

CYANIDE-INSENSITIVE RESPIRATION IN
ISOLATED WHEAT MITOCHONDRIA

A Thesis
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of
Graduate Studies
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by
Thomas Neil McCaig

In Partial Fulfillment of the
Requirements for the Degree

of

Doctor of Philosophy
Department of Plant Science

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THOMAS NEIL McCAIG

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DOCTOR OF PHILOSOPHY

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ABBREVIATIONS

| | |
|--------|--|
| ADP | adenosine-5'-diphosphate |
| Anti-A | antimycin A |
| ATP | adenosine-5'-triphosphate |
| BSA | bovine serum albumin |
| DNP | 2,4-dinitrophenol |
| EDTA | ethylenediaminetetraacetic acid |
| mCLAM | m-chlorobenzhydroxamic acid |
| Mito. | mitochondrial suspension |
| NADH | β -nicotinamide adenine dinucleotide (reduced form) |
| Oligo. | oligomycin |
| R.C. | respiratory control |
| TMPD | N,N,N',N'-tetramethyl-p-phenylenediamine |

ABSTRACT

McCaig, Thomas Neil. Ph.D., The University of Manitoba, May, 1977.

CYANIDE-INSENSITIVE RESPIRATION IN ISOLATED WHEAT MITOCHONDRIA.

Major Professor: Robert D. Hill.

Cyanide-insensitive respiration was studied under a variety of conditions in the mitochondrial fraction isolated from etiolated coleoptiles of four wheat cultivars. The intent of this study was to further our understanding of the function of cyanide-insensitive respiration in plant metabolism.

The wheat mitochondria were found to oxidize α -ketoglutarate, exogenous NADH and succinate rapidly. The oxidation of exogenous NADH did not appear to involve the hydroxamic acid-sensitive alternate electron transport pathway. Succinate oxidation was incompletely inhibited by either cyanide or the hydroxamic acid, m-chlorobenzhydroxamic acid (mCLAM), but was normally completely inhibited in the presence of both cyanide (or antimycin A) and mCLAM; for this reason succinate was used in all subsequent studies.

While the state 3 and state 4 rates of succinate oxidation were similar in the varieties studied, the cyanide-insensitive rates demonstrated a marked cultivar dependence; the cyanide-insensitive capacities of Kharkov and Neepawa mitochondria represented 34% and 8%, respectively, of their state 3 rates.

Arrhenius plots of the state 3 and state 4 rates were relatively linear over the temperature range 2° - 30°C . The cyanide-insensitive

rates, however, exhibited a distinct maximum between 15°C and 20°C.

Germination of the wheat in the presence of high concentrations of CO₂ (10-30%) was found to significantly increase the capacity of the cyanide-insensitive pathway without affecting the potential of the cytochrome pathway. Oxygen acted synergistically with CO₂ in promoting the alternate pathway although increased oxygen concentrations alone did not elicit a promotion. The inclusion or exclusion of ethylene had no effect on the observed results. Besides promoting the alternate pathway, the CO₂-oxygen mixtures evoked an increase in the linoleic/linolenic acid ratio of the mitochondrial lipids.

The cyanide-insensitive capacity was apparently not responsive to changes in the germination temperature; wheat germinated at low temperature (4-6°C) displayed a cyanide-insensitive rate not significantly different from that of wheat germinated at room temperature (20-22°C).

INTRODUCTION

Around 1900, cytoplasmic granules, barely discernible with the light microscope, were observed in a number of cells. With the advent of the electron microscope it became apparent that these tiny particles ($\sim 0.5 - 3 \mu\text{m}$), termed mitochondria, were similar in appearance in a variety of eucaryotic cell types; the distinctive and complex double membrane made identification of mitochondria relatively easy.

Mitochondria are now known to be the site of many cellular reactions, including those involved with respiratory metabolism. The matrix contains the oxidation enzymes of the citric acid cycle while the components of the electron transport chain are closely associated with the inner membrane. The complex nature of the electron transport chain is apparently necessary to ensure that a high proportion of the energy available from substrate oxidation is conserved in the form of ATP.

The majority of mitochondrial studies have involved animal systems. Although many enzymic reactions are common to both plant and animal mitochondria, it is a mistake to assume that plant and animal mitochondria are identical; a few of the differences were recently discussed by Palmer (1976).

One obvious difference between plant and animal mitochondria concerns the phenomenon of cyanide-insensitive respiration which is peculiar to plants. Cyanide, as a potent inhibitor of cytochrome oxidase, completely inhibits respiration of most animal systems. In contrast, plant respiration may be inhibited, unaffected or actually stimulated,

depending upon the tissue. Although the anomalous response of plant respiration to cyanide was reported as early as 1937, only in the last few years has it become generally accepted that plant mitochondria contain not only the cytochrome chain but also a second electron pathway to oxygen which is not inhibited by cyanide (Henry and Nym, 1975).

Cyanide-insensitive respiration has not been adequately studied and at present its role in plant metabolism remains a mystery. The CN-insensitive pathway apparently diverts electron transport from the ATP synthesizing machinery and releases much of the available energy in the form of heat. In the spadix of *Symplocarpus foetidus*, a highly CN-insensitive tissue, the heat resulting from the rapid respiration maintained a tissue temperature 15° to 35°C above the ambient air temperature (Knutson, 1974).

A study of CN-insensitive respiration at the mitochondrial level has not been previously reported for wheat or any of the cereals. Also, considering that this pathway may be involved with the release of energy as heat, it was believed that a general study was warranted as to the response of CN-insensitive respiration to temperature changes.

LITERATURE REVIEW

Historical Development

Cyanide inhibition of respiration was little understood until Keilin (1929) observed that oxidation of yeast cytochromes could be completely inhibited, *in vivo* and *in vitro*, by as little as 10^{-4} M potassium cyanide. Although cyanide proved to be a potent inhibitor of respiration in most mammalian systems, plant respiration was found to be variably affected (*vide infra*). Van Herk (1937) carried out a series of notable investigations of respiration in the spadix of the tropical aroid *Sauromatum guttatum*, as described and extended by James and Beevers (1950), and found that respiration was almost completely resistant to cyanide. The inflorescences of aroids have continued to serve as useful examples for the study of CN-insensitive respiration mainly due to the extremely rapid respiration of this tissue.

Several hypotheses were presented to explain the resistance of plant respiration to cyanide (van Herk, 1937; Okunuki, 1939; Yocum and Hackett, 1957; Chance and Hackett, 1959). However, a generally accepted explanation was not forthcoming until Bendall and Bonner (1971) reviewed the existing evidence and concluded that CN-resistance resulted from a branching of the mitochondrial electron transport chain on the substrate side of cytochrome b. In the same year, a significant obstacle to the study of this phenomenon was overcome when Schonbaum *et al.* (1971) reported that hydroxamic acids specifically inhibited CN-insensitive oxygen uptake. A simplified view of the

electron transport chain in plant mitochondria, which includes the possible involvement of the CN-insensitive pathway (also referred to as the alternate pathway), is outlined in Fig. 1.

It cannot be overemphasized, however, that exceedingly little is unequivocally understood of the mechanism of CN-insensitive respiration and any possible physiological role remains purely speculative.

Variations of Cyanide-Insensitive

Respiration with Plant Tissue

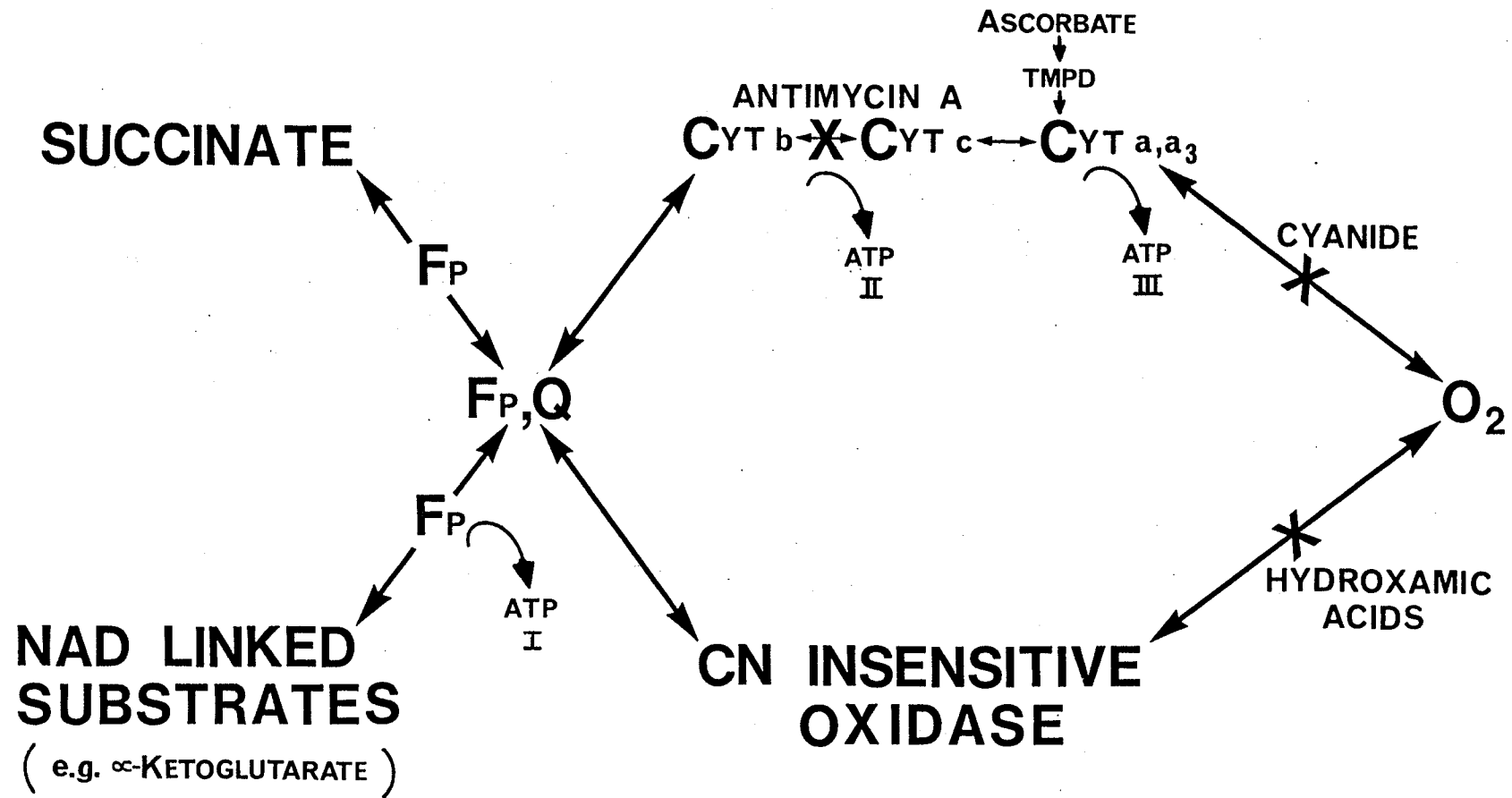
James and Elliott (1955) demonstrated that respiration of the crude mitochondrial fraction isolated from the *Arum* spadix was CN-insensitive in agreement with studies on tissue slices by van Herk (1937). Extensive study of potato tuber mitochondria revealed an anomalous dependence on physiological stage; Hackett *et al.* (1960) found that mitochondria isolated directly from whole tubers were CN-sensitive while mitochondria from tuber slices which had been aerated for 24 hr were CN-resistant.

Bahr and Bonner (1973a) studied mitochondria isolated from a variety of plant tissues and reported the percentage of respiration resistant to 0.5 mM cyanide: white potato (*Solanum tuberosum*) tuber, 1%; Alaska pea (*Pisum sativum*) hypocotyls, 10-15%; mung bean (*Phaseolus aureus*) hypocotyls, 15-20%; sweet potato (*Ipomoea batatas*) tubers, 50%; *Arum maculatum* spadix, 100%.

The climacteric, a respiratory rise which accompanies the ripening of certain fruits, may involve the CN-insensitive pathway (Solomos and Laties, 1976). Tingwa and Young (1974) concluded that the CN-resistant pathway was operating at all stages of ripening in avocados.

Figure 1

Possible electron transport pathway in plant mitochondria.



Cyanide-insensitive respiration has not been extensively studied in wheat or in the cereals generally. James and Boulter (1953) reported that approximately 25% of the respiration of barley root tips was resistant to 1 mM cyanide or azide; Elliason and Mathieson (1956) reported similar results for wheat roots. The respiration of young leaves from maize, and other plants, was inhibited by cyanide whereas respiration of older leaves was actually stimulated (MacDonald and De Kock, 1958). Although Abdul-Baki and Baker (1970) observed changes in cyanide sensitivity of the barley floret during development, they concluded that barriers to gas exchange in the mature floret were at least partially responsible. However, none of the above studies was extended to the mitochondrial level.

Only two accounts of cultivar dependence of CN-insensitive respiration were found in the literature. Bakumenko (1974) demonstrated that respiration of a frost-resistant spring wheat cultivar was inhibited 15% by 1 mM cyanide whereas two frost-sensitive spring cultivars were inhibited 45%. Passam (1976) recently demonstrated that mitochondria isolated from cassava (*Manihot esculenta*) exhibited 32-68% CN-insensitive respiration depending upon the cultivar.

Although the observed capacity for electron flux through the CN-insensitive oxidase varies markedly with tissue studied, Bonner (1965) may have been correct when he suggested that the CN-insensitive pathway could be ubiquitous among plants.

Inhibitors

Bendall and Bonner (1971) reported that CN-insensitive respiration was inhibited by iron chelators. However, the chelators chosen, α, α' -dipyridyl and 8-hydroxyquinoline, inhibited cytochrome respiration at

concentrations only slightly above those needed to inhibit CN-insensitive respiration (Bahr and Bonner, 1973a). Schonbaum *et al.* (1971) reported that hydroxamic acids, which chelate iron very tightly, were specific inhibitors of CN-insensitive respiration at extremely low concentrations. The hydroxamic acids, and specifically the substituted benzhydroxamic acids (Fig. 2), have proven exceedingly useful for the study of this phenomenon. Since the hydroxamic acids chelate ferric ions with much greater affinity than other transition metals, a non-heme iron protein was implicated (Bendall and Bonner, 1971). The only reported inhibitor of CN-insensitive respiration which is not an iron chelator is piericidin A, a classic inhibitor of electron transport in the region of phosphorylation site I (Wilson, 1971).

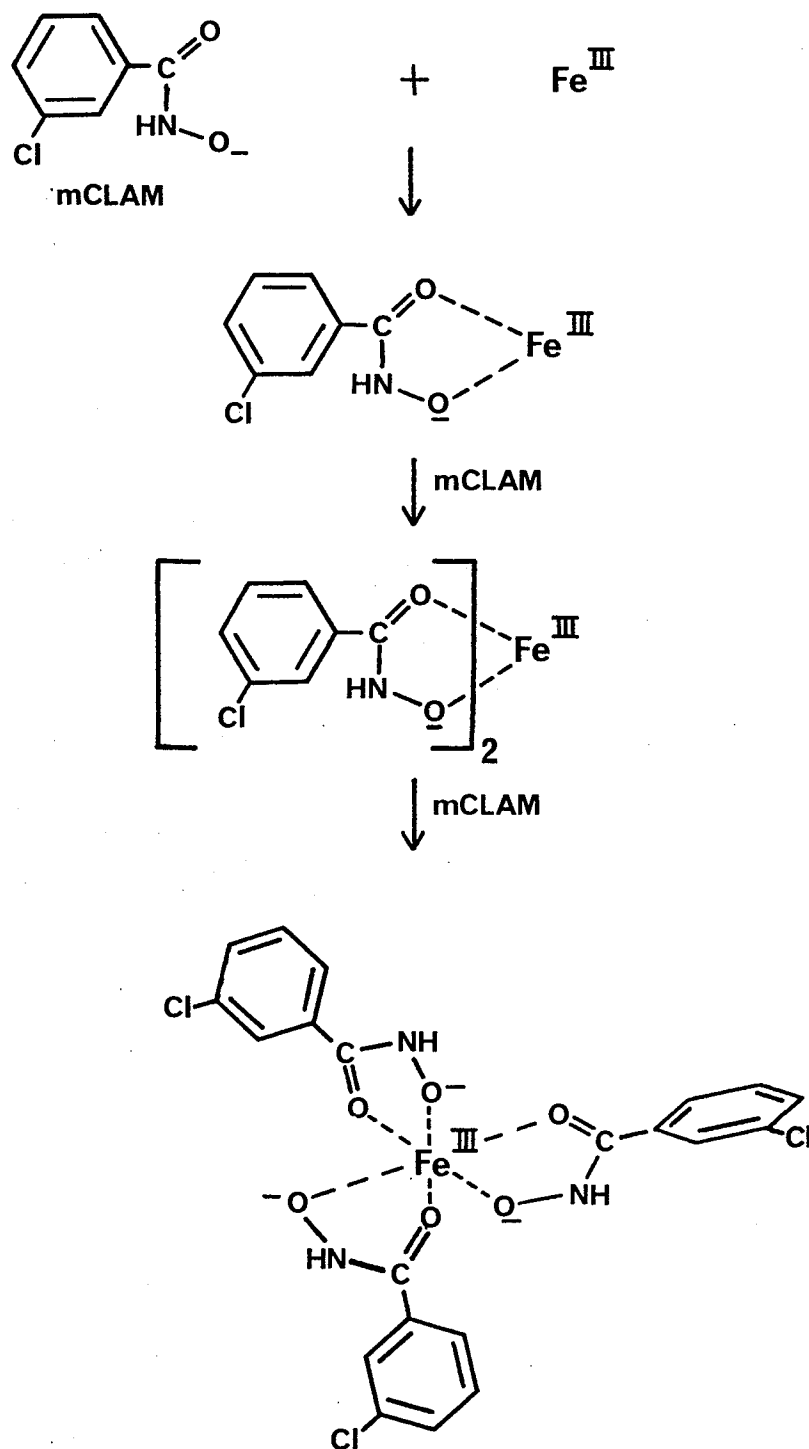
Evidence for the Location and Mechanism of Cyanide-Insensitive Respiration in the Mitochondria

The continued use of the nomenclature 'CN-insensitive' respiration is far from satisfactory but does reflect the uncertainty surrounding this topic. Respiration may continue in the presence of hydrogen cyanide for various reasons. For example, the fungi *Stemphylium loti* (Fry and Millar, 1971) and *Gloeocercospora sorghi* (Fry and Munch, 1975) synthesized an HCN-induced formamide hydro-lyase and converted the hydrogen cyanide to formamide. However, evidence presented in this section is intended to demonstrate the general existence among plants of a specific type of CN-insensitive respiration associated with electron transport in plant mitochondria.

Although the CN-insensitive oxygen uptake of plant tissue could often be accounted for in the crude mitochondrial fraction (James and

Figure 2

Complexes of an hydroxamic acid (mCLAM) with ferric ion.



Elliott, 1955; Hackett *et al.*, 1960), Nakano and Asahi (1970) purified the mitochondrial fraction from fresh and aged potato slices using sucrose density gradient centrifugation and found that the CN-insensitive respiration of aged slices resided mainly in a band of mitochondria which were thought to be newly formed during the aging process.

An early hypothesis, put forward by Chance and Hackett (1959) among others, suggested that CN-insensitive respiration resulted simply from an incomplete inhibition of cytochrome oxidase. However, Wiskich and Bonner (1963) measured the cytochrome oxidase activity of sweet potato mitochondria by observing the oxidation of ascorbate + TMPD (an artificial electron mediator) which donated electrons just prior to the cytochrome oxidase step (Fig. 1). They found that ascorbate + TMPD oxidation was almost totally inhibited by cyanide while succinate oxidation was inhibited only approximately 50%; apparently the electron flux from succinate oxidation in the presence of cyanide greatly exceeded the residual cytochrome oxidase activity. Actually the evidence indicated that cytochrome oxidase was essentially completely inhibited with as little as 0.1 mM cyanide (Hackett *et al.*, 1960; Kano and Kumazawa, 1972). Reports that a second cytochrome oxidase inhibitor, azide (Bendall and Bonner, 1971), as well as antimycin A (Ikuma and Bonner, 1967), which inhibits between cytochromes b and c, inhibited mitochondrial oxygen uptake only to the same extent as cyanide effectively refuted the excess cytochrome oxidase hypothesis.

Although the b cytochromes remained partially oxidized in the presence of cyanide, kinetic experiments by Storey and Bahr (1969) determined that the oxidation rate was far too slow to account for CN-insensitive oxidation. In the presence of antimycin A, all of the b

cytochromes were effectively reduced (Bendall and Bonner, 1971).

The flavoprotein region of the electron transport chain has been implicated in CN-insensitive respiration for a number of years; however, flavoproteins have proven difficult to study. Certain high redox potential flavoproteins which could be reduced directly by succinate remained relatively oxidized in the presence of cytochrome oxidase inhibitors (Erecinska and Storey, 1970). Bendall and Bonner (1971) rejected the possibility of a simple flavoprotein as the terminal oxidase since the expected product of such an oxidase, hydrogen peroxide, was not found and the reported K_m for oxygen ($0.5 \mu M$) was well below the K_m of other flavoprotein oxidases. In fact, H_2O_2 may be the initial product of the alternate oxidase (Rich *et al.*, 1976) although the evidence is inconclusive, and the oxygen affinity of the CN-insensitive oxidase has never been adequately studied.

The determination of ADP/O ratios ($\mu moles$ ADP phosphorylated/ μ atoms oxygen reduced) and respiratory control ratios (oxygen uptake rate in the presence of ADP/oxygen uptake rate following ADP depletion) aided in the study of the alternate pathway. According to Bonner (1973), the cytochrome containing pathway of plants probably contains three sites of ATP synthesis analogous to mammalian mitochondria (Fig. 1), with the following limiting ADP/O ratios depending on the substrate oxidized: ascorbate + TMPD, one; succinate, two; NAD-linked substrates, three (ignoring substrate level phosphorylation). However, the ADP/O and respiratory control (R.C.) ratios for skunk cabbage mitochondria were very low, but approached the expected values in the presence of an hydroxamic acid which presumably prevented oxygen uptake by the non-phosphorylating alternate pathway (Schonbaum, 1971). In contrast,

black-eyed pea mitochondria, which had little CN-insensitive respiration, yielded relatively high ADP/O and R.C. ratios which could not be improved by addition of an hydroxamic acid (Bahr and Bonner, 1973a). By using mitochondria from various plant sources and measuring the ADP/O and R.C. ratios with different substrates (mainly succinate and malate) in the presence and absence of cyanide (Storey and Bahr, 1969), evidence was obtained which was consistent with the CN-insensitive pathway branching between phosphorylation sites I and II (Fig. 1).

Information concerning the ability of Krebs cycle intermediates other than succinate and malate to donate electrons to the alternate pathway has been less conclusive. James and Elliott (1955) reported that the oxidation of citrate and α -ketoglutarate by *Arum* mitochondria was unaffected by cyanide. Skunk cabbage mitochondria oxidized α -ketoglutarate in the presence of cyanide and retained some phosphorylating capacity, presumably at phosphorylation site I (Storey and Bahr, 1969). However, since α -keto acids are capable of removing cyanide through cyanohydrin formation, the results with α -ketoglutarate are difficult to interpret. *Arum* spadix mitochondria oxidizing α -ketoglutarate yielded low ADP/O ratios (0.7 - 1.2) and poor respiratory control (Lance, 1974), while mung bean mitochondria (which had a low CN-insensitive capacity with succinate) yielded high ADP/O ratios (3.0) and good respiratory control (Bonner, 1973); although somewhat indirect, these results suggested that electrons from α -ketoglutarate did have access to the CN-insensitive pathway.

Plant mitochondria, in contrast to animal mitochondria, oxidized exogenous NADH rapidly (Douce *et al.*, 1973). The NADH apparently did not pass through the inner membrane but was oxidized by at least two

external dehydrogenases not associated with the CN-insensitive pathway (Palmer, 1976).

In summary, available evidence has suggested that electrons resulting from the endogenous oxidation of all Krebs cycle intermediates had access to the CN-insensitive pathway. Rich and Moore (1976) recently presented a model for the possible molecular-level involvement of CN-insensitive respiration in the overall scheme of electron transport. Based on kinetic information and a recent hypothesis of Mitchell (1975a, 1975b), they proposed that ubiquinone was reduced by mitochondrial dehydrogenases and reoxidized by the alternate oxidase.

Temperature Dependence of Reactions

Chemical reactions, including enzymatic reactions (within limited temperature ranges), exhibit increased rates as the temperature rises. However, not all enzymatic reactions respond identically to temperature variations and the temperature dependence of a given reaction has proven useful in the elucidation of the mechanism involved (Westley, 1969).

Arrhenius (1915) was among the first to note the relationship between temperature and reaction rates. He derived a strictly empirical equation (based on the van't Hoff equation describing the temperature dependence of the equilibrium constant) which related the rate constant, k , to the absolute temperature, T .

$$\frac{d \ln k}{dT} = \frac{E^*}{RT^2} \quad R = \text{gas constant} \quad (1)$$

Arrhenius assumed that the energy term E^* was the energy that the reacting molecules must acquire to overcome molecular repulsions and form an

'activated complex'; E^* became known, therefore, as the 'activation energy' which was dependent upon the exact nature of the reaction. As the temperature increases the fraction of the molecules which attain the activation energy increases and, hence, the rate constant increases. If E^* is independent of temperature, integration of equation (1) yields

$$\ln \frac{k_2}{k_1} = \frac{E}{R} \left(\frac{1}{T_1} - \frac{1}{T_2} \right) \quad (2)$$

in which k_1 and k_2 are the rate constants at temperatures T_1 and T_2 . A plot of the logarithm of the rate constant against the reciprocal of the temperature should yield a straight line with the slope dependent upon E^* ; this is the well-known Arrhenius plot.

Arrhenius plots of enzymic reactions are often difficult to interpret. Many reactions involve several steps with different temperature coefficients. It is perhaps surprising, therefore, that not only have many enzymic reactions yielded linear Arrhenius plots but multienzyme processes, such as respiration, often exhibited relatively good linearity (Raison, 1973). Since linearity in an Arrhenius plot indicated a constant activation energy over a selected temperature range, Crozier postulated that multicomponent reactions may often be rate-limited by a single step (reviewed by Raison, 1973).

However, the Arrhenius plots of several enzymic reactions or processes were found to be nonlinear. Various plant mitochondrial (Lyons and Raison, 1969), chloroplast (Shneyour *et al.*, 1973) and rat liver mitochondrial (Lee and Gear, 1974) reactions gave Arrhenius plots consisting of linear segments of different slopes indicating a change in the overall activation energy at some temperature. Raison (1973)

pointed out that most of the reactions exhibiting discontinuities, or 'breaks', in the Arrhenius plots were closely associated with membranes. Since the exact nature of the component membrane lipids (e.g. degree of unsaturation) partially determines the physical properties of the membranes, changes in the activation energies of membrane-linked reactions could arise from phase changes (i.e. marked changes in the fluidity) in the membrane lipids at certain temperatures which alter the conformation of the associated enzymes.

A recent study by Pomeroy and Andrews (1975) demonstrated a discontinuity between 10-14°C in the Arrhenius plots of wheat mitochondrial respiration. However, no study of the effects of temperature specifically on the CN-insensitive pathway could be found in the literature for any plant tissue.

Gases and Cyanide-Insensitive Respiration

Oxygen

James and Beevers (1950) demonstrated that respiration of slices of the CN-insensitive *Arum* spadix increased as the oxygen concentration was increased up to 100% O₂ whereas respiration of slices of the CN-sensitive peduncle saturated at 5% O₂; they designed their experiments to preclude diffusion limitations and therefore assumed that CN-insensitive oxidation depended upon a different oxidative mechanism. Wilson (1970) suggested that the dissolved oxygen concentration was a means of controlling heat production by the alternate pathway in the *Arum* spadix. MacDonald (1967) found that Arrhenius plots of respiration of fresh cut potato disks (CN-sensitive) were linear whereas aged

disks (CN-insensitive) yielded Arrhenius plots with a loss of linearity between 15-20°C; the linearity could be restored by increasing the oxygen tension. Increased oxygen concentrations initiated synthesis of the CN-insensitive pathway in suspensions of cultured sycamore cells (Wilson, 1971).

Ethylene

The ability of ethylene to stimulate respiration of plant tissues was first reported by Denny (1924) as described by Abeles (1973). Recently, Solomos and Laties (1976) presented evidence that the CN-insensitive pathway must be operative for ethylene to enhance respiration. The stimulation of respiration by ethylene was demonstrated for numerous plant tissues, including fruits, storage organs and flowers (Abeles, 1973).

Hydrogen Cyanide

Respiration of certain plant tissues, such as potato tubers (Hanes and Barker, 1931) and avocado fruits (Lips and Biale, 1966), was found to be actually stimulated by cyanide. Hydrogen cyanide was able to induce the alternate pathway in wheat and rice seedlings (Kano and Kumazawa, 1972). Stimulation of plant respiration by hydrogen cyanide and ethylene was recently reviewed by Solomos and Laties (1976).

Possible Physiological Roles

According to Meeuse (1975) the thermogenic nature of the *Arum* inflorescence was first discovered by Lamarck (1778). The philodendron inflorescence consumed oxygen at rates approaching those of flying hummingbirds; the respiratory rates correlated well with the rates of heat production (Nagy *et al.*, 1972). Knutson (1974) reported that the spadix of eastern skunk cabbage (*Symplocarpus foetidus*) maintained an internal temperature up to 35°C above the surrounding air temperature by maintaining a rapid rate of respiration fueled ultimately by starch reserves; the spadix was not frost resistant and yet survived air temperatures of -15°C. These same inflorescences maintain an extremely active CN-insensitive pathway which expends most of the free-energy available from substrate oxidation as heat, since little is conserved in the form of ATP (Henry and Nyms, 1975). Therefore, one possible physiological role for the CN-insensitive pathway, at least in certain tissues, could be heat production either to avoid freezing or simply to speed up metabolism. Although wheat seedlings are certainly not considered thermogenic, Bakumenko (1974) did report a positive correlation between CN-insensitive respiration and frost resistance in the few cultivars he studied.

Smith and Meeuse (1966) noticed that the thermogenic nature of the *Arum* spadix resulted in volatilization of chemicals that attracted insect pollinators. This information led Meeuse (1975) to propose that CN-insensitive respiration aided pollination of plants of the *Arum* family even at subfreezing temperatures.

Although the heat resulting from starch oxidation via the CN-insensitive pathway may aid frost avoidance and pollination of aroids,

the apparent ubiquitous nature of CN-insensitive respiration led to a search for other metabolic roles (Bahr and Bonner, 1973a).

Various authors noted the CN-resistant nature of respiration during the initial period of seed germination and suggested that the alternate pathway was operative (Roberts, 1969; Wilson and Bonner, 1971). Recently Yentur and Leopold (1976) reported that during the first few hours following imbibition, germination and respiration of soybeans were more effectively inhibited by hydroxamic acids than cyanide; this trend reversed approximately six hours after imbibition. Cyanide exerted a stimulating effect on germination of seeds of many different species (Major and Roberts, 1968; Hendricks and Taylorson, 1972), although the nature of stimulation was somewhat in doubt (Taylorson and Hendricks, 1973). Such evidence resulted in a proposal that the CN-insensitive pathway provided some unknown essential for completion of the earliest stages of seed germination (Yentur and Leopold, 1976).

MATERIALS AND METHODS

Plant Material and Germination Conditions

The spring cultivars Neepawa and Norquay, along with the winter cultivars Kharkov and Ulianovka were used throughout the present study. Seeds to be germinated were surface sterilized with dilute bleach (2:1, water:bleach by vol.) for 2-3 min and then rinsed for 5 min with water and finally with distilled water. Germination was carried out on moist paper in the dark either at room temperature (20-22°C) for 3-4 days or in a cold room (4-6°C) for 3-4 weeks. Under either temperature regime the tissue was harvested when the coleoptiles were no longer than 1 cm.

The static system of germination, under varied concentrations of oxygen, carbon dioxide and ethylene, employed 10 l glass chambers. Unless otherwise indicated, all germinations involving increased gas concentrations were of the static type. A gas-flow system (glass) was used when strict control of gas mixtures was desirable; the flow rates varied 100-600 ml/min depending upon the individual requirements of the experiment.

To exclude ethylene the gas was bubbled through a trap containing mercuric perchlorate solution (Abeles, 1973). Similarly, a 10% (by wt) aqueous KOH solution was used to remove carbon dioxide.

Carbon Dioxide and Ethylene Determination

Carbon dioxide concentrations were determined with a Beckman 215A infrared analyzer. To determine low concentrations (< 1.0%) the gas was passed directly through the analyzer. For higher concentrations of CO₂, a N₂ stream was passed through the analyzer and gas samples

(100 μ l - 1.0 ml) were injected into the stream. Concentrations were determined by comparing readings with those of standards.

Ethylene was determined in a 1 ml sample on a Hewlett Packard 5710A gas chromatograph equipped with a flame ionization detector. Porapak T and Q-S columns were used to separate ethylene from CO₂, water vapor and other air components. The flow rate was maintained at 20 ml/min with column and detector temperatures of 60°C and 150°C, respectively. Ethylene concentrations were calculated by comparing peak heights with those of standards.

Isolation of Mitochondria

The isolation procedure employed was a slight modification of the rapid differential centrifugation procedure developed by Sarkissian and Srivastava (1970). Their method was designed primarily to quickly separate the mitochondria from soluble proteases, lipases and other hydrolytic enzymes released when the tissue was macerated. However, in the present study the constituents and pH values of the 'grind' and 'reaction' mediums were chosen in accordance with the findings of Ikuma (1970).

Coleoptiles (1-3 g) were homogenized with a mortar and pestle for 1.0 - 1.5 min in 5 ml of grind medium (0.5M mannitol, 10 mM K₂HPO₄, 0.1% (by wt) BSA, 2 mM cysteine, 1 mM EDTA, adjusted to pH 7.5 with KOH); all operations were carried out at 0-4°C. Approximately 0.2 - 0.4 g of polyvinylpyrrolidone were added to bind polyphenols. Grind medium was then added to obtain a final ratio of 10 g grind medium per g tissue. The brei was filtered through Nitex nylon cloth

(10 μ openings; B. & S. H. Thompson & Co. Ltd.) and centrifuged for 5 min at 1000xg. The supernatant was recentrifuged for 2 min at 40,000 xg. The pellet obtained was then washed carefully twice with 1 ml of reaction medium (0.3M mannitol, 10 mM KH₂PO₄, 0.1% (by wt) BSA, 10 mM KCl, 5 mM MgCl₂, adjusted to pH 7.2 with KOH). The mitochondrial pellet was finally resuspended in 0.5 ml of reaction medium and stored on ice.

Protein was determined by the method of Lowry *et al.* (1951) after solubilizing 20 μ l samples of the mitochondrial suspension with 0.2 ml of 5% (by wt) deoxycholate.

Oxygen Measurements

Oxygen uptake was measured with a Clarke type oxygen electrode (Yellow Springs Instruments Biological Oxygen Monitor model 51). The polarizing voltage was adjusted to 0.65v. Both the standard and the high-sensitivity membranes were used. Oxygen concentrations at various temperatures ($^{\circ}$ C) were calculated using the following equation for air-saturated water solutions, determined by Truesdale and Downing (1954).

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

In a typical assay, 2.5 ml of reaction medium were allowed to equilibrate with air for at least 5 min at the desired temperature. The oxygen electrode was then lowered into the solution and all bubbles were expelled. The electrode output was adjusted to give full scale deflection of the recorder (1.0 mv). Substrate e.g. succinate, and ADP were added to give final concentrations of 10 mM and 80-100 μ M,

respectively. Oxygen uptake was initiated by adding 100 μ l of mitochondrial suspension (containing 0.3 - 1.0 mg protein). Following the trial, the baseline (i.e. $[O_2] = 0$) was obtained by addition of aqueous sodium hydrosulfite.

Mitochondrial Lipid Analysis

The lipid extraction procedure was that of Bligh and Dyer (1959) as modified by Miller *et al.* (1974). The mitochondria were isolated as described previously. However, following the second centrifugation the pellet was resuspended in 5 ml of BSA-free grind medium and then centrifuged at 40,000xg for 2 min. This pellet was then resuspended in 5 ml of boiling 2-propanol, incubated for 5 min at 82°C (to inactivate any lipolytic enzymes) and centrifuged at 3000xg for 5 min. The supernatant was collected and the pellet extracted again with 5 ml of boiling 2-propanol and three times with 5 ml chloroform-methanol-water (1:2:0.8, v/v/v); following each extraction the suspension was centrifuged at 3000xg for 5 min. The organic supernatants were combined and the solvents removed under reduced pressure.

For analysis, the lipids were hydrolyzed and the methyl esters of the free fatty acids were prepared and identified by the method of Hougen and Bodo (1973). The methyl esters were separated on a 5720A Hewlett Packard gas chromatograph equipped with a flame ionization detector. The column (1/8" x 8', copper) was packed with SP-222 polyester (Supelco, Inc.) and maintained at 230°C.

RESULTS AND DISCUSSION

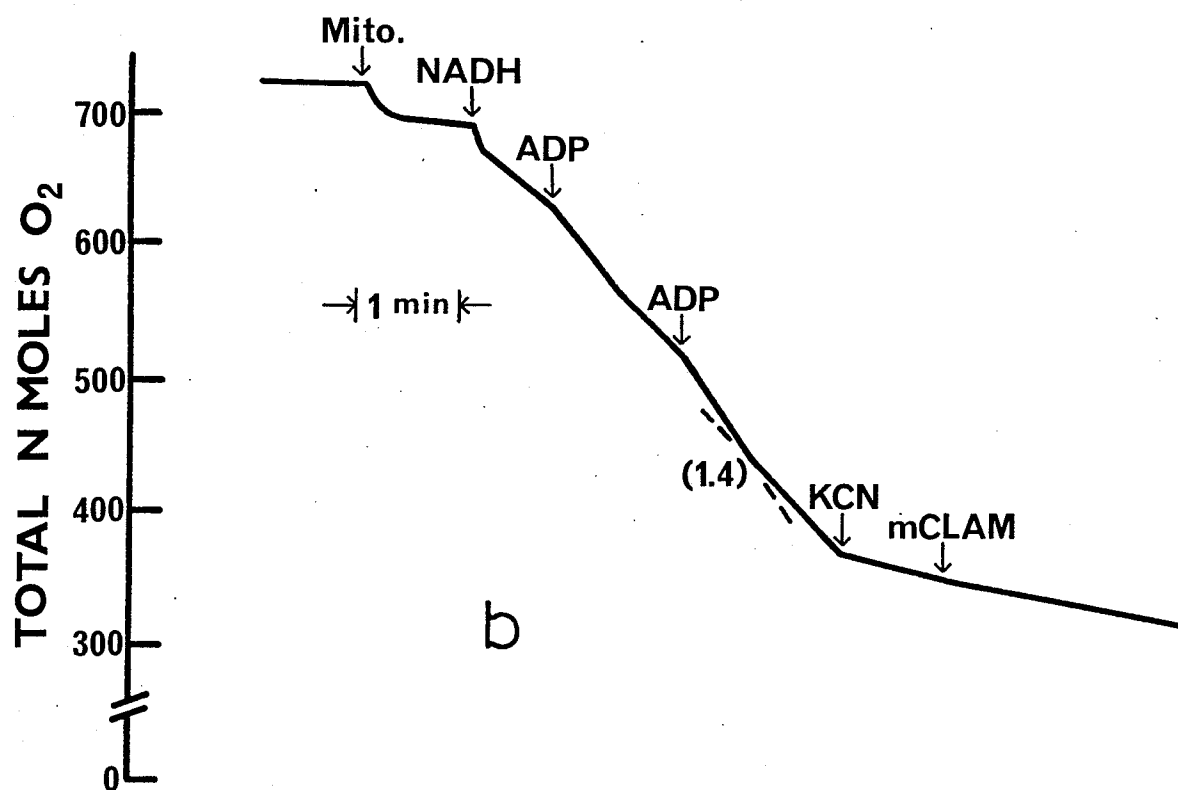
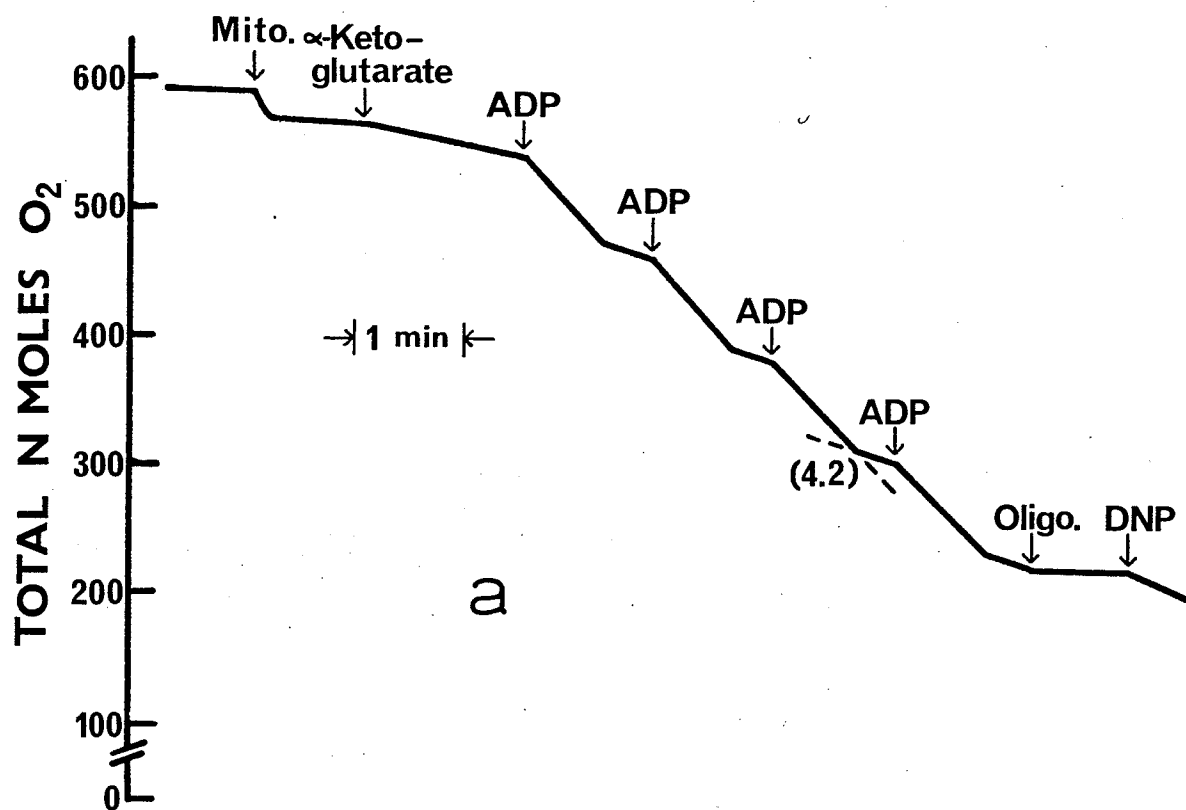
Mitochondrial Reactions

The mitochondria isolated from etiolated wheat coleoptiles were shown to be capable of several reactions accepted as characteristic of intact, active mitochondria (Palmer, 1976). In the absence of added substrate (endogenous substrate only) the rate of oxygen uptake was low (Fig. 3). Upon addition of substrate (e.g. α -ketoglutarate, Fig. 3a) the respiratory rate was still relatively low, being limited by the availability of ADP; this was the initial state 4 rate as defined by Chance and Williams (1956). Addition of ADP immediately increased the rate of oxygen consumption to a maximum (state 3 rate) which continued until the ADP was depleted; at this point the state 4 rate resumed. The relatively high respiratory control ratios (state 3/state 4) obtained with α -ketoglutarate indicated that the ATPase activity was low and ATP synthesis was tightly coupled to electron transport. Oligomycin, an antibiotic inhibitor of phosphorylation, completely inhibited oxygen reduction (Fig. 3a); dinitrophenol reversed the inhibition presumably by uncoupling ATP synthesis from electron transport.

Although the isolated wheat mitochondria oxidized α -ketoglutarate and exogenous NADH rapidly (Fig. 3), neither substrate proved satisfactory for the present study of CN-insensitive respiration. The difficulty with α -ketoglutarate resided in its ability to react with cyanide to form a cyanohydrin. Oxidation of exogenous NADH by plant mitochondria has proven to be a complex process which is incompletely understood (Douce *et al.*, 1973; Palmer, 1976). In the present study NADH oxidation was

Figure 3

Polarographic recordings of oxygen uptake by isolated Kharkov mitochondria utilizing (a) α -ketoglutarate and (b) exogenous NADH. Reaction conditions were (a) 10 mM α -ketoglutarate, 80-100 μ M ADP, 0.1 μ M oligomycin, 10 μ M dinitrophenol, 0.3 mg mitochondrial protein, 28°C and (b) 5 mM NADH, 100 μ M ADP, 0.4 mM KCN, 0.2 mM mCLAM, 0.8 mg mitochondrial protein, 15°C.



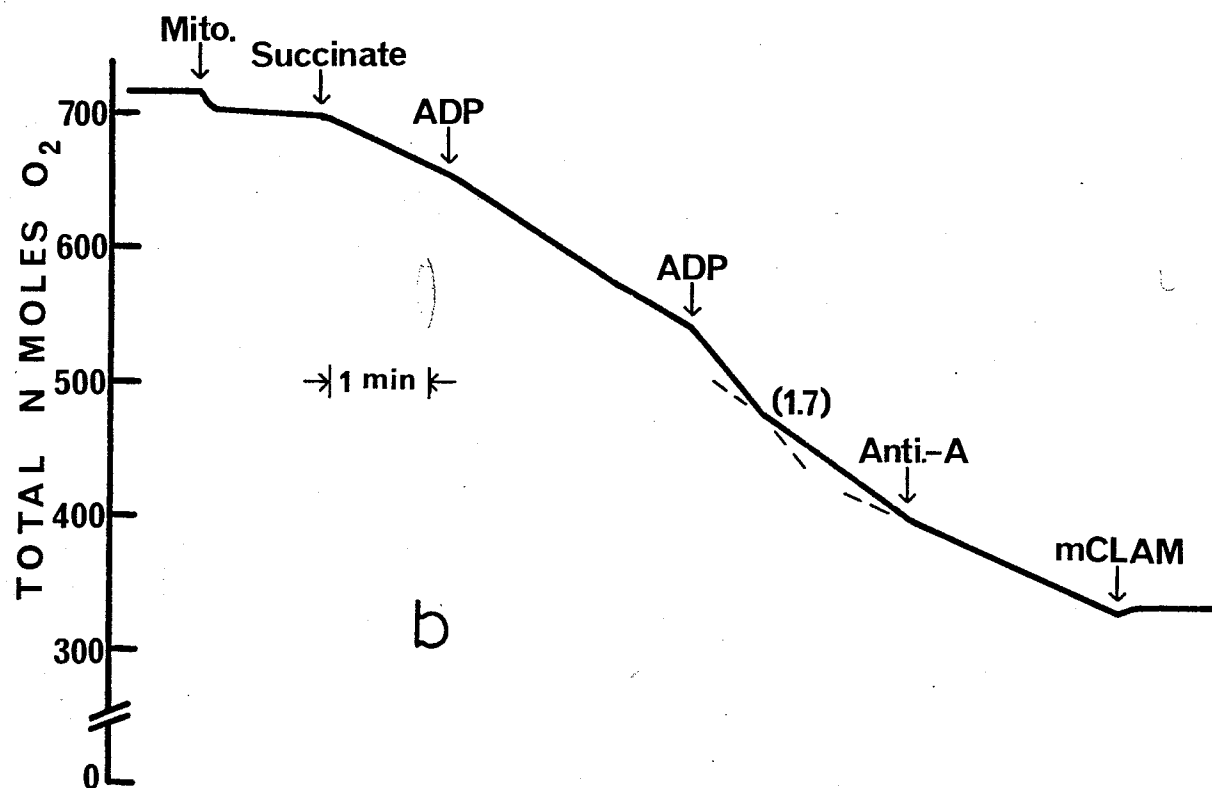
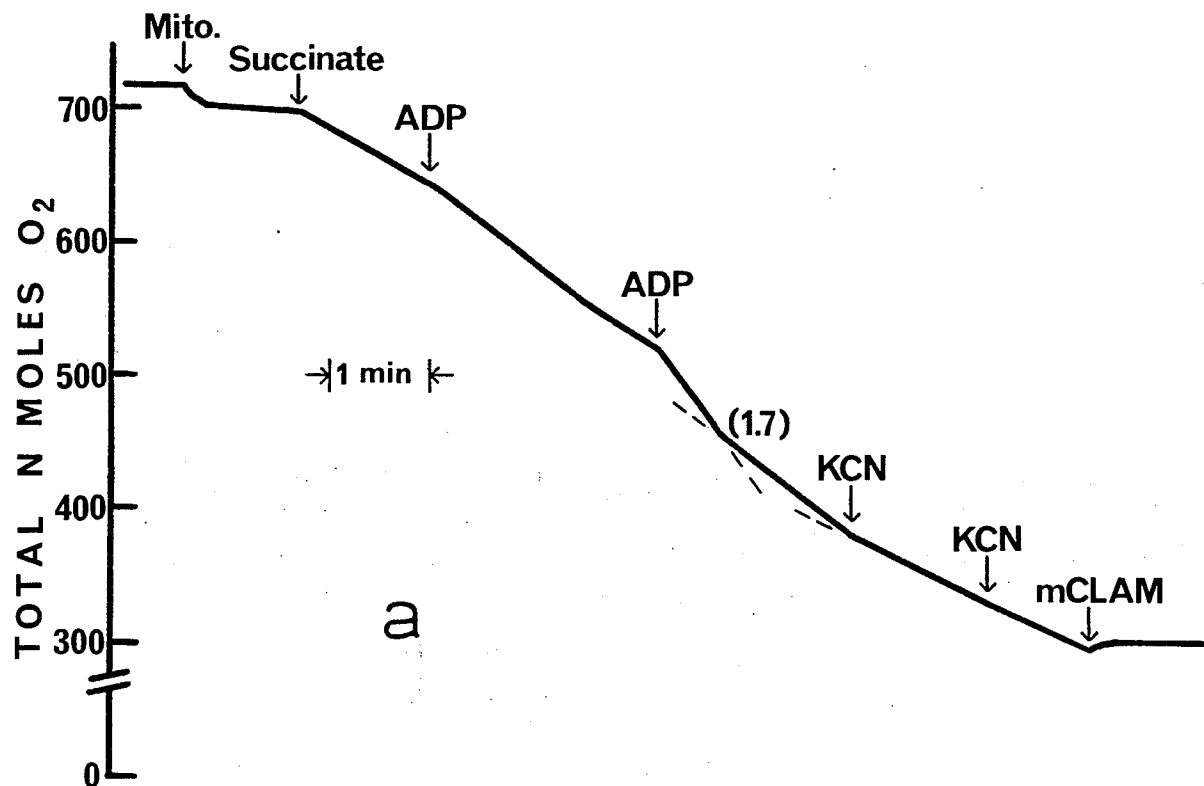
found to be variably inhibited by antimycin A and cyanide, and the residual oxygen uptake was not inhibited by the hydroxamic acid, mCLAM (Fig. 3b).

The isolated wheat mitochondria were found to oxidize succinate readily and yielded R.C. ratios reasonable for this substrate. In the trial shown (Fig. 4), Kharkov mitochondria exhibited an R.C. ratio of 1.7 following the second addition of ADP. Cyanide, at 0.4 mM, a concentration exceeding that required to completely inhibit cytochrome oxidase in plant tissue (Bahr and Bonner, 1973a; Wilson, 1971), failed to completely inhibit the respiration of the wheat mitochondria (Fig. 4a). Doubling the cyanide concentration (0.8 mM) caused only a slight increase in the inhibition which was probably attributable to a non-specific effect on the mitochondrial reactions. However, the hydroxamic acid mCLAM completely inhibited the residual oxygen uptake. Antimycin A was also found to inhibit succinate oxidation only to the same extent as did cyanide (Fig. 4b); once again the residual oxygen uptake was completely inhibited by mCLAM. From the above evidence it was concluded that the CN-insensitive pathway was operative in the wheat mitochondria.

A slight disadvantage of using succinate should be mentioned. The state 3 rate resulting from the initial addition of ADP was much lower than that observed with subsequent ADP additions (Fig. 4). According to Singer *et al.* (1973) and Oestreicher *et al.* (1973) succinate dehydrogenase of plant mitochondria required activation by ADP, ATP or succinate for several minutes to achieve maximal activity, which was accompanied by the release of tightly bound oxaloacetate. Raison *et al.* (1973) also discussed this problem of succinate dehydrogenase. In the

Figure 4

Inhibition of succinate oxidation in Kharkov mitochondria by (a) cyanide and (b) antimycin A. Both trials were run simultaneously on aliquots from the same mitochondrial preparation. Reaction conditions were 10 mM succinate, 100 μ M ADP, 0.4 mM KCN (first addition), 0.8 mM KCN (second addition), 0.03 μ M antimycin A, 0.9 mg mitochondrial protein, 15°C.



present study it was observed that the inhibition was overcome more quickly if the succinate and ADP were added to the reaction chamber prior to the addition of the mitochondria (e.g. Fig. 8a); this technique allowed completion of two state 3 - state 4 cycles and measurement of the CN-insensitive rate before the oxygen was seriously depleted.

Cultivar Variations in Cyanide-Sensitivity

The rates of oxygen uptake observed with mitochondria isolated from four cultivars of wheat are listed in Table 1. While the state 3 and state 4 rates varied slightly, the rates after addition of cyanide were distinctly dependent upon variety. The CN-insensitive oxygen uptake rate of Kharkov mitochondria was almost four times greater than the CN-insensitive rate of Neepawa. The two spring cultivars, Neepawa and Norquay, were almost entirely sensitive to cyanide whereas the winter wheat Ulianovka was intermediate between Kharkov and the spring cultivars. The slightly lower respiratory control ratios which were consistently observed with Kharkov and Ulianovka probably reflected the greater contribution of the alternate pathway.

An apparent capacity for electron flux through the alternate pathway was determined from the rate of succinate oxidation (oxygen uptake) in the presence of cyanide. However, in the absence of cyanide the alternate pathway may not be fully operative (Bahr and Bonner, 1973b). Therefore, the state 3 and state 4 rates were observed in the presence and absence of the hydroxamic acid, mCLAM (Table 2). The addition of mCLAM significantly lowered the oxygen uptake rates of Kharkov mitochondria while rates for Norquay were hardly affected. The effect of

TABLE 1. Oxygen uptake by isolated wheat mitochondria utilizing succinate

| Cultivar | Respiratory Control Ratios | Oxygen Uptake ^a | | |
|-----------|-------------------------------|----------------------------|----------------------|---------------------------|
| | | State 3 ^b | State 4 ^c | State 4 + CN ^d |
| Norquay | 1.99 | 70.4 | 35.4 | 6.3 (8.9) |
| Neepawa | 2.00 | 71.0 | 35.4 | 5.9 (8.3) |
| Kharkov | 1.75 | 67.9 | 38.3 | 23.3 (34.3) |
| Ulianovka | 1.75 | 61.3 | 35.0 | 12.7 (20.7) |

^a Rates are in nmoles O₂/min/mg protein at 15°C following the second addition of ADP and are the averages of at least three isolations.

^b 10 mM succinate, 100 μM ADP.

^c 10 mM succinate.

^d 10 mM succinate, 0.4 mM KCN. Numbers in parentheses represent the percent of state 3 respiration which is insensitive to cyanide.

TABLE 2. Effects of mCLAM^a on oxygen uptake of wheat mitochondria

| | Kharkov | Norquay |
|------------------------------|---------|---------|
| State 3 ^b | 67.9 | 70.4 |
| State 3 + mCLAM ^b | 57.4 | 68.5 |
| % Inhibition (with mCLAM) | 15.5 | 2.7 |
| State 4 ^b | 38.3 | 35.4 |
| State 4 + mCLAM ^b | 26.4 | 33.0 |
| % Inhibition (with mCLAM) | 31.1 | 6.8 |
| R.C. Ratio | 1.75 | 1.99 |
| R.C. Ratio (+ mCLAM) | 2.17 | 2.08 |

^a m-chlorobenzhydroxamic acid.

^b Rates are for succinate oxidation in nmoles O₂/min/mg protein at 15°C and are the averages of at least two isolations. Initial concentrations (where applicable) were 10 mM succinate, 80-100 μM ADP and 0.2 mM mCLAM.

mCLAM was apparently the same on both state 3 and state 4 respiration (i.e. both state 3 and state 4 rates for Kharkov decreased by 10-12 nmoles O_2 /min/mg protein). In addition, the R.C. ratio of Kharkov improved noticeably upon addition of mCLAM whereas the R.C. of Norquay improved only slightly. It should be emphasized that the inhibition of oxygen uptake by mCLAM was much less than the apparent capacity of the alternate pathway measured in the presence of cyanide; this suggested that either the alternate pathway was not fully operative in the absence of cyanide or that inhibiting the alternate oxidase with mCLAM simply shifted the electron flux through the cytochrome pathway.

Temperature Dependence of Mitochondrial Respiration

Arrhenius plots of oxygen uptake by wheat mitochondria were relatively linear for both state 3 and state 4 respiration over the temperature ranges studied (Figs. 5-7). This linearity indicated that the activation energy for succinate oxidation was relatively independent of temperature; the rate constant for the overall process increased logarithmically with temperature. Activation energies were similar for both state 3 and state 4 respiration as well as similar among the cultivars studies (9-12 kcal/mole). The slight variations in the activation energies reported here were probably within experimental error and were not considered significant, especially when compared to the four-fold increases observed by Lyons and Raison (1969) for activation energies of succinate oxidation by sweet potato or tomato mitochondria as the temperature decreased below 10°C.

In contrast to the overall respiration, the CN-insensitive succinate oxidation of Kharkov and Ulianovka exhibited a rather anomalous

Figure 5

Arrhenius plot of state 3 (■) and state 4 (▲) respiratory rates for Neepawa mitochondria oxidizing succinate. Activation energies E^* (kcal/mole) are indicated beside each line. Values represent the averages of at least 2 isolations.

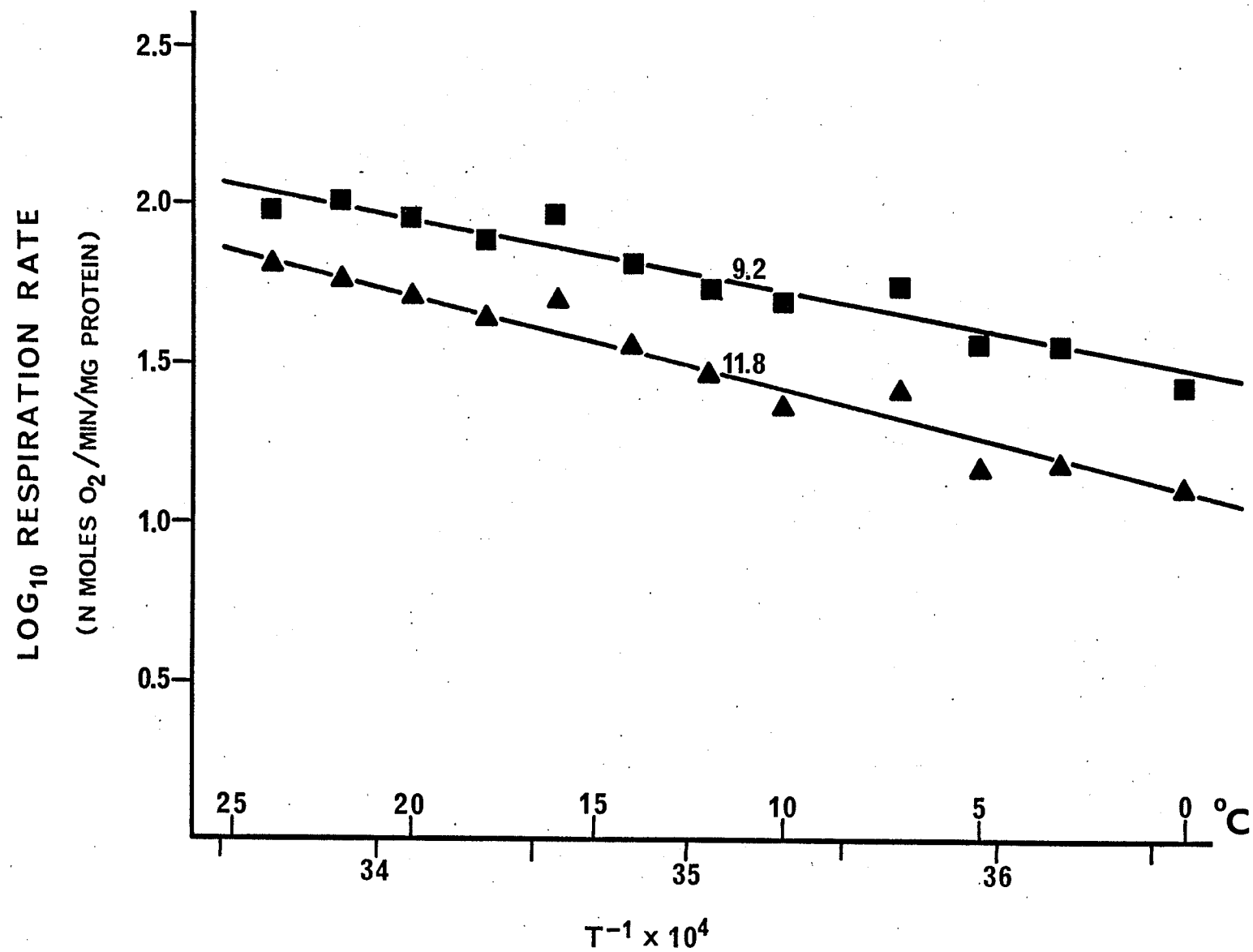


Figure 6

Arrhenius plot of state 3 (■), state 4 (▲) and state 4 + CN (i.e. CN-insensitive) (●) respiratory rates for Kharkov mitochondria oxidizing succinate. Activation energies E^* (kcal/mole) are indicated beside each line. Values represent the averages of 3 isolations.

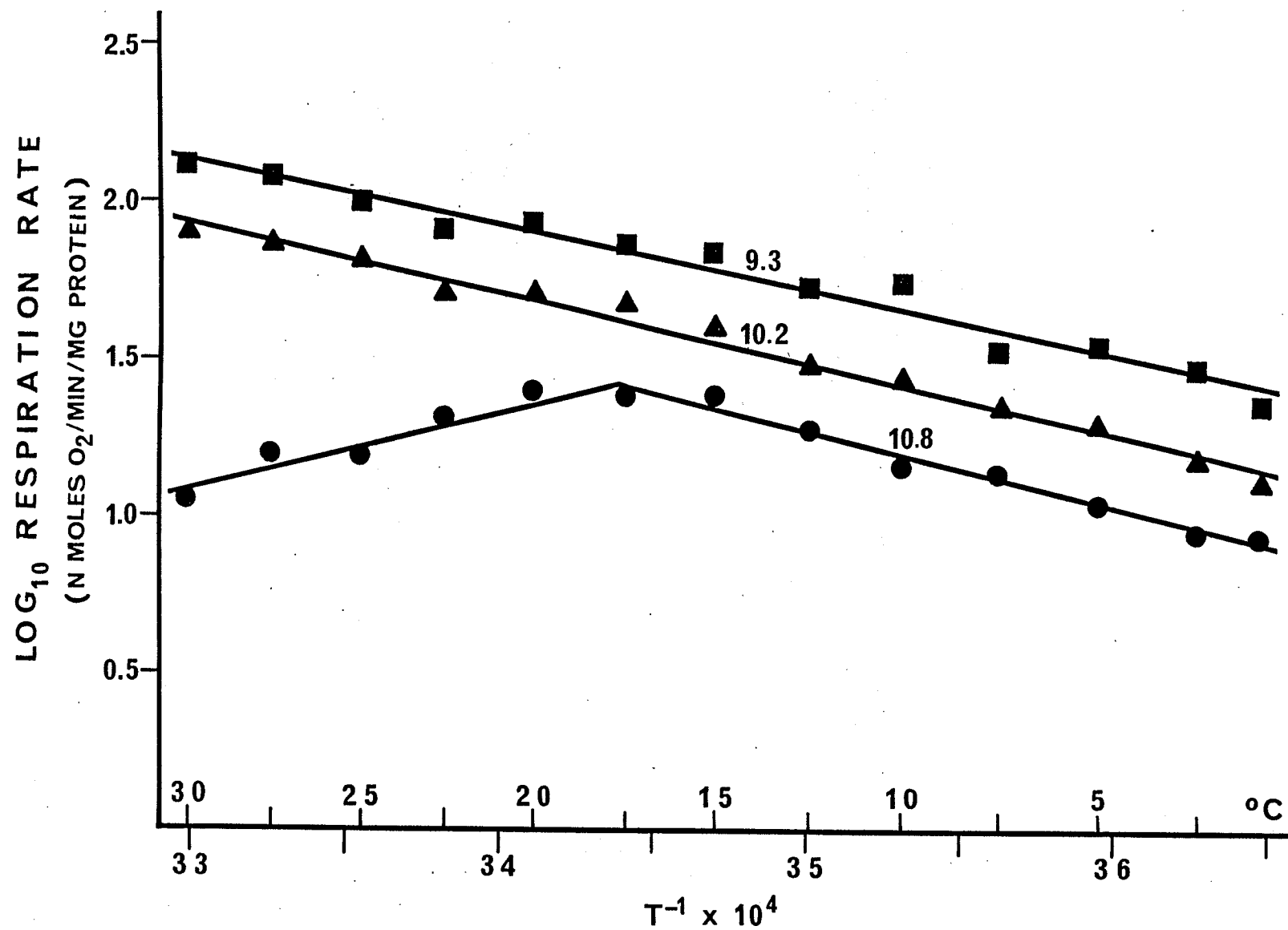
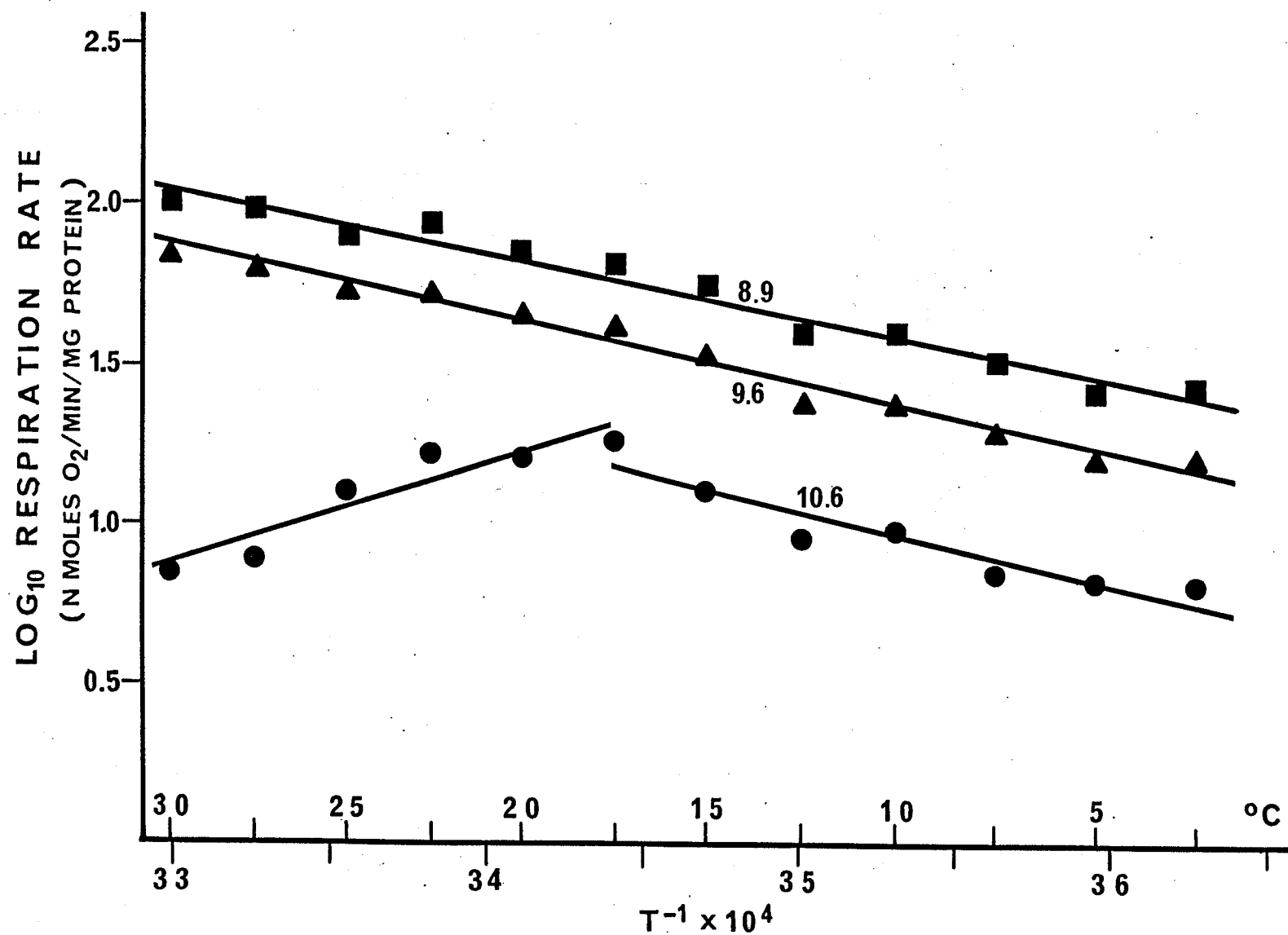


Figure 7

Arrhenius plot of state 3 (■), state 4 (▲) and state 4 + CN (i.e. CN-insensitive) (●) respiratory rates for Ulianovka mitochondria oxidizing succinate. Activation energies E^* (kcal/mole) are indicated beside each line.

Values represent the averages of at least 2 isolates.



temperature dependence. As observed in the Arrhenius plots (Figs. 6 and 7), the CN-insensitive oxygen uptake increased over the temperature range 2.0°C to 17-18°C with an activation energy (10.6 - 10.8 kcal/mole) comparable to those of state 3 and state 4 respiration. However, as the reaction temperature was increased above 17-18°C the CN-insensitive rate failed to increase and actually decreased in both Kharkov and Ulianovka mitochondria. Arrhenius plots for the CN-insensitive respiration of the spring wheats were not obtained due to the difficulties involved in measuring very low rates of oxygen uptake.

Over the temperature range 2.0°C to 17-18°C the CN-insensitive respiration exhibited a single activation energy similar to the activation energies of state 3 and state 4 respiration. The change in the temperature dependence of the CN-insensitive pathway above 17-18°C indicated that the rate-limiting step changed or was altered in some manner.

The possibility existed that the availability of oxygen became rate-limiting at higher temperatures due to the decreased solubility. Various authors presented indirect evidence which suggested that the alternate oxidase had a much lower affinity for oxygen than did cytochrome oxidase (James and Beevers, 1950; Wilson, 1970; MacDonald, 1967). Ikuma *et al.* (1964) reported that two oxidases were present in plant mitochondria; one had a K_m for oxygen $\approx 0.1 \mu M$, which was probably cytochrome oxidase, while the second oxidase had a $K_m \approx 0.5 \mu M$ and was assumed to be the alternate oxidase. However, the second oxidase was never confirmed as part of the CN-insensitive pathway and

later results indicated that it may have been an impurity (Plesnicar *et al.*, 1967).

Attempts in the present study to establish if decreased oxygen concentrations at higher temperatures were responsible for the altered temperature dependence above 17-18°C indicated that oxygen was probably not rate limiting. At 20°C the CN-insensitive rate was essentially the same when measured at 150 μM and 45 μM oxygen (Table 3); therefore, the K_m was likely well below 45 μM oxygen. However, at 30°C and 100 μM oxygen the CN-insensitive rate was much less than the rate observed at 20°C and 45 μM oxygen (Table 3). Also, it was observed that increasing the oxygen concentration at higher temperatures (by equilibrating the reaction medium with oxygen instead of air) failed to increase the contribution of the CN-insensitive pathway. As all other CN-insensitive rates reported in the present study were measured at oxygen concentrations in excess of 100 μM , the alternate oxidase should have been saturated at all temperatures studied. No attempt was made to measure the K_m with the oxygen electrode since oxygen diffusion through the membrane would probably become rate limiting at low oxygen concentrations.

The failure of the CN-insensitive rate to continue increasing above 17-18°C (in contrast to the state 3 and state 4 rates) suggested that higher temperatures were perhaps more generally deleterious to the alternate pathway than to the cytochrome pathway. In an attempt to determine the effects of higher temperatures on the mitochondrial reactions a preparation from Kharkov was divided into three samples which were treated as follows: sample #1 was used to measure the

TABLE 3. Cyanide-insensitive respiration of Kharkov mitochondria as a function of oxygen concentration and temperature

| Temperature | Oxygen | CN-Insensitive Respiration ^a | % State 3 CN-Insensitive |
|-------------|-------------|---|--------------------------|
| 20°C | 150 μ M | 27 | 30 |
| 20°C | 45 μ M | 26 | 34 |
| 30°C | 108 μ M | 14 | 10 |

^a Rates are for succinate oxidation in nmoles O₂/min/mg protein and are the averages of at least two isolations. Initial concentrations are given in Table 1.



respiratory reactions at 30°C (trial #1); sample #2 was incubated at 30°C for the time required for trial #1 (5 min) and then the respiratory reactions were measured at 15°C (trial #3); sample #3 was stored on ice throughout and then the respiratory reactions were measured simultaneously with sample #2 at 15°C (trial #2). From the results in Table 4, the 30°C preincubation apparently resulted in a decrease in both the state 3 rate and the CN-insensitive (state 4 + CN) rate. The relatively low respiratory control ratios of trials #1 and #3 suggested that the 30°C treatment probably speeded the general degradation or 'aging' of the mitochondria. However, the mitochondria which were preincubated at 30°C and measured at 15°C (trial #3) retained a much higher percentage of CN-insensitive respiration than did the mitochondria which were measured directly at 30°C (trial #1). These results implied that the decreased temperature dependence of the CN-insensitive rate at higher temperatures was probably brought about by some reversible rather than irreversible process.

Effects of Carbon Dioxide, Oxygen and Ethylene

The CN-insensitive capacity of the etiolated coleoptiles was found to be markedly affected by the germination atmosphere. In air-germinated wheat the cytochrome capacity greatly exceeded the capacity of the CN-insensitive pathway (Table 5). However, it was discovered that if high concentrations of CO₂ (10-25%) were added to the germinating chambers, the capacity of the alternate pathway in the isolated mitochondria increased dramatically (Table 5, Fig. 8b).

Oxygen was found to exert a synergistic effect with CO₂ in

TABLE 4. The effects of a 30°C preincubation on the respiratory reactions of Kharkov mitochondria^a

| | Measurements at 30°C | Measurements at 15°C | |
|-----------------------------|----------------------------|-------------------------------|--|
| | | mitochondria stored on ice | mitochondria preincubated at 30°C ^b |
| | (trial #1) | (trial #2) | (trial #3) |
| State 3 | 112 | 55 | 42 |
| State 4 | 84 | 34 | 36 |
| State 4 + CN | 17 | 20 | 16 |
| % State 3 CN-Insensitive | 15% | 36% | 38% |
| % State 4 CN-Insensitive | 20% | 58% | 44% |
| R.C. ratio | 1.3 | 1.6 | 1.2 |

^a Rates are for succinate oxidation in nmoles O₂/min/mg protein. Concentrations are given in Table 1.

^b Mitochondria were preincubated at 30°C for 5 min, the time required for trial #1.

TABLE 5. Oxygen uptake rates of the two electron transport pathways of wheat mitochondria^a

| Germination Atmosphere | Cytochrome Oxidase ^b | | CN-Insensitive Oxidase ^c | |
|----------------------------------|---------------------------------|---------|-------------------------------------|---------|
| | Kharkov | Norquay | Kharkov | Norquay |
| Air | 57.4 | 68.5 | 23.3 | 6.3 |
| Oxygen | 58.9 | 71.0 | 24.8 | 9.8 |
| 25% CO ₂ in Air | 54.6 | 60.5 | 46.4 | 40.6 |
| 25% CO ₂ in Oxygen | 66.2 | 73.2 | 82.6 | 91.2 |

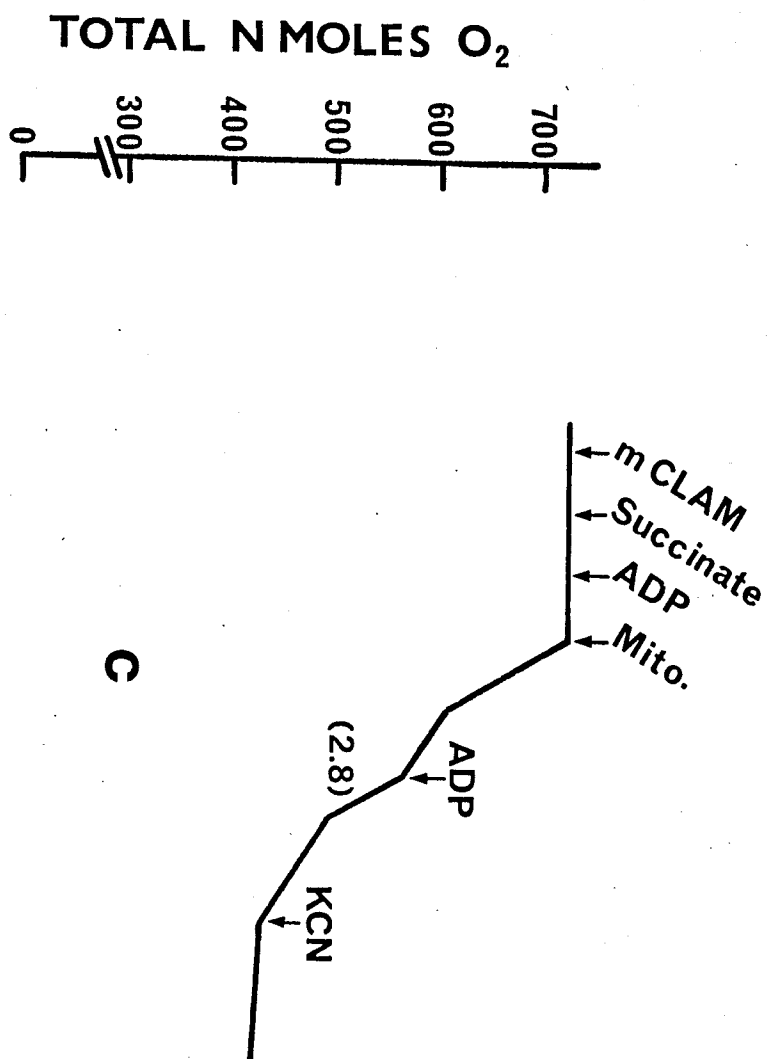
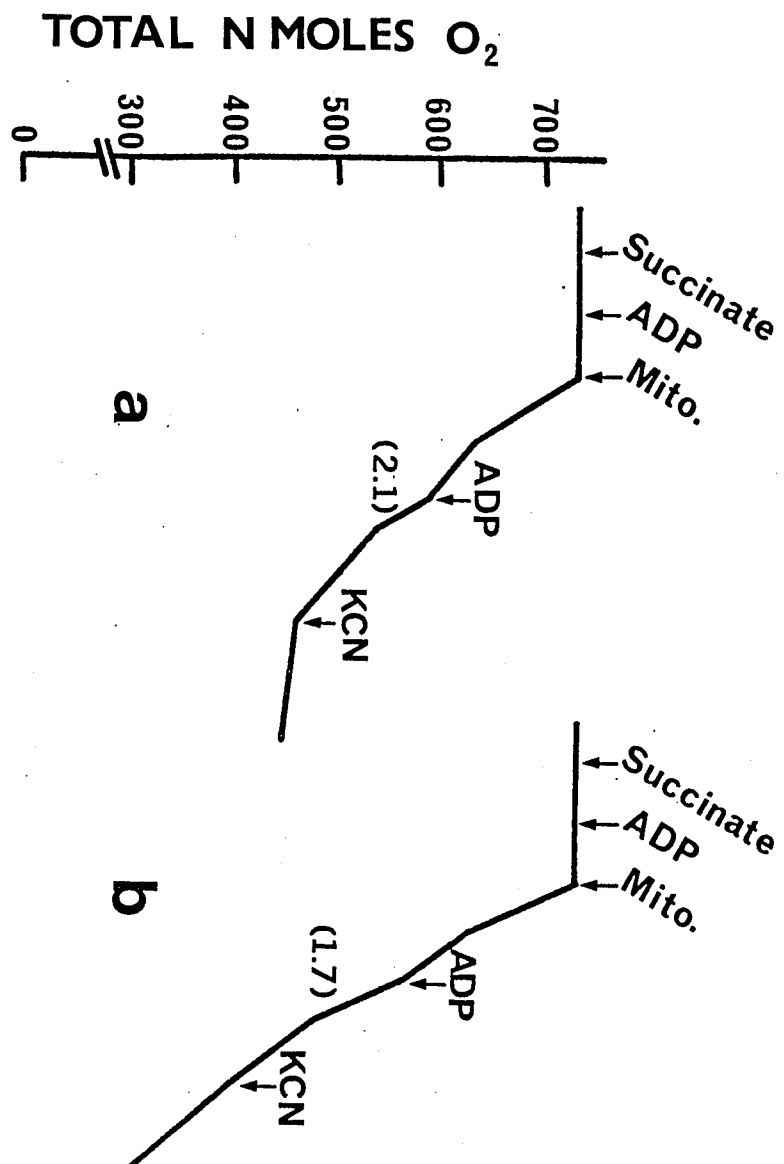
^a nmoles O₂/min/mg protein at 15°C (the average of at least two isolations).

^b 10 mM succinate, 100 μM ADP, 0.3 mM mCLAM.

^c 10 mM succinate, 0.4 mM KCN.

Figure 8

Polarographic recordings of oxygen uptake by mitochondria from Norquay germinated in (a) air, (b) (c) 20-25% CO₂ in air. Traces have been normalized to represent 1 mg of mitochondrial protein. R.C. ratios are indicated in parentheses to the left of the traces. Reaction conditions were 10 mM succinate, 80-100 μ M ADP, 0.4 mM KCN, 0.2 mM mCLAM, 15°C.



promoting the alternate pathway. Mitochondria isolated from wheat germinated in 100% oxygen showed no significant variation in the contribution of either electron transport pathway when compared to air-germinated wheat (Table 5, Fig. 9a). However, germination in 20-25% added CO_2 in oxygen resulted in mitochondria with almost twice the CN-insensitive capacity of wheat germinated in 20-25% CO_2 in air (Table 5, Figs. 8 and 9). In fact, under conditions of 20-25% added CO_2 in oxygen the capacity of the CN-insensitive pathway exceeded that of the cytochrome pathway in both wheats studied. It was noted that the CN-insensitive pathway was promoted equally well in both Norquay and Kharkov, despite the much lower CN-insensitive rate observed for air-germinated Norquay.

Mitochondria isolated from wheat germinated in the presence of high concentrations of CO_2 appeared uncoupled, exhibiting little or no respiratory control (Figs. 8b and 9b,c). However, when the hydroxamic acid mCLAM was added to inhibit the contribution of the alternate pathway, relatively good respiratory control was visible which indicated that the cytochrome system was functionally operative (Figs. 8c and 9d).

The effects described above for CO_2 were not elaborated after short periods of exposure. Placement of air-germinated etiolated seedlings in 20% added CO_2 in oxygen for 24 hr resulted in only a slight increase in CN-insensitive respiration (Table 6). Conversely, when seedlings which had been germinated in the high CO_2 -oxygen mixture were opened to the air for 24 hr the alternate pathway capacity still greatly exceeded that of air-germinated wheat. Such results

Figure 9

Polarographic recordings of oxygen uptake by mitochondria from Norquay germinated in (a) oxygen, (b) 10-15% CO₂ in oxygen, (c) (d) 20-25% CO₂ in oxygen. Traces have been normalized to represent 1 mg of mitochondrial protein. R.C. ratios are indicated in parentheses to the left of the traces. Reaction conditions were 10 mM succinate, 80-100 μ M ADP, 0.4 mM KCN, 0.2 mM mCLAM, 15°C.

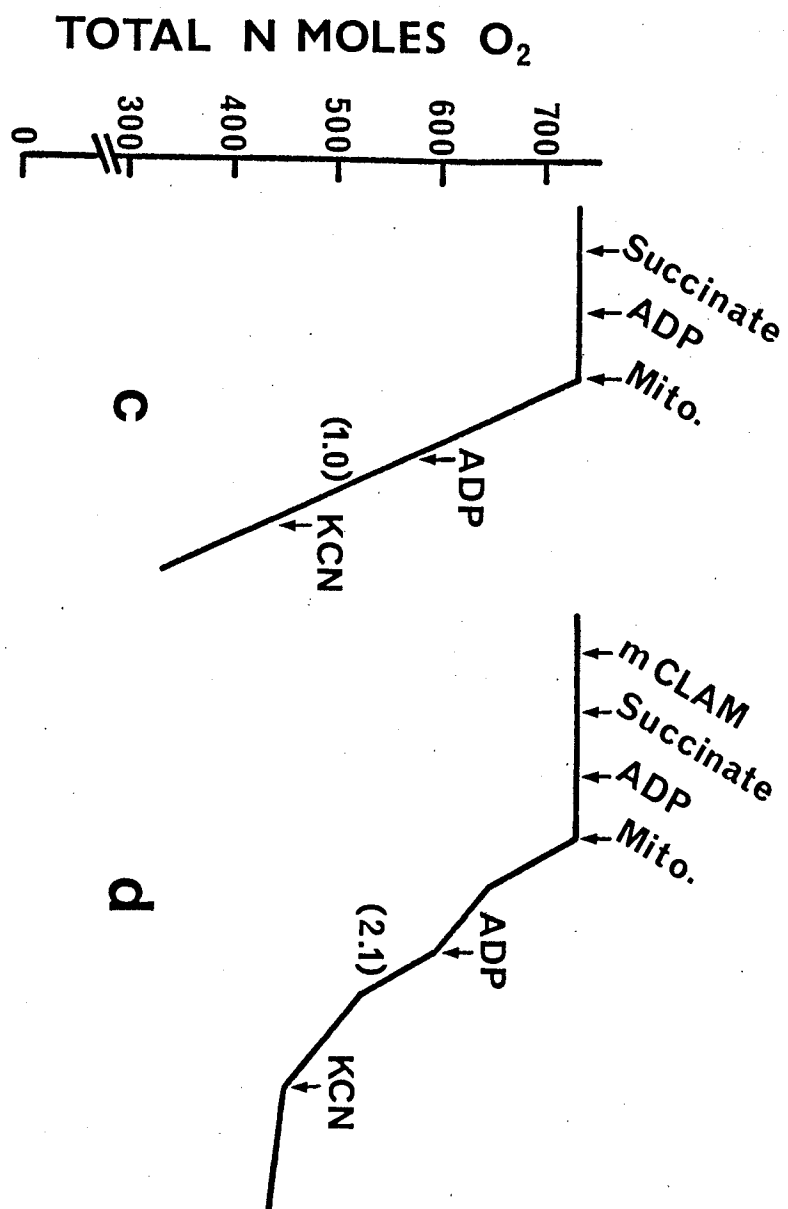
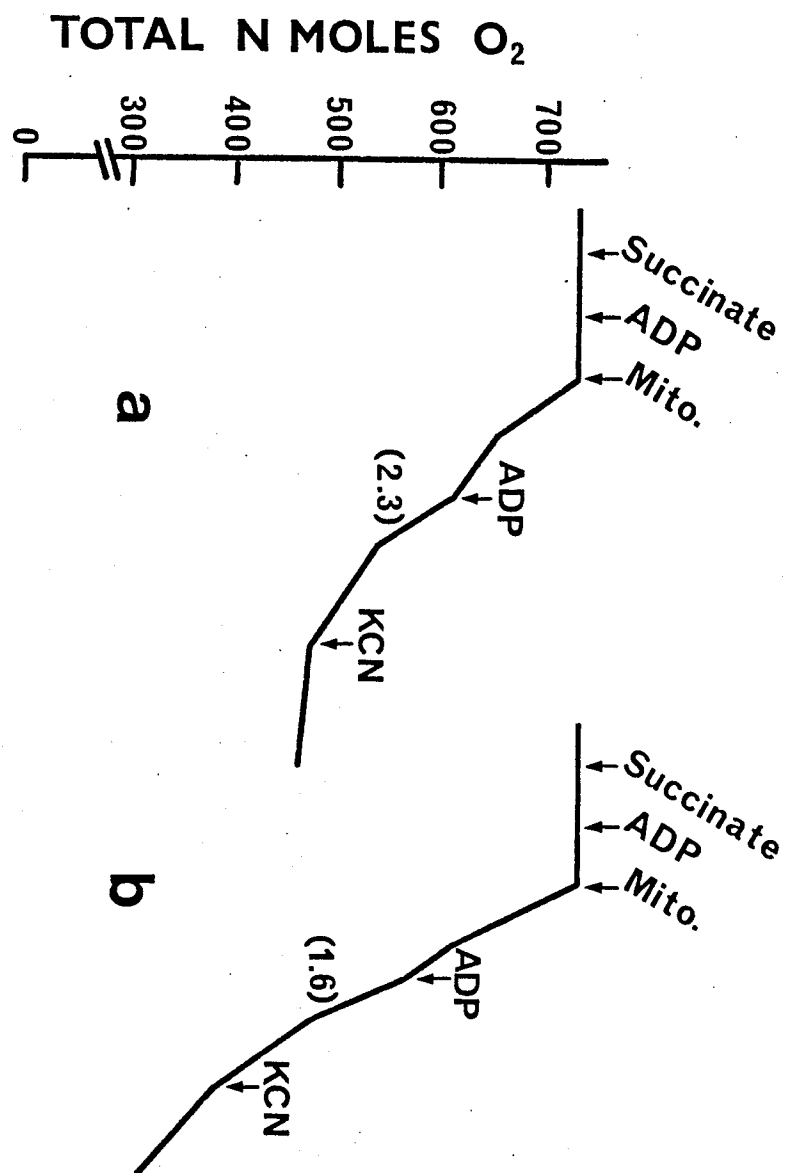


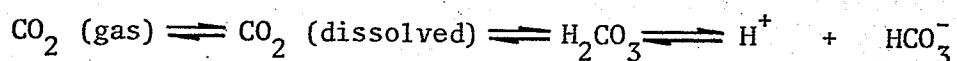
TABLE 6. The effects of altering the atmosphere during germination of Norquay

| Germination Atmosphere | | Oxygen Uptake ^a | | |
|----------------------------------|----------------------------------|----------------------------|---------|--------------|
| Initial | Final 24 hr | State 3 | State 4 | State 4 + CN |
| Air | Air | 70.4 | 35.4 | 6.3 |
| Air | 20% CO ₂ in Oxygen | 75.7 | 36.9 | 12.1 |
| 20% CO ₂ in Oxygen | Air | 88.9 | 53.2 | 42.2 |

^a Rates are for succinate oxidation in nmoles O₂/min/mg protein at 15°C. Concentrations are given in Table 1.

implied that protein synthesis was probably involved rather than a simple activation; protein synthesis was apparently necessary to the development of the alternate pathway in other tissues (Edwards *et al.*, 1976).

The CO_2 in the germination chambers would have interacted with the water surrounding the seeds according to the following reaction sequence (Umbreit *et al.*, 1972).



Therefore, an attempt was made to determine if the promotion of the CN-insensitive pathway by CO_2 was a consequence of the bicarbonate ion produced or perhaps the decreased pH. All calculations considered only the interaction of CO_2 with pure water. From the tables and equations outlined by Umbreit *et al.* (1972), at 20°C and 20% CO_2 (gas), the dissolved CO_2 concentration should approximate 7.8 mM. Since the chamber water was found to be pH 5-6 following germination in 20% added CO_2 , and using the value of 7.8 mM for CO_2 (dissolved), the bicarbonate ion concentration was calculated not to exceed 5 mM.

Germination of Kharkov at pH 4.0 (10 mM acetate buffer) failed to promote the CN-insensitive pathway (Table 7), although it did appear to slow the growth as did the high concentrations of CO_2 . In a second experiment Kharkov was germinated while continually moistened with a 10 mM bicarbonate solution; once again no promotion of the CN-insensitive pathway was observed (Table 7). Although inconclusive, these results supported the contention that the active species was CO_2 , either in the gaseous or dissolved state.

TABLE 7. Oxygen uptake by mitochondria isolated from Kharkov germinated either at low pH or in the presence of bicarbonate

| Imbibition Solution | Oxygen Uptake ^a | | |
|-------------------------|----------------------------|---------|--------------|
| | State 3 | State 4 | State 4 + CN |
| H ₂ O | 67.9 | 38.3 | 23.3 |
| pH 4.0 ^b | 65.9 | 37.2 | 19.7 |
| 10 mM KHCO ₃ | 88.9 | 47.0 | 22.0 |

^a Rates are for succinate oxidation in nmoles O₂/min/mg protein at 15°C. Concentrations are given in Table 1.

^b 10 mM acetate buffer.

to 30% CO_2 due to the respiratory gases and lack of circulation.

Although the capacity of the alternate pathway was substantially amplified by germination with CO_2 , the shape of the Arrhenius plot was not apparently altered (Fig. 10). The CN-insensitive rate was still maximal near 15-20°C while the overall state 3 rate continued to increase at least up to 30°C.

Mitochondria from Cold-Germinated Wheat

Kharkov germinated in the cold room (4-6°C) required 3-4 weeks to attain the same apparent physiological age as wheat germinated at 20-24°C for 3-4 days. It was established that the respiratory characteristics of the mitochondria from cold-germinated wheat were similar to those of mitochondria from wheat germinated at room temperature; apparently the cold treatment did not promote the CN-insensitive pathway. The Arrhenius plot for succinate oxidation by mitochondria from cold-germinated Kharkov revealed that the CN-insensitive rate again exhibited a maximum between 15°C and 20°C (Fig. 11).

Oxygen uptake by mitochondria from the spring cultivar, Neepawa, was similarly unaffected by the cold treatment (Fig. 12). The state 3 and state 4 rates yielded linear Arrhenius plots. As with Neepawa germinated at room temperature, the CN-insensitive rates of cold-germinated Neepawa were too low to be consistently measured accurately at all temperatures. However, the CN-insensitive respiration of cold germinated Neepawa never exceeded 14 nmoles O_2 /min/mg protein at any temperature studied.

Figure 10

Arrhenius plot of succinate oxidation by mitochondria from Kharkov germinated in 20% CO₂ in oxygen. The lines represent state 3 (■) and state 4 + CN (●) rates and activation energies E^* (kcal/mole) are indicated beside each line. Values are the average of 2 isolates.

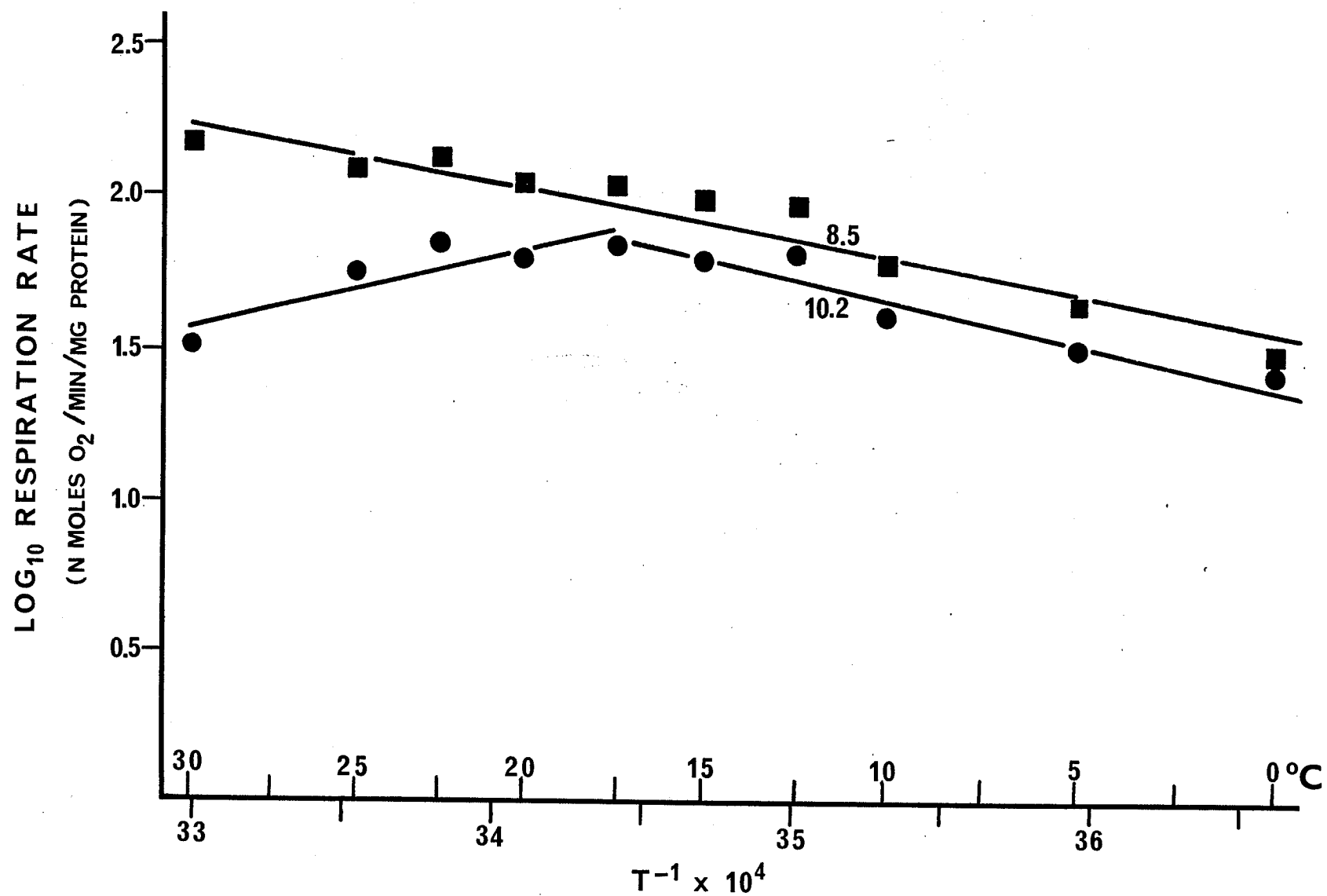


Figure 11.

Arrhenius plot of succinate oxidation by mitochondria from Kharkov germinated at low temperature ($4-6^{\circ}\text{C}$).

The lines represent state 3 (■) and state 4 + CN (●) and activation energies E^* (kcal/mole) are indicated beside each line.

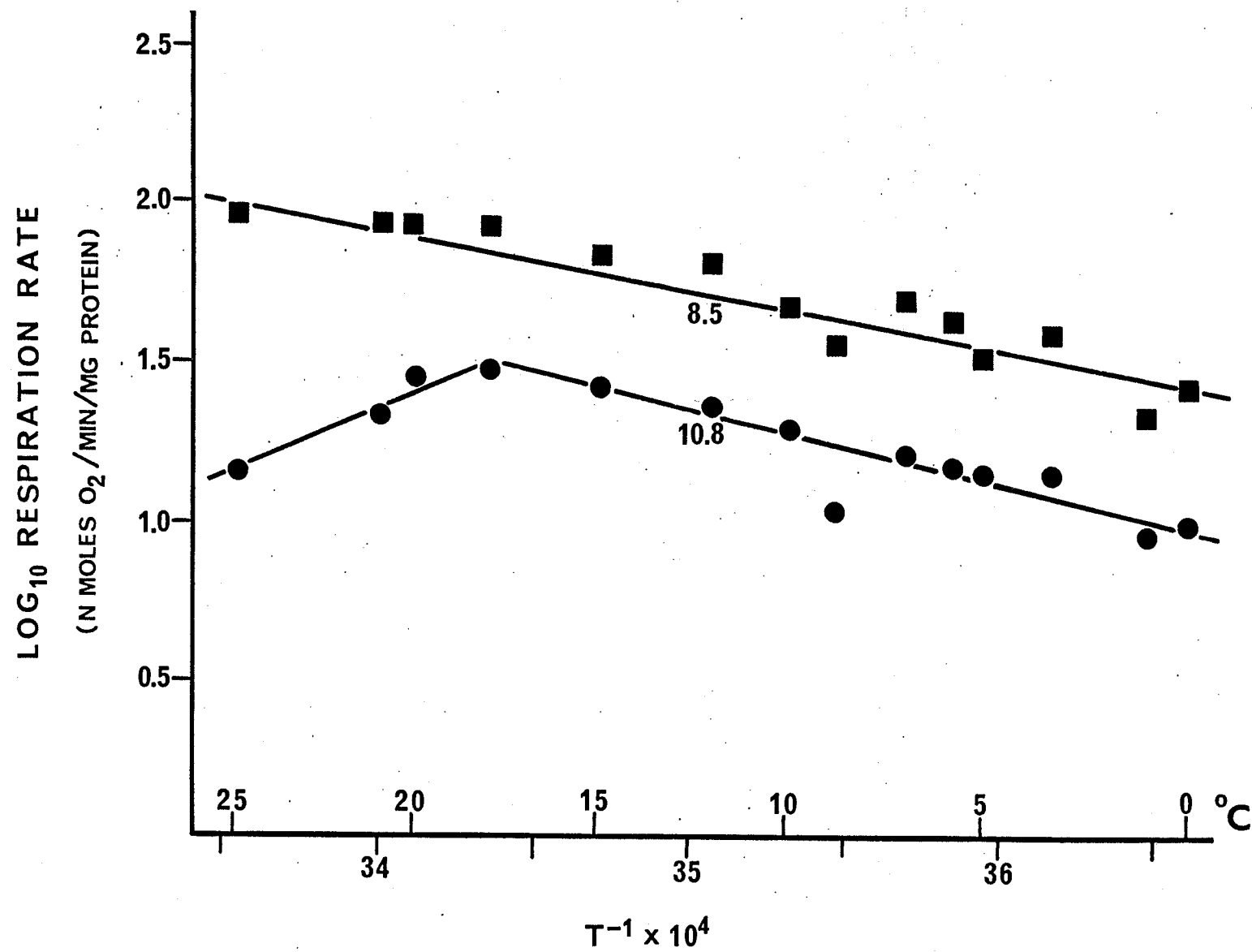
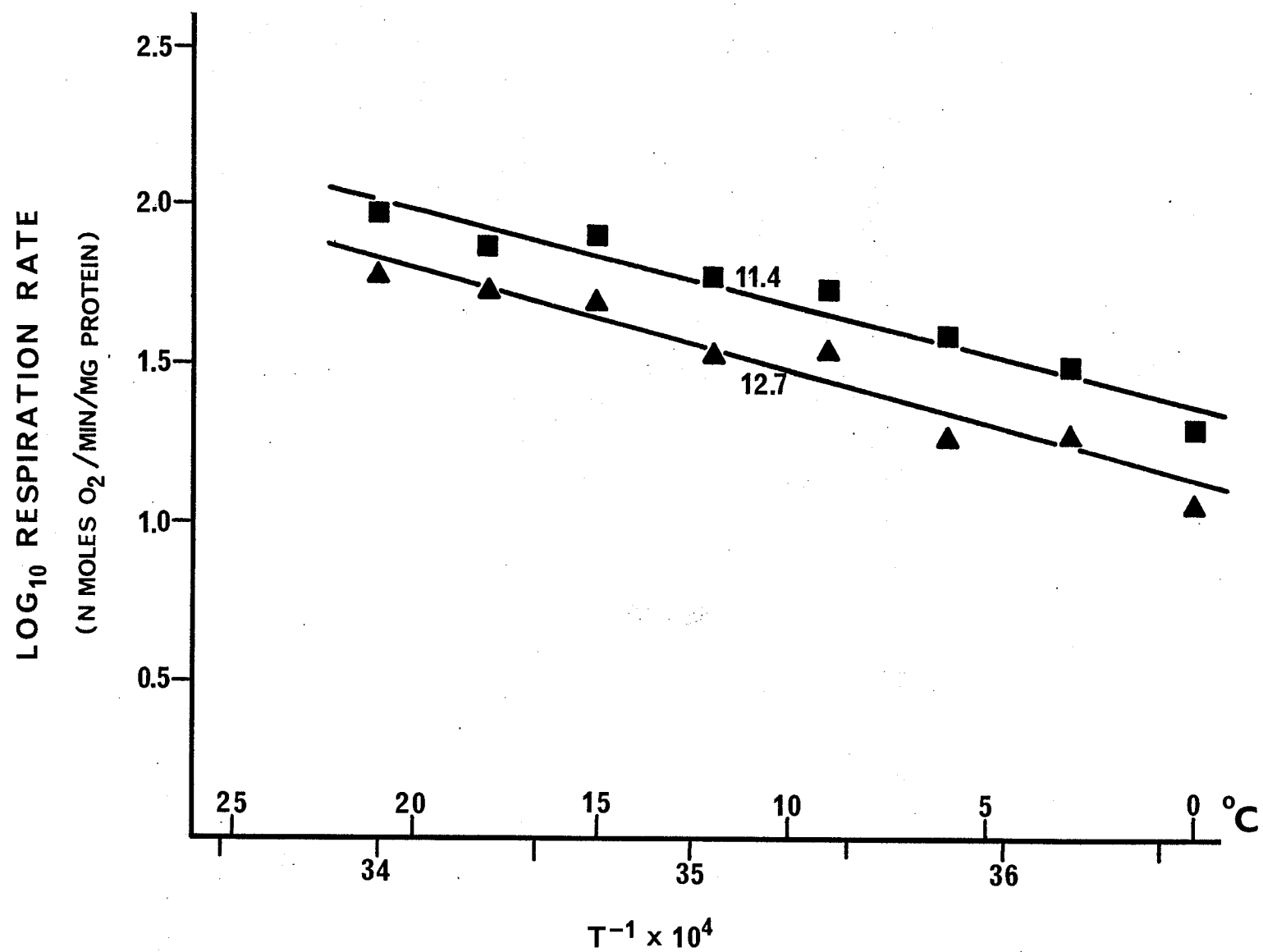


Figure 12

Arrhenius plot for the oxidation of succinate by mitochondria isolated from Neepawa germinated at 4-6°C. The lines represent state 3 (■) and state 4 (▲). Activation energies E^* (kcal/mole) are indicated beside each line.



Mitochondrial Membrane Lipids

The fatty acid composition (Table 8) of the mitochondrial membrane fraction isolated from wheat germinated at room temperature was found to be similar to the composition reported by Miller *et al.* (1974). The major fatty acid components included palmitic (19%), oleic (5%), linoleic (37%) and linolenic (37%) acids. Except for minor variations, the composition was similar for both Kharkov and Neepawa mitochondria.

Germination of the wheat in the presence of 20-25% CO₂ in oxygen resulted in a marked alteration in the unsaturated fatty acid components (Table 8). Linoleic acid increased by 15%, under conditions of high concentrations of CO₂, apparently at the expense of linolenic acid which decreased by 12%. However, the relative degree of unsaturation of the membranes was constant.

TABLE 8. Fatty acid composition of wheat mitochondrial lipids

| Cultivar | Germination Atmosphere | Fatty Acids (%) | | | | |
|----------|-------------------------------|-----------------|----------------|--------------|-----------------|------------------|
| | | Palmitic (16:0) | Stearic (18:0) | Oleic (18:1) | Linoleic (18:2) | Linolenic (18:3) |
| Kharkov | Air | 18.7 | 0.4 | 6.6 | 33.6 | 39.4 |
| Kharkov | 20% CO ₂ in Oxygen | 18.1 | 0.2 | 4.4 | 48.4 | 26.5 |
| Neepawa | Air | 18.3 | 0.3 | 4.2 | 39.6 | 35.2 |
| Neepawa | 20% CO ₂ in Oxygen | 16.5 | 0.2 | 2.5 | 54.7 | 24.3 |

GENERAL DISCUSSION

The isolation of mitochondria from plant tissues has classically been considered more difficult than from animal tissues, due to the presence of the tough cell wall, high concentrations of organic acids and oxidation products of polyphenols; differences observed between plant and animal mitochondria were often attributed to artifacts of the isolation of the plant mitochondria. However, as recently reviewed by Palmer (1976), many 'anomalies' observed in plant mitochondria apparently reflect inherent differences between plant and animal systems.

The wheat mitochondria isolated in this study were found to oxidize exogenous NADH in addition to the Krebs cycle acids (Figs. 3 and 4). Although cyanide and antimycin A failed to completely inhibit NADH oxidation, the residual oxygen uptake was not inhibited by the hydroxamic acid mCLAM. This suggested that either electrons from exogenous NADH did not have access to the alternate pathway or they entered after the point of hydroxamic acid inhibition. During the course of this study it was reported that exogenous NADH oxidation, by isolated mitochondria, did not involve the CN-insensitive pathway in sweet potato (Tomlinson and Moreland, 1975) or aged potato slices (Dizengremel and Lance, 1976). Lambowitz *et al.* (1972) found that in mitochondria isolated from 'poky' type *Neurospora crassa*, a cytochrome deficient mutant which uses the CN-insensitive pathway extensively, electrons from exogenous NADH did reach the hydroxamic acid-sensitive alternate pathway; this may indicate an interesting difference between the alternate pathway in the fungus mutant and that of higher plants.

Succinate proved to be conducive to the study of CN-insensitive respiration in wheat mitochondria; succinate oxidation was incompletely inhibited by either cyanide or mCLAM but was normally completely inhibited in the presence of both cyanide (or antimycin A) and mCLAM. This indicated that all of the oxygen uptake observed with succinate was due to the operation of the cytochrome and alternate pathways only.

The cultivar dependence of CN-insensitive respiration (*in vitro*) suggested that certain varieties of wheat may respire more efficiently than others, i.e. they may conserve more of the free-energy of substrate oxidation in the form of ATP rather than expending it as heat. Kharkov mitochondria apparently had the ability to 'waste' 34% of the energy available from succinate oxidation at 15°C whereas Norquay and Neepawa could only 'waste' 8-9% (Table 1). The possibility of increasing plant productivity by eliminating the alternate pathway has been discussed previously by Zelitch (1975).

Little is understood of CN-insensitive respiration and it is certainly possible, if not probable, that the plant may gain some advantage by maintaining both respiratory pathways; the apparent ubiquitous nature of the alternate pathway supports this concept. However, that cultivar differences should exist implies that the plant need not maintain a high level of CN-insensitive respiration, whatever role it fulfills.

The state 3 and state 4 respiratory rates demonstrated a temperature dependence typical of many enzymic reactions and yielded relatively linear Arrhenius plots, i.e. the activation energies were constant over the temperature ranges studied. In contrast to the results of the

present study of succinate oxidation, Pomeroy and Andrews (1975) reported a discontinuity in the Arrhenius plot of α -ketoglutarate oxidation by Kharkov mitochondria. The above authors observed that the respiratory rates decreased abruptly below 10-14°C with a resultant increase in activation energy. Their results, together with those of the present study, indicate that the rate-limiting steps may not be same for α -ketoglutarate and succinate oxidation, at least not at lower temperatures.

Lyons and Raison (1969) obtained linear Arrhenius plots for succinate oxidation by mitochondria isolated from chilling-resistant plants while plots for chilling-sensitive tissues were nonlinear and exhibited an increased activation energy at lower temperatures. Since wheat could be considered extremely chilling-resistant, the constant activation energies obtained in the present study agreed with the concepts of Lyons and Raison (1969).

An inverse relationship between temperature and respiratory rate, as observed for CN-insensitive respiration above 17-18°C (Figs. 6 and 7), has not been previously reported for isolated mitochondria although similar relationships have been observed for whole tissues. The respiratory rates of the philodendron (Nagy *et al.*, 1972) and the eastern skunk cabbage (Knutson, 1974) inflorescences increased as the air temperature was lowered, enabling the tissue to maintain a relatively constant temperature; both of these tissues are highly CN-insensitive.

The cause of the inverse relationship between temperature and CN-insensitive respiration at higher temperatures was not determined.

Certainly enzymic reaction rates decrease as the enzyme denatures; but 15-20°C was relatively low for denaturation, especially considering that succinate oxidation (state 3) exhibited no sign of decreased rates even at 30°C. Although the decreased oxygen solubility at higher temperatures appeared a possible explanation, it was established that the oxygen concentrations employed at all temperatures were likely well above the apparent K_m .

Minor changes in the slopes of Arrhenius plots of mitochondrial respiration have generally been attributed to phase changes in the membrane phospholipid and protein-lipid complexes which could alter the conformation of membrane-associated enzymes (Raison, 1973); any distinct change in the enzyme conformation would probably be reflected in a change in the activation energy of the reactions involved. Perhaps, therefore a change in the physical state of the mitochondrial membranes takes place at elevated temperatures which substantially alters the conformation or accessibility of some component of the CN-insensitive pathway so as to hinder the reaction. Recent publications indicated that CN-insensitive respiration of potato tubers was sensitive to the state of the mitochondrial membranes, with emphasis on the phospholipid fraction (Athanasios and Laties, 1976; Waring and Laties, 1976; Nakamura and Asahi, 1976). Although the exact cause of the rather anomalous temperature response of the CN-insensitive pathway was not determined, the results indicated that the effect was predominantly reversible (Table 4).

Some consideration should be allotted to a recent proposal that the CN-insensitive reduction of oxygen proceeds by a one-electron

transfer to form superoxide ($O_2^{\cdot -}$) or a two-electron transfer to form hydrogen peroxide (H_2O_2), which would require a less sophisticated oxidase than one capable of a four-electron transfer to form water (Rich *et al.*, 1976). Catalase and peroxidase activities were reported to be significant in plant mitochondria, even after density gradient purification (Plesnicar *et al.*, 1967; Rich *et al.*, 1976), and recently a CN-insensitive superoxide dismutase was demonstrated in the matrix of plant mitochondria (Arron *et al.*, 1976). If, for example, the initial product of the CN-insensitive pathway was hydrogen peroxide, then the oxygen concentration (as measured with the oxygen electrode) would depend not only upon the rate of production of hydrogen peroxide but also upon the method of breakdown of the hydrogen peroxide, e.g. the hydrogen peroxide could be decomposed to water and oxygen by catalase. Therefore, measurement of oxygen concentrations alone would not be sufficient to adequately determine the course of reactions and the anomalous temperature response of the CN-insensitive pathway may reflect a change in the manner of hydrogen peroxide utilization. However, the evidence of Rich *et al.* (1976) that the initial product is hydrogen peroxide is far from conclusive.

During the course of the present study it became apparent that if gases released by plant tissue were allowed to accumulate over germinating wheat seeds, the capacity of the CN-insensitive pathway was greatly enhanced. Although the plant hormone ethylene was initially suspected as being the active component, it was subsequently found that addition or exclusion of ethylene from the germination atmosphere did not affect the alternate pathway. However, high concentrations of

CO₂ (10-25%) were found effective in promoting CN-insensitive respiration of wheat seedlings. This promotion was even more dramatic if the high CO₂ concentrations were coupled with increased oxygen concentration. These results indicated that oxygen plays a vital role in the development or promotion of the CN-insensitive pathway of wheat; others have found that oxygen was necessary to the development of CN-insensitive respiration in tuber slices (MacDonald, 1968) and chloramphenicol - grown rice sprouts (Maslov and Vartapetyan, 1974). Germination of wheat in pure oxygen did not increase the CN-insensitive rate above that observed for wheat germinated in air, indicating that oxygen was necessary but not sufficient to promote the alternate pathway. In contrast, Wilson (1971) reported that increased oxygen concentrations alone were sufficient to promote synthesis of the CN-insensitive pathway of cultured sycamore (*Acer pseudoplatanus*) cells.

Recently, Yentur and Leopold (1976) presented evidence that during soybean germination the CN-insensitive pathway predominated for the first 4-8 hr until a transition was made to CN-sensitive respiration. Yu *et al.* (1976) also found with lettuce seeds that salicylhydroxamic acid effectively inhibited germination only in the first hours of imbibition. Respiration of lettuce seed was previously known to be only partially inhibited by cyanide (Mayer and Shain, 1974). When lettuce seeds are warmed to 30°C many will enter a dormant state known as thermodormancy. Keys *et al.* (1975) recently outlined a rather elaborate scheme for overcoming thermodormancy of lettuce seeds; one point at which the germination was blocked could be overcome by adding 15% CO₂ to the germination chamber. Increasing the temperature from

20°C to 30°C caused a decrease in the percent germination of lettuce seeds (Keys *et al.*, 1975), and in the present study, decreased the CN-insensitive respiration of isolated wheat mitochondria; both the percent germination and the CN-insensitive rate were increased, in the respective tissues, by addition of high concentrations of CO₂. Carbon dioxide, often in high concentrations, has frequently been reported to stimulate germination of various seed types (Koller *et al.*, 1962). The response of the CN-insensitive pathway of wheat to gases and reaction temperature appears to support the contention that the alternate pathway may play a significant role in the initial stages of seed germination.

The lipid composition of the mitochondrial membranes was examined for various reasons. Recent publications linked CN-insensitive respiration of potato tubers with the phospholipids of the mitochondrial membrane (Athanasios and Laties, 1976; Waring and Laties, 1976; Nakamura and Asahi, 1976). Raison (1973) suggested that the Arrhenius plots of succinate oxidation by plant mitochondria could reflect the state of the membrane lipids. Therefore, it was felt that the promotion of the CN-insensitive pathway by CO₂ would perhaps parallel changes in the mitochondrial lipid content. As shown in Table 8, germination in the presence of CO₂, besides promoting the alternate pathway, did significantly increase the percentage of linoleic acid (18:2), apparently at the expense of linolenic acid (18:3).

Willemot and Stumpf (1967) reported that during the aging of potato slices the *de novo* synthesis of linoleic acid from acetate-1-¹⁴C increased from zero in the CN-sensitive fresh slices to one-third of

the total fatty acid synthesis in the aged CN-insensitive slices. Abdelkader *et al.* (1969) examined the mitochondrial membranes during aging of potato slices and also reported a dramatic rise in the synthesis of di-unsaturated fatty acids. Although one cannot directly compare the promotion of the CN-insensitive pathway in potatoes by aging, with the promotion of the CN-insensitive pathway in wheat by CO_2 , it is interesting to note that the mitochondrial membranes respond to both promotions with an increase in linoleic acid.

Although the increased linoleic/linolenic acid ratio may have paralleled the promotion of the CN-insensitive respiration of wheat mitochondria, it is doubtful the CN-insensitive capacity directly depended upon this ratio. Germination of wheat in the cold ($4-6^\circ\text{C}$) substantially decreases the linoleic/linolenic acid ratio (Miller *et al.*, 1974) and yet, as presently demonstrated, the CN-insensitive capacity was unaffected.

The fact that germination in the cold ($4-6^\circ\text{C}$) did not promote the capacity of the CN-insensitive pathway in either the spring (Neepawa) or winter (Kharkov) cultivars suggested that the alternate pathway was not directly involved in cold tolerance in wheat. Even the increase in the linoleic/linolenic acid ratio accompanying the promotion of CN-insensitive respiration by CO_2 was opposite the change in this ratio that accompanied cold hardening of wheat (Miller *et al.*, 1974).

SUMMARY AND CONCLUSIONS

Cyanide-insensitive respiration was found to be active in the mitochondrial fraction isolated from etiolated wheat coleoptiles. Although the mitochondria oxidized α -ketoglutarate and exogenous NADH rapidly, succinate was found to be the more suitable substrate for the study of this phenomenon.

In contrast to the cytochrome pathway, the CN-insensitive pathway displayed a marked cultivar dependence (*in vitro*). Of the varieties examined, Kharkov maintained the highest capacity for CN-insensitive respiration. Kharkov mitochondria could divert up to 34% of the electron flux from succinate oxidation (during state 3) through the non-phosphorylating alternate pathway; Ulianovka could divert 21% while Neepawa and Norquay could only divert 8-9%. Therefore, on the basis of ATP production, some cultivars of wheat may respire more efficiently than others.

The CN-insensitive pathway of wheat was not promoted by exposure to low temperatures. The capacity of the CN-insensitive pathway, as observed in the isolated mitochondria, was similar in wheat germinated either at 4-6°C or 20-22°C. Also, when succinate oxidation rates were measured at various temperatures, from 18°C down to 2°C, it was observed that the contribution of the alternate pathway to the overall respiration did not increase as the temperature was lowered. The above results suggested that thermogenesis was not a primary function of CN-insensitive respiration in wheat.

However, the CN-insensitive respiratory rate did exhibit a rather unusual temperature dependence; the rate was maximal between 15° and 20°C.

As the reaction temperature was further increased, the CN-insensitive rate actually decreased. Decreased oxygen solubility at higher temperatures did not appear to be responsible. Although no explanation for this peculiar temperature dependence could be ascertained, two (of the many) possibilities which deserve further investigation are: (1) the conformation of some membrane-associated component of the alternate pathway responds to a phase change in the mitochondrial membrane, or (2) the initial product of the alternate pathway is not H_2O , but H_2O_2 or $O_2^{\cdot -}$ and several oxygen-involving reactions (with different temperature coefficients) compete simultaneously.

Germination of wheat in the presence of high concentrations of CO_2 (10-30%) dramatically increased the CN-insensitive capacity of the mitochondria. This promotion was apparently dependent upon oxygen since germination of wheat in CO_2 -oxygen mixtures resulted in mitochondria with approximately twice the CN-insensitive capacity of mitochondria from wheat germinated in corresponding CO_2 -air mixtures. Although oxygen was necessary for this promotion, germination in oxygen alone did not promote the alternate pathway. Germination of the wheat with increased CO_2 -oxygen mixtures also brought about a significant change in the mitochondrial lipids; the percentage of linoleic acid increased substantially, mainly at the expense of linolenic acid. The increase in the linoleic/linolenic acid ratio and the promotion of the alternate pathway, although both brought about by high CO_2 -oxygen mixtures, are likely coincidental rather than interdependent effects. Although ethylene may be intimately involved in the promotion of CN-insensitive respiration of various plant tissues, no role could be established for

ethylene in the promotion of CN-insensitive respiration of wheat, either in air or CO₂-oxygen mixtures.

Although the concentrations of CO₂ used in the present study greatly exceed concentrations normally present in plant tissues, it should be remembered that these concentrations were chosen in order to elicit the maximal (and undoubtedly completely abnormal) effect. It is readily conceivable that a physiological role for CN-insensitive respiration may exist and minor promotions of the alternate pathway could significantly affect that role; such minor promotions could be provided by the levels of CO₂ which are known to accumulate in plant tissues under certain conditions.

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