LIPOXYGENASE; THE CYANIDE-INSENSITIVE RESPIRATORY COMPONENT IN CRUDE WHEAT MITOCHONDRIAL PREPARATIONS

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Richard Shingles

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ABBREVIATIONS

ADP	adenosine-5' diphosphate
ATP	adenosine-5' triphosphate
BHAM	benzhydroxamic acid
BSA	bovine serum albumin
EDTA	ethylenediaminetetraacetic acid
LOX	lipoxygenase
NADH	β -nicotinamide adenine dinucleotide (reduced form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
PG	n-propyl gallate
Pi	inorganic phosphate
PMF	proton motive force
PVP	polyvinylpyrrolidone
RC	respiratory control
SHAM	salicylhydroxamic acid
TES	N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid
Tris	tris(hydroxymethyl)aminomethane

ABSTRACT

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When wheat seedlings were exposed to freezing and icing conditions they accumulated anaerobic metabolites such as methanol, ethanol and acetaldehyde. These same plants also showed increased levels of cyanide-insensitive oxygen consumption.

Cyanide-insensitive respiration and lipoxygenase activity were measured in crude and purified wheat mitochondrial preparations. High CO_2 in O_2 growth conditions stimulated development of cyanide-insensitive respiration and lipoxygenase activity. Utilization of a percoll gradient resulted in cyanide-insensitive respiration and lipoxygenase activity being distributed in the non-mitochondrial portions of the gradient. Cyanide-insensitive respiration and lipoxygenase activity were unaffected by the addition of disulfiram but were inhibited by salicylhydroxamic acid and propyl gallate.

L-malate, succinate and valinomycin which induced swelling in mitochondria stimulated cyanide-insensitive oxygen uptake. The fatty acid content of mitochondria decreased when they were swollen and this effect was enhanced by the presence of phospholipase A_2. Reconstitution of cyanide-insensitive oxygen uptake was achieved by adding commercial soybean lipoxygenase and phospholipase A_2 to percoll purified mitochondria. The correlation of malate oxidation to measured oxygen consumption of mitochondria in the presence of cyanide was low.

INTRODUCTION

Polemics are discussions involving controversy or dispute. Every rapidly expanding area of investigation can generate polemics. These are usually based upon unrecognized differences in experimental conditions or misinterpretation of results due to the presence of artifacts. To the people working in such areas of dispute these conflicts can become confusing and misleading. Arguments can become biased just by the sheer volume of published material alone. However the ability to recognize a polemic and to divide it into its component parts in terms of all the arguments involved is the first step towards resolving such controversies.

Cyanide-insensitive respiration has been extensively studied over the past 20 years. Reports of its occurence in plants were so widespread that some researchers believed it to be ubiquitous among plants. After being first discovered in the aroids this path was believed to be responsible for the thermogenic response allowing the odiferous principles to be volatilized (Meeuse, 1975). With cyanide-insensitive respiration being subsequently discovered in germinating seeds (Yentur and Leopold, 1976), ripening fruits (Theologis and Laties, 1978b) and wounded tissues (Kinraide and Marek, 1980), its function has been directed away from that of heat production. Other functions of this pathway were proposed but to date none of them have become widely accepted. Cyanide-insensitive respiration is considered to be of mitochondrial origin however many of the observed effects have been measured using crude mitochondrial preparations or slices of plant material. Another problem with the studies on cyanide-insensitive respiration is the failure to isolate and determine what the terminal electron acceptor is and whether H_2O or peroxides are formed as

final products.

Lipoxygenase activity has been implicated with cyanide-insensitive respiration in some reports (Siedow and Girvin, 1980). The confusion arises due to the fact that lipoxygenase is cyanide-insensitive, SHAM sensitive, forms peroxide products and consumes oxygen not unlike the alternative pathway (Parrish and Leopold, 1978). Although lipoxygenase is a cytosolic enzyme it does appear in crude mitochondrial preparations. Its distribution coincides with cyanide-insensitive respiration being found in germinating seeds, ripening fruits and in wounded tissues (Eskin et al., 1977).

There are some problems in trying to account for cyanide-insensitive respiration as being due to lipoxygenase. The first of these is the difficulty in distinguishing between lipoxygenase activity and the cyanide-insensitive path since they are both affected in a similar manner by the same inhibitors (Siedow and Girvin, 1980). There is also the problem of accounting for the observed stimulation of the cyanide-insensitive path upon the addition of Krebs cycle substrates, more typical of a mitochondrial response (Goldstein et al., 1980).

To this extent it was believed that a general study was warranted to contribute to the understanding of this polemic of plant science as it relates to wheat mitochondria.

LITERATURE REVIEW

Cyanide-Insensitive Respiration

The observation that the respiration of Krebs cycle substrates by mitochondria was not fully inhibited by concentrations of cyanide high enough to completely block cytochrome oxidase led to the discovery of cyanide-insensitive respiration. Formulation of the dual pathway concept goes back to 1939 when Okunuki discovered the cyanide and carbon monoxide insensitivity of respiration in Lilium auratum pollen. This phenomenom was also reported in slices of Sauromatum spadix by Van Herk (1937). Since then many plant tissues have yielded mitochondria with respiration insensitive to inhibition by cyanide. The two most notable tissues that have been studied are the spadices of the flowers of Arum maculatum (James and Beevers, 1950) and the skunk cabbage Symplocarpus foetidus (Hackett and Haas, 1958).

Mitochondria isolated from resistant tissues were found to contain a "dual pathway" for respiratory electron transfer: the classical cyanide sensitive electron transport chain which is coupled to phosphorylation and a cyanide-insensitive pathway which branches from the respiratory chain at the flavoprotein level on the substrate side of cytochrome b (Bendall and Bonner, 1971). Storey and Bahr (1969) postulated that the alternative oxidase was a non-heme protein and might actually be a flavoprotein itself.

In 1971 Schonbaum et al. reported the specific inhibition of the cyanide-insensitive respiratory pathway in plant mitochondria by hydroxamic acids. Using these inhibitors it was possible to study the mode of action of the two pathways independently. The relative contr-

ibution of each pathway to total respiration could also be determined (Bahr and Bonner, 1973). In general cyanide-insensitive respiration is said to occur when tissues or isolated mitochondria show cyanide insensitivity and SHAM sensitivity.

Energy Conservation of the Alternative Pathway

In terms of energetics the cyanide-insensitive path is considered to be uncoupled and incapable of energy conservation (Wilson, 1980). With malate as a substrate cyanide addition reduces the ADP/O ratio by 2/3 as would be expected if the branch point of the alternative pathway occured after the first site of phosphorylation (Solomos, 1977). With succinate as a substrate cyanide addition completely abolishes ATP formation (Storey and Bahr, 1969). In a fully operational alternative pathway with ATP production reduced by 2/3 there would be a greater requirement for electron flux down the path to generate all the necessary ATP's for metabolic purposes. This raises the question as to what happens to all the potential energy when electrons travel via the alternative pathway. One way in which this energy may be dissipated is by heat evolution. In Symplocarpus this heat is believed to volatilize the odiferous principles that attract the pollinators which is why this plant is commonly known as skunk cabbage. Sometimes the temperature difference with the environment may be as much as 63^{o}C (Knutson, 1979).

This interesting thermogenic response led investigators to think of cyanide-insensitive respiration as being involved in cold-tolerancy and cold-hardiness in plants especially since the mitochondria from all plants were structurally similar whether they were cyanide-

sensitive or insensitive (Bonner, 1961).

Distribution of Cyanide-Insensitive Respiration

Cyanide-insensitive respiration was soon found to be quite widely distributed both in terms of the different species of plants it was found in and in terms of its location in various parts of the plant.

During the ripening of fruits there is a sharp rise in respiration called the climacteric which leads to the loss of integrity of the cells of the fleshy fruit (Solomos and Laties, 1974). Ethylene is evolved during the climacteric and is implicated in the fruit ripening process. Solomos and Laties (1974) studied the climacteric of ripening avocados and found that cyanide gas also induced a rapid increase in the rate of respiration followed by a rise in ethylene production. Later studies by Theologis and Laties (1978b) revealed that although the cyanide-insensitive path was present in ripening avocado and banana fruits there was no direct evidence for its operation being essential for the respiratory climacteric and fruit ripening.

A further search for similarities between ethylene and cyanide action revealed an operative cyanide-insensitive pathway in potato tubers (Solomos and Laties, 1975). In particular it was found that the cyanide-insensitive electron transport pathway would develop upon aging of potato tuber slices (Dizengremel and Lance, 1976). These same observations were also made on sweet potato slices (Theologis and Laties, 1978a).

It was shown by Wilson and Bonner (1971) that the mitochondria of peanut seeds actually lacked cytochrome c for a period of some 16 hours after germination. Hence it was proposed that during this period respiration of the seeds must be entirely through the alternative pathway.

A survey of seven different types of seeds revealed changing sensitivity to cyanide and SHAM (Yentur and Leopold, 1976). The seeds studied were mungbean, oat, lettuce, corn, tomato, pea and soybean. Some question as to the activity of a mitochondrial cyanide-insensitive pathway in germinating seeds still occurs since it was suggested by Roberts (1969) that such seeds have active pentose phosphate pathways which would also be cyanide-insensitive.

Cyanide-insensitive respiration has also been discovered in the cells of the yeast-like fungus Moniliella tomentosa (Vanderleyden et al., 1978), in pea cotyledons (James and Spencer, 1979) and in wounded Bryophyllum tubiflorum leaves (Kinraide and Marek, 1980).

Effectors

The synergistic effect of ethylene and cyanide has already been mentioned. Along with these compounds several others have been found to induce the alternative pathway.

Carbon dioxide was very effective in causing cyanide resistance to develop in aged potato slices (Lange, 1970). McCaig and Hill (1977) found that carbon dioxide could increase cyanide-insensitivity in wheat coleoptiles however a combination of 20-25% CO_2 in O_2 stimulated cyanide-insensitive respiration to a very large extent.

Rychter et al. (1979a) reported a similar stimulation of cyanide-insensitive respiration in potato tubers with ethylene in O_2. Application of many volatiles such as ethanol, acetaldehyde and acetic acid also lead to a climacteric-like upsurge in respiration (Rychter et al., 1979b). This increase was also markedly enhanced when the volatiles were applied in 100% O_2.

Inhibitors

Cyanide-insensitive respiration was first shown to be inhibited by 2,2'-dipyridyl and 8-hydroxyquinoline (Bendall and Bonner, 1971). These compounds are iron chelators hence it was initially believed that the alternate oxidase contained an iron centre. However these compounds also inhibited cytochrome electron transfer at concentrations only slightly above those needed to inhibit cyanide-insensitive respiration (Bahr and Bonner, 1973). The first real breakthrough came when Schonbaum (1971) reported that hydroxamic acids were specific inhibitors of cyanide-insensitive respiration at very low concentrations.(Figure 1).

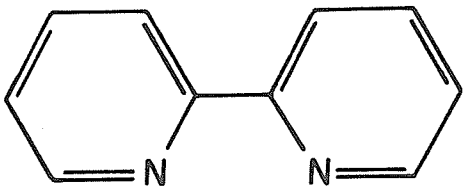
Piericidin A, a classic inhibitor of electron transport in the region of phosphorylation site I also inhibited the alternative pathway but the cytochrome path was also inhibited with its use (Wilson, 1971).

One of the more recent inhibitors found was disulfiram (Grover and Laties, 1978) (Figure 1). This compound has a very low Ki of 15uM and has minor effects on the cytochrome chain. Inhibition of the alternative path was not reversible by transition metal ions although thiol compounds such as mercaptoethanol could reverse and prevent inhibition by disulfiram. Kinetic analysis indicated that SHAM and disulfiram have separate and independent binding sites.

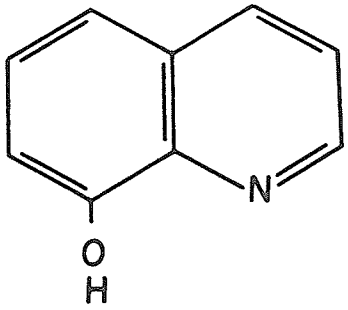
Parrish and Leopold in 1978 reported that the respiration of soybean particles was characterized by a burst of O_2 consumption during the first few minutes of imbibition. This respiratory burst was not inhibited by cyanide but was inhibited by SHAM. More important however was that the initial respiratory activity was markedly inhibited by propyl gallate, being some ten times more effective than SHAM. Propyl gallate is a known inhibitor of the lipoxygenase reaction (Yasumoto et al.,

Figure 1.

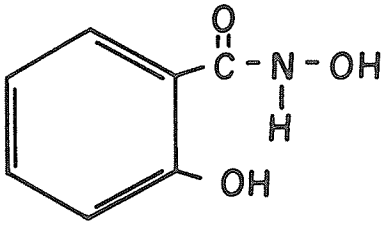
Some inhibitors of cyanide-insensitive respiration and their structures.



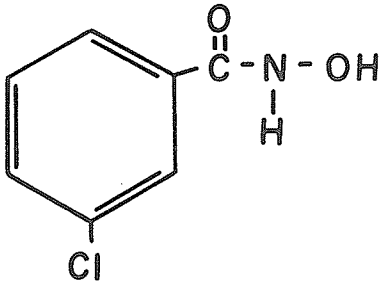
2 , 2 dipyridyl



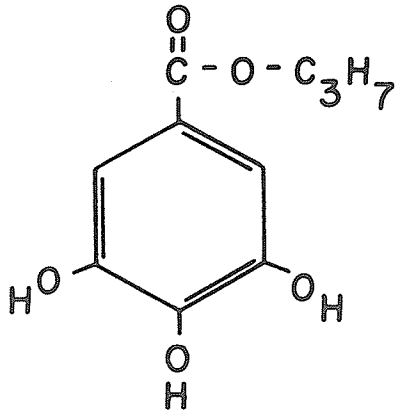
8 - hydroxyquinoline



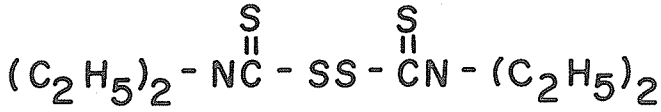
SHAM



m-CLAM



n - Propyl Gallate



Disulfiram

1970) presumably by acting as a free radical quencher.

Initially it was thought that one could distinguish between the alternative pathway and lipoxygenase activity through the use of PG and SHAM. However Siedow and Girvin (1980) indicated through kinetic studies that PG seemed to act at the same site as SHAM to inhibit the alternative pathway. Rich et al. (1978) suggested that inhibition of the alternative pathway by hydroxamic acids might be due to competition with the reducing substrate of the alternative oxidase, the most reasonable candidate being some species of reduced ubiquinone. The phenolic similarities of PG and SHAM could result in similar inhibition at a common site.

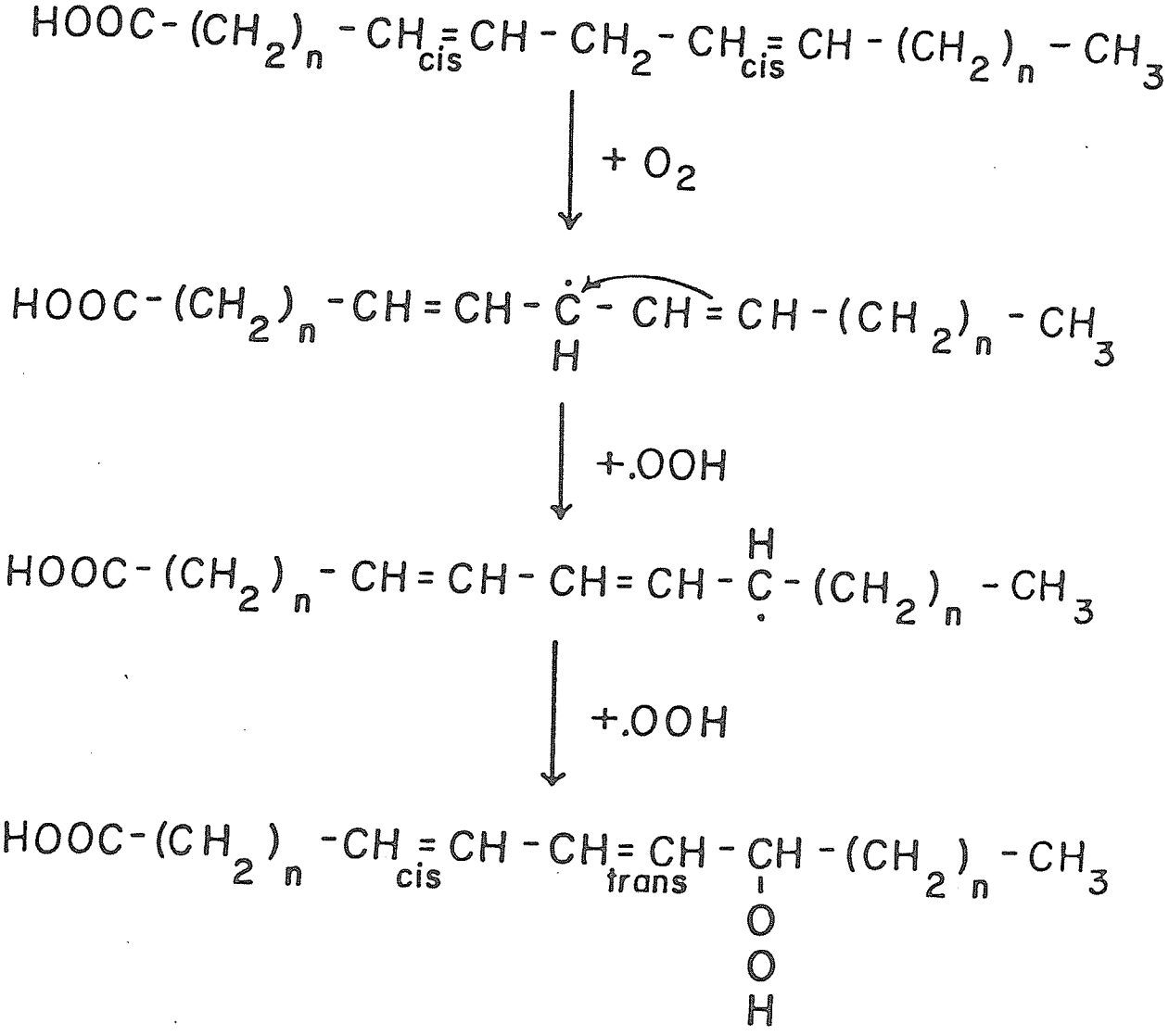
While the competition theory for the inhibition of the alternative pathway is backed by some kinetic data one possibility still not extensively studied is the antioxidant properties of SHAM itself which have been shown to inhibit the lipoxygenase reaction in much the same manner as PG (Parrish and Leopold, 1978). Siedow and Bickett (1981) have recently reported that for PG and 19 of its analogs the trihydroxy substituent and not the ester functional group was required for inhibition (Figure 1).

Lipoxygenase

Lipoxygenase, formerly called lipoxidase, catalyzes the hydroperoxidation of polyunsaturated fatty acids containing a cis,cis-1,4 pentadiene system (Eskin et al., 1977). The possible reaction mechanism is outlined in Figure 2. The first references to lipoxygenase concerned the bleaching of wheat flour and dough following the addition of small amounts of soybean flour (Haas and Bohn, 1934). The reaction involves the consumption of oxygen as shown in preparations of Lupinus albus by

Figure 2.

Possible mechanism of the lipoxygenase reaction with linoleic acid as the substrate. (From Goodwin and Mercer, 1972).


HOOC-(CH2)n-CH=CH-CH2-CH=CH-(CH2)n-CH3
cis
cis
+ O2
HOOC-(CH2)n-CH=CH-C-CH=CH-(CH2)n-CH3
H
+.OOH
HOOC-(CH2)n-CH=CH-CH=CH-C-(CH2)n-CH3
H
+.OOH
HOOC-(CH2)n-CH=CH-CH=CH-CH-(CH2)n-CH3
cis
trans
O
O
H

Craig (1936). Sumner and Sumner (1940) recognized the enzyme as being identical to carotene oxidase. The enzyme was first isolated in crystalline form from soybean by Theorell in 1947. Since then lipoxygenase has been isolated and purified from many other plant tissues and more recently from animal tissues (Greenwald et al., 1980). Most of the interest in lipoxygenase is generated in the food industry where it exerts effects on baking technology, lipid binding, dough development, lipid peroxidation and gluten oxidation (Eskin et al., 1977).

Distribution of Lipoxygenase

Lipoxygenase is widely distributed in plant tissues. The enzyme has been found in the seeds of small faba beans (Eskin and Henderson, 1974), lentils and lupins (Eskin and Henderson, 1977) and in germinating watermelon seedlings (Vick and Zimmerman, 1976). Other tissues which have lipoxygenase activity and show cyanide-insensitive respiration include potato tubers (Galliard and Phillips, 1971), soybeans (Guss et al., 1967), peas (Eriksson and Svensson, 1970) and wheat (Irvine and Anderson, 1953). An indication of the widespread occurence of lipoxygenase is a report on its presence in human platelets (Greenwald et al., 1980).

Lipoxygenase activity is prominent during the early stages of the climacteric in fruits but falls off rapidly in the later stages (Mapson and Wardale, 1971). This pre-climacteric burst of lipoxygenase activity preceeds ethylene synthesis. Addition of lipoxygenase to isolated systems containing linolenic acid was demonstrated to enhance ethylene production (Galliard et al., 1968). This and other evidence suggested that ethylene was either synthesized or regulated by the lipid hydroperoxides formed by the lipoxygenase reaction. However ^{14}C-linolenate

studies failed to confirm fatty acid hydroperoxides as precursors of ethylene (Mapson et al., 1970). Further research is still required in this area to determine the involvement of lipoxygenase in the production of ethylene in vivo.

Properties of Lipoxygenase

The major substrates in plants for lipoxygenase activity are linoleic and linolenic acids (Eskin and Henderson, 1974). To a lesser degree trilinolein, dilinolein, monolinolein and methyl linoleate serve as substrates. Arachidonic acid can also act as a substrate but its level in plants is fairly low (Edwards, 1964).

The product formed is a conjugated cis-trans hydroperoxide (Christopher and Axelrod, 1971). Although lipoxygenase requires the cis, cis-1,4 pentadiene system the position of this structure within the fatty acid will also affect the activity of the enzyme. For instance the 9, 12 isomer of linoleic acid is twice as reactive as the 13,16 isomer (Holman et al., 1969). The oxygenation reaction was very specific with respect to the position attacked. In all cases where an unsubstituted cis,cis-1,4 pentadiene system was attacked by soybean lipoxygenase the oxygen was positioned on the $\omega 6$ carbon of the fatty acid (Hamberg and Samuelsson, 1967). The following factors affect the site of O_2 insertion: source of lipoxygenase, type of isozyme, substrate and experimental conditions such as temperature and O_2 tension (Eskin et al., 1977).

Isozymes of lipoxygenase have been demonstrated in many plant species including soybean and wheat which have 4 isozymes each (Guss et al., 1967). The isozymes of soybean have been the best characterized and it is believed the different positions of oxygen insertion are a

function of the isozyme (Christopher and Axelrod, 1971). One of the isozymes of soybean lipoxygenase has a Ca^{2+} requirement for activity (Christopher et al., 1972). Wherever Ca^{2+} stimulated, EDTA normally inhibited lipoxygenase activity.

In addition to Ca^{2+} promotion of some lipoxygenase isozymes, Fe^{3+} had a general stimulatory effect (Axelrod, 1974). In fact Chan (1973) indicated that iron was closely involved in the catalysis by lipoxygenase possibly as a means of lowering the activation energy of the reaction. Fe^{2+} has also been implicated in lipid peroxidation however this has been shown to occur non-enzymically (Hunter et al., 1963).

The kinetics of the lipoxygenase reaction are somewhat complex. The oxygenation of fatty acids by the enzyme occurs with a lag period which can be abolished by adding product hydroperoxides (Smith and Lands, 1972). These findings convey a distinct role for both the product and the substrate in a manner allowing product binding only at a product site while the substrate may bind to the product site as well as the catalytic site.

Generally most lipoxygenases have maximal activity in the neutral pH range although some are active down to pH 5.0 and up to pH 9.0 (Koch et al., 1958).

Temperature effects on lipoxygenase are not well documented and are in some dispute. Koch et al. (1958) showed an inflection of activity at about 20°C. However Irvine and Anderson (1953) indicated that lipoxygenase activity was linear in the range 10-30°C.

Inhibitors

Some of the promoters of lipoxygenase activity have already been

discussed, namely Ca^{2+} and Fe^{3+}. SHAM and PG have already been identified as lipoxygenase inactivators. A number of other inhibitors have also been found such as diphenylthiocarbazone, 2,2' dipyridyl and 8-hydroxyquinoline (Chan, 1973). Cyanide was also reported to be inhibitory by Chan but this may have been due to contamination possibly by a peroxidase since lipoxygenase is known to be cyanide-insensitive. Flurkey et al. (1978) outlined a method whereby lipoxygenase and peroxidase could be separated by hydrophobic chromatography.

High concentrations of cysteine could inhibit lipoxygenase presumably by covalent binding through a free SH group to an important amino acid involved in the active site (Zakut, 1974). Other inhibitors of the lipoxygenase reaction include hydrogen peroxide and α tocopherol (Nakano et al., 1980) and anaerobic conditions which decrease the formation of free radicals (Witting, 1980).

The aforementioned inhibitors are chemical in nature. Of particular interest is an enzymic inhibitor called glutathione peroxidase. This enzyme catalyzes the reduction of lipid peroxides in the presence of glutathione (Tappel, 1980). It is believed that the observed inhibition occurs due to the removal of hydroperoxide products which are essential for eliminating the lag phase of the lipoxygenase reaction (Smith and Lands, 1972). This lag phase could be extended almost indefinitely with adequate levels of glutathione peroxidase present.

Physiological Role

The physiological role of lipoxygenase is as yet not clearly defined. Since the hydroperoxides formed are debilitating to intact cells due to their inactivation of enzymes (Matsushita, 1975) then effective

controls must be placed either on the lipoxygenase reaction itself or else on the hydroperoxides. To this extent it is believed that the lipoxygenase reaction is merely a means of activating unsaturated fatty acids for subsequent metabolism.

Hydroperoxide Metabolism

A number of aliphatic aldehydes, ketones and alcohols are thought to be synthesized from fatty acids. Some of the more important compounds include acetaldehyde, methanol, ethanol, hexanol and hexenal (Eskin et al., 1977). Ethane and ethylene are also enhanced in the presence of linolenic acid although some of the processes by which they are formed may be non-enzymic in nature (Schobert and Elstner, 1980).

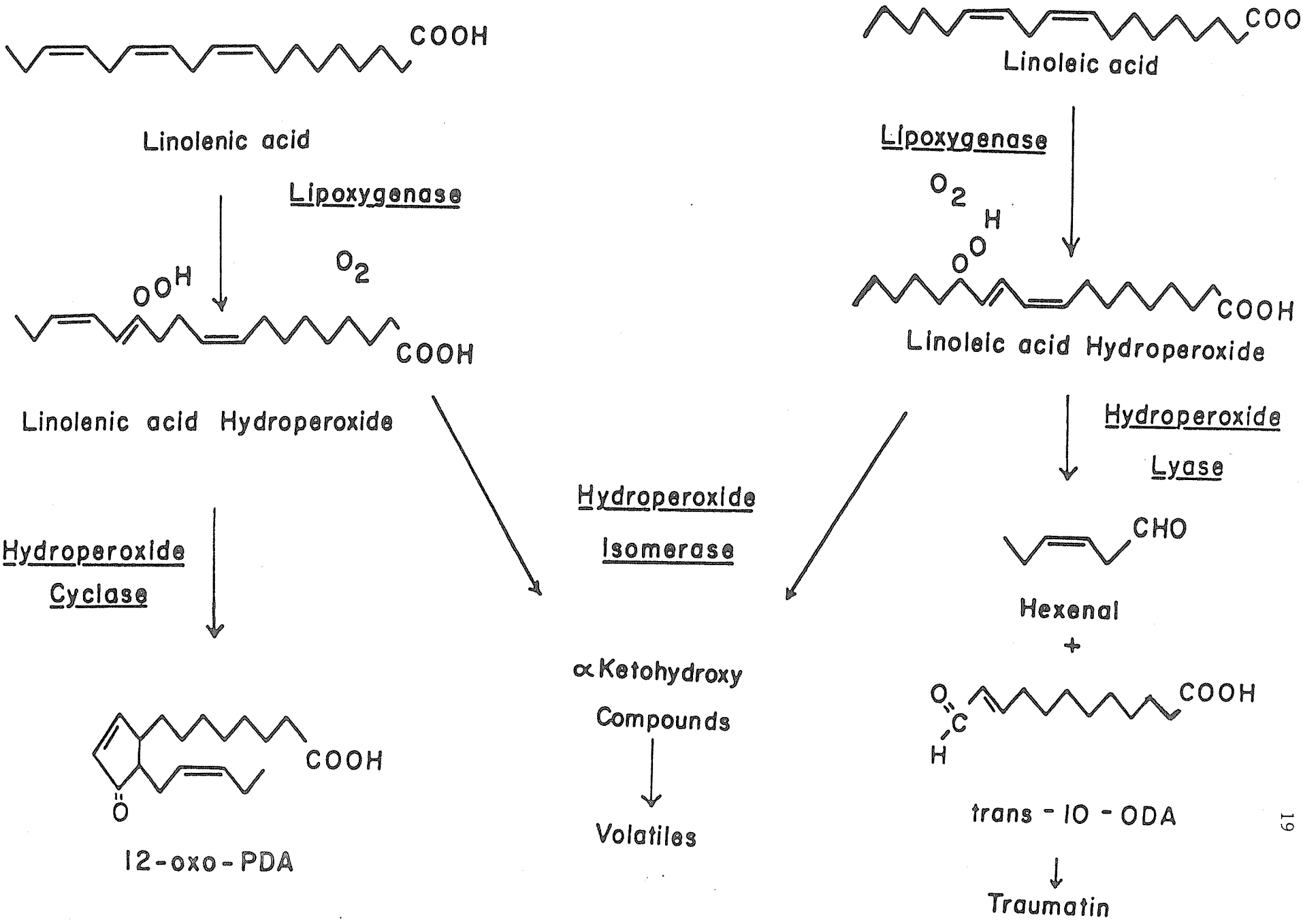
Generally plants possess three major enzymic pathways for metabolizing hydroperoxides (Figure 3). Individual species may have any or all of these pathways (Vick and Zimmerman, 1979).

The first enzyme discovered which utilizes the products of lipoxygenase for its substrate was hydroperoxide isomerase (Zimmerman and Vick, 1970). This enzyme converts the conjugated diene hydroperoxide of linoleic or linolenic acid to the corresponding monoenoic ketohydroxy fatty acid (Figure 3). Its activity has been demonstrated in crude extracts of flax, barley, soybean, corn, mungbean and wheat germ (Zimmerman and Vick, 1970). Hydroperoxide isomerase, like lipoxygenase, is insensitive to cyanide and occurs predominantly in the cytosol of cells. The α ketohydroxy compound formed is believed to be metabolized further to produce volatile compounds.

The second pathway discovered involved an enzyme called hydroperoxide lyase, first found in germinating watermelon seedlings (Vick and

Figure 3.

Possible routes for hydroperoxide metabolism (Taken from Zimmerman and Vick, 1976 and 1979).


COOH
Linolenic acid
Lipoxygenase
O_2
OOH
COOH
Linolenic acid Hydroperoxide
Hydroperoxide Cyclase
Hydroperoxide Isomerase
COOH
O
12-oxo-PDA
∝ Ketohydroxy Compounds
Volatiles
COOH
Linoleic acid
Lipoxygenase
O_2
H
O
O
COOH
Linoleic acid Hydroperoxide
Hydroperoxide Lyase
CHO
Hexenal
+
O
C
H
COOH
trans - 10 - ODA
Traumatin

Zimmerman, 1976). This enzyme converted the hydroperoxides to a compound called 12-oxo-trans-10-dodecenoic acid (trans-10-ODA). In addition to this compound hexanal and hexenal were formed from linoleic and linolenic acid hydroperoxides respectively (Zimmerman and Coudron, 1979). The trans-10-ODA compound is of interest due to its similarity to trans-2-dodecen-edioic acid also called traumatin. Traumatin is a little known compound which was identified as a plant wound hormone in 1937 (Bonner and English). This compound induces the formation of an intumescence of dividing and enlarging cells on the parenchymatous lining of seed chambers (Bonner and English, 1938). Intensive investigation by Zimmerman and Coudron (1979) suggested that trans-10-ODA was a precursor of traumatin. However trans-10-ODA was even more active as a wound hormone than was traumatin. They also found that the amount of endogenous trans-10-ODA increased when bean pod tissue was sliced indicating that its formation was a wounding response. The only plants so far surveyed which have hydroperoxide lyase activity are watermelon, cucumber and cantaloupe. No activity was detected in barley, corn, flax, green beans, peas, pumpkin, squash or sunflower (Vick and Zimmerman, 1976).

The most recent pathway discovered for hydroperoxide metabolism utilizes only linolenic acid. The product obtained is 12-oxo-cis,10,15-phytodienoic acid (12-oxo-PDA) and the reaction is catalyzed by an enzyme called hydroperoxide cyclase (Vick and Zimmerman, 1979a). The catalyzed reaction is very similar to the prostaglandin synthesis scheme found in animals which use arachidonic acid as a substrate (Hemler and Lands, 1980). Prostaglandins act as hormone modulators in animals but as yet there have been no reports of the effects of 12-oxo-PDA in plants. The enzyme is quite widely distributed being found in barley, corn, flax,

lettuce, peas, sunflower and wheat. No activity was found in apple fruit, beans, watermelon or soybeans (Vick and Zimmerman, 1979b).

Control of Lipid Peroxidation

The enzymic production of free-radicals is a normal ongoing process. Multiple defense systems exist which prevent or minimize free-radical initiated tissue damage (Witting, 1980).

Oxygen can exist as various active species (Fridovich, 1975). In the ground state molecular oxygen (O_2) has two unpaired electrons in its outer orbitals. The addition of one electron produces the superoxide anion (O_2^-). This can occur by autoxidations of hydroquinones, leucoflavins, catecholamines, thiols, dyes, ferredoxins and hemoproteins or by the enzymic reactions of xanthine oxidase, aldehyde oxidase, dihydroorotic dehydrogenase and some flavoprotein dehydrogenases (Fridovich, 1975). The superoxide anion can function both as an oxidant, in which case it will gain an electron and produce H_2O_2, or as a reductant where it will lose an electron to produce molecular oxygen.

Two superoxide anions can react with one another in such a fashion to produce a reduced and an oxidized product.

$$O_2^- + O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

This is called the dismutation reaction and is catalyzed by the enzyme superoxide dismutase (McCord and Fridovich, 1969). The production of the superoxide anion is believed to occur within all actively respiring organisms (Fridovich, 1975). The superoxide anion is the conjugate base of the hydroperoxyl radical ($HO_2^{\cdot}$) (Witting, 1980). The hydroperoxyl radical is an intermediate involved in the lipoxygenase reaction.

The superoxide anion can interact with hydrogen peroxide to produce the very active hydroxyl radical (.OH) by the Haber-Weiss reaction (Haber and Weiss, 1934).

$$O_2^- + H_2O_2 \xrightarrow{\text{metal}} {}^1O_2 + .OH$$

A consequence of this reaction is the production of singlet oxygen, a short-lived but extremely active oxygen species (Badwey and Karnovsky, 1980). Both the hydroxyl radical and singlet oxygen may initiate lipid peroxidation. Singlet oxygen is known to attack the π electron systems which are found in unsaturated phospholipids (Nakano et al., 1980).

The peroxidized fatty acids themselves are also capable of initiating secondary oxidations as suggested by Witting (1980) and Smith and Lands (1972). These secondary reactions have also been implicated in the binding of hydroperoxides to proteins (Matsushita, 1975).

Several enzymic defense systems occur in cells to prevent lipid peroxidation. Superoxide radicals are primarily removed by superoxide dismutase (McCord and Fridovich, 1969). The hydrogen peroxide formed in the reaction can be subsequently removed by catalase and/or peroxidase. Superoxide dismutase also quenches the Haber-Weiss reaction by reducing the number of reactive hydroxyl radicals and singlet oxygen formation. Secondary oxidation of lipid hydroperoxides can be minimized or prevented by glutathione peroxidase (Tappel, 1980) or by any of the other hydroperoxide metabolizing enzymes previously discussed.

An important non-enzymic control of lipid peroxidation is α tocopherol. This compound can scavenge the superoxide anion (Yagi et al., 1977). In addition α tocopherol quenches singlet oxygen thus protecting lipids from oxidative damage (Foote, 1977). The active group of the α tocopherol

molecule is an OH group which has been found to form hydrogen bonds with lipid hydroperoxides thus reducing the extent of secondary peroxidation reactions (Nakano et al., 1980).

Phenolic-like compounds such as propyl gallate can act to quench free radicals as does α tocopherol.

These multi-level controls, both enzymic and non-enzymic, emphasize the importance of limiting lipid peroxidation reactions within living cells.

Mitochondrial Swelling and Transport of Metabolites

Mitochondria have two membrane systems: an outer membrane and an extensive highly folded inner membrane. The inner membrane encloses a space called the matrix. The matrix contains all the enzymes of the Krebs cycle as well as the fatty acid oxidation enzymes. The inner membrane contains NADH and succinate dehydrogenases, iron-sulfur proteins, cytochromes b, c and a as well as the ATPase complex. The outer membrane has a few limited electron transfer components (Lehninger, 1975).

Most of the processes of electron transfer, oxygen uptake and energy conservation occur on or within the inner membrane. The outer membrane is permeable to solutes such as sugars, organic acids, nucleotide sugars and amino acids. It is the inner membrane which controls the passage of solutes into and out of the matrix (Wiskich, 1977). No discussion of mitochondrial respiration would be complete without an understanding of the processes by which substrates are transported into mitochondria and the effects which would result from such incorporations.

Any indication that a substrate has reacted with a matrix enzyme is

evidence that the substrate has penetrated the inner membrane. This could be detected by fluorescence, substrate interconversions or by oxygen consumption (Wiskich, 1977). Another commonly used method to detect solute penetration is a light scattering technique (Chappell and Haarhoff, 1967). As mitochondria swell and enlarge they scatter less light and their light transmission increases. This process can be followed spectrophotometrically.

Two types of swelling responses have been observed with isolated mitochondria: passive swelling, which is substrate and energy independent, and active swelling which is substrate and energy dependent (Lee and Wilson, 1972). Several ion translocators have been associated with passive and active swelling in plant mitochondria.

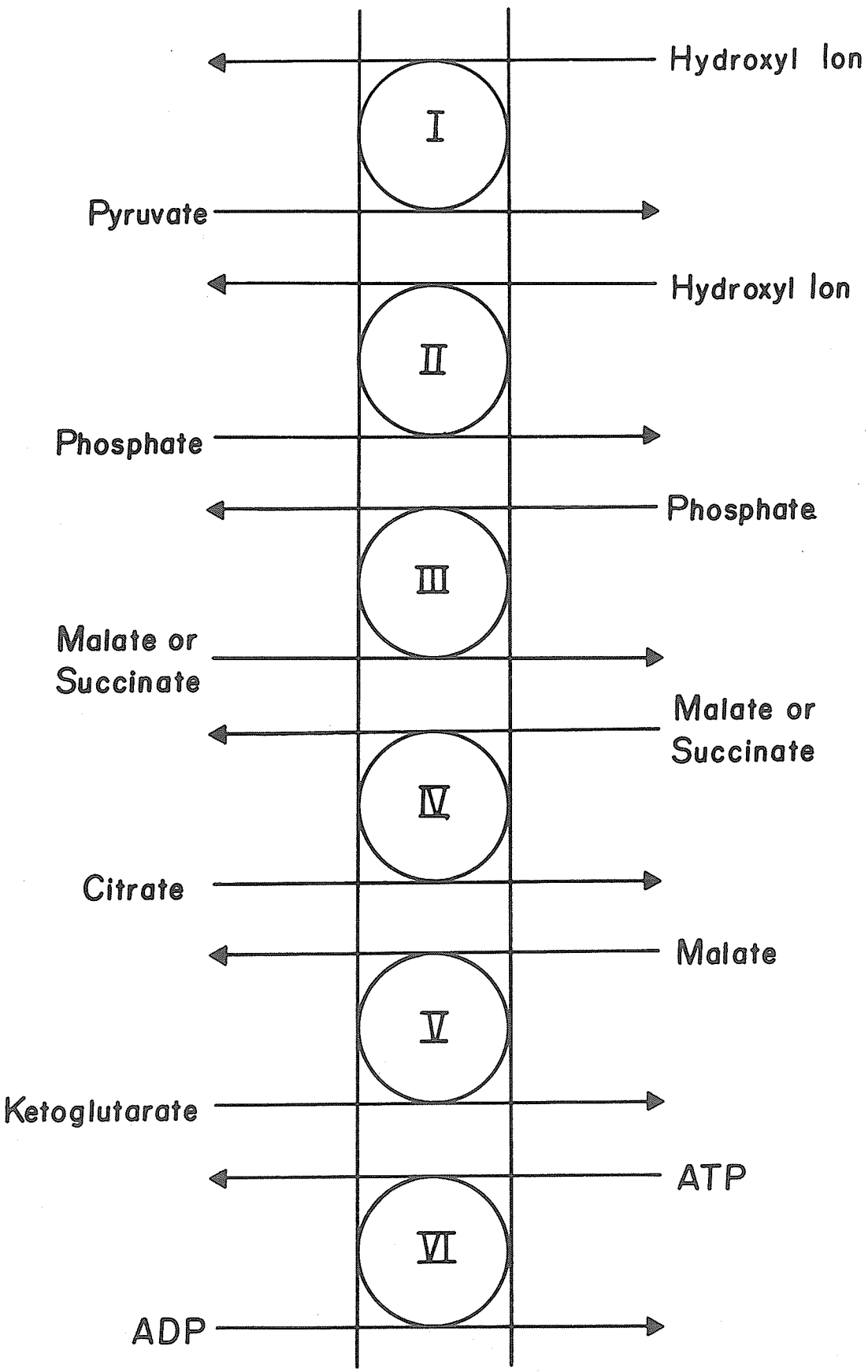
Passive swelling is thought to occur via cation/H^+ exchangers in the inner membrane. Such exchangers have been found in potato (Jung and Brierley, 1979) and turnip mitochondria (Moore and Wilson, 1977). The physiological role of passive swelling may be to prevent osmotic volume increases by pumping out excess cations which leak into the matrix in response to a concentration gradient or the mitochondrial membranes' negative potential (Mitchell, 1970). This mechanism is believed to be operative during the observed respiration-dependent contraction of mitochondria (Jung and Brierley, 1979).

Active swelling occurs as a result of several transporters including phosphate, monocarboxylate, dicarboxylate and tricarboxylate carriers (Figure 4). The transport of these anions is dependent on coupled mitochondrial respiration presumably as a result of the gradient set up by the Proton Motive Force (PMF) (Wiskich, 1977).

Phosphate transport is pH dependent since its movement neutralizes

Figure 4.

Some of the known mitochondrial transporters; (I, pyruvate; II, phosphate; III, dicarboxylate; IV, tricarboxylate; V, α ketoglutarate; VI, adenine nucleotide. (Adapted from Wiskich, 1977).


Outside Mitochondria
Inside Mitochondria
Hydroxyl Ion
I
Pyruvate
Hydroxyl Ion
II
Phosphate
Phosphate
III
Malate or
Succinate
Malate or
Succinate
IV
Citrate
Malate
V
Ketoglutarate
ATP
VI
ADP

the chemical pH gradient but maintains the electric membrane potential (McGivan and Klingenberg, 1971). Phosphate uptake by mitochondria is regarded as an HPO_4^-/OH^- exchange (Chappell and Haarhoff, 1967). The transport of phosphate is required to a) provide a substrate for ATP synthesis, b) adjust and maintain pH balance, c) induce cation uptake, d) permit net translocation of organic acid ions and e) act with the nucleotide transporter in maintaining an asymmetric phosphorylation potential.

Organic acid transporters can change the distribution of particular organic acids but cannot achieve net accumulation (Wiskich, 1977). Generally dicarboxylic acids enter the mitochondria in an undissociated form in exchange for phosphate (Figure 4). In addition tricarboxylic acids enter mitochondria in exchange for malate and/or succinate (Pressman, 1970). Monocarboxylate transporters have also been identified for pyruvate and acetoacetate (Papa and Paradies, 1974).

The first carrier mediated transport mechanism identified in mitochondria was a translocator of adenine nucleotides (Chappell and Crofts, 1965). The transporter is specific for adenine nucleotides and is asymetric, favoring the transport of ADP inwards and ATP outwards in a strict 1:1 exchange (Figure 4). The electrochemical potential being negative inside the mitochondria would favor ADP^{3-} import for ATP^{4-} export. This transporter is considered to be the rate limiting step in the energetics of oxidative phosphorylation (Davis and Lumeng, 1975).

Isolated plant mitochondria can accumulate divalent ions, such as Ca^{++}, by a phosphate dependent process (Kenefick and Hanson, 1966). Cation movement may be diffusive in response to an electrochemical gradient thus allowing H^+ ejection, but does not involve any special carrier mechanism. In fact many of the Ca^{++} effects may be due to Ca^{++} binding

sites rather than carrier mediated transport (Reed and Bygrave, 1974).

Spontaneous swelling of mitochondria was shown to be caused by another class of compounds namely free fatty acids (Earnshaw et al., 1970). The released fatty acids were also shown to have uncoupling effects on the energy-linked functions of mitochondria (Baddeley and Hanson, 1967). The participation of fatty acids in swelling of mitochondria was first demonstrated by Wojtczak and Lehninger in 1961. They indicated that Ca^{++} and thyroxine induced swelling resulted in the release of fatty acids possibly by an enzymic activation. However these results could not be reproduced using phosphate induced swelling.

Pfeiffer and McCay in 1972 reported that mitochondrial oxidation of NADPH in the presence of physiological quantities of Fe^{3+} resulted in a loss of fatty acids from rat liver mitochondria. Hunter et al. (1963) demonstrated that Fe^{2+} induced mitochondrial swelling and it was shown that NADPH will reduce Fe^{3+} to Fe^{2+}. This activity resulted in a marked decline in the ability of mitochondria to oxidize Krebs cycle intermediates. Hence the activity of certain membrane-bound oxidoreductase systems can affect the structure and function of the membranes in which they are situated and may account for some of the turnover of lipids in mitochondrial membranes.

MATERIALS AND METHODS

Plant Material and Germination Conditions

The winter wheat cultivar Norstar was used throughout the present study. For the field experiments the cultivars Kharkov, Alabaskaja, Ulianovka and Fredrick were used in addition to Norstar.

Seeds to be germinated were surface sterilized in a 2% sodium hypochlorite solution for 2-3 minutes and then washed with water for an additional 2-3 minutes. Germination was carried out in seed trays placed in the dark for 2 days at 24°C.

For seeds germinated under conditions of high CO_2 in O_2 a 10-litre static chamber was utilized. Seeds were placed in seed trays and stacked within the chamber. The chamber was then evacuated and CO_2 was introduced by reacting a known amount of sodium bicarbonate with phosphoric acid. Following this pure O_2 was fed into the chamber until it was equilibrated with atmospheric pressure. The chamber was placed in the dark at room temperature for approximately 4-5 days or until the coleoptile length was the same as those from normal atmosphere grown seedlings. The concentration of CO_2 in the static chamber was determined by withdrawing a sample of air with a syringe and injecting it into an Infrared Gas Analyzer (Model 225-2B-SS, Analytical Development Company). This was compared to a standard sample of pure CO_2 generated in a gas trap system again utilizing sodium bicarbonate and phosphoric acid. CO_2 concentration was within the 20-25% range.

Anaerobic Metabolite Analysis

The winter wheat cultivars Norstar and Kharkov were analyzed for

the levels of methanol, ethanol and acetaldehyde under various conditions of cold-hardening, freezing and icing.

Norstar wheat was germinated for 2 days then transplanted to pots (30 plants/pot) containing a peat moss: sand: soil mixture of 1:1:1 and supplemented with 10:52:10 fertilizer. These plants were grown for 1 week at room temperature. The seedlings were cold-hardened according to the conditions set out by Andrews and Pomeroy (1975) utilizing a growth chamber set at 2-4^{o}C and growing the plants for 3-4 weeks with a 16hr photoperiod and a light intensity of 200-350 uEinsteins.

Plants to be frozen and iced were removed after this period, the pots soaked to capacity and placed in a cold room in the dark for 3 weeks at an average temperature of -10^{o}C. These plants were thawed at 2-4^{o}C prior to analysis.

Approximately 5g of plant tissue was homogenized in 3 volumes of isolation media (0.5M mannitol, 10mM K_2HPO_4, 0.1% BSA, 2mM cysteine, 1mM EDTA and 2% soluble PVP at pH 7.5) using a polytron homogenizer (Brinkman Instruments). The brei was filtered through Nitex cloth (Tetko inc) and centifuged at 3,000 xg for 5 minutes. The supernatant was analyzed directly by gas chromatography using a Poropak Q column as outlined by the method of Baker et al. (1969). A model 5710A Hewlett Packard gas chromatograph with a flame ionization detector was used. The column was filled with 60/80 mesh Poropak Q pre-conditioned for 18 hrs at 230^{o}C. The oven temperature was 130^{o}C and the inlet and detector were maintained at 150^{o}C. The flow rates of nitrogen, hydrogen and air were 35 mls/min, 60 mls/min and 240 mls/min respectively. The detector response was recorded on a Spectra Physics SP4100 computing integrator.

Oxygen uptake was also measured for these plants using 1 cm segments

from the crown tissue. These segments were weighed, cut into 0.2 cm sections and infiltrated under reduced pressure with the appropriate substrates and/or inhibitors as by the method of Kinraide and Marek (1980). Oxygen uptake was followed polarographically.

Isolation and Purification of Mitochondria

The buffer systems used were chosen in accordance with the findings of Ikuma (1970). Coleoptiles (2-3g) were homogenized using a mortar and pestle for 1 minute in 3 volumes of isolation media (0.5M mannitol, 10mM K_2HPO_4, 0.2% BSA, 2mM cysteine, 1mM EDTA and 2% soluble PVP adjusted to pH 7.5 with KOH); all operations were carried out at 0-4°C. The brei was filtered through Nitex nylon cloth (10u openings) and centrifuged for 5 minutes at 1,000 xg. The supernatant was then centrifuged for 5 minutes at 20,000 xg. For crude mitochondria the pellet was resuspended in 0.5 mls of reaction media (0.3M mannitol, 10mM K_2HPO_4, 0.1% BSA, 10mM KCl, 5mM $MgCl_2$ adjusted to pH 7.2). The mitochondrial suspension was stored on ice until use.

Two methods of purifying mitochondria were tried. The first was a phase partition extraction utilizing dextran and polyethylene glycol by the method of Gardestrom et al. (1978). The second method was a percoll gradient separation as used by Jackson et al. (1979).

The percoll method was generally used but with the following modifications. The percoll gradient consisted of 13.5%, 20% and 45% (v/v) percoll made up in gradient buffer (0.25M sucrose, 0.2% BSA and 10mM K_2HPO_4 at pH 7.5). Centrifugation was for 30 minutes at 7,500 xg utilizing an angle head rotor. The band demonstrating the greatest mitochondrial activity was collected and resuspended in 10 mls of isolation media

and then centrifuged at 20,000 xg for 5 minutes. The pellet was resuspended in 0.5 mls of reaction media.

For experiments involving mitochondrial swelling and lipid analysis 10mM Tris was substituted for 10mM K_2HPO_4.

Protein was determined by the method of Lowry (1951) after solubilizing the mitochondria in 5% deoxycholate.

Oxygen Measurements

Oxygen uptake was measured with a Clarke-type oxygen electrode (Rank Brothers, Cambridge England). Standard membranes were used with the polarizing voltage adjusted to 0.65 volts. Oxygen concentrations at various temperatures were determined from the Truesdale and Downing equation (1954) for air saturated water conditions.

$$\text{Oxygen (ppm)} = 14.16 - 0.3943T + 0.007714T^2 - 0.0000646T^3$$

In a typical assay 1.5 mls of reaction media was allowed to equilibrate for at least 5 minutes at the desired temperature. The electrode output was adjusted to give a full scale deflection of the chart recorder (10 mv). All substrates soluble in aqueous solution were made up in reaction buffer. Water insoluble compounds were made up in 9.5% ethanol. Succinate or malate were added to give final concentrations of 10mM and 25mM respectively. ADP was added in a concentration ranging from 20uM to 100uM. Oxygen uptake was initiated by adding 100 ul of mitochondrial suspension containing 0.5-1.0 mg of protein. Following a trial the baseline (ie. $O_2 = 0$) could be obtained by the addition of aqueous sodium hydrosulfite. ADP/O and RC ratios were determined by the method of Estabrook (1967). Oxygen measurements were carried out at 25°C except where otherwise stated.

Lipoxygenase Assay

Lipoxygenase activity of both the mitochondrial preparations and commercial soybean lipoxygenase were assayed polarographically in the standard reaction buffer (pH 7.2). Linoleic acid (4mM), linolenic acid (4mM) and trilinolein (0.16mM) were solubilized in Tween 80 according to the method of Surrey (1964). The reaction was initiated by the addition of one of the fatty acid substrates to a 1.5 ml cell containing either a mitochondrial suspension or commercial lipoxygenase. Soybean lipoxygenase typically contained 1,000 units of activity per trial or approximately 30ug of protein.

Lipoxygenase Purification

A lipoxygenase purification procedure was followed similar to that outlined by Grossman et al. (1972). A crude wheat homogenate was prepared in 5mM acetate buffer (pH 7.0). After centrifugation for 5 minutes at 3,000 xg ammonium sulphate was added to the supernatant to produce 35%, 55% and 70% precipitates. Lipoxygenase activity was recovered in the 35-55% fraction. This was dialyzed overnight at $4^{o}C$ against 5mM acetate buffer (pH 5.5) and applied to a CM-cellulose column equilibrated with the same buffer. Protein was eluted with 100mM acetate buffer (pH 5.5). The protein peak demonstrating the greatest lipoxygenase activity was collected and applied to a linolenic acid-aminoethyl agarose affinity column equilibrated with 5mM acetate buffer (pH 5.5). The first peak was eluted with 50mM acetate buffer (pH 5.5) and subsequent peaks with 200mM acetate buffer (pH 5.5). Fractions showing lipoxygenase activity were collected and stored in the cold at $4^{o}C$.

Swelling Experiments

Mitochondria were isolated as previously described. For enhanced swelling effects the mitochondria were suspended in a swelling media consisting of 20mM TES, 0.2M KCl and 0.1% BSA at pH 7.2. Aliquots containing about 1 mg of mitochondrial protein were suspended in solutions of 10mM succinate, 25mM malate or 0.2uM valinomycin in a final volume of 2.0 mls. Inorganic phosphate (10mM) was needed to activate swelling in malate and succinate. Absorbance at 520 nm was followed for 5 minutes using a Cary 15 recording spectrophotometer.

Mitochondrial Lipid Analysis

The phospholipid extraction procedure followed was a modification of the Bligh and Dyer method (1959). The mitochondria were isolated as previously described. Approximately 1 mg of mitochondrial protein was added to the appropriate reaction systems containing those components necessary for active respiration ie. 10mM K_2HPO_4, 100uM ADP and 10mM succinate or 25mM malate. Inhibitors and phospholipase A_2 were added when required. The reaction was allowed to proceed for 5 minutes then the mitochondria were pelleted at 20,000 xg for 5 minutes and separated from the supernatant.

An internal standard of triheptadecanoate (1,000 ug) was added to each mitochondrial extract. The pellet was then suspended in 5 mls of boiling 2-propanol and incubated for 30 minutes at 80°C. After centrifugation at 3,000 xg for 5 minutes the supernatant was collected and the pellet extracted twice with 5 mls of chloroform-methanol-water (1:2:0.8 v/v/v); following each extraction the suspension was centrifuged at 3,000 xg for 5 minutes. The organic supernatants were combined and the solvents

removed under reduced pressure using a rotary evaporator. For analysis the lipids were hydrolyzed and methylated by a trans-esterification process (Hougen and Bodo,1973) using 0.4N sodium methoxide in methanol.

The free fatty acids in the supernatant of the reaction were separated by the method of Dittmer and Wells (1969) after first adding an internal standard of 1,000 ug of heptadecanoate. To the supernatant was added 2 mls of petroleum ether. This was extracted 4 times with 1 ml of 4% Na_2CO_3. The Na_2CO_3 fractions were washed 2 times with 2 mls of petroleum ether. Next the Na_2CO_3 fractions were acidified to pH 2.0 with H_2SO_4. This was washed 3 times with 1 ml of petroleum ether. The petroleum ether extract was then taken to dryness. Methyl esters of the free fatty acids were prepared by the boron-trifluoride method of Metcalfe and Schmitz (1961).

The methyl esters were separated using a 6' x 1/8" stainless steel column packed with 3% SP2310/2% SP2300 on 100/120 mesh Chromosorb WAW utilizing a 5710A Hewlett-Packard gas chromatograph equipped with FID. The gas flow rates were: N_2 carrier gas- 20 mls/min, H_2- 30 mls/min and air- 240 mls/min. The column temperature was 210°C , the injector temperature 300°C and the detector temperature 250°C. The detector response was followed on a Spectra Physics SP4100 computing integrator.

Oxaloacetate and Pyruvate Determinations

After mitochondria were assayed polarographically using malate as a substrate the contents of the reaction vessel were placed in test tubes and the reaction terminated by adding 100 ul of 71.2% perchloric acid. The precipitated protein was removed by centrifugation for 15 minutes at 10,000 xg. The supernatants were neutralized with 1M KOH and the potass-

ium perchlorate formed was removed by centrifuging for 15 minutes at 10,000 xg. The supernatant was divided into equal volumes to determine oxaloacetate and pyruvate using enzymic reactions coupled to the oxidation of NADH (Williamson and Corkey, 1969). Pyruvate was assayed with lactate dehydrogenase and oxaloacetate was assayed using malate dehydrogenase. NADH oxidation was followed spectrophotometrically at 340 nm. From the amounts of oxaloacetate and pyruvate formed the amount of malate oxidized could be determined and the expected O_2 uptake calculated.

Field Experiments

Lipoxygenase activity was measured in five winter wheat cultivars; Ulianovka, Alabaskaja, Norstar, Kharkov and Fredrick with decreasing cold-hardiness levels respectively. Planted on 13/Sept/80 the cultivars were sampled immediately after the first snowfall and at intervals thereafter. Plants were homogenized in 50mM acetate buffer (pH 7.5) using a Brinkman Polytron. The brei was filtered through Nitex cloth and centrifuged at 3,000 xg for 5 minutes. The supernatant was then assayed polarographically for lipoxygenase activity in 50mM acetate buffer using 4mM linoleic acid as a substrate. Soluble protein was determined by the method of Lowry (1951).

RESULTS AND DISCUSSION

Anaerobic Metabolite Analysis

The effect of ethanol, acetaldehyde, acetic acid and ethylene on the respiration of potato tubers has been previously studied (Rychter et al., 1979b). Generally, all of these compounds stimulated the respiration rate when applied in air or 100% O_2. Acetaldehyde appeared the most effective in stimulating respiration compared to the other volatiles. Ethylene has previously been implicated in the stimulation of the alternative or cyanide-insensitive pathway (Solomos and Laties 1974,1975). The studies of Rychter et al. (1979b) support this hypothesis and also suggest that increased ethanol, acetaldehyde and acetic acid levels can also lead to the development of the cyanide-insensitive pathway.

Ethanol accumulations are known to occur in ice-encased winter cereal seedlings (Andrews and Pomeroy, 1977) hence a study was undertaken to see if the levels of anaerobic metabolites were related to the development of the cyanide-insensitive pathway.

The first part of the study was to determine the levels of methanol, acetaldehyde and ethanol found in plants grown under control, frozen, iced and overwintering conditions (Table 1). The relative amounts of anaerobic metabolites were quite low in control and overwintered seedlings while frozen and iced plants had the greatest accumulations of the metabolites. Iced seedlings had the highest levels of ethanol in agreement with the results of Pomeroy and Andrews (1978).

The accumulation of these anaerobic metabolites raises the question of their toxicity. Andrews and Pomeroy (1977) indicated that the levels

TABLE 1. Anaerobic metabolite analysis of winter wheats grown under various environmental conditions

Conditions	Methanol	Acetaldehyde	Ethanol
		(mg/g.f.w.)	
Control Grown	0.03	0.01	0.02
Cold-hardened & Frozen	0.04	0.03	0.88
Cold-hardened & Iced	0.08	0.12	1.63
Spring harvested Kharkov	0.03	0.02	0.01

of ethanol found in ice-encased plants were not high enough to be toxic. Increasing ethanol levels suggests that during ice-encasement pyruvate utilization shifts from aerobic oxidation via the Krebs cycle and electron transport to anaerobic reduction. As a result of a stimulated glycolytic rate CO_2 production is also increased (Andrews and Pomeroy, 1977). CO_2 is a toxic component and it, like ethanol, is known to change the permeability of cell membranes (Glinka and Reinhold, 1972). Another toxic component is lactic acid which accumulates to some extent in ice-encased seedlings (Andrews and Pomeroy, 1979). The toxicity of methanol and acetaldehyde has not been previously reported although the levels of these two metabolites are relatively low compared to ethanol levels (Table 1).

A condition which often precedes ice-encasement is flooding. Pomeroy and Andrews (1979) have shown that ethanol, CO_2 and lactic acid all accumulate in cereal seedlings exposed to flooding. Thus the production of anaerobic metabolites is probably a result of the partial or total anoxia conditions which occur during flooding and ice-encasement.

Wheat which has overwintered will usually have passed through the cold-hardening and frozen stages. Low levels of anaerobic metabolites found in spring Kharkov wheat (Table 1) indicates that at some time the plants remove the levels of metabolites which must have accumulated over the winter season. Early spring is the most likely time period for this to occur since the plants are removed from the conditions of anoxia and mitochondrial respiration is allowed to resume to its fullest extent (Table 2). The metabolism of anaerobic metabolites may follow the reverse path of their synthesis (Cossins, 1978).

Respiration of Wheat Segments

The second part of the study was to relate the accumulation of anaerobic metabolites to the level of cyanide-insensitive respiration present in the wheat tissues. The respiration of segments of wheat crown tissue was measured polarographically. The inhibitors cyanide and BHAM were added, under reduced pressure to increase penetration into the tissues, and from this an estimation could be made of the relative amounts of mitochondrial respiration occuring via the cytochrome and the cyanide-insensitive pathway.

The results indicate that O_2 uptake in control grown and spring Kharkov occurs predominantly by the cytochrome path as judged by the higher cyanide sensitivity (Table 2). Spring Kharkov had the greatest amount of respiration via the cytochrome chain indicating that during early spring there is a rapid development of this pathway in order to provide the energy equivalents needed for growth to resume.

Cold-hardened and frozen plant material had a stimulated cyanide-insensitive oxygen uptake rate accounting for approximately 30% of the total respiration (Table 2). This suggests that upon exposure to low temperatures wheat plants develop a more operative cyanide-insensitive path. The cyanide-insensitive path was believed to be involved in the regulation of NADH levels in the plant cells (Bahr and Bonner, 1973). Previous work has shown that when the cyanide-insensitive path is brought into operation the rate of oxidation of exogenous NADH increases markedly (Dizengremel and Lance, 1976). Plants which have been subjected to low temperatures do have an increased glycolytic rate (Andrews and Pomeroy, 1977) which could result in an increase in NADH levels. However the results obtained in the present study indicate that a stimulated

TABLE 2. Oxygen uptake of wheat segments taken from plants grown under various environmental conditions

Conditions	Total Respiration	CN-Insensitive[a] Respiration	Residual[b] Respiration
	(nmoles O_2/min/g.f.w.)		
Control Grown	184	57(12%)[c]	35(19%)
Cold-hardened & Frozen	115	50(30%)	15(13%)
Spring harvested Kharkov	102	22(7%)	15(15%)

[a] Oxygen uptake rate with 5mM cyanide present

[b] Oxygen uptake rate in the presence of 5mM cyanide and 5mM BHAM

[c] Numbers in parentheses represent the fraction of total respiration that is CN-insensitive or residual respiration

glycolytic rate results in a shift towards anaerobic respiration and ethanol production, rather than through the aerobically linked NADH-cytochrome oxidase system.

The anaerobic shift would conserve oxygen which could be a limiting factor under the anoxic conditions of ice-encasement. In addition the conversion of acetaldehyde to ethanol utilizes NADH so this compound should not accumulate to any great extent under these conditions.

An operative cyanide-insensitive pathway in plants which is of mitochondrial origin would be detrimental as far as energy conservation is concerned. Since both the alternative and cytochrome oxidases are linked to oxygen consumption it would not be beneficial to a plant subjected to anoxic conditions to have an operative alternative path which reduces ATP production by 2/3 while consuming the same equivalent of oxygen. Since little heat production was found in winter wheat (McCaig and Hill, 1977) the function of the alternative path is not related to increased frost resistance by thermogenicity.

An increase in the levels of anaerobic metabolites (Table 1) did occur with an increase in cyanide-insensitive respiration (Table 2) in cold-hardened and frozen wheat seedlings. This relationship may be coincidental rather than dependent as a 44 fold increase in ethanol levels occurred with only a 2½ fold increase in cyanide-insensitive respiration over control levels. Ethanol may stimulate respiration (Rychter et al., 1979b) however total respiration was reduced in cold-treated plants possibly by an inactivation of the cytochrome components due to membrane changes (Miller et al., 1974).

Some residual respiration was evident even after inhibition with cyanide and BHAM. Some of this respiration may be due to polyphenol

oxidase activity as addition of 2% PVP inhibited oxygen uptake approximately to the same extent as the oxygen consumed by the residual respiratory rate (Table 3). PVP is a compound which binds phenols hence polyphenol oxidase activity is reduced (Loomis, 1974). The average inhibition of the total respiration by PVP was 12% and the observed residual respiration was 14%. Inhibition of phenol oxidases by cyanide has also been reported (Wong et al., 1971) however this was a differential effect as not all phenol oxidases were inhibited to the same extent.

The activity of glycolate oxidase was also checked by selectively inhibiting with isethionic acid (2-hydroxy ethane sulfonic acid). Little activity of glycolate oxidase was present.

The effect of disulfiram, a known inhibitor of the cyanide-insensitive respiratory component in red sweet potatoes (Grover and Laties, 1978), was investigated with the wheat segments. This compound had no inhibitory effect even though cyanide-insensitive respiration was determined to be present to the extent of 17% of the total respiration (Table 3). In fact an actual stimulation of respiration was observed. Since disulfiram was solubilized in ethanol the effect of this organic solvent on respiration was also determined. Ethanol stimulated respiration about 10% quite in agreement with the results obtained by Rychter et al., (1979b). Therefore much of the observed stimulation of respiration upon the addition of disulfiram may be due to ethanol effects.

The inability of disulfiram to inhibit suggests that its mode of action in wheat segments may be quite different from that of the red sweet potato. Disulfiram was reported to chelate transition metal ions particularly copper ions (Grover and Laties, 1978). The binding sites of

TABLE 3. Oxygen uptake of wheat segments in the presence of various respiratory inhibitors[a]

	Respiration Rate	% of Control Rate
	(nmoles O_2/min/g.f.w.)	
Control	217	100
BHAM (5mM)	182	84
Cyanide (5mM)	67	31
Cyanide + BHAM	31	14
PVP (2%)	191	88
Ethanol (5%)	239	110
Disulfiram (5mM)	248	114

[a] Results are averages of duplicate runs

the hydroxamic acids and disulfiram are separate hence inhibition by BHAM does not necessarily mean that disulfiram will inhibit to the same extent as BHAM. Since disulfiram had no effect on the cyanide-insensitive respiration of wheat segments this implies one of two possibilities. Either the disulfiram was not able to penetrate the tissues to reach its binding sites or the disulfiram binding sites are lacking in wheat tissue.

Mitochondrial Isolation and Purification

Working with wheat segments was considered to be unsatisfactory although some idea of the overall respiratory processes could be obtained. Since non-mitochondrial oxidases were evident conclusions could not be drawn on the extent of mitochondrial respiration proceeding via the cytochrome or cyanide-insensitive pathways. One of the problems working with wheat segments is that infiltration with substrates and inhibitors is difficult and the extent to which they penetrate is unknown.

Three techniques were used to isolate and purify mitochondria from green and etiolated tissues: (1) differential centrifugation, (2) phase partition extraction and (3) a percoll density gradient method.

Differential centrifugation involves a low speed spin followed by a high speed spin and results in mitochondria which are likely contaminated. Mitochondria from green tissues had chloroplast components present and little respiratory activity when isolated by differential centrifugation. However wheat coleoptiles did provide a source of mitochondria which were physiologically active as measured by the RC ratios and ADP/O ratios (Figure 5A). This source of mitochondria was utilized in

the previous studies of McCaig and Hill (1977).

A phase partition extraction was added after the differential centrifugation consisting of a two phase system containing dextran and polyethylene glycol (PEG) (Gardestrom et al., 1978). Mitochondria collected predominantly in the bottom (dextran) phase and could be washed several times with fresh top phase (PEG). Although this method was rapid mitochondria isolated from green tissue were not physiologically active. This method was not applied to the wheat coleoptile tissue. The technique relies on the surface properties of materials for separation (Pharmacia publications, 1971) so problems can occur due to ionic effects and the shape of mitochondria in solution.

The percoll density gradient technique was judged the most favorable due to ease of use, yields of mitochondria and physiological activity of the isolated mitochondria. Percoll gradients separate macromolecules and organelles on the basis of their buoyant densities (Pharmacia publications, 1977). With a coating of PVP the silica sol gel (percoll) has low viscosity, low osmolarity and is non-toxic; three important considerations when isolating intact organelles. A three step discontinuous gradient was utilized and resulted in mitochondria banding at the interface of the bottom two layers. The heavy bottom layer acted as a cushion into which the mitochondria collect. When this technique was used to isolate mitochondria from green spinach leaves cytochrome-c oxidase activity was retained and the mitochondria were considered to be 90% intact for both the inner and outer membranes (Jackson et al., 1979).

In trying to isolate mitochondria from green wheat tissue the percoll gradient technique had its drawbacks. The mitochondria banded well and their brownish colour was quite distinct from the contaminating

chlorophylls which collected in the upper layers of the gradient. However physiological activity of the mitochondria was very low. This was not too surprising since the original work using a percoll gradient employed 800g of spinach leaves to obtain a mere 10-20 mg/ml of mitochondria (Douce et al., 1977 and Jackson et al., 1979). This suggests that a great deal of tissue is required in order to isolate enough mitochondria from green tissues to show physiological activity in terms of substrate oxidation. Since wheat coleoptile tissue appears to be enriched with mitochondria this source of tissue was used for most subsequent studies.

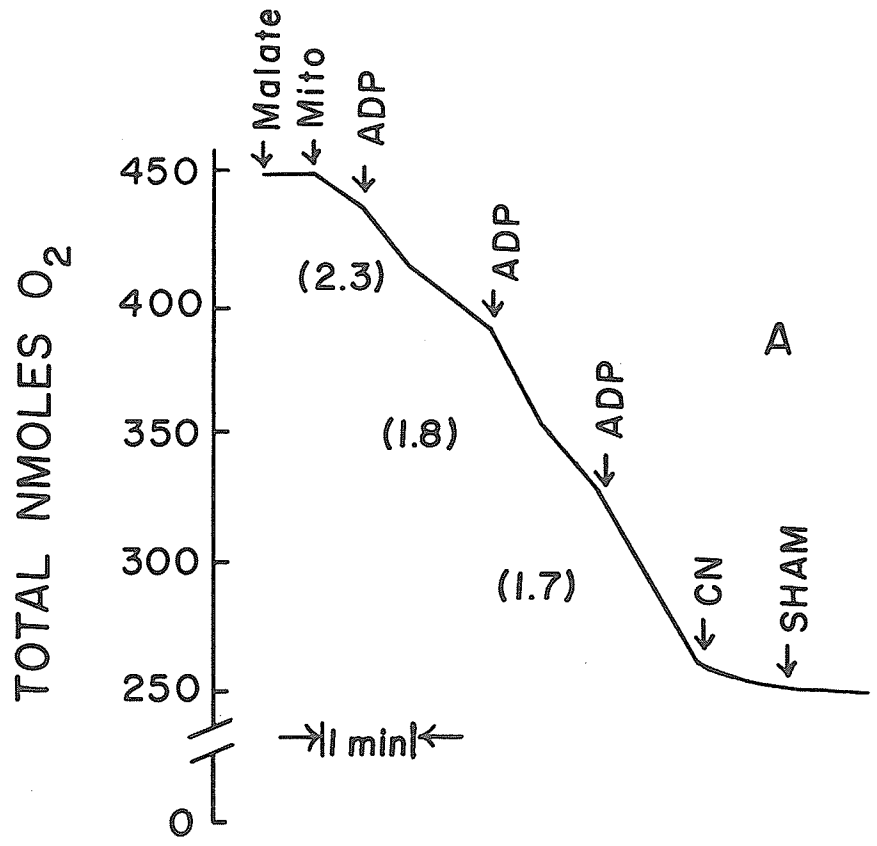
Respiratory Measurements

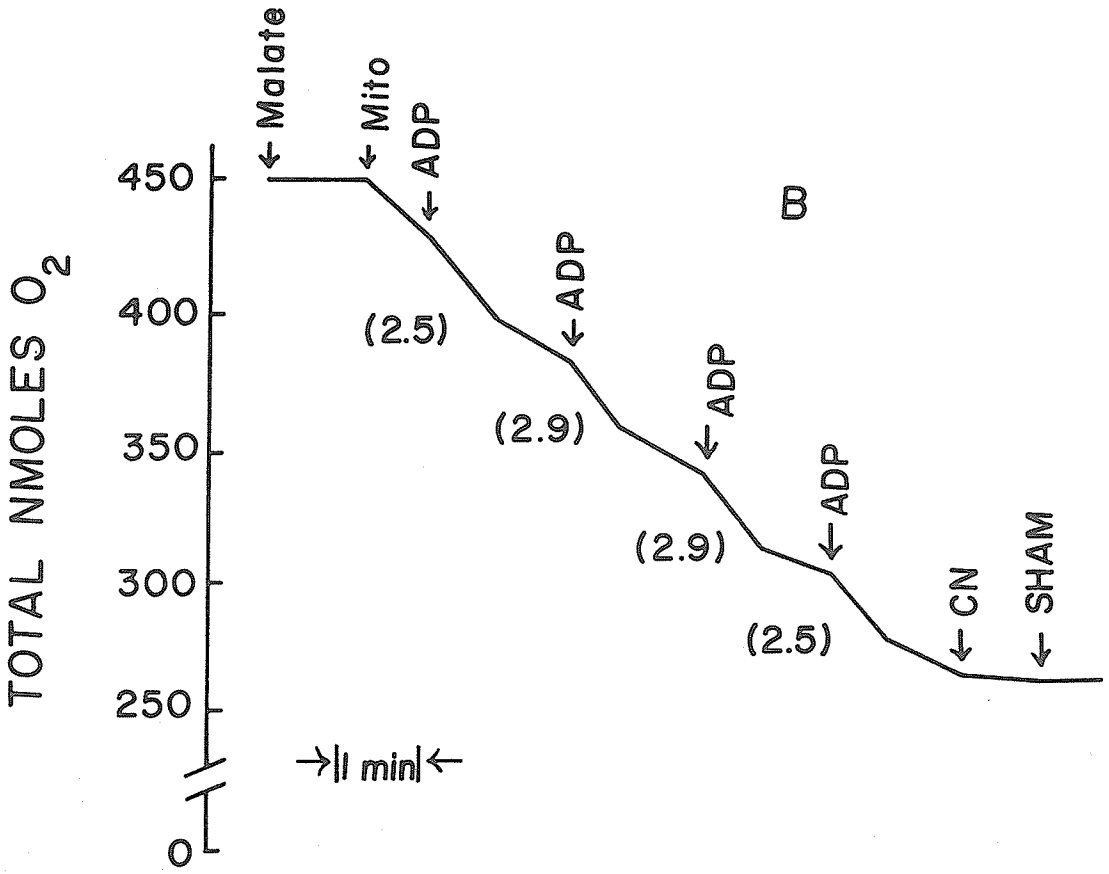
Respiratory characteristics of crude mitochondria obtained by differential centrifugation were compared to percoll purified mitochondria (Figure 5). With both crude and purified mitochondria substrate oxidation was evident in the absence of added ADP. Addition of ADP coupled the mitochondria to phosphorylation and resulted in distinct state 3 and state 4 respiration rates. Generally the ADP/O and RC ratios were much better in the purified mitochondria than from the crude (Figure 5B). Decreasing ADP/O ratios were observed with successive cycles in both the crude and percoll purified mitochondria (Figure 5). This may be due to oxaloacetate accumulation which will inhibit malate oxidation by malate dehydrogenase (Day and Wiskich, 1974).

The addition of cyanide to actively respiring mitochondria will inhibit cytochrome-linked respiration. The resultant oxygen uptake can then be considered as cyanide-insensitive respiration. The results show that a small portion of total respiration was cyanide-insensitive

Figure 5.

Polarographic recordings of oxygen uptake by isolated mitochondria from control grown Norstar coleoptiles. (A) crude mitochondria and (B) percoll purified mitochondria. Numbers in parentheses are the calculated ADP/O ratios. Reaction conditions were 25mM malate, 50uM ADP, 0.5mM cyanide, 0.5mM SHAM and 0.5-1.0 mg mitochondrial protein. Cyanide-insensitive rate in (A) was 7 nmoles O_2/min/mg protein and (B) 6 nmoles O_2/min/mg protein.


TOTAL NMOLES O_2
450
400
350
300
250
0
Malate
Mito
ADP
ADP
ADP
CN
SHAM
(2.3)
(1.8)
(1.7)
A
1 min


TOTAL NMOLES O_2
450
400
350
300
250
0
Malate
Mito
ADP
ADP
ADP
ADP
CN
SHAM
(2.5)
(2.9)
(2.9)
(2.5)
B
1 min

(Figure 5A). Percoll purified mitochondria had a lower cyanide-insensitive rate than the crude mitochondria. This was surprising since overall mitochondrial activity improved significantly in the purified preparation. Inhibition by the hydroxamic acid SHAM indicated that the cyanide-insensitive respiration observed was physiological in nature and not due to "electrode drift".

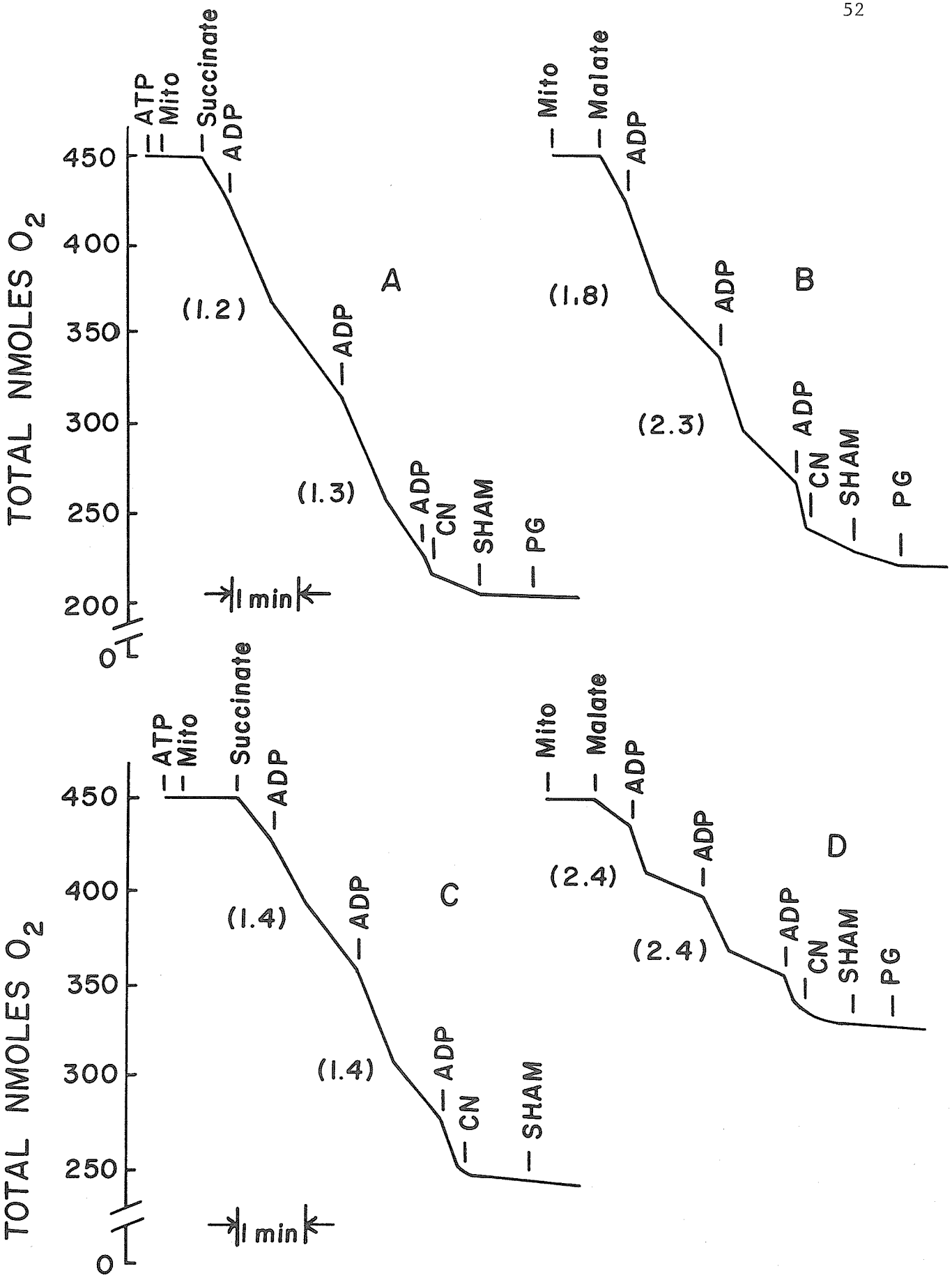
The phenomenom of reduced cyanide-insensitive respiration being obtained from percoll gradients was investigated further under a condition where cyanide-insensitive respiration was previously shown to be stimulated (McCaig and Hill, 1977 and Day et al., 1978).

The condition employed was a germination atmosphere of 20-25% CO_2 in O_2. The results show general improvements in ADP/O and RC ratios when the mitochondria were percoll purified (Figure 6). Comparing malate oxidation with succinate oxidation the respiratory characteristics were much better with malate as substrate (Figure 6A and 6B). This is due to the fact that malate oxidation is coupled to three sites of phosphorylation as opposed to only two sites for succinate. Hence the theoretical maximum for ADP/O ratios with malate and succinate are three and two respectively. Percoll purified mitochondria do approach these limits therefore ADP/O ratios could be used as a measure of the purity of a mitochondrial isolation.

The cyanide-insensitive rates of crude mitochondria isolated from the high CO_2 in O_2 grown seedlings were generally higher than those for control grown seedlings (Figure 5A and 6B). Clearly evident was a reduction in the level of cyanide-insensitive respiration when these same crude mitochondrial preparations were percoll purified (Figure 6C and 6D). The addition of SHAM completely inhibited the cyanide-insensitive

Figure 6.

Polarographic recordings of oxygen uptake by isolated mitochondria from high CO_2 in O_2 grown Norstar coleoptiles. (A) succinate oxidation by crude mitochondria, (B) malate oxidation by crude mitochondria, (C) succinate oxidation by percoll purified mitochondria and (D) malate oxidation by percoll purified mitochondria. Reaction conditions were 25mM malate, 10mM succinate, 50uM ADP, 0.5mM cyanide, 0.5 mM SHAM, 0.5 mM PG and 0.5-1.0 mg mitochondrial protein, 25°C. Cyanide-insensitive rates were (A) 24, (B) 13, (C) 3 and (D) 5 nmoles O_2/min/mg protein.


TOTAL NMOLES O_2
450
400
350
300
250
200
0
ATP
Mito
Succinate
ADP
(1.2)
ADP
(1.3)
ADP
CN
SHAM
PG
1 min
A
Mito
Malate
ADP
(1.8)
ADP
(2.3)
ADP
CN
SHAM
PG
B
TOTAL NMOLES O_2
450
400
350
300
250
0
ATP
Mito
Succinate
ADP
(1.4)
ADP
(1.4)
ADP
CN
SHAM
1 min
C
Mito
Malate
ADP
(2.4)
ADP
(2.4)
ADP
CN
SHAM
PG
D

respiration present in these mitochondria in accordance with the findings of Schonbaum et al. (1971). Percoll itself had no effect on respiration when it was added to crude mitochondrial preparations.

This preliminary evidence suggested that cyanide-insensitive respiration in wheat may actually be non-mitochondrial in origin. These results have been confirmed by the experiments of Goldstein et al. (1980 and 1981) when they showed that antimycin A insensitive respiration was removed from wheat mitochondria when they were purified on a percoll gradient.

Temperature Dependence of Mitochondrial Respiration

Arrhenius plots were constructed for mitochondrial respiration with succinate and malate as substrates (Figure 7 and 8) and comparing crude with percoll purified mitochondria (Figure 8 and 9). In all plots inflections occurred at temperatures below 15°C for state 3, state 4 and cyanide-insensitive respiration. The breaks observed here are different from those previously noted in wheat mitochondria. These differences might be a reflection of the growth conditions of the wheat seedlings. The seedlings were germinated in a closed seed tray which might lead to a buildup of CO_2 due to active respiration and this may affect lipid components and membrane permeability (Glinka and Reinhold, 1972).

The transport of substrates into mitochondria is not a rate-limiting step in terms of its effects on oxidative phosphorylation (Wiskich, 1977). However most studies on substrate transport have been performed at temperatures between 15 and 30°C. No literature is available showing the effects of low temperatures on substrate transport into mitochondria. This raises the possibility that substrate uptake by mito-

Figure 7.

Arrhenius plot for the oxidation of succinate by crude mitochondria isolated from Norstar. The lines represent state 3 (■), state 4 (●) and cyanide-insensitive (▲) respiration rates. Reaction conditions were 10mM succinate, 50uM ADP, 0.5mM cyanide and 0.5-1.0 mg mitochondrial protein.

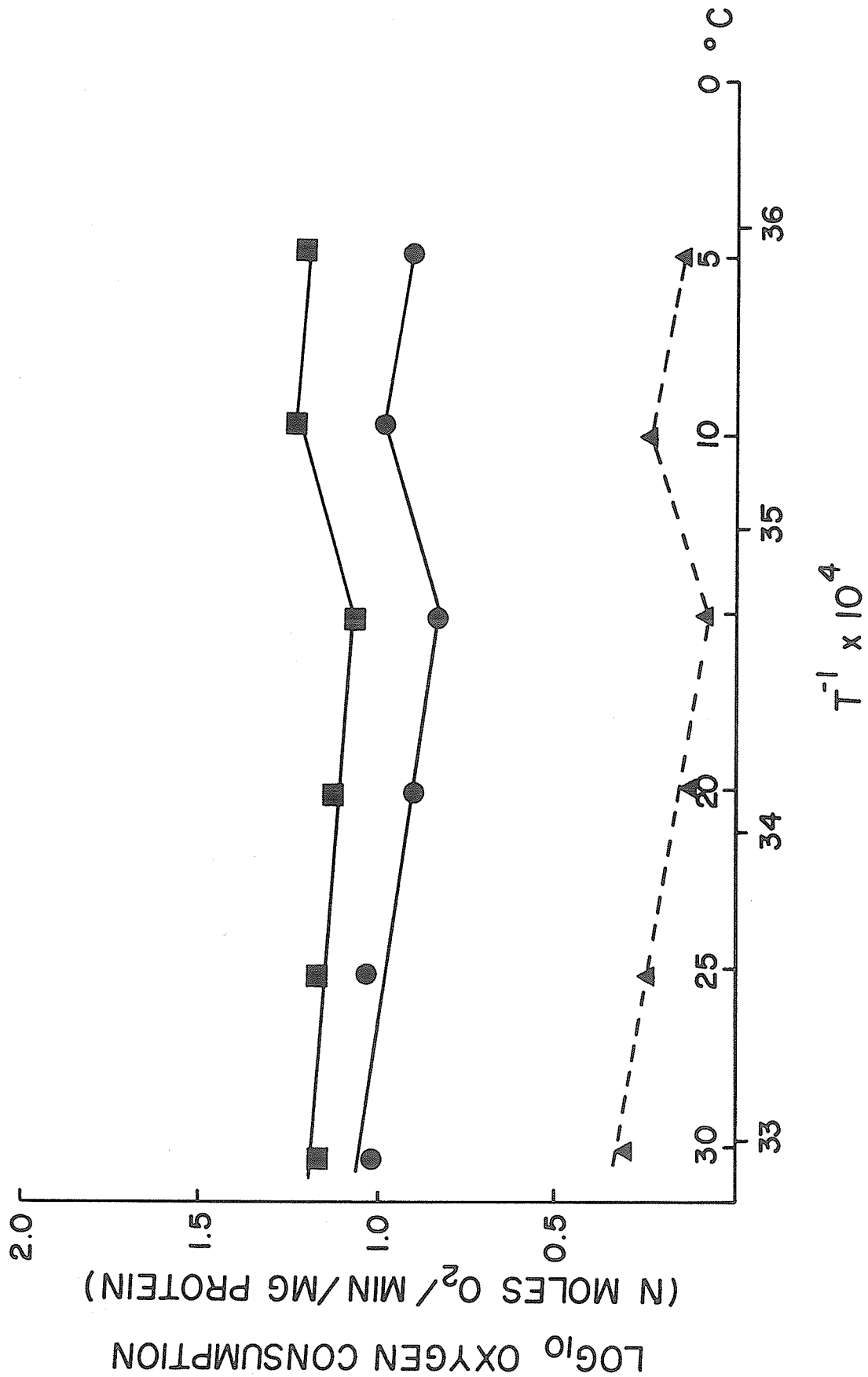

LOG10 OXYGEN CONSUMPTION
(N MOLES O2/ MIN /MG PROTEIN)
2.0
1.5
1.0
0.5
0 °C
5
10
20
25
30
33
36
35
34
T-1 x 10^4

Figure 8.

Arrhenius plot for the oxidation of malate by crude mitochondria isolated from Norstar. The lines represent state 3 (■), state 4 (●) and cyanide-insensitive (▲) respiration rates. Reaction conditions were 25mM malate, 50uM ADP, 0.5 mM cyanide and 0.5-1.0 mg mitochondrial protein.

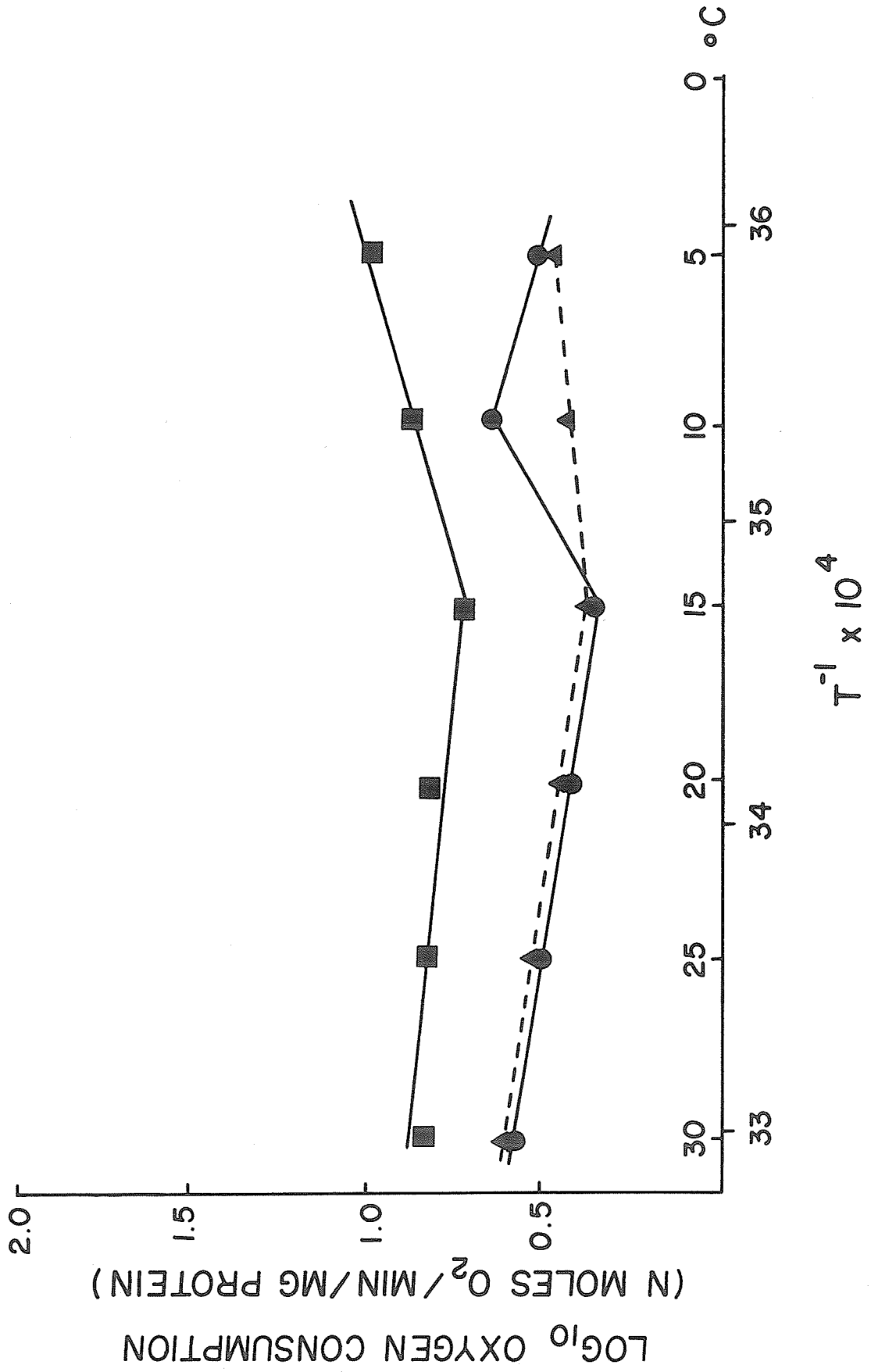
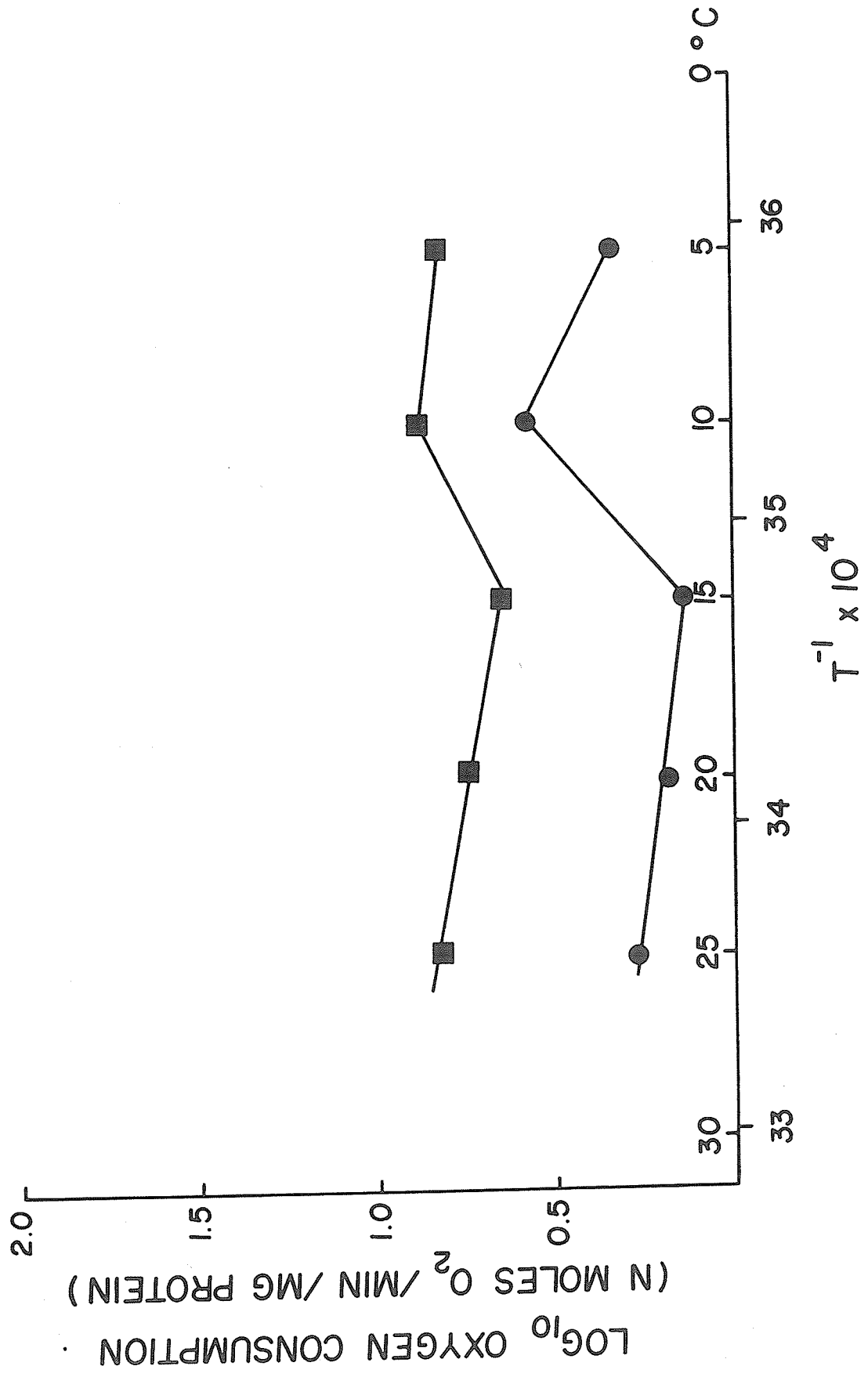

LOG10 OXYGEN CONSUMPTION
(N MOLES O2/MIN/MG PROTEIN)
2.0
1.5
1.0
0.5
0 °C
5
10
15
20
25
30
36
35
34
33
T-1 x 10^4

Figure 9.

Arrhenius plot for the oxidation of malate by percoll purified mitochondria isolated from Norstar. The lines represent state 3 (■) and state 4 (●) respiration rates. Reaction conditions were 25mM malate, 50uM ADP and 0.5-1.0 mg mitochondrial protein.


LOG10 OXYGEN CONSUMPTION
(N MOLES O2 /MIN /MG PROTEIN)
2.0
1.5
1.0
0.5
30
25
20
15
10
5
0 °C
33
34
35
36
T-1 x 10^4

chondria at low temperatures could influence their oxidation rates.

As a corollary to the above suggestion it is also possible that the spatial arrangements of the components of electron transport and phosphorylation could change with temperature.

The results presented here (Figure 7) are representative of two phase changes; one at 10°C which reduces oxidative efficiency and one at 15°C which results in an improvement in oxidation rates. These two phase changes may not be independent since phase changes in a mixture of phospholipids could occur over a broad temperature range (Shimshick and McConnell, 1973). The maximum in O_2 consumption at 10°C may be due to the inability to reach steady state rates at higher temperatures as suggested by Raison (1977) over the short time period of the assay.

The temperature effects on mitochondria oxidizing succinate and malate are very similar (Figure 7 and 8). Succinate oxidation as a whole was higher than malate oxidation over the temperature range studied.

Comparing crude with percoll purified mitochondria two pieces of information became evident (Figure 8 and 9). First of all purified mitochondria showed the same temperature inflections as crude mitochondria suggesting that the discontinuities were actually of mitochondrial origin. Secondly, purified mitochondria had much lower state 4 rates possibly due to the removal of cyanide-insensitive respiration which may have contributed to the basal state 4 rate in crude mitochondria.

Cyanide-insensitive respiration was present in crude mitochondria and showed inflections similar to state 3 respiration (Figure 7 and 8). The Arrhenius plots of cyanide-insensitive respiration are quite different from those obtained by McCaig and Hill (1977). The percentage of state 3 respiration that is cyanide-insensitive is constant indicating

that the cyanide-insensitive path may be Krebs cycle substrate linked. However purified mitochondria had no cyanide-insensitive respiration (Figure 9) which indicates that the cyanide-insensitive path may not be mitochondrial in origin. This presents somewhat of an anomaly since on the one hand cyanide-insensitive respiration is substrate linked but on the other hand it is not an integral component of the mitochondria.

Lipoxygenase Activity

The presence of lipoxygenase in mitochondrial preparations has been previously reported (Parrish and Leopold, 1978). This enzyme has similar characteristics to those used to identify the cyanide-insensitive pathway. Therefore an investigation was warranted to determine if lipoxygenase may be the component which accounts for cyanide-insensitive respiration. Many characteristics of lipoxygenase were studied including its activity in wheat mitochondrial preparations, its distribution through a percoll gradient, effects of inhibitors, affinity for substrate, temperature effects and purification properties.

Lipoxygenase activity can be conveniently measured polarographically using linoleic acid as a substrate (Parrish and Leopold, 1978). This was done for crude and percoll purified mitochondria isolated from control and high CO_2 in O_2 grown wheat seedlings (Table 4). Crude mitochondria had much higher lipoxygenase activity than percoll purified preparations. A stimulation of lipoxygenase activity was also noted to occur in high CO_2 in O_2 grown material. Since cyanide-insensitive respiration was previously reported to be stimulated by high CO_2 in O_2 conditions (McCaig and Hill, 1977) then it follows that the development of lipoxygenase activity could account for part of the observed stimula-

TABLE 4. Lipoxygenase activity in crude and percoll purified mitochondrial preparations

Germination Condition	Linoleic Acid Oxidation	
	crude	purified
	(nmoles O_2/min/mg protein)	
Control Grown	25	5
High CO_2 in O_2	90	3

tion. The removal of cyanide-insensitive respiration concomitant with lipoxygenase activity has been reported with wheat tissue (Goldstein et al., 1980).

Following the discovery that cyanide-insensitive respiration was being removed from mitochondria purified on a percoll gradient it was of interest to determine where on the gradient the cyanide-insensitive respiratory component was distributed and whether this region corresponded to lipoxygenase activity. Oxygen uptake rates were measured for crude mitochondria and for the parts of the percoll gradient corresponding to the 13.5%, 20% and 45% percoll layers (Table 5). High CO_2 in O_2 grown plant material was utilized for the stimulation of the cyanide-insensitive respiratory component.

Almost 50% of the state 3 respiration in crude mitochondria could be attributed to the cyanide-insensitive portion of respiration compared to only 15% in the percoll purified portion (45% layer). Measured respiration was highest in the 20% percoll fraction which also corresponded to the highest cyanide-insensitive rate and lipoxygenase activity.

The sum of the lipoxygenase activities in the three percoll fractions almost equals the activity measured in the crude extract indicating almost a 100% recovery of enzyme activity. The cyanide-insensitive activity was also recovered about 100% paralleling the situation with lipoxygenase. The total state 3 respiration rate was higher in that collected from the percoll gradient than it was in the crude extract. This may indicate that mitochondrial respiration was stimulated possibly as a result of the removal or dilution of inhibitors of cytochrome-linked respiration by the percoll gradient purification step. Broken membranes and fatty acids may be possible sources of inhibitory compounds (Jackson

TABLE 5. Distribution of mitochondrial and lipoxygenase activity through a percoll gradient[a]

	crude	Percoll Gradient 13.5%	20%	45%
	(nmoles O_2/min/mg protein)			
State 3 Rate[b]	82	42	63	51
CN-Insensitive Rate[c]	41	15	20	7
Lipoxygenase Rate	94	37	49	1

[a] High CO_2 in O_2 grown wheat coleoptiles

[b] Conditions employed were 100uM ADP and 10mM succinate

[c] Conditions the same as in (b) plus 0.5mM KCN

et al., 1979).

The major point to be gained from the study of the distribution of respiratory components in a percoll gradient was that the cyanide-insensitive respiratory component appeared to be more closely linked to lipoxygenase rather than the mitochondria. The continuous percoll gradients used by Golstein et al. (1980) separated the crude mitochondria into two bands; one with contamination free mitochondria and the other band showing lipoxygenase activity upon the addition of linoleic acid.

Properties of Wheat and Soybean Lipoxygenase

Inhibitors

Commercial soybean lipoxygenase was used in a study of several inhibitory compounds in order to compare the characteristics of lipoxygenase with those of the cyanide-insensitive pathway.

Propyl gallate was an good inhibitor of lipoxygenase activity being some 50% effective at a concentration of 0.5mM (Table 6). A concentration of 10mM was almost completely effective in quenching the lipoxygenase reaction. The ability of propyl gallate to inhibit lipoxygenase has generally been associated with its antioxidant properties to remove free radicals which are necessary for the lipoxygenase reaction (Eskin et al., 1977). In addition propyl gallate has been shown to chelate ferric ions which may be another means of inhibiting lipoxygenase since Chan (1973) indicated that the enzyme contained one atom of iron per molecule. Inhibition of cyanide-insensitive respiration by propyl gallate has been reported in soybean seeds (Parrish and Leopold, 1978) and in mitochondria isolated from mung bean (Siedow and Girvin, 1980).

SHAM was also shown to be an inhibitor of lipoxygenase (Parrish

TABLE 6. List of compounds and their effects on commercial soybean lipoxygenase activity

Compound	Concentration	% Inhibition
Propyl Gallate	0.5mM	57
Propyl Gallate	5mM	89
Propyl Gallate	10mM	97
SHAM	0.5mM	34
SHAM	5mM	64
SHAM	10mM	76
Ethanol	0.5%	10
Bovine Serum Albumin	0.1%	40
Valinomycin	0.2uM	23
Cobalt Chloride	5mM	35
Cyanide	0.5mM	0
Disulfiram	0.5mM	0
Calcium Chloride	5mM	0
Potassium Phosphate	10mM	0
ADP	100uM	0

and Leopold,1978). The effectiveness of SHAM as an inhibitor of soybean lipoxygenase was not as great as propyl gallate at equivalent concentrations (Table 6). SHAM has long been used as an inhibitor of cyanide-insensitive respiration in plants (Schonbaum et al., 1971). Its ability to inhibit lipoxygenase may be linked to its iron chelation capacities. The possibility of SHAM acting as an antioxidant like propyl gallate has not yet been investigated although Siedow and Girvin (1980) demonstrated that SHAM and propyl gallate inhibit cyanide-insensitive respiration at, or very near the same site.

Ethanol was tested for its effects on lipoxygenase activity since SHAM, PG, valinomycin and disulfiram were all solubilized in this solvent. Ethanol had little inhibitory effects at the concentrations employed (Table 6).

Bovine serum albumin which is used as a free fatty acid stabilizer in buffers did inhibit the lipoxygenase reaction (Table 6). By effectively binding to fatty acids it renders these substrates unobtainable for the lipoxygenase reaction. When BSA was added to wheat mitochondrial preparations there was a reduction in the cyanide-insensitive rate implicating the involvement of fatty acids in that rate.

Valinomycin, which was used to study the swelling response of wheat mitochondria, inhibited lipoxygenase action by 23%. Part of this inhibition could be attributed to the ethanol in which valinomycin was dissolved.

Lipoxygenase has been implicated in the formation of ethylene (Galliard et al., 1968). Grover and Purves (1976) indicated that cobalt increased hypocotyl elongation of cucumber seedlings by inhibiting ethylene production. Cobalt chloride was shown in these results to have an

inhibitory effect on lipoxygenase (Table 6). Therefore the possibility exists that the inhibition of ethylene formation by cobalt may be due to inhibition of the lipoxygenase reaction.

Cyanide was shown to have no effect on lipoxygenase activity and it is through the use of this compound that the cyanide-insensitive rate in mitochondrial preparations can be determined. Chan (1973) reported that soybean lipoxygenase was inhibited by cyanide but only after pre-incubation of the enzyme with the chelating agent. Generally lipoxygenase is considered to be a cyanide resistant enzyme (Eskin et al., 1977).

Disulfiram, which is a compound reported to inhibit the cyanide-insensitive respiratory component in red sweet potatos (Grover and Laties, 1978) had no effect on soybean lipoxygenase (Table 6). Nor did this compound affect the cyanide-insensitive respiration rate observed in wheat mitochondrial preparations.

Since it has been reported that one isozyme of soybean lipoxygenase has a calcium requirement for activity (Christopher et al., 1972) and not knowing whether the commercial soybean preparation contained any significant proportion of this isozyme, it was necessary to see if calcium affected lipoxygenase activity. No effect was observed (Table 6) hence the commercial lipoxygenase preparation did not require the addition of calcium for activity. Wheat lipoxygenase may have an isozyme which is stimulated by calcium although no reports of any such enzyme being present in wheat have been previously reported.

Other compounds which were present in wheat mitochondrial reactions included potassium phosphate and ADP. Neither of these components had an effect on lipoxygenase activity (Table 6).

Michaelis Constant of Lipoxygenase

The affinity of soybean lipoxygenase for linoleic acid was investigated (Figure 10). A Lineweaver-Burk plot was used to determine the Km of lipoxygenase. From the graph the Km of soybean lipoxygenase was calculated to be 0.9mM. This value is similar to that obtained in previous work by Holman (1948) who indicated that at pH 9.0 soybean lipoxygenase had a Km of 1.35mM for linoleic acid. The small deviation in the present study may reflect a difference in the working pH of the assay, 7.2 instead of 9.0, or may be due to isozyme differences in Km.

In contrast to this the determined Km for linoleic acid with wheat lipoxygenase was 5.0uM hence wheat lipoxygenase has a high affinity for substrate (Irvine and Anderson,1953).

The Km of lipoxygenase for oxygen varies between 30uM to 300uM and is dependent on the concentration of linoleic acid (Tappel,1962).

Temperature Effects on the Lipoxygenase Reaction

An Arrhenius plot of crude wheat lipoxygenase activity was constructed for the oxidation of linoleic acid (Figure 11). The plot was linear over the temperature range 10-40^{o}C with an inflection of activity occuring below 10^{o}C. This inflection may be due to oxygen concentration, oxygen being a cosubstrate with linoleic acid for lipoxygenase activity. Oxygen concentrations are greater at lower temperatures however at higher temperatures oxygen availability should not be much of a factor since lipoxygenase has such a high affinity for oxygen (Tappel,1962). However this does raise the question of the value of Arrhenius plots when oxygen acts as a substrate and is changing in concentration with the different temperatures. A system keeping oxygen concentration constant would be

Figure 10.

The Km of commercial soybean lipoxygenase for linoleic acid oxidation. Rate is in nmoles O_2/min.

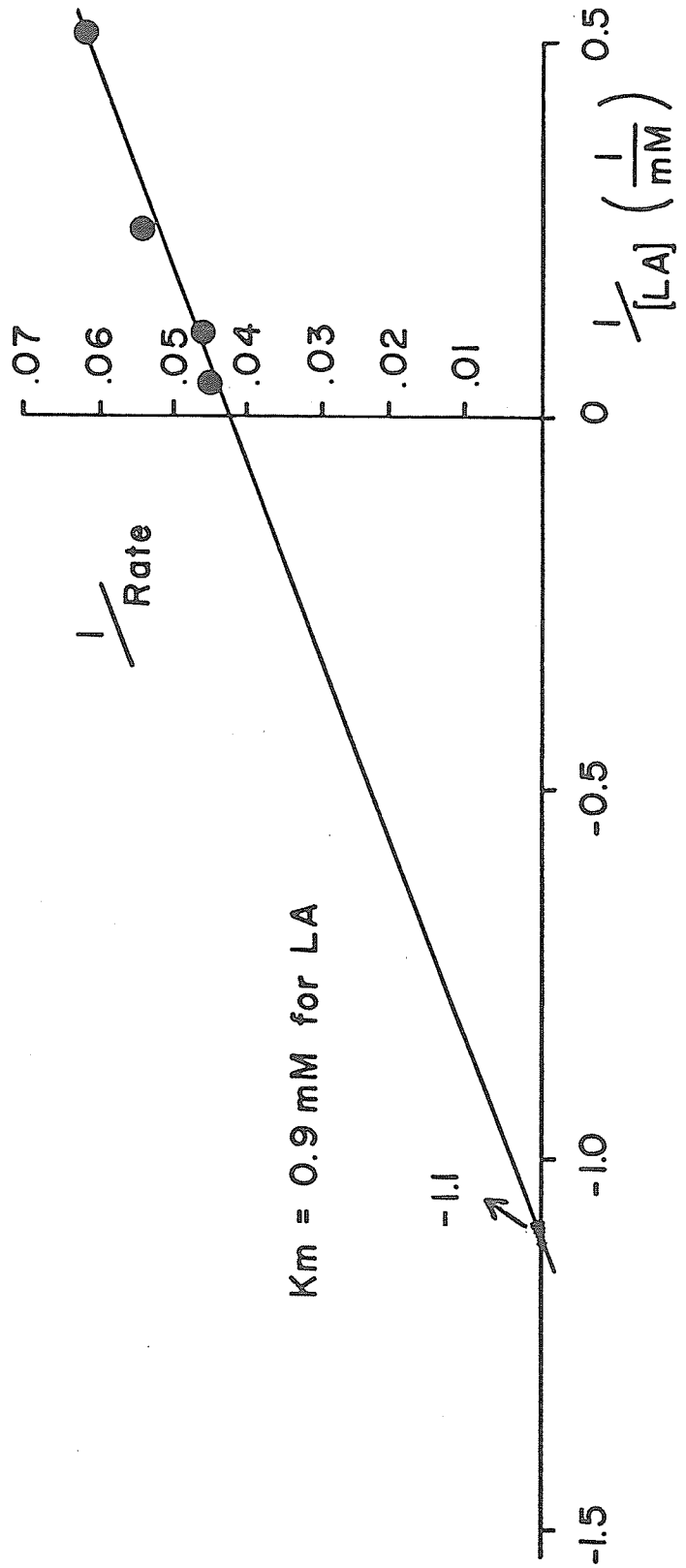
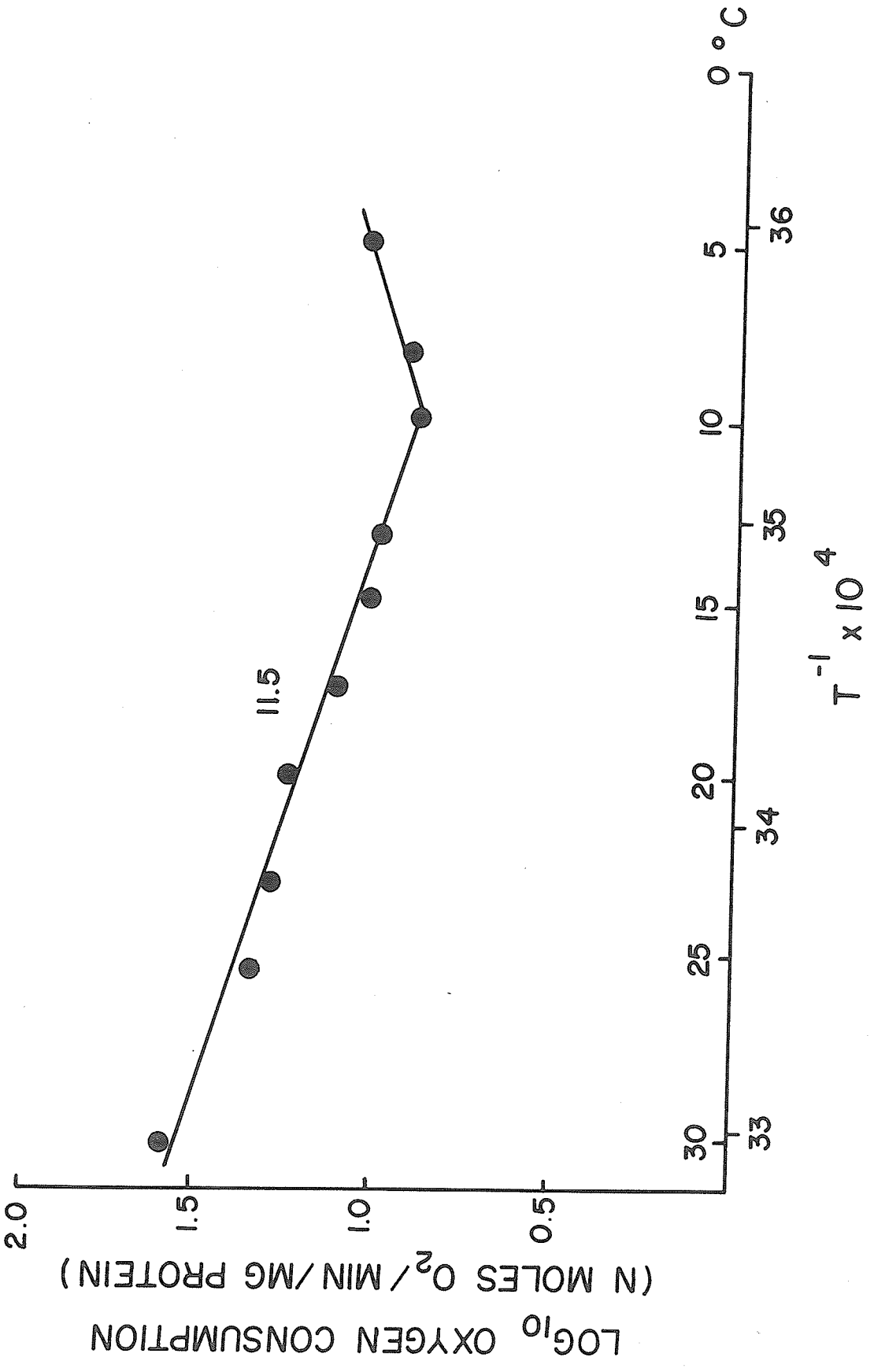

.07
.06
.05
.04
.03
.02
.01
0
0.5
-0.5
-1.0
-1.5
1/Rate
1/[LA] (1/mM)
Km = 0.9 mM for LA
-1.1

Figure 11.

Arrhenius plot of crude wheat lipoxygenase oxidation of linoleic acid. Reaction conditions were 4mM linoleic acid and 1.0 mg soluble protein.


LOG10 OXYGEN CONSUMPTION
(N MOLES O2/MIN/MG PROTEIN)
2.0
1.5
1.0
0.5
11.5
30
25
20
15
10
5
0 °C
33
34
35
36
T-1 x 10 4

highly desirable for these type of experiments.

The activation energy of crude wheat lipoxygenase was determined to be 11.5 kcal/mole. Previous studies determined the activation energy of wheat lipoxygenase to be 6.5 kcal/mole (Irvine and Anderson, 1953) and soybean lipoxygenase to be 4.3 kcal/mole (Tappel, 1962). The higher activation energy for wheat lipoxygenase which was observed in these experiments may be due to pH differences. Irvine and Anderson (1953) determined that wheat lipoxygenase activity is optimal at pH 6.5. By working away from the optimal pH it follows that more energy of activation may be required for the lipoxygenase reaction to proceed.

Purification of Wheat Lipoxygenase

An attempt to purify wheat lipoxygenase by affinity chromatography was performed.

A crude wheat homogenate was first subjected to ammonium sulphate extraction. Protein precipitates were formed with 35%, 55% and 70% ammonium sulphate. Most of the lipoxygenase activity collected in the 35-55% precipitate.

After dialyzing the precipitate overnight against 5mM acetate buffer pH 5.5 the fraction was placed on a CM-cellulose column. This step allows removal of many of the acidic proteins. After elution of protein with 100mM acetate buffer lipoxygenase activity was determined to be in the largest protein peak which eluted from the column (Figure 12).

This fraction was collected and placed onto a linolenic-agarose affinity column. The first protein peak was eluted with 50mM acetate buffer and the second and third peaks were eluted with 200mM acetate buffer (Figure 13). The elution of several peaks all showing lipoxygenase activity may reflect isozyme differences since wheat has been report-

Figure 12.

CM-cellulose purification of lipoxygenase. Wheat homogenate was applied to a (2 cm x 10 cm) CM-cellulose column in 5mM acetate buffer pH 5.5. The peaks were eluted with 100mM acetate buffer. Lipoxygenase activity was assayed polarographically using 4mM linoleic acid as substrate.

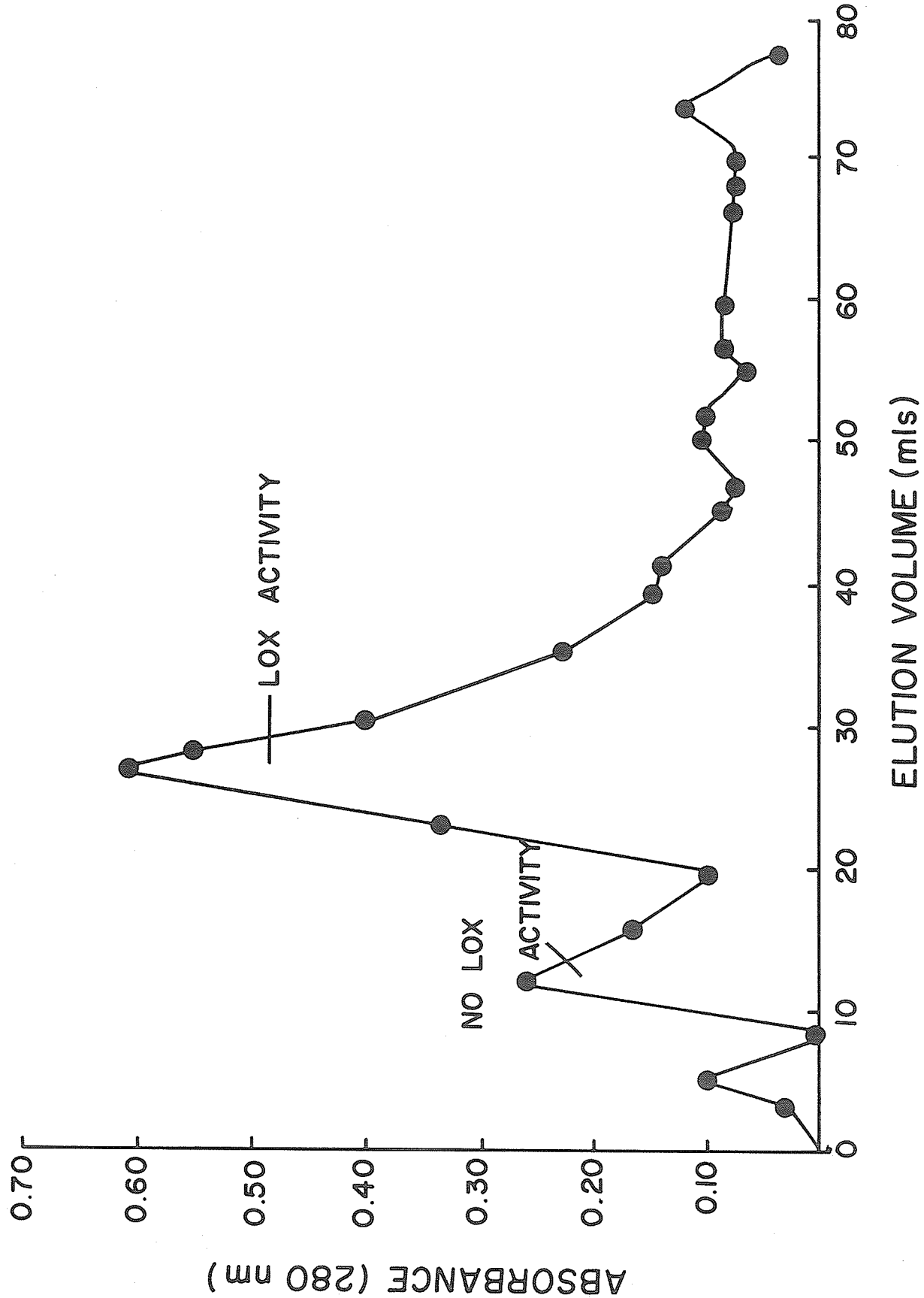
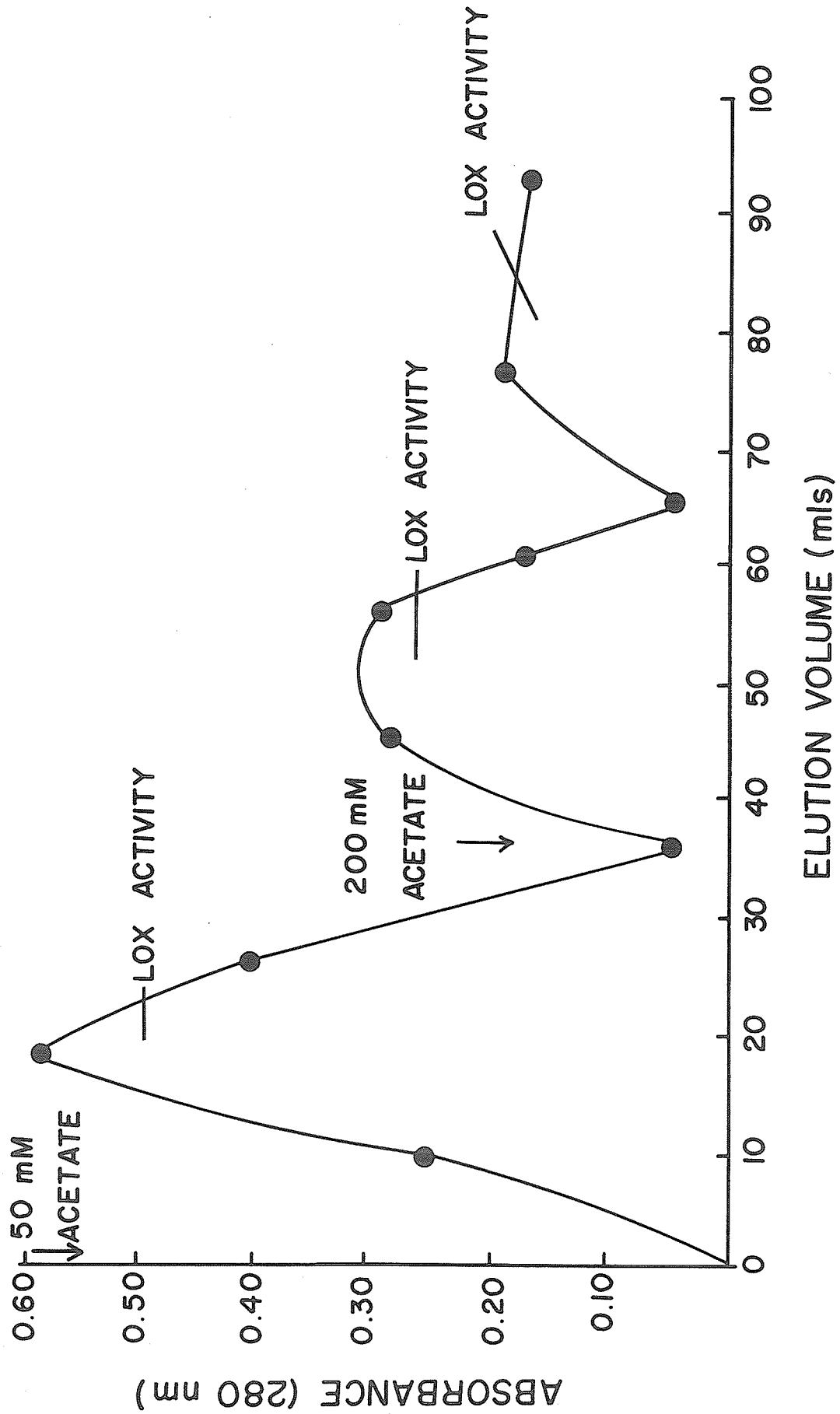

ABSORBANCE (280 nm)
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0
10
20
30
40
50
60
70
80
ELUTION VOLUME (mls)
LOX ACTIVITY
NO LOX ACTIVITY

Figure 13.

Affinity chromatography of lipoxygenase on a linolenic acid aminoethyl agarose column. The enzyme was adsorbed with 5mM acetate buffer (pH 5.5) and the first peak eluted with 50mM acetate buffer (pH 5.5). The subsequent peaks were eluted with 200mM acetate buffer (pH 5.5). Lipoxygenase activity was assayed polarographically with 4mM linoleic acid.


ABSORBANCE (280 nm)
0.60
0.50
0.40
0.30
0.20
0.10
50 mM
ACETATE
LOX ACTIVITY
200 mM
ACETATE
LOX ACTIVITY
LOX ACTIVITY
0
10
20
30
40
50
60
70
80
90
100
ELUTION VOLUME (mls)

ed to have 4 isozymes of lipoxygenase (Guss et al., 1968).

Unfortunately storage of this preparation overnight at 4°C resulted in a total loss of enzyme activity. This indicates that purified wheat lipoxygenase is unstable during storage unlike soybean lipoxygenase which is stable for months at -20°C (Tappel, 1962).

Reconstitution Experiments

Since lipoxygenase activity was shown to be removed from mitochondria by a percoll gradient step this component was thought to contribute to cyanide-insensitive respiration in wheat. Using highly purified mitochondria an attempt was made to reconstitute cyanide-insensitive respiration by adding purified soybean lipoxygenase.

No reconstitution of cyanide-insensitive respiration occured when lipoxygenase was added (Table 7). However when phospholipase A_2 was added with lipoxygenase the cyanide-insensitive oxygen uptake rate did increase to levels approaching those in crude mitochondria. Phospholipase A_2 addition alone did not have any effect on oxygen uptake. Since phospholipase A_2 releases fatty acids from phospholipids it appears that the production of free fatty acids for lipoxygenase activity to occur is enzyme mediated. The enzymic release of free fatty acids from mitochondria has been previously reported in rat liver (Wojtczak and Lehninger, 1961).

In a preliminary experiment the ability of lipoxygenase to use linoleic acid, linolenic acid and trilinolein as substrates was determined. Trilinolein was a poor substrate for lipoxygenase. Eskin and Henderson (1974) also found trilinolein to be a poor substrate for lipoxygenase activity in small faba beans. Therefore it is very doubtful

TABLE 7. Reconstitution experiments with percoll purified mitochondria

	Oxygen Uptake
	(nmoles O_2/min/mg protein)
CN-Insensitive Rate	1.2
+ Lipoxygenase	1.2
+ Phospholipase A_2	1.2
+ Phospholipase A_2 & Lipoxygenase	6.4
CN-Insensitive Rate of Crude Mitochondria	7.5

that lipoxygenase acts directly on the membranes of mitochondria, relying instead upon the release of free fatty acids from the membranes. The activities of lipoxygenase action on linoleic and linolenic acid were similar.

Calcium Effects on Lipoxygenase Activity

In rat liver mitochondria the effect of calcium was to cause the mitochondria to swell resulting in a release of free fatty acids presumably by an enzymic process (Wojtczak and Lehninger, 1961). Such a release could provide a source of substrate for lipoxygenase activity provided calcium affects wheat mitochondria in a similar fashion.

Lipoxygenase activity in crude and purified wheat mitochondrial preparations was stimulated by 5mM $CaCl_2$ (Table 8). Since it is not known whether any of the lipoxygenase isozymes of wheat require calcium for activity it can not be determined whether the stimulation is due to calcium activation of an isozyme or calcium stimulated release of fatty acids. Calcium has been reported to aid lipase activity on triglycerides by removing the freed fatty acids as insoluble calcium soaps (Goodwin and Mercer, 1972). In addition phospholipase A_2 requires Ca^{2+} for maximum activity and this activity can be mediated by the Ca^{2+}-binding protein calmodulin (Wong and Cheung, 1979).

Calcium was reported to act as an uncoupler in animal mitochondria but not in plant mitochondria (Hanson et al., 1965). The action of an uncoupler would not be seen in the absence of an oxidizable substrate. Since no endogenous oxidation rate was observed in the wheat mitochondria it would not be expected that calcium promoted mitochondrial activity hence its only effect would likely be to increase lipoxygenase activity.

TABLE 8. Effect of calcium on crude and percoll purified mitochondrial preparations

	Oxygen Uptake	
	crude	purified
	(nmoles O_2/min/mg protein)	
Linoleic Acid Oxidation	65	16
+ $CaCl_2$ (5mM)	113	21

The swelling responses of mitochondria with calcium are known in rat liver mitochondria (Wojtczak and Lehninger, 1961). In corn mitochondria calcium has been shown to contract swollen mitochondria (Hanson et al., 1965). Whether calcium is transported into mitochondria in plants is unknown. However calcium binding sites with high and low affinities have been identified in several species of plants (Chen and Lehninger, 1973).

Swelling of Isolated Wheat Mitochondria

The possibility that swelling changes in isolated mitochondria might result in the release of free fatty acids prompted a study on the effect on mitochondrial swelling of various substrates and the ionophore valinomycin.

Potassium salts of L-malate and succinate caused swelling of wheat mitochondria in both the reaction and swelling media (Table 9). Lee and Wilson (1972) demonstrated that bean shoot mitochondria could swell in the presence of potassium salts of several organic anions in a medium similar to the reaction medium used here. The swelling response was considerably amplified by use of the swelling medium which has a lower osmolarity than the reaction medium.

The swelling of mitochondria with malate was phosphate dependent since no swelling was observed in the absence of added phosphate. This fits in with the idea of a dicarboxylate transporter for malate which exchanges malate with phosphate (Wiskich, 1977). Hence in the absence of phosphate, malate would not be taken up by the mitochondria and a swelling response would not be observed.

Cyanide was noted to have no effect on the swelling response in-

TABLE 9. Swelling changes in isolated mitochondria

Additions[a]	Absorbance change over 5 min	
	Swelling media[b]	Reaction media[c]
Crude mitochondria		
L-malate	0.220	0.030
L-malate + 0.1% BSA	0.110	0.015
Cyanide + L-malate	0.240	0.028
D-malate	0.000	0.000
Succinate	0.220	0.024
NADH	0.060	0.000
Valinomycin	0.250	0.040
Ethanol	0.300	-
Passive swelling	0.050	0.015
Purified mitochondria		
L-malate	-	0.040
L-malate (Pi free)[d]	0.000	0.000
Passive swelling	-	0.000

[a] Additions were 25mM D or L-potassium malate, 10mM potassium succinate, 0.2uM valinomycin, 0.5mM cyanide, 200uM NADH and 3% ethanol

[b] 20mM TES, 0.2M KCl, pH 7.2

[c] 0.3M mannitol, 10mM K_2HPO_4, 10mM KCl, 5mM $MgCl_2$, pH 7.2

[d] Assayed in the absence of K_2HPO_4

duced by L-malate indicating that substrate transport is a cyanide-insensitive process.

BSA was shown to be inhibitory towards the swelling response thereby implicating the involvement of fatty acids in the swelling of mitochondria (Table 9). Earnshaw et al., (1970) noted that BSA inhibited swelling of Phaseolus mitochondria and suggested that endogenous free fatty acids may be involved in the swelling response by increasing membrane permeability.

D-malate was reported to be transported but not metabolized in rat liver mitochondria therefore a swelling response occurs (Chappell and Haarhoff, 1967). No swelling response was observed in wheat mitochondria using D-malate suggesting that this compound is not transported.

Lee and Wilson (1972) showed that NADH produced swelling in bean shoot mitochondria. In contrast to this Jung and Brierley (1979) showed that NADH caused mitochondria from potato tubers to contract. These results may depend upon whether the mitochondria are in a swollen or contracted state after isolation. The wheat mitochondria isolated in this system are probably in a contracted state hence NADH addition could not produce any further contraction. However the slight swelling which was observed was not much more than that resulting from passive swelling.

Valinomycin is an ionophore which allows K^+ ions to be transported into mitochondria where they accumulate and induce swelling. The swelling response of wheat mitochondria with valinomycin was maximal when compared to the responses obtained with succinate and malate (Table 9).

High concentrations of ethanol also promoted swelling in wheat mitochondria possibly by increasing the permeability of the membranes (Glinka and Reinhold, 1972). However compounds solubilized in ethanol rarely had

ethanol concentrations exceeding 0.5% in a typical mitochondrial reaction so the effects of this solvent are considered to be negligible.

Percoll purified mitochondria could also be induced to swell with L-malate indicating that crude and purified mitochondria possess the same swelling characteristics.

Swelling responses were first linked to the release of free fatty acids from mitochondria by Wojtczak and Lehninger (1961). Pfeiffer and McCay (1972) showed that mitochondria oxidizing exogenous NADPH underwent membrane alterations resulting in a loss of lipid from the mitochondria. The release of mitochondrial components in the presence of substrate has been further demonstrated by Rendon and Packer (1976) when addition of succinate resulted in the release of malate dehydrogenase activity into the intermembrane and extramitochondrial spaces. Malate dehydrogenase is typically situated in the matrix of mitochondria (Day et al., 1979). The addition of succinate was subsequently shown to induce mitochondrial swelling indicating that osmotic swelling changes of mitochondria can result in a loss of lipid and protein components from the mitochondria.

Mitochondrial Lipid Analysis

Membrane lipid changes in fatty acid composition were determined for mitochondria oxidizing succinate in the presence and absence of phospholipase A_2.

The addition of succinate resulted in the production of swollen mitochondria and associated with this was a decrease in mitochondrial lipid (Table 10). The addition of phospholipase A_2 enhanced the fatty acid decrease (Tables 10 and 11). If a fatty acid decrease is equated

TABLE 10. Fatty acid analysis of percoll purified mitochondria isolated from control grown wheat coleoptiles

Fatty Acid	Unswollen Mitochondria	Swollen[a] Mitochondria	Swollen Mitochondria + Phospholipase A_2
		(ug)	
Palmitic	3.8	3.5	3.0
Palmitoleic	1.7	1.4	1.3
Stearic	0.3	0.1	0.1
Oleic	1.6	1.1	0.9
Linoleic	3.0	2.0	1.8
Linolenic	1.5	0.7	0.6
Total	11.9	8.9	7.7

Total free fatty acids of crude extract was less than 10ug/0.1 mls

[a] Swelling was initiated by adding 10mM succinate and 10mM K_2HPO_4 mitochondrial protein was 0.57 mg added

TABLE 11. Fatty acid analysis of percoll purified mitochondria isolated from high CO_2 in O_2 grown wheat coleoptiles

Fatty Acid	Unswollen Mitochondria	Unswollen Mitochondria + Phospholipase A_2	Swollen Mitochondria[a] + Phospholipase A_2
		(ug)	
Palmitic	6.1	5.3	4.2
Palmitoleic	2.0	1.3	1.0
Stearic	0.4	-	-
Oleic	1.5	1.2	1.0
Linoleic	7.9	6.4	5.8
Linolenic	3.8	3.5	2.7
Total	21.3	17.7	14.7

Total free fatty acids of crude extract was 97ug/0.1 mls

[a] Swelling was initiated by adding 10mM succinate and 10mM K_2HPO_4 mitochondrial protein was 0.54 mg added

with a free fatty acid release then the liberated linoleic and linolenic acids can act as substrates for lipoxygenase activity. Since it was not known whether these mitochondria contained any appreciable amount of endogenous phospholipases it could not be determined whether the fatty acid release was enzyme dependent or not. The rat liver mitochondria system apparently relied on the enzymic release of free fatty acids (Wojtczak and Lehninger, 1961).

Phospholipase A_2 was shown to act on nonswollen mitochondria (Table 11) however the swollen condition appeared much more favorable for phospholipase A_2 action in terms of the fatty acid decrease in the mitochondria. The relative percent of each fatty acid remains largely unchanged in swollen and nonswollen mitochondria, whether phospholipase A_2 is present or not, indicating that there is no specific decrease in any one fatty acid.

Based upon the amounts of linoleic and linolenic acids released from the mitochondria, isolated from high CO_2 in O_2 grown wheat, the expected oxygen uptake rate is similar to that measured in the reconstitution experiments (Table 7).

The fatty acid content was generally higher in mitochondria isolated from high CO_2 in O_2 grown seedlings (Table 10). This difference is reiterated by the free fatty acid content of the crude homogenate. The high free fatty acid content of the high CO_2 in O_2 grown material may allow for a greater incorporation of fatty acids into mitochondrial lipids.

The resulting loss of free fatty acids from wheat mitochondria, when they are swollen by addition of a substrate, could provide a likely source of linoleic and linolenic acids for the lipoxygenase reaction.

Stimulation of Cyanide-Insensitive Respiration

Goldstein et al. (1980) suggested that wheat seedling mitochondria lack alternative oxidase respiration and that lipoxygenase activity was sufficient to account for all the observed cyanide-insensitive oxygen uptake. However they noted that while there was no lipoxygenase activity in the absence of Krebs cycle substrates its activity was stimulated by the addition of such substrates.

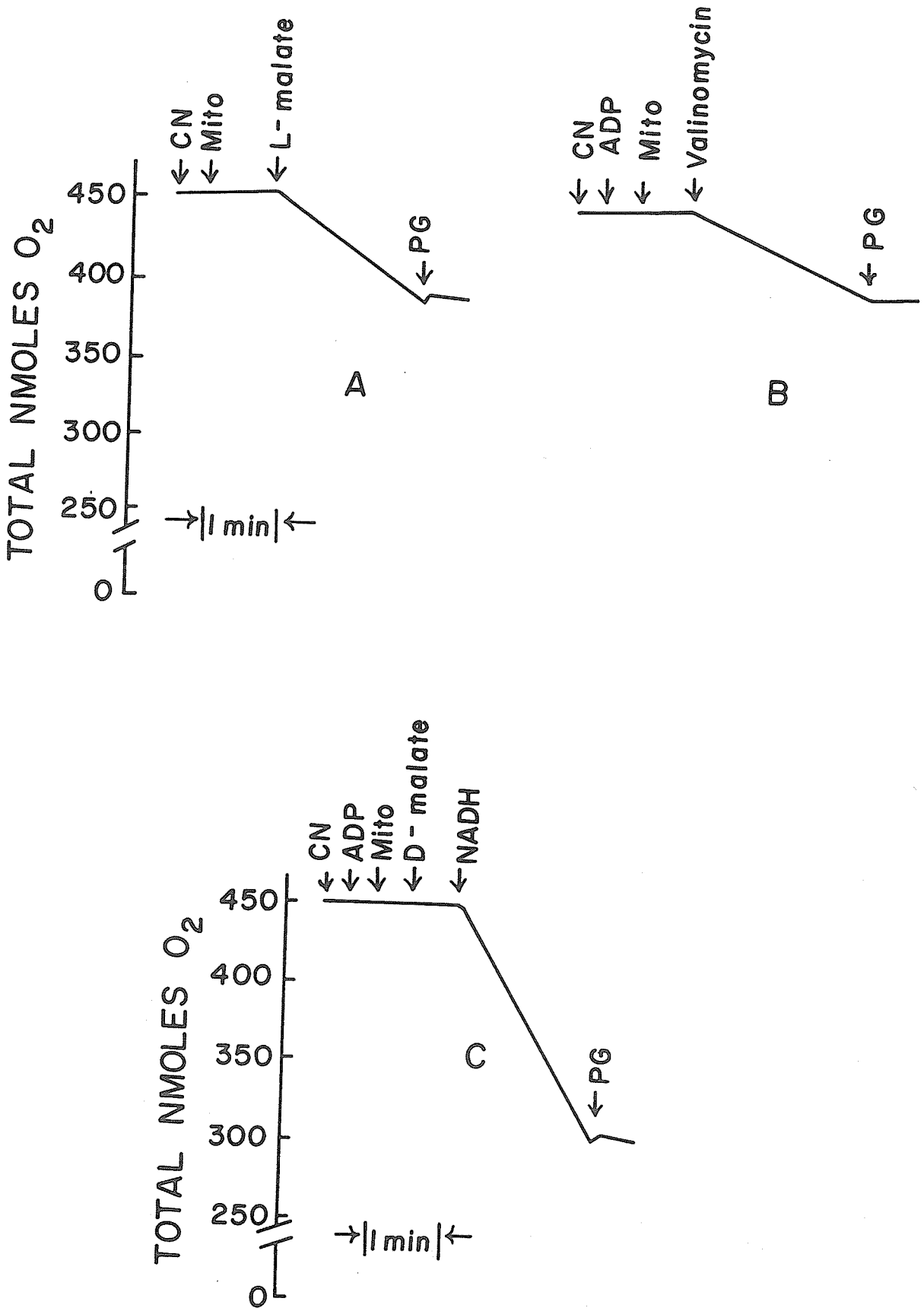
Similar to these observations it was found that crude wheat mitochondria incubated in the presence of cyanide had no endogenous oxygen uptake rate. When L-malate was added there was a stimulation of oxygen uptake which was cyanide-resistant (Figure 14A). Knowing that L-malate induced swelling and release of fatty acids from wheat mitochondria it was thought that the swelling process could initiate lipoxygenase activity.

Valinomycin is a compound which was shown to promote mitochondrial swelling (Table 9). This compound has the added advantage of not being oxidized by actively respiring mitochondria. The addition of valinomycin to mitochondria in the presence of cyanide did induce cyanide-insensitive oxygen uptake (Figure 14B). In the absence of any endogenous oxygen uptake rate it is assumed that no oxidizable substrates were present. Therefore the oxygen consumption observed upon the addition of valinomycin can be attributed to a release of free fatty acids for lipoxygenase activity as the mitochondria swell.

Addition of detergents such as deoxycholate to disrupt mitochondria had no effect on oxygen consumption possibly because such detergents inhibit phospholipase activity and because the membrane components released

Figure 14.

Polarographic recordings of cyanide-insensitive oxygen uptake by mitochondria isolated from Norstar coleoptiles. (A) L-malate induced respiratory increase, (B) valinomycin induced respiratory increase and (C) effects of D-malate and exogenous NADH. Reaction conditions were 25mM D,L-malate, 0.2uM valinomycin, 200uM NADH, 0.5mM cyanide, 50uM ADP, 0.5mM PG and 0.5-1.0 mg mitochondrial protein.


TOTAL NMOLES O_2
450
400
350
300
250
0
CN
Mito
L-malate
PG
1 min
A
CN
ADP
Mito
Valinomycin
PG
B
CN
ADP
Mito
D-malate
NADH
PG
C

would likely be triglycerides and phospholipids rather than free fatty acids. Complex lipids have been shown to be poor substrates for lipoxygenase activity (Eskin and Henderson, 1974).

When D-malate was added to crude mitochondria no increase in oxygen uptake was observed (Figure 14C). This is quite in agreement with the results from the swelling experiments which indicate that D-malate did not cause mitochondria to swell (Table 9). The effects of D-malate noted here are quite different from those noted in rat liver mitochondria where this compound could induce swelling (Chappell and Haarhoff, 1967).

The addition of NADH to wheat mitochondria stimulated cyanide-insensitive oxygen uptake (Figure 14C). The effect of NADH on mitochondria remains questionable. NADH has been shown to contract swollen potato mitochondria (Jung and Brierley, 1979) however NADH had little effect on the swelling response of wheat mitochondria (Table 9).

The oxidation of exogenous NADH and NADPH was demonstrated in potato mitochondria (Arron and Edwards, 1980). Exogenous NADH oxidation was also noted to occur in crude wheat mitochondrial preparations (McCaig 1977). In rat liver mitochondria oxidation of exogenous NADPH caused phospholipid damage and release of free fatty acids dependent upon a component having the properties of a free radical (Pfeiffer and McCay, 1972). Although oxidation of substrates can cause membrane alterations in mitochondria, in the presence of cyanide NADH oxidation should not occur.

The stimulated cyanide-insensitive oxygen uptake was inhibited by propyl gallate (Figure 14) which acts as a free radical quencher of the lipoxygenase reaction. In the mitochondrial lipid analysis the inclusion

of propyl gallate inhibited the release of free fatty acids hence free radicals may be involved in the fatty acid release similar to the system outlined by Pfeiffer and McCay (1972).

Malate Oxidation by Isolated Mitochondria

To further demonstrate that malate was not being oxidized in the presence of cyanide a check for its oxidation products was performed. Malate can be oxidized by the malic enzyme, to produce pyruvate, or by malic dehydrogenase producing oxaloacetate. NAD^+ is reduced to NADH in both of these reactions. Two molecules of malate have to be oxidized by mitochondria in order to consume one molecule of O_2. From the determined amount of malate oxidized the expected O_2 consumption can be calculated and compared to the observed O_2 uptake measured on a polarograph.

In air grown wheat seedlings the amount of malate oxidized in the presence of cyanide accounted for 25% of the observed O_2 uptake (Table 12). Much of the oxaloacetate and pyruvate thought to have been produced from malate may have been endogenous since a check of endogenous levels could more than account for the levels of these metabolites.

In comparison to this the levels of oxaloacetate and pyruvate in state 3 respiration could account for 42% of the observed O_2 uptake in crude mitochondria and 55% in percoll purified mitochondria. In addition the levels of pyruvate and oxaloacetate were significantly greater than endogenous levels. Presumably the reason why all the O_2 consumed could not be accounted for by malate oxidation is that the products are further metabolized.

The extent to which malate oxidation could account for the observed oxygen uptake was even lower in high CO_2 in O_2 grown plant material.

TABLE 12. Determination of malate oxidation products in isolated mitochondria and their relation to oxygen cosumption

	Oxaloacetate produced	Pyruvate produced	Malate oxidized	O_2 uptake Expected	O_2 uptake Observed
	(nmoles/5 min)				
Air Grown					
Endogenous[a]	20	32	52	26	12
CN-Insensitive Rate[b]	32	0	32	16(25%)[d]	64
State 3 Respiration[c]	208	42	250	125(42%)	296
State 3 Respiration (purified mitochondria)	184	128	312	156(55%)	284
High CO_2 in O_2 Grown					
Endogenous	0	12	12	6(10%)	60
CN-Insensitive Rate	25	0	25	12(5%)	216
State 3 Respiration	19	16	35	17(7%)	240
State 3 Respiration (purified mitochondria)	64	137	201	100(53%)	188

[a] Mitochondria with no additions

[b] 25mM malate, 1mM ADP and 0.5mM NaCN

[c] 25mM malate and 1mM ADP

[d] Numbers in parentheses represent the fraction of the observed O_2 uptake accounted for by production of malate oxidation products

Due to a high endogenous rate of O_2 uptake in crude mitochondria the relation between malate oxidized and O_2 consumed was low (Table 12). However in percoll purified mitochondria a high proportion of O_2 uptake could be accounted for by malate oxidation indicating that much of the endogenous rate, which was not associated with malate oxidation, was removed. Lipoxygenase activity is a likely source contributing to the endogenous rate since free fatty acid levels were shown to be high in CO_2 in O_2 grown plant material (Table 11).

Although the products of malate oxidation were significantly greater than endogenous levels in purified mitochondria the cyanide-insensitive oxygen uptake rate could not be attributed to malate oxidation. This experiment indicates that little malate oxidation occurs in mitochondria in the presence of cyanide and that most of the observed cyanide-insensitive respiration is due to the activity of lipoxygenase.

Lipoxygenase Activity in Winter Wheat

The development of lipoxygenase activity in five winter wheat cultivars of varying cold-hardiness was investigated during an overwintering period. All wheats were left for a long enough period of time to become cold-hardened.

When first sampled lipoxygenase activity was relatively high in the Ulianovka and Fredrick cultivars (Table 13). However over a period of some 10 days lipoxygenase activity developed rapidly in the Alabaskaja, Norstar and Kharkov cultivars. In all wheats lipoxygenase activity increased after 10 days of snow cover. No correlation could be drawn comparing lipoxygenase activity with the degree of cold-hardiness since Fredrick, being the least hardy, had relatively low lipoxygenase activity.

TABLE 13. Lipoxygenase activity in overwintered winter wheat cultivars[a]

Cultivar	Days after first snowfall			
	1	8	10	31
	(nmoles O_2/min/mg protein)			
Ulianovka	1.3	1.2	1.9	-
Alabaskaja	0.7	2.1	2.5	-
Norstar	0.6	2.4	3.4	3.7
Kharkov	0.4	1.4	3.9	-
Fredrick	1.4	2.1	2.3	-

[a] Cultivars were planted 13/Sept/80 and the first snowfall occured on 11/Nov/80. Cultivars are listed in decreasing order of cold-hardiness

Since the activity of lipoxygenase appears to be stimulated under conditions where free fatty acid levels are high then the general stimulation of lipoxygenase in these overwintering wheats could be due to some degree of membrane damage. However since these experiments were performed on a field plot,conditions were not constant for all cultivars in terms of the drainage of the land and snow and ice cover. Such conditions could determine the extent to which these wheat cultivars are damaged while they are overwintering.

GENERAL DISCUSSION

Ethanol has been shown to accumulate in ice-encased winter cereal seedlings (Andrews and Pomeroy, 1977). Methanol and acetaldehyde were shown to accumulate in frozen and iced winter wheat seedlings but not to the extent which ethanol accumulates (Table 1). The production of these metabolites was believed to occur by anaerobic glycolysis induced by the anoxic conditions of flooding, freezing and ice-encasement (Andrews and Pomeroy, 1977).

The respiration of cold-hardened and frozen wheat seedlings was shown to have an increased proportion of cyanide-insensitive respiration (Table 2). On the basis of the results presented here it appears that cyanide-insensitive respiration in wheat is due to lipoxygenase activity. Lipoxygenase products can be further metabolized to eventually form, amongst other compounds, acetaldehyde and ethanol (Eskin et al., 1977).

On the basis of the anaerobic metabolite and respiration studies the accumulation of ethanol in cold-abused wheat seedlings may in part be due to breakdown products of fatty acids. In addition the stimulated glycolytic rate thought to occur in cereal plants grown under low temperature conditions may be the result of lipoxygenase activity which increases under these conditions possibly as a result of membrane alterations.

The utilization of a percoll density gradient to purify wheat mitochondria proved to be fortuitous in that cyanide-insensitive respiration was shown to be lacking in the purified mitochondria (Figure 5). However overall mitochondrial activity as judged by the ADP/O and RC ratios was greatly improved. These same observations were made by Goldstein et al. (1980) also working with wheat mitochondria.

Growth conditions of high CO_2 in O_2 were previously shown to stimulate the development of cyanide-insensitive respiration in crude wheat mitochondria isolated from wheat coleoptiles (McCaig and Hill, 1977). Using a germination atmosphere of 20-25% CO_2 in O_2 a similar stimulation was observed in isolated crude wheat mitochondria using either succinate or malate as substrate (Figure 6). Purification of the crude mitochondrial preparation with a percoll gradient again resulted in mitochondria lacking the cyanide-insensitive respiratory pathway.

With the report that cyanide-insensitive respiration can be confounded by the presence of lipoxygenase activity (Parrish and Leopold, 1978) assays for lipoxygenase were performed. Using linoleic acid oxidation as being indicative of lipoxygenase activity it was noted in crude mitochondrial preparations that lipoxygenase activity was stimulated by the high CO_2 in O_2 growth conditions (Table 4). Little lipoxygenase activity was found in purified mitochondrial preparations.

Following the discovery that cyanide-insensitive respiration was being removed from percoll purified mitochondria it was determined where on the percoll gradient the cyanide-insensitive respiratory component was distributed. Cyanide-insensitive respiration was lacking in the 45% percoll layer, corresponding to the purified mitochondria (Table 5). Most of the cyanide-insensitive oxygen uptake was distributed between the 13.5% and 20% percoll layers which corresponds to those fractions of the gradient showing high lipoxygenase activity. The sum of the cyanide-insensitive rates and lipoxygenase rates through the gradient approximates the rates found in the crude mitochondrial preparations. This indicates that the cyanide-insensitive respiratory component and lipoxygenase activity can be recovered from a percoll gradient.

The cyanide-insensitive respiration of isolated crude wheat mitochondria could be inhibited by SHAM and PG (Figure 6). Disulfiram, which inhibits the cyanide-insensitive respiratory components of red sweet potato mitochondria (Grover and Laties, 1978), had no effect on the cyanide-insensitive respiration of wheat. Lipoxygenase activity was also noted to be inhibited by SHAM and PG but not by disulfiram (Table 6). BSA, which binds to free fatty acids, inhibited cyanide-insensitive respiration and lipoxygenase activity.

As a result of the inhibitor effects and the removal of cyanide-insensitive respiration concomitant with lipoxygenase activity from mitochondria purified on a percoll gradient it appears that the cyanide-insensitive respiration in wheat is due to contaminating lipoxygenase activity.

Lipoxygenase is an enzyme which has been implicated in the formation of ethylene (Galliard et al., 1968). It was shown by Grover and Purves (1976) that cobalt could inhibit ethylene production. Cobalt chloride was shown to inhibit lipoxygenase activity (Table 6) suggesting that the inhibition of ethylene production by cobalt may be indirectly due to inhibition of lipoxygenase activity.

The Michaelis constant (Km) of soybean lipoxygenase was determined to be 0.9mM for linoleic acid (Figure 10). Wheat lipoxygenase was shown to have a much higher affinity for linoleic acid with a Km of 5.0uM (Irvine and Anderson, 1953). The lower Km of wheat lipoxygenase would make this enzyme more significant, in terms of activity, than its soybean counterpart.

Temperature effects on crude wheat lipoxygenase activity was linear over the 10-40^{o}C range. McCaig and Hill (1977) reported an inflection

of activity at 17°C for the cyanide-insensitive oxygen uptake rate of isolated wheat mitochondria. If lipoxygenase accounts for cyanide-insensitive respiration then the temperature effects are not consistent. The difference may occur because crude wheat lipoxygenase was assayed with linoleic acid whereas the cyanide-insensitive respiration rate was dependent upon mitochondrial substrates.

Purification of wheat lipoxygenase was achieved through ammonium sulphate fractionation, CM-cellulose chromatography and linoleic acid-agarose affinity chromatography. Unfortunately the purified lipoxygenase activity was very labile as overnight storage at 4°C resulted in a complete loss of activity.

The control of lipoxygenase activity in isolated tissues presents itself as somewhat of a problem. BSA can complex the free fatty acid substrates of lipoxygenase (Table 6) however membrane alterations in organelles could lead to a further release of free fatty acids which the BSA might not be able to absorb. Antioxidants such as PG can inhibit lipoxygenase activity by quenching free radicals (Siedow and Girvin, 1980) but such compounds may pose problems to electron transfer systems. The enzymic control of lipoxygenase activity by glutathione peroxidase is quite effective (Smith and Lands, 1972). Unfortunately when cyanide is added glutathione peroxidase activity is inhibited (Tappel, 1980) thus eliminating an endogenous control of lipoxygenase activity. Perhaps the best way to control lipoxygenase activity in mitochondrial preparations is to remove it by centrifuging through a density gradient as done by Goldstein *et al*. (1980).

The major problem inherent in suggesting that lipoxygenase is responsible for cyanide-insensitive respiration is that in the absence of any endogenous oxygen uptake rate the addition of a Krebs cycle substrate to

isolated wheat mitochondria can initiate a cyanide-insensitive rate (Figure 14A). This could be interpreted as substrate oxidation via the alternative pathway. However compounds such as L-malate have another effect on mitochondria, besides being a source of oxidizable substrate, and that is they can induce isolated wheat mitochondria to swell (Table 9). The effect of the swelling response of mitochondria in initiating cyanide-insensitive oxygen uptake was further demonstrated using the ionophore valinomycin. Valinomycin induces rapid passive swelling of mitochondria in the presence of K^+ ions (Jung and Brierley, 1979) and this compound was shown to stimulate cyanide-insensitive oxygen consumption (Figure 14B).

Swelling of mitochondria is indicative of metabolite transport across the membrane (Wiskich, 1977). Transport of L-malate was dependent upon inorganic phosphate (Table 9) in accordance with the demonstration of a phosphate/dicarboxylate transporter system in mitochondria. BSA inhibited the degree of mitochondrial swelling possibly by fatty acid complexation. The involvement of fatty acids in the swelling response was shown by Earnshaw et al. (1970) with mitochondria isolated from bean hypocotyls which were induced to swell when oleic acid was added in a process inhibited by BSA. Mitochondrial swelling by L-malate can occur in the presence of cyanide (Table 9). D-malate which was reported to be transported in rat liver mitochondria but not oxidized (Chappell and Haarhoff, 1967) had no effect on mitochondrial swelling. This correlates with the observation that D-malate did not initiate a cyanide-insensitive oxygen uptake rate in crude mitochondria (Figure 14C). NADH caused a small swelling response in wheat mitochondria (Table 9) in conjunction with the observations of Lee and Wilson (1972). Although the swelling response was small NADH was able

to stimulate cyanide-insensitive oxygen uptake to a fairly large extent in the mitochondrial preparation (Figure 14C). Large swelling responses were observed with succinate, malate and valinomycin and it was shown that swelling could occur in percoll purified mitochondria (Table 9).

Mitochondrial swelling changes have been implicated in the release of lipid (Pfeiffer and McCay, 1972) and protein (Rendon and Packer, 1976) components from the membrane. The fatty acid content of percoll purified wheat mitochondria was shown to decrease upon the addition of Phospholipase A_2 (Table 11). This decrease was enhanced by the swollen condition of the mitochondria which was induced by the addition of succinate and K_2HPO_4. Swelling of the mitochondria in the absence of added phospholipase A_2 also resulted in a decrease in fatty acid content (Table 10). If a fatty acid decrease in the mitochondria is associated with a free fatty acid release by lipase activity then any liberated linoleic and linolenic acids could become available for lipoxygenase action.

Mitochondria isolated from high CO_2 in O_2 grown wheat seedlings were shown to have a high fatty acid content (Table 11). This correlates with a high free fatty acid content in the crude wheat homogenate. Such a high free fatty acid content may be conducive towards the development of lipoxygenase activity. A stimulation of lipoxygenase activity under high CO_2 in O_2 conditions was noted to occur (Table 4).

Reconstitution experiments with percoll purified mitochondria suggest that the observed increase in cyanide-insensitive oxygen consumption may be dependent upon phospholipase A_2 activity to release free fatty acids for the lipoxygenase reaction (Table 7).

The correlation between the amount of malate oxidized and the measured cyanide-insensitive oxygen uptake was low (Table 12). The correlation was

much higher with the state 3 respiration rate of percoll purified mitochondria. One of the problems with this product determination experiment was that pyruvate and oxaloacetate can be further metabolized. However this experiments did indicate that cyanide-insensitive respiration in isolated wheat mitochondria was not due to malate oxidation.

In a final field related experiment lipoxygenase activity was measured in five cultivars of winter wheat of varying cold-hardiness (Table 13). There was no obvious correlation between lipoxygenase activity and the level of cold-hardiness of the wheat cultivars. However there was a development of lipoxygenase activity in all cultivars over the time spent under snow cover. Since lipoxygenase appears to develop under situations where membrane damage or alterations are occuring then its development may be indicative of the degree to which these plants are being abused by the winter conditions.

SUMMARY AND CONCLUSIONS

Germination of wheat seeds under atmospheric conditions of 20-25% CO_2 in O_2 resulted in a stimulation of the development of cyanide-insensitive respiration and lipoxygenase activity in mitochondria isolated by differential centrifugation. Purification of the crude mitochondrial extract on a discontinuous percoll gradient led to a removal of the cyanide-insensitive respiratory component concomitant with lipoxygenase activity. However both the cyanide-insensitive respiratory component and lipoxygenase activity could be recovered from the non-mitochondrial fractions of the percoll gradient.

Cyanide-insensitive respiration and lipoxygenase activity showed similar responses to inhibitors. Both components were inhibited by PG and SHAM but were unaffected by the addition of disulfiram.

On the basis of the mitochondrial purification studies and the effects of inhibitors the cyanide-insensitive respiratory component in wheat is believed to be due to contaminating lipoxygenase activity.

Cyanide-insensitive respiration could be stimulated in crude mitochondrial preparations by the addition of L-malate and NADH. Valinomycin also stimulated cyanide-insensitive respiration implicating the involvement of mitochondrial swelling in releasing fatty acid substrates for lipoxygenase activity. Subsequent to this L-malate was shown to induce mitochondria to swell even in the presence of cyanide indicating that the addition of Krebs cycle substrates stimulated lipoxygenase activity through the swelling response.

An analysis of mitochondrial fatty acid content showed that when mitochondria were induced to swell by the addition of succinate there

was a general decrease in fatty acid content of the mitochondria. The addition of phospholipase A_2 promoted the fatty acid decrease indicating that the fatty acid decrease may be enzyme mediated. If this fatty acid decrease is equated to a free fatty acid release then the liberated linoleic and linolenic acids can act as substrates for the lipoxygenase reaction.

In a reconstitution experiment the cyanide-insensitive oxygen uptake rate with malate as a substrate could be stimulated by the addition of lipoxygenase and phospholipase A_2. This is further evidence for the enzymic release of free fatty acid substrates for lipoxygenase action.

As further proof that malate stimulation of cyanide-insensitive respiration was through the swelling response and not by oxidation it was shown that very little malate is oxidized in the presence of cyanide by isolated mitochondria. Certainly not enough malate was oxidized to account for the large stimulation of oxygen uptake it induces when added to crude mitochondrial preparations.

The proportion of respiration in cold-hardened and frozen wheat seedlings that is cyanide-insensitive may be due to lipoxygenase activity. If this is so then the accumulation of anaerobic metabolites in these tissues may be partially due to the breakdown products formed as a consequence of the lipoxygenase reaction and not necessarily due to anaerobic glycolysis.

The function of cyanide-insensitive respiration in plants is unclear. The identification of cyanide-insensitive respiration in wheat as being due to lipoxygenase activity does clarify its function somewhat since lipoxygenase activates fatty acids for subsequent metabolism. Lipoxygenase activity becomes evident in tissues undergoing membrane alterations which

coincidently are tissues showing cyanide-insensitive respiration. Further work on cyanide-insensitive respiration will certainly have to take into consideration the extent to which mitochondrial preparations are contaminated with lipoxygenase.

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