative pathway is blocked by SHAM there is not a switch of these electrons down the cytochrome pathway, then a plot of \( V_{T'} \) against \( g(i) \) should give a straight line, the slope of which will be \( \rho \). A good correlation coefficient \( r \) for such a plot indicates that this assumption is probably valid and thus enables the determination of the actual activity of the alternative pathway with some confidence.

The function \( g(i) \) can be determined by titration with SHAM in
the presence of $10^{-4}$ M KCN (which eliminates $V_{\text{cyt}}$). When $\text{SHAM} = 0 \ g(i) = V_{\text{alt.}}$, the maximum capacity through the alternative pathway.

The titration with SHAM is then repeated but in the absence of KCN. The rates in the presence of each given concentration of SHAM, expressed as % initial uninhibited rate ($V_{T,i}$) are then plotted against $g(i)$ to estimate $\rho$.

The titration curves for SHAM in the presence of KCN, against oxygen uptake $g(i)$ are shown in Figures 9, 10 and 11.
Figure 11 Plot of respiration rate against the concentration of SHAM in the presence of KCN ($10^{-4}$ M) for hypocotyls 24 hours after chilling.

---

- ○ non-AVG treated
- × AVG treated
Figure 9  Plot of the respiration rate (expressed as % initial uninhibited rate) against the concentration of SHAM in the presence of KCN \(10^{-4} \text{ M}\) for unchilled hypocotyls.

- **O** non-AVG treated
- **X** AVG treated
Figure 10  Plot of respiration rate against the concentration of SHAM in the presence of KCN ($10^{-4}$ M), for hypocotyls 6 hours after chilling.

- O non-AVG treated
- × AVG treated
APPENDIX II

Calculation of ADP : O and R. C. Ratios

A characteristic trace of oxygen uptake by mitochondria isolated from unchilled cucumber hypocotyls is shown in Figure 12.

ADP : O ratios can easily be calculated since a known amount of ADP is added and the amount of oxygen consumed during the State 3 respiration can be determined from the trace. The oxygraph was calibrated such that a 0 - 100% deflection represented 690 nmoles of O₂.

Respiratory control ratios (R.C.) are expressed as State 3 rate / subsequent State 4 rate of oxygen uptake.
Figure 12 A representative oxygraph trace of mitochondrial oxygen uptake