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Mitochondrial Activity of Hardened and Nonhardened Rye (*Secale cereale*) Plants Exposed to Freezing Temperatures

Bryce D. Bennett
Utah State University

MITOCHONDRIAL ACTIVITY OF HARDENED AND NONHARDENED RYE (*SECALE CEREALE*) PLANTS EXPOSED TO FREEZING TEMPERATURES

by

Bryce D. Bennett

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Bryce D. Bennett

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Major Professor: Dr. Frank B. Salisbury
Department: Botany

Five day old dark-grown seedlings of Secale cereale variety "cougar" grown at 20 C were subjected to hardening at 2 C in daily increments from 0 to 7 days, to temperature stress at -5 C for 0, 1, and 3 days, and to recovery at 20 C for 0, 1, and 3 days. Unhardened plants were killed by temperature stress but as the time of hardening increased fewer plants were killed. After 5 days of hardening all plants survived subsequent freezing stress.

Mitochondria were isolated from the plants after they received various combinations of the three temperature treatments. There were 8 hardening levels, 3 stress levels, and 3 recovery levels giving a total of 8x3x3=72 treatments. Mitochondria from unstressed plants exhibited steady ADP:O ratios, RC ratios, and rate of state 3 respiration over the whole range of hardening and recovery times. Mitochondria from stressed plants were totally inactive with 0 or one day of hardening but as the time of hardening increased so did

ADP:O ratio, RC ratio, and rate of state 3 respiration. After 5 days of hardening mitochondria from hardened then stressed plants reached a maximum level of activity.

Evidently there was some change in the mitochondria during the hardening phase.

(85 pages)

INTRODUCTION

In recent years plants able to grow under the snow and during the alternate freezing and thawing of early spring have received increasing interest. Included in this group are many species of mountain and alpine ephemerals and some commercially grown grasses and cereals. The ability to grow actively at cold temperatures (at or very near freezing) is certainly the exception rather than the rule, since most plants endemic to the temperate zone remain dormant at such cold temperatures. How these plants manage to grow under these conditions is of interest in the area of exobiology, because extreme conditions on earth may approach the norms of other planets. Information from studies of cold temperature growth could have practical applications in agriculture, and there is also a purely academic interest in how certain species seem to "break the rules."

Research relative to cold temperature growth has largely been concerned with cataloging cold temperature species and describing the environmental conditions involved. Attempts to get at the basic mechanisms of cold temperature growth have been few. We are still a long way from understanding why plants of one species are able to grow at cold temperatures while plants of another species growing at the same location are not.

The normal approach to the problem of cold temperature growth is to compare a species that grows at cold temperature with a

species that does not. Such a comparison may do little more than compare the genetic variability of the two species while shedding little light on the problem at hand. A study of the changes or mechanisms invoked within one species as it is exposed to cold temperature could be more informative. The approach in this research was to use one species in both a cold-temperature-hardened and an unhardened state.

It is known that plant growth requires energy, so a growing plant must be respiring. Since part of respiration takes place within the mitochondria of the cell, it seemed appropriate to investigate cold temperature growth on the cellular or subcellular (mitochondrial) level. This research project involves a study of the mitochondria from plants that have been cold-temperature-hardened and from plants that have not been hardened.

REVIEW OF LITERATURE

It seems that the development of scientific knowledge in general or in any specific area follows a prescribed pattern: observations are made and noted, and theories follow. This gradually-accumulating store of knowledge aids in assembling facts and advancing further speculation. The proven facts are at first general and then more specific. Finally investigations are being done on a very detailed level. This pattern goes forth regardless of who makes the discoveries or tests the theories. As scientific knowledge grows, someone somewhere will make the next step. The mass of knowledge about the existence, nature, and function of mitochondria is no exception. It began with early observations followed by descriptions, isolations, discovery of function, and elucidation of structure-function relationships. In the latter area work is continuing.

It was natural for early mitochondrial studies to be done with animal tissues. Apparently the feeling existed that plant and animal mitochondria were much the same, so why study plant mitochondria? However, there are some differences as indicated by the following quotation.

> We must consider the possibility that the lipoprotein structures of plant cells that can function happily (we suppose) either in ice banks or in tropical deserts display characteristics possibly not found in the pampered lipoprotein structures of the constant-environment warm-blooded animal system where at least 90 per cent of mitochondrial studies, and for that matter studies of lipoprotein systems, have been concentrated. (Crane, 1961, p. 13)

The following review pertains to the history of the discovery of mitochondria, the history and development of isolation techniques,

and research involving mitochondria of plants subjected to cold temperatures. The voluminous literature on the structure and function of mitochondria will be alluded to but not discussed.

Discovery and Isolation of Mitochondria

Discovery

It is not possible to specifically pinpoint the discovery of mitochondria or to name the discoverer. During the years from 1850 to 1890, many cytologists observed granular elements and inclusions in the cytoplasm of cells, some of which were undoubtedly artificats, while some were most likely authentic. Lehninger (1964) makes particular mention of Kölliker as being first to describe some characteristically-arranged granules which were later named sarcosomes. Perhaps Kölliker should also be credited with the first separation of mitochondria from the cell, since he teased some of his granules from the cell and found them to be membranous bodies capable of swelling in water. Jensen and Salisbury (1972) gave the great German cytologist, Richard Altmann, credit for being the first to actually see mitochondria, in about 1890. Lehninger (1964) also recognizes Altmann, but only for developing a greatly superior stain which proved specific for the mitochondrial granules, thus making it possible for him to see them.

The next major development in the discovery of mitochondria, reported by both Lehninger (1964) and Cowdry (1918), was the development of a crystal violet stain by Benda in 1898 and the almost-simultaneous development of the supravital staining of

mitochondria with Janus green by Michaelis. This was a major step in the right direction, since it showed that living mitochondria could bring about oxidation-reduction changes in a dye. Lehninger (1964) gives Benda the credit for coining the name "mitochondrion" from the Greek "mitos," a thread, and "chondros," a grain. These developments paved the way for a flurry of work on the study of the cell during the early part of the twentieth century. During this period some extensive and erroneous theories on the role of mitochondria were proposed. Lehninger (1964) stated that it was Kingsbury who, in 1912, first called attention to the possibility of a cellular oxidation role. At the same time Warburg found respiration to be associated with granular, insoluble elements of the cell, and Regaud concluded, in 1908, that mitochondria contain phospholipid and protein.

The next event of major significance, as reported by Lehninger (1964), was the work of Lewis and Lewis who studied the behavior of individual mitochondria in living cells of a tissue culture. They found that with time the mitochondria changed their shape, size, and location suggesting a dynamic role. Now the task was to discover that role. Research on cell respiration had progressed well, and it now appeared evident that there could be some relationship between respiration and mitochondria. As mentioned earlier, Michaelis had shown that mitochondria reduced Janus green dye, and Warburg had demonstrated that the capacity to consume oxygen resided in the particulate elements of the cell. Now it was imperative to find a way to isolate intact mitochondria from the cell.

First isolation from animal tissue

The first known attempt at isolation of mitochondria was that made by Bensley and Hoerr (1934). They used broken cell dispersions of liver tissue and a differential centrifugation technique. They were not successful because they lacked appropriate suspending media and centrifugation procedures. However, their pioneering work inspired others, and Claude (1946) reported the successful isolation of mitochondria from mammalian liver. His method involved extraction of the tissue in an isotonic sodium phosphate buffer and sodium chloride solution at pH of 7.0 followed by differential centrifugation at 1500xg, 2,000xg, and 18,000xg. He even went so far as to prepare electron micrographs of his mitochondria, and it is now known that they were badly damaged and that the preparation was impure. Claude's procedure was improved by Hogeboom et al. (1948), who replaced the saline element of Claude's isolation medium with sucrose and thus were able to recover mitochondria easily. These mitochondria were elongated and shaped like those seen in intact cells instead of being spherical and swollen as were those obtained from saline media. This group continued to refine the isolation method, and for many years the method of Hogeboom (1955) was the standard procedure employed for the isolation of mitochondria from animal tissues. Presently the procedures for isolation of animal mitochondria are many and varied.

First isolation from plant tissue

The earliest isolation of plant mitochondria may have been that of Bhagvat and Hill (1951), who isolated what they called a "preparation" from various bulbs, roots, tubers, and germinating

seeds. This "preparation" exhibited characteristics now attributed to mitochondria, but these two researchers did not specifically say they had mitochondria, so they failed to get the credit. About this same time Millerd et al. (1951) isolated "particles" from etiolated hypocotyls of mung bean, Phaseolus aureus. These isolated particles carried out reactions of the Krebs cycle, stained nicely with Janus green B, and looked like mitochondria, so they certainly must have been mitochondria. The procedure for this first isolation included disruption of tissue in a solution of 0.1 M phosphate (pH 7.1) and 0.4 M sucrose, straining of the brei through muslin, centrifugation at 500xg with the pellet discarded, centrifugation at 10,000xg, resuspension of the pellet in fresh isolating solution, a second centrifugation at 10,000xg, and final suspension in isolation solution. The workers specified that all steps must be done at a temperature as near 0 C as possible, that pH was critical, and that phosphate was essential to success of the isolation.

Uses of Isolated Mitochondria

Krebs cycle and electron transport

Perfection of a method for isolating mitochondria was prerequisite to determining the details of their functions. It was already believed that mitochondria were the cellular sites of the Krebs cycle and respiration in general. Millerd et al. (1951) used the fact that their isolated particles oxidized Krebs cycle acids as proof that the particles were mitochondria. Isolated mitochondria were and are used extensively to work out the details of respiration and electron transport. The literature pertaining

to this is voluminous. A few excellent references based on plant mitochondria are the articles by Green (1964), Green and Young (1971), Lance and Bonner (1968), Storey (1969, 1970a, 1970b, 1970c), and Storey and Bahr (1969a, 1969b).

P:O, ADP:O, and respiratory control

Mitochondria are capable of catalyzing the synthesis of ATP from ADP and inorganic phosphate coupled with the oxidation of a number of oxidizable substrates. This characteristic provides a useful tool for determining intactness of isolated mitochondria and also for study of the numerous reactions involved in the process. Oxygen uptake is readily measured by use of a manometric procedure such as that described by Slater (1967). By adding a known amount of inorganic phosphorous to a suspension of mitochondria and measuring oxygen consumption, a P:O ratio is obtained. This ratio is a good measure of the steady state rate of oxidation but is not reliable for comparing the accelerated state 3 with the steady state 4 or for measuring any rapid change. With plant tissues the P:O ratio is commonly obtained by use of a Warburg manometer apparatus.

Since the manometric method does not adequately measure rapid changes in oxygen consumption, a method was sought that would do so. It was Chance and Williams (1955) who first used an oxygen electrode to study mitochondrial respiration and oxidative phosphorylation by a polarographic method. Based upon an earlier design by Davis and Brink (1942) they fashioned an 8 mil gloss-coated open type of vibrating electrode that fit nicely into a small cuvette. They found that by adding a known amount of adenosine diphosphate (ADP) to a preparation of tightly coupled mitochondria, it was

possible to accurately measure the amount of rapid respiration. Based upon their work an ADP:O ratio was developed and also a ratio of the rapid (state 3) to steady (state 4) stage of respiration, which they called respiratory control ratio (RC). Hagihara (1961) developed a rotating platinum electrode, which he reported gave excellent ADP:O and RC ratios with animal mitochondria.

The polarographic apparatus as explained by Estabrook (1967) consists of a platinum or gold wire sealed in a glass or plastic cathode with a calomel electrode connected by a KCl agar bridge as reference electrode. When a voltage is passed across the two electrodes immersed in an oxygen containing solution, oxygen undergoes an electrolytic reduction. With proper voltage (0.6 volt) the current is directly proportional to the oxygen concentration of the solution. An appropriate recording mechanism completes the apparatus. In the mid 1960s a commercial laboratory instrument company developed a complete apparatus that is now used widely. It consists of a membrane-covered oxygen electrode, which is mounted in a plunger that in turn fits snuggly into a cuvette chamber. A magnetic stirring bar is placed in the bottom of the chamber, and when the chamber is placed in a constant temperature water bath apparatus the solution in the cuvette is under constant agitation. A slit in the side of the electrode plunger is used for adding substrates and ADP with a hyperdermic syrings. The unit is commonly attached to a recorder. This instrument is described more fully by Estabrook (1967).

Essentially all work done with plant mitochondria since the introduction of the polarographic method has utilized the oxygen monitor instrument, and P:O ratios have been replaced

by ADP:O ratios, which are calculated according to the formulae of Estabrook (1967). It is also possible to determine the oxygen consumption in microatoms of oxygen utilized per minute per milligram of mitochondrial protein. For this determination a measure of mitochondrial protein is usually made according to the Folin-phenol method of Lowry et al. (1951).

Swelling and contraction

Another characteristic of mitochondria that has been used as a research parameter is their ability to swell and contract. This phenonemon is not well understood, however, and seems to be used very litt e at the present time, especially with plant mitochondria. Volume changes in animal mitochondria have been extensively studied according to the excellent review by Lehninger (1962). It is generally agreed that swelling of mitochondria is a spontaneous physical process, while contraction is essentially an active process that requires an external energy source. A close relationship apparently exists between contraction and phosphorylative ability. Certainly contraction (and also phosphorylation) depends upon membrane integrity.

Longo and Arrigoni (1964) were possibly the first to report on mitochondrial volume changes. They found that oxidizable substrates promoted swelling, while electron transport inhibitors abolished it. The ability of mitochondria to swell or shrink is highly dependent on the existence of coupling between oxidation and phosphorylation, since swelling properties are very pronounced in tightly coupled mitochondria and become much less obvious when loose coupling is evident. Stoner and Hanson(1966) and Earnshaw

and Truelove (1968) adquately demonstrated that mitochondria of corn and bean swelled spontaniously in buffered KCl but not in sucrose. Contraction was initiated by addition of either an oxidizable substrate or ATP plus magnesium. The conditions under which the mitochondria became swollen may affect their subsequent contraction. Earnshaw and Truelove (1968) reported that bovine serum albumin in the solution retarded the rate of swelling and promoted substrate oxidation, contraction, and ion accumulation. Stoner and Hanson (1966) reported that mitochondrial contraction could not be uncoupled from respiration. However, Hanson et al. (1968) later found that low, uncoupling concentrations of dinitrophenol were able to uncouple contraction and respiration, and the earlier statement was retracted.

Some limited use of mitochondrial volume changes has been made in other studies. Lyons et al. (1964) studied the relationship between mitochondrial swelling and the chilling sensitivity of plant tissues. This work will be discussed more completely later in this review. Ku and Leopold (1970) measured the swelling response of mitochondria of cauliflower (flower buds), pea seedlings, and tomato fruit to ethylene gas. The ethylene caused swelling in all cases, but so did propylene, propane, ethane, acetylene, butane, and butene-1.

Physiological age of plant tissue

An interesting application of the use of isolated mitochondria is that of comparing mitochondrial activity of tissues at different stages of growth. From various anatomical studies it is known that the number of mitochondria present in the cell changes with

age of the cell. As a general rule young, undifferentiated cells have numerous mitochondria, while older mature cells have relatively few. This natural variation in number, and possible activity, of mitochondria must always be considered in any comparative study. Ikuma and Bonner (1967) isolated mitochondria from the hypocotyls of dark-grown mung beans and found there were no differences in mitochondrial activity from germination to 15 days of age. It is unfortunate that they did not go beyond this time limit.

Mitochondrial activity in the endosperm or cotyledons of germinating seeds of large seeded legumes has been studied by several workers. Opik (1955) used the cotyledons of dark-grown Phaseolus vulgaris L. and found that manometric oxygen uptake rose to a peak on the fourth day of germination and then declined. Malhotra and Spencer (1970) also used P. vulgaris but measured ADP:O and RC ratios polarographically. They found both parameters to be fairly good on days one and two after planting. On days three, four, and five the ADP:O ratio was 0, and the RC was completely lost. There was recovery on days six and seven followed by complete loss again. Beevers and Walker (1956) did a manometric study of the mitochondria of germinating castor bean cotyledons and found that mitochondrial activity declined after the fourth day of germination. Akazawa and Beevers (1957), using the same tissue, reported that the bulk of mitochondria increased during the first five days of germination and then rapidly decreased.

Many studies have been done on the ripening or climacteric cycle of fruits. Tomato fruits in the mature green stage and three, seven, and fourteen days past the "turning" stage were

studied by Dickinson and Hanson (1965). Oxidative rates for mitochondria isolated from the green fruits were good, while there was a steady decline in rates during the ripening process.

Extensive studies involving the isolation of mitochondria from different regions of the avocado fruit as well as from fruits in various stages of ripening have been done. Apparently isolation of mitochondria from avocado is difficult due to the high mixture of other particles. Lance et al. (1965) isolated mitochondria from avocado fruits at four stages of ripening from mature green to dead rype. They found that active mitochondria could be obtained from all stages if pH of the isolation medium was kept within close limits. Cells were disintegrated in a carefully controlled way, and a small quantity of bovine serum albumin (BSA) was present at all times. In contrast to the report that ripening tomatoes lose respiratory control, they found RC to increase with ripening in avocado. Hobson et al. (1960, 1966b) found that mitochondria isolated from the outer green-colored layer of green avocado fruit exhibited no respiratory control, but that in ripe fruit the same layer exhibited excellent control. Mitochondria from the yellow-colored inner layer of both green and ripe fruits had good control, but the ripe fruit was better.

Application to plant breeding

Perhaps one of the most interesting discoveries with respect to isolated mitochondria is that of mitochondrial complementation. McDaniel and Sarkissian (1966, 1968), who were working with hybrids and inbred lines of corn, (Zea mays), found ADP:O and RC ratios exhibited by the mitochondria of the inbred parents to be

considerably lower than those of the hybrid offspring. When isolated the mixture produced ADP:O and RC ratios very near those of the hybrid offspring! This was called mitochondrial complementation by the two discoverers. They further found that the combined mitochondria from two inbreds whose offspring did not show heterosis did not show complementation. Sarkissian and Srivastava (1969a) investigated complementation in wheat. Respiratory control ratios for mitochondria of two inbred wheat lines were 3.7 and 4.6. For the hybrid the ratio was 6.0 and for the 1:1 mixture of the two inbreds--5.6. They also measured respiration of intact wheat shoots and found that the hybrid had a respiratory rate (oxygen consumption) about 1.5 times higher than the inbreds. Hobson (1971) was able to duplicate the work of Sarkissian and Srivastava only when very strict attention was paid to the details of the isolation.

Doney et al. (1972) investigated mitochondrial complementation in sugar beets. Their results were not as conclusive as previous works with wheat and corn, possibly because the sugar beet inbreds were not as highly inbred as were those of wheat and corn. Very recently Ellis et al. (1973) reported that they were unable to demonstrate any mitochondrial complementation in hybrid barley and wheat cultivars even though they accurately followed the methods of workers who reported complementation. They deduced, obviously, that mitochondrial complementation cannot be used as a tool in hybrid cereal breeding programs until it is more fully proven and understood.

An investigation of the kinetics of mitochondrial complementation

was done by McDaniel and Sarkissian (1970). They found complementation to occur immediately upon mixing of the two extracts, which suggested that physical contact between mitochondria was necessary for complementation to occur. In further proof of the physical aspect, extracts from mitochondria of one inbred did not cause complementation when mixed with intact mitochondria of the other inbred. Further, serial dilution of mitochondrial mixtures rapidly reduced mitochondrial oxidation, while similar dilutions of unmixed mitochondria caused no such reduction. The general consensus at present is that complementation is the result of increased mitochondrial efficiency which is somehow brought about by physical contact.

Other uses of isolated mitochondria

Another use of mitochondria in research as described by McDaniel and Frans (1969), is that of the testing of herbicides. They tested Prometryne and Flurometuron on isolated mitochondria of soybean seedlings and found that Prometryne caused a decrease in the state 3 (rapid state) oxidation, while Flurometuron caused a decrease in state 4.

Methods of Isolating Plant Mitochondria

The methods used for isolating mitochondria from plant tissues are many and varied. Searchers of the literature who look for an isolation method are apt to become greatly confused, for one group of workers will recommend a certain procedure as being best, while the next group will denounce that same procedure as being totally unreliable. Most likely part of this confusion has been caused by

variations in the plant tissues being subjected to the isolation procedure. Perhaps Axelrod (1955) hit at the root of the problem when he said:

> Basically, the winning of mitochondria from any tissue depends on its disintegration to a subcellular level and a separation of the desired particules by differential centrifugation under suitably mild physical conditions. In actual practice there is a considerable variation in these conditions, depending on the particular plant tissue involved, and each material must be considered individually. (pp. 19-20)

He then proceeded to outline two procedures, one for fragile tissues such as mung bean and one for harder tissues such as avocado fruit. To demonstrate the wide variety of plant tissues that have been used for mitochondrial isolations, Table 1 is presented.

Table 1. A list of plant tissues from which mitochondria have been isolated and literature references reporting the isolation.

Plant tissue	References
Apple fruit	Romani et al. (1969), Wiskich (1966, 1967)
Apple peel	Hulme et al. (1964), Jones (1961)
Artichoke, Jerusalem, tubers	Palmer (1967)
Avocado fruit	Baker et al. (1968), Axelrod (1955), Biale et al. (1957), Raison and Lyons (1970), Hobson et al. (1966a, 1966b), Lance et al. (1965) Wiskich (1967).
Banana fruit	Haard and Hultin (1968)
Barley seedlings	Hobson (1971)
Bean, bush, cotyledon	Stinson and Spencer (1968), Malhotra and Spencer (1970, 1971), Opik (1965)

Table 1. Continued

Plant tissue	References
Bean, bush, hypocotyl	Lyons et al. (1964), Millerd et al. (1951), Earnshaw and Truelove (1968)
Bean, mung, hypocotyl	Douce et al. (1972), Ikuma and Bonner (1967), Axelrod (1955), Storey (1969, 1970a, 1970b, 1970c), Ikuma (1970)
Beet, red table, leaves	Gronebaum-Turck and Willenbrink (1971)
roots	Lyons and Raison (1970), Raison and Lyons (1970)
Beet, sugar, root	Doney et al. (1972)
Broad bean hypocotyl	Matlib et al. (1971)
Cantalope fruit	Ku et al. (1967)
Carrot root	Dalgarno and Birt (1962, 1963)
Caster bean cotyledon	Beevers and Walker (1956), Akazawa and Beevers (1957)
Cauliflower buds	Lyons et al. (1964), Lyons and Raison (1970), Ku and Leopold (1970), Raison and Lyons (1970)
Corn etiolated shoots	Hanson et al. (1968), Stoner and Hanson (1966), McDaniel and Sarkissian (1966, 1968, 1970), Miller et al. (1970), Koeppe and Miller (1971)
Cotton seedlings	Stewart and Guinn (1971), Killion et al. (1968), Throneberry (1961)
roots	Stewart and Guinn (1971)
Cucumber fruit	Lyons and Raison (1970), Raison and Lyons (1970)
"Honey Dew" melon	Ku et al. (1967)
Lemon peel	Bogin and Erickson (1965)
Peas etiolated seedlings	Lyons et al. (1964), Ku and Leopold (1970), Longo and Arrigoni (1964)
Pear fruit	Romani et al. (1969), Raison and Lyons (1970)

Table 1. Continued

Plant tissue	References
Potato, Irish, tubers	Lyons and Raison (1970), Douce et al. (1972), Verleur (1965), Raison and Lyons (1970)
Potato, sweet, tubers	Lyons et al. (1964), Lyons and Raison (1970), Baker et al. (1968) Wiskich and Bonner (1963), Raison and Lyons (1970)
Soybean seedlings	McDaniel and Frans (1969)
Spinach leaves	Parish (1971), Gronebaum-Turck and Willenbrink (1971), Nordhorn and Willenbrink (1972)
Tobacco leaf	Pierpoint (1962)
Tomato fruit	Lyons et al. (1964), Lyons and Raison (1970), Dickinson et al. (1970), Ku and Leopold (1970), Ku et al. (1967, 1968), Drury et al. (1968), Raison and Lyons (1970), Kickinson and Hanson (1965)
Turnip root	Lyons et al. (1964)
Wheat etiolated shoots	Sarkissian and Srivastava (1968, 1969a, 1969b), Hobson (1971), Ellis et al. (1973)

General differential centrifugation methods for mitochondrial isolation

While there are wide differences in isolation methods, it is still possible to outline a general pattern. A more detailed discussion, however, will follow in the next section. The tissue to be used for isolation should be suitable, which means easily obtainable in sufficient quantity, free from hardened or lignified tissue, preferably free from chloroplasts, and should not release large amounts

of vacuolar acids upon rupture of the cells. Also, plant tissues should be cold, and those containing large amounts of starch should be held in the dark for a period of time so that the amount of starch will be reduced by the process of respiration. Without exception, all steps of the isolation are done at a temperature of 0 to 4 C. The isolation begins with maceration of the tissue sufficient to rupture the cell wall, but not drastic enough to damage the mitochondria. This is normally done in a mortar or a blender containing an isolation solution consisting usually of an osmoticum (sucrose or mannitol or both), a buffer, a chelating agent such as ethylenediamine tetra acetic acid (EDTA), bovine serum albumin (BSA), and a reducing agent such as cysteine. The pH is held between 7.2 and 7.4. Following tissue maceration, the brei is strained through muslin, cheese cloth, or dacron to separate the solution containing cell particules from the macerated tissue. The solution is first centrifuged at a low speed of 500-1,000xg to remove cellular debris, then at 8,000 to 12,000xg. The resulting pellet is washed either with more of the original isolation solution or with a washing solution of different composition. Finally, the pellet is resuspended and again centrifuged at the higher speed. The resulting pellet is resuspended in the final suspension mixture, which is most often a solution of mannitol, and the mitochondria are ready for use.

The first general method for the isolation of mitochondria to be published was that of Axelrod (1955) in Methods in Enzymology. A later volume of the same set contained a general method designed by Bonner (1967). Other good references on general methods are those by Verleur (1965), Ikuma and Bonner (1967), Haard and Hultin

(1968), Romani, et al. (1969), and Ikuma (1970).

Density gradient isolation of mitochondria

The general method of isolating mitochondria by differential centrifugation often gives mitochondrial suspensions which are heavily contaminated with cellular debris and other organells. It is also useless for isolating mitochondria from green tissues because of the contamination of the mitochondrial pellet with chloroplasts. A method of purifying the mitochondrial suspension by means of density gradient centrifugation has been devised. In this method the mitochondrial solution obtained from the differential centrifugation procedure is layered on top of a sucrose gradient and further separated by additional centrifugation.

Pierpoint (1962) reported that a sucrose gradient layered in a centrifuge tube in the specific order of 1.76 M, 1.40 M, 1.10 M, 0.80 M, 0.50 M, and 0.30 M sucrose gave a good separation of tobacco-leaf chloroplasts, chloroplast fragments, and particles presumed to be mitochondria. Baker et al. (1968) reported that a mitochondrial extract from avocado was improved greatly by density gradient centrifugation. Parish (1971) used a density gradient containing sucrose, sorbitol, and Ficoll (a trade name for a nonionic synthetic polymer of sucrose) to obtain an excellent separation of chloroplasts and mitochondria from spinach. He also used the sorbitol and Ficoll in his isolation solution. Groenbaum-Turck and Willenbrink (1971) and Nordhorn and Willenbrink (1972) purified mitochondrial extracts of spinach and beet leaves by use of a silica sol "Ludox H S 40" density gradient. Finally, Douce et al. (1972) used a discontinuous sucrose gradient of 1.8 M, 1.45 M, 1.2 M, 0.9 M, and 0.6 M sucrose

to purify mitochondria from mung bean hypocotyls and from potato tubers. They found that the mitochondria collected in a narrow layer at the boundary between 1.2 and 1.45 M sucrose. It is interesting to note that with mung bean mitochondria there was always a pellet of debris in the bottom of the gradient tube following centrifugation, while the potato mitochondria produced no such pellet.

The method of density gradient purification is certainly an important new tool. It requires relatively expensive equipment and very careful technique but is the only way to separate mitochondria from green tissues.

Discussion of Isolation Procedure

Tissue disruption

The method of disrupting the cells of the plant tissue may greatly affect the amount and condition of mitochondria in the final suspension. The ideal system is one that will break the cell wall but not damage the mitochondria. It may be necessary to settle for a low yield in order to avoid such damage. The most gentle method that will produce results should be used. Certainly there is some marked difference between tissues. Estabrook (1967) suggested that bulky or hard tissues such as those in carrot, beet root, sweet potato, etc. can be cut with a salad maker, stainless steel grater, or in a Waring Blendor, but more tender tissues should be gently cut into small pieces and ground in a porcelain mortar. (It is imperative that the cutting or grating be done in the isolation solution so that disrupted cells are immediately exposed to the neutralizing substances of the solution. This is necessary in order to insure that damaging substances, such as phenolic

compounds and fatty acids, are neutralized by the isolation solution before they can damage the preparation.) Dalgarno and Birt (1962) ground carrot tissue in a Waring Blendor because it was not possible to grind the tissue in a mortar. They found that the time of grinding in the blender was very important, since mitochondria from tissue ground for 60 seconds were 70% less efficient at oxidation than those from tissue ground for only 20 seconds. Axelrod (1955) proposed two general methods for mitochondrial isolation from plants. He recommended that tender tissues such as those present in mung bean be ground in a mortar, while tougher tissues, which are normal in avocado, be disrupted in a blender. When using a blender for grinding tissue, it is common practice to reduce the motor speed and to operate the apparatus in several bursts of 2 to 5 seconds duration separated by a 10 to 30 second time interval.

Ku et al. (1967, 1968) compared three methods of tissue disruption for isolation of mitochondria from mature green tomato fruit. The outer and radial wall tissues of chilled fruits were disrupted by: 1) cutting into very thin slices with a stainless steel razor blade, but with no further manipulation, 2) grinding with a mortar and pestle, and 3)grinding in a Waring Blendor at full speed for 15 seconds. In all these procedures the tissue was kept submerged in an identical isolation solution. Mitochondria from the razor-cut sections had an RC value of infinity and an ADP:O value of 1.92, while the mortar-ground mitochondria had an RC value of 2.2 and an ADP:O of 1.45. The blended preparation had no respiratory control at all. The same results were reported for cantalope and Honey Dew melons. Bogin and Erickson (1965) macerated samples of lemon peel in both a mortar and in a blender and found

the mitochondria from the mortar-ground tissue to be superior to those from the blended tissue. Throneberry (1961) was the only worker to report that mitochondria from blender-disrupted tissue were superior to those from mortar-disrupted tissue. He did his work with hypocotyls from cotton seedlings. Wiskich and Bonner (1963) maintained that blender disruption of sweet potato presented no problems. However in a modification of the Wiskich procedure, Hobson et al. (1966b) found mortar disruption of sweet potato to be superior to blender disruption.

Longo and Arrigoni (1964) and Hobson et al. (1966b) compared the effects of using quartz sand or silica in the mortar as opposed to using nothing. They found that sand and silica were unnecessary and undesirable for tissue maceration in mitochondrial extraction.

Jones and Hulme (1961) developed a special roller mill to be used for disruption of apple peel in mitochondrial isolation. The peel was fed through the rollers which were constantly bathed in isolation solution. Further testing of the roller mill by Hulme et al. (1964) proved the superiority of the roller mill over mortar and blender disruption. The mitochondria from the mill exhibited oxygen uptake about six times higher than those from the blender and two times higher than those from the mortar. The apple peeling roller mill evidently was not tried on any other tissue.

Haard and Hultin (1968) developed an excellent method for isolation of mitochondria from green and ripe banana fruit. Slices of fruit pulp about 1 mm thick were immersed in liquid nitrogen, drained, and pulverized to a powder in six one-second treatments in a Waring Blendor at high speed. The powdered tissue was then mixed with isolation solution. Another novel method of disruption

was that of Romani et al. (1968) who placed a stainless steel screen, which had been slightly ground to provide shearing edges, in a low container. The screen was supported a small distance from the bottom of the container, and a magnetic stirring bar was placed below the screen. With the isolation solution slightly above the screen and the stirrer in operation the tissue (apple and pear fruit) was grated through the screen. A pH electrode placed in one side of the container facilitated constant monitoring and adjustment of pH.

The brei obtained by disrupting tissue in the isolation solution is commonly strained through muslin or cheesecloth prior to centrifugation. Palmer (1967) found that use of 2 layers of nylon fabric (mesh 50 strands/cm) strained the brei so well that it was not necessary to subject the extract to a low speed centrifugation to remove large particles and debris. Raison and Lyons (1970) coated the nylon material (Miracloth) with a two to four mm layer of Dicalite 4200 and found the Dicalite to effectively remove large starch grains and cell debris.

In passing, it is interesting to note that Ohnishi et al. (1966) extracted mitochondria from yeast by first dissolving the cell walls with snail gut juice.

Osmoticum

Following the first successful isolation of plant mitochondria, Millerd et al. (1951) pointed out that one of the important conditions of the isolation is proper osmotic concentration of the isolation solution. The osmoticum used in their isolation was sucrose. Today sucrose and mannitol are commonly used, either along or in combination. Axelrod (1955) in his general isolation method published in Methods in Enzymology, recommended use of sucrose at a concentration

of 0.4 M for soft tissues and 0.5 M for harder tissues. Bonner (1967), in a later volume of the same set recommended the use of 0.3 M mannitol in place of sucrose. For isolating sweet potato mitochondria, Wiskich and Bonner (1963) found a mixture of 0.37 M mannitol and 0.37 M sucrose to be better than either mannitol or sucrose alone.

The only comparative study of osmotica found during the course of this review was that of Ikuma (1970) who used mung beans for his work. He used mannitol at concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 M, sucrose at concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0 M, and a combination of 0.25 M sucrose and 0.3 M mannitol. Sucrose at concentrations greater than 0.35 M reduced respiratory activity and partially uncoupled the mitochondria. Baker et al. (1968) had previously reported that 0.4 M sucrose in the isolation solution produced mitochondria with dilated cristae and condensed matrix, while 0.25 M sucrose produced intact mitochondria with the membranes lying close together. Ikuma (1970) further found that mannitol concentrations above 0.3 M gave mitochondria with good respiratory and phosphorylative abilities. He concluded that with mung beans, mitochondrial preparations made in sucrose were of inferior quality, while those made in mannitol at high concentrations were highly preferable.

pH

The pH of the isolation solutions must be carefully controlled. In fact many workers monitor the pH with either a pH probe or narrow range pH paper during the tissue disruption procedure and maintain

a definite pH value by dropwise addition of KOH. Romani et al. (1969) reported that accurate pH control of the disruption mixture was of vital importance when isolating mitochondria from acidic tissue such as pear and apple fruit. They designed an apparatus for constant control of pH during the grinding of the tissue. A pH in the range of 7.0 to 7.4 is usually considered very good though there are some exceptions.

Millerd et al. (1951) listed proper pH control as one of the conditions they found to be extremely important. In proposing a general method for isolation of plant mitochondria, Axelrod (1955) recommended a pH of 7.1 and Bonner (1967) recommended 7.2. Jones and Hulme (1961), while isolating mitochondria from the peel of apple, investigated the effects of pH over the range from 6.0 to 10.0 and found that maintaining the pH between 7.0 and 7.5 gave best results. Wiskich and Bonner (1963) reported that at pH 8.0 sweet potato mitochondria lost all oxidative activity, but mitochondria isolated at pH 7.2 to 7.5 exhibited good oxidative properties. Ikuma (1970) found that a pH range of 7.0 to 7.5 was best for isolating mitochondria from mung bean. He concluded that a pH above 7.2 was essential for mitochondrial isolations.

In contrast to the above mentioned results, Longo and Arrigoni (1964) found that a pH of 7.0 to 7.4 was too low for isolation of mitochondria from pea seedlings. They found a pH value of 8.0 to be best. Verleur (1965) investigated the effect of pH on potato mitochondria and found the most active mitochondria were obtained at pH 6.5. Haard and Hultin (1968) maintained a pH of 8.0 during their grinding of banana fruit. However, they reported that meaningful pH readings were not obtained at the temperature of the

isolation, and upon warming of the mixture the pH was found to be 7.3.

The general consensus of all workers in the field is that pH is of vital importance and must be closely monitored and maintained. The only exception found was that of Matlib et al. (1971) who used a pH range from 6.0 to 7.5 for isolations from mung bean and reported little variation.

Buffers

Since plant cells normally contain large acidic vacuoles, which are ruptured during maceration, a buffer is commonly included in the isolation solution in order to stabilize its pH. Millerd et al. (1951) reported that the inclusion of phosphate buffer in the isolation solution was essential to the success of the procedure. Axelrod (1955) included a phosphate buffer in his general method for isolation of plant mitochondria. Biale et al. (1957) questioned the use of phosphate buffer, since they found little difference between mitochondria isolated from avocado fruit with or without it. Dalgarno and Birt (1962) found that tris-HCl buffer caused a clumping of mitochondria when used for isolation from carrot root. No added buffer was better than tris, and the use of phosphate buffer gave oxidation values of 50 to 70% greater than tris.

In his outline of a general method for extracting mitochondria from plants, Bonner (1967, p. 129) stated that, "The addition of phosphate buffer to the media...is to be avoided. Not only does phosphate extract cytochrome C, but it also leads to mitochondrial rupture." Wiskich and Bonner (1963) had already done isolation of mitochondria without the use of a buffer. When a buffer is

not used it is necessary to maintain the pH at the desired level by dropwise addition of KOH. This is often done even when a buffer is employed.

In recent years two very good comparative studies pertaining to the use of buffers in isolation of plant mitochondria have been reported. Stinson and Spencer (1968) compared the effects of phosphate, tris (hydroxymethyl) aminomethane [Tris], N-tris (hydroxymethyl) methylglycine [Tricine], N-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid [TES], and N-2-hydroxyethylpiperazine-N-2ethanesulfonic acid [HEPES] on isolations of mitochondria from cotyledons of snap bean seedlings. TES was found to be superior to all other buffers in all parameters measured (RC, ADP:O, oxygen consumption, and response to cytochrome C) except in response to cytochrome C. HEPES, phosphate, and Tricine were very similar, and tris was poorest in all parameters. In the second study, Ikuma (1970) compared isolations from hypocotyls of mung bean done with no buffer, phosphate buffer, Triethanolamine buffer, Mono-tris (hydroxymethyl)aminemethane maleate [tris-maleate], and tris-HCl. He found the phosphate and triethanolamine buffers to be more effective in preserving the respiratory and phosphorylative activities than either of the tris buffers or no buffer. Tris-maleate was the poorest of all. He condluded that phosphate or triethanolamine buffer should be used in isolating mitochondria from plant tissue.

Bovine serum albumin

One of the earliest substances found to enhance the quality of mitochondrial isolation, both animal and plant, was bovine serum

albumin (BSA). Since the early 1960s BSA has been used in virtually every isolation of plant mitochondria. The one and only report that BSA was of no value was that made by Dickinson and Hanson (1965). Axelrod (1955) did not include BSA in his basic method, and Wiskich (1967) advocated using BSA for isolations from apple fruit but not from avocado fruit. Ikuma and Bonner (1967) did not use BSA in isolating mitochondria from mung bean hypocotyls. However, Bonner (1967) recommended use of BSA in his general method, and Ikuma (1970) later reported BSA to be essential in mitochondrial isolations.

For some back ground on the action of BSA, references on work with rat liver mitochondria are being included with this review. Boyer et al. (1947) found that BSA combines with fatty acids, the amount of combination being much greater for long chaid acids than for short chain ones. The reversal of aging in rat liver mitochondria was demonstrated by Helinski and Cooper (1960), who isolated mitochondria without BSA then added it to the suspension at intervals following the isolation. Whan added up to 20 minutes following isolation, BSA restored the oxidative activity of the mitochondria to the original level. The mechanism of this restoration apparently involved some of the sites on the BSA molecule responsible for binding of anions.

The effect of BSA on free fatty acid content of mitochondrial preparations and of tissue homogenates from carrot root was studied by Dalgarno and Birt (1963). In homogenates made without BSA and kept at 4 C the free fatty acid concentration was 0.015 mM after 25 minutes and 0.11 mM after 60 minutes. In homogenates with BSA, corresponding values were 0.013 mM after 25 minutes and 0.041 mM

after 60 minutes. In a mitochondrial preparation made without BSA the free fatty acid concentration was 1.1 mM, but with BSA the concentration was 0.56 mM, the BSA reducing the free fatty acid concentration by more than half. These same researchers further found that the ADP:O ratios for mitochondria isolated with BSA were in the range of 1.3 to 2.0, while those for mitochondria isolated without BSA were less than 0.1, a thirteenfold difference! Certainly, then, the presence of fatty acids during the isolation of plant mitochondria could account for differences observed with and without BSA. Weinbach and Garbus (1965, 1966) found that the uncoupling effect of pentachlorophenol and 2, 4-dinitrophenol on rat liver mitochondria was reversed and/or prevented by addition of BSA. This property of BSA lies in its capacity to bind these reagents and remove them from the solution.

The effect of BSA on swelling and contraction of mitochondria was studied by Longo and Arrigoni (1964). It was found that BSA modified the kinetics of spontaneous swelling of pea seedling mitochondria and increased the efficiency of contraction.

An isolation solution containing 0.1% BSA gave Verleur (1965) a better RC for potato mitochondria than an otherwise identical solution containing no BSA. Hobson et al. (1966b) found avocado fruit mitochondria posessed no respiratory control when isolated without BSA, but its inclusion gave good respiratory control. Ku et al. (1967, 1968) found BSA to have a protective action on their tomato fruit mitochondria. Killion et al. (1968) found that high concentrations of BSA (1%) were essential in the extraction of oxidatively-active mitochondria from hypocotyls of cotton seedlings.

When Drury et al. (1968) allowed isolated tomato fruit mitochondria to stand in an ice bath, they found that those isolated with a solution containing BSA increased in respiratory control for 8 hours then steadily decreased to a zero point. Mitochondria isolated without BSA did not show an increase in respiratory control but decreased rapidly to a zero reading. Matlib et al. (1971) observed that the incorporation of 0.1% BSA into the isolation solution provided a preparation of tightly ccupled mitochondria, while omission of the BSA resulted in mitochondria with low RC ratios.

Efforts to find a substitute for BSA have not been successful. Throneberry (1961) tried replacing BSA with cysteine, ethylenediamine tetra-acetic acid (EDTA), polyvinylpyrrolidone (PVP), reduced glutathione, succinate, gelatin, and egg albumin in his isolations of mitochondria from hypocotyls of cotton seedlings. None of these, however, were of any value in replacing BSA. Lance et al. (1965) in their experiments with BSA found it to be superior to Ficoll, dextran, Thiogel, and polyvinylpyrrolidone for extracting mitochondria from avocado fruits.

Ethylenediamine tetraacetic acid

The history of the use of Ethylenediamine Tetraacetic Acid (EDTA) in the isolation of plant mitochondria is obscure. It seems that the use of EDTA is accepted without question, and it is presently used in all isolations. Millerd et al. (1951) did not recommend the use of it for the first isolation, and Axelrod (1955) did not recommend the use of it in his general method for isolation of plant mitochondria. Biale et al. (1957) used EDTA when extracting mitochondria from avocado fruit

and found it to be essential. Jones and Hulme (1961) reported that the use of EDTA was important in isolating intact mitochondria from acidic tissues such as those in apple fruit. Accordingly, Bogin and Erickson (1965) found increased activity in mitochondria isolated from lemon peel with EDTA present over those isolated without it. In their work with carrot tissues, Dalgarno and Birt (1962) found that adding EDTA to the isolation solution increased the respiratory values of isolated mitochondria greatly.

The only report of EDTA proving ineffective in mitochondrial isolation was that of Throneberry (1961). In isolation from cotton seedlings he found that EDTA in the isolation solution produced no better results than the solution without it.

It is believed that EDTA protects mitochondria by removing potentially injurious cations from the environment during tissue disruption. Verleur (1965) felt that while EDTA was effective in protecting mitochondria by removing harmful cations, it might also remove too much of the catalytic metals. Therefore, he used a very low concentration of 0.001 M EDTA and obtained excellent mitochondria from Irish potato tubers. Ku et al. (1967, 1968) were also concerned about the loss of catalytic metals when EDTA was used, and they tested EDTA concentrations ranging from 0.1 mM to 50 mM. They found an 8 mM concentration gave the best results on tomato fruit mitochondria. Ikuma (1970) used EDTA at concentrations ranging from 0 to 5 mM and found that EDTA concentrations higher than 0.5 mM reduced state 3 respiration rate and ADP:O ratios by 15 to 25%. However, the inclusion of EDTA in the isolation solution was better than leaving it out.

Polyvinylpyrrolidone

The use of polyvinylpyrrolidone (PVP) in mitochondrial isolations is not widely practiced. There are contradictory reports as to its merit. Some reports state that it is of great value in obtaining good mitochondria, while others state that it is of no value. The tissue being used for isolation may be an important factor in the effectiveness of PVP.

Jones and Hulme (1961) used PVP with good results in the isolation solution for isolating mitochondria from apple peel, but they did not comment on results obtained without it. Hulme et al. (1964) found PVP to greatly improve the mitochondria isolated from apple fruit. Wiskich (1967) recommended that PVP be used in isolating mitochondria from apple but not from avocado. Apparently PVP is of some value when isolating mitochondria from acidic fruits such as apple. Perhaps the pH has some effect, since Romani et al. (1969) reported that PVP was less effective at an alkaline pH than at an acidic one. They recommended that PVP be used with acidic plant tissues.

PVP was used by Dickinson and Hanson (1965) for isolating mitochondria from tomato fruit and found to be of little value. Ku et al. (1968) tested PVP at the range of 0.5 to 1.5 g per ml in the isolation medium for tomato fruits and found no effect on respiratory control. Nordhorn and Willenbrink (1972) reported that in their isolation from spinach leaves, 1% PVP remarkably reduced respiration rate and destroyed respiratory control of mitochondria.

Magnesium

Magnesium is sometimes included in the solution for isolation of mitochondria. It is not widely used, however, and reports concerning

its effectiveness are mixed, being both good and bad. Magnesium was not recommended by either Axelrod (1955) or Bonner (1967). Dalgarno and Birt (1962) used magnesium chloride in the isolation solution when isolating mitochondria from carrot tissues and reported an increase in values by a factor of fifteen. Lance et al. (1965) used magnesium at a "greatly reduced" concentration in order to avoid aggregation of avocado-fruit mitochondria and subsequent loss during centrifugation. Ikuma (1970) compared mitochondria of mung bean isolated with magnesium, without magnesium, and with magnesium plus phosphate. In every instance where magnesium was present during the isolation, respiratory rates were reduced below those of the control. He stated that these results "clearly indicate that magnesium ion exerts a deleterious effect on the maintenance of mitochondrial activity." (p. 776). Matlib et al. (1971) isolated mitochondria with and without magnesium from germinating broad bean seeds. In their results magnesium abolished tight coupling with complete loss of state 4 of respiration.

Speed of isolation and special techniques

Recently a few researchers have become concerned over the speed of mitochondrial isolations. The feeling is that a rapid isolation may provide more active mitochondria and make it easier to perform comparative studies. The first attempt at speeding up the isolation time was made by Palmer (1967). He introduced a nylon fabric for straining the brei obtained from the grinding of artichoke tubers and thus eliminated the need for the first low speed centrifugation, shortening the isolation time from 70 to 37 minutes. By increasing

the speed of the second and third centrifugation from 15,000xg to 40,000xg (for 5 minutes) the time was reduced to 15 minutes. Further reduction to 7.5 minutes was obtained by shortening the time of the second centrifugation to 1.5 minutes and by eliminating the third one altogether. He reported that the ADP:O ratio was identical for all procedures but that the respiratory control value was increased as the isolation time was shortened. Sarkissian and Srivastava (1968) used Palmer's method for wheat seedlings but found they could not eliminate the wash and final centrifugation. They also reported that the fast isolation gave good ADP:O ratios and increased respiratory control. Sarkissian and Srivastava (1968, 1969b) reported that the washing step was essential to separate the mitochondria from starch. They found a simple trick that aided the separation even further. The mitochondrial pellet was left undisturbed in the centrifuge tube during the washing, and when the tube was replaced in the centrifuge it was rotated 90° from its orientation during the previous centrifugation. Following the final centrifugation they found two sediments in each tube, a white starch pellet and a darker mitochondrial pellet. The mitochondrial pellet was easily removed with a small spatula.

Mitochondria and Cold Temperature Stress

The relationship of mitochondrial activity to cold temperature growth and stress has been studied very little. There are a few interesting studies comparing mitochondria of cold- and warm-blooded animals, and there are several good references comparing mitochondria of chilling-resistant and chilling-sensitive plant species. Some

studies on chilling or freezing injury in plants have made use of isolated mitochondria. Apparently there has been no comparative study of mitochondria from the same plant species grown under normal temperature and under temperature stress.

Differences in mitochondria of cold- and warm-blooded animals

Richardson and Tappel (1962) demonstrated a difference in flexibility between mitochondria of cold- and warm-blooded animals. They found, by using a light scattering technique, that mitochondria from fish liver (cold-blooded) had the ability to swell at a rapid rate over a wide range of temperatures down to 0 C, but mitochondria from rat liver (warm-blooded) did not swell at the lower temperatures. They also showed that a correlation existed between membrane flexibility (mitochondrial swelling) and fatty acid composition of the mitochondrial membrane. The more flexible membranes from the cold-blooded animals had a higher proportion of unsaturated fatty acids than the less flexible membranes from the warm-blooded animals. Lyons and Raison (1970a) determined Arrhenius plots for succinate oxidation by mitochondria from the livers of homeothermic (warm-blooded) and poikilothermic (cold-blooded) animals. For homeotherms there was a phase change at about 23 C with increased activation energy below this temperature, but for poikilotherms there was no phase change over the entire temperature range from 4 to 30 C. They postulated that there was a phase change in the membrane lipids of homeotherms but not in poikilotherms.

Mitochondria and freezing injury in plants

In an effort to study the effect of freezing on plant membranes, plant mitochondria have been frozen in liquid nitrogen. Dickinson et al. (1967) showed that tomato fruit mitochondria frozen in liquid nitrogen and rapidly thawed suffered severe loss of respiratory control and decrease in efficiency of oxidative phosphorylation unless they had been treated with dimethyl sulfoxide (DMSO) prior to being frozen. The DMSO served as a cryoprotective agent that preserved mitochondrial integrity. These same authors, Dickinson et al. (1970), later found that the DMSO was not necessary if the mitochondria were frozen very quickly and thawed rapidly to 30 C. Marked reduction in respiration and loss of respiratory control occurred when mitochondria were transferred from liquid nitrogen to -5, -10, or -18 C for fifteen minutes prior to thawing at 30 C. DMSO prevented freezing damage when mitochondria were incubated at -5 C but did not prevent freezing damage at -10 or -18 C.

Mitochondria and chilling injury in plants

The metabolic injury that occurs in some plants at low (0 to 10 C) temperatures has been studied recently by a few workers who used isolated mitochondria. In a study reported by Stewart and Guinn (1971a), young cotton plants were hardened at 15 C (control was 30 C) for two days then exposed to 5 C for two and four days. Another group was exposed to 5 C without hardening. Mitochondria were extracted from the roots of all treatments, and mitochondrial oxygen consumption was determined. The hardened plants had slightly more oxygen consumption than the controls but dropped to 78% of control

after two days of chilling and to 72% of control after four days of chilling. Plants not hardened at all had a value of 53% of control after four days of chilling. The hardening process provided some protection against chilling injury, but it was incomplete.

Lieberman et al. (1958) examined the oxidative and phosphorylative activities of mitochondria derived from sweet potato roots that had been stored at 7.5 and 15 C. Oxidative and phosphorylative values of mitochondria from roots stored at 7.5 C began to decline by the fifth week of storage and were at the zero level by the tenth week. Activity of mitochondria from roots stored at 15 C showed little change during the same period. Minamikawa et al. (1961) showed that mitochondria from sweet potato roots stored at 20 C maintained oxidative and phosphorylative activities while those from roots stored at 0 C showed a sharp decline in oxidation and phosphorylation after ten days of storage.

Mitochondria from plants sensitive and resistant to chilling

It has been shown that the physical nature of mitochondrial membranes of chilling-sensitive and chilling-resistant plant species can differ. This difference appears to be correlated with susceptibility of the tissues to chilling injury. Lyons et al. (1964) isolated functional mitochondria from tissues of chilling-sensitive species, including tomato fruits, sweet potato roots, snap bean seedlings, and corn seedlings; and from tissues of chilling-resistant species, including cauliflower buds, turnip roots and pea seedlings. Mitochondria from chilling-resistant tissues showed a striking ability to swell in either sucrose or KCl, but mitochondria from chilling-sensitive tissues showed very little ability to swell. It should be noted that in the chilling-

sensitive group the corn and bean mitochondria swelled more than those from tomato and sweet potato. The change in optical density during swelling of the mitochondria of the chilling-resistant species averages 0.13% while that for the chilling-sensitive species averaged 0.04%--a threefold difference. These authors also examined the content of unsaturated fatty acids in the membranes of isolated mitochondria and found that those of chilling-resistant species showed a higher content of unsaturated fatty acids than did those from chilling-sensitive species. These differences in unsaturated fatty acid content of the membranes may explain the differences in membrane flexibility.

Lyons and Raison (1970b) determined oxidation ratio, ADP:O, and RC ratios for mitochondria isolated from chilling-sensitive and chilling-resistant plant species. Their sensitive group was composed of mature green tomato fruit, sweet potato root, and cucumber fruit; the resistant group included Irish potato tubers, cauliflower buds, and table beet roots. After the mitochondria were isolated, their ability to carry out succinate induced oxidation was checked at temperatures ranging from 25 to 1.5 C, and the results were plotted in an Arrhenius plot. Mitochondria from the chilling-resistant tissues showed a linear decrease over the entire temperature range from 25 to 1.5 C, and Q_{10} values were 1.7 to 1.8. Mitochondria from the chilling-sensitive tissues showed a linear decrease from 25 C to about 9 or 12 C at which point there was a marked deviation with increased slope as temperatures were reduced to 1.5 C. The Q_{10} values were 1.3 to 1.6 above the breaking point and 2.2 to 6.3 below it. It is obvious that mitochondria from chilling-sensitive tissues

show a marked depression in respiratory rate below the critical temperature for chilling injury (10 C), which is not observed with mitochondria from resistant tissues. These same authors further observed that phosphorylative efficiency of mitochondria from all tissues, as measured by ADP:O and RC ratios, was not influenced by temperatures from 25 to 1.5 C. They concluded that the phosphorylative efficiency of mitochondria from sensitive as well as resistant tissues is not affected directly by low temperatures, although the rate of phosphoyrlation is reduced. The immediate response of chilling-sensitive plant tissues to temperatures below 10 C was to depress mitochondrial respiration. These results are consistent with the hypothesis that a phase change occurs in the mitochondrial membrane as the result of a physical effect of temperature on some membrane component such as membrane lipids.

METHODS OF PROCEDURE

Plant Tissue

Plant species utilized for study

Since it was the intent of this study to compare plants of the same species grown under normal temperature and under cold temperature stress it was important to find a species that could be used under both conditions and one for which seed was readily available. Several species were considered, and after some discussion with faculty members of the Plant Science Department, it was decided to use a variety of common rye, Secale cereale, known to be winter hardy. Seed of the variety "cougar" was obtained from a commercial source and was stored dry at 20 C from the time of purchase until planting. Only one lot of seed was used.

Plant tissue used for analysis

All analyses of the study were done on shoots grown in the dark. Seeds and roots were separated from the shoots, and the entire shoot was used.

Method of Growing Plants

Containers and growing media

All plants were grown in plastic pots that were about four inches square and four inches deep. The seeds were planted on a layer of sterile vermiculite and covered with sterile horticultural grade perlite.

Planting and growing of plants

A one half inch layer of vermiculite was placed in the bottom of the pot and about three hundred seeds, which had been previously treated with Captan, were evenly scattered on top. The pot was then filled to the top with perlite, thoroughly watered from the top and allowed to drain. Drained pots were placed in metal trays and watered from the bottom lightly every other day. By this method the perlite was easily shaken from the plants, and harvesting of the shoots was relatively easy. Plantings were made daily according to a randomly determined schedule of analysis.

Design of Experiment

Temperatures used

All plants were grown at 20 C for five days before temperature treatments were begun. The 20 C chamber was a walk-in room. Hardening was at 2 C in a refrigerator; stress was at -5 C in a deep freeze; and recovery was at 20 C in the walk-in room. These temperatures were selected on the basis of a preliminary experiment. Pots of plants were moved from chamber to chamber on a daily basis as required for each treatment.

Combinations of temperature treatments

There were a total of eight hardening treatments, which included 0, 1, 2, 3, 4, 5, 6, and 7 days of hardening. There were three stress treatments of 0, 1, and 3 days and three recovery treatments of 0, 1, and 3 days. All possible combinations of these three treatments were made giving a total of 8 X 3 X 3 = 72 treatments. Each treatment was replicated 5 times.

Isolation and Testing of Mitochondria

Mitochondrial isolation procedure

Plants were removed from the temperature chamber immediately prior to the mitochondrial isolation. After discarding the perlite, 20 to 23 g of shoots were carefully separated from roots and seeds and placed in a beaker in an ice bath. All glassware and solutions used were chilled at 0 to 4 C before use. The shoots were washed 3 times in deionized water, drained slightly on a paper towel and immediately placed in a pre-chilled porcelain mortar with 150 ml of cold isolation solution. The isolation solution consisted of 0.25 M sucrose, 0.001 M EDTA, and 0.06 M potassium phosphate buffer (pH 7.2), 0.002 M mercaptoethanol, and 0.75 mg/ml BSA. The tissue was clipped into short lengths with a pair of scissors while submerged in the isolation solution and ground for 45 seconds with a pestle. The resulting brei was strained through two layers of dacron cloth into centrifuge tubes in an ice bath. The tubes were centrifuged in a refrigerated centrifuge (0 C) at 500xg for 5 minutes. The pellet was discarded and the supernatant centrifuged again at 12,000xg for 10 minutes. The supernatant was removed by suction and the pellet rinsed three times with 5 ml of a cold wash medium and finally covered with 5 ml of wash medium but not resuspended. The wash medium was composed of 0.4 M mannitol, 0.01 M potassium phosphate buffer (pH 7.2), and 0.75 mg/ml BSA. The centrifuge tubes were replaced in the centrifuge with the pellet oriented 90° from the previous centrifugation and centrifuged again at 20,000xg for 5 minutes. The supernatant was again removed by suction and the pellet suspended in 1 ml of 0.3 M mannitol. This was the final mitochondrial

suspension that was used immediately for determination of mitochondrial activity. Total time required for one isolation was two hours.

Measurement of mitochondrial activity

All measurements were carried out in a Model 53 oxygen monitor with a Clark oxygen electrode manufactured by Yellow Spring Instrument Co., Yellow Springs, Ohio. The reactions were carried out at a constant temperature of 27 C.

Three ml of reaction mixture containing 0.3 M mannitol, 0.01 M potassium phosphate buffer (pH 7.2), 0.01 M tris-HCl buffer (pH 7.2), 0.01 M KCl, 0.005 M $MgCl_2$, 0.72 mg/ml BSA, 0.03 mg/ml cytochrome C, 0.01 mM TPP, and 0.01 mM NAD were placed in the reaction cuvette and allowed to equilibrate for five minutes. The reaction mixture was prepared minus the cytochrome C, TPP, and NAD and stored frozen until the day of use. The cytochrome C, TPP, and NAD were made up separately and also stored frozen until the day of use. The pH of all solutions was adjusted to 7.2. After addition of 0.3 ml of mitochondrial suspension, the solution was allowed to equilibrate for one minute and then the plunger and oxygen probe were inserted into the cuvette. Immediately 0.03 ml of 1 M alpha Keto glutaric acid were added through the slit in the plunger, and one minute later 0.01 ml (300 micromoles) of ADP were added. ADP additions were repeated at about two minute intervals until anaerobiosis was reached. Usually three or four additions of ADP were made. Results were recorded on a Beckman recorder at a chart speed of one inch per minute. A typical tracing is shown in figure 1.

The initial oxygen content of the air saturated reaction mixture was determined to be 230 micromoles by using intact mitochondria in

the above reaction mixture minus NAD and adding known quantities of limiting NADH according to the method of Estabrook (1967).

Calculation of ratios

All ratios were calculated from the oxygen electrode tracing produced by the recorder. Each isolation produced only enough mitochondria for one run on the oxygen monitor. Most runs produced a tracing with 3 or 4 good cycles (Fig. 1) but in some treatments where plants were severely injured fewer cycles were obtained, and if plants were dead there was no cyclic variation at all. Whenever possible, 3 cycles were used to arrive at an average value for the particular run for all parameters examined.

To calculate the ADP:O ratio the slopes of the state 3 and state 4 states of respiration were extended so that the changes from state to state were easily seen (see Fig. 1). The calculation done according to the method of Estabrook (1967) was: oxygen content of reaction medium per recorder unit X recorder units utilized in state 3 (rapid state) X ml reaction medium X 2 = microatoms of oxygen utilized = ADP:O ratio. For example for cycle 2 in figure 1 the calculation of the ADP:O ratio was as follows. There are 2.3 micromoles of oxygen per recorder unit X 9.5 recorder units utilized in state 3 X 3 ml of reaction medium X 2 which equals 131.1 micromoles of oxygen utilized. There were 300 micromoles of ADP added which divided by 131.1 microatoms of oxygen utilized gives an ADP:O value of 2.29. This ratio expresses the efficiency of the mitochondria.

The RC ratio was determined by dividing the number of units of oxygen utilized (recorder units) in one minute during state 3 by the number of units of oxygen utilized in one minute during

Cycle	ADP:O	RC	State 3
1	not used	----------	
2	2.29	2.78	1.131
3	2.42	3.05	1.244
4	2.42	3.11	1.267
Average	2.38	2.98	1.214

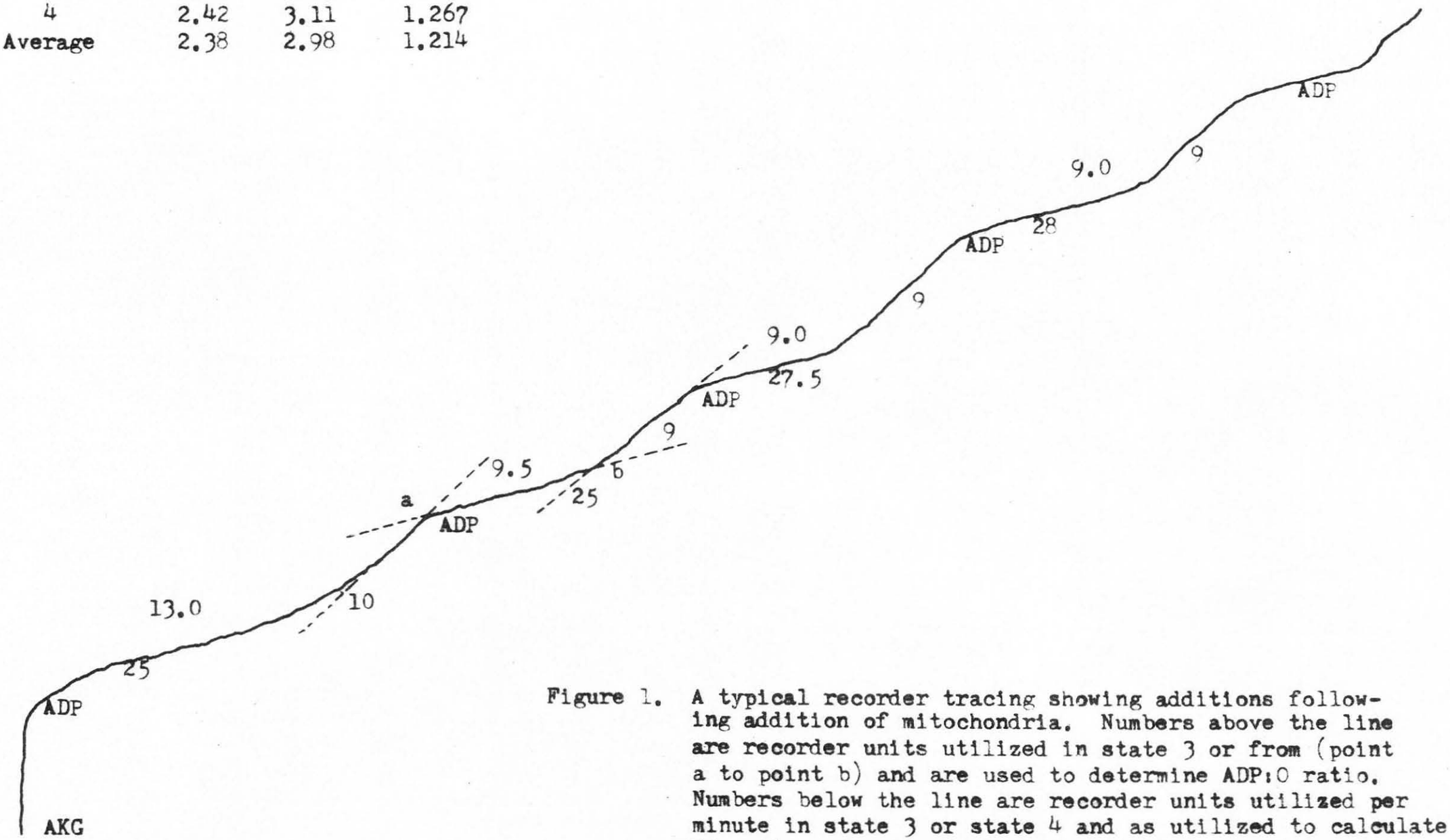


Figure 1. A typical recorder tracing showing additions following addition of mitochondria. Numbers above the line are recorder units utilized in state 3 or from (point a to point b) and are used to determine ADP:O ratio. Numbers below the line are recorder units utilized per minute in state 3 or state 4 and as utilized to calculate RC ratio and rate of state 3 oxidation in units oxygen utilized per minute per mg protein. See text for examples of calculations.

state 4. For example the RC ratio for cycle 2 is 25 recorder units utilized in state 3 divided by 9 recorder units utilized in state 4 which equals 2.78. This ratio gives an indication of the degree of coupling maintained by mitochondria.

The rate of oxidation is expressed by the units of oxygen consumed during state 3 per unit of time per unit of protein. For this determination it was necessary to measure the amount of mitochondrial protein present in the mitochondrial suspension. In accordance with the method of Lowry et al. (1951), samples of the mitochondrial suspension (0.01, 0.02, and 0.03 ml) were measured into centrifuge tubes, 5 ml of 0.25 M sucrose added to each tube, and tubes centrifuged for 10 minutes at 20,000xg. The resulting pellet was resuspended in 5 ml of a solution containing 2% sodium carbonate, 0.1 N sodium hydroxide, 0.01% copper sulfate, and 0.02% potassium sodium tartrate. After standing at room temperature for one hour, 0.5 ml of Folin Ciocalteu reagent (2 N) was added. After one more hour at room temperature, absorbance of light at 750 manometers was read on a spectrophotometer. The results were compared to a standard curve made by the same procedure using bovine serum albumin in place of mitochondria. To determine the state 3 value of oxidation, the units of oxygen consumed per minute during state 3 were divided by the mg of protein per ml of mitochondrial suspension. For example for cycle 2 shown in figure 1 there were 25 units of oxygen consumed per minute and the mitochondrial solution contained 22.1 mg protein per ml so the state 3 rate is 25 divided by 22.1 or 1.131 units of oxygen per minute per mg protein.

RESULTS AND DISCUSSION

Selection of Experimental Conditions

Method of growing plants

Since the experiment required the use of large numbers of dark-grown seedlings, it was necessary to find a way to grow them easily in small units that could be readily moved from one chamber to another. It was felt that growing the seedlings in the bottom of a container such as a flower pot would provide some protection and support for the plants. Soil could not be used as a growing medium since it would be heavy, messy, and might introduce bacteria and fungi that would rot the plants under the dark, moist conditions of the experiment. Sterile vermiculite and horticultural grade perlite used straight from the bag as purchased were tried as possible candidates for a growing medium.

Seeds were planted on a half inch layer of vermiculite or perlite in the bottom of a 4 inch square plastic pot and covered from depths of $\frac{1}{2}$ to $3\frac{1}{2}$ inches with either material. It was found that plants grew well through the deep covering, and that this covering around the young plants provided necessary support and protection. Plants grown on a shallow layer of vermiculite and covered with perlite proved to be best, so that was the method used throughout the experiment. In pots containing all vermiculite there seemed to be too much weight, and the seedlings did not grow straight. The vermiculite clung to the seedlings and was almost impossible to remove. On the

other hand there seemed to be too little moisture in the all perlite pots, and germination was poor. Seedlings penetrated the perlite easily and grew straight. The perlite was readily removed by shaking and rinsing.

Since seedlings reached the surface of the planting medium 5 days after the seeds were planted, 5 days became the standard length of time for growing seedlings before beginning hardening and stress treatments. Less than 5 days did not allow sufficient time for growth, and more than 5 days produced inconviently tall plants. Since there was no nutrient in the growing medium, plants grown longer than 7 days began to show mineral deficiency symptoms.

It was also found that when more than 300 seeds were planted in a pot, germination and growth were slower. The only apparent reason for this slowing of growth was competition due to crowding. In all subsequent tests no more than 300 seeds were planted per pot.

It was also found, quite by accident, that excessive moisture in the growing medium inhibited or even prevented germination. In the beginning of the work the freshly planted pots were moistened by placing them in a pan of water. Usually only enough water was added to thoroughly soak the planting medium. However one week end a batch of pots was left standing in a shallow layer of water. Germination in these pots was very poor. A quick trial proved that pots should be thoroughly soaked after planting, allowed to drain, and subsequently watered just enough to keep the growing medium moist but not wet.

In the early plantings some seed rot was evident. Treating the seed with Captan prior to planting eliminated this problem.

Treatment temperatures

Before beginning the actual experiments it was necessary to determine what temperatures were to be used for the growing, hardening, and stress treatments. Twenty degrees (centigrade) was arbitrarily selected as the growing and recovery temperature. As a starting point, 2 C and -5 C were selected as the hardening and stress temperatures respectively. Variation of these temperatures gave no apparent change in results, and therefore they were selected as final temperatures for the experiment.

Duration of stress period

Stress periods of 3, 5, and 7 days were tried with little difference being observed. Within the limits of the experiment, 3 days of stress were sufficient to bring about the desired death or survival of hardened and unhardened plants (Table 2).

Table 2. Percent of plants dead following hardening at 2 C for 1, 3, 5, or 7 days and stress at -5 C for 3, 5, or 7 days.

Days Hardening	Days Stress		
	3	5	7
1	52%	48%	47%
3	27%	29%	30%
5	3%	2%	6%
7	6%	5%	2%

Time required for hardening

Results of hardening trials are shown in Figures 2 and 3. Five-day old seedlings were hardened for 0, 1, 2, 3, 4, 5, or 7 days, stressed for 3 days, and allowed to recover at 20 C for 3 days. Control plants received neither hardening nor stress but did get 3 days recovery. All unhardened stressed plants died. With increasing time of hardening more plants survived the stress. For 1, 2, 3, and 4 days of hardening, the percent of plants surviving subsequent stress was 57, 31, 24, and 10 respectively (Figure 2). With 5 days of hardening only 5% of the plants were dead, which was equal to the unstressed control. Hardening longer than 5 days did not impart any more resistance to stress (Figure 2). On the basis of this information it was decided to use hardening treatments of daily intervals from 0 to 7 days.

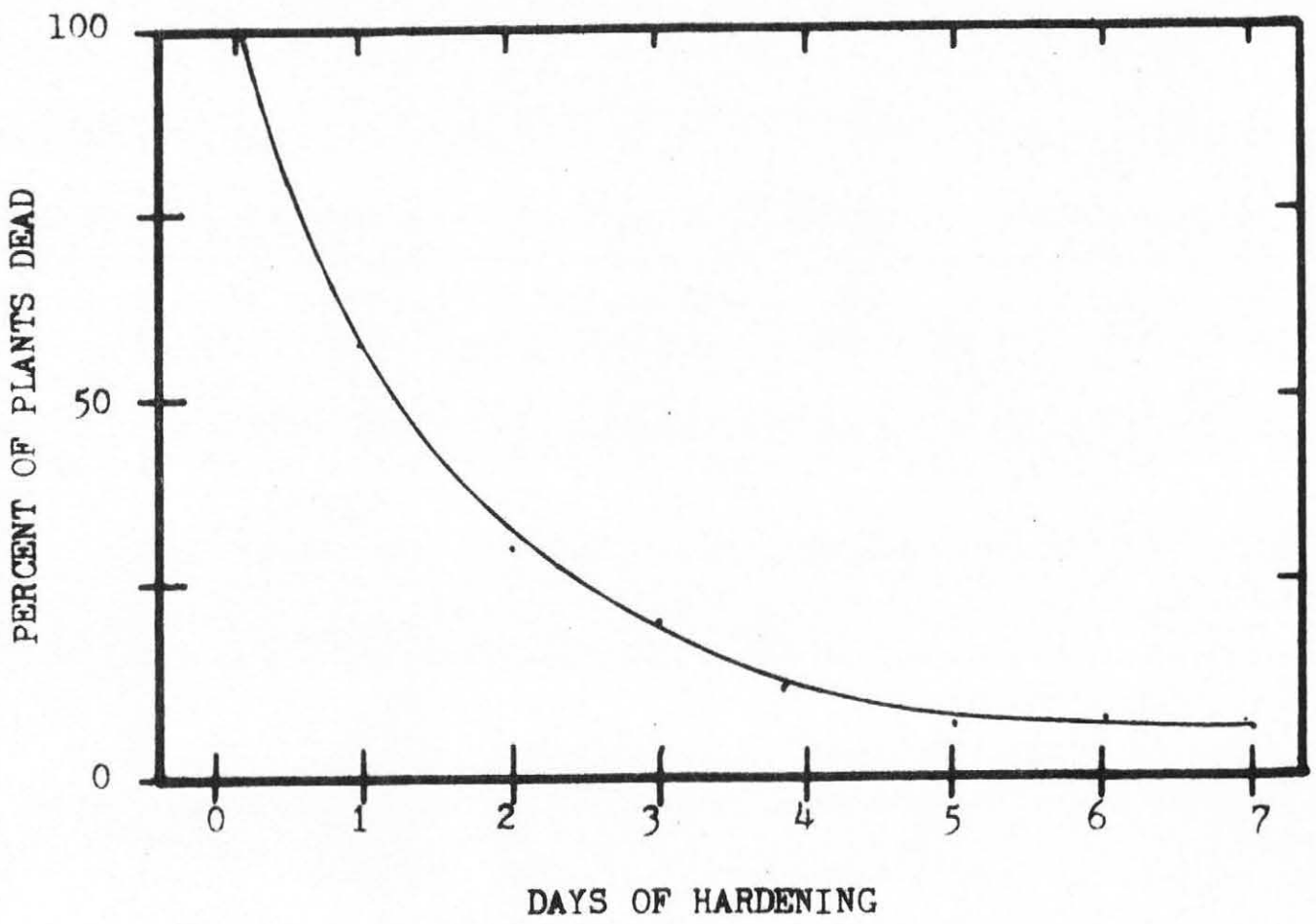
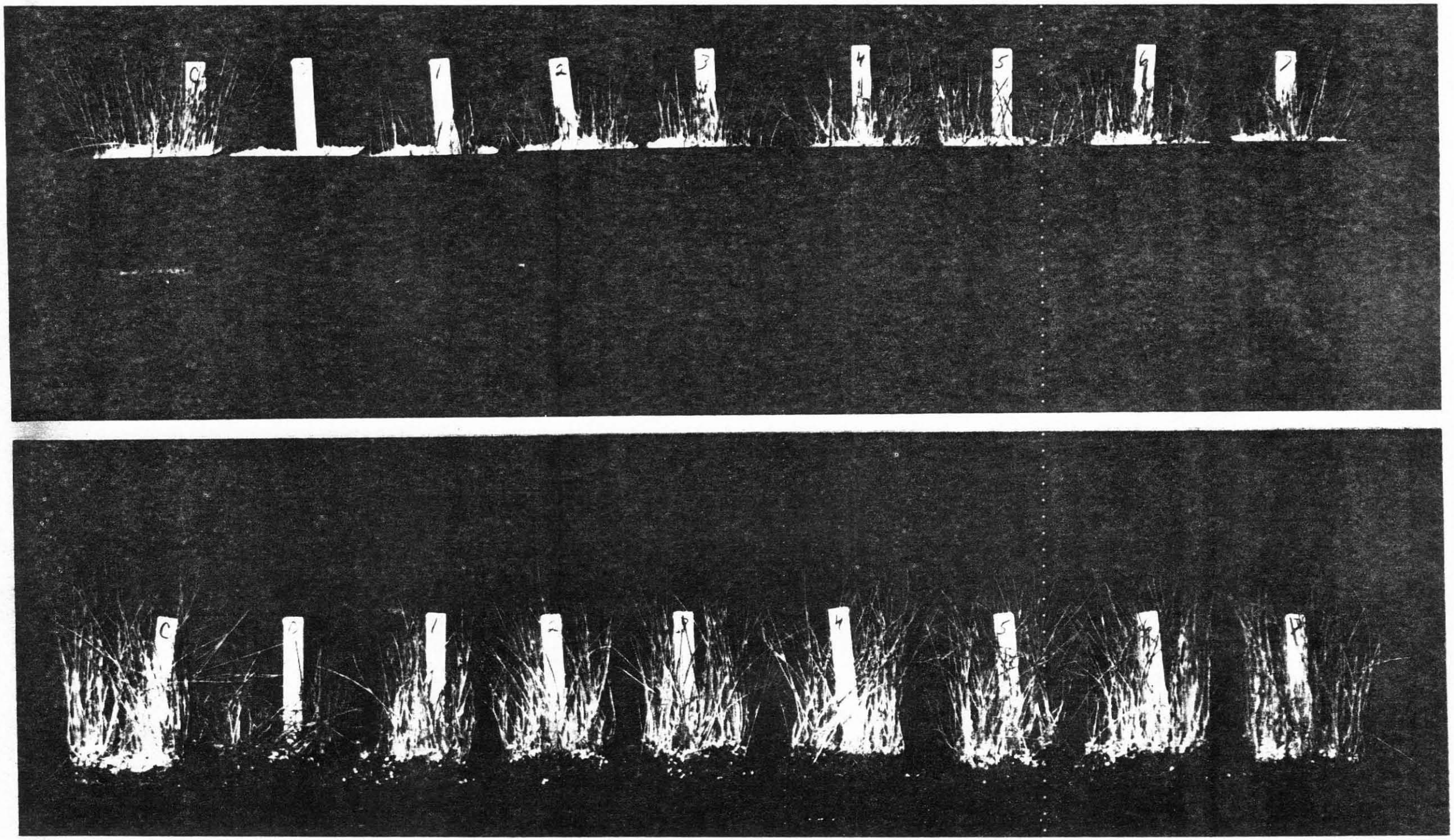


Figure 2. Percent of plants killed by 3 days of stress at -5 C following various days of hardening at 2 C. Averages of 9 replications.



Even though plants hardened for 5 days and stressed for 3 days were not killed, their subsequent growth was not as vigorous as that of the hardened, unstressed control (Figure 3). As will be shown later this lack of vigor was also expressed in the comparison of mitochondrial activity.

The demonstration of a complete resistance to stress following a period of hardening and lack of resistance to stress without the hardening completed the first phase of the experiment. Now the second phase, that of comparing mitochondrial activity between hardened and unhardened plants of the same species, could be carried out.

Analysis of mitochondrial activity

A complete analysis of variance was done for this part of the experiment. The analyses of variance for ADP:O ratios, RC ratios, and rate of state 3 respiration of isolated mitochondria are shown in Tables 3, 4, 5.

Table 3. Analysis of variance for ADP:O ratios for all treatments.

Source of Variation	Degrees of Freedom	Mean Squares	F Test Value
Hardening	7	7.33	3.78*
Stress	2	143.95	74.20*
Recovery	2	173.51	89.43*
Hardening X Stress	14	4.61	2.66*
Hardening X Recovery	14	1.55	0.80
Stress X Recovery	4	8.71	4.49*
Hardening X Stress X Recovery	28	1.54	0.79
Error	217	1.94	

*Significant at 5 percent level

Table 4. Analysis of variance for RC ratios for all treatments.

Source of Variation	Degrees of Freedom	Mean Squares	F Test Value
Hardening	7	13.33	5.95*
Stress	2	132.55	59.17*
Recovery	2	152.37	68.02*
Hardening X Stress	14	5.96	2.66*
Hardening X Recovery	14	3.14	1.40
Stress X Recovery	4	3.77	1.68
Hardening X Stress X Recovery	28	1.62	0.72
Error	217	2.24	

*Significant at 5 percent level

Table 5. Analysis of variance for state 3 rate of respiration for all treatments.

Source of Variation	Degrees of Freedom	Mean Squares	F Test Value
Hardening	7	2.66	0.96*
Stress	2	64.72	23.36*
Recovery	2	62.45	22.55*
Hardening X Stress	14	9.80	3.54*
Hardening X Recovery	14	5.13	1.85
Stress X Recovery	4	9.91	3.58*
Hardening X Stress X Recovery	28	1.78	0.64
Error	217	2.77	

*Significant at 5 percent level

Comparisons of the averages for all treatments and interactions shown to be significant by the analysis of variance are shown in Tables 6, 7, and 8.

Table 6. Comparison of overall ADP:O, RC, and rate of state 3 respiration averages for hardening, stress, and recovery.

No of Days	ADP:O			RC			State 3		
	Hard.	Stress	Rec.	Hard.	Stress	Rec.	Hard.	Stress	Rec.
0	1.04	1.63	1.61	1.20	1.83	1.81	0.93	1.26	1.21
1	1.16	1.27	1.36	1.34	1.51	1.59	0.97	1.07	1.13
2	1.23	--	--	1.40	--	--	0.98	--	--
3	1.40	0.94	0.86	1.67	1.16	1.11	1.06	0.80	0.78
4	1.34	--	--	1.60	--	--	1.15	--	--
5	1.41	--	--	1.68	--	--	1.12	--	--
6	1.35	--	--	1.59	--	--	1.07	--	--
7	1.29	--	--	1.52	--	--	1.05	--	--
LSD	(0.18)	(0.11)	(0.11)	(0.19)	(0.12)	(0.12)	(0.22)	(0.13)	(0.13)

Table 7. Comparison of ADP:O, RC, and rate of state 3 respiration averages for hardening X stress interaction.

Days Hard.	ADP:O			RC			State 3		
	Days Stress			Days Stress			Days Stress		
	0	1	3	0	1	3	0	1	3
0	1.65	0.99	0.47	1.80	1.23	0.57	1.53	0.96	0.29
1	1.70	1.09	0.68	1.89	1.31	0.81	1.44	1.05	0.43
2	1.62	1.33	0.74	1.82	1.52	0.88	1.43	0.97	0.53
3	1.74	1.51	0.95	2.04	1.66	1.30	1.15	1.10	0.94
4	1.64	1.23	1.13	1.80	1.66	1.36	1.18	1.22	1.04
5	1.68	1.35	1.19	1.92	1.61	1.52	1.04	1.21	1.12
6	1.58	1.32	1.15	1.74	1.55	1.47	1.16	1.00	1.05
7	1.42	1.28	1.18	1.63	1.51	1.41	1.15	1.02	1.00
	(LAD=0.32)			(LSD=0.34)			(LSD=0.38)		

Table 8. Comparison of ADP:O and rate of state 3 respiration averages for stress X recovery interaction.

Days of Stress	ADP:O Days recovery 0	1	3	State 3 Days recovery 0	1	3
0	1.82	1.68	1.39	1.63	1.28	0.88
1	1.62	1.40	0.77	1.12	1.17	0.91
3	1.39	1.00	0.43	0.88	0.95	0.56
	(LSD=0.19)			(LSD=0.23)		

Raw averages of 5 replications for all 72 treatments are shown in Table 9. The effect of the three factors of hardening, stress, and recovery and their interactions upon the activity of mitochondria isolated following the treatments will be discussed separately.

The effect of hardening

The analysis of variance showed significance between hardening treatments for the ADP:O and RC ratios but not for the rate of state 3 respiration (Tables 3, 4, and 5). When the averages for all hardening treatments and interactions are compared, it is seen that ADP:O and RC values for zero and one day of hardening were significantly lower than those for 2 or more days of hardening (Table 6). Evidently mitochondria of plants exposed to one to two days of hardening had increased efficiency (ADP:O ratio) and tighter coupling (RC ratio), but the rate of state 3 respiration was unchanged. It should be kept in mind that averages from the analysis of variance include all treatments that are alike in the one factor being considered regardless of the other

Table 9. ADP:O, RC, and state 3 respiration rate values for all treatments. Average of 5 replications.

Days of Stress	ADP:O Days of recovery 0	1	3	RC Days of recovery 0	1	3	State 3 Days of recovery 0	1	3
	(0 days hardening)			(0 days hardening)			(0 days hardening)		
0	1.91	1.78	1.28	2.11	1.94	1.34	2.19	1.55	0.85
1	1.62	1.24	0.12	1.89	1.53	0.27	1.31	1.35	0.22
3	1.05	0.37	0.00	1.119	0.51	0.00	0.57	0.30	0.00
	(1 day hardening)			(1 day hardening)			(1 day hardening)		
0	1.93	1.65	1.53	2.11	1.93	1.64	1.87	1.51	0.94
1	1.37	1.39	0.53	1.62	1.55	0.76	1.38	1.14	0.63
3	1.53	0.51	0.00	1.64	0.78	0.00	0.62	0.68	0.00
	(2 days hardening)			(2 days hardening)			(2 days hardening)		
0	1.78	1.57	1.50	1.99	1.79	1.67	2.33	1.13	0.83
1	1.78	1.39	0.83	1.93	1.53	1.10	0.98	1.07	0.86
3	1.23	0.88	0.12	1.23	1.12	0.28	0.65	0.75	0.20
	(3 days hardening)			(3 days hardening)			(3 days hardening)		
0	1.81	1.91	1.51	2.27	2.08	1.77	1.32	1.21	0.92
1	1.57	1.70	1.26	1.82	1.71	1.47	1.14	0.99	1.18
3	1.34	1.14	0.39	1.64	1.49	0.77	0.91	1.21	0.69
	(4 days hardening)			(4 days hardening)			(4 days hardening)		
0	1.81	1.68	1.44	1.96	1.77	1.65	1.34	1.18	1.02
1	1.59	1.40	0.77	1.95	1.67	1.35	1.00	1.15	1.51
3	1.62	1.13	0.64	1.51	1.65	0.91	1.16	1.16	0.80
	(5 days hardening)			(5 days hardening)			(5 days hardening)		
0	1.79	1.60	1.56	2.21	1.74	1.80	1.21	1.03	0.89
1	1.82	1.35	0.88	1.88	1.59	1.37	1.00	1.38	1.23
3	1.37	1.24	0.76	1.60	1.60	1.07	1.14	1.12	1.71
	(6 days hardening)			(6 days hardening)			(6 days hardening)		
0	1.77	1.67	1.50	2.00	1.76	1.48	1.36	1.30	0.82
1	1.73	1.36	0.87	1.89	1.60	1.16	1.11	1.16	0.74
3	1.51	1.23	0.73	1.73	1.61	1.06	1.01	1.29	0.83
	(7 days hardening)			(7 days hardening)			(7 days hardening)		
0	1.73	1.61	0.52	1.91	1.84	1.44	1.14	1.30	0.73
1	1.51	1.45	0.89	1.67	1.65	1.22	1.02	1.09	0.94
3	1.45	1.30	0.79	1.57	1.61	1.04	1.01	1.14	0.84
	(LSD=.54)			(LSD=.58)			(LSD=.65)		

factors involved. Thus, for the average on zero days hardening, all treatments receiving zero hardening, regardless of what other treatments they have received, were included.

If the raw averages of activity of mitochondria from plants receiving only hardening and no other treatment were considered, there was no difference between times of hardening. For example, ADP:O ratios for plants receiving hardening but no stress or recovery ranged from 1.93 to 1.73, which range was not significant (Table 9). In figure 4 it is shown that ADP:O values did not vary significantly over the seven day period of hardening. The same was true for RC and state 3 respiration values (Table 9, Figures 5 and 6).

The effect of stress

Mitochondria from plants exposed to either of the three levels of stress proved to be highly different in all three parameters measured (Tables 3, 4, and 5). No stress was much better in all 3 parameters than was one day of stress, and one day was much better than 3 days (Table 6). Of course this was the expected result. With no stress, all plants were alive, but with stress of even one day some of the plants were dead. After 3 days stress all plants that had not been hardened were dead (Table 2). Stress was so closely tied to hardening and recovery that it cannot be discussed as an independent variable but must be considered from the standpoint of interaction with hardening and recovery. This will be done in subsequent paragraphs.

The effect of recovery

The three levels of recovery also proved to be significantly different for all parameters measured (Tables 3, 4, 5, and 6).

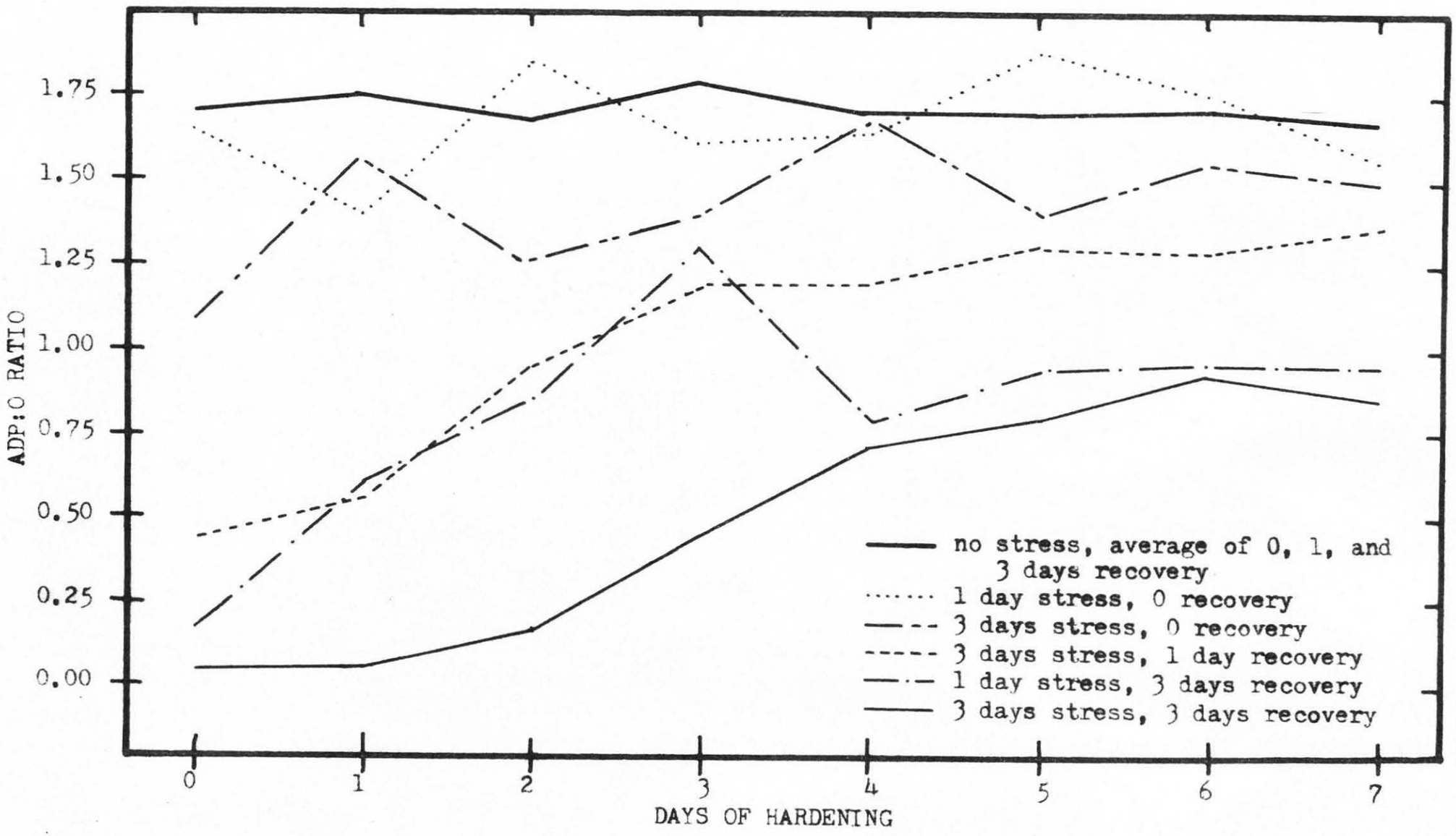


Figure 4. ADP:O ratios of mitochondria from plants receiving varied combinations of stress and recovery following 0 to 7 days of hardening.

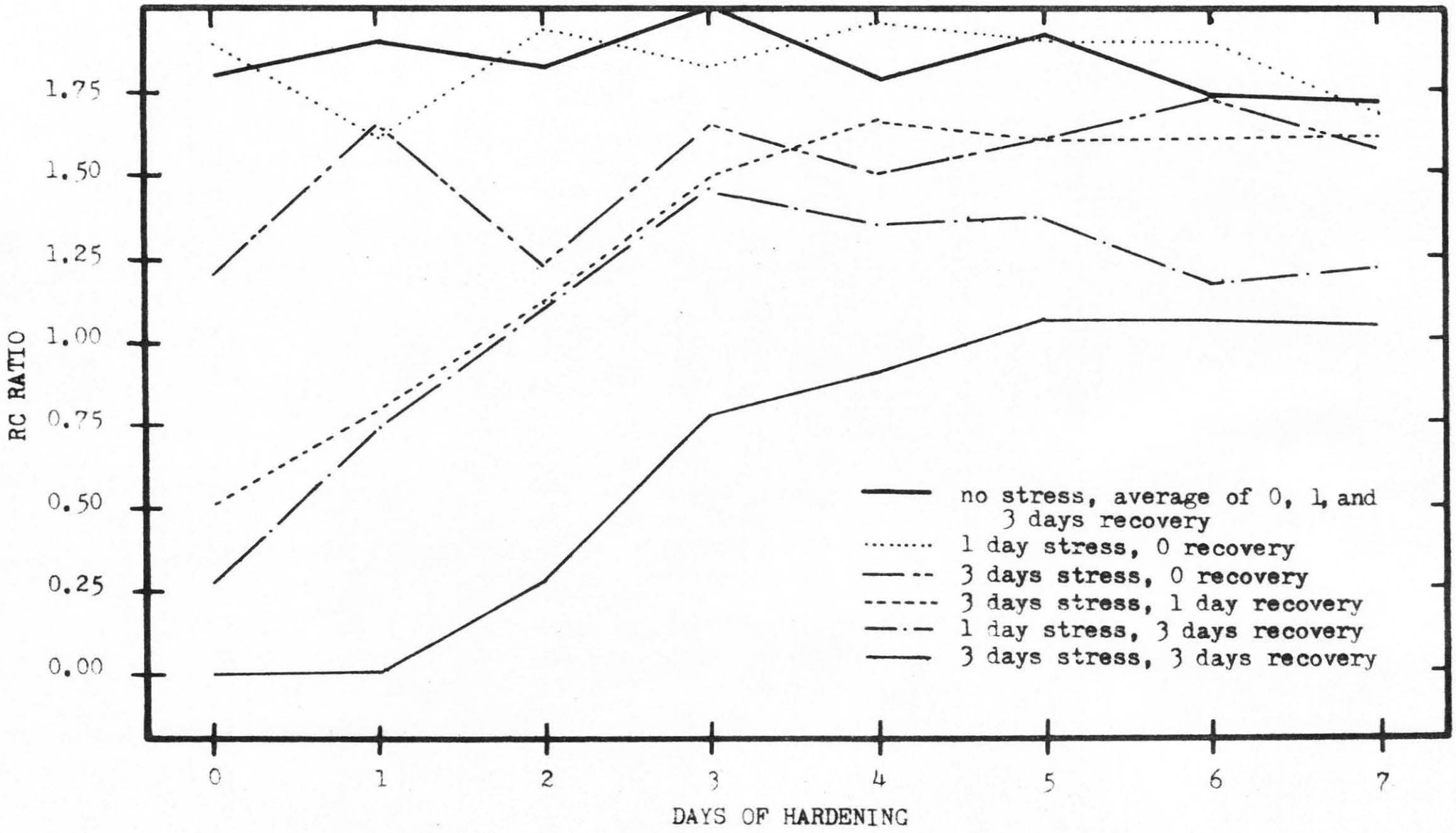


Figure 5. RC ratios of mitochondria from plants receiving varied combinations of stress and recovery following 0 to 7 days of hardening.

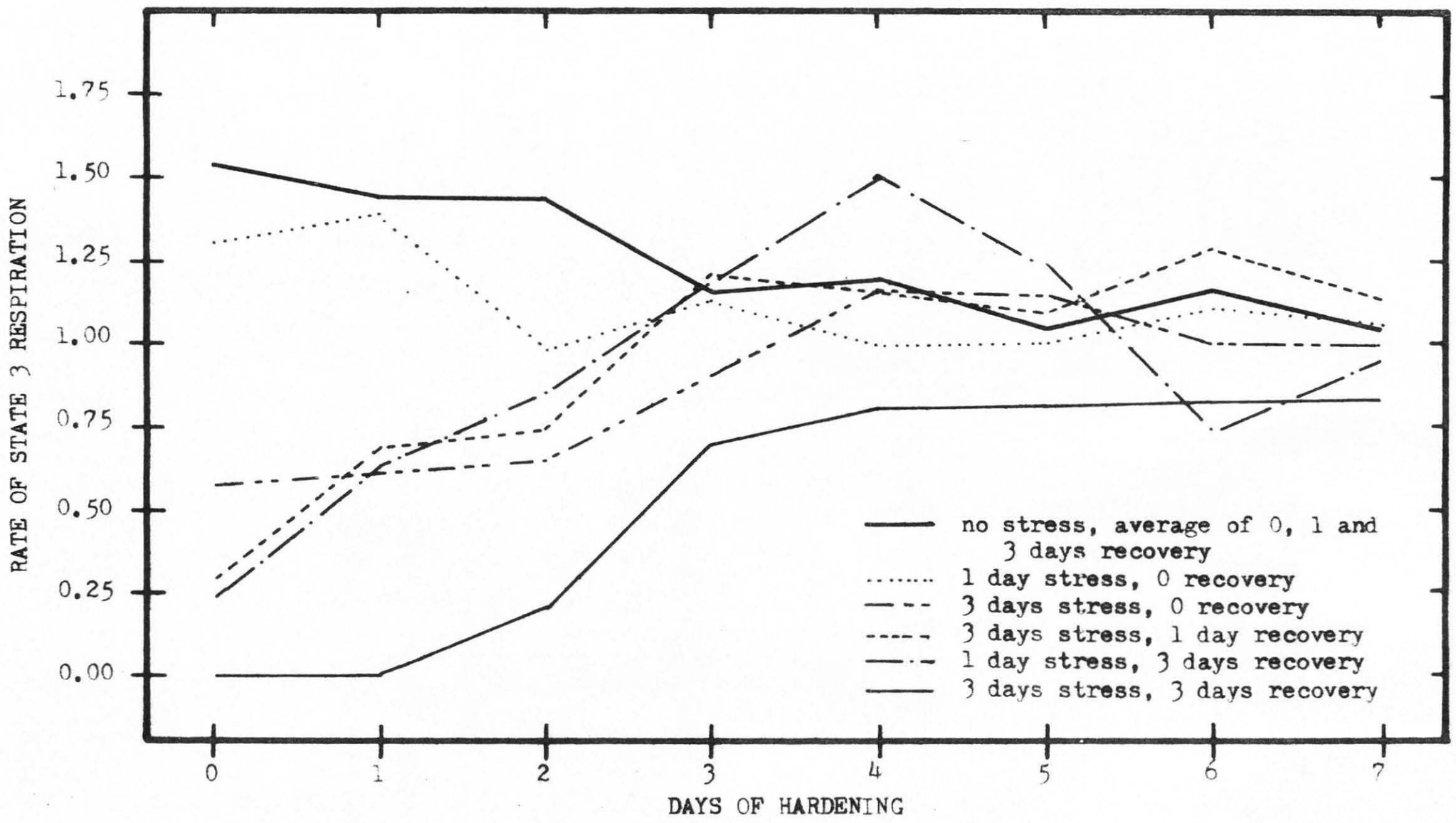


Figure 6. Rate of state 3 respiration for mitochondria from plants receiving varied combinations of stress and recovery following 0 to 7 days of hardening.

There was a decline in all values with increasing time of recovery. It was not expected that mitochondria would decline in activity, since Ikuma and Bonner (1967) had found no change in mitochongrial activity of bean hypocotyls from germination to 15 days of age. The decline shown in this experiment may have been due in part to increasing age of the plants, but most likely it was due to other factors.

It seems that some time is required for the mitochondria to break down after the plants are removed from the stress treatment. In fact, the break down of mitochondria observed in this experiment appears to be the result of other changes which take place within the cell during the stress period and not a direct effect of the stress upon the mitochondria. Unhardened plants, which had been stressed for 3 days, appeared soft and dead upon removal from the stress chamber, but still they exhibited fairly good mitochondrial activity. But after these plants sat in recovery for one day, the mitochondrial activity dropped significantly (Table 6), and after three days the mitochondria were totally inactive (Table 9).

The effect of recovery is easily seen when the raw averages are graphed (Figures 4, 5, and 6). Plants which received no recovery between the stress period and extraction of mitochondria consistently showed higher values in all parameters than did plants which sat in recovery for 3 days. In the case of ADP:O and RC ratios, 1 day of recovery produced values lying about mid-way between 0 and 3 days recovery (Figures 4 and 5) but in the case of state 3 respiration rate the 1 day recovery value was higher than the 0 day recovery value (Figure 6).

Another problem associated with recovery was the apparent invasion of dead or weakened plants by microorganisms. Fungus growth

was observed in some 3-day stress treatments where the plants were dead. In Table 8, which shows the interaction between stress and recovery, it can be seen that the significant difference was in the low averages from treatments receiving 1 or 3 days stress and 1 or 3 days recovery. It was in these treatments where death of plants occurred.

The relationship of hardening and stress

The central purpose of this research was to study the mitochondrial activity of plants subjected to various levels of cold temperature hardening followed by a period of cold temperature stress. It has already been shown that unhardened plants were killed by a period of stress, while hardened plants survived (Table 2, Figures 2 and 3). In the analysis of variance the interaction between hardening and stress treatments was significantly different for all three parameters measured (Tables 3, 4, and 5).

Table 7 shows the comparison of averages for hardening X stress interaction. Mitochondria from plants receiving no stress did not vary greatly over the range of hardening. There was a slight decrease in ADP:O, RC, and state 3 respiration values as hardening time increased, with the greatest decline in the state 3 value. With one day of stress the protective mechanism of hardening began to show up in the ADP:O and RC measurements where increased periods of hardening resulted in significantly higher values. After 3 days of stress the fully hardened plants exhibited values for all parameters 2.5 to 3 times higher than unhardened plants. The values increased daily from 0 to 4 days hardening (followed by 3 days stress) but then increased no further. Apparently the fullness of hardening protection

was reached after 4 days. This agrees with the earlier statement that plants were not killed or injured by stress following at least 5 days of hardening (Figure 2).

When raw averages of hardening and stress were plotted in graph form, the same relationships between hardening and stress were observed (Figures 4, 5, and 6). The ADP:O, RC, and state 3 respiration rate values were constant through the ranges of hardening from 0 to 7 days if no stress was given. One or 3 days of stress with no recovery produced values nearly equal to those of unstressed plants in the case of ADP:O and RC ratios but not in the case of state 3 rate of respiration (Figures 4, 5, and 6). Only after 3 days of recovery following the stress is there a significant difference between the values for stressed and unstressed plants. Again it becomes apparent that the change in mitochondria observed following stress was not an immediate effect of the stress but rather the result of other changes within the cell. When subjected to 3 days of stress and 3 days of recovery, plants which were unhardened or hardened for one day showed no activity in any of the three parameters. After 2 days of hardening the values began to rise, however, and continued to rise until the 5th day of hardening for ADP:O and RC and until the 4th day of hardening in the case of state 3 respiration. Beyond 5 days there was no increase of values with increased hardening (Figures 4, 5, and 6).

It is interesting to note that while the values for hardened then stressed plants reached a plateau after 5 days of hardening, this plateau was below the values for non-stressed plants in all cases except that of state 3 respiration rate (Figures 4, 5, and 6). The efficiency (ADP:O ratio) and degree of coupling (RC ratio) of

mitochondria from non-stressed plants, but there is no difference in respiration rate. This is not in agreement with the findings of Lyons and Raison (1970) who reported that phosphorylative efficiency of mitochondria from both cold-sensitive and cold-resistant species was not affected by low temperatures.

SUMMARY AND CONCLUSIONS

Hardening of plants

It was possible to harden plants so that they survived a period of cold temperature stress that killed unhardened plants. The fullness of hardening was achieved in 4 to 5 days at 2 C, as shown in tests with intact plants (Figures 2, 3, and Table 2) and also in isolated mitochondria (Figures 4, 5, and 6). Between the zero hardening where all plants were killed and the full hardening where all plants survived, there was a gradual steady change, also observed in both intact plants and isolated mitochondria.

Mitochondrial activity

It was shown that mitochondrial activity was destroyed when unhardened plants were stressed (killed). Mitochondrial activity increased gradually with increased hardening just as intact plants increased in resistance to stress (in ability to survive and grow). After 5 days of hardening the mitochondria from hardened and stressed plants reached a stable level of values that was lower than that of hardened unstressed plants.

Evidently there is some change in the mitochondria during the hardening period. This change corresponds to the hardening of intact plants. This change in the mitochondria may be the result of other cellular changes which preceed those of the mitochondria since unhardened stressed plants exhibited good mitochondrial activity immediately following the stress treatment but lost all mitochondrial

control if plants sat in recovery for 3 days. It may be that the mitochondrial change observed during hardening is also the result of other cellular changes and not a direct effect of the hardening treatment upon the mitochondria. The nature of the change which mitochondria undergo during hardening is not known. It may be a phase change in the mitochondrial membranes as reported by Richardson and Tappel (1962) for fish mitochondria and by Lyons and Raison (1970) for plant mitochondria. It may also be a complete destruction of the original mitochondria and production of new ones. The nature of the change would provide an interesting theme for further study.

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VITA

Bryce D. Bennett

Candidate for the Degree of

Doctor of Philosophy

Dissertation: Mitochondrial Activity of Hardened and Nonhardened Rye (Secale cereale) Plants Exposed to Freezing Temperatures.

Major Field: Botany

Biographical Information:

Personal Data: Born at Thatcher, Idaho, on August 1, 1937, a son of A. Dale and Farrel R. Bennett; married JoAnn Stucki, June 4, 1962; four children--Christina, Blake, Braun, and Cameron.

Education: Attended elementary school in Thatcher, Idaho; graduated from Grace High School in 1955; received the Bachelor of Science degree from Colorado State University, with a major in floriculture, in 1962; did graduate work in biology at California State Polytechnic College in 1969; received Master of Science degree, majoring in Plant Physiology, at Utah State University in 1971; completed requirements for the Doctor of Philosophy degree in Botany at Utah State University in 1973.

Professional Experience: 1971 to present, NDEA Fellow at Utah State University. 1969 to 1971, graduate research assistant and NDEA Fellow at Utah State University. The spring and summer of 1969, graduate student of biology at Cal. Poly. College in San Luis Obispo, California. 1965 to 1969, head grower and chief of quality control at 16-acre greenhouse range of Yoder Brothers Inc., Salinas, California; 1964-1965 assistant grower of pot plants at Rod McLellan's Acres of Orchids, South San Francisco, California. 1962-1964, served as an officer in the United States Army. 1960-1962 worked at research greenhouse at Colorado State University while attending that school. 1957-1960 served as missionary for the L.D.S. Church in New York State. 1956 received Alpha Leta award for outstanding scholarship at Utah State University. 1961 received a scholarship from Florists Telegraph Delivery. 1962 received superior ROTC Cadet Award at Colorado State University.