HETEROSIS IN WHEAT MITOCHONDRIA

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MASTER OF SCIENCE

by

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SUMMARY

This study investigates reports that heterosis may be detected in mitochondria from wheat (Sarkissian and Srivastava, 1969, 1970). It also examines the mechanisms of mitochondrial heterosis proposed by McDaniel and Sarkissian (1966, 1967a) - i.e. complementation of unlike parental mitochondria in hybrids and the existence of a new hybrid type of mitochondria.

Rapidly isolated wheat mitochondria were found to be contaminated with peroxisomes and microsomes but well coupled oxidation was observed with malate, KGA, succinate and NADH, although the respiratory control ratios were lower than those normally obtained with plant mitochondria. ATP-ase activity was responsible for the decreased control as demonstrated by the inhibition of state 4 by oligomycin and the gr dual acceleration of state 4 rates with successive cycles of phosphorylatio as well as the lessening of ADP:O ratios in later c cles. Two types of ATP-ase were identified, one oligomycin sensitive and the other not. The latter was thought to be a Ca^{++} or Mg^{++} stimulated ATP-ase contributed by microsomal contaminants.

No evidence was gained to support claims of abnormal ADP:O ratios in hybrid mitochondria although high first cycle values were irregularly observed with KGA as substrate. This was not restricted to hybrids and was KGA-specific so was considered an artifact of preparation.

Mitochondrial performance was variable because of the above, so that mitochondria from different cultivars showed no significant difference in performance. However the age of the coleoptiles at harvest was an

important determinant of subsequent mitochondrial ADP:O ratios. These wheat mitochondria are intact as their outer membrane was shown to be impermeable to cytochrome *c*. This has serious implications for testing enzyme systems which require bulk movement of exogenous cytochrome *c* to the inner membrane, whether it acts as an electron donor or acceptor.

Mitochondrial heterosis and complementation were studied in conjunction with seedling germination and tissue respiration trials for several hand-made wheat crosses. The relative expressions of hybrid vigour in each of these trials was compared to yield data provided by P. Wilson. No single estimate agreed consistently with the yield figures in all crosses. Mitochondrial malate oxidation, whole tissue respiration and seedling growth trials frequently agreed, but the predicted agreement of these with yield heterosis was not found. Mitochondrial KGA oxidation frequently agreed with yield heterosis thus conflicting with malate oxidation results.

The De Kalb cross 31MS by 28 which is reported to show strong heterosis and complementation in many different trials was found, here, to show slight positive seedling heterosis but strong negative tissue respiration heterosis. No significant enhancement of coupling could be shown to exist between the hybrid and its highest parent or between parental mixtures and the mid-parent values. Positive heterosis was recorded for NADH-cytochrome *c* reductase under all conditions and for malate and succinate cytochrome *c* reductase in swollen mitochondria.

Ascorbate/TMPD oxidation showed slightly negative heterosis but cytochrome *c* oxidase activity was strongly positive in intact mitochondria. These assays are governed more by the availability of cytochrome *c* than by the kinetic properties of the enzyme concerned.

No overall correlation between mitochondrial complementation and heterosis could be found by regression analysis, either for local cultivars or 31MS by 28. The use of physiological and biochemical assays to predict heterosis was concluded to be unreliable.

DECLARATION

The investigations described in this thesis were performed in the Botany Department, University of Adelaide, from September, 1972 to March, 1976.

To the author's belief and knowledge, this thesis contains no material previously submitted for a degree in any university, or published elsewhere, by the author or by any other person, except where due reference is made in the text.

R. L. BLACKWOOD

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Jai Guru Dev

ABBREVIATIONS

A	absorbance
ADP	adenosine diphosphate
AMP	adenosine monophosphate
ATP	adenosine triphosphate
ATP-ase	adenosine triphosphatase
BSA	bovine serum albumen
cyt.	cytochrome
CCCP	carbonyl cyanide m-chloro-phenylhydrazone
CoA	co-enzyme A
CoQ	co-enzyme Q
DNP	2, 4-dinitrophenol
DOC	deoxycholate
E_{mM}	extinction (mM)
EDTA	ethylene diaminetetra-acetate
EGTA	ethyleneglycolbis (amino ethyl) tetra acetate
Fp	flavoprotein
Fr. wt.	fresh weight
K_M	Michaelis constant
KGA	α-ketoglutarate
31MS	male sterile line 31
M_w	washed mitochondria
M.W.	molecular weight
NAD(H)	nicotinamide adenine dinucleotide (reduced)
OAA	oxaloacetate
osmosity	molar concentration of NaCl solution having the same freezing point or osmotic pressure as the given solution, g-mol/l
Pi	inorganic phosphate
RCR	respiratory control ratio

Abbreviations (cont'd)

r_s	Spearman's rank coefficient of concordance
TMPD	tetramethyl-p-phenylene diamine
TPP	thiamine pyrophosphate
Tris	tris (hydroxymethyl) aminomethane
W	Kendall's coefficient of concordance

CH. I. INTRODUCTION

PART A PROPERTIES OF MITOCHONDRIA

A mitochondrion is a subcellular organelle which is ubiquitous in eucaryotes. It has been identified as the sole site of aerobic oxidation of pyruvate with concomitant phosphorylation and thus is responsible for the production of most of the cellular energy in the form of ATP. Since ATP is required for all synthesis, its supply can be considered as one of the physical limits to growth. This has led to a study of the properties of mitochondria in parent and hybrid strains of several plant species in order to determine whether mitochondria alone can constitute a real difference between individuals of different genotypes.

Morphology of mitochondria

Mitochondria are often seen in transmission electron microscopy to be oval or circular vesicles with a diameter of 0.5-1 μ (Hanson et al. 1959). However a variety of shapes ranging from filamentous to cup-shaped or, more rarely, annular forms have been reported (Munn, 1974). In any one cell, mitochondria may be all one shape but there have been several reports of polymorphism within a particular cell (Hagopian, 1967).

Despite the variation in shape all mitochondria are bound by a double membrane. The outer membrane is a simple enclosing envelope, in which 25-30 Å pits have been described (Bonner, 1965; Parsons et al., 1965; Parsons, Williams and Chance, 1966). These pits are

only described for plant mitochondria. The inner membrane is highly convoluted into cristae which may be tubular or plate-like. Fernandez-Moran (1962) reported a series of projections or knobs on the inner surface of the cristae which have now been identified as the site of ATP-ase activity (Racker et al. 1965; Kagawa and Racker, 1966; Racker and Horstman, 1967). Although the organelles appear as separate entities there is evidence that they are not static phenomena in vivo and that rapid exchange of material may occur between the mitochondria and other cellular organelles, especially chloroplasts (Wildman et al., 1962). Chapman et al. (1975) and Gracen et al. (1972), have shown that chloroplasts of both C_4 and C_3 plants seasonally have a mobile peripheral reticulum which can enclose mitochondria. Exchange of material was postulated but has not yet been verified.

Hoffman and Avers (1973) have reported on serially sectioned cells in which the organelle was found to be one single giant network rather than discrete vesicles, and that it was sections through this network of tubules which gave circular profiles to the particles normally known as mitochondria. The branching structure was approximately 50-60 μm in length and 200-600 nm in width and was continuous between mother and budding daughter cells.

These observations have been extended to several algal genera (Atkinson et al., 1974) and to rat liver mitochondria (Brandt et al., 1974) where either a single giant mitochondria or several branched mitochondria have been observed, also by serial sectioning.

If this concept of a mitochondrion as a single reticulate system rather than a collection of discrete organelles were to be proved correct, it would have serious implications for many aspects of mitochondrial physiology and genetics and would necessitate a reappraisal of the methods of studying mitochondria as well as current concepts of their growth and development.

The outer surface and shape of mitochondria has been studied by freeze-fracture techniques and has tended to support the existence of a vesicular form in animal and higher plant tissue (Wrigglesworth et al., 1970).

The appearance of mitochondria which have been extracted from their cellular environment is often altered from their in vivo appearance and reflects their intactness and method of preparation. Penniston et al., (1968) and Hackenbrock (1966) described distinct conformational states for isolated mitochondria and correlated these with the physiological states defined by Chance and Williams (1956). The condensed state where the matrix space is reduced and the intermembrane space is expanded is found under phosphorylating conditions and changes to the orthodox configuration with the exhaustion of ADP and the cessation of phosphorylation. This latter state resembles mitochondria seen in whole tissue sections, having an expanded matrix and small intermembrane space. When mitochondria are first isolated they have the condensed configuration, due more to the osmotic effects of the hypertonic isolating medium than phosphorylation, with the expanded conformation occurring after a cycle

of phosphorylation initiated by ADP. Hackenbrock (1966) and Harris et al. (1968) claimed these conformational changes represented primary events in energy transduction. However it is now considered unlikely that these changes alone drive the formation of ATP (Deamer et al., 1967; Stoner and Sirak, 1969; Blair and Nunn, 1972).

The respiratory chain of animal mitochondria

The organization of the respiratory chain in mammalian mitochondria has been extensively studied and recently reviewed by the following: Klingenberg, 1968; Chance, Bonner and Storey, 1968; Van Dam and Meyer, 1971; Pullman and Schatz, 1967.

The arrangement presented by Lehninger (1970) shown in Fig. 1-1, summarises the present state of knowledge and expresses the consensus of opinion at this time. He places membrane-bound NAD, as the point of entry of reducing equivalents from soluble dehydrogenases, as the start of the chain. Associated with NAD is a reversible NADP-NAD transhydrogenase which can also contribute reducing equivalents from the NADP linked enzymes. The NAD is associated with flavoprotein and non-haem iron and sulphur complexes.

Electrons pass from this complex to ubiquinone which also receives reducing equivalents from succinate dehydrogenase and its associated non-haem iron and sulphur complex. Thus ubiquinone acts as a point of confluence of the NADH and succinate systems (Van Dam and Meyer, 1971) and is on the main pathway of electron transfer (Ernster et al., 1969; Klingenberg and Kroger, 1970). This was shown by Ernster et al. (1969)

who demonstrated that the ubiquinone depletion of particles completely inhibited the reduction of cytochrome *b* by NADH and caused a 75 percent inhibition of its reduction by succinate. The succinate powered reduction that did occur was no longer inhibited by malonate and could be considered as abnormal (Van Dam and Meyer, 1971).

The cytochrome chain after ubiquinone is arranged in the order *b*, c_1, *c* and the *a*, a_3 complex as the terminal enzyme. Several lines of evidence indicate there are two *b* cytochromes (Bryla et al., 1969 a, b; Berden and Slater, 1970; Wilson and Dutton, 1970; Chance et al., 1970). Slater concluded there may be a true complex composed of two molecules of cytochrome *b*, one of cytochrome *c*, and one antimycin binding site. This complex (complex III) has allosteric properties as shown by the sigmoidal binding kinetics of antimycin A (Berden and Slater, 1970).

From redox potential data, there are three sites where the free energy change between components is sufficient to allow energy conservation. These occur between NAD and cytochrome *b*, (site I) between the *b* and *c* type cytochromes (site II), and between *a* and a_3 components of cytochrome oxidase (site III). Thus NAD-linked substrates are capable of phosphorylating three ADP molecules per pair of reducing equivalents while succinate which donates reducing equivalents at a more positive redox potential (bypassing site I) can phosphorylate two ADP molecules per pair of reducing equivalents. Introduction of electrons directly to cytochrome *c* (as with ascorbate), can power the synthesis of one ATP per electron pair. Since one electron pair reduces one half of a molecule of oxygen

the ratio of the number of ADP molecules phosphorylated to the amount of oxygen taken up (the ADP:O ratio), becomes a convenient measure of the phosphorylation efficiency of the chain.

Thus it can be seen that each substrate has a maximum value for the ADP:O ratio assuming the chain is working at full efficiency. For NAD-linked substrates this value is 3, for succinate it is 2 and ascorbate 1. KGA, which is linked to a substrate level phosphorylation reaction in reduction to succinyl - CoA, has a maximum value of 4. The mechanism of phosphorylation will be considered in another section of the introduction.

The vectoral organization of the inner membrane

The chemiosmotic hypothesis of phosphorylation, to be discussed later, predicts that the inner membrane should have a definite vectoral organization. The components should be ordered so that proton carriers alternate with electron carriers, and they should be arranged across the membrane in such a way that protons are passed in a directional manner during respiration (Mitchell 1961, 1966). That such a sidedness exists has been demonstrated by many workers, in particular (Racker 1970; Racker et al., 1970; and Tyler 1970) and has been reviewed recently by Harman et al. (1974). Several techniques have been used to determine the location of individual members of the chain. These are the reaction of molecules known to be impermeable, e.g. ferricyanide, the binding of inhibitors and macromolecular probes to specific components, and histochemical staining combined with electron microscopic examination. Disruption

of mitochondria by sonic oscillation, detergent or enzyme action produces vesicles which have different orientations so that use of sub-mitochondrial particles allows examination of both surfaces of the cristae membrane. Sonic oscillation forms inside-out particles (Lee and Ernster, 1966; Mitchell, 1966) as determined by their inability to reduce ferricyanide (Harmon et al., 1974), their insensitivity to the antibody specific for cytochrome *c* (Racker et al., 1970) and their appearance in electron micrographs with the inner membrane spheres outwards (Löw and Vallin, 1963; Racker et al., 1965). Phospholipase C breaks up only the inner membrane forming a collection of small inverted vesicles inside an intact outer membrane as is illustrated by the addition of ferritin as a marker (Racker et al., 1970). Combining of such hydrolytic treatments with other techniques mentioned has provided most of the information about membrane topography.

Racker defines the faces of the membrane as the "M" or matrix side and the "C" side, so named because of the location of cytochrome *c* on this surface (Tyler, 1970). The components of the respiratory chain can be divided into four groups, the soluble matrix enzymes, those bound to the M side and those bound to the C side, and those inside the membrane (intra membrane). Fig. 1-2 lists the location of the major components.

The location of cytochrome *c* on the outer surface enables it to pass electrons to several artificial acceptors facilitating the study of partial reactions of the chain such as the reduction of exogenous

cytochrome *c* by respiratory substrates. The location of the substrate dehydrogenase on the M surface e.g. succinate dehydrogenase or in the matrix (Fessenden-Raden, 1970; Lee et al., 1969) e.g. malate dehydrogenase necessitates the transport of all substrates across the inner membrane, and in both animal and plant mitochondria substrate transporters have been reported, (Chappell 1968; Wiskich 1974). The exception to this is the reduction of exogenous NADH by plant mitochondria by means of the external NADH dehydrogenase which will be discussed later. The location of the ATP-ase on the M side, in the knob-like protrusions as mentioned previously, means that adenine nucleotides must be transported across the membrane and such a transport system, which is inhibited by atractyloside has been described (Pfaff et al., 1965; Souverijn et al., 1970; Klingenberg and Pfaff, 1966; Henderson and Lardy, 1970).

The respiratory chain of plant mitochondria

Neither the nature nor the order of the members of the plant mitochondrial respiratory chain has been fully interpreted at this stage, for, although the overall organization is along the same lines as has been described for animal mitochondria there are several significant differences, as detailed by Ikuma (1972). The cytochromes are characterized by their spectra, both at room temperature and at 77 K, by their oxidation kinetics and their oxidation reduction potentials. Using these three sources of data, measured for plant tissue (Chance et al., 1968 Chance and Bonner, 1965; Chance and Hackett, 1959; Bonner and Plesnicar, 1967; Bonner and Slater, 1970; Lance and Bonner, 1968;

Storey and Bahr, 1969a, b; Storey, 1969), Ikuma arranges the cytochromes in the following order:

$$b_{553} \rightarrow b_{557} \rightarrow c_{549} \rightarrow c_{547} \rightarrow a \rightarrow a_3.$$

However these are only tentative placements from conflicting data. The dehydrogenase complex also differs from that of animal tissue (Erecinska and Storey, 1970; Storey, 1970 ; Storey 1971) and the exact number and identity of the flavoproteins, as determined by their absorption and fluorescence properties, is at present unclear. The situation is further complicated in plants by the presence of an alternate pathway containing a flavoprotein and a postulated unknown oxidase X, (Storey and Bahr, 1969a, b; Ikuma, 1972) with oxygen as the final acceptor. Since cytochrome oxidase is not involved, such electron transfer is insensitive to azide, cyanide or CO and is thus termed the "cyanide insensitive pathway". However there are substances which will inhibit, specifically this alternative oxidation, e.g. hydroxamates or KSCN. This pathway is a non-phosphorylating pathway and its operation has the effect of reducing the overall phosphorylation efficiency so that ADP:O ratios measured for these mitochondria are the maximum values obtained with animal mitochondria. Evidence for such a pathway comes from work with antimycin A (Wakiyama and Ogura, 1970) which partially inhibits oxygen uptake with NAD-linked substrates. The alternative oxidase accepts electrons before ubiquinone, agreeing with the proposal of Storey and Bahr (1969a, b).

Ikuma (1972) suggests that such a non-phosphorylating pathway is

needed by rapidly respiring tissue to increase the metabolic flux of the cells especially glycolysis and the Krebs cycle, and that its activities are controlled by the levels of adenylate. Ikuma (1972) proposes that it is not found in storage tissue but is induced in this tissue on ageing to produce two populations of mitochondria, heavy and light, the former containing the ectra components of the cyanide insensitive chain and the latter without them, being completely cyanide sensitive. This presupposes that mitochondria can be synthesized de novo in ageing tissue slices.

It is well established that the outer membrane also has the capacity for electron transport (Douce et al., 1973; Moreau and Lance, 1972; Wilson and Hanson 1969; Wiskich et al., 1960) by means of a flavoprotein and cytochrome b_{555}, which can reduce exogenous cytochrome *c*. This is similar to the outer membrane chain found in animal mitochondria (Schnaitman et al., 1967; Sottocasa et al., 1967) and the microsomal chain reported by Rungie and Wiskich (1972).

Plant mitochondria are unique in their ability to oxidize externally added NADH. Several reports (Von Jagow and Klingenberg, 1970; Palmer and Passam, 1970; Douce et al., 1973; Day and Wiskich, 1974) suggest that mitochondria have a second NADH-dehydrogenase located on the external side of the inner membrane which is linked with the respiratory chain below site one. Thus plant mitochondria oxidize NADH in a rotenone insensitive but antimycin A sensitive manner giving an ADP:O ratio of two (Cunningham, 1964; Ikuma and Bonner, 1967; Wilson

and Hanson, 1969; Palmer and Passam, 1970; Wakiyama and Ogura, 1970; Coleman and Palmer, 1971; Douce et al., 1973; Day and Wiskich, 1974). There is interaction between the inner and outer chains as is illustrated by the relief by cytochrome *c* of antimycin A inhibition of NADH oxidation (Day and Wiskich, 1974; Douce et al., 1973; Moreau and Lance, 1972). In intact mitochondria this is only a partial relief due to the impermeability of the outer membrane to cytochrome *c* (Wojtczak and Zaluska, 1969; Wojtczak and Sottocasa, 1972) and may be a reflection of the proportion of broken outer membranes in a preparation (Day and Wiskich, 1974). Where reduced cytochrome *c* can penetrate to the intermembrane space it can reduce cytochrome oxidase in an antimycin in a sensitive manner.

Inhibitors of respiration

Some of the most useful tools for studying the respiratory chain are specific inhibitors of electron transport, where the sites of action are known. The sites of action of various inhibitors and uncouplers have been reviewed recently, (Lardy and Fergusson, 1969; Ernster and Lee, 1964). These inhibitors divide the chain into segments which can be studied individually thus helping to elucidate the sequence of components. Although there is a wide range of inhibitors it appears that their activity is restricted to three sites, which may be the same as the phosphorylation sites (Klingenberg, 1970), and which are included in Fig. 1-1 In animal mitochondria the most potent inhibitor is rotenone (Lindahl and Oberg, 1961; Wakiyama and Ogura, 1970) which acts at site I (Chance et al., 1967; Chance and Hollunger, 1963).

It completely inhibits electron transport from NAD-linked substrates when added at 25 nmoles per gram protein (Ernster et al., 1963), by binding irreversibly to the FMN component of the NADH dehydrogenase (Pharo and Sanadi, 1964; Lindahl and Oberg, 1961). Amytal, another site I inhibitor, is less specific and less potent in its activity (Ernster et al., 1955; Chance, 1956).

The second group of inhibitors act on the oxygen side of ubiquinone and the substrate side of cytochrome *c*, between the *b* and *c* type cytochromes, at site II. The most important in this group is antimycin A first reported for animal mitochondria by van Potter and Reif (1952) and for plant mitochondria by Martin and Morton (1957). It appears to bind to one of the *b* cytochromes, exhibiting sigmoidal binding kinetics (Van Dam and Meyer, 1971). Thus this inhibitor is effective against all NAD and flavin linked substrates.

The third class of respiratory inhibitors are the classical respiratory poisons, KCN, CO and azide which all bind to the cytochrome oxidase complex preventing oxygen uptake (Warburg, 1946; Keilin, 1929).

Inhibitors and uncouplers of oxidative phosphorylation

Another class of inhibitors acts on the phosphorylation process rather than on the redox reactions of the chain itself. The most important of these is the antibiotic oligomycin which was introduced by Lardy et al., (1958). It is capable of inhibiting substrate reduction or oxygen uptake where mitochondria are tightly coupled (Lardy and McMurray, 1958) and the inhibition of oxidation is released by uncouplers such as

DNP and CCCP. From a study of the partial reactions of phosphorylation Lardy and Connelly (1961) suggested that oligomycin acted near the site of phosphate incorporation. Fractionation studies (Racker, 1970) have shown that a specific membrane protein is needed to confer oligomycin sensitivity to mitochondrial ATP-ase (F_1) preparations. This fraction has been termed OSCP or CF_0 by workers in this field and has been shown to be part of the stalk region of the ATP-ase knob.

Respiration can also be inhibited by blocking the substrate, phosphate or adenine nucleotide carriers with specific inhibitors e.g. mersalyl and atractylate or by direct competition by inhibitors for the substrate dehydrogenases, e.g. malonate inhibition of succinate (Quastel and Whetham, 1925). However as these inhibitors are indirect they will not be discussed here.

Uncouplers of oxidative phosphorylation such as 50 μM DNP and 0.5 μM CCCP cause a rapid increase in the rate of substrate utilization or oxygen uptake with a simultaneous cessation of phosphorylation. This is a result of freeing the electron transfer process of the control exterted by phosphorylation, the mechanism of which was considered in the section on oxidative phosphorylation.

Oxidative phosphorylation

One of the distinctive properties of the respiratory process and oxidative phosphorylation is that it is very precisely regulated by the availability of ADP. It is this which enabled Chance and Williams (1956) to define

steady states of respiratory activity, recognized on the basis of availability of oxygen, of substrate, of ADP and of phosphate. Each state is characterized by the particular redox states of the carriers and the rates of oxygen consumption measured polarographically (Chance and Williams, 1956). A hypothetical oxygen electrode trace showing the states of respiration is shown in Fig. 1-3. In the terminology of Chance and Williams (1956) five states are recognized. The two important states involved in evaluating mitochondrial quality are states three and four.

In state 3 oxygen is non-limiting, substrate, ADP and phosphate levels are high and the requirements for oxidative phosphorylation are met. Respiration occurs at a rapid rate which is limited by the capacity of the respiratory chain and the rate of substrate and nucleotide transport across the membrane. State 4 is reached when all the ADP present has been phosphorylated to ATP and ADP becomes limiting. The degree of ADP control over respiration was suggested as the best criterion for intactness of isolated mitochondria (Chance and Williams, 1956). It is measured as the respiratory control ratio (RCR) which is obtained by dividing the state 3 rate by the state 4 rate. For respiratory control to be demonstrated it is necessary to show that repeated cycles of state 3 - state 4 transitions can be obtained. Uncouplers break this control and cause oxidation to proceed at a rapid pace while phosphorylation is prevented.

The calculation of true control ratios and ADP:O ratios requires

a sharp transition from state 3 to state 4 at the point of ADP exhaustion. Lack of a sharp transition probably indicates that ADP is being recycled as a result of ATP-ase activity (either mitochondrial or extra mitochondrial ATP-ase). The rate of hydrolysis may be considerably less than the rate of synthesis so such ATP-ase activity may only provide a slight increase in the state 4 rate initially. However with successive additions of ADP initiating phosphorylation cycles the amount of phosphorylated adenine nucleotide in the system increases and the ATP-ase activity can supply ADP at an increasing rate. Thus the typical pattern of respiration in preparations with some ATP-ase activity is that the state 4 rates become faster with each cycle and the ratio of state 3 to state 4 becomes correspondingly less (Wiskich *et al.*, 1964). Under these conditions the first cycle of phosphorylation is the most indicative of actual mitochondrial performance.

The mechanism by which the processe of energy transduction is linked to energy conservation is still unresolved. There are two major hypotheses purporting to explain this phenomenon; the chemical hypothesis first proposed by Slater in 1953, and the chemiosmotic hypothesis put forward by Mitchell in 1961. The chemical hypothesis has been formed by analogy with substrate level phosphorylation, and proposes an energy rich molecular intermediate common to the oxido-reduction chain and the ATP synthesizing system. The formation of the high energy intermediate can be represented as

$$AH_2 + B + C = A \sim C + BH_2$$

where A and B are members of the respiratory chain either of which could be reduced during transfer. The high energy compound A ~ C is postulated to form prior to the entry of phosphate (Slater, 1953; Lehninger, 1965) as phosphate was shown not to be necessary for DNP action. A second high energy intermediate C ~ D was postulated when studies with arsenite showed that A ~ C does not react directly with phosphate (Eastabrook, 1961; Huijing and Slater, 1961). The series of oxido-reductions shown in Fig. 1-4 are necessary to account for the action of oligomycin and uncouplers. The chemical hypothesis has been extensively reviewed (Ernster and Lee, 1964; Sanadi, 1965; Racker, 1965; Slater, 1958; Lehninger, 1962; Lehninger and Wadkins, 1962).

The chemiosmotic hypothesis proposes that the primary energy conservation event is separation of charges across the inner membrane. This builds up an electrochemical gradient which becomes an energy store (Mitchell, 1961). For this to be possible Mitchell and Moyle (1967) stipulate the following as necessary conditions: a proton translocating respiratory chain, a reversible proton translocating ATP-ase, and an exchange - diffusion system coupling proton translocation to that of anions and cations. All these components must reside in an ion impermeable coupling membrane. Fig. 1-5 is a schematic representation of this hypothesis.

The respiratory chain is seen to operate anisotropically with proton carriers alternating with electron carriers so that as electrons move down the chain, protons are passed unidirectionally across the membrane.

(In mitochondria this movement is to the outside of the cristae membrane). Once established the gradient or poroton motive force (PMF) exerts a back pressure on proton movement. Thus respiration is limited so that proton translocation due to oxido-reduction is equal to the return flow of protons through exchange-diffusion systems. The ATP-ase utilizes the PMF to drive ATP synthesis. The condensation reaction moves negative charge out or positive charge in, so that under conditions of phosphorylation the oxido-reductase system is stimulated by removal of the back pressure or control. The proton translocating properties of the ATP-ase mean that hydrolysis of ATP will also cause a build up of PMF which will prevent large scale breakdown of ATP.

The supporting evidence for this hypothesis will not be discussed here as it has been extensively reviewed (Slater, 1966; Schatz, 1967; Pullman and Schatz, 1967; Greville, 1969; Racker, 1970; Boyer, 1968). However certain phenomena are explained by quite different mechanisms under the two hypotheses and the implications of these will be discussed.

The action of uncouplers, under the chemical hypothesis is attributed to the dissipation of the energy rich bond A ~ C. Known uncouplers form a chemically diverse group yet it is claimed that they are all capable of acting on this bond in the same way. The chemiosmotic hypothesis proposes that uncouplers act by destroying the PMF by neutralizing charge differences across the membrane. Uncouplers such as DNP and FCCP are all lipid soluble in their protonated form and can

enter the matrix and accumulate under the influence of the proton gradient. Outward movement of the uncoupler occurs through its anionic form and it is the rate of transit of negative charge out which Mitchell says determines the potency of the uncoupler (Greville, 1969). With the back pressure removed electron transport proceeds very rapidly but phosphorylation cannot occur. Any chemical which can allow the back flow of protons across the cristae membrane can thus be an uncoupler.

There have been occasional reports in the literature of ADP:O ratios in excess of the theoretical maxima (Smith and Hanson, 1964; Gurban and Cristea, 1965; Lynn and Brown, 1965; Wand and Bacigalupo, 1965; Sarkissian and McDaniel, 1969; Sarkissian and Srivastava, 1970). Although many of these have been shown to be due to error in estimation of oxygen consumption (Lenaz and Beyer, 1965; Haslam, 1965), others are still unexplained and have been the basis of claims of supermaximal efficiency in hybrid tissues. The chemical hypothesis cannot accommodate more than the theoretical number of phosphorylations per molecule of oxygen consumed without proposing more sites of phosphorylation. As the respiratory chain organization appears to be constant this is unlikely to occur. A more flexible approach is possible with the chemiosmotic hypothesis. Fig. 1-6 illustrates alternative models, (Mitchell's ATP-ase I and ATP-ase II) where either one or two protons are translocated per ADP phosphorylated. The latter model was initially favoured by Mitchell as it conformed with empirical measurement of stoichiometry of $2H^+/ATP$

(Mitchell and Moyle, 1965a, 1968). ATP-ase I has been used more recently by Mitchell (1969) to account for adenine nucleotide transport in conjunction with ATP-ase activity. It also provides a mechanism whereby ADP:O ratios of 6 for NAD-linked and 4 for succinate can occur.

The chemiosmotic hypothesis is taken as the conceptual model for discussion in this thesis.

Preparation of intact mitochondria

The preparation of mitochondria from higher plants is inherently more difficult than their preparation from animal tissue. The presence of an acid vacuole and a tough cell wall, together with the low yield normally obtained from plant material, necessitates the use of large quantities of plant tissue and buffered extraction media . A further problem is the presence in the cytoplasm and vacuole of many inhibitory or potentially inhibitory substances (Lieberman, 1961; Jones and Hulme, 1961; Hulme and Jones, 1964). These are released or formed on disruption and act by physical adsorption, chemical interaction or denaturation (Lieberman and Baker, 1965). Harsh methods of tissue disruption and an extended preparation time further complicate the procedure. The variety of tissue types has led to a proliferation of extraction methods, so that there is no established method and many new methods have been published (Hobson, 1971; Ikuma,1970; Killion et al., 1968; Ku et al., 1968; Malhotra and Spencer, 1971; Matlib et al., 1971; Palmer, 1967; Raison and Lyons, 1970; Romani et al., 1969; Sarkissian and Srivastava,

1968).

The method of isolation of plant mitochondria in general consists of tissue disruption in an appropriate medium, isolation of the mitochondrial fraction from filtered tissue homogenate by means of differential centrifugation and final washing of the resultant pellet. However a detailed comparison of the individual methods reveals wide variety in the means of disruption, in the media and in the centrifugation procedure.

There are many aspects of preparation which affect the activity of mitochondria as assessed by respiratory control and ADP:O ratios. These are the method of disruption (Ku et al., 1968; Romani et al., 1969; Haard and Hultin, 1968), the composition of the extraction medium (Sarkissian and Srivastava, 1968), the pH of the extraction medium (Ikuma, 1970; Verleur, 1965; Watson and Smith, 1967; Romani et al., 1969), the type of buffer (Ikuma, 1970; Stinson and Spencer, 1967), the centrifugal force (Ikuma and Bonner, 1967; Verleur, 1965; Palmer, 1967; Sarkissian and Srivastava, 1968), the presence of BSA in the extraction medium (Verleur and Uritani, 1965; Sactor et al., 1958; Helinski and Cooper, 1960; Dalgarno and Birt, 1963; Chrispeels and Simon, 1964; Lance et al., 1965; Kobaysshi, 1965). In addition the type and age of tissue appears to be important in tissue which is rapidly growing or from an organ which goes through rapid maturity and senescence, e.g. cotyledonary tissue (Sarkissian and Srivastava, 1968; Beevers and Walker, 1956; Hanson et al., 1965; Matlib et al., 1971; Ikuma and Bonner,

1967) however found that the age of the tissue had no effect with mung bean and broad bean hypocotyl preparations.

The nature of the osmoticum (mannitol or sucrose), the presence of metal ions particularly Mg^{2+} (Matlib et al., 1971; Ikuma, 1970) and the nature of the substrate during assay also affect the respiratory control and ADP:O ratios.

Procedures designed to counteract these difficulties are centred on the initial steps of tissue disruption and on the extraction medium. It is important that plant cells should be broken gently to release organelles so that these are not themselves destroyed by the disruption procedure (Bonner, 1965, 1967; Earnshaw and Truelove, 1968; Hanson et al., 1968; Ikuma and Bonner 1967; Kenefick and Hanson, 1966; Lance and Bonner, 1968; Sarkissian and Srivastava, 1968; Matlib et al., 1971) produced good mitochondria from hypocotyl and shoot material using a mortar and pestle, and Ikuma (1970) found that regrinding the material improved the yield by 50 percent. Mechanical grinding is more rapid and has been successfully used for the more bulky material of storage tissue and some fruits (Wiskich & Bonner, 1963; Hawker and Laties, 1963; Verleur, 1965; Verleur and Uritani, 1965; Yoshida and Saito, 1968).

The grinding medium should be hypertonic to the cell sap to bring about shrinkage of the vesicles giving them greater stability. Either sucrose or mannitol can be used. Bonner (1967) claimed that the latter prevented the leaching of phosphate and is recommended by recent workers for wheat tissue, (Sarkissian and Srivastava, 1968;

Pomeroy, 1974). Sucrose is now widely used. To counteract the endogenous inhibitors, several additions have been made to the extraction medium. Chelating agents, (Lieberman and Biale, 1965; Tager, 1954; Honda et al., 1958) especially EDTA have been used to bind heavy metals and to prevent swelling (Verleur, 1965). Replacement of EDTA by EGTA was suggested by Sage and Hobson (1973) to prevent damage from calcium uptake and used by Ellis et al. (1973) where its use showed little improvement. Matlib et al. (1971) showed that excess Mg^{2+} stimulated ATP-ase activity and seriously reduced coupling and suggested that EDTA is preferable to EGTA as the latter is a Ca^{2+} specific chelator.

The beneficial effects resulting from the inclusion of BSA in the isolating medium are well documented. Weinbach and Garbus (1965, 1966) have shown that uncoupling by substituted phenols can be completely overcome with BSA. It is now widely accepted that BSA exerts its beneficial effects by binding endogenous uncouplers such as fatty acids implicated by Dalgarno and Birt (1963) in carrot preparations. Lehninger and Remmert (1959) and Hulsmann et al., (1960) have also shown that fatty acids uncouple oxidative phosphorylation.

Reducing agents, especially cysteine have often been added to the isolation medium to protect mitochondria from the action of polyphenoloxidase and phenolic compounds (Hulme et al., 1964; Verleur, 1965). However opinion is divided on the use of cysteine, many workers (Bonner, 1965; Bonner, 1967; Dawson et al., 1968; Dickinson et al., 1967; Drury and McCollum, 1968; Drury et al., 1968; Hawker and Laties, 1963; Hobson

et al., 1966a, b; Ikuma and Bonner, 1967a, b; Killion et al., 1968; Ku et al., 1968; Lance and Bonner, 1968; Lance et al., 1965; Verleur, 1965; Verleur and Uritani, 1965) favour the use of this reductant, but several other workers do not use it and obtain good mitochondria (Earnshaw and Truelove, 1968; Hanson et al., 1968; Kenefick and Hanson, 1966; Sarkissian and Srivastava, 1968; Yoshida and Saito, 1968). Ikuma (1970) in an attempt to clarify this discrepancy compared preparations made with and without cysteine and reported no difference. He suggests that its concentration be kept low if used (0-0.5 mM) and be excluded from the washing media as the oxidized product has been shown to inhibit cytochrome oxidase (Wiskich and Morton, 1960; Hulme et al., 1964; Verleur, 1965).

The preferred pH of isolation media is in the range 7.2 to 7.5. Alkalinity in excess of pH 8.0 is detrimental to mitochondrial performance. The need to keep the pH above 7.2 during isolation has been stressed by many workers (Wiskich, 1967; Pomeroy, 1974; Ikuma, 1970), while the optimum pH for assay is 7.2 (Ikuma, 1970).

A conventional preparation normally takes an hour to an hour and a half to complete, during which time the mitochondria are exposed to the endogenous uncouplers, centrifugal forces and changes in media which could lead to disruption of the vesicles. Palmer (1967) and Sarkissian and Srivastava (1968) have developed techniques for rapid isolation of plant mitochondria which gave improved performance and allowed many more extractions to be carried out on a daily basis. These procedures have been adapted and refined by Matlib et al. (1971), Pomeroy (1974), Sage

and Hobson (1973) and Ellis et al. (1973) and it is thought that the advantages of rapid removal of the mitochondria from their cellular environment outweighs possible damage from the high centrifugal forces they experience. Palmer (1967) employed a single 1.5 minute high speed spin (40,000 g) while Sarkissian and Srivastava (1968, 1969a) employ a similar technique with an additional centrifuge run at 20,000 g for two minutes without disturbing the pellet. Matlib et al (1971) included a further wash step to ensure complete removal of the endogenous uncouplers which delayed mitochondrial ageing for up to 4 hours. Pomeroy prepared tightly coupled wheat mitochondria by combining the conventional and rapid methods.

Precipitation of mitochondria at high speed increased the contamination of the pellet by starch and other organelles. As contamination from either of these sources can severely reduce mitochondrial performance (Douce et al., 1972; Ikuma, 1970; Sarkissian and Srivastava, 1969a: Ikuma and Bonner, 1967), steps must be taken to minimise this problem. Good separation from starch has been reported by Matlib et al (1971), and Sage and Hobson (1973) who changed the orientation of the centrifuge tube as recommended by Sarkissian and Srivastava (1969a).

A conventionally prepared mitochondrial pellet has been shown to be contaminated with cellular debris by electron microscope studies (Hanson et al., 1959). Douce et al. (1972) proposed that preparations from a variety of tissues were contaminated by microsomes, vacuolar membrane fragments and etioplasts. The crude mitochondrial pellet

can be fractionated by means of sucrose density gradient centrifugation and in this way isolated mitochondria of high purity and activity have been obtained (Douce et al., 1972; Hanson et al., 1959). As these measures were not taken in studies using rapidly prepared wheat mitochondria (Sarkissian and Srivastava, 1968) the pellets used contain varying proportions of non-mitochondrial protein. The extent and nature of the contamination in such pellets will be considered in Chapter IIIA.

Mitochondrial biogenesis

The biogenesis of mitochondria in plant cells is pertinent to any study linking hybrid vigour and mitochondrial performance. Explanations of heterosis, as proposed by other workers and detailed in section B of this introduction, depend upon different concepts of mitochondrial inheritance. In order to hypothesise on the mechanism of heterosis, it is necessary to know whether mitochondria are inherited cytoplasmically or by a process controlled by the nuclear genome.

There are three main hypotheses proposed:- firstly, that new mitochondria are formed by addition of proteins and phospholipids and other components into pre-existing mitochondria which multiply by division. Secondly, mitochondria may be synthesized de novo on the cytoplasmic ribosomes under nuclear control. Thirdly, they may be derived from other precursor structures such as the nuclear membrane (Brandt and Pappas, 1959; Hoffman and Grigg, 1958), the cell membrane (Robertson, 1959), the endoplasmic reticulum (de Robertis, 1954), or

the golgi complex (Lever, 1956).

The reviews of Gibor and Granick (1964), Work et al. (1968), Wagner (1969) and Ashwell and Work (1970) focused attention on the presence in mitochondria of nucleic acids and ribosomes capable of protein synthesis (McLean et al., 1958). Amino acid incorporation was shown to be sensitive to actinomycin D which indicated that RNA is being transcribed from a DNA template (Kroon, 1959) although the apparatus resembles the procaryotic system having 70 s not 80 s ribosomes (Wintersberger, 1965). The mitochondrial DNA and protein synthesizing system appears to be separate from the nuclear system, on the following criteria. They are physically separated, they do not have any components in common, they have no base sequences in common (Gross and Rabinowitz , 1968; Borst, 1972). There is no evidence also that m RNA transcribed in one system is translated in the other (Borst, 1972; Schatz and Mason, 1974).

The existence of two seemingly independent genetic systems has supported the hypothesis that mitochondria are cytoplasmically inherited and that they can code for their own proteins independent of the nuclear genome.

The first quantitative estimates of DNA present were thought to provide sufficient nucleotides to code for all the mitochondrial components using a triplet code (Lindberg and Ernster, 1954). The actual size of the DNA molecules has been determined more accurately by electron microscopy and renaturing data and animal mitochondria have

been found to contain 5 μ diameter circular DNA molecules with a molecular weight of 10 X 10^6 daltons (Nass, 1969). Plants have linear DNA which is in longer 10-20 μ strands with M.W of 119 X 10^6 daltons (Wolstenholme and Gross, 1968; Mukulska et al., 1970). There are several strands of DNA per mitochondrion, from 4 - 6 in vertebrates, more in yeast, (Borst et al., 1967). However it is generally assumed that all the strands are identical, so there are few actual genes present and that there is considerably less information present than is necessary for coding for a complete mitochondrion. The first estimates of the quantity of nucleic acids present were thought to be sufficient.

To support the first hypothesis the mitochondria must be shown to be self-duplicating bodies that do not arise de novo and results from work with mutant cells of yeast and fungus have been used for this purpose. The "petite" mutant of Saccharomyces cereviseae (Ephrussi, 1953) and the "pokey" mutant of Neurospora crassa (Mitchell and Mitchell, 1952) are slow growing mutant strains with altered mitochondria with part or all of the mitochondrial DNA missing (Mounolou et al., 1966; Mehrotra and Mahler, 1968; Nagley and Linnane, 1970) which results in changes in cytochrome oxidase, cytochrome *b* and ATP-ase properties (Kovác and Weissova, 1968; Schatz, 1968; Work et al., 1968). The mitochondrial mutants are inherited only through the female parent (Wagner, 1969; Ephrussi, 1953) as mitochondria are not passed on by the male gamete. Thus nuclear fusion does not repair the mutant mitochondria

or provide intact mitochondria through de novo synthesis. However there is nothing to say that the mutation is at the level of the mitochondria and not the nucleus. Recent evidence that mitochondrial DNA is synthesized by the cytoplasmic ribosomes under nuclear control (Schatz and Mason, 1974; Scragg, 1971; Parisi and Cella, 1971; Richter, 1971) weakens the case for independent cytoplasmic mutations.

Luck (1963) used autoradiographic techniques to illustrate that mitochondria of Neurospora crassa grow by addition of material into pre-existing structures and divide by binary fission. He labelled the mitochondria of a choline deficient mutant with ^{14}C choline (which is incorporated into lecithin, an important componant of the phospholipid membrane) and observed the fate of the label in cells in a non-labelled medium over 24 days during the logarithmic growth phase. He found that activity decreased uniformly throughout the mitochondrial population with the lable distribution per mitochondrion decreasing in a manner which suggested replication by division. These experiments, however, assumed that mitochondria divide equally whereas there is evidence to show that small pieces bud off mature organelles (Hoffman and Avers, 1973) in a manner which would be consistent with a more conservative distribution of any label.

If mitochondria cannot be synthesized de novo then continuity must be maintained, thus in structures lacking mitochondria, pro-mitochondria bodies should be evident.

The search for mitochondrial precursors has been carried out in particular in facultative aerobes, e.g. S. cereviseae where anaerobic

cultures have been exposed to air and the subsequent development of mitochondria has been followed with electron microscopy, through the increase in oxidative activity and the accumulation of cytochromes (Wallace and Linnane, 1964; Linnane et al., 1962; Plattner and Schatz, 1969). It has been proposed that a mitochondrial precursor structure exists (Wallace and Linnane, 1964; Schatz, 1963; Schatz, 1965; Plattner and Schatz, 1969; Breidenbach et al., 1967) and that these precursors have succinic dehydrogenase but no cytochrome oxidase activity. The introduction of oxygen induces rapid synthesis of cytochrome a, a_3 to form fully functional mitochondria (Breidenbach et al., 1967; Linnane et al., 1962).

In higher plants the evidence for precursors has been sought from studies of germinating seedlings and cotyledonary tissue where very rapid mitochondrial generation is occurring. Solomos et al. (1972) after studying pea cotyledons, concluded that mitochondria must already be present in the ungerminated seed as they are detected after only three hours of imbition and pro-mitochondrial structures were not detected. However the mitochondria were not fully developed as they underwent significant changes during the first four days of germination. The specific activity of succinic dehydrogenase and succinoxidase increased during that time, with little change in the total protein, indicating that the changes are due to structural rearrangement rather than protein synthesis. However there was no protein inhibitor studies done to confirm this interpretation. Changes in the properties of mitochondria

in germinating tissue have been reported by other workers (Akazawa and Beevers, 1957; Simon and Chapman, 1961; Bain and Mercer, 1966) and these studies favour the interpretation that mitochondria are developed through the improvement of pre-existing mitochondria rather than de novo synthesis.

The hypothesis of de novo synthesis was proposed and supported by early workers such as Wagner (1927) who worked with pollen grains and also Harvey (1946), Gustafsen and Lenicque (1952), Gustafsen (1954) and Harvey (1953) on the grounds of their research on sea urchin eggs. Harvey (1953) fractionated eggs by centrifugation into parts containing the nucleus, mitochondria, fat particles and pigments. Despite little apparent transfer of mitochondria from the male gamete on fertilization as determined by vital staining, the different mitochondria-less fractions all divided and differentiated. Thus the blastula stages had developed mitochondria in a manner similar to the whole egg. The mitochondria were said to arise de novo under nuclear direction. This work was criticized by Lansing (1953), who on studying the polarized egg found two layers of mitochondria, one in the clear or nuclear portion of the egg, not previously taken into account.

The current work as reviewed by Schatz and Mason (1974), Green and Silman (1967), Pullman and Schatz (1967) and the work of Leenders et al. (1974), Tzagoloff et al. (1973) strongly suggests that mitochondria are formed as a result of the interaction of the nuclear and mitochondrial genomes and that the bulk of mitochondrial protein components are synthesized

by the cytoplasmic ribosomes. The exceptions to this are the three large subunits of cytochrome oxidase and parts of the mitochondrial ATP-ase molecule which are synthesized on the mitochondrial ribosomes and coded by the mitochondrial nucleic acids. Schatz and Mason (1974) suggest that the two ribosomal systems are closely co-ordinated and that the results of experiments with respiratory deficient mutants show that production of protein subunits depends on their ability to be assembled into the complete molecule. Cytoplasmic synthesis will stop if the mitochondrial system is blocked. This research depends heavily on the use of bacterial antibiotics such as chloramphenicol and erythromycin which specifically inhibit mitochondrial protein synthesis (Linnane et al., 1968; Wilkie, 1970; Grivell et al., 1971; Mahler and Perlman, 1971) in conjunction with cycloheximide a specific inhibitor for the cytoplasmic ribosomal system (Nawa and Asahi, 1973; Von Reucker et al., 1974; Mason and Schatz, 1973). When antibiotic studies are combined with radioactive tracer techniques (Kadenback, 1968, 1969; Kadenback and Hadvary, 1973; Nawa and Asahi, 1973) a picture of the site of synthesis of the individual components of mitochondria can be obtained. It is still not known how these components are assembled, whether they are incorporated into pre-existing structures or formed into new mitochondria. Since the soluble enzymes such as malate dehydrogenase are synthesized externally on the endoplasmic reticulum (Bingham and Campbell, 1972; Roodyn et al., 1962; Roodyn and Wilkie, 1968) the mechanism for including them inside an impermeable vesicle is unknown.

The mechanism for communication between the two genomes is also unknown.

In the light of this new evidence, the derivation of mitochondria from other already specialized membranes seems less credible. These claims were made on limited evidence based on chance observations of mitochondria in close proximity with the membranes concerned. They have not been followed up by observations of the intermediate stages which could be expected to be prevalent if this were the major form of synthesis.

PART B HETEROSIS AND COMPLEMENTATION

This project is concerned with investigating a specific heterosis, that of seedlings and examining the proposition that this heterosis is caused by the combination of unlike mitochondria in hybrids. This combination, it is hypothesized, (McDaniel and Sarkissian, 1966) produces a complementation reaction which is either wholly or in part responsible for seedling, and eventually, whole plant heterosis.

This hypothesis assumes that enzyme and organelle activities are capable of individual heterosis. To accept mitochondria as a casual agent of heterosis is to negate another prevalent attitude, that heterosis is the result of optimum metabolic balance (Hageman et al., 1967). The observation that many enzymes, when isolated from heterotic hybrids and compared to isolates from inbreds, show intermediate activity supports

this latter view. Knight (1973) proposed a model of interaction between genotype and environment by which a hybrid that is intermediate to its inbred parents in its response to an environmental factor is automatically heterotic over a certain range of environmental conditions (see Fig. 1- 7). The expression of heterosis is thus intimately tied up with the environment as has been demonstrated many times by other workers (Manwell and Baker, 1970; McDaniel, 1971; Whaley, 1952).

The hypothesis of mitochondrial heterosis and complementation was formulated from seedling studies using superior growth rates as the only criterion for heterosis. There have been only two reports providing a positive correlation between mitochondrial complementation and yield heterosis. The mechanism operating in seedlings to produce more rapid growth, need not be the same as that responsible for producing a large grain yield from adult plants. It would seem that when seedlings became independent of parental seed reserves, the rate of carbon fixation would become a more critical limiting factor for growth than mitochondrial energy conversion. Studies of photosynthetic phosphorylation rates between inbred and hybrid corn have shown the hybrid to be intermediate in activity in all cases (Hageman et al., 1967).

To examine McDaniel and Sarkissian's hypothesis and its underlying assumptions, it is necessary to define heterosis and outline how it may operate on the genetic and enzymic levels. The rationale behind the use of seedling material will be considered and the incidence of mitochondrial heterosis will be discussed and compared to other studies on heterosis in

isolated enzyme activity. The evidence for complementation between unlike mitochondria of inbreds producing heterotic hybrids and the theoretical implications of these findings will be given, and the relevance to plant breeding of these findings will be discussed.

Definition of heterosis

The term heterosis was coined by Shull (1948) as a contraction of the word heterozygosis to be synonomous with the older term hybrid vigour. Heterosis was said to occur if a hybrid from an out-cross was better than the highest parent in any measurable characteristic. Thus Shull applied heterosis in a positive sense. As all inbred lines do not produce the same vigour when crossed, Stern (1948) introduced the concept of negative heterosis to describe those in which the character measured decreased in comparison with the highest parent. Typically, the vigour associated with the F_1 generation is transient, being lost if the F_1 is selfed for further generations. This is known as inbreeding depression and appears to be associated with high levels of homozygosity. Hybrids are also reported to have phenotypic stablity in that they are said to be more resistant to changes in the environment than their parents, a phenomenon also termed homeostasis.

Effective manipulation of heterosis is an important breeding tool but its use is hampered by the present lack of knowledge in three fundamental areas. The problems were outlined by Hageman et al., (1967) as: (1) How do genes produce effects on growth and metabolic processes?

(2) What genetic mechanisms are involved in heterosis? (3) By what criteria can plant breeders select genotypes to obtain maximum heterosis in desired characters (such as yield) and is there any method of doing this more efficiently.

The first question covers the field of molecular genetics and was not studied here. It is a subject that has been studied intensively and has been recently reviewed (Watson, 1965; Bonner, 1965; Lock , 1963; Manwell and Baker, 1970). The second and third questions are considered in the following section.

Since heterosis is a phenomenon of growth, resulting from the interaction of genes, metabolism and environment (Whaley, 1952) it is unlikely that one unifying genetic mechanism will be found to answer question two. As it appears impossible to determine the exact genotype of any higher plant hybrid, Sarkissian suggested that seeking an understanding of how heterosis is accomplished in physio-chemical terms might provide an alternative approach to purely genetic studies. It is through such studies that possible biochemical criteria for plant selection have been proposed.

Mechanisms for heterosis

Many attempts have been made to describe the mechanism by which heterosis operates, resulting in two main classes of hypothesis. The first rests on the assumption that an as yet unexplained physiological stimulation results from the union of two gametes of unlike origin (Gowen,

1952). The interaction of unlike mitochondria as proposed by McDaniel and Sarkissian (1966, 1967 , . 1970), may provide some basis for this type of explanation. Such an hypothesis is supported by differences in vigour in reciprocal crosses showing the influence of maternal cytoplasm (Jones, 1952; Oehlker, 1964; Wagner, 1969). However the weight of evidence from Drosophila studies (Gowen et al., 1946; Gowen, 1952) suggests that hybrid vigour is a genetic phenomenon, and that is is in some way related to heterozygosity. This is the assumption on which the second class of hypotheses are based.

The pre-Mendelian explanation of hybrid vigour rested on the observation that both parents of an inbred have the same defect which is intensified in the offspring. Outbreeding parents have different defects which tend to compensate for each other in the immediate progeny. To explain this observation many modern genetic mechanisms have been proposed. The simplest was the dominance theory (Bruce, 1910; Jones, 1917; Crow, 1948; Hayes, 1952) which proposed that favourable dominant genes were brought together in the hybrid. If this were the case it would be a relatively easy matter to fix heterosis by crossing and selection for these dominants. Thus simple dominance cannot be the explanation as it takes no account of inbreeding depression (Manwell and Baker, 1970). Lerner (1958) and Falconer (1960) postulated that heterosis was merely the reverse of inbreeding depression since out-crossing will reverse the deleterious effects of inbreeding. However this explanation does not take account of plant species, such as wheat,

in which inbreeding is obligatory and high levels of homozygosity are maintained. It also does not give an explanation of inbreeding depression and cannot be regarded as a mechanism for heterosis.

Other workers used the term overdominance (Crow, 1948, 1952) when referring to heterotic behaviour. This description, elaborated by East (1936), is equivalent to single gene heterosis, and describes that condition where a heterozygote at a particular locus, is superior to both homozygotes. Overdominance at several loci might add up to positive heterosis, with an increase in vigour being proportional to the amount of heterozygosity. Epistatis or gene interaction has also been implicated as a mechanism for heterosis (Brewbaker, 1964; Manwell&Baker 1970). These are all descriptive terms of genetic interaction. As Sarkissian (1972) points out, they are circular definitions stating that a hybrid shows vigour because it is superior or not inferior. Thus they are not explanations of the mechanism by which a heterozygotic combination of alleles exerts it superiority.

The pre-Mendelian concept of covering parental deficiencies in the hybrid has been the basis of a scheme of complementation of genotypes proposed by Irwin (1932, 1952) and later by Manwell and Baker (1963, 1970). Such complementation provides a testable hypothesis for discovering the physiological and biochemical means by which hybrid vigour is expressed. Complementation is here used to denote completeness, or fullness. It can also be used to describe the interaction of two systems to give a result greater than the mean of those two systems.

McDaniel and Sarkissian, (1966) in coining the term mitochondrial complementation, were using it in this second sense.

Complementation as a mechanism for heterosis

The complementation approach suggests two possible molecular mechanisms as the basis of heterosis. Firstly, complementation can occur between the component chains of proteins to give hybrid molecules which have enhanced activity. Secondly, complementation may occur between enzymes in metabolic pathways, in particular, interaction between neighbouring enzymes in a macromolecular assembly. These will be considered in turn.

Hybrid proteins often have highly advantageous properties. Such hybrids may arise from single gene heterosis, in which case proteins must have a quaternary subunit structure which is coded for by a polymorphic allele. Examples of such proteins are malate dehydrogenase and enolase. The hybrid proteins can only be found in a hybrid individual.

A more complex series of hybrid proteins results from epistatic gene action on biquaternary proteins. These have different polypeptide chains, each coded for by a distinct genetic locus. Examples of such proteins are vertebrate haemoglobin and the regulatory enzymes characterized by an isoenzyme series such as lactic dehydrogenase. Here the hybrid is found in all individuals of a species as a fixed heterozygote.

Enzymes are often part of a larger structural unit or macro-

molecular assembly which has a precise stoichiometry. The components of the respiratory chain and the mitochondrial ATP-ase are good examples of this quintary structure (Manwell and Baker, 1970). The heterozygotic condition can also influence this organization with enzymes being brought together in a hybrid allowing greater reactivity. Complementary interactions of this type have been reported among mitochondrial enzymes in hybrids of yeast (Sels and Jakob, 1967). They observed accelerated cytochrome oxidase synthesis in crosses of respiratory mutants. Interactions between the enzymes of glycolysis were reported by Kwon and Olcott (1965). They observed that the activity of isolated aldolase was enhanced by addition of adjacent glycolytic enzymes (apart from effects caused by removal of products) but not by unrelated proteins. Thus enzymes have been shown to influence each other by their proximity.

It is necessary to view all complementary interactions from both a positive and negative aspect if they are to be considered as a basis of heterosis. The hybrid proteins of lactate dehydrogenase produced by the combination of the H and M subunits (i.e. H_3M, H_2M_2, HM_3) have greater reactivity than either H_4 and M_4 (Bridges and Freier, 1966). This must be set against the decreased stability of the hybrid molecules (Anderson and Weber, 1966) which is negatively heterotic.

Complementation between biquaternary proteins and different enzymes are examples of gene interaction or epistasis, while complementation between alleles at one locus to give hybrid quaternary molecules is single gene heterosis or overdominance (Manwell and Baker, 1970). Thus

complementation can provide a mechanism for the genetic descriptions of heterosis already discussed.

Seedling heterosis

In an attempt to explain the phenomenon of heterotic growth, recent studies have looked for characteristic enzyme systems which elicit vigour during the early growth of organisms. Attention has been focused on the metabolic steps which would be of primary importance to a growing organism and on events controlling these steps or reactions. Since growth is limited by the rate of turnover of cellular energy, respiratory and mitochondrial studies seemed an obvious starting point. Other lines of approach have been to study glycolytic enzymes, the first steps in nitrogen metabolism, and carbon fixation. Whaley, (1952) suggested that genetic and physiological studies concerned with the early stages of development would be most likely to reveal the cause of hybrid vigour because studies of growth rates over longer periods in mature plants had failed to reveal any significant difference between parents and hybrids. Also it was in accordance with the "initial capital" concept of Ashby (1930, 1937, 1949), Hatcher (1939, 1940) and Luckwill (1937, 1939) who emphasize that the hybrid advantage in their material was either present in the resting embryo or became manifest in the early stages of post-germination growth. With this advantage, or increased "initial capital", hybrids were better because they had a better start, even though the condition of a seed may be a direct reflection of the maternal parents vigour and nutritional status. However Groszmann and Sprague (1948) clearly showed that "initial

capital" differences have little, if any, basic influence on early heterotic vigour.

Whaley's work (1952) provided guidlines for a physiological-genetic approach but specific examples, especially from enzyme studies, were not available. Germinating seedlings have since been used extensively for the study of heterosis in the attempt to determine whether enhancement of enzymic activity acts as the mechanism of heterosis.

Germinating seedlings provide a convenient biochemical material which can be grown free from environmental fluctuations (Hageman, Land and Dudley, 1967). Heterosis in the morphological characteristics of seedlings is well documented for maize and is expressed in terms of faster germination, faster elongation and weight gain. Morphological development has been correlated with physiological development (Woodstock and Feeley, 1965; Kittock and Law, 1968), and a comparison between hybrid and parental rates of both aspects of development have been presented (Sarkissian, Kessinger and Harris, 1964; Sarkissian and Srivastava, 1967). In particular, the respiratory rate, measured even 3 to 4 hours after imbition begins, has highly positive correlation with later seedling growth (Woodstock and Combs, 1964, 1965; Woodstock and Grabe, 1967; Woodstock and Feeley, 1965). Thus seedling respiration can be used as a parameter for the assessment of vigour with hybrid tissue respiring faster than parental tissue, (Sarkissian, Kessinger and Harris, 1964)

The respiratory rate of germinating tissue goes through a cycle of rise and decline during the first few days of germination (Hanson et al.,

1959; Akazawa and Beevers, 1956, 1957). The respiratory change is reflected in the activity of mitochondria isolated from the seedling over this period. It appears that mitochondria develop rapidly during early post-germination growth, enlarging and increasing in enzyme activity (Mittendorf, 1939; Akazawa and Beevers, 1957 Hanson et al., 1959). This is associated with a decrease in organelle density and it is possible that different mitochondria are at several stages of development at any one time. This may have implications when separating seedling mitochondria on density gradients (McDaniel and Sarkissian, 1967). It also means that the age of tissue used for the extraction of mitochondria is critical (Sarkissian, 1972; McDaniel, 1971; Hanson, 1959) and should be kept constant. Hybrid seedlings are often as much as a day ahead of parent seedlings in morphological characteristics and this may influence comparisons of mitochondrial preparations between genotypes.

The study of morphological heterosis in wheat seedlings may be invalidated by the incorporation of the "norin" genes for dwarfness (Allen et al.). Dwarfness is an agriculturally favoured characteristic but this gene will prevent fast increases in coleoptile length, masking germination heterosis in forms of length. Norin genes are not present in the Australian wheat used in this study, but it is not known whether they are incorporated in exotic lines. Although morphological heterosis has been correlated with mitochondrial heterosis in maize, this relationship has not been repeated for wheat, nor has there been any attempt to

correlate yield and morphological factors. Thus for the studies on wheat heterosis to be described in the following section, the observation of heterosis in mitochondrial activity is taken to indicate good combining ability as it does in maize (McDaniel and Sarkissian, 1966; Sarkissian and Srivastava, 1967) and barley (McDaniel, 1971) but no evidence has been presented to show that such a relationship exists for wheat.

Ellis & Hanson (1974) have warned that problems may arise in seedling tests of heterosis, as it is possible for seed quality to interfere with physiological and biochemical properties of seedlings. While developing a screening test for yield heterosis with a series of F_1 hybrid barleys, Ellis reported a significant positive correlation between scutellum respiration rates and grain yield of glasshouse grown plants. However a similarly significant correlation was observed between scutellar oxygen uptake and percentage germination using nine seed lots from a single cultivar.

The age and method of storage of the seed, reflected in percentage germination and field emergence values, will have as much influence as genotypic differences on tissue respiratory rate and may equally effect mitochondrial performance, although Ellis did not examine this latter point.

Such observations on the influence of the environmental conditions during ripening, harvest, drying and storage on cereal seed performance have been reported previously (Heydecker, 1972; Woodstock and Grabe, 1967; Parmar and Moore, 1966; Kittock and Law, 1968; Finlay, 1960). By enlarge no mention was made of seed quality by McDaniel and Sarkissian in their work on maize, wheat and barley, although McDaniel (1973) mentioned that

mitochondrial trials were performed annually with fresh seed for four years.

Mitochondrial heterosis

The faster respiratory rates of hybrid seedlings can be explained in one of two ways. On the assumption that the mitochondria are responsible, the higher rates could be due to, either, a larger number of mitochondria per seedling, i.e. a quantitative increase, or else it could be due to an increase in their activity, a qualitative improvement. The latter could arise if the hybrid mitochondria are different from parent mitochondria in some way, or if normal mitochondria are released from metabolic constraints such as allosteric enzyme control, substrate availability and feedback inhibition that are normally operating. The possibility of quantitative increases will be considered first.

It is not possible to determine the number of mitochondria per seedling or per unit weight of tissue, and the best attempts are only rough estimates. The proportion of total mitochondrial protein extracted in a normal preparation is known but is likely to be low, and could be expected to vary considerably with each preparation (Hanson, 1963), making comparison between preparations difficult. Thus experiments which correlate large seed size with the quantities of extractable mitochondrial protein (McDaniel, 1969) cannot be taken as meaningful. As will be discussed more fully in Chapter V, the possibility of respiration being limited by the number of mitochondria present is remote. Tissue respiration has

been shown to be stimulated by the addition of uncouplers (Beevers, 1961). If tissue respiration can be stimulated by DNP, its mitochondria are not working at full capacity and are limited by extramitochondrial controls. Having more mitochondria in such a case would be of no consequence in speeding respiratory activity. In an attempt to isolate the point of control of hybrid vigour several workers have studied the properties of isolated mitochondria in developing seedlings. That mitochondria might be implicated in heterosis had been suggested by Sarkissian et al., (1964) from indirect studies. To substantiate this suggestion it was necessary to demonstrate that mitochondria of hybrids were superior to those of inbred lines. The first comparative study of mitochondria from parental and hybrid genotypes of maize (McDaniel and Sarkissian, 1966) supported this suggestion. The mitochondria of all genotypes were assayed and the hybrids were found to be significantly higher than the parents for the rate of oxygen uptake, though not for P:O determinations. Non-heterotic hybrids had mitochondria which were not significantly different from the highest parental type. The criticism has been raised however (Hageman, Lang and Dudley, 1967) that the parents of the non-heterotic hybrid, Ohio 43, by Ohio 45, were so closely related that it could be considered as an inbred cross, rather than an example of negative heterosis. The claim for positive mitochondrial heterosis gained support from subsequent reports (McDaniel and Sarkissian, 1967) that significant differences between mitochondria of hybrids and highest parents had been obtained for other parameters. The level of cytochrome oxidase was found to be higher

in the hybrid (Sarkissian and McDaniel, 1967) but the ADP:O and respiratory control ratios were only heterotic with KGA as substrate (McDaniel and Sarkissian, 1968). Polarographic traces of the oxidation of succinate and NADH for the parents and their F_1 showed that not all were coupled. It is hard to believe that the mitochondria of two inbred lines of one species could differ so much that one could show coupled succinate and NADH oxidation while the other could not. Since tissue with uncoupled mitochondria would soon be dead, it is possibly a reflection of the isolation and assay condition, i.e. succinate dehydrogenase was not activated to attain a true state 3 rate, so coupling was not observed (Wainio, 1970).

Oxidation of a wide range of TCA intermediates was tested, none of which showed control. The rates of oxidation were compared and the conclusion reached that only NAD-linked substrates had heterotic activity, attributed to the greater control exerted by the respiratory chain when such substrates were utilized. Respiratory rate is an unreliable criterion especially where the oxidation does not exhibit control. A fast rate may indicate uncoupling and be a sign of poor mitochondrial performance, rather than the reverse. It is certainly insufficient evidence upon which to postulate a mechanism for mitochondrial heterosis. The corn mitochondrial experiments (McDaniel and Sarkissian, 1966, 1967, 1968; Sarkissian and McDaniel, 1967; Sarkissian and Srivastava, 1967) supported the concept of qualitative advantage although whether this was achieved through release from repression or although intrinsically superior enzymes was not clarified. Work on wheat mitochondria attempted to make this distinction (Sarkissian

and Srivastava, 1969, 1970, 1971; Srivastava , Sarkissian and Shands, 1969; Sarkissian, 1972).

Hybrid mitochondria from wheat were reported to be especially efficient (Sarkissian and Srivastava, 1969, 1970) as they consistently had ADP:O ratios of above theoretical values for the substrates KGA, malate, succinate and NADH. This above-normal activity was never found in parental mitochondria giving hybrids a special efficiency which would advantage a developing seedling, and could be expressed as heterotic growth. Sarkissian and Srivastava (1969, 1970) repeated these estimations manometrically and still found ADP:O ratios for KGA of 5.3 to 5.8. Heterosis in wheat mitochondria was found for all substrates and it was measured on a broad range of parameters. These included the rates of the specific enzyme, cytochrome oxidase, and of the enzyme sequence NADH-cytochrome *c* reductase.

Wheat hybrids were also said to be less sensitive to the uncoupler, DNP, as they had the highest ADP:O ratios and control ratio after addition of sub-optimal concentrations of DNP and the lowest percentage drop in these ratios (Sarkissian and Srivastava, 1969). They also showed the least enhancement of DNP-stimulated ATP-ase but only because the level of hybrid ATP-ase was much higher initially than either parent. Srivastava (1973) claimed this high ATP-ase to be a further expression of heterosis. To say that mitochondria are simultaneously more tightly coupled and have a higher ATP-ase activity is contradictory.

The above-theoretical levels of phosphorylative efficiency have

not been obtained by other workers using the same isolation procedures (Zobl et al., 1972; Sage and Hobson, 1973) and the same wheat lines (Ellis, Brunton and Palmer, 1973). Close examination of Sarkissian and Srivastava's data indicate there may be an experimental explanation for their claim; this and other criticisms will be discussed more fully in Chapter VII

Mitochondrial heterosis was established in barley by McDaniel (1971, 1972, 1973). The value of heterosis over a large number of hybrids, clusters about the 110 to 140 percent mark. McDaniel derives his percentage heterosis measurement by expressing the F_1 as a percentage of midparent which gives higher values than comparing F_1 to the highest parent, but it is a weaker measure as it does not comply with the strict definition of heterosis.

Having established the presence of barley seedling heterosis, mitochondria were isolated and those of the hybrids were shown to have statistically higher ADP:O, and respiratory control ratios and higher rates of oxygen uptake. ADP:O ratios were usually below 3 for KGA and at no time were they above the theoretical level of 4. Polarographic traces of mitochondrial oxidation showed it to be well coupled with no ATP-ase activity. Controlled oxidation of succinate, NADH and isocitrate was observed but comparisons of the hybrid and parents for these substrates were not presented. Malate was shown to support heterotic mitochondrial oxidative phosphorylation (McDaniel, 1971, 1973) so malate dehydrogenase a soluble enzyme was isolated and comparisons were made of the kinetics

of the purified enzyme from different genotypes. No difference in activity existed between genotypes for mitochondrial malate dehydrogenase assayed by cytochemical staining of electrophoretically separated preparations. This agrees with some of the other enzymological studies to be discussed. McDaniel (1971) concluded that the structural organization of the mitochondrial system was more important than the enzymic activities, per se, in determining the overall reactivity with any substrate. Conformation had been implicated previously (McDaniel and Sarkissian, 1968) in maize studies where the membrane bound NAD site was said to be responsible for the enhanced hybrid activity. That mitochondrial enzyme activities are profoundly altered on removal from their membraneous environment has been extensively documented (Senior, 1973; Woodward, 1968; Packer, 1974). This is interpretated as a result of the integrating role of the membrane which appears to be responsible for keeping molecules in juxtaposition, e.g. complex III (Van Dam and Meyer, 1971) or masking additional active sites as in cytochrome oxidase (Senior, 1973; MacLennan, 1970). The membrane can also exert control by limiting the availability of substrates and by maintaining polarity. Thus one would not necessarily expect to find heterosis in isolated enzymes of an organelle where the membrane plays a central organizational role. Sarkissian and Srivastava (1971) have reported that cytochrome oxidase from wheat maintains its hybrid advantage even after partial purification. Thus they maintain that the advantages conferred to the hybrid from having more active cytochrome oxidase are in part an

explanation for its vigour. However, as mentioned previously, cytochrome oxidase is one of the chain components most effected by disruption of the membrane structure as many more active sites become exposed. The activity when purified may bear no relation to the in vivo properties of the enzyme.

The numerous reports of McDaniel and Sarkissian (1966, 1967 1968) therefore support the proposition of qualitatively improved mitochondria, resulting from heterozygosity in the F_1 hybrids produced from unlike inbred lines. Sarkissian and Srivastava (1969, 1971) propose that the mitochondria have intrinsically better enzymes in the hybrids, while McDaniel (1971) suggests conformational or organizational changes as the cause of the hybrid mitochondrial superiority.

Mitochondrial heterosis as an indicator of yield

McDaniel (1971, 1973) claims that ADP:O ratios of mitochondria from seedlings is an accurate indicator of whole plant potential. As selection proceeds in a given line over several seasons, he suggests that the mitochondria are gradually improved, and has presented data of ADP:O ratios over the course of 4 years selection to show that the ADP:O ratio increases with each season (from 125 to 130 percent) as does the percentage yield heterosis. Thus he concludes that barley breeders, in selecting for yield are in fact selecting for superior mitochondria.

However there are only three cases where mitochondrial performance over a number of hybrids has been correlated with yield. The first was

presented by McDaniel (1972) for corn, where a positive correlation was reported with a correlation coefficient of 0.69, which was significant at the 1% confidence level. In this correlation, grain yield data was obtained from other worker's published results and personal communications and was not estimated on samples from which the mitochondria were taken. Also, heterosis and complementation were correlated jointly as though being one phenomenon and as Ellis *et al.* (1973) point out, this failure to distinguish between the two phenomena does not provide convincing evidence that either phenomenon alone is correlated with yield potential.

The second correlation was presented by Sage and Hobson (1973) who showed that for British wheat grown at a low (non-agricultural) seed density, mitochondrial complementation was significantly correlated at the 0.1% confidence limit with yield heterosis, provided the lines were fully restored and relatively disease free. This meant discarding two crosses from their trial, one which showed high susceptibility to disease and another in which the restorer genes had not been able to cover the male sterility effects resulting in poor seed set.

A third significant correlation was made (McDaniel, 1973a) for yield and mitochondrial activity in "Pima" cotton, where an association between the pounds of lint produced and ADP:O ratio per line was illustrated. Mitochondrial heterosis is not tested in this correlation of yield. There have been no correlations made with mitochondrial heterosis and wheat and barley yields. Thus the association of mitochondrial heterosis with yield heterosis depends almost entirely on the link between yield heterosis

and seedling heterosis.

Other enzymic determinations of vigour

Hageman, Leng and Dudley (1967) reviewed extensive biochemical work done on corn inbreds and hybrids to assess the role of enzymes in eliciting vigorous growth. They approached the problem of growth and yield as the sum total of a series of biochemical reactions and hoped that by studying key enzymes in the photosynthetic, glycolytic and nitrogen pathways they would find heterosis on an enzymic level. Isolated chloroplasts were assayed for cyclic phosphorylation and the glycolytic enzymes studied were aldolase and triose phosphate dehydrogenase and glucose-6-phosphate dehydrogenase. The first two are involved in the supply of metabolites to the Krebs cycle and glucose-6-phosphate dehydrogenase is the first step in the synthesis of ribulose-5-phosphate and thus is an important control point. Nitrate metabolism was studied via nitrate reductase, which has been shown to be a major control site for nitrogen metabolism, being sensitive to repressor type genetic control (Filner, 1966). Nitrate reductase activity has been positively correlated with protein formation (Hageman et al., 1961) and the growth rate of cell cultures (Filner, 1966).

The one factor most likely to limit plant growth is the rate of carbon fixation. However, Hageman et al., (1967) presenting results from a comparison of isolated chloroplast activity between inbred and hybrid lines of corn showed that the rate of cyclic phosphorylation was

not heterotic in any hybrid as all levels were intermediate or lower than the highest inbred line. The same pattern was seen in the activity of *in vitro* glycolytic enzymes which showed only slight enhancement in the hybrids and this only appeared when great care was taken to maintain the uniformity of environment and seed size. Where the environment was changed to semi-anaerobic conditions, all hybrids were intermediate to parents. On isolation and purification of the enzymes all differences disappeared and the Km values estimated for parents and hybrids were essentially identical. Similarly with nitrate reductase, studies of the partially purified enzyme over a range of genotypes (pure enzyme preparations were too unstable to be studied) have shown that the Km values vary within the level of experimental error (Beevers *et al.*, 1964). Although a survey of many inbred lines showed nitrate reductase activity to be determined by genotype, an extensive survey of hybrids showed that with few exceptions the hybrids gave an intermediate response. Yet all hybrids studied showed heterosis in grain yield and vegetative growth, regardless of the level of nitrate reductase activity. Hageman *et al.*, (1967) maintained that this clearly indicates that heterotic levels of nitrate reductase activity are not necessary for "agronomic heterosis". They conclude that most of the data does not favour either (a) more enzyme or (b) more efficient systems, but they admit this conclusion is restricted by their lack of success in obtaining highly purified enzymes and the difficulty in obtaining any assessment of the quantity of an enzyme *in vivo*. They proposed that heterosis was manifested because hybrid metabolism is better balanced as

a whole. This ties in with Whaley's suggestion that the genome of inbreds of a given species cannot differ greatly and it must be their favourable combination that causes heterosis. Hageman et al., (1967), think that intermediate enzyme activity would be more favourable than one which was extremely high or low, as it would promote a balanced metabolic system.

Mitochondrial complementation

Complementation resulting from epistatic gene action is proposed as the basis for the phenomenon termed mitochondrial complementation (McDaniel and Sarkissian, 1966). Artificial mixtures of mitochondria isolated from the inbred parents of heterotic hybrids have been tested for several biochemical parameters and have been reported to exceed the average value of the parental mitochondria measured separately. This positive interaction of respiring mitochondria has been termed mitochondrial complementation and was first described for mixtures of inbred corn mitochondria (McDaniel and Sarkissian, 1966, 1967 1968, 1970 Sarkissian and Srivastava, 1967). The observation of interaction was extended to barley (McDaniel, 1971 , 1972, 1973), wheat (Sarkissian and Srivastava, 1969 , 1971; Srivastava, Sarkissian and Shands, 1969; Sarkissian, 1972; Srivastava and Sarkissian, 1972; Sage and Hobson, 1973), "pima" cotton (McDaniel, 1973a), sugar beet (Doney, Theurer and Wyse, 1972) and Drosophila melanogasta (McDaniel and Grimwood, 1971).

However, other workers have reported that attempts to repeat this work have failed. Ellis, Brunton and Palmer (1973) could not find

statistically significant mitochondrial interaction in either wheat or barley, although they used the same breeding lines and followed the techniques reported by McDaniel and Sarkissian exactly. Similarly Zobl et al. (1972) found no correlation between yield trials and enzymic determinations of inorganic phosphate esterification, ADP:O ratio and oxygen uptake by mitochondrial mixtures. The claim for complementation with sugar beet mitochondria was based exclusively on ADP:O ratios all of which were between 1 and 2, which, with KGA as substrate, suggests the mitochondria were loosely coupled and not representative of maximally active mitochondrial preparations.

Mitochondrial complementation was proposed as a mechanism of heterosis because it occurred in the direction of heterosis. As well as the improved activity in heterotic mixtures, parents of negatively heterotic barley showed negative interaction when they were artificially combined (McDaniel and Sarkissian, 1966). However, as mentioned previously, this evidence is weakened by the observation of Hageman et al. (1967) that the negatively heterotic hybrid was a cross from two closely related lines, equivalent to an inbred cross and not an example of negative heterosis.

Stronger evidence for a correlation between mitochondrial complementation and heterosis is provided by Sarkissian and Srivastava (1967), who report that different maize hybrids exhibit differing degrees of seedling heterosis, measured in terms of germination and rate of elongation of the radicle. Mitochondria extracted from these seedlings also exhibit heterosis regards oxidative phosphorylation. The 1:1 mixtures of parental mitochondria reflected the activity of the hybrids and in most

cases the relationship was direct; high heterosis - high complementation, no heterosis - no complementation, negative heterosis - negative complementation.

McDaniel and Sarkissian (1966, 1967) proposed that the mitochondria of inbred parents of heterotic hybrids are different from each other in some way. Hybridization brings these populations of mitochondria together to form a polymorphic population in the new individual. Interaction occurs between these unlike mitochondria to increase the efficiency of energy production. The in vitro mixture of parental mitochondria is seen as a mimic of the hybrid situation. This line of reasoning assumes that mitochondria are inherited cytoplasmically as discrete units which are essentially free of the influence of the nuclear genome. The evidence for and against this concept of mitochondrial biogenesis was discussed earlier (part A). However it seems that even if cytoplasmic inheritance does occur and at syngamy whole mitochondria are contributed by each gamete, the nuclear genome is extremely important in mitochondrial synthesis as it codes for the majority of mitochondrial proteins.
Van Vallen (1967) in a criticism of the work of McDaniel and Sarkissian (1966) suggested that it was necessary to prove that the complementing mixture was as active as the hybrid mitochondria if the hypothesis was to be supported. This was done statistically by McDaniel and Sarkissian (1967). However new experiments (Sarkissian and Mc Daniel , 1967) provided for hybrid preparations being more active than complementing mixtures. Crude mitochondrial pellets from parents, hybrids and several mixtures

of parents, and parents and hybrids, were subjected to sucrose density gradient centrifugation. The pellets were fractionated into density bands each with cytochrome *c* oxidase activity. The parents had a high density band, in common, and one parent had a second low density band, which remained distinct in the 1:1 parental mixtures. The hybrid had both high and low density bands, and an intermediate band which was unique to the hybrid preparation. McDaniel and Sarkissian presented these findings to support their original hypothesis, but with an important modification. They suggested that mitochondria had been shown to be different in individual genotypes and that they represent an inheritable difference between strains. However the hybrid individual, as well as inheriting the two parental types is capable of de novo synthesis of a new hybrid mitochondrial population as a result of interaction of the parental genomes.

Thus they propose two mitochondrial mechanisms for heterosis. The mixing of unlike mitochondria is sufficient to amplify oxidative phosphorylation and oxygen uptake. However there is an additional 30 percent increase in the hybrid when compared to the mixture which is directly attributable to the mitochondria of intermediate density (Sarkissian and Mc Daniel , 1967).

Additional evidence for this conclusion came from experiments with cytoplasmic male sterile wheat lines (Srivastava, Sarkissian and Shands, 1967) which are becoming increasingly widely used as parents of inter-varietal hybrids. The activity of mitochondria were consistently

found to be superior in respiratory control and phosphorylation efficiency to activities of mitochondria of parental lines. Artificial mixtures of the mitochondria of the parents of the male sterile line (the female line with Triticum timopheevi cytoplasm and a selected genome, is crossed with a maintainer line which acts as the male parent, and has no fertility restoring genes) were reported to exhibit complementation not only for oxidative phosphorylation where over theoretical values of ADP:O ratios were reported for the male sterile lines and some mixtures, but also for ATP-ase activity in the absence of uncoupler. High ATP-ase activity usually indicates poor mitochondrial preparations and thus the same contradiction is introduced into these results as into those on mitochondrial heterosis. The greatest complementation was found when mixtures were made in the ratio 1:2 with T. timopheevi being the larger component. As T. timopheevi is used as the female parent, and as it contributed the larger share of the cytoplasm on syngamy, cytoplasmic inheritance occurred. Cytoplasmic heterogeneity is described as a persistent phenomenon, transmissible to subsequent generations (Srivastava et al., 1969) thus one would wonder whether any further complementation would occur when the male sterile line was crossed with the restorer line in the production of a hybrid. Double hybrid crosses had previously been reported as not showing strong mitochondrial heterosis. Since complementation has been acclaimed as a predictive test for favourable combining ability, its success in predicting male sterile, male fertile crosses is essential, as this is the most economically feasible way of

producing hybrid cereals. This report on male sterile superiority is somewhat at odds with a report by Srivastava and Sarkissian (1969) where the heterosis and complementation are seen in the hybrid of line 28 and the male sterile 31 MS. In this latter paper no mention is made of superior activity of the male sterile line which had lower than theoretical ADP:O ratios, and acted like other parental lines.

Measurement of heterosis

The assessment of heterosis depends on the point of view of the assessor, characters which may be considered positively heterotic in one situation may be considered deleterious in another. From one point of view, heterosis can be linked with the maintenance of an excess of heterozygotes in a population, which under the Hardy-Weinberg equilibrium, indicates the heterozygote has an adaptive advantage, i.e. exhibits heterosis. However this approach is not suitable for plant breeders who have difficulty identifying a heterozygotic condition in the particular characters of agricultural importance. This is because characters such as growth rate and yield are under polygenic control (Jones, 1952). In crops for human consumption, breeders must also select for "quality" or the processing properties, the determinants of which are still largely unknown (Rodríguez et al., 1967). Thus yield and yield heterosis are usually measured directly, and they are split into yield components. Some yield components for cereal crops are plant weight, grain weight, the number of tillers and heads per plant and

the number of grains per head.

The percentage heterosis or hybrid vigour is calculated as the difference between the hybrid and the highest parent for any measurable characteristic, expressed as a percentage of the highest parent.

$$\text{Percentage heterosis} = \frac{\text{Value of hybrid} - \text{Value of highest parent}}{\text{Value of highest parent}} \times 100$$

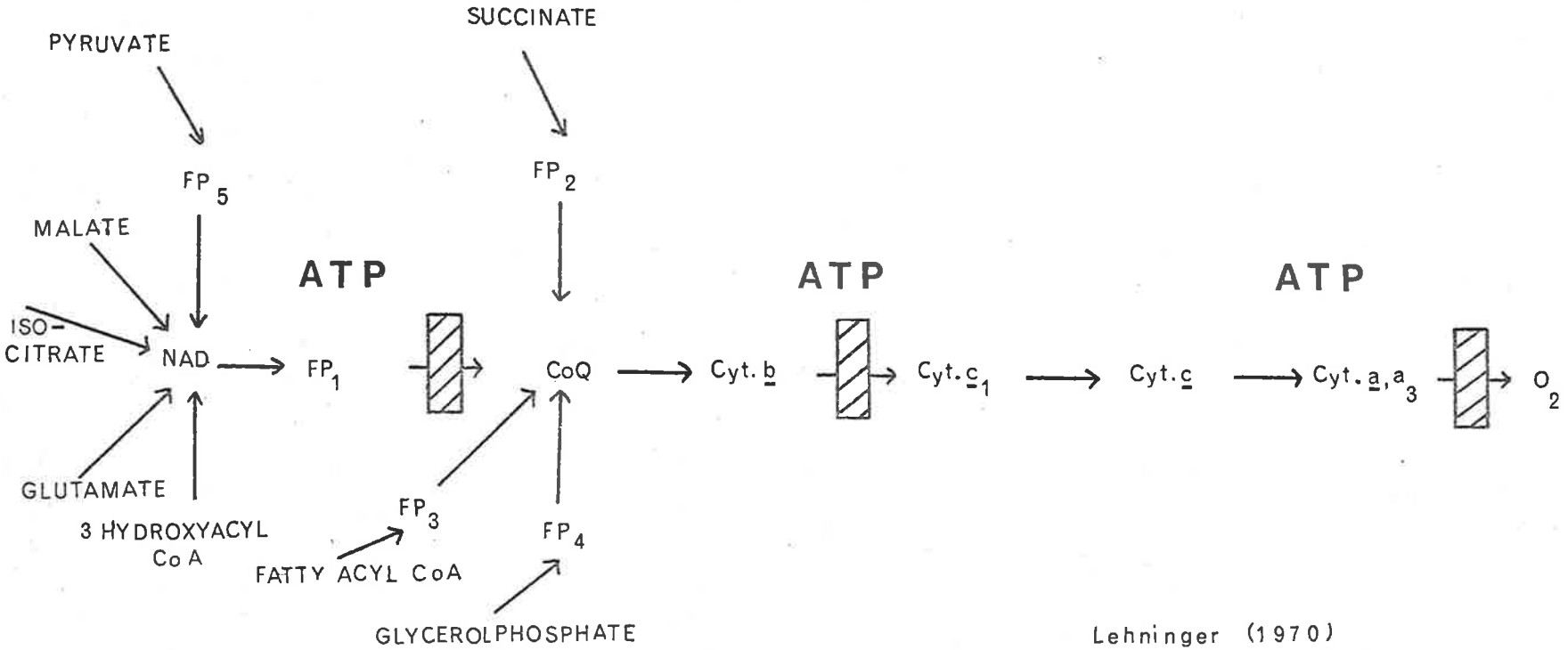
Some authors choose to express the percentage heterosis as the increase over the mid-parent value (Mather, 1949; Lerner, 1958; Falconer, 1960; Brewbaker, 1964). However this differs from the original idea of hybrid vigour as superiority over both parents and is agriculturally less significant as the parents are often high performance commercial varieties.

For heterosis to be useful, increases of 20-30% in yield must be obtained under field conditions. That wheat is capable of producing this type of increase has been demonstrated (Rodríguez et al., 1967) in studies in the Mexican (CIMMYT) wheat breeding programme. However not all wheat lines when combined produce significant heterotic effects. Thus as production of hybrid seed is difficult and time consuming when done either by conventional methods or even when cytoplasmic male sterility is employed a preliminary screening test to look for favourable combining ability has been sought. Mitochondrial complementation between inbred parental lines has been suggested as one such mechanism (McDaniel and

Sarkissian, 1966, 1967, 1970 , 1968, 1970; Sarkissian and Srivastava, 1967; Srivastava et al., 1969; McDaniel, 1971, 1972, 1973), tissue respiratory rates as another (Ellis et al., 1974). The aim of this project was to study the mitochondrial complementation phenomenon and to determine its relationship to heterosis. By studying heterosis on four levels: (a) the whole seedling growth, (b) the tissue respiration, (c) the phosphorylating efficiency of isolated, intact mitochondria and (d) the activity of mitochondrial enzyme assemblies, it was hoped that the mechanism for heterosis, or at least the site of specific heterotic activities could be distinguished.

The present work utilized wheat mitochondria exclusively in an attempt to verify the experimental results of Sarkissian and Srivastava (1969, 1971) in particular the claims of high ADP:O ratios.

FIG 1 – 1



Lehninger (1970)

Fig. 1-2

Location of inner membrane components, from Harmon, Hall and Crane (1974)

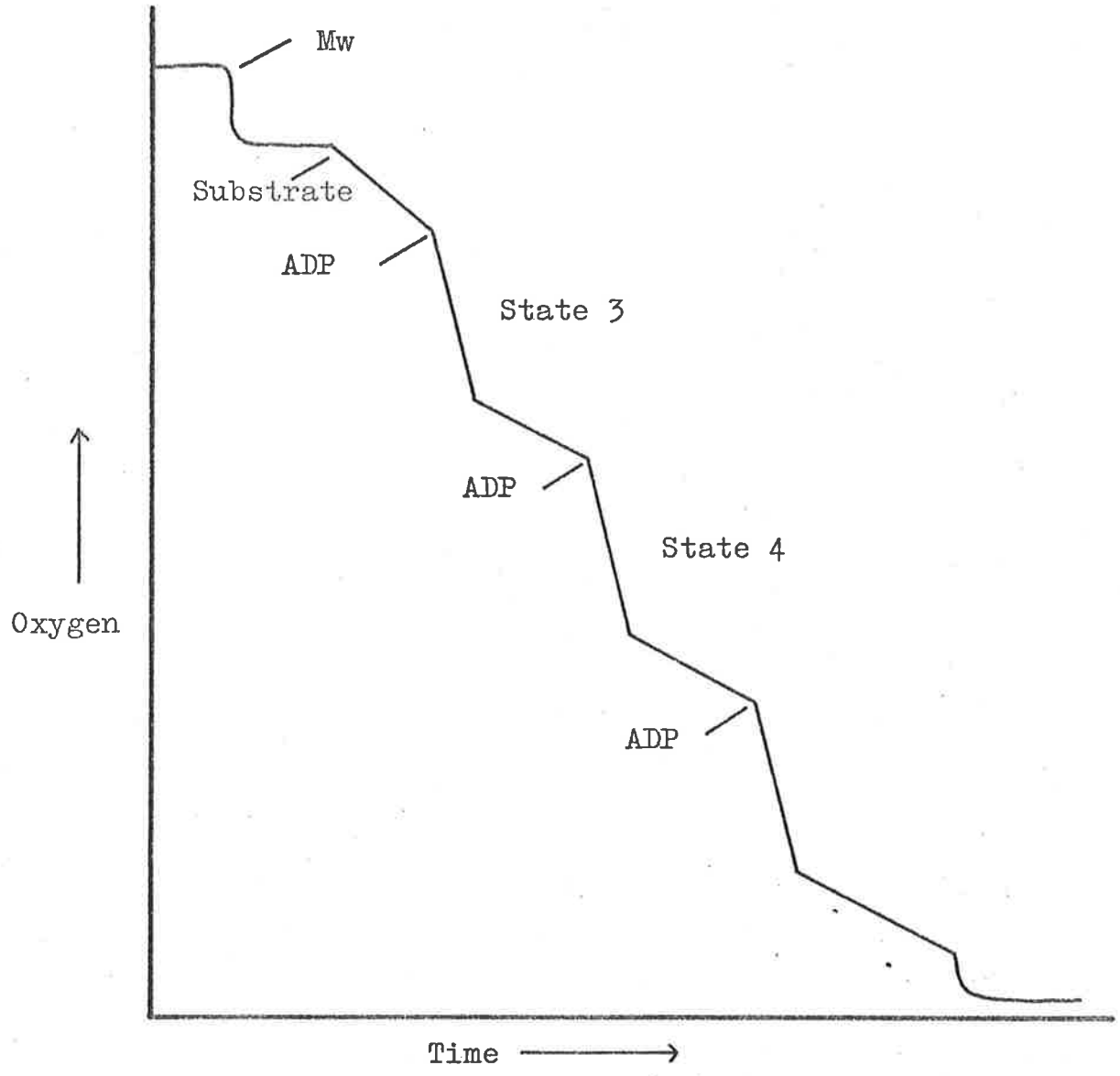
Outside = (C); Inside = (M); Intra membrane = (I)

well established		tentative		suggested		unknown	
cyt *c*	(C)	cyt c_1	(M)	NHI $_{1b}$	(M)	cyt *b*	(I)
cyt *a*	(C)	NHI III	(M)	cyt b_k	(M)	NHI_{11} 28	(C)
NADH-D	(M)	cyt a_3	(M)	CF_o	(M)	NHI_{1a}	(C)
succinate-D	(M)					transhydrogenase copper protein.	
F_1	(M)						

---

cyt = cytochrome; NHI = non haem iron; NADH-D = NADH dehydrogenase; succinate-D = succinate dehydrogenase. F_1 = fraction 1; Racker's soluble ATP-ase; CF_o = coupling factor 0, Racker.

Fig. 1-3



Diagramatic polarographic trace of mitochondrial oxidation.

Fig. 1-4

$$AH_2 + B + C \longleftrightarrow A \sim C + BH_2$$

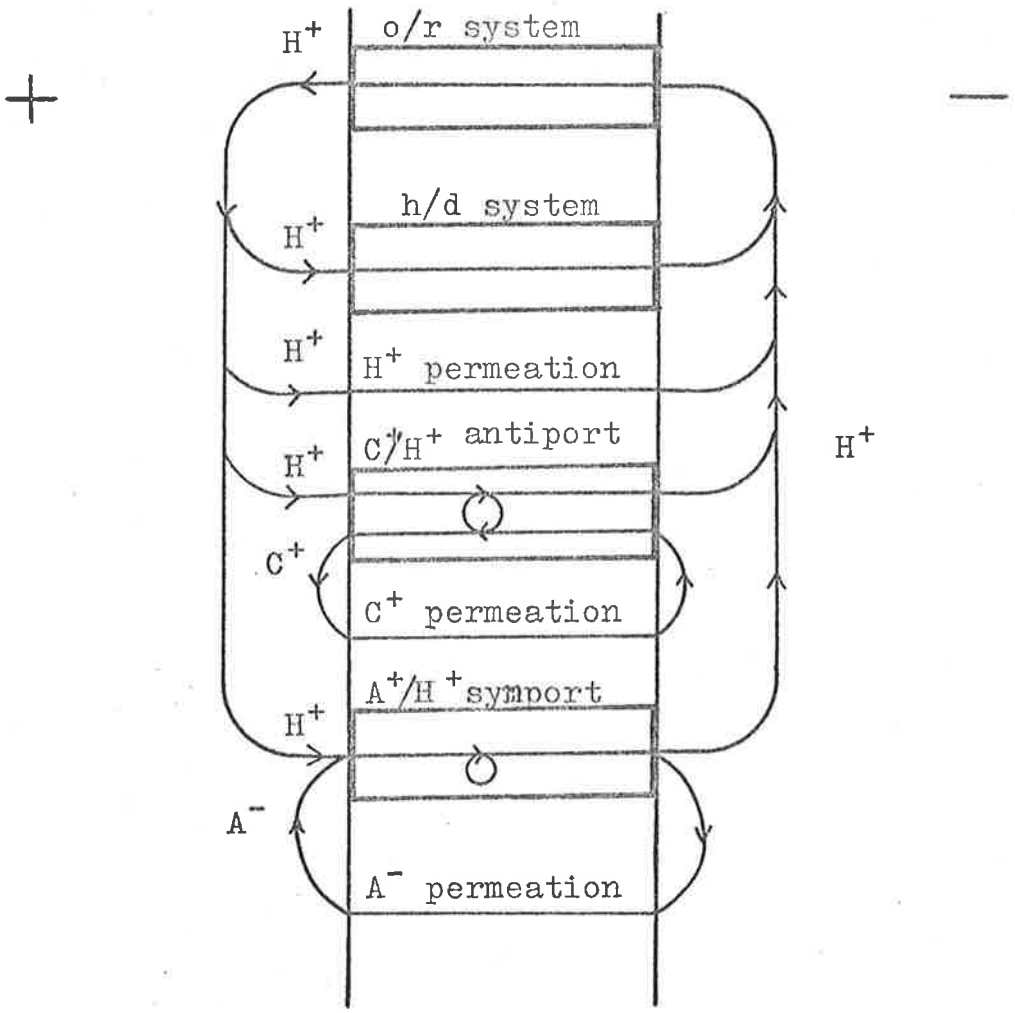
$$A \sim C + D \longleftrightarrow A + C \sim D$$

$$C \sim D + P_i \longleftrightarrow D \sim P_i + C$$

$$D \sim P_i + ADP \longleftrightarrow ATP + D$$

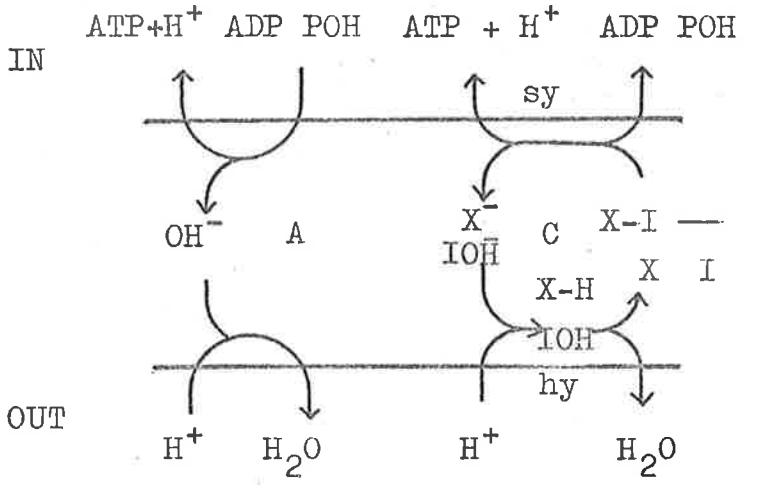
Reactions of the chemical hypothesis (Slater, 1953).

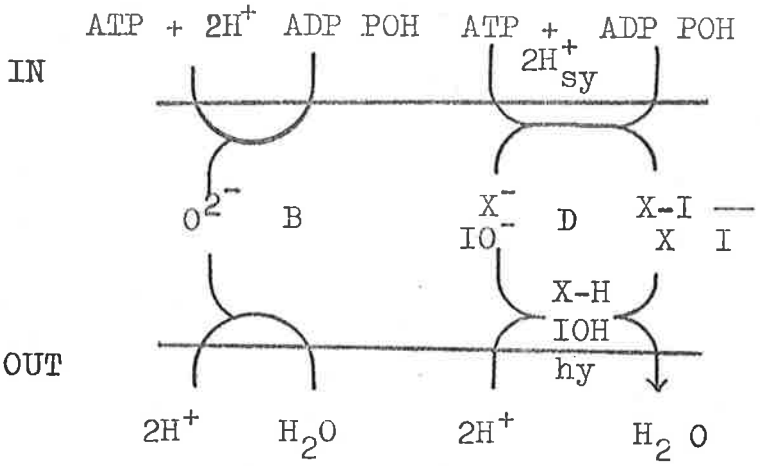
Fig. 1 - 5



Representation of the coupling membrane with proton translocating circuits, (Mitchell 1969).
o/r system = oxido-reductase system
h/d system = hydration-dehydration system
The permeant and exchange diffusion systems dissipate the proton current.

Fig. 1 - 6





(From Greville, 1969)

Alternative models for the action of mitochondrial ATP-ase. A and B are models for OH^- and O^{2-} transport. C and D are schemes by which this transport may be achieved, by means of the hypothetical ionizable groups, XH and IOH.

Hy = X-H hydrolase, Sy = X-I synthetase

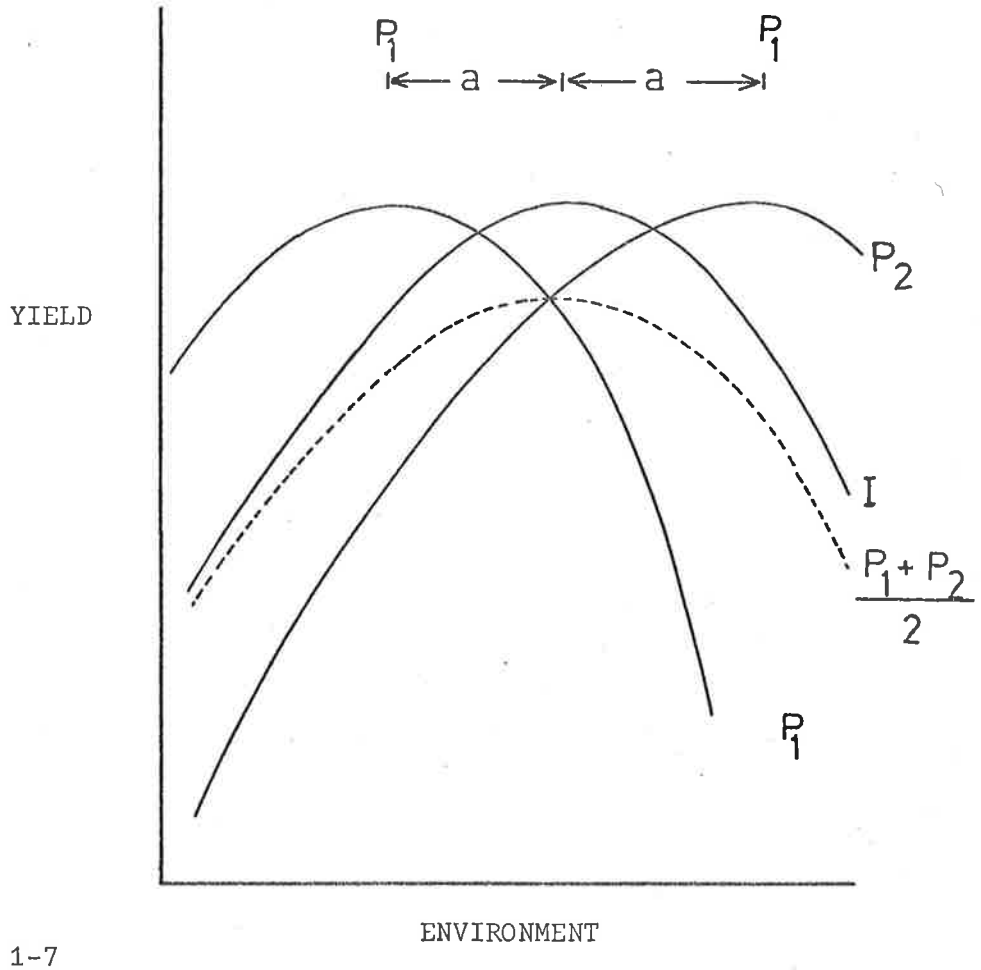


Fig. 1-7

Diagram from Knight 1973 showing the heterosis of an intermediate hybrid of two parents which have different yield responses to a given environment. P_1 if yield response of parent$_1$. P_2 if the yield response of parent$_2$. I is the intermediate response of a hybrid which exceeds both parents at its peak, and the mean parental response at all levels of environment X.

CH. IIA MATERIALS AND METHODS

MATERIALS

This project can be divided into three parts; the development of routine preparation and assay methods of wheat mitochondria; the detection of heterosis at the mitochondrial level and the verification of reports of mitochondrial complementation. For each of these three stages different sets of wheat varieties were used with material being supplied by the Waite Agricultural Research Institute, Australia or by De Kalb Shands Seed Co. U.S.A.

Readily available commercial lines (predominately Halberd, but also Heron, Gabo, Warimek and Wariquam) were used in the initial stage to establish the techniques.

Twelve parental varieties were included in the initial screening programme for complementation and heterosis. Gabo, Gamut, Festival, Falcon, Eureka, Heron, Mangavi, Chile IB and Nabawa being well established and often old varieties while Strain 52, and Pusa 4 were more recent breeding lines. The lineage of these lines is given in Appendix I, and it can be seen they are the products of complex crosses, many from common ancestors.

Seven crosses were prepared to obtain material for heterotic experiments. These were:-

Gabo x Falcon	Eureka x IRN	Gabo x Pusa 4
Heron x Gamut	Nabawa x Chile IB	

Heron x St 52 Mengavi x Festival

Crosses were made predominantly one way with small samples of reciprocal crosses being made for comparison of respiratory properties.

To confirm the validity of these trials, the lines used by Sarkissian in the majority of his experiments (28, 31 MS and 28 x 31 MS) were used as well as other imported parents and hybrids, (De Kalb lines 109 H, 261, 272 H as the parents and their hybrids 109 H x 261 and 109Hx 272 H). This material was used for heterosis and complementation experiments.

METHODS

Growth of Seedlings

Mitochondria were isolated from the etiolated coleoptiles of wheat, (*Triticum aestium* L.) which had been surface sterilized in saturated calcium hypochlorite for 5 minutes, washed thoroughly with distilled water and left to soak for 24 hours. The imbibed seeds were spread evenly between two double layers of moist paper towelling. After inverting a second tray to form a lid, the tray was placed in a dark cabinet. The cabinet was connected to an air pump and kept at 28 C. The seeds were watered daily.

Isolation of Mitochondria

A known weight of coleoptiles was harvested after 60-70 hours, placed in a petri dish on ice, and left to chill for five minutes. They were then ground by hand in a mortar and pestle with 10 ml of buffered grinding medium (Sarkissian & Srivastava 1968) containing

0.5 M sucrose, 0.067 M potassium phosphate buffer, pH 7.2, 0.001 M EDTA, and 0.75 mg/ml BSA. The pulp was filtered through two layers of nylon mesh and the filtrate was poured into a centrifuge tube. To ensure no loss of material all containers were rinsed with grinding medium and collected to give a final volume of 25 ml.

The time for this step on a single preparation was 90 - 120 secs as compared to the 60 - 80 secs claimed by Sarkissian and Srivastava (1968). Reducing the time of grinding would have meant compromising on the thoroughness of maceration.

Some criticism can be levelled at the technique of hand grinding for disintegration of tissue as this has a human element and thus could be liable to fluctuations in strength and duration. In an attempt to determine the effect of tissue disintegration, a mechanical device (the Sorval Omnimix) was tested, both with a macro and micro attachment at various speeds and for various times. The results of such preparations are shown in Table 2-1. Hand grinding produced more reliable preparations, and was chosen as the standard procedure. The ATP-ase activity which characterised all preparations was not affected by the grinding technique. Since the coleoptiles were 1 to 2 cm in length and the sample size was routinely 1 to 2 gm, other methods of mechanical disintegration were unsuitable.

A complete heterosis and complementation trial required the extraction of mitochondria from four sources, both parents, the

hybrid and a 1:1 mixture by weight of the parental shoots. These preparations were done either singly or in pairs, the latter being more convenient, although the total preparation time was increased from 14 to 17 mins. No detrimental effects on mitochondrial activity were observed as a result of this increase in time. As is shown in Table 2 - 2 it was advantageous to be able to use wheat of exactly the same age and to be able to test the two preparations simultaneously. Complementation experiments were also done by mixing equal volumes of parental mitochondria in situ in the oxygen electrode.

The centrifugation procedure was adapted from that reported by Palmer (1967), on methods of isolating mitochondria rapidly from tissue. Sarkissian and Srivastava (1968), McDaniel (1971), Mulliken & Sarkissian (1970) Sage and Hobson (1973), Ellis et al., (1973) and Pomeroy (1974) have all published variations on Palmer's method, and these are described in Table 2 - 3. These methods aim to separate the mitochondria from cell tissue in the fastest possible time to minimise damage by enzymes and facilitate comparisons between preparations. However, the methods fall into two groupings, those in which the filtrate is first cleared of large debris by a low speed spin and those where filtration is considered sufficient to trap all debris and the mitochondria are collected immediately. Methods E and F give the best comparison between these two types of preparation and Table 2 - 4 shows there was little difference between mitochondria prepared by either method. The low speed spin in F always sedimented a heterogeneous pellet larger than

the mitochondrial pellet produced by the second spin. This shows that large contaminants pass through the nylon filter and filtration should not be relied on to remove them. Rotation of the centrifuge tube through 90 degrees (Sarkissian & Srivastava 1969a) never completely separated the starch from the mitochondrial pellet necessitating careful separation with a glass rod. Thus spin 3 was omitted in a modification of method F, and this procedure was preferred as a routine method. Exception to this was when Sarkissian's work was being followed precisely and methods C and D were used.

Subfractionation of the Crude Mitochondrial Pellet

Rapid isolation techniques, especially that of Sarkissian and Srivastava (1969a), would be expected to sediment mitochondria, cell debris and other organelles. Discontinuous sucrose density gradient centrifugation can be used to separate these components from a crude pellet (Douce et al., 1972). The five layered gradients shown in Fig. 2 - 1 is made up of 1.78, 1.65, 1.5, 1.0 and 0.5 M sucrose with a one ml suspension of mitochondria in standard mannitol medium layered on top. It was centrifuged at 34,800 g for 30 mins. in a Sorvell SS-34 angle-head rotor and three pellets were obtained. The lightest pellet (fraction 1) occurred on the wall of the tube on the 1.5 - 1.65 M boundary with a second pellet (fraction 2) on the 1.65 - 1.78 M boundary, and a starch pellet (fraction 3) at the base of the tube in the 1.78 M sucrose.

The balancing tube was filled with the mitochondrial supernatant

and a gold coloured pellet (fraction 4) was also formed in this tube. It was resuspended separately and included in the assay programme.

Assay of Isolated Mitochondria

Polarographic Measurement of Oxygen Uptake

Changes in oxygen concentration by a suspension of isolated mitochondria was followed in a Rank Oxygen Electrode (Rank Bros. Bottisham, Cambridge, England) see Fig. 2-2. This vessel was chosen because it was of variable volume determined by a moving plunger sealed with an "O" ring. The contents of the vessel were maintained at 25 C by a jacket of circulating water and were stirred continuously by a glass covered metal flea and a magnetic stirrer. The electrode was covered with a teflon (0.0005" thick) membrane with a polarising voltage of 0.6 V provided by a variable voltage box. A pair of electrodes were connected to a twin channel recorder (model Electronik 194, Honeywell Controls Ltd. Great Britain), with a full scale deflection of 1 mV.

The respiratory control and ADP:O ratios defined by Chance & Williams (1956) and obtained from oxygen electrode studies formed the main criteria for intactness and the main basis of comparison between parental and hybrid mitochondrial types. The ADP-stimulated and ADP-limited rates of oxygen uptake, (State 3 and State 4 notation of Chance & Williams 1956) were measured on a per milligram protein per minute basis.

The substrates malate, KGA, succinate, pyruvate and NADH were all used at different times but the first two were chosen for

routine analysis during heterosis and complementation experiments. Malate additions were always accompanied by glutamate to prevent build up of oxaloacetic acid (OAA) which would result in product inhibition (Wiskich et al. 1964).

KGA required the presence of the cofactors thiamine pyrophosphate (TPP) and coenzyme A (CoA) for maximal oxidation rate and malonate was included to competitively inhibit succinate dehydrogenase and prevent lowering of the phosphorylation efficiency as measured by the ADP:O ratio. The protein content of each preparation was estimated by the method of Lowry et al. (1951) using BSA as a standard.

The assay medium was either the standard mannitol medium (Sarkissian & Srivastava 1968) with BSA or the standard sucrose medium (Wiskich 1974) without BSA. The sucrose medium was easier to use because there was less risk of air-bubbles being trapped within the chamber of the electrode.

The standard sucrose medium contained 0.25 M sucrose, 0.025 M EDTA and 5 mM $MgCl_2$, 0.01 M potassium phosphate buffer pH 7.2 and 0.01 M Tris/HCl pH 7.2 while the mannitol medium contained 0.3 M mannitol 0.01 M KCl, 0.075% w/v BSA, 5 mM Mg Cl_2, 0.01 M potassium phosphate buffer (pH 7-2) and 0.01 M Tris/HCl buffer (pH 7-2).

Cytochrome c Reduction

The activity of cytochrome *c* reductase was measured spectrophotometrically as described by Day and Wiskich (1974) on a Beckman spectrophotometer (model DB) connected to a Beckman linear-log 5 inch strip chart recorder. The rate of change in A at 550 nm was measured in a cuvette

with a one cm light path, at 21 C. The assay comprised 3 ml of standard sucrose medium with 25 mgm cytochrome *c* per ml, 10 mM KCN and, when used, 5 μM antimycin A. To this medium was added 0.1 - 0.2 ml of mitochondrial suspension containing approximately 0.2 mg protein and the reaction was initiated by adding 20 mM NADH, 15 mM malate or 15 mM succinate. The mM extinction coefficient for the reduced minus oxidised cytochrome *c* was taken as 19.8×10^{-3} cm^{-1} (Morton, 1958).

The cytochrome *c* reductase activity of intact mitochondria was compared to that of osmotically swollen mitochondria. The swelling was achieved by incubating 0.1 ml mitochondrial suspension with 1.5 ml of water for 45 secs and then adding 1.5 ml standard sucrose medium to restore an osmotic balance.

Cytochrome Oxidase Assay

Cytochrome oxidase can be studied in two ways, either by following the rate of disappearance of oxygen on its reduction to water, or by the rate of conversion of cytochrome *c* from the reduced to oxidised form. The former is followed polarographically with ascorbate/TMPD being the source of reducing power. Ascorbate reduces the highly lipid soluble TMPD complex which can penetrate the mitochondrial membranes to reduce the cytochrome a, a_3 complex directly, (Jacobs, 1960). Rates of oxygen uptake were observed in the presence and absence of ADP.

The procedure for osmotically shocking mitochondria has been

described for the cytochrome *c* reductase assay.

Cytochrome oxidase activity can be assayed spectrophotometrically by following the fall in absorbance associated with the oxidation of fully reduced cytochrome *c*. This was done using a Beckman Acta Spectrophotometer at 550 nm through a cuvette with a 1 cm light path. The milli molar extinction coefficient for reduced minus oxidized cytochrome *c* was taken as 19.8 (Morton, 1958). The assay medium consisted of 3 ml $\overset{\text{sucrose}}{\wedge}$ medium and a known concentration of reduced cytochrome *c* (30-50 μM), with or without ADP. The volume of mitochondrial suspension added varied from 25 to 100 μl and both intact and swollen particles were used.

The oxidation follows first order kinetics (Smith, 1955) and its rate depends on the ratio of concentrations of reduced to oxidized cytochrome *c*. The rate constant which has the units sec^{-1} is derived from the equation:

$$k = \log 10 \frac{[A_o]}{[A]} \frac{x\ 2.3}{t}$$

which is the integral of the first order rate equation:

$$\frac{d\ [A]}{dt} = k\ [A],$$

where [A] is the concentration of substrate and where k is taken as the slope of the semilog graph obtained by plotting $\log_{10}$ absorbance against time. The rate of reaction is calculated using k in the equation,

$$\text{Initial rate} = \frac{k \times [\text{cyto. } c]}{\text{mg protein}}$$

and is expressed as nmoles cytochrome *c* oxidized mg protein^{-1} min^{-1}.

Assay of Catalase activity

Catalase activity was estimated by following the rate of oxygen evolution when anaerobic particles are provided with H_2O_2. Measurements were made polarographically on the oxygen electrode system already described. Oxygen was displaced by bubbling the contents of the reaction vessel with nitrogen until less than 10% oxygen remained. At this time the vessel was sealed and H_2O_2 and a 5 ml aliquot of sample were added in quick succession to prevent a build up of oxygen due to the spontaneous breakdown of H_2O_2 with stirring. It was important to use very small volumes of particles to prevent the reaction exceeding the response time of the recorder.

Peroxidase determination

Peroxidase activity was located by the method of Gregory (1966) which used the coupled reactions shown in equation (1) and (2).

$$\text{Benzidine}_{(\text{red})} + H_2O_2 \xrightarrow{\text{peroxidase}} 2H_2O + \text{benzidine}_{(\text{ox})} \quad \text{---- (1)}$$

$$\text{Ascorbate} + \underset{(\text{blue})}{\text{benzidine}_{(\text{ox})}} \longrightarrow \underset{(\text{colourless})}{\text{benzidine}_{(\text{red})}} + \text{Ascorbate}_{(\text{ox})} \quad \text{---- (2)}$$

Hydrogen peroxide is present in excess but the quantity of ascorbate

is limited, thus the two reactions can continue until ascorbate is exhausted. This end point is signified by the appearance of a blue colour caused by the accumulation of oxidized benzidine.

The assay is set up by mixing one ml of each of the following solutions in order:

(1) A freshly prepared suspension of 2 ml saturated benzidine in ethanol, in 98 ml, 0.1 M sodium citrate buffer pH 5.3.

(2) A standard solution of 1 to 5 mM ascorbate.

(3) Approximately 0.2 M H_2O_2 (allowing for losses in dilution of the commercial stock).

The sample was injected into this mixture and the reaction timed from the time of injection to the appearance of the blue colour. As the end point was always gradual and the time of reaction in excess of that predicted by Gregory (1966), this determination was used qualitatively rather than quantitatively.

Whole tissue respiration

Warburg manometers were used to follow the rate of oxygen uptake of wheat tissue. Known weights of material were placed in the main compartment of the manometer vessels in 2 ml of 0.01 M phosphate buffer, pH 7.2. The centre-well contained 0.2 ml 20% KOH and the side arm 0.1 ml 3 mM DNP. Readings were taken every ten minutes.

Determination of heterosis

Comparisons between parents and F_1 hybrids were made on

germination, growth characteristics, whole tissue respiration (coupled and uncoupled) and on several biochemical criteria. The most important of the latter group were phosphorylation efficiency, (the ADP:O ratio) the rate of ADP-stimulated respiration, the activity of NADH - cytochrome *c* reductase and of cytochrome oxidase.

Electron microscopy of tissue

Wheat shoots which had been growing for 72 hrs were used in the etiolated condition or else exposed to light for 1½ hrs to initiate greening. One by three mm strips of tissue were cut from longitudinal sections of the shoots and fixed immediately in 2% glyceraldehyde in Millonig's phosphate buffer for 2 hrs. The material was then rinsed thoroughly, either in Millonig's buffer for post-fixation in 1% osmium tetroxide or in vernoyl acetate buffer before post-fixation in 1% $KMnO_4$. The sections were rapidly dehydrated in an alcohol series and flat-embedded in araldite for sectioning with a Reichert OmU3 ultramicrotome. The sections were mounted on "formvar" grids and stained with uranyl acetate followed by lead citrate and viewed with a Siemens Elmiskop.

Electron microscopy of pelleted material

A mitochondrial pellet was prepared by the method F, the modified method of Ellis et al. (1973) and tested on the oxygen electrode for activity before fixation. The volume of the mitochondrial suspension

was then made up to 10 ml with glyceraldehyde in manitol resuspending medium to a final concentration of 2% glyceraldehyde and left to stand for two hours. The fixed particles were re-sedimented and the fragments of the resulting pellet were well rinsed and post fixed in 1% osmium tetroxide for 12 hours. A rapid dehydrogenation in an alcohol series prepared the particles for embedding in araldite before sectioning, staining and viewing as already described.

CH. IIB RE-ASSESSMENT OF METHODS

The problem of mitochondrial quality will be discussed in detail in Chapter III. The routine procedure produced mitochondria with widely varying phosphorylative activity, partially due to the presence of ATP-ase activity. As the purpose of this study was to compare preparations among genotypes, this variation may give misleading results. Alterations were made in the method of preparation of tissue and the media used in an attempt to solve this problem.

Altering the starting material

The results of the use of mechanical disruption of the tissue have already been presented in part A, and as hand grinding was superior to the mechanical method, it was retained as the routine method of disruption. Many published methods recommend chilling tissue before disruption (Day and Wiskich, 1974; Ikuma, 1967; Wiskich et al., 1960), however in this study wheat shoots were left in a cold room for only five minutes before grinding. This was because longer chilling times (20 mins and 1 hr) inactivated mitochondria prepared from Halberd wheat. Mitochondria isolated after 20 minutes chilling had low rates of malate oxidation with no control. Mitochondria isolated after one hour showed no oxidative activity at all with malate or KGA. Brief lowering of the temperature immediately prior to tissue disruption was advantageous however, presumably because of cold-inactivation of the catabolic enzymes (Lieberman and Baker, 1965). The use of chilled grinding medium which

was in a partially frozen condition, produced somewhat more active mitochondria than the use of medium at 0 to 4C (Table 2-5). The ice facilitated the crushing of the shoot material in the shortest possible time allowing a rapid and thorough extraction of mitochondria.

Speed of isolation

Sarkissian and Srivastava (1970) stressed that the speed of separation of mitochondria from their cellular environment was an important factor in obtaining good mitochondria. Their stated time of 10 minutes for centrifugation is obviously in error as it does not allow for the centrifuge to run up and down as noted by Matlib et al., (1971). To test the validity of Sarkissian and Srivastava's (1971) claim, a comparison was made between mitochondria prepared with two centrifuges, Sorval models RC-2 and RC-2B. The latter centrifuge had a very rapid acceleration and efficient braking which could lessen the time taken by the RC-2 by up to 4 minutes, so that the time elapsed from the commencement of grinding to collection of the pellet was reduced to 13 or 14 minutes. Separation of the mitochondrial pellet from the starch pellet and resuspension took a further 3 to 4 minutes, thus the total time taken for a slow preparation is normally 21 to 22 minutes and the fast preparation, 17 to 18 minutes. Results of this comparison are presented in Table 2-6. Reducing the centrifugation time does produce some improvement in the control ratio and ADP:O ratio of mitochondria but they did not reach the values reported by Srivastava and Sarkissian

(1970).

Alterations to media

As discussed in the introduction, it is usual to include a metal chelator in disruption media to prevent damage to the mitochondria by high levels of cations, in particular calcium. Although the grinding medium of Sarkissian and Srivastava (1969) containing 1 mM EDTA, has been used by Pomeroy (1974) to prepare well coupled mitochondria, Sage and Hobson (1973) recommended changing the chelator to 2 mM EGTA which is specific for calcium (Chappell and Crofts, 1965). Ellis et al. (1973) tested the modified media and found that mitochondrial ADP:O ratios were enhanced with its use. However other changes (the addition of 1 mM TES buffer and the lowering of the sucrose concentration to 0.45 M) were made at the same time so the specific influence of EGTA as compared with EDTA had not been investigated. Wheat mitochondria were prepared in the sucrose grinding medium of Sarkissian and Srivastava (1968) with 1 mM EGTA being substituted for 1 mM EDTA in some preparations. The oxidation of malate and KGA by the two sets of mitochondria is shown in Table 2-7. Part A shows malate oxidation by two preparations made simultaneously with either EDTA or EGTA in the grinding medium. Part B shows mean respiratory control and ADP:O ratios for the first and second cycles of phosphorylation taken from 10 to 16 experiments, where tissue used was all 60 to 63 hours old at harvest. As will be discussed later, the activity of mitochondria was not determined by the genotype used but by the age of the shoots at

harvest. Thus part B combines many genotypes within one age class. Part B confirms the findings of Ellis et al. (1973) that for KGA oxidation use of EGTA medium is preferable. This did not apply to malate oxidation where the EDTA preparations had higher ADP:O ratios but lower control ratios than those prepared with the EGTA medium.

Calcium in low concentrations is known to uncouple mitochondria (Honda et al., 1958; Lehninger, 1970) and in high concentrations to inhibit electron transport entirely. It is thought that the stimulation of substrate oxidation and the lowering of control is due to competition between calcium uptake and phosphorylation, since both processes utilize the membrane potential (Mitchell, 1966). It is not known why malate oxidation should be less sensitive to calcium uncoupling than KGA oxidation. Control may be lowered in response to factors outside the mitochondria, in particular the presence of non-mitochondrial ATP-ases which are stimulated by Ca^{++} and Mg^{++} (Lehninger, 1970). Since EGTA is specific for calcium, it does not protect mitochondria from magnesium. Magnesium is an essential cofactor for the mitochondrial ATP-ase as ADP enters the reaction as Mg-ADP (Nobel, 1974). Matlib et al., (1971) found that addition of 5 mM $MgCl_2$ to their Mg^{2+} free assay medium caused complete loss of control in Vicia faba mitochondria. It is possible that the endogenous levels of magnesium are above the Km requirement of the ATP-ase in some tissues such as beans and perhaps wheat and that further additions of $MgCl_2$ only serve to stimulate other ATP-ases and obscure control. It is of interest to note that these authors used a

rapid isolation technique. High Mg^{2+} may be a feature of such preparations.

Table 2-8 shows a comparative study of Halberd mitochondria with and without 5 mM $MgCl_2$ in the standard sucrose medium. The absence of Mg^{2+} in no way impaired the oxidation of malate or KGA as the rates of oxygen uptake, control and ADP:O ratios were closely comparable in both experimental situations. In fact the ADP:O ratios for the $-Mg^{2+}$ experiments were slightly higher for both substrates. Thus there must be more than 5 mM magnesium in the mitochondrial suspension, even though EDTA was included in the isolation medium. Some of this would be contributed from the resuspending medium which had 5 mM $MgCl_2$ but the final concentration from this source would be 1.36 mM, well below the required level. Thus in routine experiments with standard sucrose medium, this high level of magnesium could be expected to stimulate ATP-ase activity which would mask control (Matlib et al., (1971). This could explain occasional preparations which, although exhibiting control with malate as substrate, completely lacked control with KGA which is oxidized at a slower rate. Although mitochondria prepared with EGTA gave improved KGA oxidation the need to protect the preparations from high levels of both calcium and magnesium led to the chelator EDTA being routinely employed with EGTA being used where indicated.

The use of cysteine in preparations

Excised wheat shoots brown rapidly on standing indicating the

presence of polyphenol oxidase activity. As discussed in the introduction, mitochondria can be protected from the harmful action of such oxidases by inclusion of the reducing agent cysteine. The effect of including 5 mM cysteine in the disintegration and resuspension medium on the activity of wheat mitochondria oxidizing malate or KGA was thus investigated. Mitochondria were prepared simultaneously in normal or plus cysteine media and were tested polarographically. The results of these trials are shown in Table 2-9.

Inclusion of 5 mM cysteine in the grinding and resuspension media did not consistently enhance the ADP:O ratio for malate or the respiratory control ratios for either substrate. The ADP:O ratios for KGA in the presence of cysteine are greater than the minus cysteine treatment for this substrate. The percentage inhibition of state 4 by oligomycin was used as a measure of ATP-ase activity which was shown to be present in both preparations, being higher in the presence of cysteine. Since cysteine did not relieve the ATP-ase problem and there was no increase in control or ADP:O ratios, it was excluded from the media in routine preparations.

Greenway and West (1973) showed that concentrations of 20 mM glutamate in their extraction media greatly enhanced the activity of Zea mays root mitochondria. Glutamate is known to prevent OAA inhibition of mitochondrial malate dehydrogenase (Wiskich& Bonner 1963). Their medium also included 100 mM phosphate, ten times the concentration

normally used for assay of mitochondria; these variations were tested independently to see whether any reduction in ATP-ase and enhancement of malate oxidation could be obtained from their use. The comparative activity of mitochondria prepared in the presence or absence of 20 mM glutamate are shown in Table 2-10. The presence of glutamate suppressed respiratory control ratios, ADP:O ratios and rates of oxygen uptake in both malate and KGA oxidation. Thus the beneficial effects of glutamate reported for maize root mitochondria did not apply to wheat mitochondria.

The disintegration medium recommended by Sarkissian and Srivastava (1968, 1969), the same as that used by Hanson (1963), contains a high concentration of phosphate buffer. Alternative methods recommend the exclusion of phosphate buffer from the isolation medium altogether (Wiskich and Bonner, 1963; Ikuma, 1970) regulating pH by dropwise addition of KOH. This is because Bonner (1967) claimed that phosphate extracts cytochrome *c* and ruptures the mitochondria. Since Greenway and West (1973) prepared good mitochondria from maize roots in the presence of 100 mM phosphate, a titration of phosphate concentration in the sucrose grinding medium against mitochondrial activity was performed. Four separate preparations were made using sucrose grinding medium in which the phosphate concentrations were 25, 50, 80, and 100 mM. Fig. 2-3 illustrates the response of the respiratory control ratio, the ADP:O ratio and the state 3 and state 4 rates of oxygen uptake to increasing phosphate concentration during malate oxidation. The

increase from 1.4 to 1.8 for the ADP:O ratio was slight and none of the preparations showed the usual phosphorylation efficiency associated with malate oxidation. The respiratory control increased because the state 3 rates increased while the state 4 rates remained constant. This faster state 3 rate may reflect enhanced substrate availability as phosphate is required for the entry of malate (Chappell and Haarhoff, 1967; Chappell, 1970 ; 1968; Chappell, 1968). Mitochondria prepared in a high phosphate media may be loaded with phosphate and thus be able to immediately exchange phosphate for malate ions. As the phosphorylation efficiency was little altered by the concentration of phosphate in the grinding buffer, the intermediate value of 67 mM was retained as part of the routine disintegration medium.

The published methods for wheat mitochondria all recommend the use of buffered manitol medium for resuspension of the mitochondrial pellet. This is a result of the recommendation of Bonner (1967) who suggested that mitochondria were easier to separate from contaminating starch when manitol was used. However sucrose may be used in all stages of preparation and testing (Wiskich and Bonner, 1963) with mitochondria resuspended in 0.4 M sucrose and assayed in the standard sucrose medium. In order to determine which media were most favourable, mitochondria were prepared in sucrose grinding medium and then resuspended and tested under the four conditions set out below.

Resuspending medium	Assayed medium
RM_1	S_1 (A) RM_1 (B)
0.4 M Sucrose	S_1 (C) RM_1 (D)

RM_1 = manitol resuspending medium

S_1 = standard sucrose medium

See appendix 2 for details of media.

Trials were carried out over several days with both malate and KGA as substrate and the results are shown in Table 2-11. These trials showed malate oxidation to be relatively insensitive to changes in osmoticum. KGA was more sensitive with four cycles of respiration only being obtained for three experiments, two from group A and one from group C. These latter cycles do not appear in the table of means. Treatments which use manitol resuspending medium (RM_1) as the assay medium had conspicuously low control ratios, ill defined state 3 to state 4 transitions and only one or two cycles of phosphorylation per experiment. Since the same mitochondria were used for treatment A and B and for C and D the differences seen for KGA oxidation were attributed to the use of RM_1 during assay. The data favoured the use of RM_1 as a resuspending medium in preference to 0.4 M sucrose. Again the choice of medium for resuspension had little influence on the pattern of malate oxidation so the decision to use RM_1 was based on the improvement in activity of mitochondria using KGA under condition A when compared to B. As the mean rates of oxygen uptake are consistently higher under condition A, it is suggested that mitochondria are most active when resuspended in a buffered manitol medium with BSA present, and when assayed in the standard sucrose medium (S_1) and these media were selected for routine wheat mitochondrial

preparations.

Conclusion

The routine procedures for preparation and testing of wheat mitochondria described in part A, were selected because they gave the most active mitochondria possible within the restriction of the rapid preparation technique. Preparations consistently showed oligomycin inhibition of state 4 after numerous changes to the media and tissue. Conventional slow preparations of wheat mitochondria may avoid some of these problems but the rapid technique was retained firstly because it allowed comparison of mitochondria of four wheat genotypes in a four hour period and secondly, because it is with techniques of this general type that all previous work on mitochondrial heterosis and complementation has been done. Since one aim of this project is to determine the feasibility of mitochondrial predictions of heterosis in wheat lines as reported (Sarkissian and Srivastava, 1969, 1971; Sarkissian, 1972; Sage and Hobson, 1973) it was necessary to follow published procedures closely for comparative experiments and thus it became necessary to characterize the mitochondria produced in this way. The activity of mitochondria and the possible causes of the ATP-ase activity will be considered in the following chapter.

TABLE 2-1

A comparison of mechanical and manual methods of tissue disintegration from 60 to 65 hour old Heron and Gabo wheat shoots.

SUBSTRATE		MORTAR AND PESTLE		MICRO ATTACHMENT		MACRO ATTACHMENT	
cycle		RCR	ADP:O	RCR	ADP:O	RCR	ADP:O
KGA	1	1.3	3.7	2.91	4.38	-	-
	2	-	-	1.83	3.82		
Malate	1	2.67	2.72	2.63	2.28	1.17	1.03
	2	2.43	1.76	-	-	-	-
	1			2.92	1.92	1.28	1.4
				39.4%		32.1%	

Manual grinding with a mortar and pestle was compared to mechanical disruption in either of the two attachments of a Sorvell omnimix. 1 gm wheat shoots were disrupted in 25 ml of a sucrose grinding medium, 0.5 M sucrose, 0.001 M EGTA, 0.067 M potassium phosphate buffer at pH 7.2, and 0.75% BSA. The protein added for manual, micro attachment and macro attachment respectively were 1.7, 1.5 and 2.34 mg protein per ml. The preparations were assayed as described in Ch. II in 1 ml standard sucrose medium to give a final volume of 1.47 ml for malate and 1.61 ml for KGA trials. Reagents were present in the following concentrations, malate 13.6 mM, glutamate 30.4 mM, KGA 18.6 mM, malonate 12.4 mM, CoA 186.5 µg/ml, TPP 124 µM. ADP additions were 220 µM. RCR - respiratory control ratio.

TABLE 2-2 Phosphorylative activity of synchronous preparations.

PREPARATION			RCR	ADP:O	STATE 3	STATE 4
1A	cycle	1	2.19	2.42	40	18
	"	2	2.14	2.21	48	23
1B	"	1	2.26	2.42	33	15
	"	2	1.88	1.97	35	18

The pattern of malate oxidation by two preparations of Heron wheat mitochondria prepared synchronously and tested simultaneously as described in chapter II. Mitochondria were incubated in 1 ml standard medium with 13.6 mM malate and 34 mM glutamate and phosphorylation initiated by additions of 214 uM ADP. 1A contained 4.9 and 1B 4.3 mg mitochondrial protein per reaction. RCR is respiratory control ratio and rates are expressed as nmoles O_2.mg protein^{-1}.min^{-1}.

Table 2-3

Variations of the Rapid Isolation Procedure for Wheat Mitochondria.

All methods use mortar and pestle for tissue disruption and filter the brei with nylon cloth. Details of media are given in appendix II.

Text Ref.	Source	Procedure
A.	Sarkissian and Srivastava (1968)	Spin 1. 40,000 g for 2 mins. Change GM_1 without dislodging the pellet. Spin 2. 20,000 g for 2 mins. Resuspend pellet in manitol medium (minus BSA).
B.	Mulliken and Sarkissian (1970)	Spin 1. 10,000 g for 1 min. Retain supernatant. Spin 2. 40,000 g for 1 min. Resuspend pellet in manitol medium (minus BSA).
C.	Modification of method B used in this study	Spin 1. 10,000 g and brake immediately. Retain supernatant. Spin 2. 40,000 g for 2 mins. Resuspend pellet in manitol medium (minus BSA).
D.	Sarkissian and Srivastava (1969) "Simple Trick"	Spin 1. 40,000 g for 2 mins. Change grinding medium (GM_1) without disturbing the pellet. Rotate centrifuge tube 90°. Spin 2. 20,000 g for 2 mins. Resuspend in manitol medium (minus BSA).
E.	Sage and Hobson (1973)	Spin 1. 40,000 g for 1 min. Change grinding medium and rotate the tube 90°. Spin 2. 10,000 g for 25 secs. Resuspend in manitol medium.
F.	Ellis et al., (1973)	Spin 1. 2,000 g for 2 mins. Retain supernatant. Spin 2. 30,000 g for 3 mins. Rotate tubes 180°. Spin 3. 6,000 g for 1 min. Resuspend in manitol medium (minus BSA).
G.	Pomeroy (1974)	Spin 1. 2,000 g for 5 mins. Retain supernatant. Spin 2. 20,000 g for 4 mins. Resuspend pellet in 10 ml grinding medium. Spin 3. 1,500 g for 5 mins. Retain supernatant. Spin 4. 48,000 g for 15 min. Resuspend pellet in manitol medium.

Table 2-4

TESTING THE NECESSITY OF AN INITIAL LOW-SPEED SPIN
(METHOD E vs METHOD F)

	Cycle	Malate		KGA	
Method E		RCR	ADP:O	RCR	ADP:O
	1	2.50	2.92	6.50	2.80
	2	2.73	2.70	-	-
	3	3.40	2.08	-	-
Method F					
	1	2.47	3.07	2.75	3.62
	2	2.18	2.70	1.54	3.16
	3	-	-	1.47	4.05

Malate oxidation was observed in 1.47ml in the presence of 13.4 mM malate and 34 mM glutamate added to 1ml standard sucrose medium. KGA oxidation was observed in 1.56ml total volume in the presence 13.7 mM KGA, 13.7 mM malonate, 13.7 µM TPP and 267 µM CoA were added to 1ml standard sucrose medium. 2.01 mg mitochondrial protein was present and 250 µM ADP initiated each phosphorylation cycle.

Table 2-5

The effect of frozen grinding medium on mitochondrial activity.

		Unfrozen			Frozen	
		RCR	ADP:O		RCR	ADP:O
A.	cycle (1)	1.73	2.28	B.	4.4	2.47
	cycle (2)	1.74	2.03		4.2	2.35
	cycle (3)	-	-		5.0	3.66

Two mitochondrial preparations from Heron wheat coleoptiles were prepared by the method of Sarkissian and Srivastava, (1969) on different days. Malate oxidation was measured polarographically with 1.05 mg and 0.93 mg protein for treatment A and B, respectively, added to 1 ml of standard sucrose medium in a total volume of 1.47 ml. Final concentrations of reagents were 13.6 mM malate, 34 mM glutamate, and cycles of phosphorylation were initiated with 238 µM additions of ADP.

Table 2-6

Speed of preparation and mitochondrial activity.

SLOW	RCR	ADP:O	State 2	State 3	State 4
Cycle 1.	1.92	2.18	17.5	53	27.5
Cycle 2.	1.79	2.01	-	58	32.0
FAST					
Cycle 1.	2.22	2.42	13.5	54	24.5
Cycle 2.	2.01	2.09	-	61.5	30.0

Mitochondria were prepared by method F (Ellis et al., 1973) with a preparation time of 21 or 17 minutes for the slow and fast preparation respectively (see text). These mitochondrial suspensions contained 2.24 and 2.92 mg protein and were added to 1.0 ml standard sucrose medium to give a volume of 1.47 ml. Reagents were added to the final concentration 13.6 malate, 34 mM glutamate and cycles of phosphorylation were initiated by addition of 214 µM ADP. Results are the means of two experiments.

Table 2-7

The effect of EDTA and EGTA on oxidative phosphorylation by mitochondria.

PART A		EDTA		EGTA	
Malate					
RCR	cycle (1)	2.78		3.37	
	cycle (2)	2.58		2.84	
ADP:O	cycle (1)	1.89		1.18	
	cycle (2)	1.75		1.03	
PART B					
KGA			n		n
RCR	cycle (1)	1.91	11	2.87	16
	cycle (2)	1.69	10	2.4	12
ADP:O	cycle (1)	5.02	11	5.01	16
	cycle (2)	2.86	10	3.68	12

Mitochondria were prepared by method D (Sarkissian and Srivastava, 1969) in part A and by either method E or F (Sage and Hobson, 1973, or Ellis et al., 1973) in part B. Malate assays were conducted in a total volume of 3.29 ml in the presence of 5 mM malate and 10 mM glutamate with successive additions of 255 µM ADP. Additions of 0.64 and 0.56 mg protein were made in the EDTA and EGTA trial respectively.

Part B. Various quantities of protein added as 0.4 ml aliquots to 1.0 ml standard sucrose medium to a final volume of 1.52 ml. KGA^{137}mM, $malonate^{137}$mM, TPP^{137}µM and CoA^{267}µM were also present. ADP concentration varied but was approximately 300 µM.

Table 2-8

The effect of Mg^{++} on the oxidation of malate and KGA. Mitochondria from Halberd wheat were prepared by method F, (Ellis et al., 1973).

		MALATE		KGA	
		$-Mg^{++}$	$+Mg^{++}$	$-Mg^{++}$	$+Mg^{++}$
RCR	cycle 1	3.07	2.82	2.29	2.48
	cycle 2	2.76	2.88	2.67	2.0
ADP:O	cycle 1	2.18	2.09	2.74	2.54
	cycle 2	2.09	1.53	1.34	1.98
State 3	cycle 1	172	144	71	77
Rate	cycle 2	204	221	102	91
State 4	cycle 1	56	51	31	31
Rate	cycle 2	74	77	76	46

Assays for oxidative phosphorylation were carried out in a total of 1.47 or 1.56 ml. To 1 ml standard sucrose medium minus MgCl was added 2.0 mg mitochondrial protein resuspended in manitol medium (RM_1) which contained 5 mM $MgCl_2$. Additions of either 13.6 mM malate and 34 mM glutamate in 1.47 ml or 18.65 mM KGA, 12.4 mM malonate, 1.24 µM TPP and 186.5 µg/ml CoA in 1.56 mls.

$+Mg^{++}$ trials contained 5 mM + 1.36 mM $MgCl_2$.

$-Mg^{++}$ trials contained 1.36 mM (from RM_1) $MgCl_2$.

Phosphorylation was initiated by additions of 240 µM ADP

Rates are expressed as nmoles O_2 · mg $protein^{-1}$ min^{-1}.

Table 2-9

The action of cysteine on wheat mitochondria

		Malate		KGA		
		- cysteine	+ cysteine	- cysteine	+ cysteine	
RCR cycle	(1)	2.83	1.81	*2.0	2.16	*1.85
	(2)	2.85	2.21	1.7	1.82	1.64
	(3)	-	2.35	1.6	-	
ADP:O	(1)	2.55	2.18	3.46	3.53	2.21
	(2)	2.10	2.25	2.65	2.89	2.21
	(3)	-	2.22	2.09	-	
State 3	(1)	76.5	92	23	47	35
rate	(2)	102.5	114.5	30	55.5	39
	(3)		115.0	33	-	
State 4	(1)	26	52	11	22.5	19
rate	(2)	34.5	50.5	18	29.5	22
	(3)	-	49.5	20	-	
% inhibition of state 4 by oligomycin		11.1%	26.5%	-	-	

Except for * which is a single experiment, results are means of two experiments, conducted with 60 hr old Heron wheat. Mitochondria were prepared by the method F (Ellis et al., 1973) in sucrose grinding medium (GM_1) and manitol resuspending medium (RM_1) with or without 5 mM cysteine in both media. Assays were conducted under the same conditions as in Table 2-8 with 0.4 ml mitochondrial protein containing 1.46 or 3.54 mg protein for the - cysteine preparations and 1.38 or 3.3 mg protein for the + cysteine preparations. Rates of uptake are expressed as nmoles $O_2 \cdot$ mg protein^{-1} min^{-1}.

Table 2-10

The effect of 20 mM glutamate on oxidative phosphorylation by Gabo wheat mitochondria.

		- Glutamate	+ Glutamate	- Glutamate	+ Glutamate
		Malate		KGA	
RCR	cycle (1)	2.75	2.4	2.24	1.9
	cycle (2)	2.75	1.53	1.84	1.78
	cycle (3)	-		1.57	1.3
ADP:O	cycle (1)	2.15	2.26	3.97	3.66
	cycle (2)	2.06	1.9	3.11	2.44
	cycle (3)			2.23	2.44
State 3 rate	(1)	112	90	43	40
	(2)	130	102	55	41
	(3)			57	43
State 4 rate	(1)	41	38	19	21
	(2)	47	67	30	23
	(3)			36	33

Mitochondria were prepared from 63 hr wheat shoots by method F (Ellis et al., 1973) with sucrose grinding medium (GM_1) and manitol reaction medium (RM_1). 20 mM glutamate was added to both these media for the + glutamate trial.

Mitochondria were assayed under the same conditions as described for Table 2-8, except that additions of 266 µM ADP were used to initiate cycles of phosphorylation. Malate and KGA experiments were done on the same preparation so that 2.0 mg and 1.72 mgm protein were added for the - glutamate and + glutamate respectively.

Table 2-11

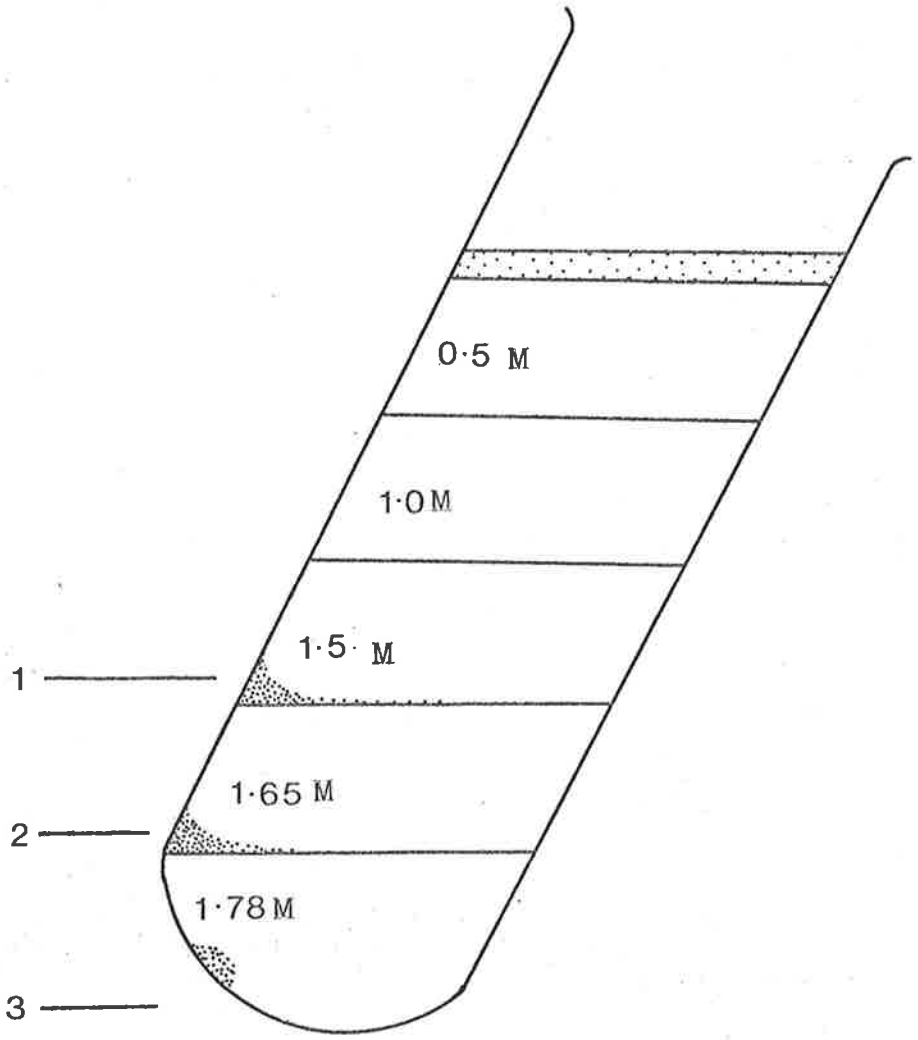
The influence of the composition of the resuspending and assay medium on mitochondrial oxidation of malate and KGA.
Conditions of assay are as described for Table 2-8 with final volumes of 1.47 and 1.61 ml for malate and KGA trials. Additions of 252 µM and 230 µM ADP were made in malate and KGA experiments to start phosphorylation cycles. Mitochondrial protein added ranged from 0.59 to 1.18 mg for the six preparations used for malate trials and from 0.8 to 1.2 mg for the four preparations used in the KGA experiments.

Table 2-11

Mitochondria Resuspended	Mitochondria Assayed in	
RM_1	S_1 A	RM_1 B
0.4 M Sucrose	S_1 C	RM_1 D

MALATE OXIDATION		A	B	C	D
RCR	cycle (1)	2.6	2.87	3.24	2.49
	cycle (2)	2.51	2.88	2.25	2.12
ADP:O	cycle (1)	2.98	2.33	2.97	2.65
	cycle (2)	2.44	2.0	1.94	2.05
State 3	cycle (1)	12.8	100.0	75.4	84.5
Rate	cycle (2)	150.4	107.5	100.7	84.5
State 4	cycle (1)	49.8	45.0	33.6	33.9
Rate	cycle (2)	68.5	50.0	47.8	36.5
		n = 3	n = 2	n = 3	n = 2
KGA OXIDATION					
RCR	cycle (1)	2.67	1.2	2.03	1.33
	cycle (2)	1.84	-	1.58	1.21
	cycle (3)	1.61	-	-	-
ADP:O	cycle (1)	4.11	5.3	5.34	4.88
	cycle (2)	3.15	-	4.42	3.25
	cycle (3)	3.08	-	-	-
State 3	cycle (1)	48.0	22.1	41.3	25.0
Rate	cycle (2)	50.8	26.1	42.9	32.3
	cycle (3)	50.3	-	-	-
State 4	cycle (1)	18.7	17.6	19.6	17.9
Rate	cycle (2)	24.9	-	26.0	27.1
	cycle (3)	30.8	-	-	-
		n = 2	n = 2	n = 2	n = 2

Fig 2 - 1



Sucrose density gradient described in Ch II and the fractions collected from a crude mitochondrial suspension which had been layered onto the gradient and spun at 34,800g for 30 minutes.

Fig.2 -2

Diagram of a Rank Oxygen Electrode.

(1) Vessel walls in which water circulates for temerpature control.

(2) Movable plunger sealed with a rubber "O" ring.

(3) Reaction chamber.

(4) Access slot.

(5) Glass covered magnetic flu.

(6) Teflon membrane (0.0005 ins. thick).

(7) Platignum cathode.

(8) Circular anode.

(9) Saturated KCl solution.

(10) Magnetic stirrer.

(11) Threaded retaining clamp.

Fig. 2 - 2

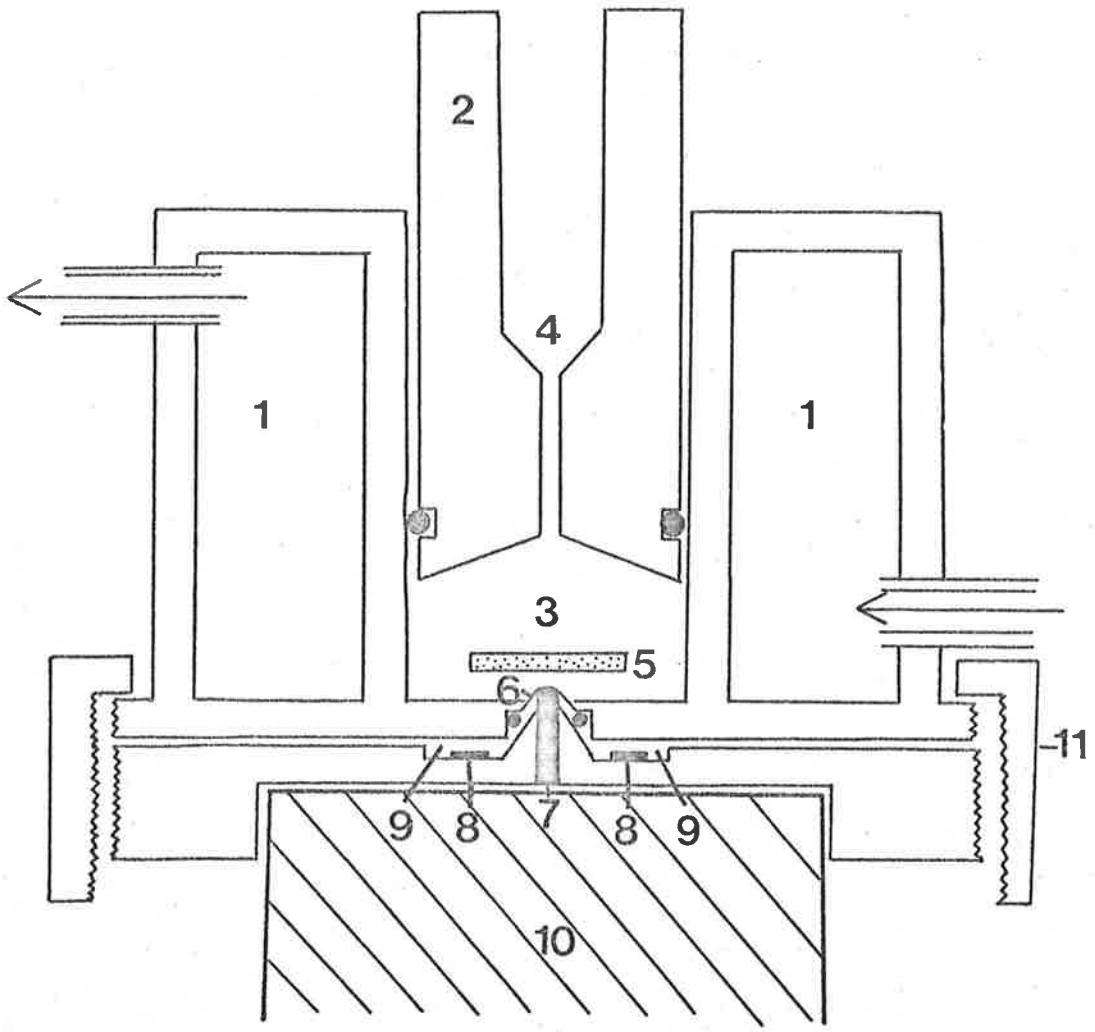
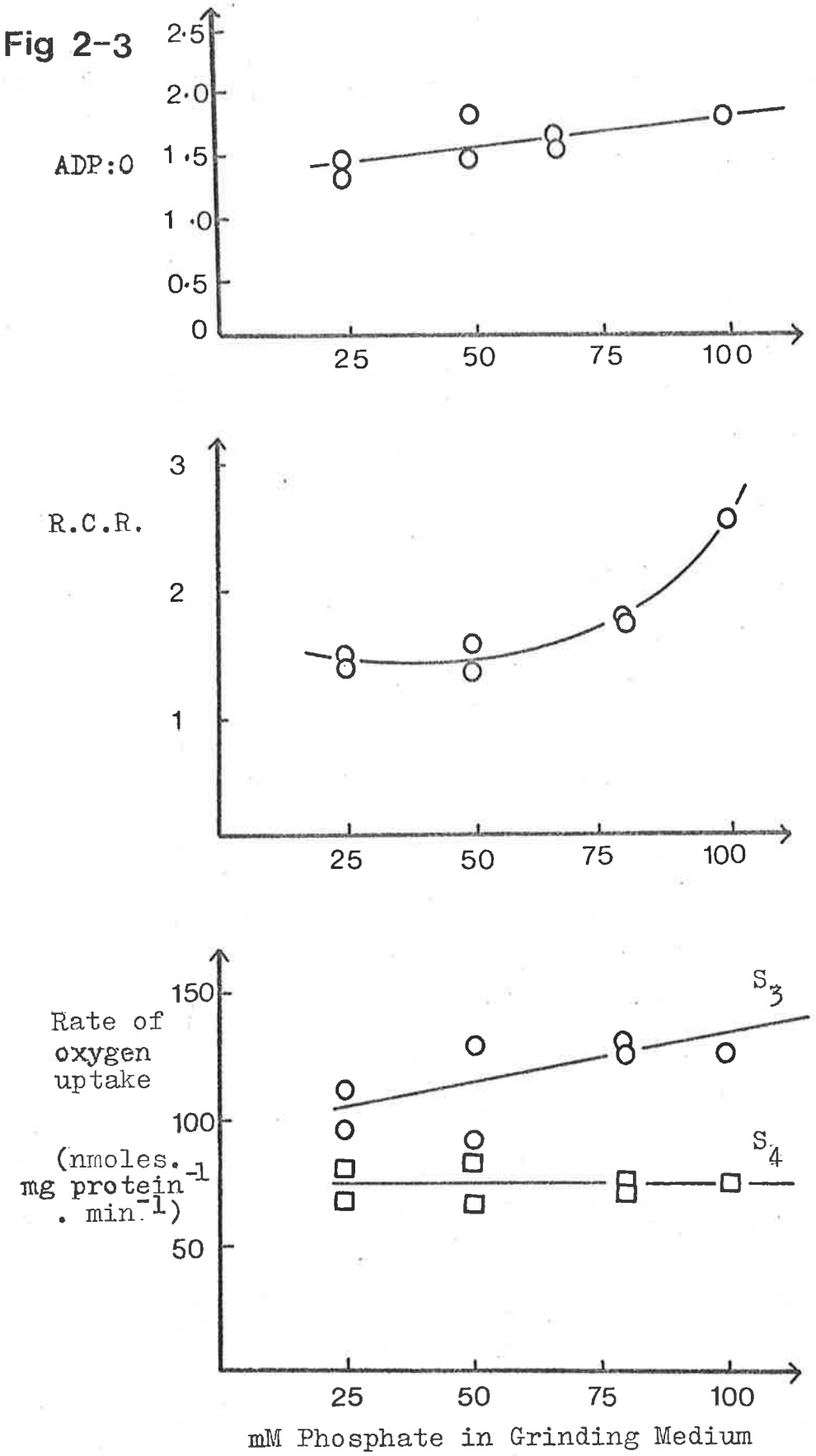


Fig. 2-3

Grinding buffer phosphate concentration on mitochondrial activity. Mitochondria were prepared from 65 hr Gabo wheat shoots by the modified method F (Ellis et al., 1973). The sucrose grinding medium (GM_2) for each preparation had 25, 50, 80 and 10 mM potassium phosphate buffer (pH 7.2). All experiments except the 100 mM phosphate preparation were duplicated and points are the cycle 1 values for the two preparations. Mitochondrial oxidation of malate in the presence of glutamate was measured polarographically as described under Table 2-8. Additions of 0.98, 0.82, 0.78 and 0.9 mg protein were made for the 25, 50, 80 and 100 mM phosphate preparations respectively to a total volume of 1.47 ml. Phosphorylation was initiated by addition of 230 µM ADP.

Fig 2-3


ADP:O
2·5
2·0
1·5
1·0
0·5
0
25
50
75
100
R.C.R.
3
2
1
25
50
75
100
Rate of oxygen uptake
(nmoles. mg protein^{-1} . min^{-1})
150
100
50
S_3
S_4
25
50
75
100
mM Phosphate in Grinding Medium

CH. IIIA

CONTAMINATION IN RAPIDLY PREPARED MITOCHONDRIAL PELLETS

INTRODUCTION

Consideration of the size and density of the organelles within a plant cell leads one to expect that mitochondria prepared by the fast technique (Palmer, 1967; Sarkissian and Srivastava, 1968) would be contaminated by particles of larger and smaller size. This would be accentuated in preparations where the high speed spin is done first. Nylon cloth or miracloth may filter nuclei and cell wall fragments but all particles of chloroplast size or smaller would pass through the filter and be sedimented in the first spin at 30,000 x g. The centrifugal force of the high speed spin is great enough to pellet particles lighter than mitochondria, even though it is maintained for only a short time.

That pellets from conventional differential centrifugation procedures are heterogeneous has been demonstrated by Parish and Rickenbacher (1971) who subjected pellets (produced by spinning a leaf homogenate successively at 1,000, 6,500, and 20,000 x g each for 20 mins) to sorbitol-ficol-sucrose gradient centrifugation. Each fraction was found to contain whole or fragmented chloroplasts, peroxisomes and mitochondria, as identified by electron microscopy and enzymic examination.

There have been no previous reports examining the purity of mitochondria prepared by the rapid technique or considering the

influence contaminants may have on mitochondrial activity and these aspects will be discussed in this chapter.

Results of Electron Microscopy

Electron microscope studies of etiolated wheat shoot cells using methods as described in chapter II (Fig. 3-1, 2 & 3) revealed the presence of large 3 to 5 μ membrane bound vesicles which sometimes contained starch grains. In tissue which had been exposed to light for 2 hours, these particles showed the beginnings of the internal membrane system of the chloroplast lamellae, including some pro-lamellar bodies. Thus the particles were identified as etioplasts. There were also 1-3 μ membrane bound particles enclosing a dense granular matrix which resembled peroxisomes previously described (Parish and Rickenbacher, 1971; Tolbert et al., 1968, 1969). These organelles being larger than mitochondria, could contribute to contamination of the pellet. The density of the pellet supports these expectations as there is a difference between the two types of preparation. Those with the high speed spin first, type E (Sage and Hobson, 1973) produce a soft heterogeneous pellet with a green centre and a gold-coloured fringe, all overlying a firm starch layer. Rotation of the centrifuge tube gives a partial separation of the soft pellet from the starch. Procedures which include a low speed spin first, method F, (Ellis et al., 1973) form a smaller green-gold coloured pellet which is assumed to be more homogeneous then the above mentioned type because there is a large

green pellet overlaying a starch pellet, produced by spin 1. This discarded green fraction was assumed to contain the etioplast mentioned above.

The type F pellet was subjected to electron microscopic examination by methods described in Chapter II, to determine the extent and nature of contamination. Mitochondria were prepared from the inbred wheat lines Gamut and Heron, their hybrid, Gamut x Heron and a 1:1 w/w mixture of the two parental types, and the preparations were tested for malate oxidation before fixation. Table 3-1 shows that, judged on the characteristics of malate oxidation, the mitochondria are coupled but control ratios are not particularly high. Thus the preparation being viewed is fairly typical of other mitochondrial preparations being used in this study. Figs 3-4, 5, 6 and 7 are representative microscopic fields taken at a magnification of 5,000, of the four preparations, and the overall similarity of the four types of preparations can be seen. Mitochondria are all small, (1μ in diameter), thinly dispersed and in the condensed form. Some are broken and although it is not possible to determine whether mitochondria are broken during isolation or fixation procedures enzymatic studies suggest that some organelles are broken in preparation . All preparations are contaminated, predominantly by the membrane bound organelle, from 1 to 3 μ in diameter with a granular matrix which corresponded to the isolated peroxisomes of Parish and Rickenbach (1971). Membrane fragments resembling the endoplasmic

reticulum seen in whole tissue sections (Fig. 3-1, 2) were also present and unbound material occurs in each view, but especially in Fig. 3-5 and was probably derived from broken organelles. Starch is another frequent contaminant and grains up to 1-2 μ in diameter can be seen. All these contaminating organelles were identified in the whole tissue micrographs (Fig. 3-1, 2, 3). Pellets produced by method F are not contaminated by the etioplast particles seen in whole tissue (Fig. 3) supporting the previous assumption that these particles are precipitated by the initial low speed spin.

Subfractionation of the mitochondrial pellet

To identify the contaminants visualized by electron microscopy, a pellet produced by method F was subjected to sucrose gradient centrifugation and the separated particles tested biochemically. The density of a peroxisome is greater than that of a mitochondrion (Parish and Rickenbacher, 1971; Tolbert et al., 1969; Tolbert, 1971) so such fractionation would be expected to separate these components with the mitochondria being in the upper layer. Peroxisomes are characterized by the presence of catalase (Hall et al., 1974; Solomos et al., 1972; Breidenbach and Beevers, 1967; Newcomb and Fredrick, 1971), and mitochondria by cytochrome oxidase and antimycin A sensitive NADH-cytochrome *c* reductase, so fractions obtained were assayed for these enzymes. Antimycin A insensitive NADH-cytochrome *c* reductase is associated with the outer mitochondrial membrane and the endoplasmic

reticulum membranes.

Results and discussion

The gradient separated three pellets which were designated 1 to 3 in order of increasing density. The first two were greeny yellow suspensions formed at the interfaces of the 1.65 M and 1.78 M sucrose layers. The third was white and sticky and was assumed to be predominantly starch while the gold-coloured pellet produced from the recentrifuged supernatant was fraction 4. The enzymic activity of the four fractions are shown in Table 3-2.

That mitochondria are in fraction 1 is demonstrated conclusively by the location of cytochrome oxidase activity in this fraction. The ascorbate oxidation was stimulated by ADP and inhibited strongly (92 percent) by KCN indicating that all but a residual activity was mediated by cytochrome oxidase. The inclusion of a blank with boiled mitochondria indicated that a certain level of non-enzymic ascorbate oxidation was occurring which was enhanced by KCN. This activity was sufficient to explain the levels of oxidation found in all other fractions especially the increase in fraction 2 on addition of KCN.

The distribution of NADH-cytochrome *c* reductase activity was uneven with the highest activity in fraction 4. This activity was antimycin A insensitive which, considering the source of fraction 4 indicates it is due to microsomal particles. Of the fractions from the crude mitochondrial pellet, only fraction 1 had a high activity

which was five times greater than the second pellet. Again all activity was antimycin A insensitive thus either the mitochondrial outer membrane chain or microsomal fragments could be contributing to the reduction. That mitochondrial pellets can be contaminated by microsomes has been observed previously (Novikoff , , 1956; Wainio, 1970). No measurable rate of the specifically mitochondrial enzyme succinate - cytochrome *c* reductase was observed, but as the presence of mitochondria in fraction 1 has already been established, these studies may be hindered by the possible impermeability of the outer membrane to cytochrome *c* (Wojtczak and Sottocasa, 1972). This would also explain the lack of antimycin sensitive, NADH-cytochrome *c* reductase activity; further evidence for this contention will be presented later in this chapter. It is possible that the succinic dehydrogenase may have been inactivated during the fractionation procedure (although ATP was present in the assay medium) as the mitochondria may be severely broken due to osmotic shocking. But this explanation is inconsistent with the lack of antimycin A sensitive NADH-cytochrome *c* reductase and the first alternative was considered more likely. The rate of antimycin insensitive NADH-cytochrome *c* reductase of a standard crude mitochondrial pellet is usually from 100 to 200 nmoles cytochrome *c* reduced per mg protein per minute. Thus the specific activity of fraction 1 is increased two to four times, which suggests that the particles which contain this activity are being separated from other particles which do not, the latter particles could not therefore be mitochondria.

Catalase activity when expressed as specific activity was evenly distributed which suggests that the peroxisomes have broken and released their activity. This enzyme adheres to membrane surfaces (Parish and Rickenbacher, 1971) and could thus be expected in all membrane fractions. It was hoped there would be a concentration of activity in fraction 2 as these particles, being denser than mitochondria were thought to be the peroxisomes seen in electron micrographs of the preparation (Fig. 3-4 to 7).

The very low peroxidase activity was restricted to fractions 1 and 4. A blank run with boiled mitochondria showed a slow rate of oxygen release from the non-enzymic breakdown of H_2O_2. This was subtracted from all observed rates.

Conclusion

The enzymic properties of the three fractions resulting from sucrose gradient centrifugation of a crude mitochondrial pellet show that there is contamination in such a pellet from particles of higher and lower density. The former are suspected to be the denser plant peroxisomes, which are known to be fragile and liable to disruption in sucrose gradients (Parish and Rickenbacher, 1971).

Fragments of microsomes appear to occur in the mitochondrial fraction as the activity of peroxidase and antimycin insensitive NADH-cytochrome *c* reductase resembles that of fraction 4. Thus the expectation of a mitochondrial pellet produced by a rapid technique, being contaminated

with other organelles and starch has been upheld. Such contamination may be responsible for the high levels of Mg^{++} and Ca^{++} stimulated ATP-ases which act to reduce respiratory control in all the wheat preparations produced by this method.

CH. IIIB

A STUDY OF THE ACTIVITY OF WHEAT MITOCHONDRIA

The pattern of malate, KGA, succinate and NADH oxidation was studied polarographically and the rates of respiration, the respiratory control and ADP:O ratios determined and, where possible, compared with the theoretical maxima (see Chapter I). The results of these studies are presented and compared with reports of wheat mitochondrial activities from other authors.

Malate oxidation

Studies of the properties of rapidly isolated mitochondria (Srivastava and Sarkissian, 1970) showed that malate is readily oxidized by these mitochondria, without added cofactors, to give near theoretical ADP:O ratios and control ratios approaching 3. Pomeroy (1974) obtained high control ratios of up to 6.4 for malate oxidation but had ADP:O ratios of 2.1 to 2.2. The reported state 3 rates of respiration associated with malate oxidation vary from 53 μmoles $\cdot$mg protein^{-1} $\cdot$min^{-1} (Srivastava and Sarkissian, 1970) to 90 to 122 nmoles O_2 $\cdot$mg protein^{-1} $\cdot$min^{-1}(Pomeroy, 1974). Oligomycin reduced the state 3 rate by 80 percent (Pomeroy, 1974) to a state 4 rate which was released by 10^{-4} M DNP, but oligomycin had no effect when added in state 4. The effects of a wide range of respiratory chain and transporter inhibitors were investigated by Pomeroy (1975) and the expected sensitivity was demonstrated. However a residual

rate was observed in the presence of all the inhibitors which Pomeroy suggested supported the idea that plant mitochondria have an antimycin A insensitive alternative oxidase (Day and Wiskich, 1974; Storey, 1971; Wiskich and Bonner, 1963).

Malate dehydrogenase is inhibited by product inhibition as oxaloacetate levels increase during the reaction. Krebs and Bellamy (1960) and Wiskich et al. (1964) have shown that inclusion of 20 mM glutamate in the reaction medium may prevent this gradual inhibition because oxaloacetate can be removed via a transamination reaction producing KGA and aspartate. Thus glutamate was present in all assays of malate activity.

Results

Malate oxidation supported good ADP:O ratios but the respiratory control rarely exceeded 4 and was more commonly between 2 and 3. The state 3 rates of oxygen uptake were generally of the order of 100 nmoles O_2 ·mg protein^{-1} ·min^{-1} which agrees with Pomeroy (1974). An oxygen electrode trace with malate as substrate is illustrated in Fig. 3-8 where it can be seen that mitochondria exhibit good control. The rate of oxidation may increase with successive phosphorylation cycles as in Fig. 3-9 where it rose from 104 to 132 nmoles O_2 ·mg protein^{-1} ·min^{-1} for state 3 and 38 to 54 nmoles O_2 ·mg protein^{-1} ·min^{-1} for state 4. The relatively greater increase in state 4 compared to state 3 led to a decrease in the respiratory control ratio. Oligomycin added to state 4

caused a significant inhibition (Fig. 3-9) in this case 30 percent, bringing the rate back to the rate of the first cycle state 4. This action of oligomycin strongly suggests that mitochondrial ATP-ase is active in the preparation, hydrolysing ATP to ADP and recycling ADP. The ATP-ase would be negligible in the first cycle when levels of ATP are low, but its activity would increase as further additions of ADP were converted to ATP.

KGA oxidation

Srivastava and Sarkissian (1970) reported that wheat mitochondria from parental lines oxidised KGA with respiratory control ratios of 5 and an ADP:O ratio approaching 4. They found the optimum concentration of substrate to be 10 mM with higher concentrations causing inhibition. They also found that the cofactors TPP and CoA did not improve the phosphorylative efficiency or rate of oxygen uptake. These authors did not include malonate in their reaction medium routinely and the activity observed would be expected to be a combination of succinate and KGA oxidation. Only where malonate was included (as in one trace in Srivastava and Sarkissian, 1970) was a true KGA rate obtained and this (given at 25 μmoles O_2 ·mg protein^{-1} ·min^{-1}) was 50 percent of the other rates with KGA. Malonate prevents succinate, produced from KGA, contributing reducing equivalents to the electron transport chain.

Pomeroy (1974) reports the highest respiratory control ratio for KGA oxidation of 6.0 with ADP:O of 3.0. Again malonate was not present

during assay which may account for the lower than expected ADP:O ratio and the high rates of 80 to 90 nmoles O_2 ·mg protein^{-1} ·min^{-1}. Sage and Hobson (1973) and Ellis *et al.* (1973) using the rapid isolation techniques reported ADP:O ratios well below theoretical at 2.3 to 3.0.

Mulliken and Sarkissian (1970) made the unusual claim that KGA oxidation was only "partially sensitive" to the uncoupler DNP present at a final concentration of 1.7×10^{-5}M. By this they mean that DNP does not completely destroy the phenomenon of respiratory control by ADP at this concentration and this was used as an argument for hybrid mitochondrial superiority (Chapter I). Ikuma and Bonner (1967) also found that DNP only stimulated state 4 of KGA oxidation in the presence of ATP. The action of uncouplers on KGA oxidation is not the same as for other substrates because KGA oxidation is limited strongly by its substrate level requirement for ADP (Wiskich *et al.*, 1964). The concentration of DNP used by Mulliken and Sarkissian (1970) was sub-optimal as a concentration of 50 µM was shown to produce the maximum stimulation of state 4 in sweet potato mitochondria. (Wiskich and Bonner, 1963). As other workers commonly use 10^{-4} M DNP, 17 µM additions of DNP could only produce a small fraction of the maximum stimulating concentrations for complete uncoupling (Pomeroy, 1974).

Results ADP:O and respiratory control ratios

The results of a typical oxygen electrode trace with KGA are

shown in Fig. 3-8 and the mean values of 21 ADP:O and respiratory control ratios are compared to those of other workers in Table 3-3. The data from each source are similar with the exception of Sarkissian and Srivastava (1969, 1970) where higher than theoretical ADP:O ratios were observed in hybrid mitochondria. The first cycle ADP:O ratios of the mitochondria in this study were also above the expected level of 4, but this was not taken to imply that the mitochondria were abnormally intact or efficient. Table 3-4 shows that this first cycle phenomenon occurred in mitochondria isolated from parents, hybrids and mixtures alike and the phosphorylation efficiency returned to below the theoretical value for the second and subsequent cycles. The rate of oxygen uptake in the first cycle was slower than the rate of later cycles as has been previously observed by Chance and Williams (1956).

Use of cofactors

In contrast to the report of Srivastava and Sarkissian (1970) the inhibitor malonate and the cofactors TPP and CoA have been included in all runs where KGA oxidation has been studied. The cofactors were included as a result of studies of their effect on the activity of KGA oxidation. Table 3-5 compares the KGA oxidation of three individual sources of mitochondria in the presence and absence of the cofactors where malonate was included in every run. From these results it can be seen that ADP:O ratios are higher and control ratios are slightly

lower in the presence of CoA and TPP. The rates of oxygen uptake (shown for one experiment only) are higher in the presence of cofactors indicating that the KGA dehydrogenase complex is more efficient under these conditions.

ATP-ase in mitochondrial preparations

A typical oxygen electrode trace of KGA oxidation by Halberd wheat mitochondria as shown in Fig. 3-8 has three cycles of ADP stimulated oxygen uptake. It can be seen that during the experiment the rates of both state 3 and state 4 increase with successive cycles and the state 3 to state 4 transition becomes less sharp while the amount of oxygen taken up increases for a constant amount of ADP added. As with malate these results indicate ATP-ase activity in the preparation. Further support for this conclusion was obtained by comparing the responses to oligomycin and uncouplers of malate and KGA oxidation. Table 3-6 presents results of such an experiment where oligomycin and CCCP were added to both malate and KGA driven oxygen uptake on the same preparation of mitochondria. The malate figures confirm that there is considerable oligomycin sensitive ATP-ase activity in state 4 as this rate is inhibited by 43 percent on addition of oligomycin. Other non-mitochondrial ATP-ases may be recycling the ADP to the mitochondrial ATP-ase thereby reducing control. Once oligomycin is present and inhibiting phosphorylation, the state 4 rate is the maximal non-phosphorylating rate and is dependent on the intactness of the inner

membrane and its ability to maintain a proton gradient. The state 4 rates of KGA oxidation (Table 3-6) are lower than either the state 4 rate or the oligomycin inhibited rate of malate, due to the presence of substrate level phosphorylation. If, in the presence of oligomycin, non-mitochondrial ATP-ases were contributing ADP to relieve substrate level phosphorylation with KGA, state 4 would gradually increase to become equivalent to that obtained in the malate trace. This tendency was seen (Table 3-6) in the increasing values of state 4 rates with successive cycles of phosphorylation.

Wiskich et al. (1964) have shown that the DNP stimulation of a KGA supported state 4 rate is completely sensitive to oligomycin. Thus in Table 3-6 there can be no uncoupling resulting from stimulation of the mitochondrial ATP-ase. In both KGA experiments addition of 1 µM CCCP stimulated oxygen uptake which implies that alternative ATP-ases are acting as a source of ADP for the substrate level site. These ATP-ases cannot supply ADP at a rate sufficient to support full uncoupling as the subsequent addition of ADP produced another stimulation of oxygen uptake.

It is therefore concluded that wheat mitochondria prepared by the rapid isolation technique are contaminated by oligomycin insensitive ATP-ase which is acting to reduce the value of control and ADP:O ratios by recycling ADP. This supply of ADP is initially small due to the low levels of ATP, but increases with each addition of ADP.

Pre-incubation of isolated mitochondria with ADP

Raison *et al.* (1973) highlighted one possible source of error which may occur when mitochondrial preparations are compared. They found that it may take several cycles of phosphorylation to develop a consistent state 3 rate of oxidation. Since in wheat mitochondria, both state 3 and state 4 rates of oxygen uptake were observed to rise with successive additions of ADP, it was thought possible that the phenomenon described by Raison could be responsible for the state 3 increases. This would mean that mitochondrial rates were being compared at less than full development. In order to examine this possibility, mitochondria were incubated in an unsealed oxygen electrode in the presence of substrate while three state 3 to state 4 transitions were initiated by addition of 400 μM ADP made over 15 minutes. The vessel was then sealed and measurements of oxidation were made after further additions of ADP. The incubated mitochondria were compared to a non-incubated control (Table 3-7). The efficiency of phosphorylation and control for malate was not markedly altered by the pre-incubation procedure but the rates of oxygen uptake were found to have been decreased by 50 percent in the incubated trial. KGA oxidation became extremely slow and uncontrolled as a result of incubation. This was contrary to Raison's findings of enhanced rates of oxygen uptake and thus it was concluded that the phenomenon reported by Raison was not observed here and the lowering of oxidation rate by incubation was attributed to mitochondrial ageing during the long period of incubation.

Loss of control with KGA

A serious hindrance to comparative work with KGA as substrate was the irregular occurrence of preparations in which coupling was completely absent, despite the fact they could be shown to oxidise malate in a controlled manner. In such experiments the first addition of ADP could stimulate a slow but gradually increasing rate of oxygen uptake which was insensitive to oligomycin or uncouplers. Two explanations can be offered for the loss of control in a trace in this manner. Firstly, the ADP-stimulated rate may be inhibited so that it is indistinguishable from the ADP-limited rate. Inhibition of state 3 occurs when the level of mitochondrial protein falls below a critical level (Raison and Lyons, 1970) and as these preparations were done on a small scale this type of inhibition was possible. Secondly, the ADP-limited rate may be stimulated by an ATP-ase until it masks the transition from unlimited to limited rates of respiration. The presence of contaminating ATP-ases has already been established. Raison and Lyons (1970) showed that for potato, tomato and beetroot mitochondria, 1 mg of mitochondrial protein must be added to the reaction vessel if a maximal state 3 rate is to be attained. In this study protein concentration was correlated graphically with ADP:O ratios and the rates of oxygen uptake for malate and KGA oxidation and the results are shown in Fig. 3-10 and 11. The data in these figures have been accumulated from 12 experiments with one cultivar, where different protein concentrations were used. The range

of protein concentrations was also obtained from a single experiment and these results are shown in Fig. 3-12 with KGA as the substrate.

In the combined results (Fig. 3-10) there was no change in rate with increasing protein content in either state 3 or state 4 respiration for either substrate. The total volume in the reaction vessel varied in these experiments so rates were plotted against the final concentration of protein. However this was not seen when ADP:O versus protein concentration was plotted (Fig. 3-11). The ADP:O ratio for malate reached a steady maximum of 3 at the low protein concentration of 0.3 mg per ml, which could be said to be the critical level. However the ADP:O for KGA oxidation continued to rise as protein increased with no plateau even at the high concentration of 1.4 mg mitochondrial protein per ml which corresponds to 2.2 mg protein added.

A similar experiment was done using beetroot mitochondria prepared by the method of Wiskich et al. (1960). Fig. 3-13 shows that neither ADP:O nor respiratory control ratio for KGA are affected by low protein concentrations which agrees with Raison and Lyons (1970) who showed that beetroot had the lowest critical level. Fig. 3-14 shows that the state 3 and state 4 rates of oxygen uptake decreased with increasing protein on a per mg basis. The state 3 rate decreased more than state 4 causing only a very slight decrease in the respiratory control ratio. The positive correlation of ADP:O with protein concentration is a feature of wheat mitochondria as it was not

observed in beetroot mitochondria.

A positive correlation of ADP:O with protein concentration does not explain the lack of control in experiments where protein levels were high. However it may have a direct bearing on the short cycle phenomenon which was found only with KGA. If ADP was being bound or used by a protein or complex in some way there could be a direct relationship between the amount of protein in the vessel and the amount of ADP bound. Thus the observed ADP:O would rise linearly with the protein concentration. Such a mechanism would be a general reaction and should be observed with all substrates. However malate ADP:O ratios follow the model proposed by Raison and Lyons (1970) so a general adsorption of ADP by protein in the preparation was not the explanation for the apparent loss of ADP in KGA oxidation.

Absence of controlled KGA oxidation in mitochondria in which controlled malate oxidation had been demonstrated may be a result of the faster rate of malate oxidation both in state 3 and state 4 when compared to KGA oxidation. The dehydrogenase enzymes are limited by the lack of ADP in state 4 and thus ATP-ase working at a constant pace can produce equal increases in state 4 for both substrates. The loss of control is therefore more obvious with the slower state 3 rates of KGA oxidation. Since the initial state 3 rate for KGA is depressed, as indicated by Chance and Williams (1956) and by the observation of state 3 in these results, some preparations may never show the state 3 to state 4 transition and an uncoupled trace would be obtained

High ADP:O ratios in cycle 1

It was necessary to determine whether the short first cycle and high ADP:O were an artifact due to some use of ADP before phosphorylation or a true ADP:O. Sarkissian and Srivastava (1970) claimed that ADP was being utilized totally for phosphorylation and thus reasoned that mitochondria incubated with ADP prior to the addition of substrate would have the same oxidation characteristics as those incubated with substrate before ADP addition. Fig. 3-15 shows such an experiment with KGA oxidation. Trace A shows the results of pre-incubation with substrate and trace B the result after pre-incubation with ADP. The addition of ADP to trace A initiated the short cycle with an ADP:O ratio of 4.21, as observed previously, while addition of KGA to trace B gave a long control cycle with an ADP:O of 2.5, well below the theoretical maximum and equivalent to the second cycle of trace A. The lengthening of the first cycle (trace B) initiated by KGA could be due to the conversion of ADP to AMP and ATP by the action of adenylate kinase during the incubation without substrate. Since wheat mitochondria have ATP-ase activity the ATP produced would be further broken down to ADP and inorganic phosphate which could again be transphosphorylated to AMP. At the time of addition of substrate a proportion of the adenine nucleotide would be in the monophosphate form, requiring the addition of two molecules of phosphate to produce

ATP. The phosphorylation efficiency would decrease by an amount dependent on the proportion of AMP present. However this does not explain the shortening of the first cycle when mitochondria are incubated with KGA. The adsorption of ADP by protein has been rejected as mentioned previously because of the restriction of this phenomenon to one substrate. An alternative explanation is that the ADP is being utilized by part of the KGA complex or in a reaction specifically involving KGA. This short cycle appears to be an artifact of these particular mitochondria as it was not observed in beetroot and has not been reported elsewhere. At this stage no experimentally verified explanation can be offered for this phenomenon.

Succinate oxidation

Succinate dehydrogenase is one of the enzymes reputed to show heterotic activity in wheat (Sarkissian, 1972) although not in barley or maize (McDaniel, 1971; McDaniel and Sarkissian, 1968). Hybrid mitochondria oxidising this substrate were reported to have above normal activities with ADP:O ratios of 3.4 (Sarkissian, 1972). The properties of succinate oxidation were studied to determine the suitability of this substrate for heterosis studies.

Results

An oxygen electrode trace (Fig. 3-16) shows a comparison of malate and succinate oxidation by one mitochondrial preparation. The malate trace shows that the mitochondria exhibited tight control with

respiratory control ratios of over 3.0 for the first two cycles of phosphorylation. The control ratios were halved with succinate, with values of 1.28 and 1.47 being recorded for the two cycles observed. Some lowering of control could be expected as succinate contributes electrons below phosphorylation site 1 (Chapter I). The respiratory rates are lower for succinate than malate in the first cycle. This was anticipated as succinate dehydrogenase requires activation by ATP and has been observed previously to give a slower first cycle (Wiskich and Bonner, 1963). The second state 3 rate attained a value equivalent to the malate rates which agrees with previous observations (Ikuma and Bonner, 1967; Wiskich et al., 1964). The addition of oligomycin to state 4 inhibited oxidation of both substrates but had a much greater effect with succinate (57 percent) than with malate (5.56 percent). The ADP:O ratios are in the expected range for succinate although the second cycle was particularly sensitive to the action of ATP-ase and decreased considerably. Thus while three cycles of controlled oxidation were demonstrated for malate, in the presence of ATP-ase activity, only two were observed for succinate and the second of these was poorly defined. It was decided that the control during succinate oxidation was too easily masked to use this substrate as part of the routine testing for heterosis.

Pyruvate oxidation

Pyruvate is oxidized by plant mitochondria only if small amounts of a sparker tricarboxylic acid is also present

to form OAA. The pyruvate is decarboxylated to acetyl CoA which enters the TCA cycle by condensation with OAA to form citric acid (Walker and Beevers, 1956).

Washed mitochondria normally contain low levels of the soluble TCA intermediates and without trigger amounts of malate very little pyruvate oxidation is observed. The theoretical maximum ADP:O ratio for pyruvate oxidation would be three if the TCA cycle operated fully.

Wheat mitochondria were shown to oxidise 30 mM pyruvate with malate, as shown in Table 3-8 where the oxidation is compared to that of malate plus glutamate. All parameters are lower than for malate oxidation and the two concentrations of malate sparker, (6 and 3 mM) gave the same response. Malate in these concentrations is below the requirement for malate dehydrogenase (Millard, 1967).

Sarkissian and Srivastava (1970) could not find a heterotic response for oxidative phosphorylation for pyruvate nor did it show oxidative heterosis in wheat mitochondria. McDaniel & Sarkissian, (1968) also failed to find heterosis with pyruvate in maize or barley (McDaniel, 1971). The only reported incidence of heterotic utilization of pyruvate is of heterotic mice referred to by Sarkissian (1972). It was therefore decided not to use pyruvate in the testing program for heterosis even though it would support coupled oxidation.

NADH oxidation

NADH has been claimed to support mitochondrial high efficiency

and heterosis (Sarkissian, 1972). Controlled NADH oxidation with control ratios ranging from 3 (Pomeroy, 1974) to 12.5 (Sarkissian and Srivastava, 1968) and ADP:O ratios from 1.3 to 2.24 (Sarkissian, 1972) have been reported. The reported rates of oxidation vary considerably, and are, in general, higher than those reported for other substrates. Ikuma et al. (1967) found state 3 rates of 60 nmoles O_2 ·mg protein^{-1} ·min^{-1} for mung bean mitochondria while Pomeroy (1974) reported rates of 100 nmoles O_2 ·mg protein^{-1} ·min^{-1} for NADH oxidation. Rates of 53 µmoles O_2 ·mg N^{-1} ·min^{-1} (Srivastava and Sarkissian, 1971) are very much higher as they are equivalent to 318 nmoles O_2 ·min^{-1} ·mg protein^{-1}. The addition of 0.05 mM cytochrome *c* as a cofactor for NADH oxidation may be the cause of this dicrepancy, as its presence caused a 30 and 43 percent enhancement of state 3 and state 4 respectively (Srivastava and Sarkissian, 1970). This can be attributed to the participation of non-phosphorylating outer membrane chain oxidation of NADH in the presence of cytochrome *c* (Day and Wiskich, 1974) and thus would be expected to alter the ADP:O ratio. In the present study NADH oxidation was observed to assess its suitability for use in heterosis studies. Rates of NADH oxidation by intact mitochondria were also required for comparison with NADH-cytochrome *c* reductase.

Results

Wheat mitochondria would support controlled oxidation of NADH but as shown in Fig. 3.17, the control ratios (1.83 and 1.94) and

ADP:O ratios (1.26 and 1.2) were low. The rate of oxygen uptake of 80 to 90 nmoles O_2 ·mg protein^{-1} ·min^{-1} agreed with those of Pomeroy (1974). Fig. 3-17 also shows that NADH oxidation was totally inhibited by antimycin A but that this inhibition could be overcome partially by addition of cytochrome *c*. The cytochrome *c* rate was 22 percent of the initial state 3 rate, indicating that either cytochrome *c* can only penetrate the outer membrane slowly or that 20 percent of the outer membranes are broken sufficiently to allow penetration. That the outer membrane is impermeable to cytochrome *c* is discussed in the following sections.

NADH proved to be an unreliable substrate for routine study of these wheat preparations as many preparations showed no control at all. The low control ratio made it susceptible to ATP-ase activity, as described for succinate and for this reason it was not included in the programme for study of heterosis. At no time was a phosphorylation efficiency of greater than 2 detected for NADH oxidation by these wheat mitochondria.

Cytochrome c reductase

The reduction of exogenous cytochrome *c* by respiratory intermediates via the respiratory chain has been discussed in Chapter I. Cytochrome *c* can be reduced by three enzyme systems, malate-cytochrome *c* reductase, succinate-cytochrome *c* reductase and NADH-cytochrome *c* reductase. The first two are specific to mitochondria as they involve the two electron transport complexes of the mitochondrial inner membrane.

They are sensitive to electron transport inhibitors which act before cytochrome *c*, in particular antimycin A (Lardy *et al.*, 1958). The third reductase is not specific to mitochondria as NADH-cytochrome *c* has been observed in preparations of mitochondrial outer membranes (Day and Wiskich, 1974) and microsomal particles (Rungie and Wiskich, 1972). This reduction is achieved by means of an electron transferring cytochrome chain, the properties of which (Chapter I) are similar in both microsomes and outer membranes. The system is insensitive to antimycin A and can be distinguished from the inner membrane reduction on this basis.

The presence in plant mitochondria of an external NADH dehydrogenase has been discussed in Chapter I where it was seen that plant mitochondria can oxidise externally added NADH in an antimycin sensitive manner giving an ADP:O ratio of two.

Exogenous cytochrome *c* can be reduced directly by the outer mitochondrial membrane or the microsome chain in an antimycin insensitive manner, or it can be reduced by the inner membrane with reducing equivalents being passed by endogenous cytochrome *c* which is situated on the outer side of the inner membrane (Racker, 1970; Tzagoloff *et al.*, 1973). Cytochrome *c* must be able to penetrate into the inner-membrane space if antimycin sensitive reduction is to occur. It is necessary to prevent the reoxidation of cytochrome *c* by the terminal oxidase if rates of reduction are to be meaningful, thus it is necessary to include a terminal oxidase inhibitor (e.g. KCN) in the experimental system.

Sarkissian and Srivastava (1971) published results for NADH-cytochrome *c* reductase which have been obtained by following the rate of change of NADH concentration with no terminal oxidase inhibitor. They observed the oxidation of NADH as a single process disregarding the two possible pathways, and have calculated kinetic data as well as conclusions relating to heterosis and complementation from these results.

It is important, if mitochondria of different genotypes are being compared on the bases of NADH-cytochrome *c* reductase activity, to know what proportion, if any, of the activity is being contributed by contaminating endoplasmic reticulum.

Results

Spectral Assay of NADH-cytochrome c reductase

Preparations of wheat mitochondria reduced added cytochrome *c* with NADH as substrate at a rate of 70 to 270 nmoles cytochrome *c* reduced $\cdot$mg protein^{-1} $\cdot$min^{-1} and all activity was antimycin A insensitive while mitochondria were untreated. This activity could result from either the outer membrane chain or microsomal contamination. The lack of antimycin A sensitivity could be due to poor penetration of the cytochrome *c* into the intermembrane space. The latter explanation is supported by the following observations. (1) Purified mitochondria from a sucrose density gradient (Chapter IIIA) showed a threefold increase in specific activity of NADH-cytochrome

c reductase activity. They exhibited the same antimycin A insensitivity with NADH as substrate as unfractionated mitochondria (Table 3-9).

(2) Malate and succinate-cytochrome *c* reductase activity could not be detected in untreated mitochondria, or in mitochondrial fractions obtained from the sucrose density gradient, even in the presence of ATP which was added to activate the succinate dehydrogenase.

(3) Osmotically shocked mitochondria (cf. Chapter IIA) exhibited a measurable rate for both malate and succinate-cytochrome *c* reduction which was totally antimycin A sensitive (Table 3-10). The rate of NADH-cytochrome *c* reduction in swollen mitochondria increased with the increment being sensitive to antimycin A (Table 3-11). A very slow rate of cytochrome *c* reduction was observed in the absence of exogenous substrate, which indicated these mitochondria have some endogenous substrates after rapid preparation.

Discussion

The technique of following the reduction of cytochrome *c* via NADH-cytochrome *c* reductase is an unsuitable method for assessing mitochondrial quality as the most intact mitochondria should have the lowest rates. The reaction rate observed depends on the intactness of the outer membranes with the most broken preparations having the highest rate. Thus Sarkissian and Srivastava's (1971) claim of hybrid mitochondrial superiority on the basis of faster rates is erroneous.

Had antimycin A been included in the assay, reduction would have been restricted to the outer membrane chain which would have prevented this problem. It would also have simplified interpretation of kinetic data which as it is presented is the amalgamation of activity of two electron transporting systems, one of which is not operating at full efficiency.

A second criticism of Sarkissian and Srivastava's (1971) experiment was that they followed the reaction by observing NADH oxidation at 340 nm. This wavelength is not a cross over point for cytochrome *c* and thus the change in absorbance observed is a combination of the absorbance shifts produced by cytochrome *c* reduction and NADH oxidation which are in the same direction. The relative contributions of the two compounds to the ΔA was determined experimentally as shown below by the reduction of cytochrome *c* ascorbate at both wavelengths.

Cytochrome *c*	340 nm	550 nm	EmM cytochrome *c*
Oxidized	0.534	0.238	= 19.8
Reduced	0.5	0.74	EmM NADH
ΔA	0.034	0.5	= 6.22

To achieve a drop of 0.5 at 550 nm is equivalent to reducing 0.0252 mM cytochrome *c*. Since NADH reduces two molecules of cytochrome *c* per molecule, 0.0126 mM NADH would be required to produce this effect. The change at 340 nm produced by the oxidation of 0.0126 mM NADH would be 0.0785 but 0.03 would be added to this as a result of the

change in cytochrome *c*. Thus the reading would be in error by 30%. As no account has been made for this influence the results of Sarkissian and Srivastava (1971) must be regarded with suspicion.

Cytochrome oxidase activity

Cytochrome oxidase, the terminal member of the mitochondrial electron transfer chain, is a complex enzyme containing two haem groups, two copper atoms and a number of different polypeptide components (Komai and Capaldi, 1973; Tzagoloff et al., 1973). The exact number of subunits is uncertain and reports range from two to seven (Lemberg, 1969; Rubin and Tzagoloff, 1973; Manson and Poytom, 1973). The active site of oxygen reduction is associated with cytochrome a_3 and is located on the inner surface of the inner membrane (Racker, 1970; Maclennan, 1970). Membrane bound cytochrome *c* is on the outer side of the inner membrane (Racker, 1970).

Observation of the reduction of oxygen by the terminal oxidase can be made using ascorbate as a substrate (Jacobs, 1960). Millard (1967) reported controlled oxidation of ascorbate/TMPD by beetroot mitochondria, giving an ADP:O ratio of one, which is the theoretically expected value. TMPD is a highly lipid soluble compound which, when present in catalytic amounts, replaces cytochrome *c* as the substrate after being reduced by ascorbate (Jacobs, 1960). This prevents problems of substrate penetration as TMPD reduces the cytochrome *a*, a_3 complex directly allowing the maximum rate of reduction by cytochrome oxidase. The oxidation of ascorbate produces a charge separation across the

membrane and is thus capable of energy conservation and the rate of ascorbate oxidation is stimulated by ADP. When exogenous reduced cytochrome *c* is the substrate it acts by reducing the membrane bound cytochrome *c* which passes electrons to the active site of cytochrome oxidase. Thus the electron must pass through the membrane, via the cytochrome *a* component (Racker, 1970). This reduction of cytochrome oxidase would be expected to have different kinetic properties from the direct supply of electrons by TMPD.

Since the substrate for cytochrome oxidase is an electron, not a molecule the affinity of the enzyme for its substrate depends on the environment of the enzyme. The capacity of the chelated iron atom to pass electrons to oxygen is far in excess of the demand of intact mitochondria and this activity is controlled by the lipo-protein sub-units which bind the complex to the membrane. Removal of the enzyme from its membrane bound condition strips the protective covering, and exposes new reduction sites. Thus one would expect an increase of specific activity to be associated with sonic, enzymatic or detergent disruption of the mitochondrial membranes and this has been shown for wheat mitochondria. There appears to be little value in extrapolating the kinetics of such disrupted systems to the intact mitochondrial system.

Results

Spectrophotometric studies of the oxidation of cytochrome *c* were combined with polarographic observations of oxygen uptake to examine the properties of the cytochrome oxidase system and assess

its suitability as a parameter for between preparation comparisons. Several observations were made which indicate a penetration barrier to cytochrome *c* at the outer membrane. These are as follows:-

(1) Ascorbate was unable to stimulate oxygen uptake in the presence of antimycin A and ADP (Table 3-12) unless an electron carrier was present. A comparison of the efficiency of cytochrome *c* and TMPD in transporting electrons to the active site is obtained by comparing the rates of ascorbate stimulated oxygen uptake in the presence of these carriers (Table 3-12). The cytochrome *c* supported rate was 5 percent of the TMPD supported rate, but the former was gradually increased to be 400 percent of this initial rate on successive additions of the detergent deoxycholate.

(2) The rates of oxidation by intact mitochondria (Table 3-13) ranged from 20 to 70 nmoles cytochrome *c* ·mg protein^{-1} ·min^{-1} which is ten times lower than the results of Sarkissian and Srivastava (1970) who report values of 0.2 to 0.6 µmoles ·mg protein^{-1} ·min^{-1}. Osmotic shocking increased the rate from 20 to 200 nmoles cytochrome *c* ·mg protein^{-1} ·min^{-1}, a tenfold increase (Table 3-13). This increase is of the same magnitude as found with cauliflower mitochondria for digitonin disruption (D. Day Personal communication).

(3) It is possible to compare the rates of cytochrome *c* oxidation and ascorbate/TMPD powered oxygen reduction when both rates are expressed in equivalent units. Such a comparison of substrates shows that, in the presence of ADP, cytochrome *c* is oxidised at a lower

rate than ascorbate/TMPD. Theoretically one would expect the former rate (expressed as nmoles cytochrome *c* ·mg protein^{-1} ·min^{-1}) to be four times faster than the latter (expressed as nmoles O_2 ·mg protein^{-1} ·min^{-1}) as four electrons are required to reduce one molecule of oxygen while cytochrome *c* oxidation is a single electron process. Thus equivalence shown in Table 3-14 is gained by reducing the oxygen uptake figures by one quarter.

(4) Ascorbate/TMPD oxidation increased 50 to 70 percent on addition of ADP, however cytochrome *c* oxidation was insensitive to ADP (Table 3-15) and must be limited by some control other than phosphorylation.

(5) Oxidation of cytochrome *c* in both intact and swollen particles ceased well before the supply of substrate was depleted. Addition of $FeCN^{\equiv}$ initiated a further drop in absorbance, indicating non-enzymic oxidation of the remaining substrate.

Alternative explanations

The early cessation of oxidation may have resulted from particles settling as the cuvette contents remain unstirred for the duration of the experiment (180 secs.). A further addition of mitochondrial protein (0.149 mg) with stirring was made to the hybrid trial in A_1 which had reached equilibrium (170 secs.). Doubling the protein content would be expected to increase the rate in itself but if settling of particles was the main factor causing early equilibrium one would expect this stirring to increase the rate to almost its initial value. A small stimulation occurred but the value of the second

rate constant (0.0276) was still very much lower than the initial rate constant, (0.069) and the rate 14.35 nmoles cytochrome *c* ·mg protein^{-1} ·min^{-1} was one quarter of the initial rate 71 nmoles cytochrome *c* ·mg protein^{-1} ·min^{-1}.

It was concluded that removal of mitochondria from the main volume of the reaction mixture by settling was not responsible for the early cessation of oxidation of cytochrome *c*.

Effect of endogenous substrates on cytochrome c oxidase activity

Rapidly isolated wheat mitochondria have been shown previously (Ch. IIIA) to contain phosphate and endogenous substrates. Oxidation of these substrates could lead to the production of reduced cytochrome *c* i.e. the reverse reaction to that being followed. Such an effect would be negligible in the early stages of the reaction but as the rate of oxidation decreased the endogenous reduction of oxidized cytochrome *c* would become increasingly noticeable The rate of oxidation would be seen to decrease sharply and in fact all the semilog plots of $\log_{10}$ A against time changed slope after an initial linear period. Inclusion of antimycin A in the reaction mixture would prevent interference from endogenous substrates, but as antimycin A was not present only the initial portion of the curve was used for the calculation of rate constants.

Discussion

The very low cytochrome *c* oxidase rates in intact mitochondria,

especially compared to ascorbate/TMPD oxidation rates, their lack of stimulation by ADP and their ten-fold increase following osmotic disruption, suggest that cytochrome *c* cannot easily pass through the outer membrane. This agrees with the conclusions from cytochrome *c* reductase studies already discussed (Ch. III B). Mitochondria isolated by the rapid technique may be better preserved as a high proportion of intact outer membranes could be expected. Thus the rate observed is a function of the intactness of the outer membrane and does not represent the properties of the oxidase complex itself.

A positive heterotic rate would indicate a more broken outer membrane, an implication inconsistent with claims of hybrid superiority in oxidative phosphorylation (Sarkissian and Srivastava, 1969, 1970). The experimental evidence that Sarkissian and McDaniel (1967) advance to substantiate heterosis and complementation with the in vivo enzyme were based on mitochondria which had been subjected to separation by sucrose density gradient centrifugation on a linear sucrose-EDTA gradient spun for 25,000 rpm for 90 mins. Their tests were performed on freshly prepared and freeze-stored samples and both the centrifugation and the freezing could be expected to cause a deterioration in membrane structure, increasing the rate of cytochrome *c* oxidase activity.

The importance of osmotic conditions on the observed oxidase activity is strongly supported by Sen (1975) who showed that although the optimum molarity of the K-Pi buffer in the assay medium for plant cytochrome *c* oxidase was 75 mM the activity depended on storage of

mitochondria in 10 or 20 mM media. He concluded the hypotonic solution enhanced swelling and subsequent substrate penetration.

Sen (1975) also emphasized the sensitivity of this assay to the strength of the assay solution. Wheat mitochondria show an increase in the rate of oxidation of cytochrome *c* oxidation which approaches four fold as buffer strength rises from 20 to 75 mM. Sarkissian and Srivastava (1971) used 100 mM buffer which Sen (1975) shows to give near optimal rates of reaction. Sarkissian and McDaniel (1967) and Srivastava and Sarkissian (1971) use below optimal concentrations, 38 mM and 0.1 mM respectively. The latter used 0.1 mM buffer to assay both intact mitochondria and purified cytochrome *c* oxidase. The activity observed in such extreme hypotonicity could be the result of swelling and easy penetration of cytochrome *c* rather than an estimation of an <u>in vivo</u> activity. The present experiments were conducted under osmotic conditions resembling those of Sarkissian and Srivastava (1971) and Srivastava and Sarkissian (1972). Phosphate concentration was low (10 mM) but 25 mM sucrose was present to act as the osmotic agent. This concentration of sucrose has an osmosity of 0.14 M (Wolf, *et al.* 1971 - 1972) close to that of 100 mM K-Pi buffer pH 7.2 which is 0.132 M. Reducing the buffer strength to 75 mM as recommended by Sen (1975) halves the osmosity (0.079 M). Thus the present experiments were conducted near the peak of activity and within the pH range recommended by Sen (1975).

Nicholls (1974) has recently drawn attention to the difficulties

associated with observation of cytochrome oxidase in intact mitochondria. He points to the futility of calculating kinetic data when the system is under severe constraints from sources other than enzyme and substrate concentration. Some of these problems may be resolved by study of purified samples of the complex. Reports of purification of animal and yeast cytochrome oxidase are numerous (Straub, 1941; Griffith and Wharton, 1961; Minneart, 1961; Yonetani, 1961). Plant cytochrome oxidase has been extracted (Miller et al., 1958; Srivastava and Sarkissian, 1971) with claims of twentyfold purification as determined by the twentyfold increase in specific activity. A better estimation of the extent of purification is the haem to protein ratio used by Komai and Capaldi (1973) who report 14.6 nmoles haem per mg protein. Since the specific activity of wheat mitochondrial cytochrome oxidase increases at least tenfold on osmotic swelling it is difficult to say how much of the increase reported by Srivastava and Sarkissian (1971) is due to concentration of the enzyme rather than substrate availability resulting from structural disruption. The heterotic activity reported in intact mitochondria was retained by the concentrated enzyme complex and also after extraction of phospholipids from mitochondria (Srivastava and Sarkissian, 1972).

As cytochrome oxidase activity exceeds other cytochromes and the stoichiometric requirements for the enzyme (Lehninger, 1970) it must be working below its maximum efficiency in situ. The actual rate should be determined by the spatial organization in the membrane so

the activity of an isolated enzyme may bear no relation to the action of the complex in intact mitochondria. Comparisons of hybrid and parental rates for isolated enzyme cannot be extrapolated in the *in vivo* situation.

Table 3-1

Oxidative Activity of the Mitochondria Observed by Electron Microscopy

Source of Mitochondria	R.C.R.	ADP:O
Heron	1.64	2.73
Gamut	1.84	2.32
Hybrid	1.88	2.25
1:1 mixture	1.4	1.27

Malate oxidation was observed by adding 0.4 ml mitochondria to 1 ml standard sucrose medium followed by 13.6 mM malate, 34 mM glutamate. Phosphorylation was initiated by additions of 256 µM ADP. The following amounts of protein were added in each assay; Heron = 1.35 mg; Gamut = 1.35 mg; Hybrid = 1.55 mg; mixture = 0.93 mg.

Table 3-2

A Cytochrome oxidase assay Aliquots (0.1 ml) of enzyme suspension from fractions 1 to 4, containing 0.455, 0.285, 0.265 and 0.685 mg protein respectively were added to 2 ml standard sucrose medium and sealed. The reaction was initiated by the addition of 4.7 mM ascorbate with 1.2 mM TMPD. 191 µM additions of ADP and 150 µM KCN were made subsequently to give a total volume of 2.125 ml. The blank sample contained 0.1 ml boiled enzyme but was otherwise the same.

B NADH-cytochrome *c* reductase activity To 3 mls standard sucrose medium was added 25.3 µM oxidized cytochrome *c*, 10 mM KCN and 0.2 ml enzyme suspension containing 0.37, 0.12, 0.104 and 0.57 mg protein from samples 1 to 4 respectively, The reaction was initiated by 14.88 mM additions of NADH to a final volume of 3.31 ml.

C. Catalase assay A total volume of 3.065 ml was composed of 3 ml standard sucrose medium, 3.26 mM H_2O_2 and 5 µl enzyme sample containing from fraction 1 to 4 respectively: - 0.322×10^{-3}, 0.515×10^{-3}, 0.565×10^{-3} and 0.215×10^{-3} mg protein. Additions were made in the above order, the standard sucrose medium had been made anaerobic with N_2. Values are corrected for non enzymic O_2 release due to breakdown of H_2O_2.

D Peroxidase assay The enzyme was assayed in a total volume of 3.1 ml made up as described in Chapter II(A). The final concentration of reagents was 1.75 mM ascorbate, 65 mM H_2O_2 and added to the 1 ml of 2% benzidine in sodium citrate pH 5.3. 0.1 ml aliquots of enzymes containing 0.455, 0.285, 0.265 and 0.685 mg protein were added as timing was commenced.

Table 3-2

Location of enzyme activity in fractions from sucrose gradient centrifugation of a pure mitochondrial pellet.

Fraction No.		1	2	3	4	Blank
A cytochrome oxidase ($nmoles\ O_2 \cdot mg\ protein^{-1} \cdot min^{-1}$)	-ADP	257.8	30.4	19.0	55.8	26.8
	+ADP	280.3	44.7	-	-	44.7
	+KCN	19.6	71.6	28.55	14.88	62.5
B NADH-cytochrome *c* reductase (nmoles cytochrome *c* $\cdot mg\ protein^{-1} \cdot min^{-1}$)		390	79.8	105.2	710	
C Catalase μ moles $O_2 \cdot mg\ protein^{-1} \cdot min^{-1}$)		7.58	7.96	6.96	9.21	
D Peroxidase		+	-	-	+	

Table 3-3

KGA Oxidation A Comparison of Data

Sarkissian and Srivastava (1969) (Table 1)		RCR	ADP:O
Means of an unstated number of determinations for the genotypes 28, 31 MS hybrid and mixture respectively		3.7	3.2 ± 0.17
		4.6	3.8 ± 0.05
		5.5	5.8 ± 0.2
		5.3	5.4 ± 0.95
Zobl et al. (1972)			
Means of 5 determinations given for 3 genotypes		3.4 ± 0.17	3.8 ± 0.15
		2.8 ± 0.07	3.3 ± 0.16
		2.9 ± 0.07	3.1 ± 0.17
Sage and Hobson (1973)			
Means of 4 determinations, over the range shown were given for different genotypes.		-	2.07 → 3.91
Ellis et al. (1973)			
Means of 3 determinations were given for different genotypes over the range shown			2.07 → 2.41
Pomeroy (1974)	(Age)		
Means were obtained by combining the values for all cycles in 5 or 5 experiments	2 days	6.5 ± 1.2	3.0 ± 0.1
	3 days	4.8 ± 1.2	2.9 ± 0.3
Present Data	(n)		
Mean of different genotypes, all cycles combined, * cycle 1 only, ** cycle 2 only.	21	2.3 ± 0.08	3.9 ± 0.24
	* 21	2.2 ± 0.12	5.19 ± 0.47
	** 20	2.3 ± 0.16	3.29 ± 0.24

Table 3-4

Oxidative phosphorylation by wheat mitochondria using KGA.

OXIDATION OF αKETO GLUTARATE

	RCR	ADP:O	
cycle (1)	2.57	6.4	Nabawa
(2)	2.46	3.81	Parent 1
(3)	2.1	1.94	
cycle (1)	2.0	6.4	Chile
(2)	1.7	2.98	Parent 2
(3)	1.75	1.78	
cycle (1)	2.0	7.65	Hybrid
(2)	1.56	3.83	(Nabawa & Chile)
(3)	1.85	3.06	
cycle (1)	2.21	5.8	1 : 1 mixture
(2)	2.2	3.5	
(3)	2.25	2.18	

To 2 ml of standard manitol medium with 7.8 mM KGA and 7.8 mM malonate, was added 0.4 ml mitochondrial suspension which contained the following quantity of mitochondrial protein:- Nabawa 1.72 mg, Chile IB 1.8 mg, hybrid 2.28 mg and a 1:1 mixture 2.36 mg. The total volume was 2.57 mls and 172.5 µM additions of ADP initiated successive phosphorylation cycles.

Table 3-5

The effect of the cofactos CoA and TPP on the oxidation of KGA by wheat mitochondria.

		- cofactors	+ cofactors
RCR	cycle (1)	2.63	2.24
(n=3)	cycle (2)	3.27	2.95
	cycle (3)	3.66	3.29
ADP:O	cycle (1)	2.53	3.11
(n=3)	cycle (2)	2.05	2.89
	cycle (3)	2.01	2.49
(nmoles O_2 ·mg protein^{-1} ·min^{-1})			
State 3	cycle (1)	48	62.8
rate	cycle (2)	45	62
(n=1)			
State 4	cycle (1)	28	31
rate	cycle (2)	26	34
(n=1)			

To 1 ml standard sucrose medium was added 18.65 mM KGA and 12.4 mM malonate and 0.4 ml mitochondrial suspension to give a final volume of 1.52 ml. Additions of 124 µM TPP and 186.5 µg/ml CoA were added to the + cofactor trials to give a final volume of 1.56 ml. ADP:O and respiratory control ratios are means of three experiments in which the protein additions were from 2.0 → 3.0 mg/ml. The rates are from one experiment where 2.06 mg protein addition was made.

Table 3-6

Demonstration of mitochondrial and non-mitochondrial ATP-ase activity in Gabo wheat mitochondria.

	Substrate	RCR	ADP:O	S_2	S_2	S_4	Oligomycin	CCCP	ADP
1	Malate	1.41	1.34	29	114	81	46	-	-
2	KGA	2.16	3.66	0	64	30			
		1.68	2.59		79	47	45	54	59
3	KGA	2.0	5.14	0	38	19			
		1.81	2.59		49	27			
		1.79	2.67		71	36	30	32	38

Results are given for successive cycles of phosphorylation for three experiments. Mitochondrial suspension was added to 1 ml standard sucrose medium to give 0.98, 0.78, 0.9 mg protein respectively for the three experiments 1 to 3. The final volume was 1.47 ml for malate and 1.61 ml for KGA trials and reagents were present at the following concentrations: 12 mM malate, 34 mM glutamate, 18.7 mM KGA, 12 mM malonate, 124 µM TPP, 187 $\mu gm.ml^{-1}$ CoA, 1 µM CCCP and 8 µM oligomycin. Phosphorylation was stimulated by 230 and 210 µM ADP for malate and KGA respectively. Rates are expressed as nmoles $O_2 \cdot mg\ protein^{-1} \cdot min^{-1}$. RCR = respiratory control ratio and S_2, S_3 & S_4 = respiration states 2, 3 and 4.

Table 3-7

Pre-incubation of mitochondria with ADP and its affect on malate and KGA oxidation

		MALATE		KGA	
	Cycle	Non-incubated	Incubated	Non-incubated	Incubated
RCR	1	2.82	3.25	2.0	8
	2	3.26	2.56	1.8	
	3	2.88	1.91	1.4	4
ADP:O	1	1.78	1.54	1.6	1.5
	2	1.44	1.57	1.8	
	3	1.94	1.7	-	
State 3 rate	1	83.0	45	28	9.0
	2	84.8	40	32	7.6
	3	80.0	36	33	-
State 4 rate	1	29	17	14	6.12
	2	26	16	18	
	3	28	19	18	

ADP = 156.1 µM

Malate = 2.55 19.61 mM Glutamate = 19.61

KGA = 2.55 15.69 mM Malonate = 19.61

Mitochondrial assays were conducted in a total volume 2.55 ml with 0.4 ml aliquots of mitochondria containing 1.2 mg protein being added to 2.1 ml standard sucrose medium. Either 19.61 mM malate and 19.61 mM glutamate, or 15.69 mM KGA and 19.61 mM malonate were present. Phosphorylation was initiated by addition of 156.1 µM aliquots of ADP.

RCR = respiratory control ratio.

Rates are expressed as nmoles O_2 ·mg protein^{-1} min^{-1}.

Table 3-8

Pyruvate oxidation by wheat mitochondria.

		(A) Malate (13.6 mM)	(B) Pyruvate + Malate 6 mM	(C) Pyruvate + Malate 3 mM
RCR	cycle 1	2.6	1.39	1.74
	cycle 2	2.48	1.79	1.44
ADP:O	cycle 1	2.83	2.2	2.28
	cycle 2	2.2	1.69	1.87
State 2 rate		11	7	8
State 3 rate	cycle 1	60	36	27
	cycle 2	73	49	35
State 4 rate	cycle 1	23	26	16
	cycle 2	30	28	24

Mitochondria isolated from the American Line 109H by method F (Table 2-3) were added to 1.0 ml standard sucrose medium with 13.6 mM malate and 34 mM glutamate (A), 30 mM pyruvate and 6 mM malate (B), and 30 mM pyruvate and 3 mM pyruvate (C). Additions of 272 µM ADP initiated each cycle of phosphorylation. 2.52 mg mitochondrial protein were added to each reaction. Rates are expressed as nmoles O_2 ·mg protein^{-1} ·min^{-1}.

Table 3-9

NADH-cytochrome *c* reductase activity.

	nmoles cytochrome *c* reduced ·mg protein^{-1} ·min^{-1}.	
	— Antimycin A	+ Antimycin A
(1) Intact Mitochondria	97	100
(1) Purified Mitochondria	390	390
(2) Swollen Mitochondria	275	168.5

(1) Mean of 5 preparations
(2) Mean of 4 preparations

0.1 ml Mw protein was added to 3 ml standard reaction medium, with 2.5 mg cytochrome *c* and 10 mM KCN. The reaction was started with an addition of 6.42 mM NADH. 5 µM antimycin A was included in the reaction mixtures where indicated. The total volume was 3.255 ml or 3.26 when antimycin A is present.

Table 3-10

Cytochrome *c* reduction by succinate and malate in osmotically swollen mitochondria.

Mitochondrial Source	Succinate-cytochrome *c* reductase		Malate-cytochrome *c* reductase	
	- Antimycin A	+ Antimycin A	- Antimycin A	+ Antimycin A
31MS	25	0	12.1	0
28	31	0	11.3	0
Hybrid	40	0	15.5	0
1:1 mix	20	0		

Aliquots of mitochondrial suspension containing between 0.445 and 0.625 mg protein in 0.1 ml were first swollen in 1.5 ml double distilled water then after 45 secs. 1.5 ml standard sucrose medium was added to restore the osmotic balance. The assay was conducted with 2.5 mg cytochrome *c*, 15.34 mM malate or 15.34 mM succinate, 10 mM KCN and 5 µM antimycin A, when present, in a total volume of 3.26 ml

All rates are expressed as nmoles cytochrome *c* reduced ·mg protein^{-1} ·min^{-1}.

Table 3-11

NADH-cytochrome *c* reductase in intact and osmotically swollen mitochondria.

Mitochondrial Source	Intact Mitochondria		Swollen Mitochondria	
	(nmoles cytochrome *c* reduced $\cdot$mg protein^{-1} $\cdot$min^{-1})			
	- Antimycin A	+ Antimycin A	- Antimycin A	+ Antimycin A
31	180	-	240	130
28	150	-	300	150
Hybrid	270	-	430	250
1:1 mix	140	-	130	90

Intact mitochondria were tested as described in Table 3-9 in a total volume of 2.26 ml. Swollen mitochondria were treated as described in Table 3-10 and Ch. II. For both treated and untreated mitochondria the reactions were started with 6.42 mM NADH. Additions of mitochondria contained between 0.445 and 0.625 mg protein.

Table 3-12

Ascorbate oxidation with cytochrome *c* and TMPD measured polarographically.

Additions	Rate (nmoles O_2 ·mg protein^{-1} ·min^{-1})	Additions	Rate (nmoles O_2 ·mg protein^{-1} ·min^{-1})
Ascorbate	0	Ascorbate/ TMPD	204
ADP	3	ADP	236
cytochrome *c*	14	KCN	13
20 DOC	34		
20 DOC	46		
20 DOC	56		
KCN	7		

A mitochondrial suspension containing 0.785 mg protein was added to 2 ml standard sucrose medium. The additions listed were added sequentially in the following concentrations: 4.5 mM ascorbate, 1.12 mM TMPD, 109 µM ADP, 5.97 µM cytochrome *c*, 135 µM KCN, 20 µl of 2% deoxycholate (DOC).

Table 3-13

Cytochrome *c* oxidation by intact and osmotically swollen wheat mitochondria.

Cytochrome *c* oxidase activity

(nmoles cytochrome *c* oxidised ·mg protein^{-1} ·min)

Source of Mitochondria	Intact Mitochondria		Swollen Mitochondria	
	A	-ADP	B	-ADP
31MS		25.1		182
28		37.1		208
31MS x 28		71.0		272

The assays were carried out under two sets of conditions; A: to 3.0 ml standard sucrose medium was added 25 µl mitochondrial suspension and the reaction started with 47 mM ferrocytochrome *c* to give a total volume of 3.225 ml. To 1.5 ml standard sucrose medium was added 0.1 ml mitochondrial suspension pre-incubated in 1.5 ml distilled water for 45 secs. The reaction was initiated by addition of 46.5 mM ferrocytochrome *c* to give a total volume of 3.3 ml. Protein added in the mitochondrial suspension was as follows: 31MS = 0.675 mg, 28 = 0.575 and 31MS x 28 = 0.595.

Table 3-14

Cytochrome oxidase activity

Source of Mitochondria	- ADP		+ ADP	
	Cytochrome *c* Oxidase	Ascorbate/TMPD Oxidase	Cytochrome *c* Oxidase	Ascorbate/TMPD Oxidase
	(nmoles cytochrome *c* oxidized ·mg protein^{-1} ·min^{-1})			
31MS	25.1	38	21	57
28	37.1	36.9	22.3	60.5
31MS x 28	71	33.5	47.7	57

A comparison of the rate of oxidation of cytochrome *c* and the rate of reduction of oxygen. The rate of oxygen uptake has been expressed in the equivalent units of nmoles cytochrome *c* oxidized ·mg protein^{-1} ·min^{-1}. Experimental details are given in Table 3-12, for ascorbate/TMPD oxidation and Table 3-13 conditions A.

Table 3-15

The effect of ADP on cytochrome *c* oxidase and cytochrome oxidase.

	Cytochrome *c* Oxidase		Ascorbate Oxidase	
Source of Mitochondria	- ADP	+ ADP	- ADP	+ ADP
31MS	25.1	21	152	228
28	37.1	22.3	148	242
Hybrid	171.0	47.7	134	228

Cytochrome *c* oxidase was assayed as described for Table 3-13 part A except where 224 µM ADP was present. Mitochondrial protein was added as in Table 3-13. Cytochrome oxidase activity was assayed in a total volume of 1.11 ml in an oxygen electrode vessel. To one ml standard sucrose medium was added 0.1 ml mitochondrial suspension containing: 31MS, 0.675 mg; 28, 0.575 mg, 31MS x 28, 0.595 mg protein. The reaction was started by addition of 9 mM ascorbate and 2.25 mM TMPD. ADP when present was 230 µM.

Fig. 3-1a, b, Fig. 3-2 and Fig. 3-3

Transmission electron micrographs of 2½ day old wheat coleoptile tissue.

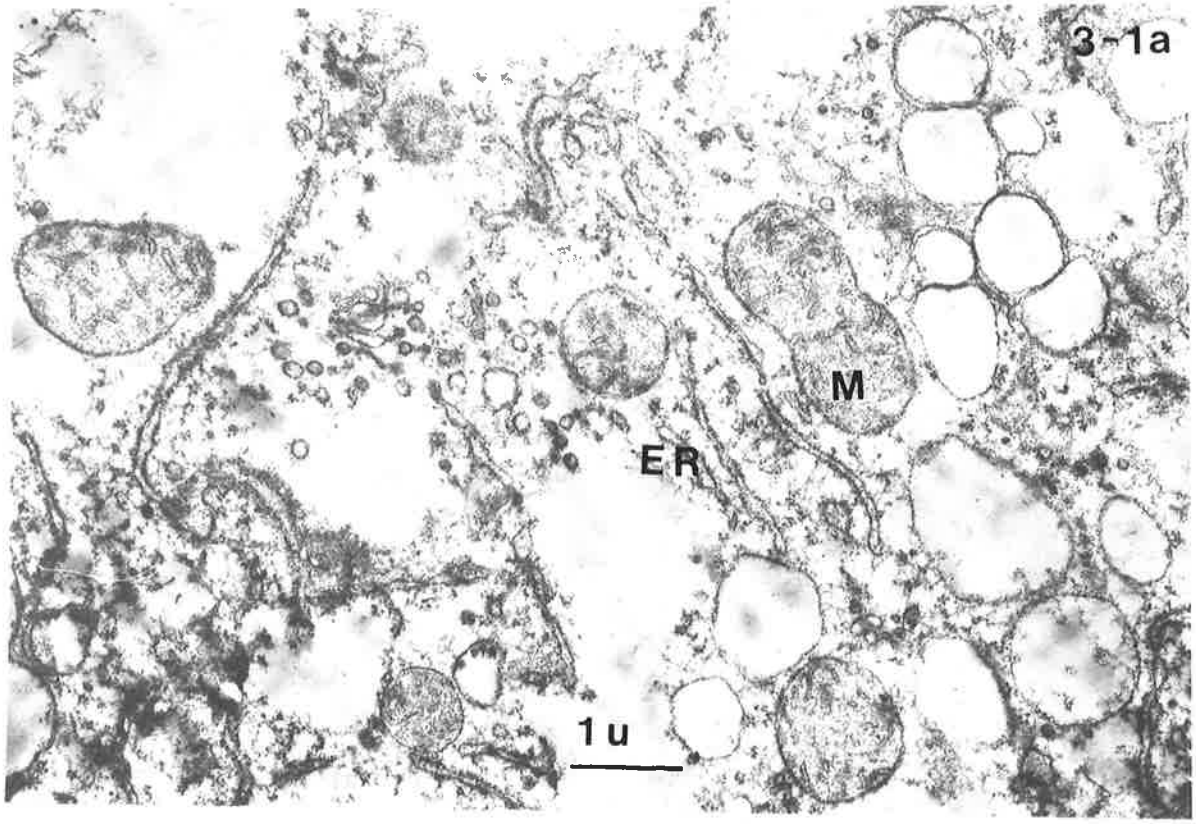
Fig. 3-1a, b and Fig. 3-2 were taken of dark grown shoot material whereas Fig. 3-3 is from shoots which had been illuminated for two hours before fixation.

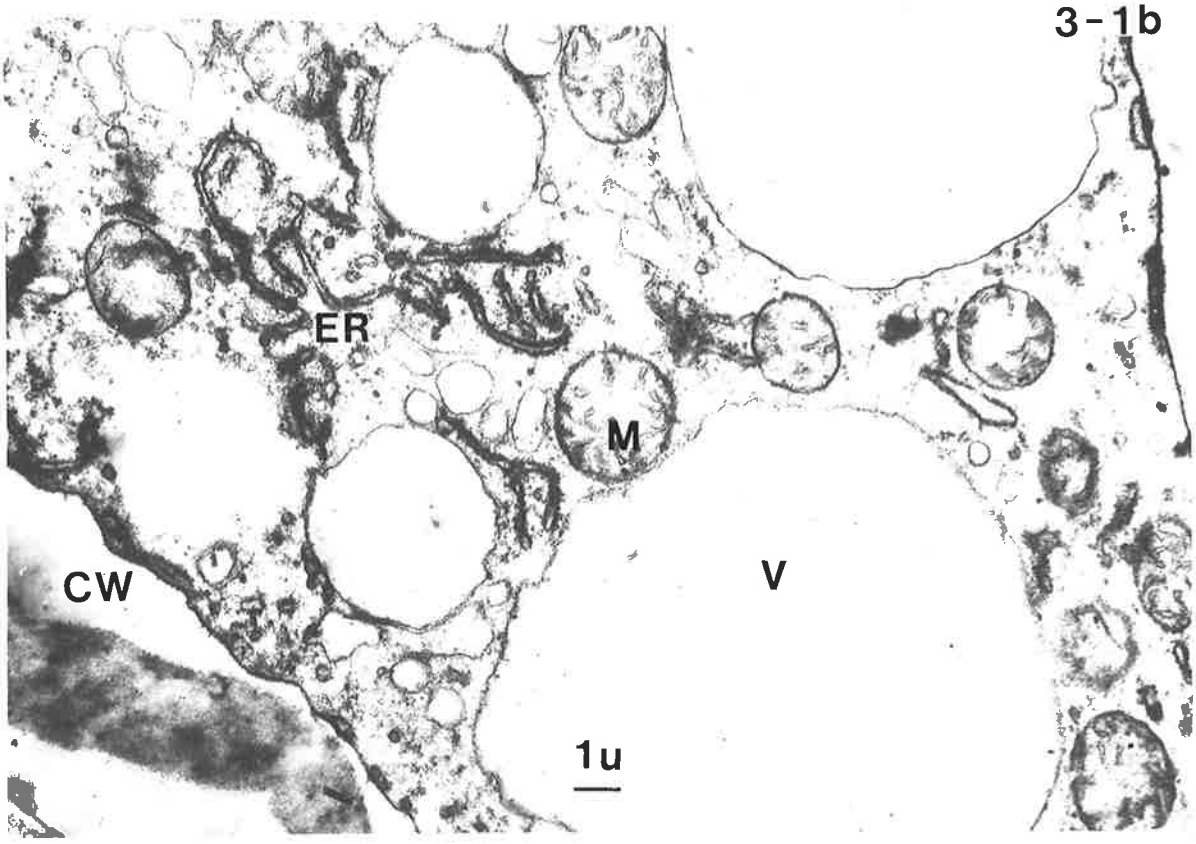
Fig. 3-1a, magnification = 6,000X, shows mitochondria (M), one of which is dumbell shaped, among endoplasmic reticulum (ER) and membrane enclosed spaces which may be transparent bodies or vacuoles.

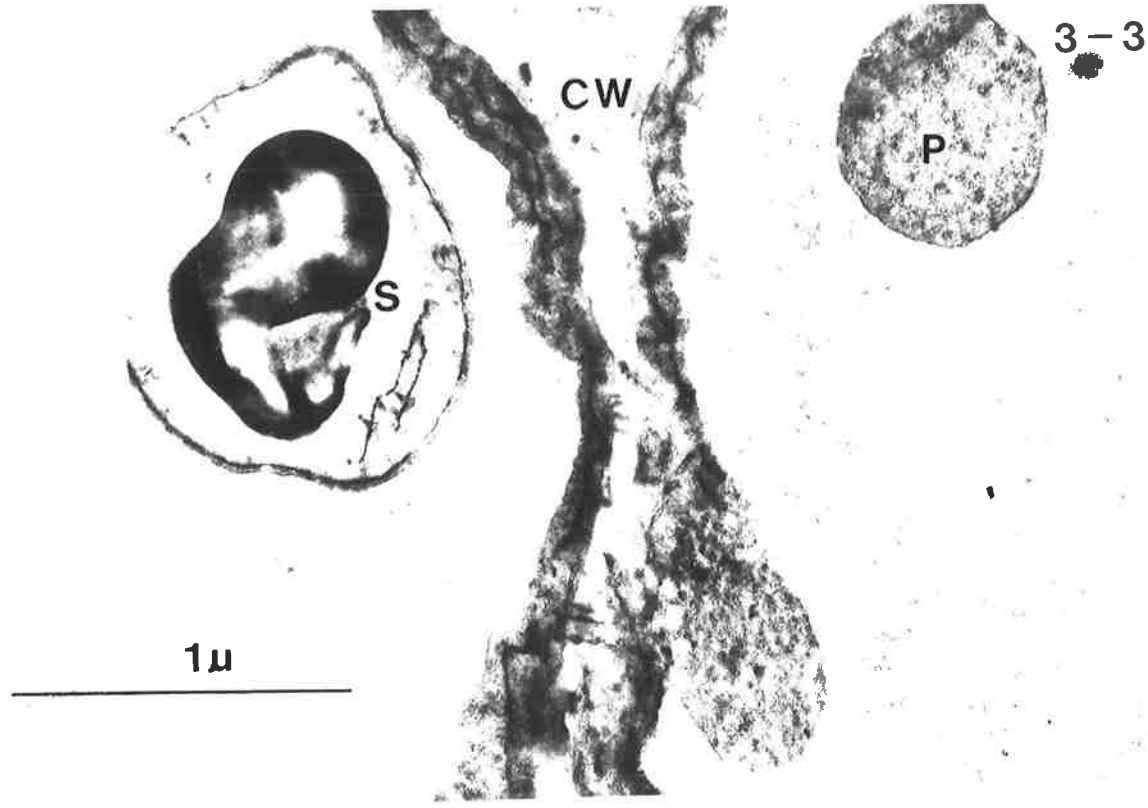
Fig. 3-1b, magnification 14,000X, shows regular, shaped mitochondria surrounded by endoplasmic reticulum and granular cytoplasmic material with larger membrane bound vacuoles (V) and a cell wall (CW).

Fig. 3-2, magnification = 10,000X, shows a thin strip of cytoplasm on each side of a cell wall bounding the central vacuoles of two cells. A transparent body of 2 μ, longest cross section, is also situated in the cytoplasm and is thought to be a proplastid (P). Endoplasmic reticulum in longitudinal and cross section is also visable.

Fig. 3-3, magnification 44,00X, shows a thin strip of cytoplasm on either side of a cell wall. In one vacuole is an etioplast (EP) with a starch grain and two strands of membrane inside which are the initiation of the lamellar system after 2 hours of illuminations. The second vacuole contains two densely granular, membrane bound peroxisomes.


3-1a
M
ER
1u


3-1b
ER
M
CW
V
1u


3－3
CW
P
S
1μ

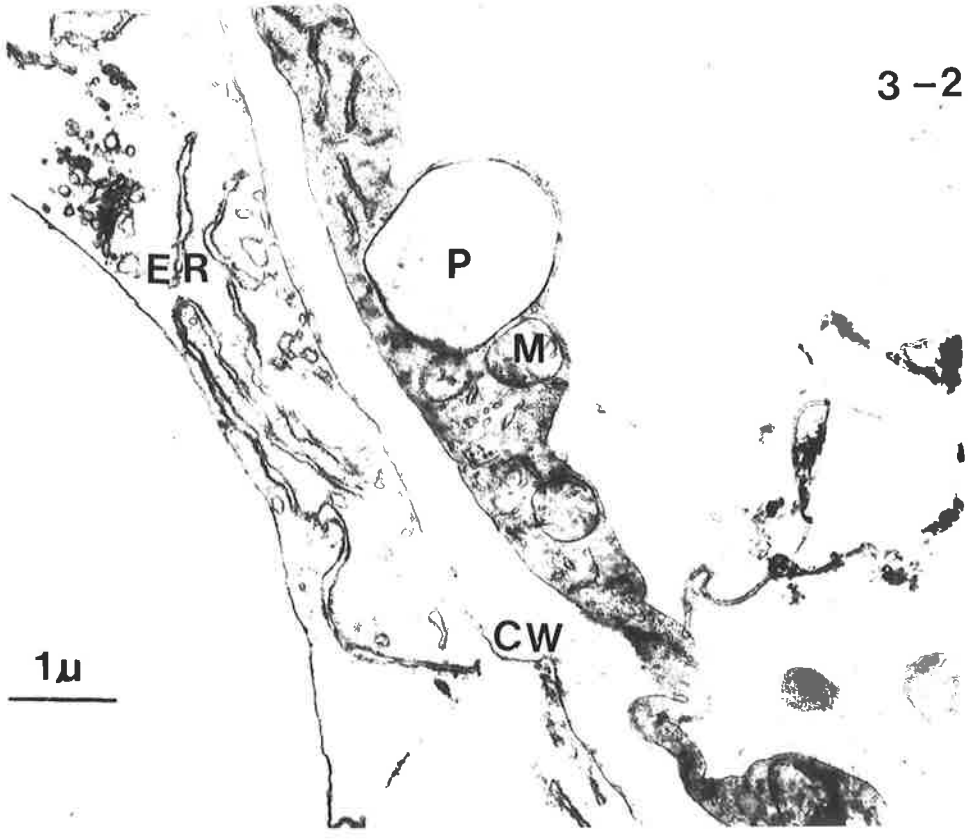

3－2
ER
P
M
CW
1μ

Fig. 3-4, 3-5, 3-6 and 3-7.

Transmission electron micrographs of mitochondrial pellets from preparations of Heron, Gamut, Gamut X Heron and Gamut X Heron (1:1 w/w) wheat cultivars.

Mitochondrial material was prepared as described in Chapter IIA. Representative fields of each preparation are shown as follows:

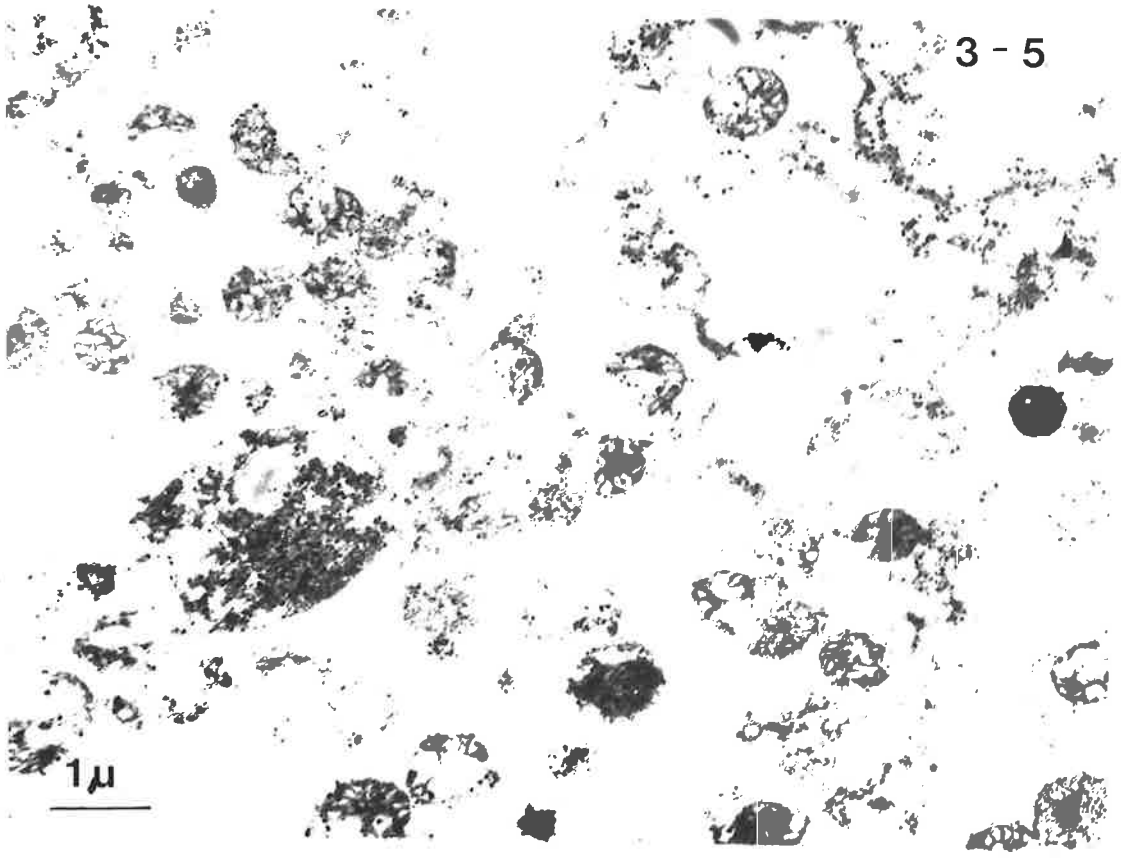
Fig. 3-5 Heron mitochondria; 12,500.

Fig. 3-4 Gamut mitochondria; X 19,600

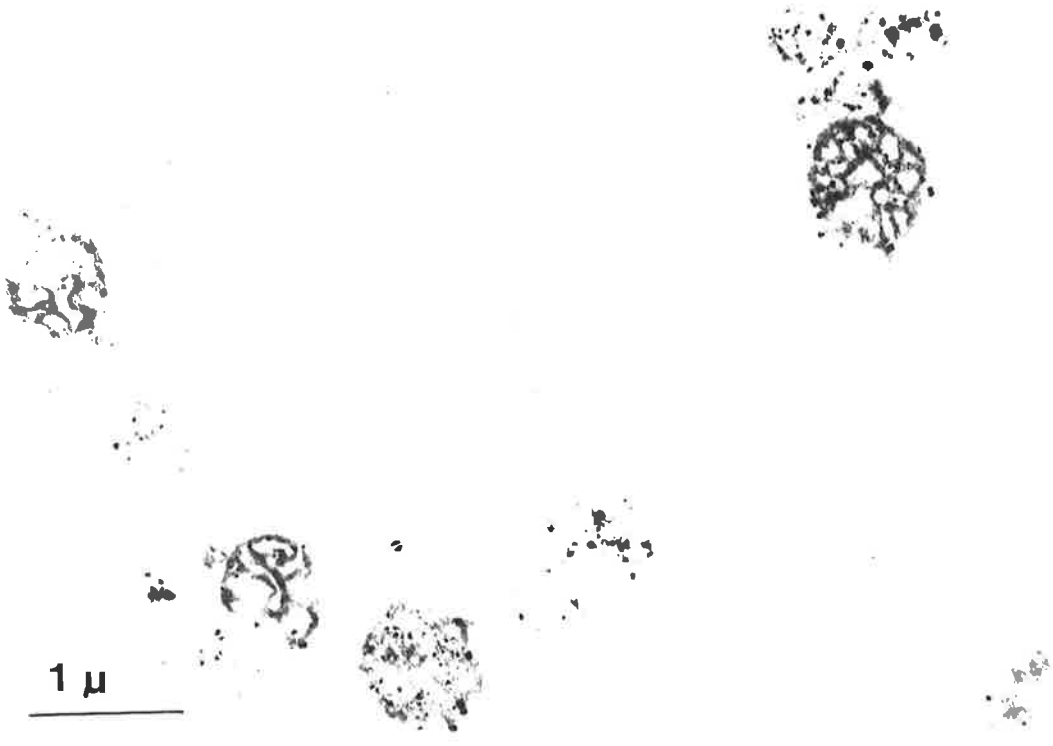
Fig. 3-6 Gamut X Heron mitochondria; X 13,000

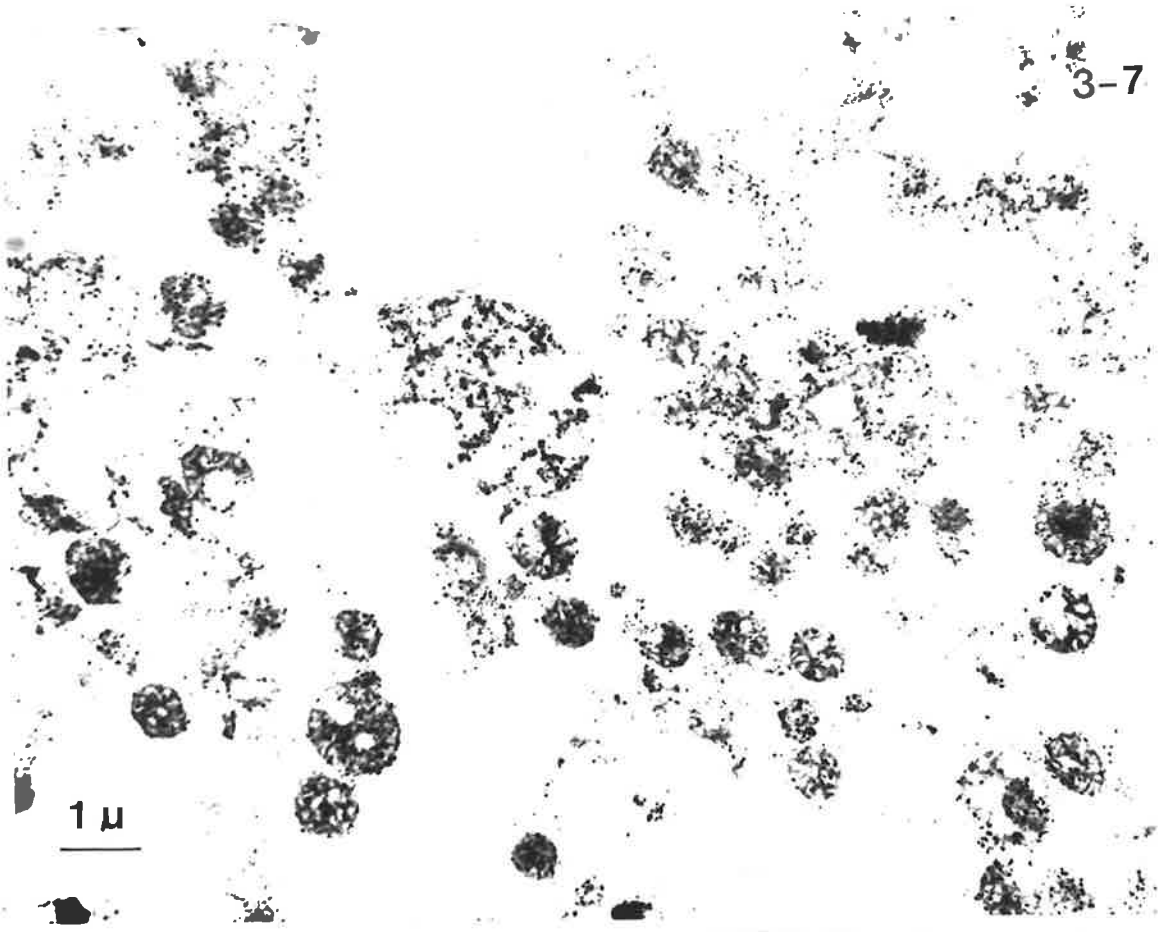
Fig. 3-7 Gamut + Heron mitochondria; X 10,000

Mitochondria (M) and peroxisomes (P) are scattered as well as undifferentiated non-bound material and membrane fragments and in Fig. 3-5 a small starch grain (S) is visable.



3 - 4




3-7
1 μ

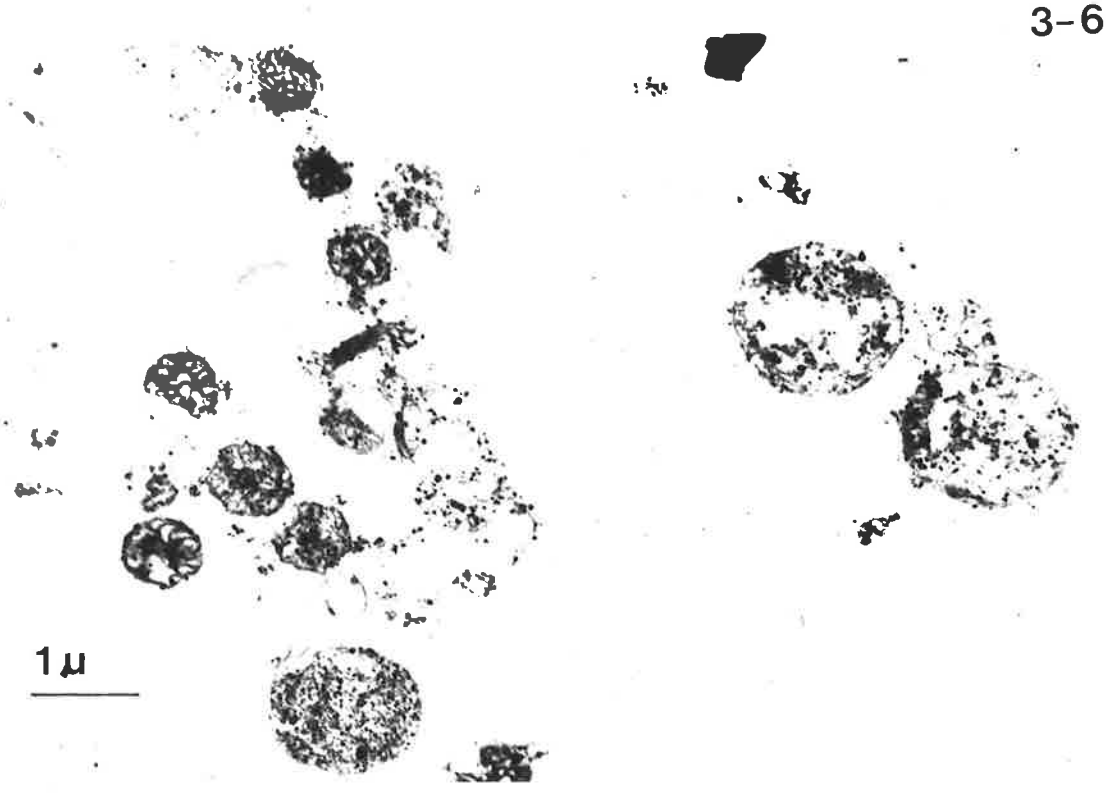

3-6
1 μ

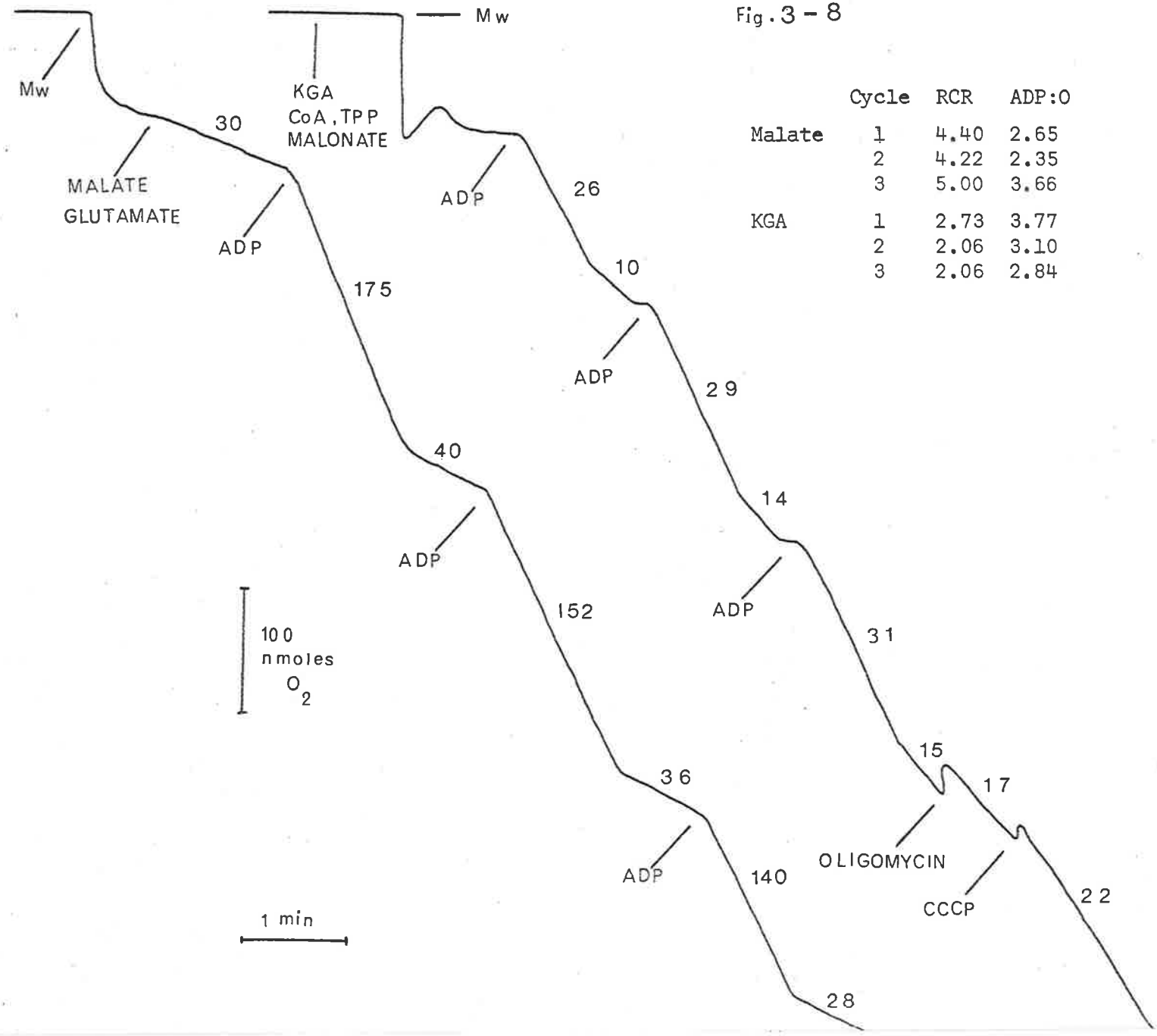
Fig. 3-8

Malate and KGA oxidation by wheat mitochondria.

Malate oxidation was determined in a total of 3.27 ml which contained 1.1 mg protein, 3 ml standard sucrose medium, 6.12 mM malate, 15.3 mM glutamate and additions of 307 µM ADP initiated the phosphorylation cycles.

KGA oxidation was observed in a total of 1.56 ml which contained 2.01 mg mitochondrial protein, 1.0 ml standard sucrose medium, 13.7 mM KGA, 13.7 mM malonate, 13.7 µM TPP and 267 µM CoA. 1 µM CCCP and 3.4 µg oligomycin/ml were added as indicated. Additions of 250 µM ADP initiated the phosphorylation cycles.

Fig. 3 - 8



	Cycle	RCR	ADP:O
Malate	1	4.40	2.65
	2	4.22	2.35
	3	5.00	3.66
KGA	1	2.73	3.77
	2	2.06	3.10
	3	2.06	2.84

Fig. 3-9

The Effect of Oligomycin and Uncoupler on Malate Oxidation

Mitochondria were assayed in a total volume of 1.47 ml composed of 1.0 ml standard sucrose medium to which had been added 1.42 mg mitochondrial protein, 13.6 mM malate, 34 mM glutamate, 8 μM oligomycin, 1 μM CCCP. Phosphorylation was initiated by additions of 231 μM ADP.

Fig 3 - 9

	R.C.R	ADP:O
Cycle 1.	2.31	3.08
Cycle 2.	1.95	1.88

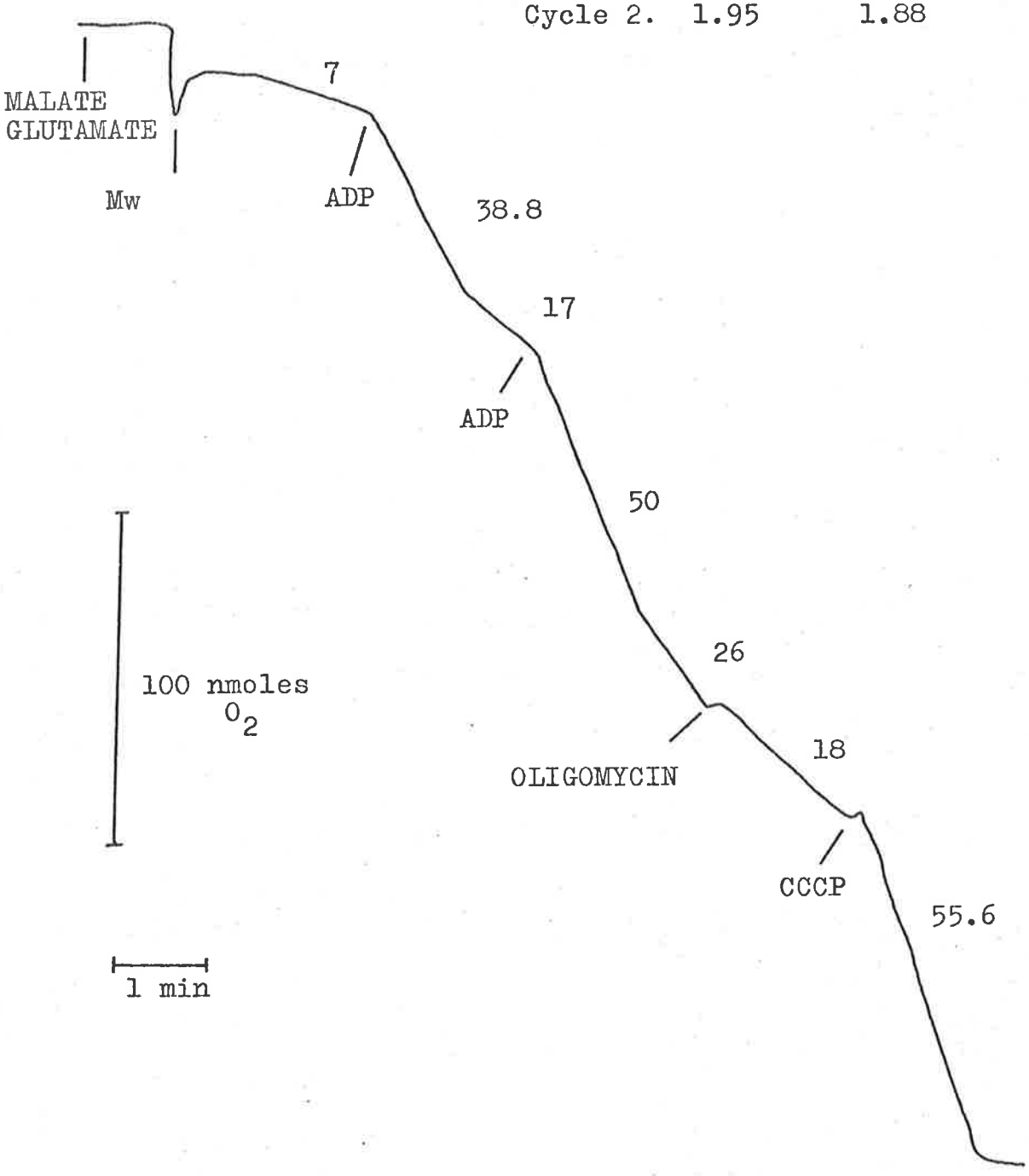


Figure 3-10

The Effect of Protein Concentration on the Rates of Oxygen Uptake for Wheat Mitochondria oxidizing Malate and KGA.

Results are taken from 12 experiments in which additions of protein from 0.8 to 2.0 mg were made. All reactions took place in standard sucrose medium. In graphs A and B, 12.8 mM KGA, 12.8 mM malonate, 186.5 µg/ml CoA and 124 µM TPP were added before 0.4 ml mitochondrial suspension to give a final volume of 0.56 ml.

In graphs C and D, 13.6 mM malate, 34 mM glutamate were added before 0.4 ml mitochondrial suspension to give a total volume of 1.47 ml. Phosphorylation cycles were initiated, for both substrates, by adding 200 to 250 µM ADP. Values for three cycles are shown.

0 = cycle 1; Δ = cycle 2; □ = cycle 3.

Fig 3 - 10

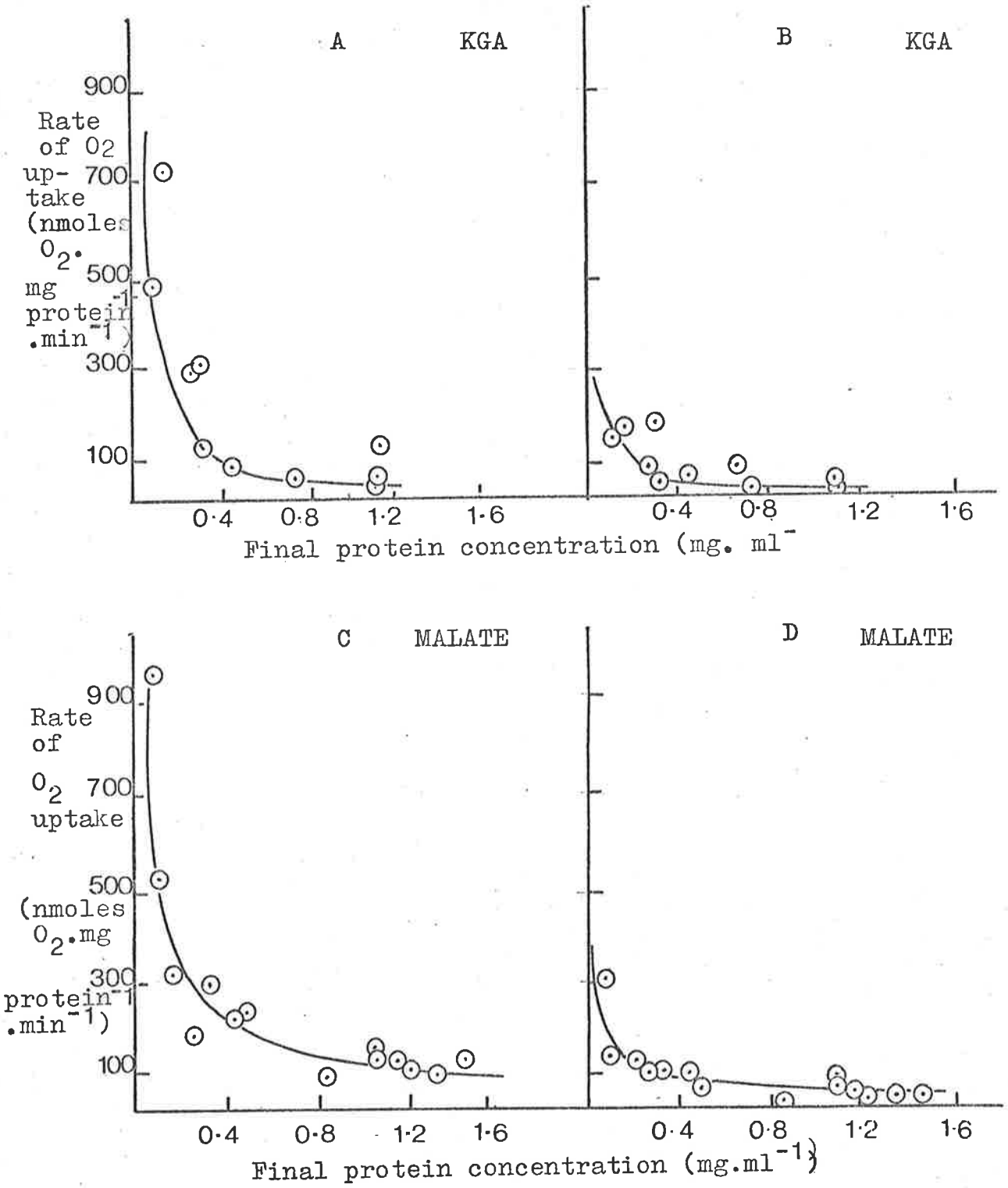

A KGA
B KGA
900
700
500
300
100
Rate of O2 up-take (nmoles O2. mg protein-1 .min-1)
0·4
0·8
1·2
1·6
Final protein concentration (mg. ml-
C MALATE
D MALATE
900
700
500
300
100
Rate of O2 uptake (nmoles O2.mg protein-1 .min-1)
0·4
0·8
1·2
1·6
Final protein concentration (mg.ml-1)

Fig. 3-11

ADP:O vs Final Protein Concentration for Wheat Mitochondria Oxidising Malate and KGA.

Results are taken from 12 experiments in which protein concentrations from 0.6 to 1.5 mg protein/ml were used. All reactions took place in 1 ml standard manitol medium.

Graph A. 12.8 mM KGA, 12.8 mM malonate, 186 µg/ml CoA and 124 µM TPP were added before 0.4 ml mitochondrial suspension to give a final volume of 1.56 ml.

Graph B. 13.6 mM malate and 34 mM glutamate were added before 0.4 ml mitochondrial suspension to give a final volume of 1.47 ml. Cycles of state 3 to state 4 transitions with both substrates were initiated by addition of 5 µl aliquots of ADP i.e. between 200 and 250 µM additions. Values for 3 cycles are shown. O = cycle 1, Δ = cycle 2, □ = cycle 3.

Fig. 3-11

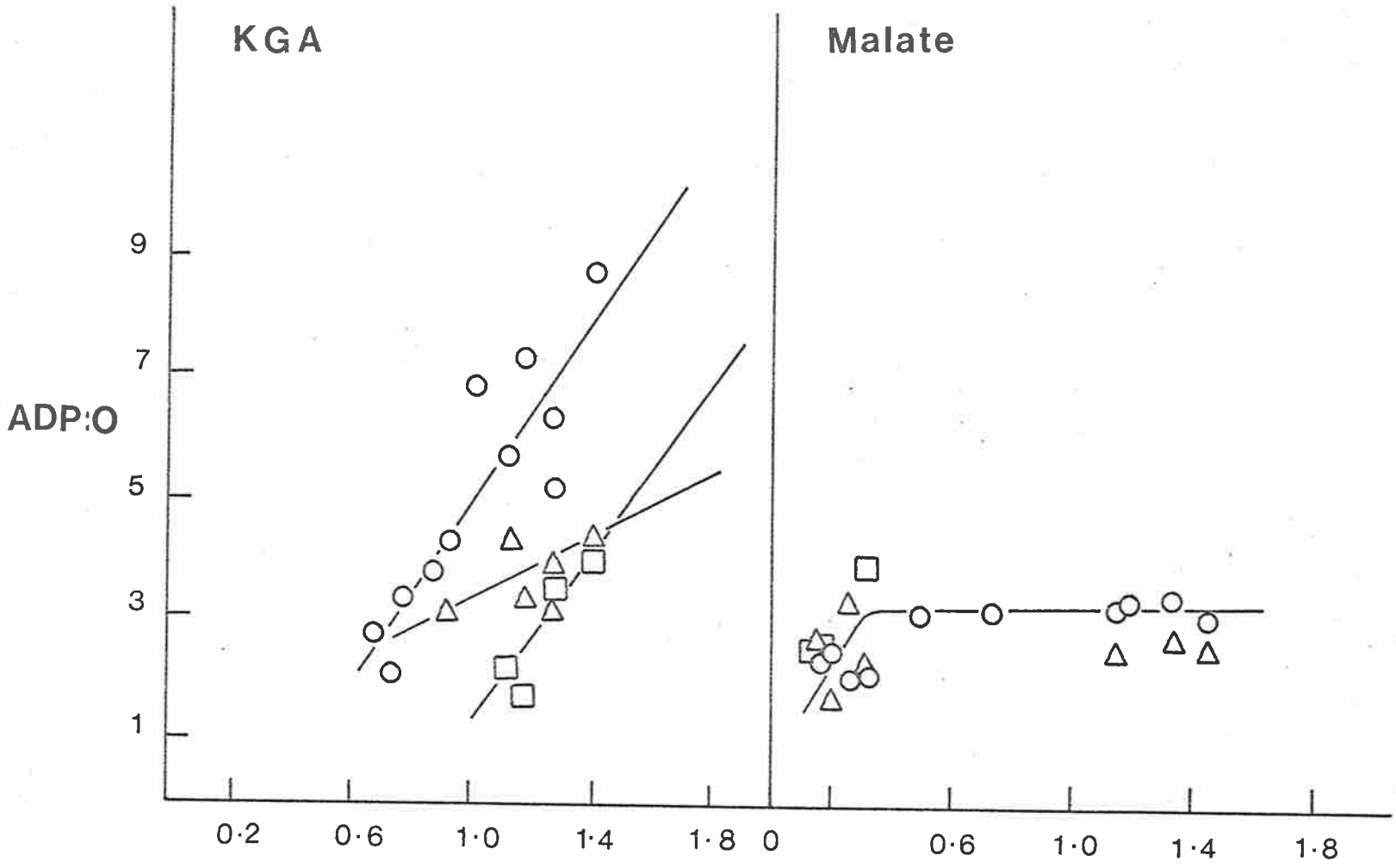


Fig. 3-12

Protein concentration vs ADP:O, RCR and rates of oxygen uptake.

ADP:O, respiratory control ratios (RCR) and rates of oxygen uptake were plotted against the final protein concentration for preparations from a single lot of wheat. Varying volumes of mitochondrial suspension containing either 2.8 or 1.8 mg protein/ml were added to standard manitol medium which contained 12.8 mM KGA, 12.8 malonate, 186.5 µg/ml CoA and 124 µM TPP so that the final volume was constant at 1.56 ml. Sequential additions of 350 µM ADP initiated state 4 to state 3 transitions and 3 cycles are shown, O = cycle 1, Δ = cycle 2 and □= cycle 3. Rates of oxygen uptake are given as nmoles $\cdot$mg protein^{-1} $\cdot$min^{-1}. Protein concentrations are in mg $\cdot$ml^{-1}.

Fig. 3 - 12

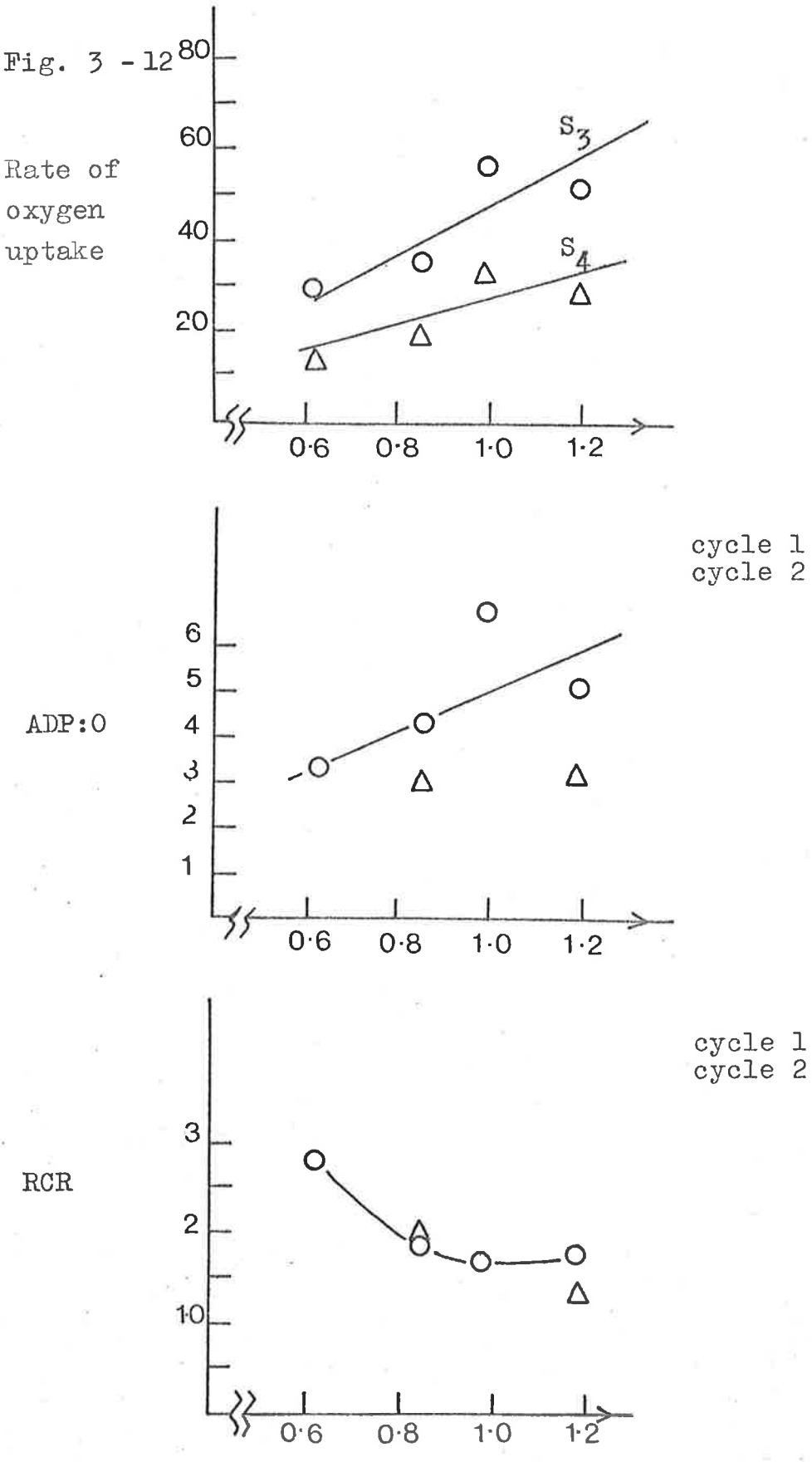

Rate of oxygen uptake
80
60
40
20
S_3
S_4
0·6
0·8
1·0
1·2
ADP:O
6
5
4
3
2
1
cycle 1
cycle 2
RCR
3
2
1·0

Fig. 3-13

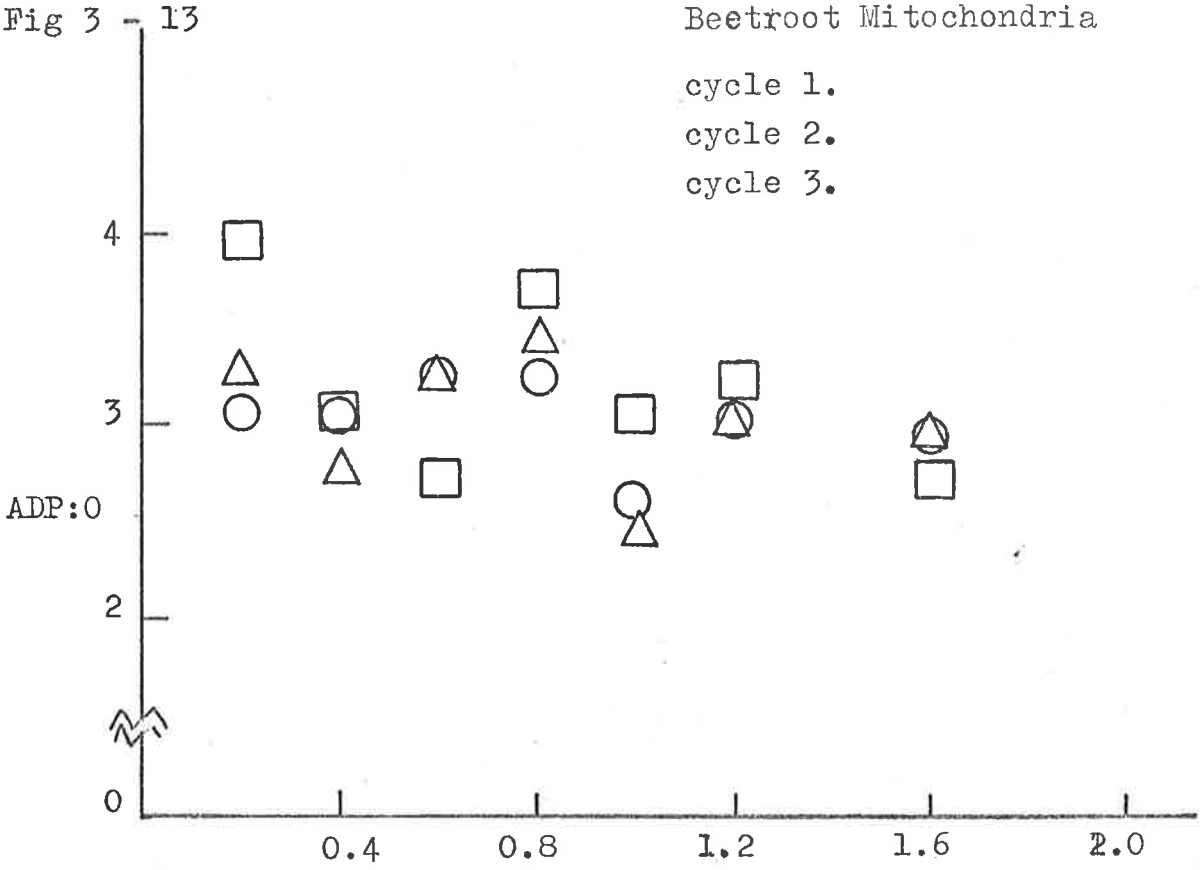
ADP:O and respiratory control ratios (RCR) vs protein concentration with beetroot mitochondria.

15 mM KGA and 10 mM malaonate were added to the standard sucrose medium and the volume made to 2 mls by addition for each run of between 0.1 to 0.7 ml of a mitochondrial suspension containing 4 mg protein per ml. ADP:O and RCR values are plotted against final protein concentration (mg $\cdot ml^{-1}$) in the reaction vessel. Values for 3 cycles of state 3 to state 4 transitions are shown:- O = cycle 1, Δ = cycle 2, $\square$ = cycle 3.

Fig 3 - 13

Beetroot Mitochondria

cycle 1.
cycle 2.
cycle 3.



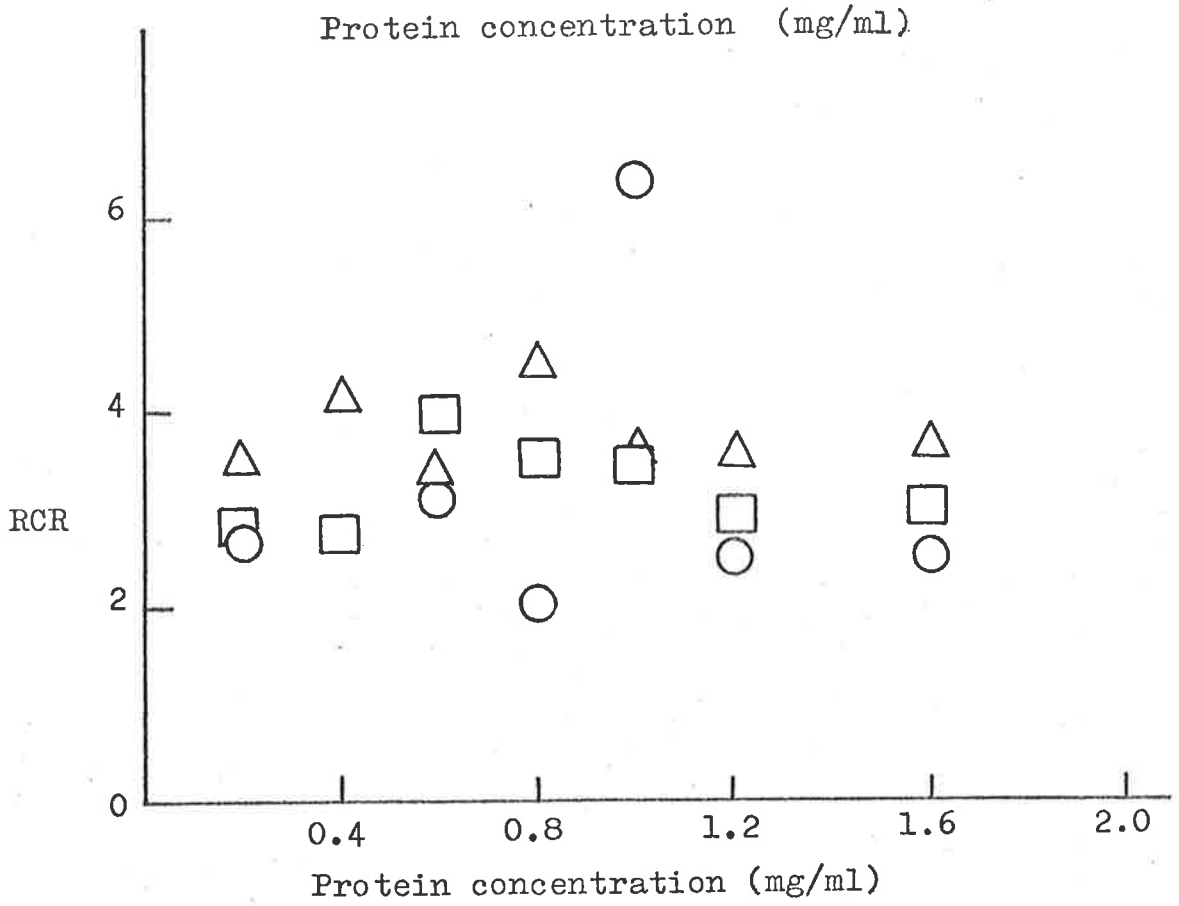
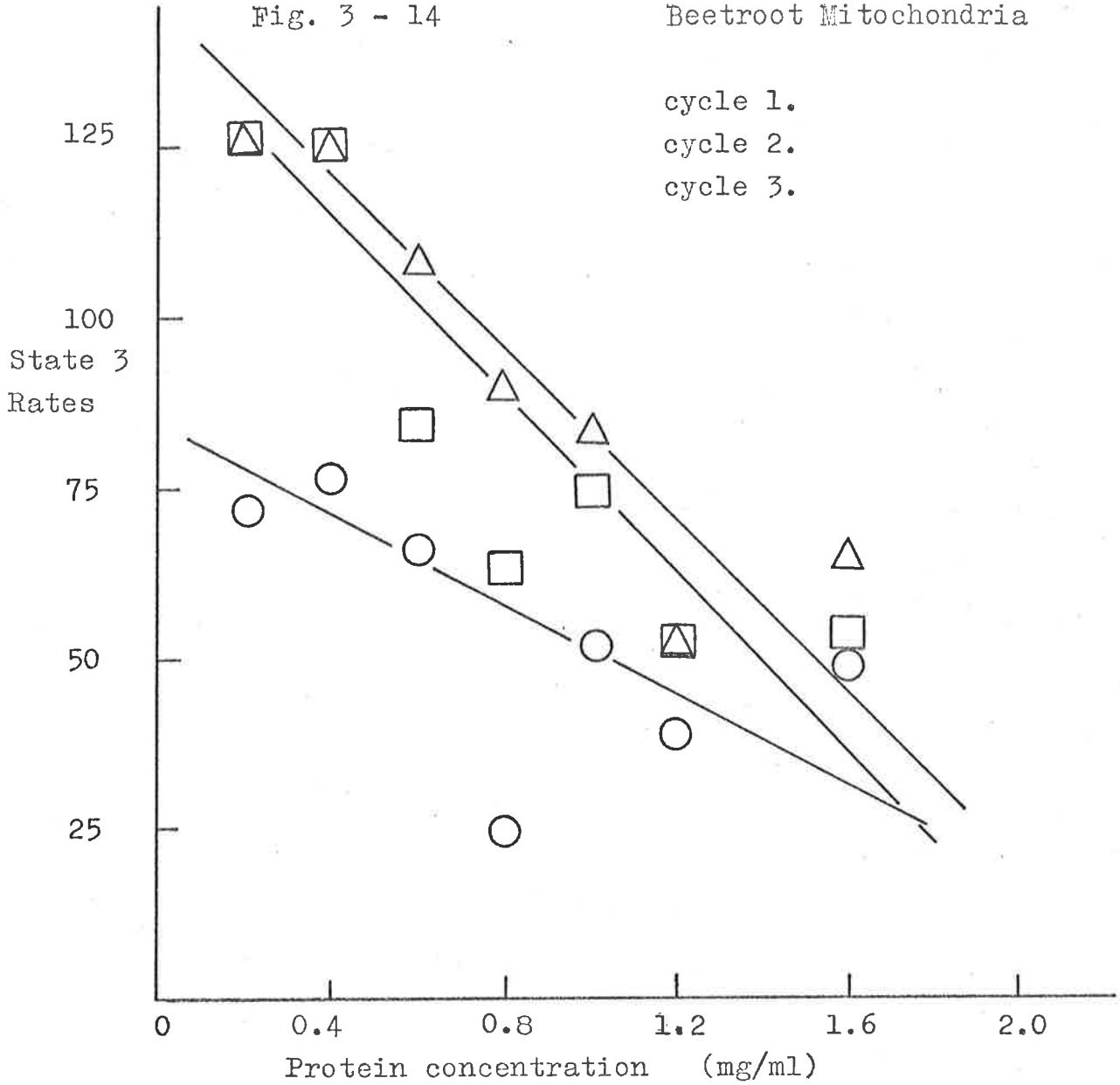


Fig. 3-14

Rates of oxygen uptake vs protein concentration with beetroot mitochondria.

15 mM KGA and 10 mM malonate were added to the standard sucrose medium and the volume was made up to 2 ml by addition for each run of between 0.1 to 0.7 ml of a mitochondrial suspension containing 4 mg protein per ml. Graphs A and B show respectively the state 3 and state 4 rates of oxygen uptake (nmoles O_2 ·mg protein^{-1} ·min^{-1}) plotted against the final protein concentration of each run (mg ·ml^{-1}). Values for three cycles of state 3 to state 4 transitions, are given:- 0 = cycle 1, Δ = cycle 2 and □ = cycle 3.

Fig. 3 - 14 Beetroot Mitochondria



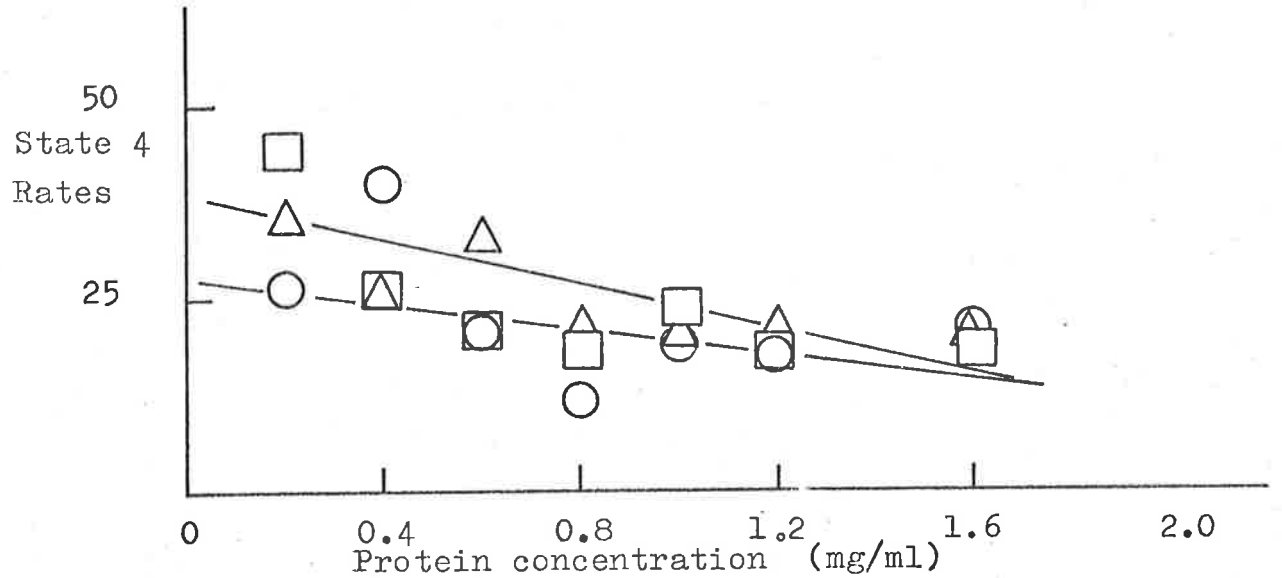
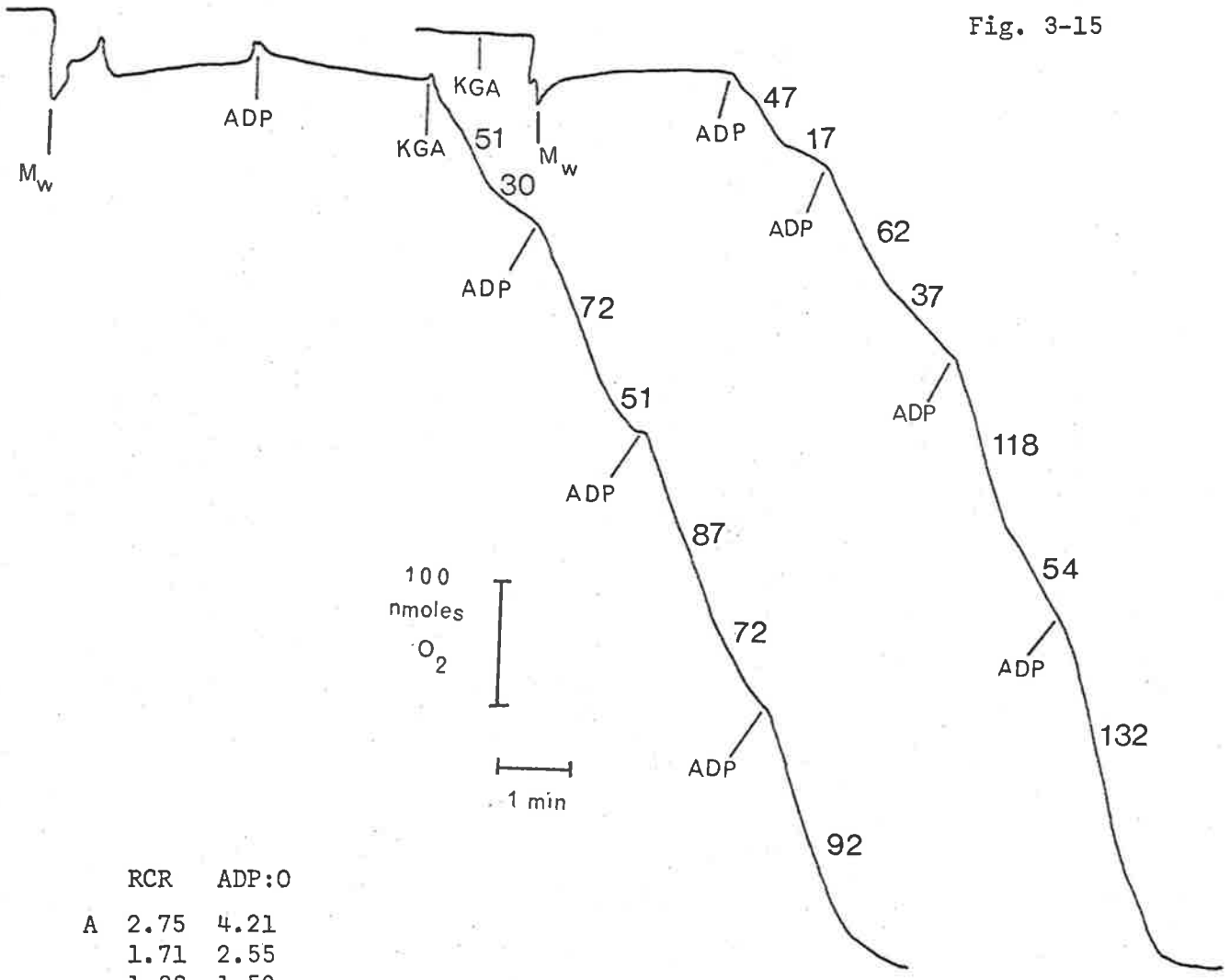


Figure 3-15

Pre-incubation of Wheat Mitochondria with ADP.

WW-15 wheat mitochondria containing 0.9 mg protein were added to the standard sucrose medium with 186.5 µg/ml CoA and 124 µM TPP. Additions of KGA and ADP were 18.6 mM and 140 µM ADP respectively in a total volume of 1.61 mls. A. Mitochondria were incubated with KGA for two minutes before phosphorylation cycles were initiated by 140 µM additions of ADP. B. Mitochondria were pre-incubated with ADP for two minutes before the first phosphorylation cycle was initiated with KGA. Subsequent cycles were initiated by ADP additions. Figures along the trace are rates of oxygen uptake in nmoles O_2 mg protein^{-1} $\cdot$min^{-1}. RCR = respiratory control ratio.

Fig. 3-15



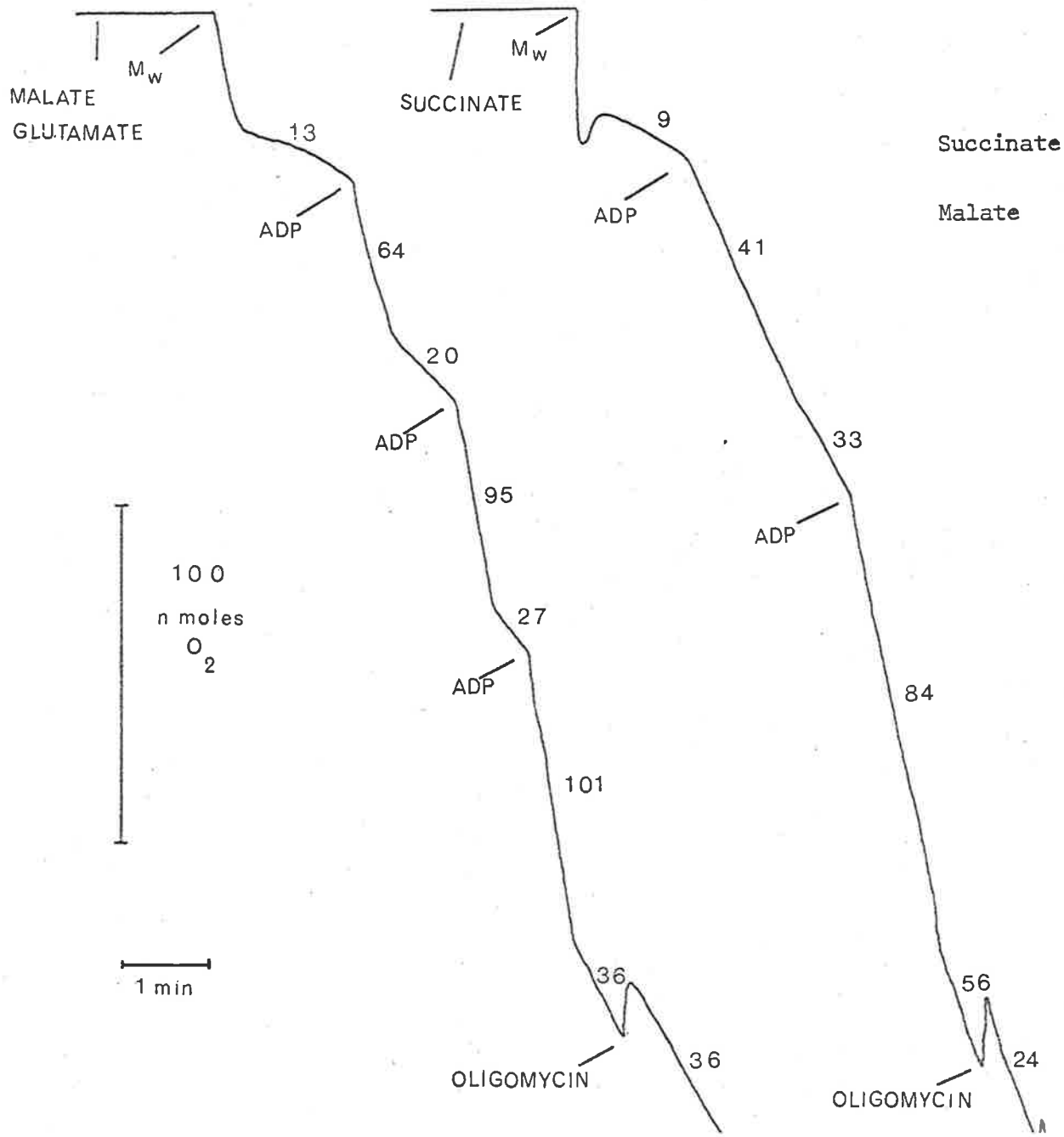
	RCR	ADP:O
A	2.75	4.21
	1.71	2.55
	1.88	1.50
	1.82	1.07
B	1.72	2.50
	1.41	2.135
	1.20	1.54

Fig. 3-16

Succinate and Malate Oxidation by Wheat Mitochondria.

A suspension of Heron wheat mitochondria, containing 1.46 mg protein was incubated in 1 ml standard sucrose medium which contained either, 13.6 mM malate and 34 mM glutamate (A) or 13.6 mM succinate (B) to give a final volume of 1.47 or 1.42 ml. 160 µM ADP, 1 µM CCCP and 5 µgm oligomycin were added where indicated. The numbers along the trace are rates of oxygen uptake and are expressed as nmoles O_2 ·mg protein^{-1} ·min^{-1}. Respiratory control ratio = RCR.

Fig. 3 – 16



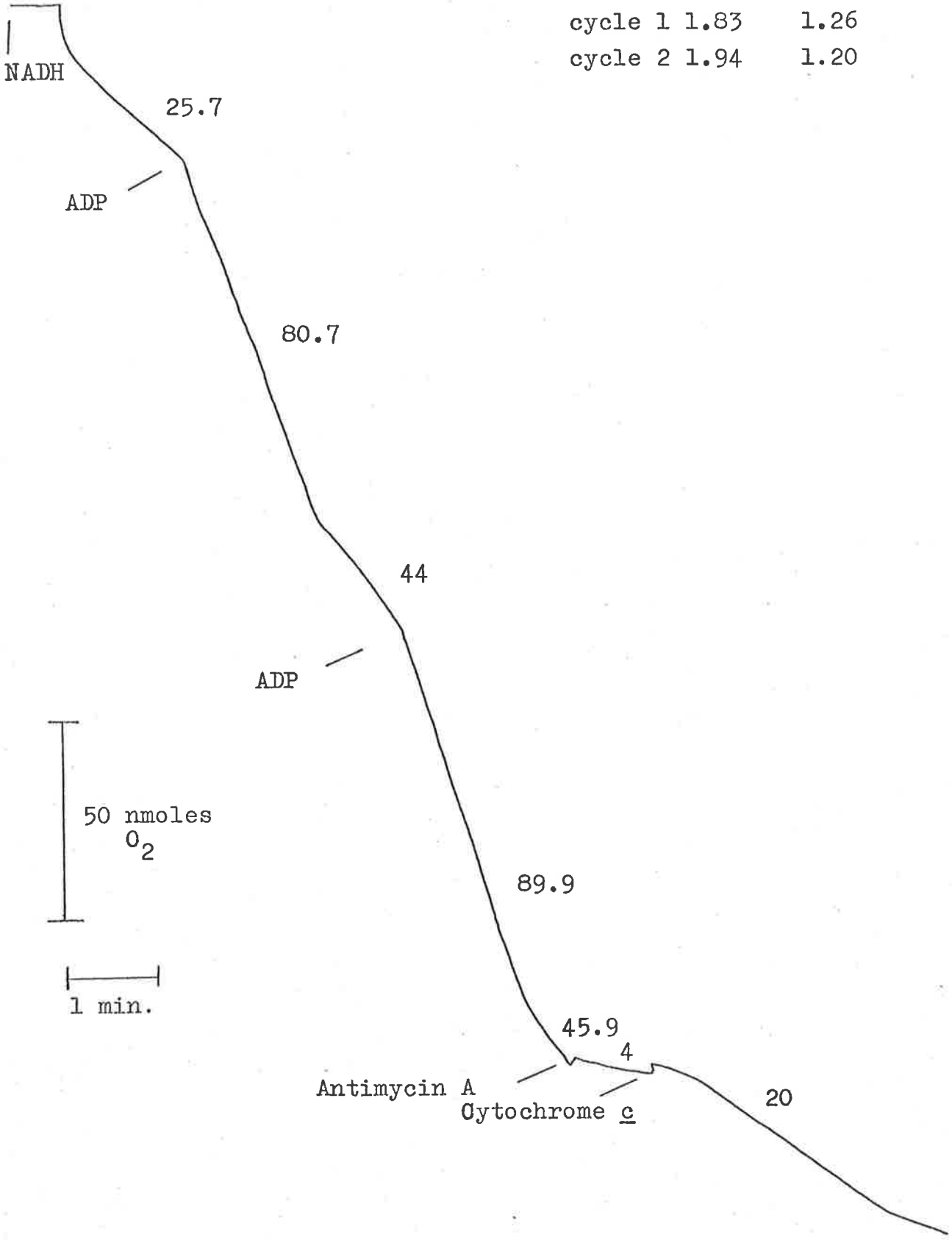
	Cycle	RCR	ADP:O
Succinate	1	1.28	2.04
	2	1.47	1.26
Malate	1	3.12	2.35
	2	3.44	1.92
	3	2.80	1.31

Figure 3-17

Oxidation of 3.2 mM NADH by 0.785 mg mitochondrial protein in a total volume of 1.2 ml was observed. Additions of 187 µM ADP, 532 µM cytochrome *c* and 204 µgm antimycin A/ml were added where indicated. Rates are expressed as nmoles O_2 ·mg protein^{-1} ·min^{-1}.

Fig. 3 - 17

	RCR	ADP:O
cycle 1	1.83	1.26
cycle 2	1.94	1.20



CH. IV SOURCES OF VARIATION AMONG PREPARATIONS

INTRODUCTION

In this study mitochondria were isolated from the shoots of several wheat varieties which had been germinated and grown for different lengths of time. The results were variable so, to determine if preparations could be compared, it was necessary to determine which factors contribute significantly to that variation. Statistical analysis was performed on the first cycle ADP:O ratio figures to elucidate whether there was as much variation within the results of a particular variety as between separate varieties, i.e. are the mean ADP:O ratios between varieties significantly different. Respiratory control ratios were not used as this parameter is greatly influenced by ATP-ase activity. Other possible sources of variation tested for significance were the preparation method used and the age of tissue at harvest. Investigations were by means of a series of t-tests, an analysis of variance not being attempted because of the widely different values of observations in each sample. The ADP:O ratios were assumed to belong to a normal distribution but it was not assumed that each population had an equal variance. This was incorporated into the formula used to calculate the combined t value and the critical t value t'. Significance was determined by comparison of t and t'; if t exceeds t' the means of each sample are said to be significantly different at the chosen level of significance (Edwards,

1970).

RESULTS AND DISCUSSION

The influence of preparation method on ADP:O ratios

The results of different varieties were first arranged in three preparation technique classes, methods D, E, and F (Table 2-3) with each substrate treated separately as shown in Table 4-1. The number of varieties in each class containing 3 or more observations was low, thus a representative cover of interaction between lines could not have been obtained by comparing means of different varieties within groups. Heron wheat was used consistently and appeared in every class so it was possible to compare the means of different isolation methods for one variety and determine whether this factor was significant. The results of these comparisons are shown in Table 4-2. Neither malate nor KGA oxidation results showed significance at the 5 percent level for the three comparisons, so one can conclude that the preparation method is not having a significant effect on the mean values ADP:O ratio of mitochondria so isolated. From this result it was decided to group the results into varieties with no regard to preparation method. In this way the sample size and the number of samples could be increased to give more reliable statistical comparisons.

The influence of wheat variety on ADP:O ratios

There were seven varieties for each substrate which had four or more observations and these were grouped as shown in Table 4-3.

These groups were compared and the results displayed in seven by seven tables (Table 4-4&4-5) The results for malate show that there is no significant difference between any of the compared means, so for malate the variety is clearly not an important factor determining the spread of results. It also suggests that no ADP:O values can be assigned to a particular variety as being characteristic of that variety, which would limit ones ability to predict yield from ADP:O ratios. In the table of comparison for KGA (Table 4-5) all comparisons are non-significant with the exception of the variety Gamut which is significantly different from Heron, Halberd and WW-15 although not from other strains. This exception can perhaps be explained by the small sample size of Gamut, n = 4 as compared to Heron with n = 24, Halberd with n = 6 and WW-15 with n = 6. The mean value of 5.74 for Gamut is well above the theoretical maximum for the ADP:O ratio with KGA and this result may only indicate that Gamut mitochondria are more susceptible to the shortened first cycle oxidation than the other varieties. Unfortunately there were only three observations for Gamut in the malate class which was insufficient to include it in the table for that substrate, however there was no indication from the malate mean ADP:O ratio of 2.85 that Gamut is in any way distinguished from the other varieties. Thus it is concluded that the significance in KGA comes either from biased sampling or as a result of the first cycle artifact in ADP utilization.

The effect of tissue age on mitochondrial activity

The respiration rate of a germinating seedling has been shown to alter with successive stages of its development (Lund et al., 1958; Beevers and Walker, 1956; Solomos et al., 1972). It was possible for these authors to find correlations between the metabolic activity of the tissue and the activity of cytoplasmic particles extracted from that tissue. Much of this work involved cotyledons which are a special case, because the organ undergoes a rapid cycle of activation and death during the first days of a seedlin'gs growth (Young et al., 1960; Bain and Mercer, 1964; Solomos et al., 1972). However studies on monocotyledons such as maize (Lund, Vatter and Hanson, 1957) showed that these correlations apply to tissue from germinating seedlings of all types. The age of tissue selected as the source of mitochondria must therefore correspond to the most active period in the germination phase. Srivastava and Sarkissian (1970) found that wheat shoot mitochondria had a peak of activity when seedlings had been germinating for 2½ to 3 days. In this study the precise age of wheat shoots at harvest was recorded for every preparation as it was anticipated that age may be a source of variation in mitochondrial performance.

A comparison of the activity of a series of mitochondrial preparations made from 2 to 5 day old Halberd wheat shoots is presented in Table 4-6. Seedling age was shown to be an important determinant of mitochondrial activity for this single variety with the 2 and 2½ day old shoots producing the most active mitochondria as judged by ADP:O

and respiratory control ratios. These observations were extended to cover all the varieties used in this study by statistical analysis of the first cycle ADP:O ratio values of all the preparations made. Since genotype is not a significant determinant of mitochondrial activity, five age classes were established as follows:

Class	Age	n	Malate mean ADP:O	n	KGA mean ADP:O
A	50-59 hrs	7	2.83	3	3.91
B	60-62 hrs	51	2.65	24	4.84
C	63-65 hrs	7	2.18	6	3.73
D	66-71 hrs	15	2.27	19	3.39
E	72 + hrs	30	2.18	18	4.38

Varieties and preparation methods were combined and the means of each group tested for significance, again by a t-test for $n_1 \neq n_2$ and $\sigma_1 \neq \sigma_2$. The results of these tests are tabulated in a two by two design shown in Table 4-7. Malate shows significant differences between the means from A and E, and B and E, with no significance for closer age groups. KGA activity also showed significant differences between age groups but they were not the extreme groups as in the malate figures, but groups B and D, and D and E, both showing significance at the 1 percent level.

Discussion

Oxidising malate, mitochondria from 59 hour or younger shoots have significantly higher mean ADP:O ratios in cycle one than those

from shoots over 66 hours old. Those mitochondria from class B (60-62 hrs) are significantly more active than mitochondria from over 72 hour old wheat. The age of tissue is therefore an important source of variation in ADP:O ratios with the highest mean values occurring in young shoots. The non-significance between adjacent classes means there must be a gradual change in activity with age making it valid to compare preparations of varying age as long as the discrepancy is less than 6 to 7 hours. Some heterosis and complementation experiments took 4 hours to complete with each preparation being done individually, so that the first and last preparation would belong to different age classes, yet these would be comparable.

For KGA oxidation the highest ADP:O ratios also occur in the younger classes, with the exception of group E. The significance found between B and D, and D and E tells us that D is lower than the adjacent classes, so once again one can only validly compare mitochondria from tissue ranging in age from 59 to 65 hours. Results from mitochondria over 72 hours are above the theoretical value and equivalent only with group B. They are probably a reflection of the high first cycle ADP:O ratios discussed in chapter III rather than an indication of high phosphorylation efficiency. The usefulness of these figures is thus limited for KGA oxidation.

Conclusion

Statistical analysis has shown that the variation in ADP:O

ratio among wheat varieties is not significantly different from that within a single variety, and similarly that variation among preparation methods is not significantly different from that within one method. Therefore it is valid to compare mitochondria preparations independent of the wheat variety or the isolation procedure used. However the age of the tissue at harvest appears to have a definite influence on the ADP:O ratios obtained and thus these results support the contention of Srivastava and Sarkissian (1970) that there is an optimal age to extract mitochondria from wheat shoots. The age of fresh tissue at harvest must be considered before comparison among preparations as a discrepancy of over six hours would introduce significant differences into the results.

Table 4-1

Mean ADP:O ratios for cycle 1 oxidation of malate and KGA, grouped into varieties and arranged in classes of isolation procedure. Only those varieties where three or more observations had been made comprise each class. Methods described in Table 2-3.

(D) Sarkissian and Srivastava method

	Malate			KGA	
Variety	n	$\bar{x}$	Variety	n	$\bar{x}$
Heron	8	2.7	Heron	10	3.64
Halberd	16	2.41	Chile	3	3.43
Nabawa	3	1.84			
Festival	3	2.39			

(E) Sage and Hobson method

	Malate			KGA	
	n	$\bar{x}$		n	$\bar{x}$
Heron	4	2.43	Heron	5	4.18
			Gamut	3	5.74
			4894	5	4.74

(F) Ellis, Bruton and Palmer method

	Malate			KGA	
	n	$\bar{x}$		n	$\bar{x}$
Heron	18	2.51	Heron	9	2.87
Gabo	9	2.18	Gabo	4	3.65
			WW-15	5	2.78

Table 4-2

t-test on the mean ADP:O ratios of cycle 1 malate oxidation of Heron wheat from three preparation classes, D, E and F (see Table 2-3).

The number of observations in each sample (n); the mean ADP:O ($\bar{x}$), and the sample value of t (t_i), from which the combined t (t) and the critical t (t') is computed, are given. Significance is taken at the 5% level (*) or the 1% level (**) for a two tailed test.

NS indicates no significance

method (D)	method (E)	method (F)
$N_1 = 4$	$n_2 = 8$	$n_3 = 18$
$\bar{x}_1 = 2.43$	$\bar{x}_2 = 2.7$	$\bar{x}_3 = 2.51$
$t_1 = 3.182$	$t_2 = 2.365$	$t_3 = 2.11$

Comparison of the Statistics t and t'

Method D vs Method E	t =	-0.83	t' =	2.922	NS
Method D vs Method F	t =	-0.766	t' =	2.25	NS
Method E vs Method F	t =	0.032	t' =	3.18	NS

Table 4-3

Malate	Combined Data		KGA		
Variety	n	$\bar{x}$ ADP:O	Variety	n	$\bar{x}$ ADP:O
Heron	30	2.55	Heron	24	3.47
Halberd	19	2.61	Halberd	6	3.21
Gabo	13	2.34	WW-15	6	3.13
Nabawa	4	2.08	4894	5	4.74
Pusa 4	4	2.09	Chile	5	4.52
IRN	4	2.895	Gabo	5	3.87
Chile	4	2.98	Gamut	4	5.74

Mean ADP:O ratios for varieties which, after combination of the isolation method classes, had $n \leq 4$.

Table 4-4

t-test on paired mean 1st cycle ADP:O ratios of each variety where $n \leq 4$. t = combined value of "t". t' = critical value of t at 5% level of significance. t" = critical value of t at 1% level of significance. NS = not significant. * = significant at 5% level. ** = significant at 1% level. Results are obtained by combining preparation method groups. Malate as substrate.

Table 4-4

MALATE OXIDATION

Variety	Nabawa						
Halberd	t = 1.27 t' = 3.46 NS						
		Halberd					
Festival	t = 0.37 t' = 4.1 NS	t = 0.2 t' = 4.13 NS					
			Festival				
Chile IB	t = 0.44 t' = 2.05 NS	t = 1.09 t' = 2.73 NS	t = 0.74 t' = 4.19 NS				
				Chile IB			
Gabo	t = 0.99 t' = 3.02 NS	t = 0.99 t' = 2.14 NS	t = 0.06 t' = 4.21 NS	t = 2.11 t' = 2.91 NS			
					Gabo		
Heron	t = 1.24 t' = 3.07 NS	t = 0.26 t' = 2.09 NS	t = 0.21 t' = 4.25 NS	t = 1.54 t' = 2.99 NS	t = 1.33 t' = 2.28 NS		
						Heron	
Pusa 4	t = 0.162 t' = 3.18 NS	t = 0.94 t' = 3.01 NS	t = 0.33 t' = 3.53 NS	t = 1.56 t' = 3.18 NS	t = 0.42 t' = 3.11 NS	t = 0.88 t' = 3.13 NS	
							Pusa 4
IRN	t = 0.95 t' = 3.98 NS	t = 0.35 t' = 3.1 NS	t = 0.46 t' = 3.72 NS	t = 0.10 t' = 3.18 NS	t = 0.67 t' = 3.08 NS	t = 0.44 t' = 3.16 NS	t = 0.86 t' = 3.182 NS

Table 4-5

t-test on paired mean 1st cycle ADP:O ratios of each variety where n < 4. t = combined value of "t". t' = critical value of t at 5% level of significance. t" = critical value of t at 1% level of significance. NS = not significant. * = significant at 5% level. ** = significant at 1% level. Results are obtained by combining preparation method groups. KGA as substrate.

Table 4-5

KGA OXIDATION

Variety	Heron					
Halberd	t = 0.61 t' = 2.49 NS					
		Halberd				
Gamut	t = 5.4 t' = 2.99* t" = 4.25** NS	t = 4.63 t' = 2.87* NS				
			Gamut			
4894	t = 2.11 t' = 2.72 NS	t = 2.29 t' = 2.52 NS	t = 1.44 t' = 2.9 NS			
				4894		
WW-15	t = 0.95 t' = 2.46 NS	t = 0.95 t' = 2.46 NS	t = 2.44 t' = 2.78 NS	t = 2.44 t' = 2.78 NS		
					WW-15	
Chile IB	t = 1.42 t' = 2.74 NS	t = 1.6 t' = 2.73 NS	t = 1.49 t' = 2.87 NS	t = 0.24 t' = 2.78 NS	t = 1.77 t' = 2.74 NS	
						Chile IB
Gabo	t = 1.42 t' = 2.74 NS	t = 0.98 t' = 2.71 NS	t = 2.79 t' = 2.91 NS	t = 1.09 t' = 2.78 NS	t = 1.77 t' = 2.74 NS	t = 0.72 t' = 2.78 NS

Table 4-6

TISSUE AGE Vs ACTIVITY

Age	means of RCR	ADP:O
2 days	1.85	2.56
2½ days	2.42	2.48
3 days	2.12	1.74
4 days	1.83	1.94
4½ days	-	-
5 days	-	-

First cycle ADP:O ratios obtained during malate oxidation by mitochondria extracted from Halberd wheat shoots of varying ages. Conditions of assay were as described in Ch. II. Mitochondrial protein per reaction ranged from 0.8 to 1.2 mg with conditions as described for figure 3-8. Mitochondria from 4½ to 5 day old shoots showed ADP-stimulated oxygen uptake but lacked control.

Table 4-7

Table of comparisons by t-test of pairs of mean ADP:O ratios from 5 age classes, where A = ⩽ 59 hrs, B = 60-62 hrs, C = 63-65 hrs, D = 66-71 hrs and E = ⩽ 72 hrs. t = combined value of t. t' = critical t at 5% level of significance. Significance determined where t' ⩽ t. NS = non-significant. * = significant at the 5% level. Results derived from data of combined wheat varieties.

Table 4-7

Malate

	A	B	C	D
B	t = 0.95 t' = 2.28 NS			
C	t = 1.95 t' = 2.45 NS	t = 1.47 t' = 2.38 NS		
D	t = 2.28 t' = 2.26 *	t = 1.69 t' = 2.09 NS	t = 0.25 t' = 2.36 NS	
E	t = 3.5 t' = 2.31 *	t = 2.97 t' = 2.0 *	t = 0 t' = 2.4 NS	t = 0.41 t' = 2.12 NS

KGA

	A	B	C	D
B	t = 1.42 t' = 3.48 NS			
C	t = 0.29 t' = 3.83 NS	t = 2.17 t' = 2.27 NS		
D	t = 0.09 t' = 4.3 NS	t = 3.09 t' = 2.08 *	t = 0.84 t' = 2.4 NS	
E	t = 0.74 t' = 3.59 NS	t = 0.85 t' = 2.09 NS	t = 1.34 t' = 2.31 NS	t = 2.25 t' = 2.11 *

CH. V HETEROSIS IN WHEAT

Introduction

In this chapter the phenomenon of heterosis is examined for a series of wheat crosses for Australian and imported lines. The investigations were carried out on three organization levels in an attempt to isolate the control for the expression of heterosis. Firstly, studies were made of the comparative rates of seed germination and seedling growth to observe the expression of heterosis when the whole organism is acting in integrated growth. Secondly, the rates of tissue respiration were compared between parent and hybrid lines under coupled and uncoupled conditions and thirdly, isolated mitochondria were compared. The third line of approach resulted from the work of McDaniel and Sarkissian (1966, 1967, 1968, 1970), Sarkissian and McDaniel (1967), Sarkissian and Srivastava (1969, 1970, 1971) and Sage and Hobson (1973) who claim that isolated mitochondria reflect the expression of whole plant heterosis, and thus are a possible controlling site for heterosis. The results of each type of study will be used to rank the crosses in order of descending heterosis and the overall agreement between the three methods of investigation will be discussed.

As mentioned in Chapter I there have been no studies which prove that mitochondrial heterosis can be associated with yield heterosis and the whole case for relating mitochondrial heterosis with yield heterosis depends on the link between seedling and yield heterosis (Sarkissian and Srivastava, 1967). Thus it is important to establish that the reported

relationship between seedling heterosis and mitochondrial heterosis exists in these trials. Yield trials were not done as part of this project but a limited amount of yield heterosis data has been provided for the Australian wheat crosses by Mr. P. Wilson of Tamworth, N.S.W. This field heterosis data will be compared to the laboratory data to assess the extent to which the latter predicts the yield performance.

Seedling heterosis

To determine the mechanism of gene action responsible for differences between a hybrid and its parents, workers studied differences in the early stages of development of hybrid and parental seedlings, and in particular the contribution of seed and embryo size to the percentage hybrid vigour expressed (Ashby, 1930; Copeland, 1940; Whaley, 1950, 1952). Reports have been conflicting as to whether hybrid vigour is associated with large seed and embryo size. Whaley (1950) points to the wide variation of seed size in ten different inbred lines of hybrid corn. The F_1 seed embryo produced from crosses of these lines were in most cases intermediate in weight between that of the two parents while the weight of other seed components followed or slightly exceeded the weight of the female parent. This indicates that seed size is not playing a large part in determining embryo vigour and the main influence is from the vigour of the maternal plant on which the seed was formed, as discussed in Chapter I.

Germination trials were performed on parental and hybrid wheat lines where the three parameters, seed dry weight and length and weight of the etiolated coleoptiles were measured. Since Whaley (1952) claims

the heterotic advantage is the result of initial advantage, measurements were made after three and four days of growth.

Results

Table 5-1 presents the percentage heterosis found in two trials for values dry seed weight, coleoptile weight and length and shows that there is not always agreement between these parameters in the direction or extent of heterosis. In several crosses, altering the length of germination period altered the heterosis observed. The crosses Eureka by IRN and Gabo by Pusa 4 were the only ones to show consistent positive heterosis. The crosses 109H by 272H, 31MS by 28, Nabawa by Chile, and 109H by 261 form an intermediate group with some positive and some negative heterosis for the different parameters. The three day trials for 109H by 272H showed growth heterosis despite smaller hybrid seed, but this was not found in the four day trials where the vigorous female parent 109H outgrew the hybrid. The other American hybrid, 31MS by 28 also had smaller seeds than the 31MS parent but still produced heavier shoots which were the same length as that of the male parent line 28, thus overcoming any possible seed size effect. Nabawa by ChileIB showed strong seed size heterosis thus the positive shoot weight heterosis in the early trial may be a reflection of the size advantage. The loss of positive shoot weight heterosis in the 4 day trial supports this view as the initial advantage of the hybrid seedling could not be maintained. Relative measurements of coleoptile length showed strong negative heterosis,

but this may be due to Nabawa being a tall variety. In 109H by 261 where only shoot weight for three day seedlings showed positive heterosis, neither the hybrid nor strain 261 could match the vigour of strain 109H. Heron by Strain 52 and Heron by Gamut form the final group of totally negative heterosis for seedling growth characteristics, and thus could not maintain the growth advantage arising from larger hybrid seed size.

To assess the possibility that fluctuations in seed quality could be causing the disparity seen in the intermediate class and that positive growth heterosis may be the result of more virilent seed, (Ellis and Hanson, 1974) percentage germination was determined for parent and hybrid seed. Table 5-2 shows the percent germination of the eight crosses studied and the percentage heterosis exhibited by the hybrid. In all but one cross (Eureka by IRN) at least one parent had full germination, thus the hybrid could be equal to or less than this parent and positive heterosis was recorded only for Eureka by IRN. The lowest hybrid germination was only thirteen percent below its highest parent indicating that all seed was of an evenly high quality and positive heterosis in the growth characters already discussed was not due to poorer quality parent seed as the hybrid had, in most cases, overcome a slight disadvantage in seed quality.

Discussion

Germination characteristics should give an indication of a seedling's initial capacity to grow using its seed reserves. Since shoot material was used exclusively in mitochondrial studies, measurements of

germination concentrated on coleoptile development. These trials indicate that the amount of shoot growth has little relationship to the initial dry weight of seeds. Heterosis observed in the length and weight of the coleoptiles was found to be related, being in the same direction in all but one cross, 109H by 261 where the shoots were shorter but heavier than the parents. This can be tested statistically by applying Spearman's rank coefficient of concordance (Siegel, 1956) to the results of these two estimations of heterosis. The crosses are ranked in descending order of percent heterosis for both length and weight. The difference between the rankings under each parameter is calculated so that the correlation coefficient (r_s) can be determined from the formula:

$$r_s = 1 - \frac{6 \Sigma_d^n i^2}{N^3 - N}$$

where N is the number of observations and d the difference between the two ranks for each cross. The significance of r_s is determined by comparison with a critical value.

The correlation coefficients for the 3 and 4 day germination trial were 0.86 and 0.89 respectively and were both significant at the five percent level. Thus the characteristics of growth are in agreement with each other within one trial. A ranking of the crosses could be obtained by taking a sum of the ranks and it was seen that there was little agreement between the two listings as shown below:

3 Day Germination Studies

Cross			Σ Ranks
Gabo	X	Pusa 4	3
109H	X	272 H	3
Nabawa	X	Chile	7
Eureka	X	IRN	8
109H	X	272H	10
31MS	X	28	11
Heron	X	Gamut	14
Heron	X	Strain 52	16

4 Day Germination Studies

Cross			Σ Ranks
Eureka	X	IRN	3
Heron	X	Strain 52	3
Nabawa	X	Chile IB	7
109H	X	261	7
109H	X	272H	10
Heron	X	Gamut	12

The presence of three tied positions in this listing also limits its usefulness.

The heterosis shown after 3 days is not always maintained after 4 days of growth (109H X 272H, Nabawa X Chile IB and 109H X 261) so an overall estimate of rank was sought using the six crosses common to both trials the growth heterosis figures were again ranked. The 4 rank values are summed as shown below.

Eureka X IRN	Nabawa X Chile IB	109H X 272H	109H X 261	Heron X Strain 52	Heron X Gamut	Shoot Heterosis
4	2	1	3	6	5	weight
2	3	1	4	6	5	length
1	3	5	4	2	6	weight
2	4	5	3	1	6	length
9	12	12	14	15	22	

It is possible to test whether the individual estimates of ranking in each test are consistent by employing Kendall's coefficient of concordance W (Siegel, 1956). If there is overall agreement among the different rankings the variance for the sum of ranks gained by each cross will be maximum while disagreement will produce approximately equal summed ranks. The coefficient can have values from 0 to 1 and is calculated by the formula:

$$W = \frac{\underline{s}}{\frac{1}{12} k^3 (N^3 - N)}$$

where k is the number of sets of rankings, N is the number of entities ranked and $\underline{s}$ is the sum of squared deviations from the mean sum of ranks.

The coefficient of concordance for these trials was 0.35 which is not significant at a five percent probability level. Thus when the four estimates are taken together they disagree as to the relative position of the 6 crosses. Any overall estimate of rank gained from these sums of ranks can only be taken as a rough guide especially as Nabawa by Chile IB and 109H by 272H share the second rank as is shown in the ordering of crosses set out below.

Cross	Rank
Eureka X IRN	9
Nabawa X Chile IB	12
109H X 272H	12
109H X 261	14
Heron X Strain 52	15
Heron X Gamut	22

This list agrees in all but two crosses with a non-statistical ranking obtained by dividing the crosses into three categories, total positive heterosis, partial positive heterosis and no positive heterosis for the different estimates (Table 5-1) as is shown below:

Total positive heterosis	-	Eureka X IRN
		Gabo X Pusa 4
Partial positive heterosis	-	109H X 272H
		31 MS X 28
		Nabawa X Chile IB
		Heron X Strain 52
No positive heterosis	-	Heron X Gamut
		109H X 261

These rankings will be compared in a general discussion to those obtained by other measurements of heterosis, and yield heterosis in particular.

Whole tissue seedling respiration

Manometric studies of the respiratory rate of whole seedling tissue were undertaken to determine whether heterotic growth was reflected by increased hybrid tissue respiration as has been suggested by Sarkissian and Srivastava (1969) for wheat tissue and McDaniel (1969, 1973) for barley. These reports claim increases of 125 and 130 percent respectively, in the respiration of a hybrid when compared to its highest parent.

Respiration of whole tissue is subject to cytoplasmic as well as

mitochondrial controls, and heterotic performance could be due to enhancement of the enzyme systems on either level. In order to determine where the rate limiting steps are located, DNP was used to uncouple mitochondia in situ. Addition of DNP to respiring tissue will increase the rate of oxygen uptake if cytoplasmic factors are limiting respiration. If DNP penetrates the tissue, yet causes no stimulation one can assume the mitochondria are already working at their maximum capacity and are limiting the rate of oxygen uptake. Wound respiration the short term rise in respiratory rate accompanying tissue disruption (Eberhardt, 1960) may mask the true rate of seedling respiration, especially in tissue which has been finely diced to facilitate penetration of uncouplers and diffusion of gases to the liquid phase. The wound response may occur unevenly, adding to the variation in results and possibly obliterating differences caused by genotype. Finally almost all measurements made in this study were expressed on a fresh weight basis whereas Sarkissian and Srivastava (1969) expressed their values on a nitrogen basis, estimating the nitrogen content from protein measurements. Faster growing seedlings at an advanced physiological age may have a higher number of elongated cells which would contain more cell sap but little more cytoplasm than the shorter, slower developing tissues. Thus material compared on a fresh weight basis may not have equivalent amounts of protein and it would be the heterotic hybrids in which the comparative rate of oxygen uptake is lowered. Trials were carried out on tissue compared on a dry weight basis using the hybrid 31 MS X 28 and its parents,

in an attempt to repeat Sarkissian and Srivastava's results.

RESULTS

(1) Influence of wound respiration

The effect of tissue preparation on respiration rates and heterosis was examined to determine what type of tissue gave consistent results and was most favourable for demonstrating hybrid superiority. An experiment was set up to determine the influence of wound respiration in diced tissue and the change in the rates when whole seedlings including the seed were used. Two treatments used intact seedlings with the seed (A) and without the seed (B). A further two groups involved diced shoot and root tissue, one left standing in phosphate buffer for 3 hours before use (C), and the other used immediately after slicing (D). The results of these trials are shown in Table 5-3. The rates of oxygen uptake are similar for treatments A and B and range from 388 to 770 µl $O_2 \cdot gm\ fr\ wt^{-1} \cdot hr^{-1}$. Group A would have less shoot and root material on a fresh weight basis than group B where the slowly respiring seed was excluded. Presumably the shoot tissue contributes significantly more to the respiration rate than the seed and this may account for the appearance of strong positive heterosis seen in B but not seen in A.

Dicing shoot and root tissue increased the respiratory rates of the parents in treatment C but not that of the hybrid. In group D where wound respiration would be expected to appear, an increase occurred in only one parent (IRN) and was again not seen in the hybrid samples. Thus

a strong negative heterosis was recorded under conditions C and D.

Since dicing tissue altered the rates of respiration of some parents, especially when tissue was left standing, whole seedlings with seeds removed were used for most experiments except with substrates and uncoupler when freshly diced tissue was preferred.

(11) The effect of DNP on tissue respiration

The action of DNP on whole tissue respiration and on diced shoot material was generally to stimulate oxygen uptake but usually by only a small percentage, whereas other fresh tissues have been shown to be stimulated by over 100 percent (Beevers, 1961). The highest stimulation observed here occurred with Gamut and Heron wheats when using diced shoots. Increases of 118, 74, and 163 percent for the two parents and hybrid were obtained. The magnitude of the response to DNP was variable in these experiments as is shown in Table 5-4, where different percentage increases are observed in two trials done on the same day with the same crop of wheat shoots. Such discrepancies are thought to be the result of penetration differences, even though the tissue had been sliced into 1 mm strips.

(111) Measurement of heterosis in tissue oxygen uptake rates

The respiratory rates and the percentage heterosis derived from those rates for a series of crosses and reciprocal crosses, ranked in decreasing order of positive heterosis are shown in Table 5-5. The respiratory performance of a hybrid is dependent on the direction of the

cross and if mitochondria are inherited cytoplasmically from the female parent this could be expected. However, it could also be a reflection of the vigour of that female parent, on which the hybrid seed developed. It would appear from this data that both positive and negative combining ability can be detected from seedling respiration. These experiments were repeated with the De Kalb lines used by Sarkissian; 31 MS, 28 and their F_1 hybrid. The resulting rates and percentage heterosis are shown in Table 5-6 where they are compared to the values published by Sarkissian and Srivastava (1969). In their data rates are expressed as $\mu l\ O_2 \cdot mg\ N^{-1} \cdot hr^{-1}$ rather than on a fresh weight basis. Since the total nitrogen content of a seedling is more closely related to its dry weight than its fresh weight, respiration was also measured on a dry weight basis and the results are inlcuded in Table 5-6.

The American wheat varieties consistently showed a small, negative heterosis when judged by whole tissue respiration. This result was unaltered when the seedlings were partially uncoupled by DNP and when the rates were expressed on a dry weight basis. The high positive heterosis of 38 percent which has been reported for the 31 MS X 28 cross was not obtained in any experiment although positive heterosis could be obtained with some Australian wheat lines.

The percentage heterosis for coupled and uncoupled rates have been calculated and comparison of the two has shown that heterosis persists in uncoupled tissue. The values obtained are different but the direction of heterosis, positive or negative, with one exception

remaining the same as shown in Table 5-7.

The cause of heterosis may be found in a seedling's ability to mobilise endosperm from the seed, thus if excised shoot material from a heterotic hybrid is suspended in a sucrose or glucose solution the ready supply of substrate may cause the hybrid to lose its advantage. Seedlings from Eureka, IRN and their hybrid, which had shown 80 percent heterosis previously were selected for this trial, as too were Gamut, Heron and their hybrid. Table 5-8 presents the results of the experiment and as can be seen, heterosis persists with a substrate as a bathing solution, thus it is not superior supply of food to the shoots which determines heterosis. To test this hypothesis completely it would be necessary to grow the seedlings in a sucrose medium so that their development at harvest could be compared.

Discussion

It appears that heterosis in whole tissue respiration is determined by cytoplasmic factors as it persists in the presence of DNP and sucrose. The wheat tissue respiration can be stimulated by DNP is evidence that the mitochondria in this tissue are not normally respiring at their maximum rate, thus they are not at full efficiency while in situ and must be limited by other cytoplasmic controls, such as the supply of ADP, of phosphate and reducing power. If the mitochondria are not the site of control it is less likely that they will exhibit meaningful heterosis when artificially isolated from the cytoplasm.

An approximate ranking of crosses in the three classes of heterosis

occurrence is given below. This ranking has been derived from the results in Table 5-5 and will be compared to rankings on other parameters in the general discussion.

High heterosis	Eureka X IRN
	Falcon X Gabo
	Strain 52 X Heron
	Heron X Gamut
	Gamut X Heron
Low heterosis	Chile IB X Nabawa
	Nabawa X Chile IB
	Gabo X Falcon
	Mengavi X Festival
Negative heterosis	Gabo X Pusa 4
	Heron X Strain 52
	IRN X Eureka
	Pusa 4 X Gabo

Mitochondrial Heterosis

The rational behind the use of mitochondria to study heterosis has been outlined in Chapter I. The mitochondrion, it is claimed, is one site of the sub-cellular expression of heterosis and thus plays a determining role in plant development. All previous studies of mitochondrial heterosis have relied heavily on the parameters of integrity and efficiency, the respiratory control ratio and the ADP:O ratio, as

a basis of comparison among mitochondria from tissue of different genotypes. In addition Sarkissian and Srivastava (1971) used the rates of NADH-cytochrome *c* reductase and cytochrome *c* oxidase to establish the heterosis of one particular De Kalb cross 31 MS by 28.

In this study the phenomenon of mitochondrial heterosis was sought in Sarkissian's cross (31 MS x 28) using the parameters he had reported. This work was extended to a survey of crosses in Australian wheat breeding programmes which was intended to show if possible use could be made of the mitochondrial heterosis technique for prediction by Australian wheat breeders. The experiments measuring oxygen uptake were restricted to two substrates, malate and KGA as they had proved most suitable for work with these mitochondria (Chapter IIIA) and because other reports were mainly based on the characteristics of KGA oxidation. It was necessary to see whether heterosis could be detected in the crosses studied and if it was consistent for both substrates and all parameters or if it had a limited or uneven occurrence.

Results

(1)

Heterosis in the cross 31 MS X 28

Polarographic studies of the parents and hybrid from the 31 MS by 28 cross were replicated six times and the data for KGA oxidation and malate oxidation was considered separately. This data was analysed by the Kruskal Wallis one way analysis of variance by ranks (Siegel, 1956), which, being a non-parametric statistical test, did not assume a normal distribution of values. This test was applied to values of ADP:O and

respiratory control ratio and the state 3 and 4 respiration rates. Each cycle of phosphorylation was treated separately. Thus replicated readings in three sets, one for each parent and the hybrid, were compared to see whether the variance between the groups was significantly different. Tables 5-9 and 5-10 show the value of the calculated statistic H and its associated probability of supporting the null hypothesis that all groups are from one population. The values from which these statistics have been calculated are in appendices III and IV.

Groups were considered significantly different at a probability level of 5 percent and lower. As can be seen there was no significance for the KGA oxidation figures for any parameter in any cycle of phosphorylation. Malate also showed no significance for both cycles of ADP:O ratio the first cycle of respiratory control. The second cycle of control showed significant variation between the groups at the 5 percent level. This effect could not be attributed to positive heterosis however as analysis of the sum of ranks for each group (from which the statistic was derived) shows that it is the parent line 28 which far exceeds the hybrid or the parent 31 MS [Σ ranks: line 31 MS = 27.5; line 28 = 49; hybrid = 28.5].

Further, when the average percentage heterosis values are taken (Table 5-11) it can be seen that hybrid advantage is never greater than 10% and may be a disadvantage of as low as - 15.8%. Thus although a mean is only a rough guide these figures would support the general conclusions from the analysis of variance. In particular the second

cycle of respiratory control for malate oxidation had an average percent heterosis of 0.15 which is effectively zero which confirms that the significant variation observed between genotype in the statistical analysis above was not due to a heterotic effect.

Since control ratios are derived from respiratory rates the highly significant variation observed in the second cycle state 3 rates for malate was to be expected. Here the sum of ranks for the hybrid and line 28 were closer than the sum of line 31 MS [Σ ranks: 31 MS = 25; line 28 = 75; hybrid = 54]. Thus significance is observed because mitochondria from line 31 MS have slower rates of oxidation than the other two lines. Heterosis could not be shown to occur statistically and these conclusions are a true reflection of the trend found in data from each experiment where low positive heterosis was only found in one set of preparations, as set out in the histogrammes of percentage heterosis (Figure 5-1). This lack of heterosis for the parameters of oxidative phosphorylation is in direct contrast to the reports of Sarkissian and Srivastava (1969, 1970) for this wheat.

Experiments were extended to NADH-cytochrome *c* reductase activity with intact and osmotically swollen preparations. The intact rate of cytochrome *c* reduction was determined and a significant hybrid advantage was recorded (Table 5-11) being highly positive, 50 percent greater than the highest parent, line 31 MS. The preparations were also assayed after mitochondria had been partially swollen which increased the rate of reduction by an average of 37 percent. The hybrid advantage was retained

by these swollen mitochondria, only dropping to 43.3 percent of the highest parent line 28. Although the overall influence of heterosis may be said to be present, it is interesting that the parental lines reversed their rank. Since osmotic swelling allows the inner electron transport chain to function (Chapter III) the activity in swollen mitochondria should show antimycin A sensitivity. The rate of NADH-cytochrome *c* reductase reaction was measured in the presence of antimycin A in swollen mitochondria and as Table 5-11 shows, the rates dropped to a level similar to that of intact mitochondria. However line 28 was again the highest parent. Thus the change in parental order was not a result of a relatively greater contribution by the inner membrane chain in line 28. The percentage heterosis was lower in this trial but was still highly positive. Thus NADH-cytochrome *c* reductase can support positive heterosis when the reaction occurs via both the inner or outer mitochondrial membrane chain.

Heterosis was also looked for in the associated reactions malate - cytochrome *c* reductase and succinate-cytochrome *c* reductase, which utilize the inner electron transport chain exclusively. Intact mitochondria showed no measurable cytochrome *c* reduction with either substrate so osmotically swollen mitochondria were used for determinations of heterosis. The rates observed were low and completely sensitive to antimycin A and, as shown in Table 5-12, the hybrid exceeded the parents for both assays. Thus this data for swollen mitochondria agrees with that for NADH-cytochrome *c* reductase for intact and swollen mitochondria. One can conclude that hybrid mitochondria reduce exogenous cytochrome *c*

more rapidly than mitochondria from the parental lines irrespective of the pathway used for this reduction.

Heterosis was also looked for in cytochrome oxidase activity as Sarkissian and Srivastava's results (1971) suggest that this oxidase complex exhibits 87 percent heterosis when assayed in intact mitochondria. The activity of the cytochrome a,a_3 complex was assessed in two ways, firstly the rate of oxygen uptake stimulated by ascorbate in the presence of TMPD was followed in the presence and absence of ADP, Secondly, the rate of oxidation of fully reduced cytochrome c was measured spectrophotometrically. Since the cytochrome c cannot readily penetrate to the inner membrane to donate electrons, additional experiments using osmotically swollen mitochondria were included for comparison with rates observed in intact mitochondria.

The results of these determinations are shown in Table 5-13. Ascorbate oxidation by the hybrid mitochondria, in the presence and absence of ADP occurred at a slower rate than that of the highest parent. In the absence of ADP both parental rates were higher with 31MS being the highest giving a percentage heterosis of minus 12 percent. In the presence of ADP the rates of all types of mitochondria increased by 50, 64, and 70 percent for 31MS, 28 and hybrid mitochondria respectively, but even though the hybrid was stimulated more than the parents its rate was equal to that of 31MS and lower than line 28. Thus the level of heterosis was negative again at minus 5.8 percent.

When the cytochrome a,a_3 complex was used to oxidize reduced cytochrome c however quite different results were obtained. As Table 5-13

shows, intact mitochondria had very strong positive heterosis for ferro-cytochrome *c* oxidase both in the presence and absence of ADP. This high level of heterosis was not consistently carried through to studies of osmotically swollen mitochondria where one hybrid increase of 31 percent and one decrease of minus 21 percent were recorded. Thus only the intact mitochondria produced the type of heterosis reported by Sarkissian and Srivastava (1971). A closer study of these results shows that the addition of ADP to intact mitochondria (Table 5-13) did not enhance the rate of reaction found in experiment A. Yet the ascorbate oxidation results had shown that the cytochrome oxidase was stimulated by ADP by over 50 percent. The rates of 21 to 71 nmoles cytochrome *c* oxidized . mg protein^{-1} . min^{-1} are very low and these two observations taken together suggest that the rate is being suppressed by the slow penetration of the substrate (reduced cytochrome *c*) to the enzyme. The rates of oxidation in the broken mitochondria (from 180 to 347 nmoles cytochrome *c* . mg protein^{-1} . min^{-1}) are very similar to those reported by Sarkissian and Srivastava (1971) for parental mitochondria. They found line 31MS and line 28 to oxidize cytochrome *c* at 371 and 265 nmoles cytochrome *c* . mg protein^{-1} . min^{-1} respectively. However at no time was their hybrid rate of 694 nmoles cytochrome *c* . mg protein^{-1} . min-1 obtained in the present experiments.

Discussion

It is thought that the hybrid advantage that has been observed by Sarkissian and Srivastava (1971) and replicated in these results is

not a result of more efficient cytochrome oxidase or cytochrome *c* reductase systems, but a reflection of more broken outer membranes in the hybrid. It is suggested that the mitochondria examined by Sarkissian and Srivastava (1971) for cytochrome oxidase were no more intact than the osmotically swollen mitochondria examined here, as both were tested in the absence of ADP and yet have similar rates. Further it is recognized that breaking the outer membrane by osmotic swelling, although performed in a routine manner, will cause different extents of damage depending on the initial integrity of the mitochondria. Although there is a wide discrepancy in percent heterosis between experiments C and D (Table 5-13) the values obtained for the hybrid are virtually the same, thus the difference comes in the response to swelling of the parents, in particular the highest parent, line 28, which was stimulated to a lesser extent in experiment C than in experiment D. This difference was enough to cause a 50 percent swing in the figure for percent heterosis.

If mitochondria from one genotype have leakier membranes than those of another, the difference in integrity should be detected in a comparison of the state 4 rates of oxidation and the effect on the ADP:O and respiratory control ratios. It is particularly interesting to compare the state 4 rates of oxygen uptake of one set of preparations with the rates of malate-cytochrome *c* reductase. This comparison is made in Table 5-14. As discussed in Chapter III, malate should support a rate of reduction of cytochrome *c* which is four times faster than the rate of oxygen uptake on a molar basis. As this is

clearly not what was obtained the conclusion that cytochrome *c* penetration is limiting the rates is validated. The hybrid mitochondria in this individual experiment show a faster state 4 rate than either parent in both cycles of ADP-stimulated oxygen uptake, which tentatively supports the conclusion that the membranes of the hybrid are more permeable. However the state 3 rates for this preparation also show the same hybrid advantage, which could indicate that the hybrid was capable of faster electron flow in the presence of ADP. This may be inconsistent with the proposal of more broken membranes unless it can be shown to be the result of uncoupling. When the respiratory control and ADP:O ratios for this set of preparations are compared, however, a small negative heterosis is found with both parameters. This further supports the initial proposition and would tend to negate high state 3 rates as an indicator of high efficiency and suggest these observations are indicative of a partially uncoupled respiratory rate, perhaps reflecting the ATP-ase component described in Chapter III.

(11)
Concordance between the different measures of heterosis for the cross 31MS by line 28

Two germination trials in which the growth characteristics of seedlings were compared both indicate that although the hybrid seed is lighter than the highest parent (negative heterosis for seed weight) the shoot grows at a rate equal to or slightly exceeding that of the highest parent. Heterosis for shoot weight in a 2.5 and a 3 day trial respectively, was 15.9 and 4.7 percent. Heterosis for shoot length was -1.5 and 0 percent respectively. Thus germination trials would suggest a small positive heterosis overcoming a disadvantageous seed size effect.

Tissue respiration trials however show an all negative relationship between the hybrid and parent tissue rates of oxygen uptake. Experiments based on tissue fresh weights gave a negative heterosis of -8 percent which persisted in the presence of DNP. Repetition with rates estimated on a dry weight basis showed a consistent and more strongly negative trend with an average percentage value of -19.13 over four experiments. Oxidative phosphorylation trials gave rise to a spectrum of percentage heterosis results which when reduced to mean values for each cycle (Table 5-15) were shown to range between 5.18 and -15.78 percent for KGA oxidation and 9.37 and -7.52 percent for malate oxidation. Half of these values were negative for each substrate and an overall average heterosis reduced to very insignificant numbers in both malate and KGA oxidation figures. KGA showed -3.32 percent and malate 0.67 percent heterosis when an average was taken of the 12 means shown in Table 5-16.

Thus the percentage heterosis as ascertained by manometric observation of tissue oxygen uptake was similar to the overall trend observed when percent heterosis was estimated by mitochondrial oxidative phosphorylation. However the extreme variation between the latter estimates makes it unwise to place too strong an emphasis on an overall or mean figure of heterosis. Contrary to reported results (Sarkissian and Srivastava, 1967; McDaniel, 1971), both types of respiratory estimates of heterosis disagree with the direction of heterosis as estimated by germination trials. However, all three estimates show that very little difference can be seen in the behaviour of the hybrid in relation to its highest parent. The only strong evidence for mitochondrial

heterosis for 31MS by 28 was obtained with enzymic studies of mitochondrial oxidation reduction reactions. Here the results were similar to those of Sarkissian and Srivastava (1971). It could be expected that the overall functioning of the mitochondrial system would have to show improvement if the hybrid advantage at the mitochondrial level were to be transferred to the whole-plant growth and yield characteristics. Thus it is difficult to see how, in the absence of improvements in oxidative phosphorylation, hybrid advantage in redox reactions of the mitochondrial chain, in isolation, could be responsible for hybrid vigour for yield. The explanation proposed previously is thought to better account for this one case of positive biochemical heterosis in the mitochondria of 31MS by 28.

(111)
Mitochondrial heterosis surveyed in a number of crosses: polarographic studies

Mitochondria were prepared from seedlings of a total of seven hybrid crosses and compared to the activity of the parental lines. The histograms, Fig. 5-2 and 5-3 display the percent heterosis for each of the four parameters, ADP:O and respiratory control ratios and the state 3 and state 4 rates, with the two substrates malate and KGA. The data from which these figures were constructed are given in appendix 3-4. Each bar in the figure is the mean of the cycles for each experiment as the majority of experiments show consistency in the direction, if not in the extent, of heterosis between cycles. However, the means for some determinations of heterosis are not as

representative of the real situation because the direction of heterosis is inconsistent. These cases where wide variation occurred have been marked with a star.

The choice of parameter for ranking the crosses for percent heterosis is important because there appears to be no fixed relationship between any of the four criteria used here. Hybrid mitochondria with higher phosphorylation efficiencies do not necessarily have higher control ratios or rates of oxygen uptake than the highest parental line. This is perhaps not surprising as a high respiratory rate can indicate lack of control. It is a serious drawback that the two parameters of integrity do not appear to show agreement as reported by Doney et al (1972), Sarkissian and Srivastava (1969), McDaniel and Sarkissian (1968). The nature of possible agreement was determined by analysing the data from the histograms (Fig. 5-2 & 5-3) further. Mitochondria oxidizing KGA showed close agreement between the percentage heterosis of ADP:O and respiratory control ratios in three trials and agreement in direction only in one further trial with two trials having larger differences in the mean heterosis both in direction and extent. Continuing this qualitative analysis with the results of malate oxidation we see that in four trials the respiratory control ratio and ADP:O are in the same direction although in only one case is the numerical value very close. Disagreement in sign occurs in two cases. The four cases where disparity exists cannot be attributed to the act of averaging individual cycles in which heterotic expression is in opposite directions because such averages show both agreement and dis-

agreement between respiratory control ratio and ADP:O ratio.

It is thus difficult to state categorically that one hybrid will produce greater heterosis than another on this type of data unless one is willing to select one criterion subjectively. There has been no attempt to combine individual experiments in this presentation as repetition of the experiment with one cross produced widely different levels of heterosis in all parameters. Results from repetitions A and B of the Nabawa by Chile IB trial (Fig. 5-2, 5-3) for both substrates illustrate this point. For KGA oxidation this cross showed positive heterosis for ADP:O ratio of 25.9 and 6.25 percent for B and A respectively, and the state 3 rates both had negative heterosis of -25.3 (B) and -10.6 (A) percent. However the heterosis for control ratio varied from -23.6 (B) to +15.7 (A) and for state 4 was 0.2 in one determination (B) and -11.2 in A. The Nabawa by Chile IB trial for malate oxidation also showed great variation with the hybrid advantage for ADP:O dropping from 15.2 in trial A to -1.07 in trial B and the state 3 rate similarly changing from -34 to 12.9 in the two trials. State 4 rates of the hybrid were -31.1 percent of the highest parent but in the repeated trial the hybrid and parents were almost identical showing -0.67 percent heterosis. Only the control ratio gave similar readings on the mean figures with -4.8 and -6.69 being the two values observed. It is possible that these results which are derived from mean values of all cycles may be misleading because of the wide variation even within the individual cycles of one test run. The histogram (Fig. 5-4) shows the detailed heterosis observed in each cycle of the

Nabawa by Chile IB trials for malate oxidation and illustrates that even if each cycle is compared separately there are still large changes in the duplicate values.

Discussion

Obtaining an overall pattern of mitochondrial heterosis from polarographic measurements is difficult because not only is there disagreement between different parameters in the percentage heterosis but it appears to be expressed unevenly in the same parameter in different trials. The large degree of subjectivity needed in interpreting data of this kind reduces its usefulness as a general technique as the results appear to depend on the parameter observed and the substrate used. This difficulty was discussed by Ellis et al. (1973) who emphasized the need for more precise knowledge of the conditions under which heterosis is expressed.

It is possible to determine whether any one cross is significantly different from any other when heterosis was measured by the four parameters. It is also necessary to test the observation that there is no relationship between the four parameters of heterosis.

Ranking crosses for each substrate on the basis of ADP:O ratios following the choice of parameter of Sarkissian and McDaniel (1966) produced the list below, which when arranged in order of decreasing heterosis, is as follows:

Malate	Eureka X IRN	KGA	Nabawa X Chile IB (B)
	Nabawa X Chile IB (A)		Nabawa X Chile IB (A)
	Heron X Gamut		Heron X Gamut
	Nabawa X Chile IB (B)		Eureka X IRN
	Heron X Strain 52		Heron X Strain 52
	109H X 272H		Gabo X Pusa 4

Further rankings can be made on the basis of the respiratory control ratio which alters the order in the following manner:

Malate	Eureka X IRN	KGA	Nabawa X Chile IB (A)
	Nabawa X Chile IB(A)		Gabo X Pusa 4
	Nabawa X Chile IB(B)		Heron X Strain 52
	109H X 272H		Eureka X IRN
	Heron X Gamut		Heron X Gamut
	Heron X Strain 52		Nabawa X Chile IB (B)

The percentage heterosis for state 3 and state 4 rates of oxygen uptake also provide data for ranking in a similar manner:

State 3

Malate	Eureka X IRN	KGA	Heron X Gamut
	109H X 272H		Heron X Strain 52
	Nabawa X Chile IB (B)		Nabawa X Chile IB (A)
	Heron X Strain 52		Nabawa X Chile IB (B)
	Nabawa X Chile B (A)		Gabo X Pusa 4
	Heron X Gamut		Eureka X IRN

State 4

Malate	Eureka X IRN	KGA	Heron X Gamut
	Heron X Strain 52		Heron X Strain 52
	109H X 272H		Nabawa X Chile IB (B)
	Nabawa X Chile IB (B)		Nabawa X Chile IB (A)
	Heron X Gamut		Gabo X Pusa 4
	Nabawa X Chile IB (A)		Eureka X IRN

It is necessary to find some way to relate these changes in order as there appears to be a tendency for certain crosses to occur at the top of the lists more frequently than others. To establish whether some crosses do have consistently higher mitochondrial heterosis than others the data presented in Fig. 5-2 and 5-3 were subjected to a Kruskal-Wallis one-way analysis of variance by ranks for independent samples. There were five trials in which both malate and KGA oxidation was observed on one preparation. These five trials thus formed five groups,

each with eight members, which were the observations of heterosis derived from the four parameters in the histogram. The analysis of variance of the total sample was non-significant with H = -11.4 which cannot occur under the χ^2 distribution. The data were also analysed for each substrate separately so that five groups of four members each were compared. Both gave values of H below the critical level at 4 degrees of freedom of 9.49 with H for malate being 8.15 and for KGA being 6.1. Since the cross 109H by 272H was tested with malate only and Gabo by Pusa 4 for KGA only these results could be included in a segregated analysis. The results of the repeated analyses with n=6 did not change the level of probability for H as the value of H for malate was 9.86 and for KGA it was -0.48 which are both below the critical value for significance at the 5 percent level with five degrees of freedom. Thus no cross is significantly different from any other when mitochondrial heterosis is being estimated from ADP:O and respiratory control ratios and state 3 and state 4 rates.

The ranking of crosses presented above can also be used to see if any correlation exists between the four measurements of heterosis. The relationship among several rankings of N objects can be determined by a non-parametric test, Kendall's coefficient of concordance, W. The ranks are numbered from one to six from highest to lowest and these values are summed for each cross individually so that six sum of ranks are obtained as is shown below:

MALATE	Eureka X IRN	Nabawa X (A)	Chile IB (B)	Heron X Gamut	Heron X Strain 52	109 H X 272 H	Gabo X Pusa 4
RCR	1	2	3.5	5	6	3.5	-
ADP:O	1	2	4	3	5	6	-
State 3	1	5	3	6	4	2	-
State 4	1	6	4	5	2	3	-
	4	14	14.5	19	7	14.5	-
KGA							
RCR	4	1	6	5	3	-	2
ADP:O	5	2	1	6	4	-	3
State 3	6	3	4	1	2	-	5
State 4	6	4	3	1	2	-	5
	21	10	14	13	11	-	15

The coefficient of concordance was calculated separately for malate and KGA measurements as is shown below:

Malate $$W = \frac{134}{\frac{1}{12}.4^2(6^3-6)} = \frac{134}{280} = 0.48$$

KGA $$W = \frac{76}{\frac{1}{12}.4^2(6^3-6)} = \frac{76}{280} = 0.27$$

Significance of the correlation is determined from the value of s by comparison with the critical value which is 143 at the 5 percent level of significance (Siegel, 1956). Thus, for both malate and KGA the association between the four parameters of heterosis is non-significant although the malate value approaches significance.

Siegel (1956) emphasizes that the presence or absence of agreement between ranks does not constitute a proof of correctness of the ranking system with reference to an external criterion. The disagreement found here must signify that some mitochondrial measures of heterosis are better predictors of plant heterosis than others and the relationship between the various estimates will be considered in a later section.

An overall estimate of rank can be obtained from the sum of rank figure. This procedure has been recommended by Kendall (1948) as a means of finding a best estimate of the order of the entities studied. When W is insignificant such an ordering would also tend to be less significant but as it is necessary for examining relative rankings by different techniques combined ranks and sum of ranks are given below:

	Cross	Σ Rank	Cross	Σ Rank
Malate	Eureka X IRN	4	Nabawa X Chile IB (A)	10
	Nabawa X Chile IB (A)	14	Heron X Strain 52	11
	Nabawa X Chile IB (B)	14.5	Heron X Gamut	13
	109H X 272H	14.5	Nabawa X Chile IB (B)	14
	Heron X Strain 52	17	Gabo X Pusa 4	15
	Heron X Gamut	19	Eureka X IRN	21

The malate trials separate the crosses more than the KGA trials for percentage heterosis. The individual parameters for malate oxidation are in closer, but still less than significant agreement as to the ranked order of crosses. Analysis of variance between crosses showed that although no significant variation is found between crosses with either

substrate the results from malate oxidation were closer to significance. This disagrees with the report that KGA oxidation is the best indicator of mitochondrial heterosis (McDaniel and Sarkissian, 1968), and the exclusive use of this substrate by several workers (Sage and Hobson, 1973; Ellis et al., 1973, Zobl et al., 1972).

The relationship between the different measures of heterosis in this project and the yield trials will be considered in the next section.

(IV)
Yield heterosis

Field trials were carried out in an independent program by Mr. P. Wilson at Tamworth, N.S.W. on many of the crosses examined for mitochondrial heterosis. Plantings were made in June, 1966 in 7" by 36" plots with 25 seeds per plot. The irrigation was complete and fertility high; thus the growth was not limited by environmental factors. The plants were laid out in a modified randomized block design with four replications so that in the field each hybrid was flanked by its parents and the crop was harvested and measured in December, 1966.

The yield components were determined from which a percent heterosis for each component was calculated. Table 5-16 shows the percent heterosis for each component calculated from the raw data supplied by Wilson from trial I for those crosses which were common to both projects. In addition, Wilson finds the average improvement in hybrid performance over the average parental value, combining all the hybrids and parents together so that over his 13 hybrids and parents the average yield of hybrids (expressed as bushels per acre) is 39.07 percent higher than the average yield of the parents, an agriculturally useful level of heterosis.

Additional field data was provided by an observation trial (trial III) where of the 47 crosses observed four crosses were common to both projects. Results for three field components and the yield in bushels per acre were provided so the hybrid advantage can be calculated for these trials also and is set out in Table 5-17.

Three crosses Gabo by Pusa 4, Heron by Gamut and Heron by Strain 52 were common to both trials I and III and it was seen that heterosis for overall yield was not consistent within one cross over the two test situations. As trial III was an observation trial with one determination for each cross the figures for trial I were taken to be more reliable, having been obtained from four replications.

The percentage heteroses for the yield components do not always agree with the heterosis figure for total yield. This is not surprising in components such as ears per plot which is a function of plants per plot and ears per plant.

Similarly, positive heterosis (weight per 100 grains and number of grains per ear) should agree with the total yield unless it is offset by opposite trends in other components. Yield components are valuable for determining in which ways a hybrid crop differs from the parental crop but this study is interested in predicting favourable combinations for overall yield. Thus the conflicting heterosis within the yield components was ignored and the percentage heterosis for yield was taken as the parameter with which to compare other estimates of heterosis.

Discussion

This lack of agreement in field trials, both among individual

trials and among the yield components and total yield (Table 5-16 and 5-17), highlights the difficulty of assessing hybrid performance. In all, seven crosses were studied by Wilson which were used in this project, six being part of trial I and one, Gabo by Falcon, being included only in the observation trial. These crosses can be ranked in order of decreasing yield heterosis as follows for comparison with rankings previously drawn up.

Nabawa X Chile IB
Eureka X IRN
Heron X Strain 52
Heron X Gamut
Gabo X Falcon
Gabo X Pusa 4
Mengavi X Festival

(V)
Relationship between different estimates of heterosis from a number of crosses

Since there is no evidence in the literature of a correlation between mitochondrial heterosis and yield heterosis, the justification for use of mitochondrial heterosis as a predictor of yield heterosis lies in the reported relationship of mitochondrial heterosis to seedling germination heterosis and of seedling heterosis to yield (McDaniel, 1969; Sarkissian and Srivastava, 1967). In discussing the relationship of the different measurements of heterosis it is therefore of particular importance firstly to ascertain whether the percentage heterosis for germination agrees closely with that of yield for the present trials and secondly that mitochondrial heterosis is indeed related to seedling heterosis.

The overall rankings presented at the end of each section have been displayed collectively in table form (Table 5-18). The germination data is presented both in the order determined from the sum of ranks used in determining the Spearman rank coefficient for each trial separately and as a combined ranking determined also from the sum of ranks. As the latter ranking is derived from tests which did not significantly correlate, the empirical grouping of crosses into the three heterosis classes of all positive, partial and no positive heterosis in the six determinations is also included. Tissue respiration data are arranged in order of decreasing heterosis using the respiratory rate as the sole parameter, while the mitochondrial heterosis rankings are obtained for each substrate by combining the rankings of the four parameters used in this study.

The relative performance of each cross for each determination will be considered individually as it is not possible to combine all the separate rankings statistically as there are different numbers of crosses in each determination.

Eureka by IRN was the most heterotic hybrid for tissue respiration, mitochondrial malate respiration and the four day germination trials. However it rated third position in the ordering for heterosis of the three day germination trial and last or sixth when assessed for heterosis in KGA respiration by mitochondria. It was second only to Nabawa by Chile IB for heterotic performance in the field trials and as these two crosses showed similarly high percentage heterosis (75.7 and 77.8 percent) which were well above the next value, this ranking could be

considered a tied rank. Thus for Eureka by IRN, three of the laboratory methods of determining heterosis are good indicators of its yield performance under optimum conditions. However, this is countered by the KGA oxidation rank which does not agree with the rank of even the lowest seedling heterosis determination of the yield data, thus limiting the usefulness of predictive experiments.

The other high yielding hybrid, Nabawa by Chile IB was consistently near the top of all the lists of rankings. It was in second rank in both the germination trials and also in the overall list. In the mitochondrial ranking with malate oxidation duplicated experiments took second and third place. The duplicates for KGA oxidation did not occupy adjacent ranks with trial A being the most heterotic hybrid of the test and trial B being in fourth rank. The tissue respiration experiments showed a lower rank as Nabawa by Chile IB and its reciprocal cross tied for fifth rank showing low positive heterosis of 3 percent. This position was below crosses which, with the exception of Eureka by IRN and Heron by Gamut, were not included in the other types of determination. This position may not be inconsistent, therefore, with the other higher placings. Thus for Nabawa by Chile IB the predicted relationship between yield, seedling and mitochondrial heterosis can be tentatively demonstrated.

The Heron by Strain 52 hybrid was significantly heterotic yielding 54 percent more than the highest parent. It appears in the tied first rank of the four day germination trials but this in in opposition to the three day trial where it is the most negatively heterotic cross. The

overall placing for seedling heterosis is second last or fifth and the cross showed positive heterosis in only three of the six determinations in these trials; thus, seedling heterosis could not be said to be a good predictor of yield heterosis for this cross. This trend is continued in the low rank obtained in the tissue respiration trials (fourth from last) and mitochondrial malate oxidation (second from last). However, KGA oxidation by mitochondria supports a higher level of heterosis as Heron by Strain 52 is second only to Nabawa by Chile IB on these results. Thus malate oxidation agrees with overall germination heterosis and tissue respiration in the lack of heterosis while KGA oxidation is the only test which agrees with the field results, of significant yield heterosis for this cross.

Heron by Gamut with a yield advantage of 28 percent was also a significantly heterotic cross in terms of yield. However, it ranked low in both germination trials being second lowest and lowest in the three and four day trials respectively with no positive heterosis shown in any characteristic. It also filled the lowest of the mitochondrial malate oxidation ranks. The only indication of possible positive heterosis was in mitochondrial KGA oxidation where it ranked third and the tissue respiration where it ranked a tied third above Nabawa by Chile IB. Thus again mitochondrial malate oxidation and germination heterosis agree as to the lack of hybrid vigour in contradiction to the field trial results, while mitochondrial KGA oxidation and tissue respiration would indicate that some positive heterosis could be expected, agreeing with the field trial result.

As Gabo by Falcon did not form part of the mitochondrial respiration or germination trials, comparison can be made only between its yield position of fifth due to small negative heterosis, and its tissue respiration position of sixth which was derived from a heterosis figure of 0.8 percent; thus the two estimates agree closely.

The yield heterosis for Gabo by Pusa 4 was negative with this cross being second last in field trial ranking. Determinations of heterosis however were widely variable and did not all reflect this yield result. The three day germination trial produced a top rank for this cross as it showed total positive heterosis, yet the tissue respiration trial placed it in eighth position with negative heterosis. Gabo by Pusa 4 mitochondria were also in a low rank being fifth or second lowest in the KGA oxidation heterosis ranking. Thus the mitochondrial KGA oxidation results and the tissue respiration results agree with the yield results for heterosis. Seedling heterosis did not concord with any of these findings giving a totally opposite estimation of heterosis.

Mengavi by Festival was only tested for whole tissue respiration where it ranked seventh, above Gabo by Pusa 4 and Heron by Strain 52 which performed better in yield heterosis trials. Both these results agree on the overall lack of heterosis for this cross (both positions are derived from negative heterosis results) but disagree on the performance relative to other crosses.

No single method of estimating heterosis in an artificial way consistently agrees with the estimates of vigour obtained by field trials. Thus no single test would be able to serve as a predictor of

yield performance. In particular, the results from malate oxidation consistently agree with those of the overall ranking of seedling heterosis but the reported link between seedling heterosis and yield heterosis (with the exception of Nabawa by Chile IB and Eureka by IRN) was not demonstrated. The results of mitochondrial KGA oxidation and yield trials agreed on several occasions (with the notable exception of Eureka by IRN), while disagreeing with malate oxidation and seedling heterosis. The tissue respiration heterosis results largely agreed with the yield heterosis results, but since many more crosses were examined for tissue respiration characteristics than for yield, it is difficult to compare these rankings precisely.

In only one cross (Nabawa by Chile IB) could the individual assessments of heterosis be said to be consistent to a useful degree and even with this cross contradictions occurred. Thus prediction of yield heterosis from laboratory experiments requires several different estimates and can at best only be a rough guide to the field performance.

Table 5-1

GERMINATION HETEROSIS

Cross	% Heterosis					
	3 days			4 days		
	Dry Weight	Shoot Weight	Shoot Length	Dry Weight	Shoot Weight	Shoot Length
Eureka X IRN	4.30	5.40	24.00	17.00	52.90	24.70
Gabo X Pusa 4	22.40	66.40	32.00	-	-	-
109H X 272H	-12.50	55.40	40.60	5.30	-27.20	-29.00
31MS X 28	-49.50	4.70	0	-	-	-
Nabawa X Chile IB	41.04	44.10	6.60	25.00	20.50	28.20
Heron X Strain 52	- 7.60	-58.10	-48.20	9.50	32.50	36.20
Heron X Gamut	16.60	-24.20	-38.30	59.30	-30.80	-38.80
109H X 272H	15.15	42.80	-14.00	-14.70	-24.60	-25.70

Seedlings from parental and hybrid lines were germinated as described in Chapter II and the relative growth of the hybrid and the highest parent compared. Indices of growth were the fresh weight and length of excised shoots. The initial dry seed weight of hybrid relative to that of the highest parent was also observed. Heterosis is expressed as a percentage value. Seedlings were allowed to germinate for 3 or 4 days. Each value is the mean of 15 to 20 measurements.

Table 5-2

SEED VIRILITY AS INDICATED BY PERCENT GERMINATION

3 day growth period			4 day growth period		
Genotype	Germination %	Heterosis %	Genotype	Germination %	Heterosis %
Eureka	100		Eureka	93	
IRN	93	-7	IRN	93	7.5
Eureka X IRN	93		Eureka X IRN	100	
Nabawa	93		Nabawa	66	
Chile IB	87	-6.4	Chile IB	100	0
Nabawa X Chile IB	87		Nabawa X Chile IB	100	
Heron	100		Heron	100	
Gamut	93	0	Gamut	66	-7
Heron X Gamut	100		Heron X Gamut	93	
Heron	100		Heron	100	
Strain 52	100	0	Strain 52	87	-13
Heron X Strain 52	100		Heron X Strain 52	87	
109H	100		109H	100	
261	87	-7	261	100	-13
109H X 261	93		109H X 261	87	
109H	100		109H	100	
272H	93	-7	272H	87	0
109H X 272H	93		109H x 272H	100	

Table 5-2 (cont'd)

Genotype	3 day growth period Germination %	Heterosis %
31MS	87	
28	100	0
31MS X 28	100	
Gabo	79	
Pusa 4	100	0
Gabo X Pusa 4	100	

Seedlings were germinated and grown as described in Chapter II. 15 seeds were used for each genotype and all seeds were pre-soaked before being planted out. Results of parents which occur in two crosses have been repeated for ease of comparison.

Table 5-3

COMPARISON OF TISSUE TREATMENTS FOR
OXYGEN UPTAKE STUDIES

Genotype	Treatment			
	A	B	C	D
IRN (female)	575	482	602	698
EUREKA (male)	388	538	750	394
HYBRID	527	770	460	505
% HETEROSIS	-8.8	44	-23	-29.4

The four types of tissue treatment were:

A - whole seedling with seed attached

B - whole seedling with no seed attached

C - diced seedling tissue without the seed, after 3 hours incubation

D - diced seedling tissue without the seed, used immediately.

Rates of oxygen uptake were expressed as $\mu l\ O_2.gm$ fresh $wt.^{-1}hr^{-1}$. The tissue was suspended in 2ml of 0.01M phosphate buffer at pH 6.0 in the manometer main compartment, with 0.2ml 20% KOH in the manometer centre well.

Table 5-4

THE EFFECT OF DNP ON TISSUE RESPIRATION
BY WHEAT SHOOTS

Genotype	-DNP	+DNP	% Change
Falcon (female)	493	700	42.00
Gabo (male)	490	750	53.00
Falcon X Gabo	560	1007	79.00
% Heterosis	16	34	
Gabo (female)	480	486	1.25
Falcon (male)	420	482	19.50
Gabo X Falcon	484	553	14.30
% Heterosis	0.8	10.5	

Rates of oxygen uptake were measured as µl O_2.gm fresh weight^{-1}.hr^{-1}. The "% change" refers to the percentage increase in rate after the addition of DNP. Diced shoots and roots were suspended in 2ml of 0.01M phosphate buffer at pH 6.0. Additions of 1.3×10^{-4}M DNP were made from the side arm after an initial rate had been established. 0.2ml of 20% KOH was present in the centre well of each flask.

Table 5-5

RATE OF O_2 UPTAKE µl O_2.GM FR.WT^{-1}.HR^{-1}

CROSS MALE X FEMALE	FEMALE PARENT	MALE PARENT	HYBRID	% HETEROSIS
Eureka x IRN	337	335	640	68.00
IRN x Eureka	485	510	434	-15.00
Falcon x Gabo	493	490	562	16.00
Gabo x Falcon	480	420	484	0.83
Strain 52 x Heron	456	513	587	12.60
Heron x Strain 52	493	428	453	- 8.00
Heron x Gamut	532	648	728	12.35
Gamut x Heron	404	470	513	9.15
Chile x Nabawa	365	336	371	3.60
Nabawa x Chile	372	282	385	3.50
Mengavi x Festival	412	416	415	0
Gabo x Pusa 4	552	476	531	- 3.80
Pusa 4 x Gabo	506	598	401	-33.20

Legend: rates of oxygen uptake in wheat seedlings were expressed as µl O_2.gm fr. wt^{-1}.hr^{-1}. Diced shoots or whole seedlings were suspended in 2ml of 0.01M phosphate buffer at pH 6.0 with 0.2ml 20% KOH in the centre well.

Table 5-6

HETEROSIS IN COUPLED AND
UNCOUPLED TISSUE RESPIRATION

Genotype	µl O_2.gm fr.wt^{-1}.hr^{-1} (A)		µl O_2.gm dry wt.$^{-1}$hr.$^{-1}$ (B)	µl O_2.mg N^{-1}.hr^{-1}
	-DNP	+DNP	-DNP	
31MS female	402.5	416	4306	145*
28 male	567.0	618	4242	110
31MS x 28	521.5	579	3933	200
% heterosis	-8.02	-6.3	-8.8	38

* Data from Sarkissian and Srivastava (1969)

A. Sliced tissue was suspended in 2ml of 0.01M of phosphate buffer at pH 6.0 and used immediately. Additions of 1.3 x 10^{-4}M DNP were made from the side arm after establishing the initial rate. 0.2ml of 20% KOH was present in all flasks.

B. In a separate experiment tissue was treated as for A but at the end of the measurements it was oven dried and the dry weight determined.

Table 5-7

HETEROSIS IN THE PRESENCE AND ABSENCE OF UNCOUPLER FOR AUSTRALIAN WHEAT CROSSES

Cross (Male x Female)	% Heterosis -DNP	+DNP
Falcon x Gabo	16.00	34.00
Gabo x Falcon	0.80	10.50
Chile x Nabawa	3.60	25.00
Nabawa x Chile	17.00	- 4.50
Eureka x IRN	68.00	13.00
Heron x Gamut	12.40	8.70
Gamut x Heron	-4.80	-14.00
Gabo x Pusa 4	-3.80	- 4.50

Measurements were made using whole or diced tissue in 2ml of 0.01M of phosphate buffer in the presence of 20% KOH. 1.3×10^{-4}M DNP was added to the side arm of vessels after an initial rate had been determined. Rates are expressed as µl O_2.gm fresh weight^{-1}.hr^{-1}.

Table 5-8

TISSUE RESPIRATION IN THE PRESENCE OF SUBSTRATE

Genotype	-Glucose	+Glucose
Eureka	347	448
IRN	335	380
Hybrid	640	752
% Heterosis	84.00	68.00
	-Sucrose	+Sucrose
Gamut	404	326
Heron	470	325
Hybrid	513	354
% Heterosis	9.15	8.50

Freshly diced tissue was suspended in 50mM sucrose or 50mM glucose solution in the main vessel compartment and readings taken in the presence of 0.2ml of 20% KOH in the centre well of the vessel. Rates are expressed as $\mu l\ O_2.gm\ fresh\ weight^{-1}.hr^{-1}$.

Table 5-9

RESULTS OF ANALYSIS OF VARIANCE

Substrate	Parameter	Cycle	H	Probability	Significance
KGA	ADP:O	1	1.83	P > 0.1	NS
		2	1.37	P > 0.1	NS
		3	0.052	P > 0.1	NS
KGA	RCR	1	2.0	P > 0.1	NS
		2	0.81	P > 0.1	NS
		3	1.35	P > 0.1	NS
Malate	ADP:O	1	2.09	P > 0.1	NS
		2	3.76	P > 0.1	NS
Malate	RCR	1	-7.42	P > 0.1	NS
		2	5.69	0.49 > P > 0.01	*

* Significance at 5% level

Comparison of preparations from line 28, line 31MS and their hybrid 31MS x 28, using the Kruskal Wallis one-way analysis of variance by ranks.

The number of observations (N) was 12 for KGA in 3 groups of 4, except for ADP:O cycle 3 where N = 11 and RCR cycle 3 where N = 10 with 2 groups of 3 and one of 4 observations. For malate N = 15 in cycle 1 and N = 14 in cy le 2.

Calculations are made using the formula:

$$H = \frac{12}{N(N+1)} \sum_{j=1}^{k} \frac{Rj^2}{nj} - 3(N+1)$$

where N = number of observations

k = number of groups

nj = number of cases in the j^{th} sample

Rj = sum of ranks in the j^{th} column

Table 5-10

RESULTS OF ANALYSIS OF VARIANCE

Substrate	Parameter	Cycle	H	Probability	Significance
KGA	State 3 rate	1	1.04	P > 0.1	NS
		2	1.42	P > 0.1	NS
		3	2.58	P > 0.1	NS
KGA	State 4 rate	1	2.35	P > 0.1	NS
		2	3.23	P > 0.1	NS
		3	3.80	P > 0.1	NS
Malate	State 3 rate	1	4.22	0.2 > P > 0.1	NS
		2	12.17	P > 0.01	**
Malate	State 4 rate	1	2.33	0.5 > P > 0.2	NS
		2	2.63	0.3 > P > 0.2	NS

** Significance at 1% level

Comparison of preparations from line 28, line 31MS and their hybrid 31MS x 28, using the Kruskal Wallis one-way analysis of variance by ranks. The number of observations (N) was 12 for KGA in 3 groups of 4, except cycle 3, state 4 where N = 10 with 2 groups of 3 and one of 4. In the malate estimations N = 18, 3 groups of 6 for both cycle 1 values but N = 17 in cycle 2 with one group of 5 and two of 6. Calculations were done as described in Table 5-8. Significance was determined by use of χ^2 tables where N > 15 and by reference to a table of probabilities provided by Siegel (1965) for small sample size.

Table 5-11

NADH - CYTOCHROME *c* REDUCTASE ACTIVITY IN LINE 28, 31MS AND THEIR HYBRID 31MS X 28

Treatment	Rate (nmoles cyt. *c* reduced.mg protein^{-1}.min^{-1})			
	31MS	28	Hybrid	% Heterosis
Intact mitochondria	180	150	270	50.00
Swollen mitchondria	240	300	430	43.30
Swollen mitochondria + Antimycin A	130	200	250	25.00

The assay was conducted as described for Table 3-9 in a total of 3.25ml. Mitochondrial protein added to each assay was 31MS, 0.565mg; 28, 0.585mg; hybrid 0.625mg. 0.2mM KCN was present throughout these trials. Antimycin A was added to a final concentration of 5 µM. Cytochrome *c* was present at a final concentration of 47 µM.

Table 5-12

MALATE- AND SUCCINATE-CYTOCHROME *c* REDUCTASE ACTIVITY

Enzyme		31MS	28	Hybrid	Heterosis %
Malate-cyt. *c* reductase	-ADP	12	11.3	15.5	29.00
Succinate-cyt. *c* reductase	-ADP	25	31	40	29.00

Malate-cytochrome *c* reductase activity was followed as described in Table 3-10. The reaction mixture combined with 47 µM ferricytochrome *c*, and 10 mM KCN in 3ml standard sucrose medium to a total of 3.23ml. The reaction was started by addition of 15.34 mM malate. Additions of 0.1ml mitochondrial suspension contained the following amounts of protein: 31MS = 0.675mg, 28 = 0.575mg, hybrid = 0.595mg.

Succinate-cytochrome *c* reducuctase was assayed as above except that 15.34mM succinate was used to initiate the reaction and 156 µM cytochrome was present. Mitochondrial protein was added in the following amounts: 31MS = 0.565mg, 28 = 0.585mg, hybrid = 0.625mg.

Antimycin A was added to both reactions in a final concentration of 5 µM.

Table 5-13

Ascorbate oxidase was followed polarographically in the presence of TMPD as described in Table 3-12. The following additions of mitochondrial protein were made. 31MS = 675 µgm, 28 = 575µgm, hybrid = 595 µgm. ADP was 300 µM in a total volume of 1.1ml. One mitochondrial preparation was used for the ascorbate oxidase assay and experiments A, B and C of the ferrocytochrome oxidase assay.

Ferrocytochrome *c* oxidase was determined in a volume of 3.125ml as described in Table 3-12. The reaction was started by addition of 47 µM ferrocytochrome *c* after addition of mitochondrial protein and 120 µM ADP. Mitochondrial protein per reaction was as follows:

A	31MS = 169 µgm.	28 = 144 µgm.	hybrid = 149 µgm.
B	31MS = 337 µgm.	28 = 278 µgm.	hybrid = 297 µgm.
C	31MS = 675 µgm.	28 = 575 µgm.	hybrid = 595 µgm.
D	31MS = 88.8 µgm	28 = 104 µgm.	hybrid = 139 µgm.

Table 5-13

HETEROSIS IN CYTOCHROME *c* OXIDASE ACTIVITY

Ascorbate oxidase (nmoles O_2.mg protein^{-1}.min^{-1})

Source of mitochondria	-ADP	+ADP
31MS	152	228
28	148	242
Hybrid	134	228
% Heterosis	-12	-5.8

Ferrocytochrome *c* oxidase (nmoles cyt. *c* oxidized . mg protein^{-1}.min^{-1})

Source of mitochondria	Intact mitochondria		Swollen mitochondria		Sarkissian & Srivastava (1971)
	A -ADP	B +ADP	C -ADP	D -ADP	-ADP
31MS	25	21	182	288	371
28	37	22.3	208	347	269
Hybrid	71	62.7	272	274	694
% Heterosis	92	181	31	-21	87

Table 5-14

MALATE-CYTOCHROME *c* REDUCTASE AND MALATE OXIDASE ACTIVITY

Malate oxidation	Cycle	31MS	28	Hybrid	% Heterosis
Intact mitochondria (nmoles O_2.mg. protein^{-1}min^{-1})	-ADP				
	1	20.00	29.00	34.00	17.00
	2	27.00	34.00	39.00	15.00
State 3	+ADP				
	1	50.00	57.00	68.00	19.00
	2	54.00	74.00	86.00	16.00
RCR	1	2.00	2.05	2.00	2.44
	2	2.19	2.20	2.19	0
ADP:O	1	2.82	2.65	2.57	- 8.80
	2	2.50	2.50	2.05	-18.00
Malate-cytochrome *c* reductase					
Intact mitochondria (nmoles cyt. *c* reduced . mg protein^{-1} min^{-1})*		12.00	11.30	15.50	29.00

* Repeated from Table 5-10

Comparative activity of mitochondria from one preparation:

Malate oxiation was followed polarographically as described in Table 3-8. ADP was 254 µM. Additions of 0.4ml mitochondrial suspension contained the following amounts of protein: 31MS = 2.7mg., 28 = 2.3mg., hybrid = 2.38mg.
Malate-cytochrome *c* reductase results are repeated from Table 5-10 for ease of comparison.

Table 5-15

MEAN HETEROSIS IN OXIDATIVE PHOSPHORYLATION FOR 31MS x 28

KGA oxidation

Cycle	RCR	ADP:O	State 3	State 4
1	-1.75	-10.14	3.45	5.18
2	2.84	-13.78	4.23	-15.78
3	-2.56**	- 2.92*	-11.87	3.21

Σ = -39.89, n = 12, $\bar{x}$ = -3.32

Malate oxidation

1	-7.52	- 7.17*	- 3.56	9.29
2	0.15	2.80	3.42	9.37

Σ = 6.78, n = 8, $\bar{x}$ = 0.57

Mean values for percent heterosis are calculated from the individual values presented in Fig. 5 (A, B, C & D). For KGA oxidation n = 4 except for * where n = 3 and ** where n = 2. Malate oxidation had n = 6 except for * where n = 5.

Two overall means have been calculated by combining all cycles and all parameters.

Table 5-16

YIELD HETEROSIS (P. WILSON, 1966, TAMWORTH, N.S.W.) TRIAL I

% Heterosis for Yield Components

Cross	Plants/ Plot	Ears/ Plot	Wt/ 100 grains (gm)	Grains/ ear	Plant height	Yield (bushels/ acre)
IRN x Eureka	20.25	8.18	10.34	19.94	-3.04	75.70
Gabo x Pusa 4	-15.40	-10.84	0.39	1.20	-2.35	- 6.17
Nabawa x Chile IB	17.89	28.80	8.10	17.97	4.68	77.78
Heron x Gamut	- 5.70	4.65	7.42	9.12	5.80	28.20
Mengavi x Festival	24.60	33.66	8.03	-39.20	-3.65	-10.20
Heron x Strain 52	34.30	39.10	- 4.30	- 5.20	-5.90	54.40
Mean % Increase	12.66	17.26	4.99	0.64	-0.74	36.60

Figures derived from means of four replications
Hybrids produced mechanically
Trials were conducted as described in the text

Table 5-17

YIELD HETEROSIS (P. WILSON, 1966, TAMWORTH, N.S.W.) TRIAL III

Cross	Plants/ Plot	Ears/ Plot	Plant Height (inches)	Yield (bushels/acre)
Gabo x Pusa 4	-30.77	-31.94	-11.05	27.50
Gabo x Falcon	-23.07	-13.55	-14.20	- 4.25
Heron x Gamut	27.30	-29.03	40.58	- 7.50
Heron x Strain 52	9.09	- 5.88	27.96	2.38
Mean % Increase	10.82	- 4.36	-20.10	2.84

Trials were conducted as described in the text
Hybrid produced mechanically
No replications, observation plot only

Fig. 5-1 (a, b, c & d)

Mitochondrial Heterosis and Complementation in the Cross 31MS X 28.

The histograms are drawn from data presented in appendices III and IV. Each bar shows the value of one cycle of phosphorylation with successive bars for one parameter representing successive cycles in one experiment. Fig. 5-1 a & b shows heterosis in respiratory control and ADP:O state 3 and state 4 rates for malate oxidation while Fig. 5-1 c and d show heterosis in the same parameters for KGA oxidation. Experiments were conducted under the routine conditions described (Fig. 3-8).

Fig 5 - 1a (31MS X 28) PERCENT HETEROSIS MALATE

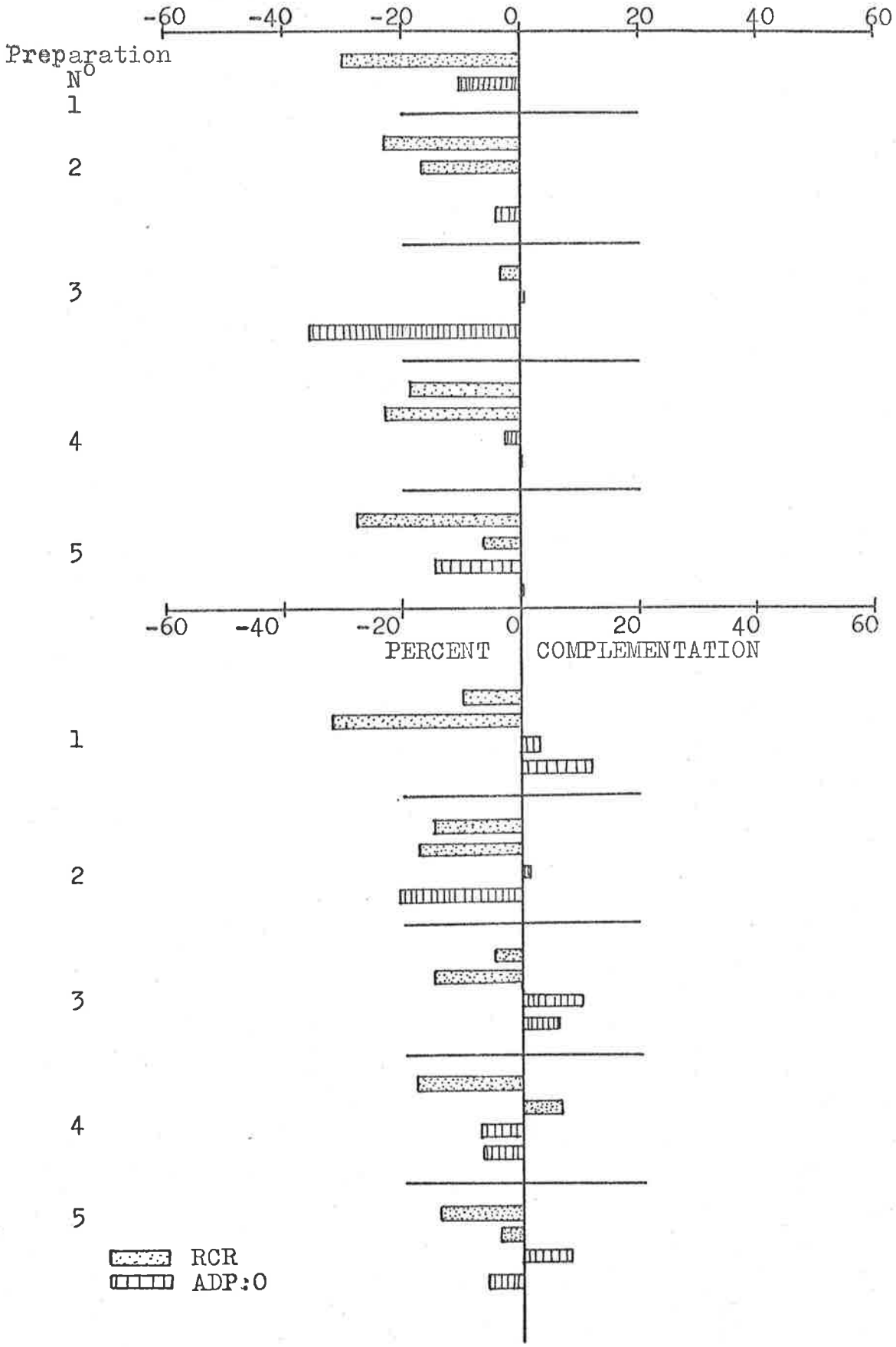


Fig 5 - 1b (31MSX28) PERCENT HETEROSIS MALATE

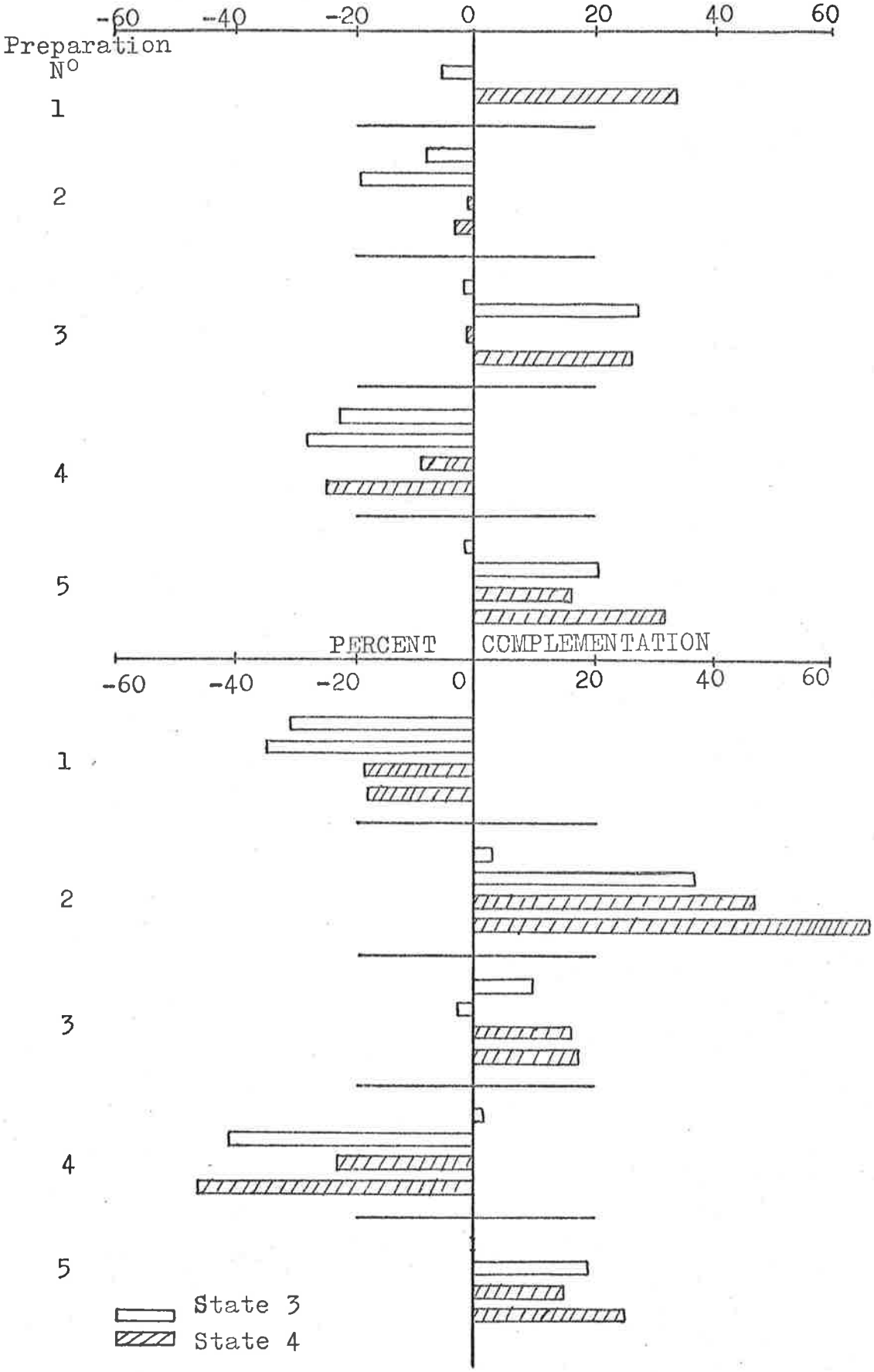


Fig 5 - 1c (31MS X 28) PERCENT HETEROSIS KGA

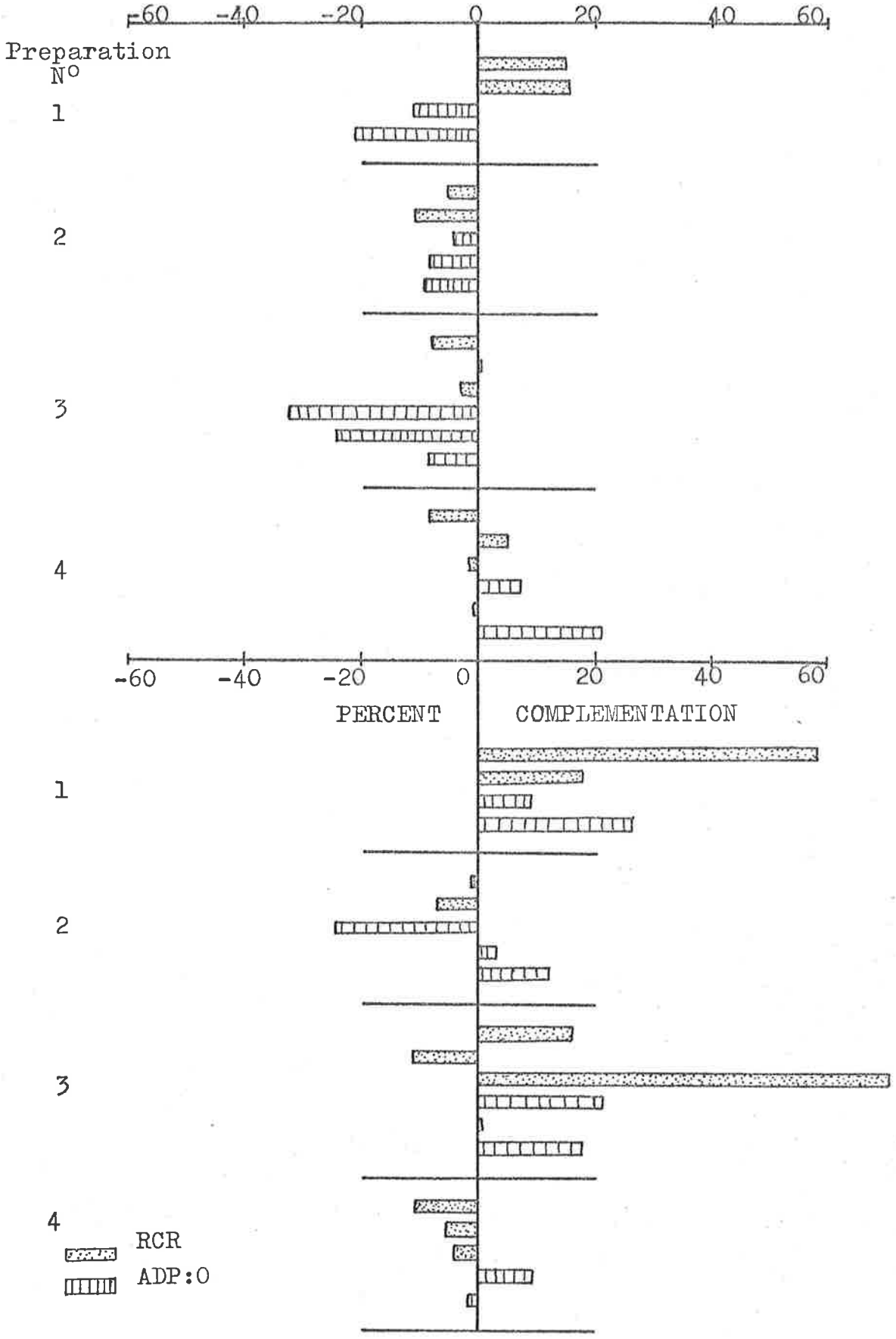


Fig 5 - 1d (31MS X 21) PERCENT HETEROSIS KGA

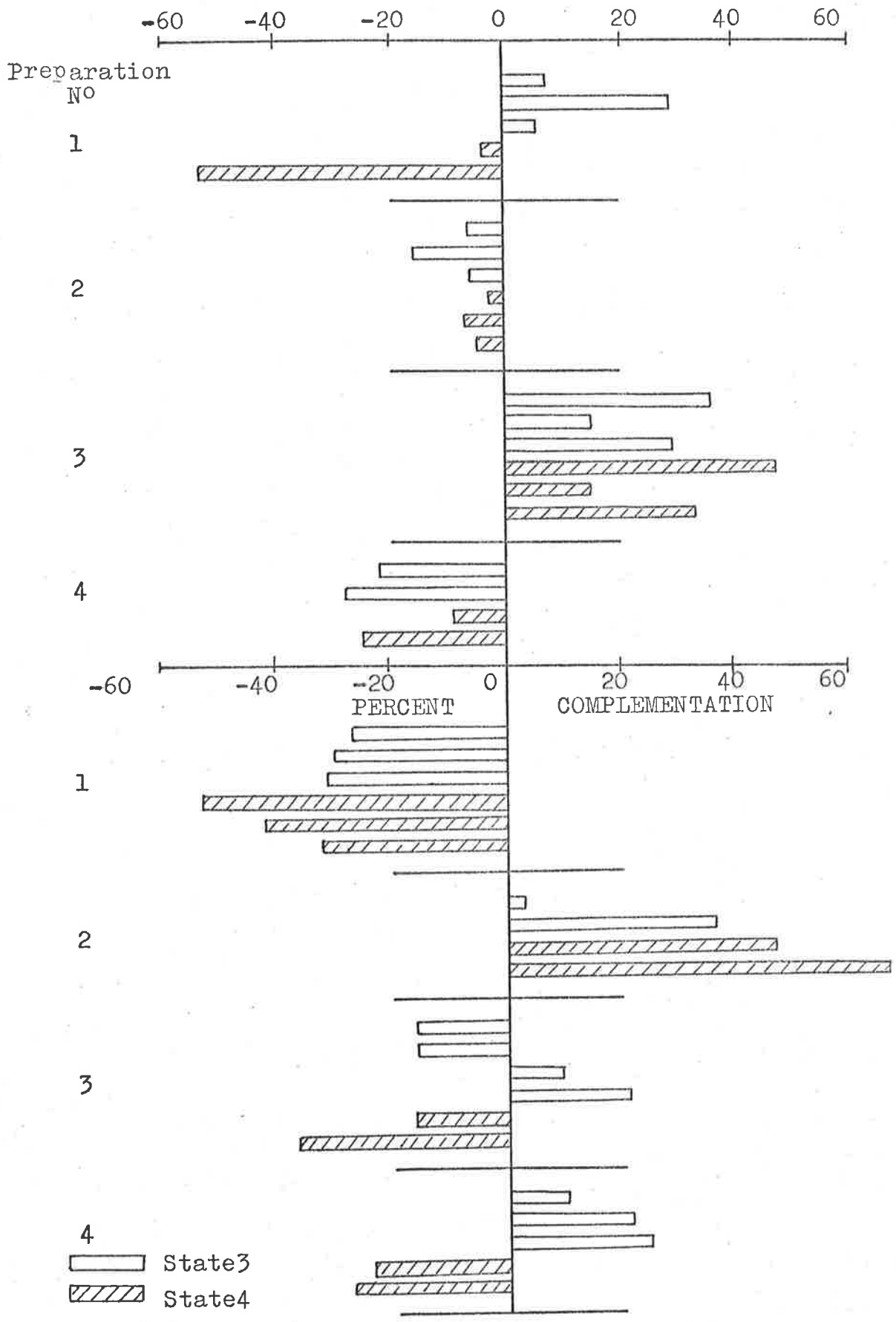


Fig. 5-2

Mitochondrial Heterosis in Several Wheat Crosses with Malate.

The histogram, drawn from the data presented in appendices III and IV, shows the percent heterosis in 6 trials where 5 different crosses were used. Malate oxidation was studied under the routine conditions described (Fig. 3-8). Each bar represents the mean of two cycles of phosphorylation with the exception of Eureka X IRN which represents one cycle. Respiratory control and ADP:O ratios and state 3 and state 4 rates are used for determining heterosis. Stars indicate where the mean has been derived from two widely different values.

Fig 5 - 2

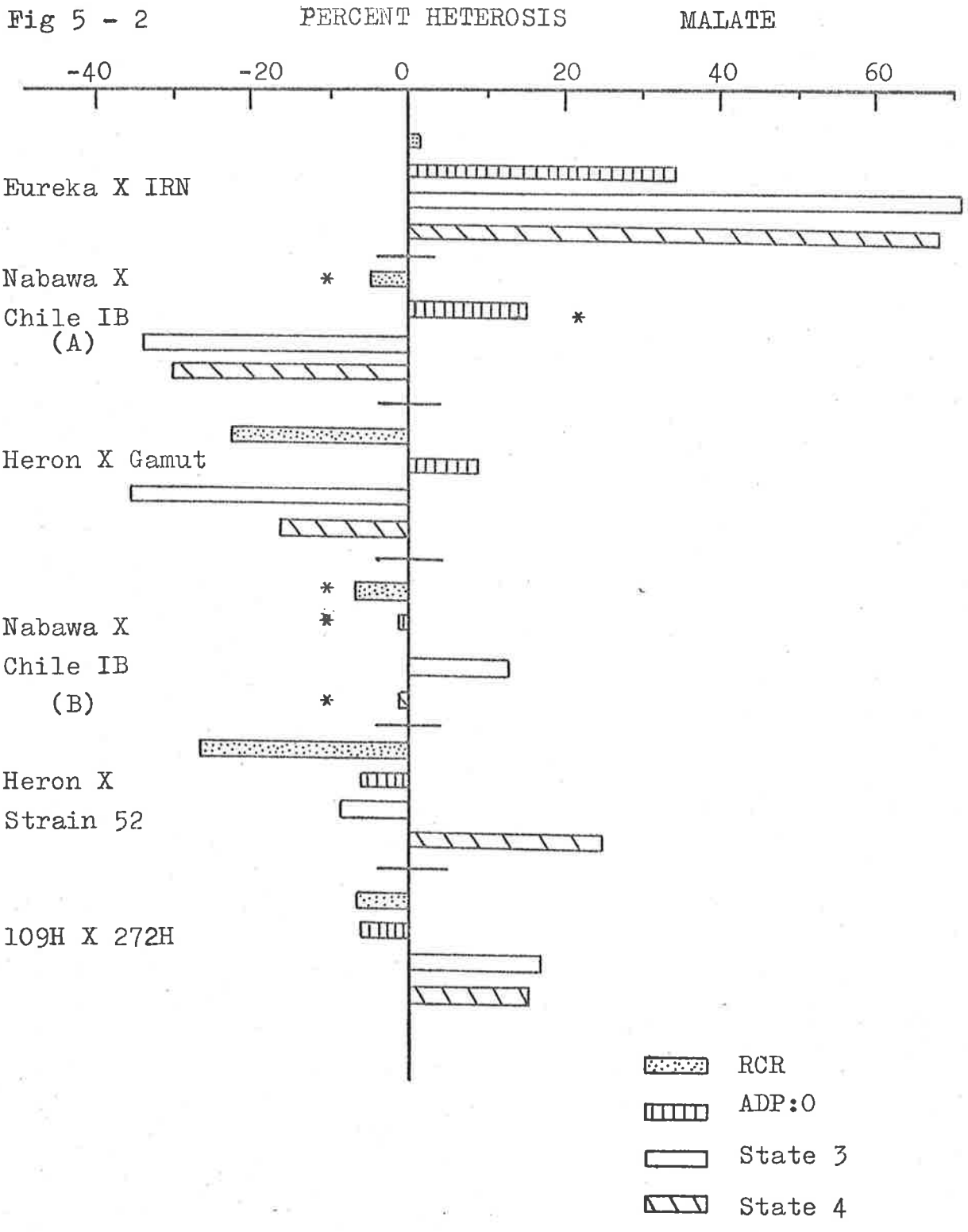

PERCENT HETEROSIS
MALATE
-40
-20
0
20
40
60
Eureka X IRN
Nabawa X
Chile IB
(A)
Heron X Gamut
Nabawa X
Chile IB
(B)
Heron X
Strain 52
109H X 272H
RCR
ADP:O
State 3
State 4

Fig. 5-3

Mitochondrial Heterosis in several Wheat Crosses with KGA.

The histogram, drawn from the data presented in appendices III and IV, shows the percent heterosis in 6 trials where 5 different crosses were used. KGA oxidation was studied under the routine conditions described (Fig. 3-8). Each bar represents the mean of three cycles of phosphorylation. Respiratory control and ADP:O ratios and state 3 and state 4 rates are used for determining heterosis. Stars indicate where the mean has been derived from two widely different values.

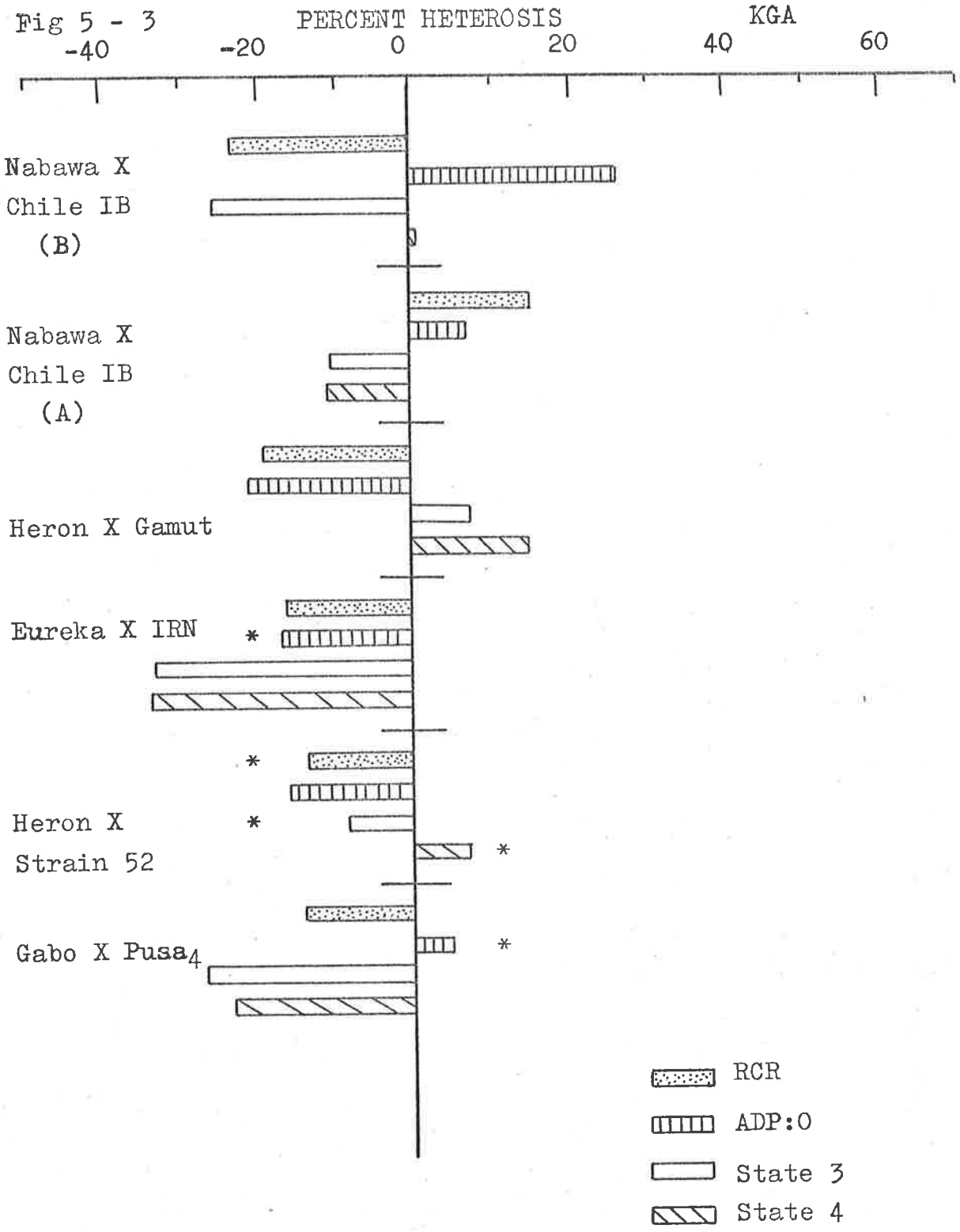


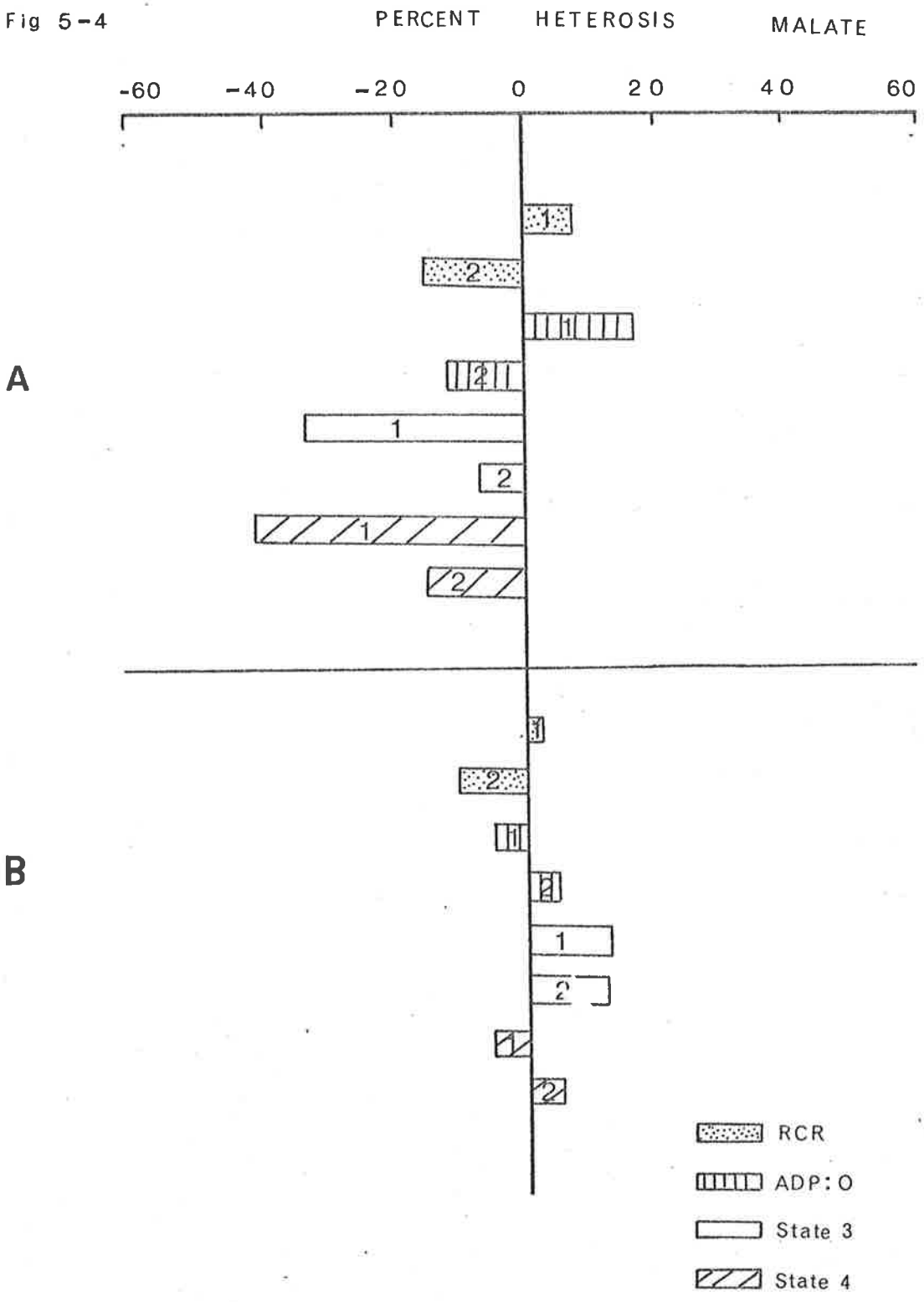
Fig 5 - 3
PERCENT HETEROSIS
KGA
-40
-20
0
20
40
60
Nabawa X
Chile IB
(B)
Nabawa X
Chile IB
(A)
Heron X Gamut
Eureka X IRN
*
*
Heron X
Strain 52
*
*
Gabo X Pusa4
*
RCR
ADP:O
State 3
State 4

Figure 5-4

HETEROSIS IN NABAWA X CHILE IB TRIALS

The histogram shows the percent heterosis for the Nabawa x Chile IB cross in two trials A and B. The four parameters respiratory control ratio (RCR), ADP:O ratio, state 3 and state 4 rates have been used for the determination. Each bar represents heterosis in a single cycle of phosphorylation the numbers of which have been included in the figure.

Fig 5-4


PERCENT HETEROSIS MALATE
-60
-40
-20
0
20
40
60
A
B
RCR
ADP:O
State 3
State 4

CH. VI MITOCHONDRIAL COMPLEMENTATION

Introduction

Mitochondrial complementation is an artificial concept (McDaniel and Sarkissian, 1966) describing the positive interaction between mitochondria of parents of a heterotic hybrid. The theoretical basis for this has been discussed fully in Chapter I. McDaniel and Sarkissian (1966) suggest that any increase in mitochondrial respiratory activity resulting from the artificial mixture of two inbred lines can be taken as an indication that a hybrid between these two lines would produce heterotic mitochondria when isolated and assayed and this should assure heterotic performance of the whole plant.

There have been only three studies which link mitochondrial complementation to agricultural heterosis, one each for barley (McDaniel, 1972), wheat (Sage and Hobson, 1973) and "Pima" cotton (McDaniel, 1973a).

Experiments comparing the activity of parental mitochondrial mixtures to both the mid-parental value and the value of the F_1 hybrid of those parents have been performed. The first comparison provided the measurement of mitochondrial complementation as defined by McDaniel and Sarkissian (1966) and the second relates complementation to heterosis. If the mitochondrial mixture has properties which approach those found in the hybrid mitochondria, then the relative increases resulting from mixing should approach the hybrid advantage in a heterotic cross. Similarly, negative interaction should indicate negative heterosis. Thus the percentile values of heterosis and complementation were compared as a further test of McDaniel and Sarkissian's (1966) hypothesis.

Artificial mixing of parental mitochondria would be expected to produce activity equal to the mean activity of the parents if the amount of protein contributed by each parent was equal. Thus any deviation from this mid-parental value can be interpreted as resulting from mitochondrial interaction in either a positive or negative direction. This assumption does not hold if the amount of mitochondrial protein contributed by each parent is different. The activity in this case would be more closely related to the most plentiful mitochondria and could be lower than this parent when tested alone if the overall mitochondrial protein levels were near the critical minimum for observing controlled respiration (Raison and Lyons, 1970). McDaniel (1970) reports that the maximum enhancement occurs when the mitochondria are mixed in proportions of 2:1, female parent to male parent, and suggests this is a proof of the synergism occurring in the mixtures.

Consideration of the reported phenomenon of mitochondrial complementation raises a series of questions. Firstly is it a general phenomenon and can it be located in a group of 10 Australian wheat varieties, or is it restricted to the cross 31MS by 28 used extensively by Sarkissian and Srivastava? Secondly, is variation from the mean significant and is it a reliable phenomenon or the product of experimental error? Thirdly, is it possible that the enhancement can be obtained by the action of mixing itself? If all the previous three have indicated a true interaction effect, a second series of questions arises. Is complementation due to one parent in particular or does it depend on both equally, and is complementation more evident with

some substrates than with others? Finally, does the reported relationship with mitochondrial heterosis exist and can it be used for predictive purposes?

An investigation of a number of random mitochondrial mixtures from the 10 Australian wheat varieties used in this program was performed to locate possible interaction. The means of testing was by means of polarographic measurements of mitochondrial performance. The percentage change of the mixture from the mid-parent for a given parameter is termed the percent value of mitochondrial complementation for that parameter. The statistical significance and reliability of observed changes was evaluated in replicated studies of three crosses, one of which was the De Kalb line, 31MS by 28. Interactions were observed during KGA and malate oxidation, and for 31MS + 28 in some specific enzymic assays. The influence of the mixing procedure was tested with Heron mitochondria where identical simultaneous preparations were mixed in vivo and their mitochondrial performance compared to the mean of the two parental preparations tested separately. The influence of other factors in creating deviation from the mid-parental activity, such as the rate of seedling growth and the method of mixing, was also considered.

It was necessary also to determine whether any relationship exists between the percentage heterosis and percent complementation figures, even though both may be non-significant as there may be a possibility of using the trend of complementation to predict the performance of hybrid combinations. This relationship will be examined fully in a following section.

Results

Australian wheat mixtures were found to exhibit the whole range of possible interaction. The results of the initial mixing program are tabulated (Table 6-1) where the respiratory control and ADP:O ratio have been used as the parameters of complementation. Respiratory rates were not used for these comparative studies because they are used indirectly in the control ratios and more importantly they give a false impression of improvement. High mitochondrial complementation in state 4 rates would be an indication of lower overall mitochondrial quality in a mixture. If positive interaction was the reflection of an enhancement of the efficiency of phosphorylation, one would expect the mitochondrial complementation in respiratory control to be at least in the same direction as that of ADP:O ratio, if not equal in value. Table 6-1 shows that almost half the experiments showed the percentage change to be in the opposite direction in the two parameters so that of 25 runs 14 agree as to the direction of change and 11 disagree. Having established that differences could be observed between the performance of mitochondrial mixtures and their parents, it was necessary to determine the reliability and significance of this variation. The extent of mitochondrial interaction in mixtures of mitochondria from the parental lines 31MS and 28 was estimated from five experiments with malate and four experiments with KGA as substrate. Thus it was possible to test statistically the observed mean activity of the mixture against the expected mean value, the mid-parent activity, with a χ^2 test. The results of this test for ADP:O ratio and respiratory control ratio

are shown in Table 6-2 where the individual cycles of phosphorylation are treated separately. All χ^2 values were low, being less than 0.1; thus they all indicate that the activity of the mixture is not significantly different from that of the mid-parental value. The highest values of the statistic were found in the ADP:O ratio with both substrates. They occurred in the second cycle of KGA oxidation which is free of the influence of the artificially high ADP:O ratios, and the first cycle of malate oxidation which is not lowered by the ATP-ase contamination in the preparations. If this work was to duplicate the findings of Sarkissian and Srivastava (1969) and Sarkissian (1972) these values would be the ones most likely to show a significant complementary effect. Although the χ^2 values for these observations were the highest in the table, they were still below the critical value at a 5 percent level of significance. Thus mixing mitochondria from the parental lines 31MS and 28 could not be shown to produce, on average, a significant interaction positively or negatively, even though the fluctuations observed in the percent complementation were quite large in some cases (Fig. 5-1) with the highest being 71.3 percent in one trial.

Additional comparison of the activity of three enzyme systems was made for the lines 31MS and 28 in conjunction with heterosis studies. The enzymes NADH-cytochrome *c* reductase, succinate-cytochrome *c* reductase and cytochrome *c* oxidase were observed in intact and uniformly swollen mitochondria. The results, shown in Table 6-3, reveal that none of the

three assays supports claims of synergistic effects of mixing on the enzymic level, as strong negative complementation was observed in all assays.

Two mixtures of local varieties were also studied repeatedly, Heron + Gamut which had shown positive complementation in the initial trial with malate and Gabo + Pusa 4 in which mitochondrial complementation had been negative with malate. Repetition of these experiments with malate and KGA showed the phenomenon to be unstable (Table 6-4) in the case of Heron + Gamut, the ADP:O ratios of the mixture were consistently 12.5 percent above the mid-parent figures but the respiratory control values of the mixture were from 8 percent above to 4.5 percent below the mid-parent figure. Gabo + Pusa 4 showed negative complementation for two trials, but a third produced very high positive mitochondrial heterosis for respiratory control while the ADP:O ratios were approaching zero. Changing the substrate oxidized altered the results drastically. Mitochondrial complementation in Heron + Gamut became consistently negative for all respiratory control ratios and in all but one value of ADP:O ratio. Gabo + Pusa 4 retained its predominantly negative characteristics with KGA as substrate alone showing one ADP:O ratio which marginally exceeded the mid-parent value (2%). In addition, in the general survey (Table 6-1) the mixtures Nabawa + Chile IB and Eureka + IRN were tested with both substrates. Again it can be seen that the two substrates support a different extent and direction of change in ADP:O ratio and respiratory control ratio. Thus in Australian lines complementation was not demonstrated consistently and

the phenomenon did not retain its characteristics with different substrates.

It was necessary to eliminate the possibility that parental mixtures varied in phosphorylative performance from the mean of their parents because of the process of mixing itself. A 1:1 mixture, made by combining equal aliquots of two identical Heron preparations (Table 2-2) in an oxygen electrode vessel, showed no significant change from the mean of the previous two assays. The phosphorylative characteristics of the mixture are compared to the mid-parent values in Table 6-5. Thus the process of mixing itself does not cause a deviation from the mean parental value.

Discussion

Mitochondrial complementation could not be detected consistently either with the lines used successfully by Sarkissian and Srivastava (1969) or in the Australian varieties mixed. Variation from the mean of the parental values did occur in almost all mixtures tested but the direction and extent of deviation was highly irregular. Furthermore the two parameters of mitochondrial integrity, respiratory control and ADP:O ratio, which could be expected to concur as to the direction of complementation within one mixture, were as often dissimilar as similar in the initial trial. Interaction between parents also apparently varied with the substrate used. If mitochondrial complementation is to be taken as an indicator of total hybrid vigour one would expect stability between the two substrates. However, McDaniel and Sarkissian (1968) claim that the interaction phenomenon for maize mitochondria is

substrate specific with NAD-linked substrates alone being capable of synergistic interaction. However, these conclusions are based on data from mitochondria of uneven quality as is suggested by the lack of control found in three of the four preparations of parents, hybrids and mixture with succinate and in one preparation with NADH. In making these claims, McDaniel and Sarkissian (1968) use respiratory rates as the means of comparison in uncoupled preparations. Thus conclusions as to the superiority of one type of mitochondrial preparation relative to another will be irrelevant to considerations of mitochondrial efficiency.

One would expect all substrates to support a complementary effect if it was the result of enhanced mitochondrial efficiency, as it is the structure of the respiratory chain, not the substrate dehydrogenase activities that is the rate limiting step in oxidation. This can be shown by the enhanced activity of in vivo dehydrogenases (Lieberman and Baker, 1965; Douce et al., 1972; Bernofsky and Utter, 1966). In addition malate and KGA are both NAD-linked substrates which gain access to the matrix dehydrogenases by means of the dicarboxylic acid transporter. Later reports (Sarkissian, 1972) show the phenomenon for KGA, malate, pyruvate + malate, succinate and NADH oxidation. In the present trials of wheat mitochondrial complementation the predominant direction of the interaction in, for example, Heron + Gamut was reversed when the substrate was changed. Apparent mitochondrial complementation observed in these trials is thus thought to be due to random variation of mitochondrial performance than to a true interaction effect.

An alternative explanation for the variation in mitochondrial performance must be sought. Firstly, it was shown in Chapter IV that individual wheat varieties do not have mitochondria which on extraction repeatedly show characteristic ADP:O ratios for that variety. Thus the difference observed between two mitochondrial preparations from parental lines is not due to a real difference in their inherent efficiency but to some other variable. The same conclusion can be reached about the alteration in mitochondrial performance when mitochondria from parental lines are mixed. Interaction can only be observed by immediate comparison. The variation could be attributed to experimental method used for comparison or the nature of the tissue being compared. Again the statistical conclusions of Chapter IV show that all three variants of the rapid extraction technique produced mitochondria in which the ADP:O ratios were not significantly different. Thus slight method changes between preparations should not produce significant changes in the value of the ADP:O ratio. Furthermore, in any one comparative trial the conditions are held strictly uniform (parents are prepared and tested synchronously), so respiratory control and ADP:O ratios should reflect accurately the relative performance of mitochondria so prepared. Thus the method should be sufficiently controlled and differences between the preparations not attributed to this source.

Yet it may be that the rapid isolation method produces inherently variable mitochondria. The statistical treatment of results (Ch. IV) showed that the variation of results obtained by changing the preparation method of variety of wheat used, is no greater than the spread of results

already existing within one preparation method or one variety. However, the presence of ATP-ase in all preparations, a result of the rapid isolation procedure, has been discussed in Chapter III.

The influence of the ATP-ase in reducing the ADP:O ratio was greater in later cycles of phosphorylation and was unevenly expressed in separate preparations. Thus it is very likely to contribute to the variation between comparative preparations. KGA oxidation exhibited a high first cycle in many preparations which was not found in conventionally prepared wheat mitochondria. This tended to appear in all genotypes prepared together as part of one comparison (e.g. see Table 3-4) and thus should not markedly affect the relative performance of preparations in a single comparison. It would be expected to influence the reliability of replicated trials, especially where only one cycle of phosphorylation was observed.

The nature of the tissue from which mitochondria are extracted is also a likely source of variation between mitochondrial preparations. In Chapter IV the only significant influence of ADP:O was the age class of the tissue used for mitochondrial extraction. It was shown that for malate six extra hours of germination was enough to produce significantly lower ADP:O ratios taken over a wide number of preparations and varieties. This effect was not as precise with KGA oxidation as material in the 65 to 71 hour age class had anomalously high ADP:O ratios and as only the first cycle had been taken, this was considered to reflect the prevalence of high ADP:O ratios in older tissue. Srivastava and Sarkissian (1970) and Sarkissian (1972) stress the

influence of tissue age on respiratory control and ADP:O ratios in wheat mitochondria suggesting that the optimal time for extraction is between 2 and 3 days after the commencement of germination. McDaniel (1973) also reports similar findings with mitochondria extracted from barley seedlings.

Germination studies made for the estimation of seedling heterosis provide information as to the weight and length of coleoptiles after three and four days of germination. Although all seedlings used in one set of preparations have been growing for the same length of time and germination and growth of seedlings is made as uniform as possible by pre-soaking the seeds, large variation may occur in the development stage reached in a given time by different varieties of wheat. In the trials conducted in this project (Appendix V) one parent of a possible mix often showed two or three times the mean coleoptile length and weight of the second parent. As comparison of the data from any one line in the three and four day germination trials showed that such an increase represented a day's growth, we can surmise that parent shoots of one age, used in a complementation trial may differ from each other by as much as a whole day in physiological age. This would be a substantial source of variation in mitochondrial quality between preparations from two wheat varieties prepared simultaneously.

McDaniel (1969) reported that large differences in developmental rate observed in barley seedlings were positively correlated with seed size. However he found that although the total amount of protein in a mitochondrial fraction extractable from the largest seedlings exceeded that of the small seedlings, the amount of protein, expressed

on the basis of fresh weight, remained constant throughout the seed classes. This result would suggest that it was not decreasing mitochondrial protein availability that was causing the lower respiratory control and ADP:O ratios in older shoots. Tissue becomes more rigid as differentiation progresses which could necessitate stronger sheering forces for cell disruption which could damage mitochondria more than in softer, less developed tissue.

Conclusion

Mitochondrial complementation was not found in these studies although the mixing of mitochondria often altered the measured respiratory control and ADP:O ratios. This variation is thought to be a product of the uneven developmental rate of parental seedlings which separates two parents into effectively different age classes. The rapid technique of mitochondrial preparation was also considered to be responsible for the variance observed within one comparative preparation, but this is attributed to the inherent pitfalls in the method rather than lack of uniformity in applying the method.

Since mixing mitochondria could not produce a significant and consistent enhancement in mitochondrial activity further experiments to determine what part individual parents played in mixing phenomena were unnecessary. If conditions are standardized so that possible variations are minimized - i.e. by testing two parents, a mixture (w/w) and a hybrid preparation in two simultaneous, consecutive preparations - it should be possible to examine the relationship between trends in

mitochondrial heterosis and complementation, even though both are insignificant in themselves.

Complementation and heterosis

Experiments where mitochondrial heterosis and mitochondrial complementation were both determined on one preparation provided the basis of a linear regression study for determining if percent heterosis was a dependent variable of percent complementation. Such a dependence must be illustrated if percent mitochondrial complementation is to be used as a predictor of potential heterosis. Initially sets of data from crosses were examined individually. Data was obtained from each cycle of ADP-stimulated respiration so that the ADP:O ratio, control ratio, state 3 and state 4 rates of oxygen uptake each provide an estimate of the heterosis and complementation in that cycle.

For the cross 31MS by 28 simple regressions were performed on all the data combined so that the equation $y = 0.04x - 3.12$ was obtained to best describe the distribution of points. The coefficient of determination, r^2, was 1.51×10^{-3}. Thus, as a perfect correlation has an r^2 value of 1, the scatter was very large and the line was not a good description of the points. Thus no correlation occurs.

Sarkissian and Srivastava (1969) reported strong heterosis with both malate and KGA oxidation and found that mitochondrial complementation approximated to mitochondrial heterosis with both substrates, thus justifying the treatment of the present data in a combined analysis. However, the present study suggests that malate produces a greater

difference between crosses than KGA and combination of results could be masking a possible relationship between the two variables. Thus the analysis was repeated for malate and KGA oxidation trials separately and again no correlation was found as the r^2 values were 0.08 and 0.017 respectively. Treating the four parameters together was valid as each provides a comparison between the hybrid, or mixture and parental mitochondrial activity. However, since the four determinations of heterosis were shown previously to disagree and the disagreement appeared greatest between measures of heterosis based on the parameters of control and those from rates of oxygen uptake, the data was also analysed by linear regression with the results derived from changes in ADP:O and respiratory control ratios separated from those derived from state 3 and state 4 rates. Results from different substrates were also separated. Again there was no correlation as the values were all below 0.1. The equations and values for r^2 are presented in Table 6-6.

Thus, although one would expect from the results of Sarkissian and Srivastava (1969) a strong positive correlation between percent complementation and percent heterosis for 31MS by 28, no correlation was found. Not only was the reported positive heterosis not obtained, the mainly negative trends in heterosis were not followed by the value of complementation and the two values appeared completely random.

Some of the experiments with other crosses also provided results which could be analysed by a simple regression. The correlation between heterosis and complementation was studied along similar lines to the

31MS by 28 cross and the results of the analysis are presented in Table 6-7. The number of readings in which the two values could be paired was different in each cross so not all analyses were partitioned as fully as has been previously described. Each cross was treated separately.

Of the all inclusive analyses, two crosses showed positive correlations. Eureka by IRN had a coefficient of determination of 0.37 which indicated that the points cluster loosely around the line of best fit, $y = 0.83x - 8.05$. Heterosis could be expected to have the same direction as complementation but the value for the former would be lower than that of the latter.

This low positive correlation did not persist when the Eureka by IRN data was partitioned and analysed on the basis of individual substrates. The malate points show completely random relationship ($r^2 = 4.74 \times 10^{-5}$) although all readings are positive, while the KGA results are also scattered ($r^2 = 0.02$) and are predominantly negative. Assuming independence of substrates and combining data, the number of observations in the total sample is increased, reducing total variance. It is only under these conditions that a slight correlation can be observed between heterosis and complementation.

The second case of positive correlation occurs with Heron by Gamut where again the all inclusive analysis showed that points tended to cluster around the line $y = 0.52x - 14.42$ with the r^2 value of 0.57. However, in this case the positive correlation is still present when the data is sub-divided on the basis of substrates with malata data

showing a slight clustering (r^2 = 0.28) around the line y = 0.69× - 17.86 but with the KGA data being strongly correlated. This latter set of data approximates to the line y = 0.47× - 10.65 with r^2 = 0.91 and is thus a very good fit. So again the analysis shows heterosis to correspond to complementation of the same direction but of lower value. Complementation has been proposed as an artificial mimic of the mitochondrial action in hybrids and it has always been described as approaching the level of the hybrid vigour and not exceeding it. That the two are reversed in this analysis may be due to the two methods used to calculate these values. The hybrid is always compared with the highest parent, while the mixture is compared to the mid-parental value which was the expected value in a 1:1 mixture. It is not meaningful to talk of heterosis in terms of the mid-parental value because in a practical situation the highest parent must be improved upon if any advantage is to be gained in crop yield. Had data for heterosis been calculated in relation to the parental average as has been done by McDaniel (1971) and Sarkissian and Srivastava (1971), there would have been a general increase in the level of heterosis and this may possibly have raised the value of the slope of the line. However, it is unlikely to alter any overall relationship which exists between heterosis and complementation.

The Nabawa by Chile IB cross showed no correlation at all when the all-inclusive regression was performed, and there was also no correlation for either substrate based subdivision. Since there were 41 readings in the total sample these results were divided further into parameters of integrity and respiratory rates as was done for 31MS by 28.

Of the four tests done in this way three showed no correlation (Table 6-7) but surprisingly the rates of malate oxidation provided a positive correlation with the points being described by the line $y = 0.66x - 0.67$ and $r^2 = 0.54$. Thus high values of percent mitochondrial complementation for rates of oxygen uptake could be used to predict positive heterosis for rates. However, a high hybrid advantage in respiratory activity has usually nothing to do with high efficiency and there is no logical reason why observing higher rates in hybrids should be related to final yield, in fact one would expect the opposite to occur.

Suitable data for a linear regression on the crosses Gabo by Pusa 4 and 109H by 272H were available with only one substrate in each case. The pairs of data for 109H by 272H was obtained with malate as substrate and showed no correlation. Heterosis was found to be negatively correlated with complementation, however, in the Gabo by Pusa 4 data obtained with KGA oxidation where the $r^2 = 0.61$ shows a reasonable fit of the points to line $y = 0.33x - 9.9$. This was the only negative correlation found to be significant in all the tests on Australian wheat and it means that for this cross the direction of heterosis could be expected to be opposite to any given direction of complementation.

Table 6-1a

MITOCHONDRIAL COMPLEMENTATION IN AUSTRALIAN WHEAT

Mixture	Cycle No.	% Mitochondrial Complementation	
		RCR	ADP:O
*Gabo + Pusa 4	1	-13	-15
Gabo + Chile IB	1	-1.5	-1.5
	2	4	9
Nabawa + Chile IB	1	-8	-12
	2	0	1
Nabawa + Chile IB	1	23	-12
	2	-18	-1
Nabawa + IRN	1	7	11
	2	-	-
Festival + IRN	1	-13	25
	2	-41	-10
Eureka + IRN	1	18	80
Festival + Heron	1	10	14
Festival + Eureka	1	56	-56
	2	51	38
	3	-	-15
* Heron + Gamut	1	5	13
Mengavi + Falcon	1	6	5

A survey of mitochondrial complementation in Australian wheat lines was conducted with malate as substrate. Heterosis was calculated on respiratory control ratio (RCR) and ADP:O ratios with each cycle of phosphorylation taken separately.

Stars indicate those sets of data which are repeated in Table 6-4.

Table 6-1b

MITOCHONDRIAL COMPLEMENTATION IN AUSTRALIAN WHEAT

Mixture	Cycle No.	% Mitochondrial Complementation	
		RCR	ADP:O
Heron + Pusa 4	1	17	12
	2	30	6
Heron + Eureka	1	4	68
* Heron + Gamut	1	-26	-36
* Gabo + Pusa 4	1	-10	2
	2	-19	-33
Nabawa + Chile IB	1	41	68
	2	4	-16
	3	4	12
Nabawa + Chile IB	1	-4	9
	2	-6	3
	3	17	19
Nabawa + Chile IB	1	59	7
	2	30	22
Eureka + IRN	1	8	-14
	2	12	-8
	3	0	+16

A survey of mitochondrial complementation in Australian wheat lines was conducted with KGA as substrate. Heterosis was calculated on respiratory control ratio (RCR) and ADP:O ratios with each cycle of phosphorylation treated separately.

Stars indicate those sets of data which are repeated in Table 6-4.

Table 6-2

TESTING FOR SIGNIFICANT MITOCHONDRIAL COMPLEMENTATION BETWEEN LINE 28 AND 31MS

Substrate	Parameter	Cycle	χ^2 value	Probability	Significance
KGA	ADP:O	1	1.66×10^{-3}	$0.975 > P > 0.95$	NS
		2	6.7×10^{-2}	$0.9 > P > 0.10$	NS
		3	3.7×10^{-2}	$0.9 > P > 0.10$	NS
KGA	RCR	1	1.3×10^{-3}	$0.975 > P > 0.95$	NS
		2	2.8×10^{-4}	$0.99 > P > 0.975$	NS
Malate	ADP:O	1	9.38×10^{-2}	$0.9 > P > 0.1$	NS
		2	2.2×10^{-2}	$0.9 > P > 0.1$	NS
Malate	RCR	1	3.7×10^{-2}	$0.9 > P > 0.1$	NS
		2	4.9×10^{-2}	$0.9 > P > 0.1$	NS

Comparison of the 1:1 mixture to the average parental activity using the χ^2 test. Mean values for each cycle are derived from 4 values in KGA experiments and 5 values in malate experiments.

RCR = respiratory control ratio.

Table 6-3

MITOCHONDRIAL COMPLEMENTATION

NADH-cytochrome *c* reductase activity

	31MS	28	Mixture	% M.C.
Intact mitochondria	180	150	140	-15.00
Swollen mitochondria -AA	240	300	130	-52.00
+AA	130	200	90	-45.00
Succinate cytochrome *c* reductase activity				
Swollen mitochondria	25	31	20	-32.00
Cytochrome *c* oxidase activity				
Swollen mitochondria	512	433	327	-31.00

NADH- and succinate-cytochrome *c* reductase rates are expressed as nmoles cyt. *c* reduced . mg protein^{-1}.min^{-1}. Cytochrome *c* oxidase rates are expressed as nmoles cyt. *c* oxidized . mg protein^{-1}.min^{-1}.

Assays were performed as described in Chapter II under the conditions detailed in Tables 3-9, 10 & 13.

Swollen mitochondria had been treated as described in Chapter II.

M.C. = mitochondrial complementation.

Table 6-4

REPLICATED MEASUREMENTS OF MITOCHONDRIAL COMPLEMENTATION WITH TWO AUSTRALIAN WHEAT CROSSES

	Mixture	Phosphorylation Cycle No.	% Mitochondrial Complementation	
			RCR	ADP:O
	Malate oxidation			
	Heron +Gamut	1	5	13
	Heron + Gamut	1	-14	12
		2	8	-
*	Gabo + Pusa 4	1	-13	-15
	Gabo + Pusa 4	1	-28	-19
		2	-1	-21
	Gabo + Pusa 4	1	46	2
		2	69	-3
	KGA oxidation			
*	Heron + Gamut	1	-26	-36
	Heron + Gamut	1	-28	14
	Heron + Gamut	1	-21	-29
		2	-14	-29
*	Gabo + Pusa 4	1	-10	2
		2	-19	-33

Mitochondrial complementation and heterosis in two Australian crosses estimated with both malate and KGA as substrates and for the parameters ADP:O and respiratory control ratio (RCR).

Assays were performed under routine conditions (Fig. 3-8).

Stars indicate results repeated from Table 6-1.

Table 6-5

MIXING MITOCHONDRIA FROM ONE WHEAT VARIETY

Means of Parental Preparations tested separately

Cycle	RCR	ADP:O	State 3 rate	State 4 rate
1	2.22	2.31	37	17
2	2.01	2.20	42	21

Values of mixed Parental Preparations (1:1 v/v)

Cycle	RCR	ADP:O	State 3 rate	State 4 rate
1	2.12	2.24	39	18
2	1.88	2.00	41	22

Two Heron preparations, prepared and tested synchronously (Table 2-2) were mixed by adding 0.2ml aliquots of each to the reaction vessel containing 1ml standard sucrose medium, with 13.6mM malate, 34 mM glutamate and phosphorylation was initiated by addition of 21.4 µM ADP. The two preparations contained 4.9 and 4.3mg mitochondrial protein per reaction; thus the mixture would have contained 4.6mg protein per reaction. Mean figures of the parental activity, from Table 2-2, are presented for comparison with the mixture. Rates are expressed as nmoles O_2 . mg protein-1.min^{-1}. RCR = respiratory control ratio.

Table 6-6

SIMPLE LINEAR REGRESSIONS OF % MITOCHONDRIAL COMPLEMENTATION ON % MITOCHONDRIAL HETEROSIS FOR THE WHEAT CROSS 31MS X 28

Conditions	n	Equation	r^2	Correlation
Malate + KGA All parameters	75	$y = 0.04x - 3.12$	1.51×10^{-3}	Negative
Malate All parameters	35	$y = 0.43x - 2.54$	0.08	Negative
KGA All parameters	40	$y = -0.09x - 1.97$	0.017	Negative
Malate ADP:O & RCR	17	$y = -0.12x - 13.5$	0.008	Negative
KGA ADP:O & RCR	19	$y = 0.6x - 6.64$	0.014	Negative
Malate S_3 & S_4 rates	18	$y = 0.18 + 0.76$	0.069	Negative
KGA S_3 & S_4 rates	22	$y = 1.49 - 0.17x$	0.045	Negative

r^2 = coefficient of determination, n = number of pairs of samples in each analysis, RCR = respiratory control ratio, S_3 & S_4 = state 3 and state 4 rates of oxygen uptake. Results are derived from results presented in Appendices III & IV.

Table 6-7

SIMPLE LINEAR REGRESSION OF % MITOCHONDRIAL COMPLEMENTATION TO % MITOCHONDRIAL HETEROSIS FOR A NUMBER OF CROSSES

Cross	Conditions of Assay	n	Equation	r^2	Correlation
Nabawa x Chile IB	Malate + KGA All data	41	y = 0.05x - 7.78	5×10^{-3}	Negative
	Malate All data	17	y = 0.26x - 7.35	0.12	Negative
	KGA, all data	24	y = -0.05x - 5.91	3.9×10^{-3}	Negative
	Malate RCR, ADP:O	8	y = 0.18x + 11.00	0.048	Negative
	KGA, RCR & ADP:O	10	y = 0.37x + 0.99	0.079	Negative
	Malate S_3 & S_4	9	y = 0.66x - 0.66	0.54	Positive
	KGA, S_3 & S_4	14	y = -0.17x - 13.49	0.16	Negative
Eureka x IRN	Malate + KGA All data	16	y = 0.83x - 8.05	0.37	Positive
	Malate All data	4	y = 0.007x + 43.42	4.74×10^{-5}	Negative
	KGA, all data	12	y = -0.18x - 14.62	0.02	Negative
Heron x Gamut	Malate + KGA All data	15	y = 0.52x - 14.42	0.57	Positive
	Malate All data	7	y = 0.69x - 17.86	0.28	Slight Positive
	KGA, all data	8	y = 0.47x - 10.65	0.91	Strong
Gabo x Pusa 4	KGA, all data	8	y = -0.33x - 9.9	0.61	Negative
109H x 272H	Malate All data	8	y = 0.14x + 3.04	0.015	Negative

CH. VII FINAL DISCUSSION

Heterosis is a phenomenon of growth, normally associated with increases in agriculturally important criteria such as yield. It can be detected in seedlings by measuring growth rates. McDaniel and Sarkissian (1966) claim that heterosis is retained on a still lower level of cellular organization, the mitochondria, and that hybrid superiority can be observed in mitochondria isolated from seedlings of heterotic coleoptiles. This claim was substantiated by further reports of mitochondrial heterosis in corn (Sarkissian and McDaniel, 1967), wheat (Sarkissian and Srivastava, 1969, 1970), barley (McDaniel, 1971, 1973) and cotton (McDaniel, 1973a) where mitochondria were compared by polarographic analysis and enzymic studies. From these reports one would expect mitochondria of any crosses showing seedling heterosis to perform better in comparison to mitochondria from their highest parent. The present study failed to find the reported persistence of heterosis to the mitochondrial level.

A mechanism for the expression of heterotic growth was proposed by Sarkissian and McDaniel (1967) who suggested that mitochondrial populations of corn are polymorphic and that a hybrid specific type of mitochondrion of superior activity to the parental types exists. This superiority was proposed to account for an additional 30 percent of the total cytochrome oxidase activity of the hybrid mitochondria (Sarkissian and McDaniel, 1967), and also for the better performance

of hybrids as compared to parental mixtures in KGA stimulated oxygen uptake rates (McDaniel and Sarkissian, 1966). This peculiarity of hybrid mitochondria was claimed to be accentuated in wheat hybrids so that twice the usual efficiency of oxidative phosphorylation was reported.

Testing these predictions, hybrid mitochondria of Australian and American wheat lines were studied but the high efficiency reported was not evident. Some above theoretical ADP:O ratios were observed in the first cycle of KGA oxidation but these were not confined to the mitchondria derived from the hybrid lines. Hybrid mitochondria were not found to be significantly different from their highest parents, prepared concurrently, either in the wheat lines used by Sarkissian, (31MS by 28)or in several crosses of local varieties.

A third claim was made concerning the complementary activity of mixtures of mitochondria. Part of the mechanism of heterosis originally proposed by McDaniel and Sarkissian (1966) centres around the apparent difference in mitochondria from pure-bred, parental lines of corn and the retention of this difference (with density gradient separation) in an F_1 hybrid corn scutellum preparation (Sarkissian and McDaniel, 1967).

The enhancement of mitochondrial activity on artificial mixing of parental types of isolated mitochondria was interpreted as an _in vitro_ demonstration of interaction which could occur within the hybrid cytoplasm between different types of parental mitochondria. As discussed in Ch. I both explanations were invoked to explain why the mixtures

approximated but not equalled hybrid activity. The expected enhancement of activity in the same direction as seedling and mitochondrial heterosis measured by several parameters, was not found in this study. Inconsistent interaction did occur with mixing but statistical analysis showed that the differences between the mixture and midparent of 31MS by 28 was not significant. Further, there was no correlation between the direction of mitochondrial complementation and mitochondrial heterosis, nor between mitochondrial complementation and seedling heterosis.

The properties of mitochondria predicted under McDaniel and Sarkissian's mechanism of heterosis thus could not be demonstrated in the present work.

Other workers have supported McDaniel and Sarkissian by reporting mitochondrial complementation. Sage and Hobson conducting a trial with European wheat lines, found that over the whole experiment there was no correlation between mitochondrial interaction and yield heterosis. However exclusion from the analysis of the cross severely infected by disease and that showing incomplete fertility restoration did produce significant correlations between field vigour and mitochondrial interaction when a low seed density was employed. Sage and Hobson's work (1973) differs from others in that they tried to compensate for different seedling germination and growth rates by sowing seed at spaced time intervals. In so doing they may have been eliminating the variance due to shoot physiological age which was shown in Ch. IV to have a

significant influence on mitochondrial activity. They also mascerated each preparation as though it was a mixture, dividing the parental shoots into two 0.45 gm samples and grinding them separately, mixing the filtered brei in equal volumes. As mixing per se produced no change in mitochondrial activity (Ch. VI) this step should not have been any more successful than the procedure of grinding equal quantities of parental shoots in one batch. However, the Sage and Hobson technique may have shortened the time the mitochondria were subjected to sheering stress and may have helped to reduce the amount of damage. There is no information as to the amount of scatter in the value of ADP:O ratios found for the four replicates. Some parents occurred in three crosses and their ADP:O ratio has been estimated each time. The mean values are different in each estimate, indicating that variation between preparations occurs. Sage and Hobson (1973) made no attempt to combine the values between sets of comparative experiments indicating that they considered it invalid.

The lack of significant yield heterosis may be responsible for the lack of correlation between mitochondrial complementation and yield at the high seed density. Sage and Hobson (1973) discuss the interaction of environment and genotype on the expression of heterosis and conclude that maximal heterotic expression is the result of optimal balance of yield components. Adverse environmental conditions which depress certain yield components, such as disease or high density can prevent the full development of heterosis. They envisage mitochondrial efficiency as a

basic yield component, but one which could only become limiting under highly favourable environmental conditions when no other stress operated. Mitochondrial complementation being a prediction of mitochondrial heterosis, could only be a useful indicator of yield heterosis under such favourable conditions, i.e. with low density, disease free, fully restored plants.

Sage and Hobson's work employs the cytoplasmic, male-sterile breeding technique which uses an already hybrid female parent (Ch. I). As Srivastava, Sarkissian and Shands (1969) found that several male sterile lines had enhanced mitochondrial activity they suggested that these lines contained complementing mitochondria. This would raise midparent values reducing the significance of the percentage complementation. Line 31MS was twice shown to be superior to line 28 (Sarkissian and Srivastava, 1969; Sarkissian, 1972).

In the present study line 31MS was not always the highest parent. For example 31MS recorded the highest rate of NADH-cytochrome *c* reduction when mitochondria were intact. This superiority was lost after osmotic swelling when line 28 was more active both in the presence and absence of antimycin A. The ADP:O and respiratory control ratios of line 28 and of 31MS (Appendix III) show that line 28 mitochondria were not more broken than those of the male sterile line. Thus conditions for observing heterosis or complementation were optimal as the mid-parent value was not influenced by especially high activity in the male sterile parent.

Successful complementation studies have also been reported for

sugar beet mitochondria (Doney et al., 1972) where both respiratory control ratios and ADP:O ratios of mixtures exceeded the highest parental value in crosses where heterosis had been observed in root growth. The values for mixtures were slightly lower than those of hybrid mitochondria as predicted by McDaniel and Sarkissian (1966, 1967). The beetroot mitochondria (Doney et al., 1972) were poorly coupled with ADP:O ratios ranging from 0.97 to 1.73, for KGA oxidation, and with control ratios of 1.38 to 2.48 indicating that the mitochondria had been damaged during isolation. Comparison of phosphorylation efficiency among preparations showing so little control cannot be meaningful as membrane integrity will influence the results far more than any inherent difference in the phosphorylation mechanisms of mitochondria from different genetic origins.

Two groups of workers, Ellis et al. (1973) and Zobl et al. (1972) could not verify the reports of mitochondrial heterosis in either wheat or barley on careful repetition of the experiments. Ellis et al. (1973) tested the barley cultivars (18-17 by Avirat) used by McDaniel (1971, 1972, 1973) and the wheat lines (31MS and 28) used by Sarkissian and Srivastava (1969, 1972) for mitochondrial complementation. They first followed the reported procedure closely then four modifications were made, some on the recommendation of Sage and Hobson. None of the experiments produced a significant or reliable complementary effect in either direction. The authors comment on the low ADP:O ratios, particularly of barley mitochondria (1.5 to 2.0), and suggest that the

cause of the discrepancy among reported ADP:O ratios may also be the cause of discrepant findings for complementation effects.

Zobl et al. (1972) investigated mitochondrial complementation and heterosis in European wheat varieties. They found no significant difference in the ADP:O ratios or respiratory rates of mixtures and parents with the calculated value for complementation never exceeding 6.5 percent. Furthermore, no correlation with the results of field trials with the F_1 hybrids was indicated, either for kernel yield heterosis or straw height heterosis. They concluded that complementation between the mitochondria isolated from their winter wheat varieties either does not exist or remains within the range of experimental error.

Since two other groups have also failed to find the reported heterosis or complementation claimed for wheat mitochondria, it is necessary to examine the original reports to determine whether some fallacy exists in the hypothesis. The evidence presented for wheat mitochondrial heterosis will be discussed and then viewed in relation to the work on barley and maize.

The first report of mitochondrial heterosis and complementation in wheat (Sarkissian and Srivastava, 1969) contained several new observations. Unlike previous reports with corn, wheat mitochondria exhibited both phenomena with flavin and NAD-linked substrates. The 1:1 mixture exhibited activity which exceeded the mid-parent and tended towards the hybrid in all cases. The hybrid activity was unusual in that the ADP:O ratios measured exceeded the theoretical maximum for

all substrates and the complementing mixture also exceeded this maximum in KGA and malate oxidation trials.

Sarkissian and Srivastava (1969, 1970) and Sarkissian (1972) placed great importance on these hybrid ADP:O ratios claiming they imply more efficient phosphorylation and thus reveal the mechanism by which heterosis is expressed.

Under the chemiosmotic theory of oxidative phosphorylation (Ch. I) it is possible to explain a more efficient mitochondrial system by employing Mitchell's ATP-ase I instead of model ATP-ase II. Closer analysis of the data presented (Sarkissian and Srivastava, 1969 , 1970) reveals another explanation of these high ADP:O ratios which makes this theoretical explanation unnecessary.

Diagrams of actual polarographic traces are presented in two papers (Sarkissian and Srivastava, 1969, 1970) to substantiate claims of high ADP:O ratios. In trace (a) Fig. 1 (Sarkissian and Srivastava, 1970) the mean rate of oxidation, was calculated from the numbers in the diagram to be 12 μM O_2 per min. Yet from the scale given, 192 μM oxygen (576 nmoles) was consumed in a time span of 16 minutes. This necessitates an even rate of uptake of 36 nmoles oxygen per min which is faster than the highest state 3 rates quoted in the diagram. If 576 nmoles oxygen were to be consumed at a maximum rate of 25 nmoles oxygen per minute as suggested, it would require 23 minutes to complete the experiment so it appears that something is wrong in the calculated rate of oxygen uptake. This discrepancy can be seen for the other

traces presented in Fig. 1 (Sarkissian and Srivastava, 1970). Trace (b) (succinate oxidation) showed a total of 675 nmoles O_2 uptake at a maximum rate of 40 nmoles $O_2 \cdot min^{-1}$, which would require 16 mins if this rate was maintained throughout. The time shown was only 12 minutes. Similarly in trace (c) with malate, 638 nmoles were consumed in 10.5 minutes which would require a maximum rate of 61 nmoles $O_2 \cdot min^{-1}$ whereas the highest state 3 rate given was 45 nmoles $O_2 \cdot min^{-1}$. It appears that Sarkissian and Srivastava (1970) are underestimating the amount of oxygen utilized and this could explain why their estimates of the ratio of ADP used per oxygen taken up are too high. This situation could be the result of a gradual leak of oxygen into the vessel chamber. Gradual replacement of oxygen reduced by the mitochondria would make rates appear slower and allow a greater amount of ADP to be added.

This explanation would be more acceptable than that of high efficiency proposed by Sarkissian and Srivastava (1969 , 1970). Previous claims of high ADP:O ratios have all been explained by errors in the determination of oxygen (Haslam, 1965; Lenaz and Beyer, 1965). The traces presented by Sarkissian and Srivastava (1969) were also examined, the mean slope of each trace being used to calculate the average rate of uptake. The rates reported were all lower in this instance than a mean rate calculated from the rate values presented in the diagram shown below as the actual mean rate

Source of Mitochondria	Actual Mean Rate	Given mean rate
31MS	36.4	55.5
28	42	61.5
Hybrid	39.6	55.8
Mixture	36.7	44.4

Either the scales presented are in error or the calculations are at fault. Since this discrepancy is in the opposite direction from that found in previously discussed figures, it appears to be some basic fault in the method of calibration of the equipment which is variable or the means of calculating the results.

Srivastava (1973) reported heterosis in ATP-ase activity. This is compatible with claims of enhanced oxidative phosphorylative ability if the ATP-ase measured is exclusively coupling factor ATP-ase. Since rapidly isolated wheat mitochondria contain another contaminating ATP-ase (Ch. IIIB) this is unlikely. Further, to obtain a meaningful measurement, ATP-ase should be operating at its maximum DNP-stimulated rate. Sarkissian and Srivastava (1969) did not fulfil this second condition when they determined the ATP-ase activity in parent, mixture and hybrid mitochondria. They showed that addition of sub-optimal concentrations of DNP (1.75×10^{-5}M) increased all rates of phosphate release, but that hybrid rates were stimulated least. This comparison is invalidated by the observation that, before uncoupling the ATP-ase

activity of the hybrid was higher than that of both parents. This initial rate also exceeded line 28 after uncoupling. The hybrid mitochondria were either more contaminated or more broken than the parents to produce this greater rate. Inhibition of the ATP-ase with oligomycin would have resolved these alternatives. It is not surprising that the percent stimulation on addition of DNP was least in the mitochondria where the initial rate was highest.

The same argument of heterotic homeostasis was applied to observations of the drop in ADP:O ratio on addition of low concentrations of DNP (Sarkissian and Srivastava, 1969). Hybrid mitochondria are said to withstand the uncoupling effects of DNP better because the ADP:O ratio falls by a marginally lower percentage than that of line 31MS and because the mitochondria of the hybrid and mixture show higher absolute values for ADP:O and respiratory control ratios than the parental lines. If one looks at the actual change in ADP:O and respiratory control ratios however, which are as follows;

Source of Mitochondria	28	31MS	Hybrid	Mixture
Change in ADP:O	1.8	1.3	2.6	2.2
Change in RCR	1.12	1.8	2.6	2.8

it is clear that DNP is having a more drastic effect in reducing ADP:O and respiratory control ratios in the hybrid and the mixture than the parents, which contradicts claims of mitochondrial homeostasis.

It is possible to compare the reported rates of ATP synthesis (Table 1) with those of ATP hydrolysis (Table 3) (Sarkissian and

Srivastava, 1969). The state 3 rate of KGA oxidation of 40 µM O_2 $\cdot mgN^{-1}$ $\cdot min^{-1}$ is equivalent in these experiments to 7.2 µmoles O_2 $\cdot mgN^{-1} \cdot hr^{-1}$ or 14.4 µatoms O_2 $\cdot mgN^{-1}$ $\cdot hr^{-1}$. Assuming a P:O ratio of 4, there should be 57.6 µmoles phosphate esterified $\cdot mgN^{-1}$ hr^{-1} or using the stated ADP:O ratio of 5.2 one could expect 75 µmoles Pi $\cdot mgN^{-1}$ $\cdot hr^{-1}$. Yet the ATP-ase of hybrid mitochondria before DNP addition was quoted as 140.6 ± 15.4 µmoles Pi $\cdot$mg protein^{-1} $\cdot hr^{-1}$. Assuming that the distinction between protein and nitrogen is a mistake, Sarkissian and Srivastava are claiming that coupling is evident when the rate of ATP hydrolysis is twice that of ATP synthesis. If the distinction between nitrogen and protein is intended [Sarkissian and Srivastava (1969) state that nitrogen was estimated from mitochondrial protein determined by a modified Lowrey method] then the value for ATP-ase activity must be adjusted. The ratio of protein to nitrogen of 6.2 for plant tissue (Pirie, 1955) is calculated from the mean percentage of nitrogen in the amino acids which is 16 percent. Then 140 µmoles P_i $\cdot$mg protein^{-1} $\cdot hr^{-1}$ becomes 840 µmoles Pi $\cdot mgN^{-1}$ $\cdot hr^{-1}$ which makes comparison with the figures for ADP esterification even more difficult.

Sarkissian and Srivastava (1971) have reported heterosis in NADH-cytochrome *c* reduction despite some faults in their experimental technique. The reaction was observed at 340 nm by following NADH disappearance. This introduces two problems. Firstly the reaction could proceed via two NADH dehydrogenases with separate pathways. Calculations of a single Michaelis constant is therefore an over-

simplification. Secondly, the rate of reaction is governed by the integrity of the outer membrane, due to its impermeability to cytochrome *c* (Ch. III). Faster hybrid rates in the absence of antimycin A, would indicate more broken outer membranes and are in direct contradiction to claims of higher phosphorylation efficiency. Thirdly, cytochrome *c* contributes to the change of absorbance at 340 nm as this wavelength is not at a crossover point in the reduced minus oxidized spectrum of cytochrome *c*. The absorbance change at 340 nm due to cytochrome *c* reduction is in the same direction as that due to NADH oxidation and accounts for 30 percent of the total change observed.

These three considerations invalidate Sarkissian and Srivastava's report (1971) of heterosis and complementation in NADH-cytochrome *c* activity. The positive heterosis found in this study can be explained by the second of these considerations.

The detection of heterosis and complementation in mitochondrial enzymes was extended to cytochrome *c* oxidase activity (Sarkissian and Srivastava, 1971) where the heterotic properties of this enzyme system were alleged to persist after partial purification of the enzymes. Michaelis constants were calculated but since it is penetration of substrate that governs the rate of reaction, these figures do not indicate the true kinetic properties of the enzyme. The difficulties of assaying this enzyme, centering around its substrate requirements, and the problems of altering the active sites when extracting the enzymes from the inner membrane have been discussed (Ch. IIIB).

In the present study positive heterosis for cytochrome oxidase activity was observed only when cytochrome *c* was the reductant. Ascorbate/TMPD, which has no penetration problems did not support this heterotic effect. The positive results can be attributed to more broken outer membranes in the hybrid mitochondria.

McDaniel and Sarkissian, (1970) tried to determine what factors control the expression of complementation in corn mitochondria. They concluded that contact between the two parental types was essential (1) by observation of reduced rates of oxidation on dilution of mitochondria. (2) By measuring activity in mitochondria separated by a dialysis membrane, before and after actual contact. (3) Adding aqueous, etheric and ethanolic extracts of one type of mitochondria to the other mitochondria and observing any changes in rates.

The rate reduction in the dilution experiment could be a result of lower protein concentration as McDaniel (1973) shows that barley mitochondrial oxidation rates drop approximately 50 percent when protein is reduced by half. The double chamber experiment has several drawbacks, firstly it is not controlled KGA oxidation which is being observed. The assay medium contained NAD and cytochrome *c* and no malonate, thus succinate oxidation and external chain NADH oxidation (Day and Wiskich, 1974) could be reducing control and ADP:O ratios. The rates of oxygen uptake (McDaniel and Sarkissian, 1970) best illustrated the complementary effect of mixing unlike parents as the respiratory control ratio dropped on mixing. The ADP:O ratio increased from 1.42

to 1.75 after mixing but as a larger increase (1.25 to 2.0) occurred in one of the controls (Ohio 45 + Ohio 45) this effect cannot be attributed to interactions of different mitochondria.

The lack of stimulation of respiratory rates on addition of ether and alcohol extracted fractions is not surprising in view of later studies (McDaniel, 1973). Structural integrity of the mitochondria was shown to be necessary for the enhancement phenomenon as malate dehydrogenase on isolation did not retain its heterotic or complementary properties.

McDaniel and Sarkissian (1968) located the site of mitochondrial heterosis in corn at the NAD-linked dehydrogenases while that of complementation was specifically at the KGA oxidase enzyme complex. This conclusion was based on the lack of control in some succinate and NADH experiments. The lack of control with these substrates could be due to the isolation and assay techniques and extrapolation from these results is not warranted. Further work on wheat (Sarkissian, 1972; Sarkissian and Srivastava, 1969 , 1970) and barley (McDaniel, 1971, 1973) showed coupled oxidation with these substrates. Succinate is claimed to reduce complementation in barley mitochondria when added as a co-substrate to KGA. Succinic dehydrogenase activity is seen as relieving the constraints in the parent KGA dehydrogenase systems. However the already high KGA rates were only increased slightly by succinate (180 to 210 nmoles O_2 ·mg protein^{-1} ·min^{-1}) and ADP:O ratios decreased slightly. KGA oxidation is always performed in the absence

of malonate (McDaniel, 1971, 1973) and must therefore be a combination of succinate and KGA oxidation so complementation is occurring in the presence of succinate in all other trials where it is observed.

The de novo synthesis of hybrid mitochondria suggested (Sarkissian and McDaniel, 1967) by the separation by density of three different mitochondrial fractions, was an age dependent phenomenon observed only in young germinated scutella. It is possible that the mitochdonrial bands reflect a stage of development rather than a unique mitochondrial type, especially as mitochondria decreased in density with age (Sarkissian and McDaniel, 1967). Hybrids may be physiologically older than inbred shoots after 12 hours gemination and may have developed the older less dense mitochondria which appear as the lighter bands unique to the hybrid.

In this project correction could not be made for seed size effects and as larger seeds produce more vigorous shoots (McDaniel, 1969) heterotic mitochondrial behaviour may be a reflection of initial capital (Ashby, 1930, 1932). McDaniel (1969, 1971) however, showed that the mitochondrial activity when expressed per mg protein is unaffected by the seed size class so this can be discounted as a source of error.

None of the published work shows an electron micrograph of a mitochondrial pellet and purity of the preparations was assumed. This assumption was found to be false (Ch. IIIA) as microscopy and density gradient fractionation showed. In the present study these contaminants were instrumental in reducing the activity of the preparation when expressed on a mg protein basis. They also introduce considerable

variation in the quality of preparations making between preparation comparisons difficult.

The present study of mitochondrial complementation and heterosis found these two phenomena difficult to quantify repeatedly because of the difficulties arising from the rapid isolation technique. Although the method of extraction was standardized to a precise routine, results varied because of contaminating particles and occasionally because of discrepancies in physiological age of the three genotypes being compared in any one trial.

This failure of mitochondrial parameters to predict yield heterosis is in accord with two other reports (Ellis et al., 1973; Zobl et al., 1972) but in partial contradiction with Sage and Hobson (1973) who found significant correlation under limited conditions. These three reports dealing only with complementation and its relation to yield all agree that the interaction of parental mitochondria was found to produce insignificant changes in activity. This supports the present findings reported in Ch. VI.

Similarly with mitochondrial heterosis, McDaniel and Sarkissian are the only workers who report significant increases in hybrid ADP:O ratios as compared to the parents. Studies with the 31MS by 28 hybrid and the seven other crosses failed to find significant heterotic changes in any of the parameters studied (Ch. V). The agreement between mitochondrial and yield estimates of heterosis was better for KGA parameters than malate parameters, but neither showed a close agreement

because of the variability of results. The failure to find agreement between seedling heterosis and yield heterosis is a serious difficulty because this is the link on which McDaniel and Sarkissian built their case. Seedling heterosis was found to agree fairly frequently with tissue respiration and malate oxidation but not often with KGA oxidation or yield. One would expect that mitochondria would be heterotic in utilization of both malate and KGA as had been reported for barley (McDaniel, 1971) and wheat (Sarkissian and Srivastava, 1969) if heterosis was the result of more efficient systems. Disagreement between these two estimates throws further doubts on this method of predicting yield heterosis. McDaniel (1971, 1973) emphasises that the ADP:O ratio is the best mitochondrial predictor of yield in barley and he presents (McDaniel, 1973a) a significant regression to show that the ADP:O ratio is positively correlated with yield in "Pima" cotton. McDaniel indicates that it is only necessary to know the hybrid ADP:O ratio for a prediction of yield to be made. This would make heterosis estimates from simultaneous preparations unnecessary. The ADP:O ratios for KGA oxidation in the present study (Appendix III) did not form a reliable indicator of yield because of the infrequent occurrence of high first cycle values. Using the relative measurement of hybrid vigour gave a more reliable figure to compare with yield.

Conclusion

The recommended procedure for isolation and testing of mitochondria from wheat tissue (Sarkissian and Srivastava, 1968) could not

be used to produce mitochondria of consistent quality for comparative studies between genotypes.

If one accepts the idea that a heterotic plant is one in which the whole plant is more efficient because it is better balanced or homeostatic, one cannot accept that the source of vigour lies in a specific heterosis such as mitochondrial efficiency. To grow faster a whole plant must have superior carbon fixation because ultimately it will need more building blocks with which to grow. Mitochondria could only be instrumental in heterosis if they were limiting the growth of the tissue *in vivo*. The stimulation of wheat tissue respiration by DNP shows that mitochondria are working below their maximum capacity in the tissue and are thus limited by external restraints. Thus more mitochondria or more efficient mitochondria would not make any difference to the rate of respiration if the limiting factor is the supply of ADP.

It is concluded that while mitochondria are an integral part of cellular metabolism they operate in conjunction with the rest of the cell. Extrapolation of whole plant properties from isolated mitochondrial properties appears to have no theoretical background and could lead to erroneous predictions.

Finally, the evidence for mitochondrial complementation and heterosis depends heavily on the evidence of Sarkissian and co-workers. Since serious errors have been found in their data it is suggested that their work be critically reassessed before conclusions as to the validity of these phenomena are made.

Appendix I

Derivation of Pure Line Wheats used for Hybrid Production (Macindoe and Walkdon-Brown, 1968).

Gabo	Released 1945	Bobin selection X Gaza (Durum Wheat) X Bobin selection.
Gamut	Released 1965	Gamenya X (Gabo X Kenya 324 X Urquiza).
Festival	Released 1936	Pusa III X (Kenya C6041 X Baringa).
Falcon	Released 1960	Gluar X (Dunda X Gluar) X Bencubbin.
Eureka	Released 1938	(Kenya X Florence) X Dundee.
Heron	Released 1959	(Ranee X Ranee X Doubbi) X Insignia 49.
Mengavi	Released 1969	(Gabo X Mentana 1124) X ($Gabo^2$ X Eureka X C.I. 12632) (Complex back cross).
Chile IB		Synonomous to Australian Club. Imported by Pugsley of Wagga in 1950's from Mexico.
Nabawa	Released 1915	Gluyas, Early X Bunyip.
Strain 52		A Gabo - Mengavi type (unreleased).
IRN 59111		Exotic Mexican introduced by Prof. Watson, Univ. of Sydney.
Pusa 4		Federation X Pusa. (Moss & Wrigley, 1974).

Appendix II

Media Used in Routine Experiments.

Sucrose Grinding Medium

0.5 M Sucrose

0.67 M K-P_i Buffer pH 7.2

0.075% BSA

Standard Sucrose Medium

0.25 M Sucrose

0.01 M K-P_i Buffer pH 7.2

5 mM Mg Cl_2

0.5 mM EDTA

0.01 M Tris/HCl

Manitol Reaction Medium

0.3 M Manitol

10 mM K-P_i Buffer

10 mM KCl

5 mM $MgCl_2$

10 mM Tris/HCl 0.075% BSA

Resuspending Buffer

0.3 M Manitol

0.01 M K-P_i Buffer pH 7.2

0.01 M Tris/HCl

Appendix III

Respiratory control and ADP:O ratios obtained in heterosis and complementation experiments

Cross & Substrate	Cycle	Parent 1	Parent 2	Hybrid	Mixture	% M.H[1]	% M.C[2]
I							
31MS x 28 (Malate)	RCR	2.06	2.57	1.80	2.08	-30.0	-10.00
		2.12	4.00	-	2.07	-	-32.00
	ADP:O	2.76	2.58	2.48	2.76	-10.1	3.37
		2.06	2.06	-	2.31	-	12.10
(KGA)	RCR	1.66	2.00	2.30	2.89	15.0	58.00
		1.73	1.67	2.00	2.00	15.6	18.00
		1.44	1.66	-	-	-	-
	ADP:O	3.50	3.39	3.10	3.76	-11.4	9.00
		2.48	2.44	3.02	3.10	-21.7	26.00
		2.22	2.32	-	-	-	-
2							
31MS x 28 (Malate)	RCR	2.60	2.17	2.00	2.03	-23.1	-14.70
		1.93	2.10	1.74	1.66	-17.2	-17.80
	ADP:O	3.59	3.48	3.59	3.48	0	1.55
		2.31	3.00	2.88	2.09	- 4.0	-21.00
(KGA)	RCR	1.43	1.92	1.83	1.64	- 4.7	- 1.70
		1.30	1.67	1.50	1.38	-10.2	- 7.07
		-	1.50	1.33	1.22	-	-
	ADP:O	4.75	4.72	4.54	3.54	- 4.42	-25.00
		2.98	3.38	3.10	3.29	- 8.3	3.00
		2.38	2.46	2.22	2.71	- 9.76	12.00
3							
31MS x 28 (Malate)	RCR	1.80	2.57	2.50	2.07	- 2.72	- 5.04
		1.88	2.08	2.09	1.67	0.48	-15.60
	ADP:O	2.56	2.80	1.47	2.95	-	9.66
		1.88	2.30	-	2.21	-36.00	4.60

1 % M.H. = % mitochondrial heterosis

2 % M.C. = % mitochondrial complementation

Note: cycles of phosphorylation in each experiment are presented.

Appendix III (cont'd)

Cross & Substrate	Cycle	Parent 1	Parent 2	Hybrid	Mixture	% M.H.	% M.C.
3							
(KGA)	RCR	1.65	2.25	2.07	2.25	- 8.00	15.40
		1.38	1.57	1.58	1.30	0.64	-11.50
		1.50	1.50	1.45	2.57	- 3.30	71.30
	ADP:O	4.70	3.39	3.19	4.93	-32.10	21.88
		3.13	2.71	2.37	2.93	-24.30	0.34
		1.52	2.01	1.82	2.09	- 9.45	18.00
4							
31MS x 28 (Malate)	RCR	2.66	3.20	2.60	2.40	-18.75	-18.08
		2.00	3.06	2.34	2.69	-23.53	6.30
	ADP:O	2.65	2.74	2.67	2.50	- 2.56	- 7.06
		1.99	2.13	2.13	1.92	0	- 6.80
(KGA)	RCR	2.57	2.13	2.33	2.09	- 9.30	-11.05
		1.90	1.76	2.00	1.75	5.30	- 5.90
		1.83	1.43	1.50	-	- 1.80	-
	ADP:O	3.39	3.39	3.64	3.69	7.37	8.80
		2.65	2.36	2.62	2.46	- 0.80	- 1.60
		1.97	2.01	2.22	-	10.45	-
5							
31MS x 28 (Malate)	RCR	3.27	4.73	3.40	3.43	-28.10	-14.00
		3.40	3.64	3.42	3.38	- 6.05	- 4.00
		2.86	3.64	-	-	-	-
	ADP:O	2.64	2.77	2.37	2.93	-14.40	8.00
		2.45	2.38	2.45	2.28	0	- 6.00
		2.32	2.32	-	-	-	-
6							
31MS x 28 (Malate)	RCR	2.00	2.05	2.00	-	- 2.44	-
		2.19	2.20	2.19	-	0	-
	ADP:O	2.82	2.65	2.57	-	- 8.80	-
		2.50	2.50	2.05	-	-18.00	-

Appendix III (cont'd)

Cross & Substrate	Cycle	Parent 1	Parent 2	Hybrid	Mixture	% M.H.	% M.C.
A							
Nabawa x Chile IB (KGA)	RCR	1.10	1.64	1.79	2.18	9.15	59.00
		1.53	1.48	1.87	1.53	22.20	3.00
	ADP:O	3.60	3.29	3.29	3.68	0	7.00
		2.40	1.79	2.70	2.56	12.50	22.00
(Malate)	RCR	1.70	2.00	2.14	2.28	7.00	23.00
		2.05	1.94	1.71	1.63	-16.60	-18.00
	ADP:O	2.39	2.18	2.78	2.00	16.30	-12.00
		1.79	1.66	2.04	1.71	14.00	- 1.00
		1.47	-	-	-	-	-
B							
Nabawa x Chile IB (Malate)	RCR	2.71	3.10	3.18	2.70	2.58	- 7.85
		2.84	3.44	3.07	3.15	- 10.8	0
	ADP:O	2.82	2.88	2.72	3.00	- 5.55	5.27
		2.20	1.77	2.275	2.36	3.41	19.20
(KGA)	RCR	2.57	2.00	2.00	2.21	-22.20	- 3.95
		2.46	1.70	1.56	2.20	-36.60	- 5.77
		2.10	1.75	1.85	2.25	-11.90	17.00
	ADP:O	6.40	6.40	7.65	5.80	19.60	- 9.40
		3.81	2.98	3.83	3.50	0.53	3.25
		1.94	1.72	3.06	2.18	57.70	19.00
Eureka x IRN (Malate)	RCR	2.06	1.92	2.08	2.34	0.97	17.60
		-	2.03	2.06	1.72	-	-
	ADP:O	2.88	4.00	5.38	6.18	34.50	80.00
		-	2.60	1.52	6.58	-	
(KGA)	RCR	2.63	2.28	2.10	2.26	-20.20	- 7.94
		2.90	2.00	2.33	2.75	-19.65	12.24
		2.00	2.00	1.83	2.00	- 8.50	0
	ADP:O	4.68	4.80	5.19	4.10	8.13	-13.50
		3.20	3.60	2.11	3.12	-41.40	- 8.24
		2.79	2.23	2.31	2.91	-17.20	16.00

Appendix III (cont'd)

Cross & Substrate	Cycle	Parent 1	Parent 2	Hybrid	Mixture	% M.H.	% M.C.
109H x 272H (Malate)	RCR	1.44	2.47	2.25	2.07	- 8.90	6.00
		-	2.00	1.91	1.68	- 9.50	-16.00
		-	2.00	-	-	-	-
	ADP:O	2.02	2.68	2.36	1.80	-11.95	- 2.30
		-	2.38	2.36	1.93	- 0.84	-19.00
		-	2.02	-	-	-	-
Gabo x Pusa 4' (Malate)	RCR	3.84	2.61	-	2.33	-	-27.70
		2.33	2.42	-	2.36	-	- 0.63
		2.27	-	-	-	-	-
	ADP:O	3.46	2.57	-	2.45	-	-18.70
		3.07	2.44	-	2.17	-	-21.20
		2.65	-	-	-	-	-
(KGA)	RCR	2.60	2.16	2.00	2.14	-23.10	-10.00
		1.78	2.32	2.20	1.67	- 5.18	-18.50
		-	2.45	1.90	1.09	-22.50	-
	ADP:O	4.76	4.04	4.50	4.47	- 5.46	1.60
		4.57	2.93	5.29	2.52	15.75	-32.80
		-	2.93	2.99	2.78	2.04	-
Heron x Gamut (Malate)	RCR	3.50	3.30	2.22	2.94	-36.60	-13.50
		3.28	2.70	3.00	2.76	- 8.54	7.70
	ADP:O	2.38	2.46	2.77	2.67	12.60	11.72
		2.29	2.40	2.52	-	5.00	-
(KGA)	RCR	2.16	1.85	1.80	1.58	-16.50	-21.00
		2.16	1.45	1.67	1.56	-22.70	-13.57
		1.60	-	-	-	-	-
	ADP:O	5.16	6.68	5.23	4.22	-21.70	-28.70
		3.04	3.39	2.65	2.29	-21.80	-28.70
		4.60	-	-	-	-	-

Appendix III (cont'd)

Cross & Substrate	Cycle	Parent 1	Parent 2	Hybrid	Mixture	% M.H.	% M.C.
WW-15 x 4894 (Malate)	RCR	3.80	3.82	5.14	4.58	34.60	20.20
	ADP:O	2.40	2.67	2.40	2.75	10.10	8.70
1							
Heron x Strain 52 (KGA)	RCR	1.38	1.54	1.64	-	9.30	-
		-	1.40	1.70	-	-	-
	ADP:O	3.39	11.20	4.04	-	-63.90	-
		-	2.88	3.70	-	-	-
2							
Heron x Strain 52 (Malate)	RCR	2.29	2.45	1.68	-	-26.30	-
		2.11	2.55	1.84	-	-27.80	-
		-	2.50	-	-	-	-
	ADP:O	1.90	2.30	2.08	-	- 9.56	-
		1.47	1.61	1.54	-	- 4.35	-
		-	1.32	-	-	-	-
(KGA)	RCR	1.75	2.18	1.67	-	-23.40	-
		1.61	2.33	1.53	-	-31.40	-
		1.25	1.44	1.62	-	12.50	-
	ADP:O	3.15	3.47	2.95	-	-15.00	-
		1.40	2.03	1.69	-	-16.70	-
		1.59	1.96	1.64	-	-16.30	-

Appendix IV

State 3 and State 4 rates obtained in heterosis and complementation experiments

Cross & Substrate	Cycle	Parent 1	Parent 2	Hybrid	Mixture	% M.H[1]	% M.C[2]
I 31MS x 28							
Malate	S_3	94.50	120	113	74.50	- 5.80	-30.54
		108	160	-	86.50	-	-35.50
	S_4	45.80	46.60	62.40	37.20	33.90	-19.50
		48.80	53.30	-	41.70	-	-18.32
KGA	S_3	45.40	71.50	76.70	42.60	7.27	-27.12
		57.40	65.00	83.60	42.60	28.62	-30.40
		54.40	65.00	68.40	41.00	5.23	-31.30
	S_4	27.20	35.70	34.20	14.80	- 4.20	-52.90
		33.20	89.00	41.80	21.30	-58.00	-41.00
		37.80	39.00	-	26.10	-	-32.00
2 31MS x 28							
Malate	S_3	57.00	94.75	86.70	98.50	- 8.50	2.98
		81.60	122.50	98.80	139	-19.40	36.20
		81.60	-	-	-	-	-
	S_4	21.90	43.70	43.40	48.40	- 0.69	47.60
		42.40	58.40	56.80	84.00	- 2.74	66.70
KGA	S_3	30.60	41.00	38.10	53.20	- 7.07	48.60
		39.20	49.30	41.30	57.60	-16.20	30.17
		44.30	59.00	55.40	58.40	- 6.10	14.73
	S_4	18.70	21.40	20.80	32.60	- 2.80	6.26
		27.20	29.60	27.70	41.10	- 6.40	44.72
		34.00	39.40	41.50	48.00	- 5.33	30.80

1 % M.H. = % mitochondrial heterosis

2 % M.C. = % mitochondrial complementation

Note: cycles of phosphorylation in each experiment are presented.

Appendix IV (cont'd)

Cross & Substrate	Cycle	Parent 1	Parent 2	Hybrid	Mixture	% M.H.	% M.C.
3 31MS x 28							
Malate	S_3	56.80	79.38	77.85	74.82	- 1.93	9.88
		67.28	119.07	152.24	90.30	27.90	- 3.09
		71.76	167.58	-	-	-	-
	S_4	31.40	30.84	31.14	36.12	- 0.83	16.00
		35.88	57.33	72.66	54.18	26.70	16.25
KGA	S_3	17.70	44.28	60.17	26.00	35.90	-16.10
		28.98	54.12	62.25	34.68	15.00	-16.53
		43.47	51.66	66.40	52.00	28.50	9.32
	S_4	9.66	19.68	29.05	11.56	47.60	21.00
		20.93	34.44	39.43	23.12	14.50	-16.50
		28.98	34.44	45.65	20.23	32.55	-36.20
4 31MS x 28							
Malate	S_3	87.50	100.80	77.90	95.10	-22.70	1.01
		106.90	131.00	93.90	69.30	-28.30	-41.70
		-	136.00	-	-	-	-
	S_4	32.80	31.50	29.80	39.60	- 9.15	-23.17
		53.50	42.80	40.10	25.70	-25.10	-46.60
KGA	S_3	48.10	42.70	37.40	50.14	-22.30	10.40
		50.73	49.40	45.40	61.00	-10.50	21.80
		58.74	53.40	48.10	69.80	-18.10	24.50
	S_4	18.70	20.00	16.02	24.00	-19.90	24.00
		26.70	28.00	22.70	34.90	-18.20	27.60
		32.10	37.40	32.00	-	-14.40	-
5 31MS x 28							
Malate	S_3	89.50	110.50	108.00	100.00	- 2.20	0
		84.50	108.50	130.50	113.00	20.30	18.95
		78.30	108.50	-	-	-	-
	S_4	27.30	23.41	31.80	29.20	16.50	15.19
		24.80	29.00	38.20	33.40	31.70	24.20
		27.30	29.00	-	-	-	-

Appendix IV (cont'd)

Cross & Substrate	Cycle	Parent 1	Parent 2	Hybrid	Mixture	% M.H.	% M.C.
6 31MS x 28							
Malate	S_3	73.20	83.50	100.00	-	19.80	-
		78.70	108.50	126.50	-	16.60	-
	S_4	29.30	43.00	50.00	-	16.00	-
		40.30	49.70	57.80	-	16.00	-
A Nabawa x Chile IB							
KGA	S_3	75.80	58.50	66.90	57.00	-11.70	-15.10
		83.00	71.20	75.00	61.80	- 9.64	-23.40
		83.00	89.00	77.50	76.00	-12.90	-11.60
		93.80	96.60	75.00	80.70	-22.40	-15.20
	S_4	36.10	35.60	37.40	26.10	3.60	-27.20
		54.20	48.30	40.10	40.40	-26.00	-21.20
		61.40	63.50	48.20	47.50	-24.10	-23.90
		43.30	71.20	56.20	52.40	-21.10	- 8.50
Malate	S_3	178.00	161.00	116.00	100.00	-34.80	-41.00
		208.00	152.00	139.00	124.00	-33.20	-31.10
		114.00	154.00	182.50	110.00	-12.30	-17.90
	S_4	91.60	80.30	52.80	44.00	-42.40	-48.80
		101.60	78.00	81.60	76.00	-19.70	-15.40
		96.60	-	-	52.00	-	-
B Nabawa x Chile IB							
Malate	S_3	79.00	67.10	89.10	51.40	12.80	-29.60
		88.50	74.50	100.00	96.40	13.00	18.28
	S_4	29.10	21.60	27.50	19.70	- 5.50	-22.30
		31.20	20.60	32.50	30.40	4.17	17.40
KGA	S_3	17.00	16.00	12.38	31.20	-27.20	89.10
		34.00	22.30	21.60	30.40	-36.50	79.90
		44.54	28.50	38.60	41.50	-13.30	13.60
	S_4	7.40	8.12	6.17	30.70	-24.00	29.60
		13.80	13.20	13.90	13.80	0.72	2.22
		9.60	16.30	20.10	18.50	23.30	4.29

Appendix IV (cont'd)

Cross & Substrate	Cycle	Parent 1	Parent 2	Hybrid	Mixture	% M.H.	% M.C.
Eureka x IRN							
Malate	S_3	51.40	83.00	142.00	94.00	71.10	39.88
		80.50	-	173.00	88.00	-	-
	S_4	28.00	40.40	68.00	40.00	68.30	17.00
		36.00	-	84.00	49.40	-	-
KGA	S_3	29.60	13.00	24.60	30.20	-16.90	2.00
		37.00	15.60	32.80	38.20	-11.50	3.24
		44.40	22.20	36.20	43.80	-18.50	- 1.35
	S_4	13.00	9.30	11.60	13.80	-10.80	6.16
		15.60	10.40	14.00	13.80	- 9.00	-10.30
		22.20	15.00	20.00	22.60	- 9.90	1.80
109H x 272H							
Malate	S_3	50.50	62.20	72.00	56.70	15.70	0.62
		43.40	60.50	70.40	58.60	16.40	12.80
		-	60.50	71.00	-	-	-
	S_4	35.20	25.20	32.00	27.50	- 9.40	- 8.94
		-	30.20	36.80	34.80	21.80	-15.20
		-	30.20	-	-	-	-
Gabo x Pusa 4							
Malate	S_3	36.50	52.50	-	41.00	-	- 8.00
		33.50	61.50	-	45.00	-	- 5.00
		34.00	-	-	-	-	-
	S_4	9.00	19.30	-	17.50	-	24.00
		14.00	25.00	-	19.50	-	0
		15.00	-	-	-	-	-
KGA	S_3	10.50	18.00	14.00	18.00	-22.20	26.00
		13.00	21.00	14.00	24.00	-33.30	41.20
		-	19.00	15.00	23.00	-	-
	S_4	4.00	8.50	7.00	8.40	-17.70	34.00
		7.00	9.00	6.40	14.50	-28.90	81.30
		-	7.50	7.70	21.00	-	-

Appendix IV (cont'd)

Cross & Substrate	Cycle	Parent 1	Parent 2	Hybrid	Mixture	% M.H.	% M.C.
Heron x Gamut							
	S_3	270.00	194.00	160.00	185.00	-40.70	-20.30
		254.00	205.00	175.00	190.00	-31.10	17.70
Malate	S_4	78.00	59.00	72.00	63.00	- 7.60	- 8.02
		78.00	77.00	59.00	68.50	-24.40	-11.60
	S_3	59.00	43.50	53.50	58.50	- 9.32	14.20
		59.00	43.50	62.00	70.00	5.09	36.60
		59.00	-	-	-	-	-
KGA	S_4	28.00	23.50	29.50	37.00	5.36	43.70
		28.00	30.00	37.00	45.00	23.30	55.20
		36.00	-	-	-	-	-
1 Heron x Strain 52							
	S_3	21.00	23.00	27.00	-	17.40	-
		-	33.00	32.70	-	-	-
		-	34.00	-	-	-	
KGA	S_4	15.50	15.50	16.20	-	4.52	-
		-	23.00	19.00	-	-	-
2 Heron x Strain 52							
	S_3	127.00	93.50	110.00	-	-13.40	-
		147.00	157.50	149.00	-	- 5.40	-
		-	192.00	-	-	-	-
Malate	S_4	55.70	46.80	65.30	-	17.20	-
		44.00	61.60	81.00	-	31.50	-
		-	76.50	-	-	-	-
	S_3	50.20	61.30	62.80	-	2.45	-
		69.30	125.00	77.00	-	-38.40	-
		76.50	84.00	94.00	-	11.90	-
		81.20	120.00	-	-	-	-
KGA	S_4	28.70	28.00	37.65	-	31.18	-
		43.00	56.00	50.20	-	10.40	-
		57.40	58.60	58.00	-	-	-
		47.80	69.00	-	-	-	-

Appendix V

GERMINATION TRIAL DATA FROM WHICH
% HETEROSIS WAS CALCULATED

3 day Germination Trial

Variety	Dry wt. of seed (mgm)	Fr. wt. shoots (mgm)	Length of shoots (mm)
Eureka	351	186	7.00
IRN	611	163	10.00
Eureka x IRN	637	196	12.40
Nabawa	506	127	10.60
Chile IB	558	143	12.10
Nabawa x Chile IB	787	206	12.90
31MS	635	143	11.20
28	321	149	13.10
31MS x 28	475	156	13.10
Heron	580	215	14.10
Strain 52	458	100	8.50
Heron x Strain 52	536	90	7.30
Gamut	483	48	4.40
Heron x Gamut	676	163	8.70

Appendix V (cont'd)

3 day Germination Trial

Variety	Dry wt. of seed (mgm)	Fr. wt. shoots (mgm)	Length of shoots (mm)
Gabo	398	102	10.30
Pusa 4	492	11	8.50
Gabo x Pusa 4	602	183	13.60
109H	640	100	8.00
261	250	112	14.30
109H x 261	543	160	12.30
272H	417	130	10.60
109H x 272	560	202	14.90

4 day Germination Trial

Eureka	423	289	19.40
IRN	572	478	30.40
Eureka x IRN	670	731	37.90
Nabawa	559	653	40.50
Chile IB	600	290	23.90
Nabawa x Chile IB	750	519	29.10

Appendix V (cont'd)

4 day Germination Trial

Variety	Dry wt. of seed (mgm)	Fr. wt. shoots (mgm)	Length of shoots (mm)
Heron	452	640	43.10
Strain 52	425	278	19.70
Heron x Strain 52	495	432	27.50
Gamut	367	156	16.10
Heron x Gamut	720	440	26.40
109H	538	537	35.10
261	351	467	38.90
109H x 261	452	405	28.90
272	459	357	29.80
109H x 272	567	391	27.60

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