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PEA MITOCHONDRIAL ADENOSINE TRIPHOSPHATASE ACTIVITY AND ETHYLENE

BY

© DONALD ROBERT PHILLIPS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "Pea Mitochondrial Adenosine Triphosphatase Activity and Ethylene" submitted by Donald Robert Phillips in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

Recent research efforts have been undertaken to establish the relationship between respiration and ethylene evolution from plant tissue. The purpose of the present dissertation was to examine this relationship by way of a study of the involvement of ethylene with the membrane bound mitochondrial ATPase activity.

The respiratory and ATPase activities of mitochondria isolated from the cotyledons of etiolated pea seedlings were found to initially increase with the age of the tissue and to reach maximal values in preparations from five-day old tissue. However, the ATPase activity was observed to increase several fold when the respiratory parameters of the mitochondria from older tissue indicated the existence of uncoupled mitochondria. Mitochondria isolated from five-day old tissue displayed respiratory parameters that were indicative of particles with a high degree of integrity.

The use of sucrose as osmoticant was observed to cause an increase in State IV respiration when compared to the use of mannitol. Additions of the monovalent cations, sodium and potassium, were also found to stimulate State IV respiration.

Since mannitol was observed to interfere with the detection of phosphate, the ATPase activity studies were conducted in the presence of sucrose. The ATPase activity of pea mitochondria displayed a requirement for a magnesium to ATP ratio of unity. Under such conditions, the activity was found to have a Km of about 1.0 mM. In addition, the activity showed a high degree of substrate specificity, was inhibited by ADP, displayed a lack of pH optimum, and an

inhibition by azide and oligomycin.

The presence of sodium or potassium ions was observed to differentially and hyperbolically stimulate the ATPase activity. In the presence of a low fixed concentration of potassium, the activity was sigmoidally stimulated by the addition of sodium, This stimulation was found to be maximal in the presence of a sodium to potassium ratio of about five to one. The sodium plus potassium stimulated ATPase activity was found to be moderately sensitive to ouabain and to be insensitive to oligomycin. The addition of valinomycin induced potassium stimulated activity to a value that was comparable to the sodium plus potassium activity value. The novel similarity between the ATPase activity described herewithin and that of the red cell membrane system has been described.

The addition of ethylene to mitochondrial preparations was shown to be ineffectual in inducing any change during oxygen polarographic analysis. However, it was observed that ethylene would stimulate magnesium dependent ATPase activity. In the presence of monovalent cations, ethylene was shown to have no effect on potassium stimulated activity but to enhance sodium stimulated activity and to alter the sigmoidicity of sodium plus potassium induced activity. In addition, the effect of ethylene was inhibited by oligomycin and was found to affect the inhibition by dinitrophenol of the ATPase activity in the presence of sucrose.

It was established that intact mitochondria display a sodium plus potassium stimulated ATPase activity, that respiration is affected by the presence of these monovalent cations and that ethylene

can allosterically alter mitochondrial ATPase activity. It may be possible that ethylene and respiration are related through a mitochondrial ATPase.

ACKNOWLEDGEMENTS

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To my wife, Elaine, I wish to express my heartfelt gratitude and thanks for her enthusiasm, understanding and patience during this program as well as for the typing of the preliminary drafts and final copy of this manuscript.

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LIST OF ABBREVIATIONS

ADP	adenosine-5'-diphosphate
ATP	adenosine-5'-triphosphate
ATPase	adenosine triphosphatase
ADP:O	ratio of μ moles ADP esterified to μ atoms O_2 consumed
DNP	2,4-dinitrophenol
GC	gas chromatograph
GTP	guanosine-5'-triphosphate
K_i	inhibition constant
Km	Michaelis constant
αkg	α-ketoglutarate
LDH	lactate dehydrogenase
nm	nanometers
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
PEP	phosphoenolpyruvate
PK	pyruvate kinase
P_i	inorganic phosphate
ppm	parts per millions
RCR	respiratory control ratio
SLS	sodium lauryl sulphate
TES	N-tris (hydroxymethyl)-methyl-2-aminoethanesulphonic acid
TPP	thiamine pyrophosphate
Tris	N-tris (hydroxymethyl)-aminomethane
Mg^{++} ions	This term is used with the realization that it may not all be in the form of free ions.

INTRODUCTION

Recent studies from this and other laboratories have shown that ethylene is produced by enzyme processes from sub-cellular fractions that contain mitochondria. Moreover, ethylene and respiration are intimately related. For example, in a variety of tissues ethylene and carbon dioxide more or less simultaneously reach maximal peaks of evolution. In the light of these observations it is not unreasonable that a site of action for ethylene may be on mitochondrial membranes.

Further studies from this laboratory have revealed an effect of ethylene on the rate of mitochondrial volume changes induced by ATP or ADP, as well as stimulation of the ATPase activity of these preparations from bean cotyledons. By computer simulation of the effect of ethylene on ATPase activity it was shown that the increased rate of release of phosphate acceptor was sufficient to cause a major change in the respiration of the mitochondria. The effect of ethylene on an isolated ATPase preparation was found to be negligible. It was suggested that, for ethylene to have an effect on it, the enzyme had to be in a particular environment, such as that found in vivo.

Thus, with the realization of the high solubility of ethylene in lipoidal material and the possibility of attainment of high concentrations of the gas in membranes, the present study was undertaken to characterize the membrane bound mitochondrial ATPase activity and further investigate the effects of ethylene on this activity.

Since volume changes of mitochondria may be intimately related to ion transport, this study undertook to look at these possibilities. Therefore, because of the foregoing, it was absolutely imperative that

the mitochondria used in the present study show a high degree of integrity.

CHAPTER I

REVIEW OF LITERATURE

A. Isolation of Mitochondria from Plant Tissue

Since the present investigation was undertaken to examine the adenosine triphosphatase activity (a terminology that does not imply the existence of a specific enzyme) of intact plant mitochondria, it was of paramount importance that techniques be adopted that would yield mitochondria with a high degree of integrity. One index of mitochondrial integrity and the index employed in the present study was the respiratory control ratio (RCR) as defined by Chance and Williams (1956). This ratio describes the degree of coupling between oxidative and phosphorylative activities (a high value representing a high degree of intactness) of the respiratory chain of mitochondria.

Since Millerd *et al*. (1951) first described the phenomenon of oxidative phosphorylation in plant mitochondria, many hundreds of reports on it have appeared in the literature. Concomitant with these reports have come improvements in procedures used for the isolation of these sub-cellular particles. Modifications of cell disruption procedures and in compositions of solutions employed, have been made. However, each laboratory that works with mitochondria has seemingly developed its own art of isolation by trial and error.

1. Cell Disruption Procedures

Tissue maceration techniques vary with plant species and specific organs, unlike techniques used with animal tissue. The chemical

compositions of plant cytoplasmic and vacuolar inclusions that are detrimental to mitochondrial integrity, as well as the rigid cell wall of plant tissue, have caused these various modifications to be made.

Disruption of plant tissue has, most commonly, been accomplished with the use of a mortar, or blendor. However, wide fluctuations in yields and activity of the mitochondria thus obtained have been reported. Such procedures have been used to advantage when rapidity of isolation of mitochondria has been essential (bean cotyledons) or when the homogenate contained high quantities of organic acids, tannins or polyphenols (tomatoes, apples).

Procedures involving less harsh conditions for the rupturing of cells have also been employed. Physically rupturing the cell by hydraulic pressure has been found to be quite successful (Olson, 1967). Isolations utilizing enzymic digestion of cell wall material have been reported (Sato, 1968), as well as techniques that recommend pulverization of tissue in the frozen state (Haard and Haltin, 1968). In addition, the presence of ion exchange resins during grinding of tissues that possess high concentrations of phenolic materials has been found to be beneficial (Lam and Shaw, 1970). Throughout any of the above procedures, maintenance of a low temperature (0-5^{o}) has been imperative.

2. Chemical Composition of Media

Various components of the media used for isolation of intact mitochondria have been suggested. Certain components have come to be recognized as being essential for successful isolations. A buffer (if

present at all), osmoticant and specific compounds such as cysteine (to protect sulfhydryl groups), bovine serum albumin (to complex inhibitory free fatty acids) and EDTA (to complex metal cations) are routinely employed.

The presence of buffer in the medium has been found to be unnecessary if the solution is continuously monitored and adjusted for pH change during isolation (Bonner, 1965). However, as this is difficult to achieve in practice, most investigators utilize a buffer of one type or another for pH maintenance. Phosphate buffer is routinely used in some laboratories; however, this ion induces swelling of mitochondria (Lipsett and Corwin, 1959) so that its inclusion only presents another variable. A comprehensive evaluation of various buffers used for mitochondrial isolation and suspension media has been reported (Stinson and Spencer, 1967). These workers indicated that Tris (tris-(hydroxy-methyl)-amino-methane) was detrimental to mitochondrial respiratory parameters, and found the Good buffers, notably TES (N-Tris (hydroxymethyl)-methyl-2-amino-ethanesulphonic acid), to be useful.

The most routinely used osmoticants in mitochondrial investigations are mannitol, sucrose or a combination of both. The osmolarity of isolation media has ranged from 0.2 to 0.7. It has been observed that sucrose has somewhat of a detrimental effect on mitochondrial integrity compared to mannitol; however, the reasoning behind such observations is not at all clear (Tedeschi, 1965). Perhaps the mitochondria exhibit different spaces available for diffusion of mannitol and sucrose so that observed differences at similar

molarities cannot be equated. The effective osmolarity of each may be sufficiently different so as to give different conformational states. Alternatively, the ability of mannitol to complex phosphomolybdate is well known (Vreman and Jobsis, 1966) and this ion complexing ability may remove or inactivate deleterious endogenous compounds from tissue homogenates. Mannitol has been recommended for use with tissue that has a high starch content as this facilitates centrifugal separation (Wiskich and Bonner, 1963). In investigations where the use of mannitol or sucrose is prohibited, potassium chloride (KCl) is the osmoticant employed. However, KCl has been reported to be the cause of some clumping of animal mitochondria (Kennedy and Lehninger, 1949).

B. Oxidative and Phosphorylative Activities of Plant Mitochondria

Mitochondria isolated from plant tissue have been shown to have the ability to metabolize pyruvate, isocitrate, α-ketoglutarate, succinate and malate. The requirement for malate addition prior to α-ketoglutarate or isocitrate oxidation has not been shown for plant mitochondria as it has for animal (Chappell and Haarhoff, 1967). Wiskich and Bonner (1963) have suggested that the lower rates of succinate and malate oxidation by plant mitochondria than by animal mitochondria could be a result of inhibitory levels of oxalacetate. Otherwise, plant mitochondria oxidize Krebs cycle acids at very high rates compared to animal mitochondria.

The report that plant mitochondria oxidize exogenous NADH (Luberman and Baker, 1965), which contrasts with observations on animal mitochondria, has been subjected to criticism (Packer *et al.*

1969). However, the consistency of these observations must not be overlooked.

The majority of studies on plant mitochondria (substrate oxidation and oxidative phosphorylation) have been initiated with physiological questions in mind. However, it must be recognized that different physiological states or ages may themselves induce the cell to either produce compounds that are beneficial or detrimental to the actual isolation of active mitochondria, or have different physical properties. Hanson et al. (1965) have noted that the uncoupling of mitochondria isolated from aged corn scutellum was a result of inadequate isolation procedures; once deleterious proteins and fatty acids were removed, this uncoupling activity was not observed.

Oxidative phosphorylation in animal and plant mitochondria shows similar traits of P:O ratios of 3 with NAD^+ linked substrates, 3 coupling sites at similar positions along the electron transfer chain (ETC) and similar sites of response to ETC and oxidative phosphorylation inhibitors.

C. Energy-Linked Functions of Plant Mitochondria

The energy-linked functions of plant mitochondria, namely, ion transport, reversed electron transport, and transhydrogenation are in a state of necessary evaluation. Swelling and contraction of plant mitochondria have been somewhat elucidated. It has been in this area of investigation that the major differences between plant and animal mitochondria have been revealed.

When plant mitochondria are added to buffered KCl there occurs

a spontaneous swelling that is about five times faster than in rat liver mitochondria (Lyons and Pratt, 1964). Swelling induced by DNP, or phosphate is observed to be an effect on the rate rather than the extent of swelling (Stoner and Hanson, 1966). However, calcium, which induces spontaneous swelling of animal mitochondria, has been observed to inhibit swelling of corn (Truelove and Hanson, 1966) and cauliflower (Lyons and Pratt, 1964) mitochondria.

Contraction of plant mitochondria can be initiated by addition of ATP and Mg^{++} or oxidizable substrate (and enhanced by the presence of BSA)(Lehninger, 1964). ATP induced contraction is accompanied by extrusion of phosphate (Stoner and Hanson, 1966) and is related to the ATPase activity of these mitochondria. Sucrose, shown to inhibit swelling, also suppresses the ATPase activity. The substrate induced contraction is inhibited by a low phosphate concentration (Truelove and Hanson, 1966), and this inhibition is relieved by addition of calcium. Calcium and phosphate addition to respiring mitochondria results in Ca + P_i transport and increased oxygen utilization (Kenefick and Hanson, 1967).

Differences between animal and plant mitochondria may not be of major importance, and more similar behaviors may be found with improvements in experimental techniques. Recent reports (Wilson et al. 1967) have indicated active swelling (as occurs in animal mitochondria (Blair and Stollar, 1967)), of corn mitochondria on addition of substrate to mitochondria partially swollen in potassium acetate. The importance of the anion, and anion carriers, has been emphasized by these workers.

D. Oxidative Phosphorylation

During the late 1930's, experimentation had resulted in the observation of oxygen dependent ATP formation, wherein the molecules of ATP formed, stoichiometrically exceeded the number of atoms of oxygen consumed. Oxidative phosphorylation is of paramount biological importance and has received an unimaginable amount of study.

1. Oxidative Phosphorylation Hypothesis

The possibility of ATP formation being a result of a concerted reaction between ADP and P_i was considered by Boyer (1968). Such a mechanism would involve the rearrangement of a ternary complex and would dictate that the first covalent compound formed is ATP itself. Recent observations from Boyer's laboratory (Jones and Boyer, 1969) on the absolute requirement of ADP for the $P^{18}O_4 \rightleftharpoons H_2{}^{18}O$ exchange reaction is consistent with this theory. From experimental evidence, it can be stated that a non-phosphorylated, high-energy intermediate does exist. It has been observed (Slater, 1966; Ernster and Lee, 1964; Pullman and Shantz, 1967; and Lehninger, 1967) that substrate oxidation provides energy for ion transport and the reduction of NAD^+ by succinate in the presence of oligomycin, which inhibits ATP formation, and in the absence of P_i. Thus, formulations of the mechanisms of oxidative phosphorylation include a non-phosphorylated high energy intermediate or state.

There have been two major proposals to account for the energy coupling and transfer reactions of oxidative phosphorylation. The first and most widely known mechanism is the chemical intermediate

hypothesis of Slater (1953), which is analogous to the mechanism of action of glyceraldehyde-3-P-dehydrogenase and has been subjected to many reviews and modifications (Slater, 1965; Pullman and Shantz, 1967; Boyer, 1968; Racker and Ferguson, 1969; and Storey, 1970). The second hypothesis is the chemiosmotic hypothesis (Mitchell, 1961, 1963; Mitchell and Moyle, 1965). It is to be realized that both hypotheses account for the formation and utilization of a non-phosphorylated high energy intermediate (X~Y). However, the chemical hypothesis involves a phosphorylated intermediate and the Mitchell chemiosmotic hypothesis does not, (postulating a concerted reaction).

The 'vectorial metabolism' of the chemiosmotic hypothesis substitutes a pH gradient and membrane potential for a chemical intermediate with the respiratory carrier (A_{ox}~X). The production and utilization of X~Y are quite different in the two theories and rely upon differences in the function of the mitochondrial membrane. The membrane of the chemical hypothesis serves as a site for organization of the respiratory electron transport chain. The chemiosmotic theory postulates a membrane that is impermeable to ions and translocates protons across this barrier. A cyclic proton current is established by the operation of a reversible ATPase. The oxidation of substrate expels protons to the outside whereby this proton motive force now drives the reversible ATPase reaction. The proton translocating respiratory chain and the proton translocating reversible ATPase are separately 'plugged through' (Mitchell, 1967) the non-conducting osmotic barrier.

Because of the ion impermeability of the membrane, the

necessity of a pH differential, osmotic stability and substrate accessibility, it was necessary to postulate a very rapid exchange-diffusion system for certain ions (H^+ / cation antiport and H^+ / anion symport).

The necessity of a vesicular structure for the operation of Mitchell's hypothesis may lead to its abandonment if oxidative phosphorylation is ever demonstrated in a vesicle-free system. However, Williams (1970), in order to account for volumes of aqueous phases into which the H^+ is expelled, the lack of effect of buffers on P/O ratios (buffers do not mop up generated protons), and to circumvent the necessity of a vesicular system, has suggested modifications of the chemiosmotic hypothesis. According to him, the production of protons and condensation of ADP and P_i occurs in a non-aqueous region of the lipoidal phase of the membrane thereby facilitating the stabilization of ATP.

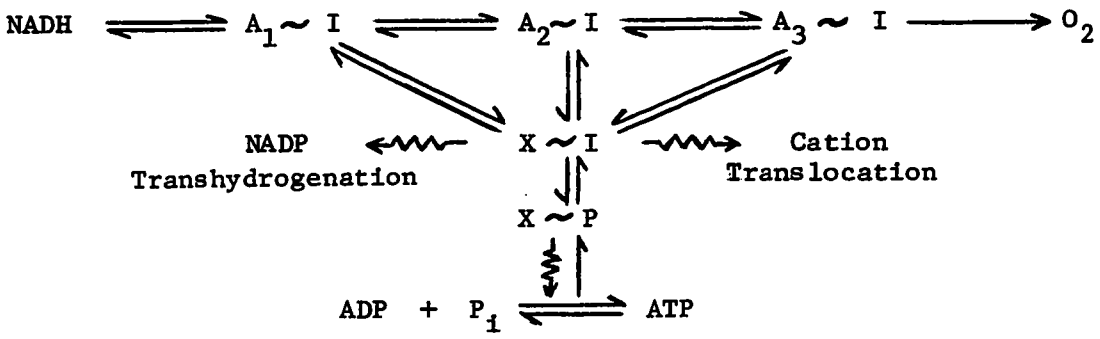
The scheme, displayed in Figure 1, shows the essential features of the chemical intermediate and chemiosmotic hypothesis. In drawing a comparison to the work of Racker (1967, 1968) on the coupling factors F_1 and F_o of oxidative phosphorylation, Mitchell has likened Sy ($X\sim I$) synthetase) to F_1 (the ATPase factor) and Hy ($X\sim I$) hydrolase to F_o (confers oligomycin sensitivity on F_1).

Evidence in support of the Mitchell hypothesis has been mounting recently with results from the light-driven H^+ uptake and pH gradient-driven ATP synthesis experiments with chloroplasts (Jagendorf and Hind, 1963; Jagendorf and Uribe, 1967; Jagendorf, 1967), from respiration-driven proton translocation by oxygen pulse experiments (Mitchell and Moyle, 1967), from experiments demonstrating proton

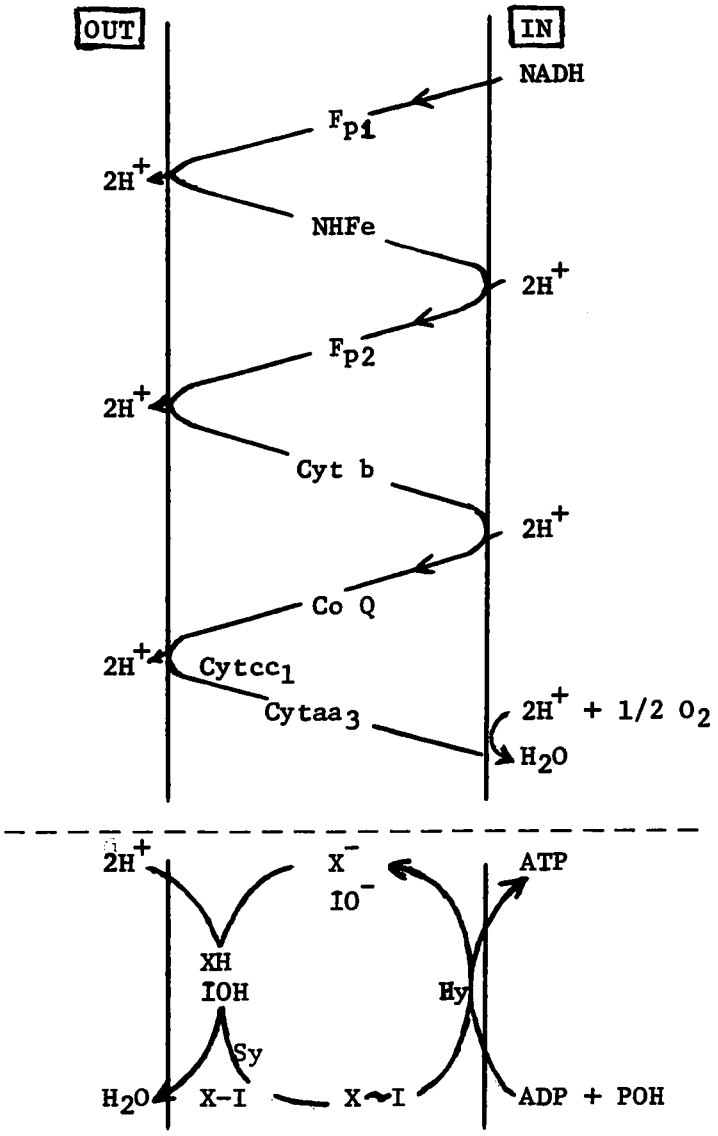
Figure 1. Diagrammatic representation of the chemical intermediate and chemiosmotic hypothesis of oxidative phosphorylation after Greville (1968).

A. Chemical intermediate hypothesis. Energy linked functions of the high energy intermediate (X~I) derived from the three coupling sites (A~I) (~~~>).

B. Chemiosmotic hypothesis. The build up of hydrogen ions on the outside of the mitochondrial membrane by transfer of reducing equivalents along the electron transport chain (upper portion) drives the production of a high energy intermediate by Sy (X~I synthetase) in the reversible ATPase (lower portion). ATP formation is coupled with the hydrolysis of X~I by H_y (X~I hydrolase).



A. Chemical intermediate hypothesis



B. Chemiosmotic hypothesis

translocation associated with ATP hydrolysis (ATP pulse experiments) (Mitchell and Moyle, 1968), from results of the proton impermeability of the inner mitochondrial membrane (Mitchell and Moyle, 1967) as measured by HCl-pulse experiments, and from data derived from experiments with uncouplers of oxidative phosphorylation and ionophorous antibiotics (which will be discussed later). Despite these results, as well as many more which display characterization of the chemiosmotic hypothesis, there are a number of workers like Chance, Slater, Ernster, Pressman and Avron who are inclined to be critical of this hypothesis whereas other prominent workers including Klingenberg, Lehninger, Packer and Racker adopt a neutral attitude. Because of these conflicting hypotheses, much research effort has been stimulated during the last decade. The recent report of Storey (1970) on the involvement of lipoprotein enzyme complexes at each coupling site has given some impetus to the chemical hypothesis. This scheme has also involved the use of conservation of free energy by conformational change as proposed by Boyer (1965).

2. Action of Uncouplers of Oxidation and Phosphorylation

The various theories of oxidative phosphorylation have had to be explained or re-examined in the light of the action of uncouplers and ionophorous antibiotics. These dynamic investigations have led to very fruitful but sometimes confusing results if one is not involved directly in this dispute. It seems that as one group of workers is about to invalidate a hypothesis on the basis of work with these compounds, the opposing forces invariably 'slip from this grasp' on

the basis of slightly differing experimental techniques.

The two theories of oxidative phosphorylation discussed above (Section D.1.) offer different explanations for the phenomenon of uncoupling. The chemical intermediate hypothesis postulates that the uncouplers induce the hydrolysis of a non-phosphorylated high energy intermediate generated during electron-transport (Lehninger, 1955; Slater, 1958). The proponents of the chemiosmotic coupling hypothesis, on the other hand, propose that uncoupling agents increase the permeability of the mitochondrial membrane to H^+ ions, thereby collapsing the pH or electrochemical gradient across the mitochondrial membrane (Mitchell, 1966). The lack of evidence for the existence of a high energy intermediate, the demonstration by Bielawski et al. (1966) that 2,4-dinitrophenol (DNP), the classical uncoupler, decreases the electrical resistance of phospholipid bilayer membranes (later shown to result from an increase of protonic conductance (Hopfer et al. 1968) and the experiments of Carafoli et al. (1969) on the pH and K^+ ion dependency of the DNP effect have led to the strengthening of the chemiosmotic approach. The primary uncoupling by DNP was thought by Slater (1958) to be a result of the uncoupler being actively taken up as a weak acid anion and passively diffused out as the free acid. The basic tenet of this approach rested upon the respiratory inhibition by high DNP concentrations and the idea that an essential coupling factor was bound by DNP. However, when it was shown that high substrate reversed the effect, this theory was modified. An alternative view is now held by these workers to the effect that the uncouplers inhibit substrate penetration (Van Dam and Slater, 1967). Opposing

views of this hypothesis suggest that uncouplers conduct protons into the hydrophobic phase of membranes causing acid hydrolysis of a high-energy intermediate at low concentrations and protonation of the active site of dehydrogenases at high concentrations (Skulochev et al. 1967). Wilson and Merz (1967) have indicated that uncouplers compete with substrate at the active centre of dehydrogenases. In addition, the reports of Weinbach and Garbus (1968, 1969) indicating that couplers may act by causing conformational transitions in mitochondrial catalytic proteins, must be given consideration, especially in the light of Boyer's (1965) proposal for energy transduction.

The views of Van Dam and Slater (1967) are the reverse of those held by Mitchell, in support of the chemiosmotic hypothesis, whereby the uncoupler is taken up as the free acid and moves out as an anion. The release of protons within the mitochondria discharges the pH gradient that is essential for energy coupling. Involvement of proton and potassium permeability in the action of uncouplers has been recently outlined by Caswell (1969). Ion movements following uncoupler administration to mitochondria may, indeed, lead to the elucidation of its mode of action; however, he indicates that uncouplers do not induce a very high permeability of the membrane to H^+ ions. The induction of K^+ permeability of the membrane by uncouplers is felt to be "through its action in depleting the energy reserves of the mitochondria rather than through a specific activating effect on permeability by the uncoupler itself".

Various chemical structure-activity relationships of uncouplers have been proposed and include organic compounds that contain readily

dissociable hydrogen atoms (Parker, 1958) and have elaborate delocalized electron orbitals (Szent-Györgi, 1957), compounds that display a high degree of lipophilicity (Hemker, 1962) and have the ability to combine with proteins (Weinbach and Garbus, 1966). These compounds include the nitrated (dinitrophenol) halogenated (pentachlorophenol) and oxygenated (dicoumarol) phenols in addition to derivatives of carbonylcyanidephenylhydrazones.

E. Monovalent Ion Transport of Mitochondria

There have been four recognized energy-linked functions (besides oxidative phosphorylation) that intact mitochondria display; namely, ion transport, transhydrogenation, reversed electron transport, and swelling-contraction phenomena. By far, the majority of research in the area of energy-linked functions has been conducted on animal mitochondria.

Recognition of these functions has led to a more thorough understanding of the mechanism of energy transfer reactions of oxidative phosphorylation. In addition, they have prompted intensive investigations on the part of the proponents of the two major theories of oxidative phosphorylation.

Studies of ion transport in plant mitochondria are still somewhat in their infancy and the majority of work has been done by two groups of workers, namely, Hanson and his colleagues (e.g. Hodges and Hanson, 1965) with corn mitochondria and Yoshida *et al.* (1968, a,b,c) with castor bean mitochondria. Cation transport in plant and animal mitochondria has been almost universally emphasized with two notable

exceptions. The work of Chappell and colleagues based on the acceleration of tricarboxylic acid entry into animal mitochondria by malate (Chappell, 1964), has evolved around anion transport. Active anion transport in plant mitochondria has been intensively investigated by Hanson and co-workers (Stoner et al. 1964; Wilson et al. 1969) with work on acetate and phosphate transport. Cation transport has been recently reviewed by Lehninger et al. (1967) and anion transport by Lardy and Ferguson (1969). Work on anion transport has substantiated the chemiosmotic hypothesis. These results (Chappell, 1968; Chappell and Robinson, 1968) have indicated the existence of specific exchange-diffusion carriers, namely, phosphate/OH^-, malate/phosphate and substrate/malate "antiports". Mitchell (1968) suggests that these results may also be interpreted as cotransportation of anion and proton ("symport"). The ADP-ATP exchange-diffusion system of mitochondria, another example of an anion carrier, has been studied by Klingenberg and Pfaff (1968).

Investigations that have shown that cations are taken up actively and anions passively have been done by Papa et al. (1969). The use of the ionophorous antibiotics of the valinomycin-nigericin type has substantiated the views of active cation transport. The valinomycin-type includes cyclic depsipeptides (valinomycin and the enniatins), open-chain peptides (gramicidins) and macrotetralides. Valinomycin (as well as enniatins and macrotetralides) increases the permeability of negatively charged (Andreoli et al. 1967) or uncharged (Lev and Buzkinsky, 1967) artificial thin lipid membranes as revealed by electrical conductivity and potential changes. In the presence of

uncouplers that permit proton transfer, valinomycin induces a rapid K^+/H^+ exchange. Valinomycin increases membrane permeability to K^+, Rb^+ and Ca^{++} whereas gramicidins act with H^+, Li^+ and Na^+ as well as the above (Wenner and Hackney, 1969). However, transport probably depends on the size of the hydrated cation. Ohnishi and Urry (1970) have recently reported the solution conformation of the valinomycin-potassium ion complex.

According to Harris et al. (1967), the addition of valinomycin to mitochondria respiring in a K^+ containing medium causes uptake of K^+, extrusion of H^+ and stimulation of respiration with the K^+ uptake being reversed by uncouplers. The uptake of K^+ is accelerated when permeant anions as P_i or acetate are in the medium (Azzi and Azzone, 1966). Cockrell et al.(1967) found that mitochondria suspended in a medium with valinomycin, rotenone and no K^+, rapidly lost K^+ with accompanying synthesis of ATP. They suggested that "valinomycin increases access of potassium to an energy-linked cation transport system tightly coupled to ATP synthesis". However, Mitchell (1968) suggests the efflux of K^+ creates a membrane potential that drives protons through the reversible ATPase. Nigericin, which catalyzes a non-electrogenic K^+ efflux, caused no ATP synthesis in the experiments of Cockrell et al.

Nigericin addition to mitochondria that have taken up K^+ or Na^+ under the influence of a valinomycin-type antibiotic, causes rapid efflux of the alkali metal ion and uptake of H^+ (Graven et al. 1966). It also facilitates K^+ / H^+ exchange across the red cell membrane (Harris and Pressman, 1967).

Since nigericin catalyzes an electrically neutral K^+/H^+ antiport and does not decrease the membrane potential when it collapses the pH gradient, the membrane potential is sufficient to drive ATP synthesis (Mitchell, 1968). With the addition of both valinomycin and nigericin, nigericin dissipating the pH gradient (by K^+/H^+ antiport) and valinomycin the membrane potential (pK^+ gradient), oxidative phosphorylation would be nullified. These additions have been shown to uncouple chromatophore photophosphorylation by Jackson et al. (1968) and oxidative phosphorylation in mitochondria (Pressmen et al. 1967) and submitochondrial particles (Cockrell and Racker, 1969). Recent investigations of Montal, Chance and Lee (1970) on ion transport and energy conservation in submitochondrial particles (SMP) are consistent with both the chemical and chemiosmotic hypothesis of energy coupling and, regardless of the primary coupling event, SMP possess an energized state associated with a membrane potential and pH gradient.

Work with SMP has shown that the movement of ions (respiration induced H^+ uptake, valinomycin induced K^+ efflux) is opposite to that in intact mitochondria. These results as well as electron-microscopic work supports the view that these particles are 'inside-out' with respect to intact mitochondria.

There has been a large volume of work published on the movements of K^+ ion in combination with valinomycin and uncouplers. Mitchell and Moyle (1967) have shown that mitochondria in the presence of uncoupler (FCCP), valinomycin and K^+ ions give a rapid relaxation of proton transport initiated by pH perturbation in the external medium. Observations of endogenous K^+ ion release on addition of DNP was first reported by Judah et al. (1965).

F. Interaction of Ethylene with Biological Systems

Ethylene, its production by and effects on plant tissue, has recently received much attention due in part to its activity as a plant hormone and in part because of its generation from a commercially available compound (2-chloroethanephosphonic acid). This compound has been and will be of immense commercial interest for it provides a means of handling and applying the hormone. The observations of change in sex expression of cucumbers by application of it (McMurray and Miller, 1968) will be one of many, commercially useful,applications. Recent reviews that have dealt with various aspects of ethylene as a plant growth regulator have been written (Pratt and Goeschl, 1969; Phan, 1969; Mapson, 1969 and Spencer, 1969). It was not the aim of the author to be repetitious of these comprehensive reports so that brief descriptions were felt to be sufficient.

1. Biological Production of Ethylene

Ethylene production by biological systems has been documented for all forms of life, that is, from animals (Chandra and Spencer, 1963), plants (see Olson, 1966), and microbes (Pratt, 1944). The biogenesis of ethylene has been found to be by enzymic as well as non-enzymic reactions. Precursors of ethylene have been associated with fatty acids (Abeles, 1966), dicarboxylic acids (Jacobsen and Wang, 1968), alcohols (Phan, 1962), aldehydes (Mapson, 1969) and amino acids (Lieberman, 1969; Thompson and Spencer, 1967). Some non-enzymic systems seem to involve light and riboflavin with participation of peroxide (Lang *et al*. 1967).

Ethylene evolution from intact plant tissue has been shown to be dependent on the physiological age of the tissue from seedling stage (Spencer and Olson, 1965) to maturity (Knight, 1969) and has been associated with respiration in climacteric tissue (Spencer, 1965). However, endogenous levels of ethylene may be entirely different from that which is evolved and has led to studies involving the internal atmosphere of tissue (Phan, 1963). The production of ethylene has been shown to be enhanced by treatment with auxin-herbicides (Morgan and Hall, 1962), radiation (Young, 1965), by tissue damage (McGlasson and Pratt, 1964) and pathological invasion (Smith et al. 1964).

The amounts of ethylene evolved by tissue are in the order of nℓ/kg/h. Concentrations of ethylene in air necessary to elict a physiological response are recorded in parts per billion.

One site of production of ethylene has been found to be a sub-cellular fraction that includes the mitochondria (Chandra and Spencer, 1963; Gibson, 1963). The transformation of β-alanine to ethylene by enzyme powder from bean cotyledon mitochondria (Thompson, 1966) has led to the furtherance of the former suggestion. In addition an enzyme system that converts uracil to β-alanine has been observed (Knight, 1969) to be involved with ethylene formation. A basic requirement of ethylene production for an energy source (ATP) has been found in preclimacteric fruit (Spencer, 1959) as well as sub-cellular preparations (Thompson, 1966).

A close association of carbon dioxide and ethylene production, by fruit displaying a climacteric rise of respiration, has been observed. As to whether the maximum in ethylene production precedes or follows the climacteric peak is not thought to be of major

significance. However, the competitive interaction of these two gases is of particular interest with respect to production and mode of action of ethylene.

2. Physiological Responses to Ethylene

Treatment of mature fruit with exogenous ethylene has been shown to greatly enhance the rate of senescence of the tissue. This aspect of ethylene usage has led to commercial applications and is by far the most typical of ethylene responses. The use of ethylene as an abscission accelerator will undoubtedly find commercial advantages in the near future (harvesting of such crops as cotton is facilitated by defoliation of the plant). The association of ethylene and cellulase activity in petiolar abscission zones has been suggested by Horton and Osborne (1967). Other beneficial effects of ethylene such as initiation of adventitious roots, breaking of dormancy, flower induction and inhibition of stem elongation have been well documented. However, induction of 'anesthetic' type responses ('sleepy' carnations) and root swelling has also been reported to follow ethylene treatment.

The variety of responses caused by treatment of tissue with ethylene as well as dependence on physiological age and CO_2 levels must suggest a site of action common to all tissue and of a basic nature. The effects of ethylene on such parameters as membrane permeability, energy production or utilization, nucleic acid metabolism and enzyme induction or repression have received little attention. However, if the type of response observed when humans are subjected to anesthetic dosages of ethylene can be used as an

example of rapid response time, the effects on regulation or disruption of nucleic acid or protein synthesis can be negated. The view that the primary site of ethylene action is on membranes (site of action of indole acetic acid (Royle et al. 1970) and site of oxidative phosphorylation in mitochondria) can be somewhat expected in that ethylene would tend to accumulate here because of its high solubility in lipoidal material.

The physiological response of pea tissue to ethylene is often quoted as a typical ethylene effect and is the 'triple response' of leaf epinasty, stem swelling and inhibition of stem elongation (Pratt and Biale, 1944). Thus the tissue used in this study is well known as being able to respond to ethylene.

3. Mode of Ethylene Action

As discussed above, the site of ethylene action may, indeed, exist in membrane structures. However, the mechanism by which the activity expresses itself is unknown. The effects of ethylene on various enzymic processes has been noted and would suggest effects on cytoplasmic contents apart from lipoidal membranes. Enhancement of cellulase activity in Phaseolus abscission zones (Horton and Osborne, 1968) and pectin methylesterase of ripening tissue (Biale and Young, 1962) has been noted. However, such studies do not specify a primary site of ethylene action. Alteration of membrane permeability could conceivably cause a similar induction of activity.

As with many plant growth regulators, knowledge of the mechanisms of action of ethylene is still in the future. The recent

review of Spencer (1969) has discussed possible sites of action, namely, modifiers of enzyme synthesis or activity (activation, inhibition and feedback regulation). As discussed by her, the chemical structure and physical properties of ethylene would suggest its involvement with light (free radical formation), metals (free as well as complexed in electron transfer reactions) and membrane structure (ethylene is fourteen times more soluble in lipid than water).

The main purpose of a previous dissertation from this laboratory was to investigate an effect of ethylene on the ATPase activity of mitochondria (Olson, 1967). These results indicated that ethylene had little activity on 'soluble ATPase' prepared from bean cotyledons but did affect the rate of mitochondrial volume changes caused by ATP or ADP but not by calcium or phosphate. Lyons and Pratt (1964) showed that the rate of mitochondrial swelling caused by calcium or phosphate was increased by ethylene treatment. This effect was displayed only while mitochondria were suspended in potassium chloride. The present thesis was concerned with the effects of ethylene on intact mitochondria from pea cotyledons in relation to ion induced ATPase activity. Ion-induced activity would be directly related to volume changes of mitochondria through maintenance of a Gibbs-Donnan equilibrium inside the mitochondrion.

G. Adenosine Triphosphatase Activity in Biological Systems

The enzymic hydrolysis of ATP is the major pathway for the biological transformation of chemical as well as mechanical energy. This hydrolysis with its accompanying large change in standard free

energy accounts for the majority of the energy supply for metabolic pathways. Not only is this energy utilized in biochemical endergonic reactions but also for ion transport across biological membranes and for muscle contraction.

1. Red Cell Membrane ATPase

The ATPase activity displayed by erythrocytes is undoubtedly concerned with the maintenance of an internal ionic composition of high potassium and low sodium ions. In so doing, the cell is prevented from haemolysing when small anions diffuse across the membrane. The reverse of these concentrations is present in the plasma surrounding the cell.

The ATPase activity of these membranes consists of two components, one requiring magnesium ions and the other requiring magnesium, sodium and potassium ion (and inhibited by the cardiac glycoside, ouabain). The interaction of the glycoside-sensitive component with sodium and potassium has been found to follow allosteric-type kinetics (Skou, 1965). It has been subsequently shown that the first step of the dephosphorylation is sensitive to sodium ions and the second step (production of phosphate and free enzyme) sensitive to potassium ions (and inhibited by ouabain). The sodium-potassium transport in erythrocytes has been shown to be inhibited by anesthetics (Wilcox and Fuchs, 1969). It may be recalled that ethylene has been used as an anesthetic.

Two postulations have been made with respect to the actual mechanism of action of this transport ATPase. Opit and Charnock (1965) proposed a model, based on the Danielli-Davson membrane model,

which allowed for conformation change of the protein in the membrane. The protein exhibits differential affinity for sodium or potassium ion depending on the cationic environment. Re-distribution of electron density along the macromolecule resulting from the binding of sodium ions to cationic sites, leads to the formation of a phosphorylated intermediate. This latter formation induces further conformational change resulting in elongation of the inner protein layer and rotation of it about the central lipid core, thereby exposing the sodium sites to the outside. A preference of the cationic site for potassium is now observed and sodium is exchanged from potassium. The resultant accompanying change of electron density facilitates the hydrolysis of the phosphorylated complex, which subsequently allows the protein to return to its original conformation. Sodium ions have been transferred to the outside and potassium ions to the inside of the cell.

The model of Jardetzky (1966) has introduced allostery into the mechanism. The macromolecule is envisaged as containing two possible configurations such that a cavity is open to one side of the membrane in one configuration and open to the other side in the alternate configuration. Sodium ions are preferred at the binding site when the cavity is open to the inside, and this triggers a phosphorylation near the site. This phosphorylation causes an allosteric alteration of the site so that the cavity is now open to the outside. This rearrangement lowers the affinity of the binding site for sodium and sodium diffuses out of the cavity. Replacement of sodium by potassium in the cavity triggers dephosphorylation and rearrangement

of the macromolecule so as to generate a cavity on the inside.

These two proposals account for the majority of the experimental observations (kinetic as well as vectorial movements). However, the strength, specificity and number of binding sites has not been clearly defined. Recent evidence (Robinson, 1970) suggests multiple sites for both sodium and potassium with probable competition between sodium and potassium, and allosteric interactions with the possibility of alternative reaction pathways dependent on the level of activation. The involvement of a potassium dependent phosphatase with the potassium dephosphorylation step of ATPase has been suggested (Askari and Koval, 1968). This potassium dependent dephosphorylation has been found not to be inhibited by oligomycin (Stahl, 1968). The latter observation may have significance with respect to ion transport in mitochondria wherein potassium stimulated ATPase has been observed to be insensitive to oligomycin (Fisher and Hodges, 1969). The possibility of ion transport in mitochondria may come about by conformational changes of the membrane resulting from electron transport or from hydrolysis of ATP through a phosphorylated intermediate giving a similar induction of conformational change.

The ion movements during nerve cell excitation are similar to the ATPase activity of erythrocytes (Mann, 1968).

2. Mitochondrial ATPase

In order to elucidate the mechanism of oxidative phosphorylation, several workers (notably Racker, Pullman, and Penefsky) have isolated, from disrupted mitochondria, factors that display cold labile ATPase activity (F_1). Additional phosphate transferring factors

(solubilized ATPase, Factor A, F_1 - X, etc.) indicate similar properties and are specific for the techniques employed. Factors required for energy transfer (F_2, F_3, F_4, F_5, Factor B) and necessary for the oligomycin sensitivity (F_o, CF_o, F_c) of F_1 are likewise technique specific. These factors and their role in oxidative phosphorylation have been reviewed by Lardy and Ferguson (1969).

The present dissertation was more intimately concerned with the ATPase activity displayed by intact mitochondria. The amount of study that plant mitochondrial ATPase activity (both from intact and disrupted mitochondria) has received has been negligible in comparison to animal mitochondria.

One of the original investigations of ATPase activity of plant mitochondria was undertaken only thirteen years ago on pea mitochondria. Forti (1957) observed an ATPase activity that was dependent on the presence of phosphate and stimulated by dinitrophenol. Wedding and Black (1962) found plant mitochondrial ATPase activity to be insensitive to DNP. With further improvement of techniques, this lack of DNP induced activity has been equated with the use of sucrose as osmoticant.

Plant mitochondrial ATPase activity has been observed to be magnesium dependent and either not stimulated by monovalent metal cations (Yoshida, 1969) or stimulated to a small degree (Fisher and Hodges, 1968; Gomez-Puyou, 1965). This stimulation by potassium was found to be insensitive to oligomycin. Potassium stimulation of animal mitochondrial ATPase was noted by Lardy and Wellman (1953).

Other characteristics that have been shown for plant mito-

chondrial ATPase include oligomycin sensitivity, a lack of pH optimum, as well as a dependence on the osmolarity of the suspending media (Reid et al. 1964).

This lack of oligomycin sensitivity of potassium stimulated activity may be indicative of an altered pathway apart from the reverse of oxidative phosphorylation. If this altered pathway is reminiscent of ion transport in erythrocytes, a phosphorylated intermediate would be suggested. A recent report (Cross et al. 1970), which has described detection of a phosphorylated intermediate in animal mitochondria, may perhaps be the intermediate associated with ion transport as the assay medium in these studies contained potassium.

CHAPTER II

MATERIALS AND METHODS

A. Plant Material and Growth Conditions

Previous studies from this laboratory involving sub-cellular particles have utilized tomatoes or Kinghorn waxbeans. Because of problems inherent in preparation of sub-cellular fractions from these tissues (high acidity of tomatoes (Meheriuk, 1965) and the relative instability of bean cotyledon mitochondria (Malhotra and Spencer, 1970)) it was decided that for this project, etiolated cotyledonary tissue of pea (Pisum sativum L. var. Homesteader) would be used as a source of material. This material was readily available, of sufficient size to facilitate harvesting procedures, reproducibly grown in a short period of time, easily macerated and yielded mitochondrial preparations that had a relatively long in vitro life and showed no loss of respiratory chain components.

The seed used in this study was purchased from a local seedhouse (Seed Centre Ltd., Edmonton) and was stored in a cold room prior to use. Sufficient dry seeds to yield 100 g fresh weight of material were placed in tap water for a total of eight hours prior to planting. In an attempt to standardize the stage of growth of the experimental material the seeds that had imbibed water in one hour (because of ruptured seed coats) were discarded and the seeds that had not imbibed water in eight hours were similarly discarded. The remaining seeds were planted over a 3 in layer and under a 3/4 in layer of horticultural grade vermiculite in plastic trays and allowed to germinate at

26° in the dark. The vermiculite was kept moist during the growth period with tap water. The tissue was called one day old, 24 hr after the dry seeds had been placed in the tap water.

B. Preparation of Mitochondria

Pea cotyledons were harvested at daily intervals by removing the seed coat, hypocotyl and epicotyl. They were weighed, rinsed with distilled water, blotted dry and kept chilled in a beaker on ice prior to the maceration of the tissue.

Cotyledons were ground with a chilled mortar and pestle in 'grind medium' (3 ml medium per g cotyledons) of the following compositions: 0.5 M mannitol, 2 mM EDTA, 0.3% BSA (w/v), 0.05% cysteine (w/v) and 50 mM TES pH 7.4 (Tris) at 0°. (The terminology TES (Tris) or TES (KOH) is used to indicate the neutralization of TES to the desired pH by Tris or KOH, respectively). Every attempt was made to be as gentle as possible during the maceration step so as not to damage the mitochondria. The pestle was allowed to fall softly with each stroke in an attempt to apply only enough pressure to rupture the cell walls. The brei obtained after 3 min of grinding was filtered through four layers of cheesecloth to remove the uncrushed tissue. The supernatant layer after centrifugation of the filtrate at 2,500 x g for 10 min in a refrigerated centrifuge (International Centrifuge Model B-20) was strained through cheesecloth into another set of cold centrifuge tubes (taking care to exclude the fluffy layer on top of the starchy pellet). The mitochondria were subsequently separated from this layer by centrifugation at 20,000 x g for 10 min. Suction was used to remove the resulting supernatant layer and the

light coloured material surrounding the dark brown mitochondrial pellet.

The mitochondria were either used after this centrifugation or washed by suspension and recentrifugation. For the latter, the pellet obtained from the second centrifugation was suspended in 'wash medium' (0.5 ml/g cotyledons) of the following composition: 0.3 M mannitol, 3.0 mM $MgCl_2$, 0.3% BSA (w/v), 50 mM TES pH 7.2 (Tris) at 0°. A uniform suspension was made by gently sucking the solution up and down a wide bore pipette. The supernatant layer after a slow speed centrifugation (1,000 x g for 10 min) was centrifuged at 6,000 x g for 16 min in order to sediment the washed mitochondria.

The mitochondria (either unwashed or washed) were suspended in a small quantity of 'suspend medium' of the following composition: 0.3 M sucrose (or 0.3 M mannitol) (see Chapter IV, Section F) 3.0 mM $MgCl_2$ and 25 mM TES, pH 7.2 (Tris) at 0°. In order to give a protein concentration of 20 to 25 mg mitochondrial protein per ml, mitochondria from 100 g of cotyledons (ground in two lots) were surface washed and suspended in 2.0 ml of 'suspend medium'.

Further purification procedures for the mitochondria thus obtained, was accomplished by means of sucrose density gradient centrifugation. Preliminary studies involving linear sucrose density gradients, made by a Buchler gradient maker, established the equilibrium density at which the mitochondria, as well as the other particulate bodies, banded. With this knowledge, it was possible to adapt the separation procedures to the more easily prepared sucrose density discontinuous gradient. The volumes and compositions of the layers introduced into a 5.0 ml cellulose nitrate tube were as

follows: 0.5 ml 1.75 M sucrose, 1.4 ml 1.40 M sucrose, 1.7 ml 1.31 M sucrose and 1.4 ml 1.11 M sucrose. Approximately 100 to 150 µl of the washed mitochondrial suspension (stored at 0^{o}) were layered on the top of the prepared tubes. The separation into bands was accomplished using an SW 50.1 rotor in a Spinco L-2-65B ultracentrifuge set at 42,000 rpm (approx. 150,000 x g) for 210 min at 4^{o}. The fractions with a high protein content were separated, and diluted with water to give a final sucrose molarity of 0.5. The pellets obtained by centrifugation at 20,000 x g for 10 min were suspended in small quantities of 'suspend medium'.

C. Measurement of Mitochondrial Respiratory Parameters

Mitochondria after being prepared by the above procedures were analyzed for their rate of oxygen utilization, respiratory control ratio (RCR) and ADP/O values. These parameters were measured at 25^{o} with a Model 53 Biological Oxygen Monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio) equipped with a Clark fixed-voltage polarographic probe. A continuous tracing of oxygen concentration of the 3.0 ml 'assay medium' used was provided by use of a Beckman 100 mv potentiometric recorder set with a chart speed at 0.5 in per min. The 'assay medium' was composed of: 0.3 M mannitol (or 0.3 M sucrose), 4 mM $MgCl_2$, 5 mM H_3PO_4, 0.075% BSA (w/v) 25 mM TES pH 7.2 (KOH or Tris) at 25^{o} with either 8 mM α-ketoglutarate, (with 5 mM malonate and 0.07 mM thiamine pyrophosphate) or 8 mM succinate as respiratory substrate. RCR was determined from the ratio of the respiration rates in State III (phosphate, oxygen, substrate and ADP in excess) and

State IV (ADP limiting). ADP/O values were obtained by calculating from the tracing, the amount of oxygen consumed during the respiratory rate increase caused by the addition of 600 nmoles of ADP. These values were calculated in accordance with the definitions of Chance and Williams (1955, 1956).

For all of the above measurements, it was found by the method of Winkler (1914) and in accord with Stinson (1968) that the concentration of oxygen in air saturated 'assay medium' was 225 μM at 25°.

D. Measurement of Mitochondrial ATPase Activity

The hydrolysis of ATP was assayed by methods estimating the inorganic phosphate and/or ADP liberated during an incubation period at 25° with shaking. The reactions were carried out in 15 ml conical glass centrifuge tubes in a total volume of 1.0 ml. The composition of the usual reaction mixture was: 0.3 m moles sucrose (or mannitol), 3.0 μ moles $MgCl_2$, 3.0 μ moles Tris-ATP, 25μ moles TES pH 7.2 (Tris) at 25° and approximately 0.5 mg mitochondrial protein. After preincubation of the mitochondria in the assay mixture for 3 min the reactions were started with the addition of the 3.0 μ moles ATP in 30 μl. To terminate the reaction the tube was plunged into ice followed by the immediate addition of a protein precipitant (5% trichloroacetic acid or 5% perchloric acid final concentration) or sodium lauryl sulphate (SLS). The samples treated with a protein precipitant were kept at 0° for 5 min and subsequently centrifuged at 1200 x g for 5 min in a Model HN International Centrifuge to sediment the protein. Analyses for phosphate or ADP content of the solutions were performed

immediately in order to minimize acid hydrolysis of ATP. Blank samples (mitochondria and/or ATP absent) as well as zero time samples (where the addition of the reaction terminating compound preceeded the addition of ATP) were also prepared.

E. Analysis of Inorganic Phosphate

A variety of methods for quantitatively estimating inorganic phosphate liberated by the hydrolysis of ATP were investigated. Procedures involved the measurement of the reduced or oxidized form of the phosphomolybdate complex.

For the spectrophotometric measurement of the oxidized form of the complex, the procedure, adapted from Mozersky, Pettanti and Kolman (1966), involved termination of the reaction with the addition of 4.0 ml of 0.75 M $NaClO_4$, 0.05 M glycine, and 0.125 N HCl. After a 5 min precipitation time at 0^{o}, protein was removed by centrifugation at 1200 x g for 5 min. To a 4.0 ml aliquot of the supernatant layer was added 2.0 ml of a molybdate solution (2.1 N H_2SO_4, 0.6 M $NaClO_4$ and 12.5 mM ammonium molybdate). After the addition of 5.0 ml isobutanol:benzene (1/1 v/v), the 15 ml test tubes were capped (with polyethylene caps), vigorously shaken by hand for 15 sec and centrifuges for 45 sec. The optical density at 313 nm of an aliquot of the upper (organic) phase was measured in stoppered quartz cuvettes in a Beckman DU-2 spectrophotometer.

A procedure for the analysis of the reduced phosphomolybdate complex was according to the method of Rathbun et al. (1968). Trichloroacetic acid (2.0 ml of 7.5%) was used as a protein precipitant

and sedimentation conditions were as in the previous procedure. After centrifugation, a 2.0 ml aliquot was taken and to it was immediately added 0.7 ml acetate:formaldehyde mixture (3 N acetate/37% formaldehyde (1/0.1)). The reduced phosphomolybdate complex was formed by the sequential addition of 0.1 ml 2% molybdate and 0.2 ml 6.75 mM stannous chloride (clarification of this solution was achieved by addition of 0.1 ml glacial acetic acid to a prepared volume of 25 ml). Additions of the latter two solutions were made down opposite side walls of the tube. After mixing and a 20 min incubation period at room temperature, the optical density of the reduced complex was measured at 735 nm.

In addition to the foregoing methods of reaction termination and phosphate analysis, a procedure that involved the addition of 1.5 ml of 1.5% SLS to stop the reaction, and reduction of the phosphomolybdate complex by ferrous sulphate was devised. The reduced complex was measured spectrophotometrically as above after the addition of 1.0 ml 0.2% molybdate in 0.5 N H_2SO_4 and 0.5 ml 0.2 M ferrous sulphate in 0.2 N H_2SO_4 followed by a 20 min incubation period at room temperature.

F. Estimation of ADP

The amount of ADP produced by the hydrolysis of ATP was estimated by use of a coupled enzymic reaction. Phosphoenolpyruvate (PEP) in the presence of ADP and pyruvate kinase (PK) is converted to pyruvate. Reduced nicotinamide adenine dinucleotide (NADH) in the presence of pyruvate and lactate dehydrogenase (LDH) is converted to

the oxidized form (NAD+) so that there is a decrease in the optical density of the solution at 340 nm (absorption maximum of NADH). The decrease in the A_{340nm} is proportional to the amount of pyruvate present, which is proportional to the amount of ADP present in the solution. The sequence as outlined by Adam (1965) is summarized in the following scheme.

$$ATP + H_2O \rightleftharpoons ADP + P_i$$

$$ADP + PEP \xrightleftharpoons{PK} ATP + Pyruvate$$

$$Pyruvate + NADH \xrightleftharpoons{LDH} Lactate + NAD^+$$

The ATPase reaction was terminated by the addition of 1.0 ml 1.2 M $NaClO_4$, in 0.2 M HCl and the protein sedimented by centrifugation after 5 min at 0^o. The insoluble potassium perchlorate that formed by the addition of 1.0 ml 2.0 M KCl and 0.22 M Tris was removed by centrifugation; the resulting solution had a pH of 7.2. A 2.0 ml aliquot of the final supernatant layer was introduced into a 3 ml stoppered quartz cuvette. Addition of the coupled enzyme system with required cations minus PEP was made in a 0.9 ml volume. The 0.9 ml PK-LDH addition included 5.4 µg of pyruvate kinase (Calbiochem 300 I.U./mg), 80 µg lactate dehydrogenase (Sigma Chem Co. 63 I.U./mg, 30 µ moles $MgCl_2$, 150 µ moles KCl and 1.10 µ moles NADH. The reaction was initiated with the addition of 4.5 µ moles of PEP in 0.1 ml. The change in optical density at 340 nm was measured with a DU-2 spectrophotometer. Blank samples were prepared by the addition of 0.1 ml water in place of PEP.

G. Estimation of Mitochondrial Phosphatase Activity

The unspecific phosphatase activity of the mitochondria was estimated using the identical conditions to those in the ATPase assay. The reaction was initiated by addition of 3 μ moles p-nitrophenyl-phosphate to the assay mixture that included mitochondria. The reaction was terminated by the addition of 1.5 ml of 1.5% sodium lauryl sulphate. In order to form the yellow p-nitrophenylate ion 0.5 ml of 0.1 N KOH was added. The amount of p-nitrophenylate produced from the hydrolytic cleavage was determined by measurement of the optical density of the solution at 400 nm. Standard and blank solutions were prepared so as to equate the spectrophotometric readings to μ moles of p-nitrophenylate ion present.

H. Methodology of Studies Involving Ethylene

1. Detection of Ethylene

The measurement of the ethylene content of air was made with the use of a Perkin-Elmer Model 811 flame ionization gas chromatograph as described by Stinson (1968) and Knight (1970). The absorption column was 50 cm x 6 mm I.D. and contained activated alumina coated with 2+1/2% silicone 550. The injection port was on an external U-tube circuit. The carrier gas (helium) could be directed either through the internal column or through the external circuit and then into the internal column by means of a two-way valve.

With the carrier gas flowing only through the internal column, samples were injected with a Hamilton (1.0 ml) gas-tight syringe

through a silicone plastic septum into the U-tube. The measurement was commenced by allowing the carrier gas to sweep through the U-tube and take the sample onto the absorption column and to the detector. A calibration curve of the instrument was obtained by plotting peak height vs. nl ethylene injected. Peak heights were recorded on a Beckman 100 mv potentiometric recorder at a setting of 1 mv.

The carrier gas, helium, was passed through the column at the rate of 35 ml per min. The flow rates of the hydrogen and air were 30 and 400 ml per min respectively. The temperature of the flame ionization detector was set at 125° and the column was maintained at room temperature.

2. Treatment of Mitochondria with Ethylene

Prior to the addition of mitochondria and ATP to the incubation mixture, the assay medium was treated by purging the individual solutions (contained in 15 x 100 mm test tubes) with a 100 ppm ethylene mixture (Matheson of Canada Ltd.) at a rate of 50 ml per min for three min.

It was found that this treatment gave an equilibrium state in that the solution had attained the maximum concentration of ethylene to be achieved with this gas mixture (as determined by the constancy of the ethylene content of the air space above the liquid). The control tubes were treated with compressed air in a similar fashion. The tubes were quickly stoppered with rubber septum stoppers and kept in ice.

Mitochondria were injected through the septa, and the tubes

and contents were incubated in a 25° water bath for 5 min prior to the injection of ATP. The individual ATPase assays were spaced 3 min apart in order that all mitochondria were exposed to ethylene for the same length of time.

I. Estimation of Mitochondrial Protein

The Lowry method (Lowry et al. 1951) for the quantitative determination of protein was modified to determine mitochondrial protein concentration. To remove compounds that would interfere with the protein assay, the mitochondria were first precipitated by addition of 0.3 ml 10% TCA to 0.01 ml of the mitochondrial suspension followed by centrifugation at 1200 x g for 5 min. The supernatant layer was poured off and the pellet was dissolved in 0.5 ml 1N NaOH, and the volume was made to 1.0 ml with distilled H_2O. Addition of 5.0 ml of 0.02% $CuSO_4$, 0.04% potassium tartarate and 4% Na_2CO_3 in 0.1 N NaOH was followed by an incubation period of 15 min at 25°. Subsequently, 3.5 ml water and 0.5 ml of a 1.0 N phenol-water mixture (1:1 v/v) was added to each tube. The optical density of the solution at 750 nm was observed on a Beckman DU-2 after a 30 min incubation period at room temperature. Samples were assayed in triplicate. Human serum albumin was used as a standard.

CHAPTER III

EVALUATION OF TEST SYSTEM EMPLOYED

For each assay procedure used in this study, a separate project was undertaken in an effort to fully evaluate conditions with respect to variability between replicates as well as establish sensitivity and factors affecting the assay procedure. The final methods adopted in the study were determined primarily by sensitivity and reproducibility and secondarily by rapidity of each determination. Acknowledged procedures were not accepted per se but were modified so as to attain optimum conditions of the test systems to be used under prevailing conditions.

It was determined that there exist many variables that profoundly affect the sensitivity and reproducibility of the various test systems employed. An attempt was made to minimize these variables.

A. Estimation of Inorganic Phosphate

1. Comparison of Various Methods

Since the major portion of this research project involved the enzymatic hydrolysis of ATP measured by the production of orthophosphate, an accurate quantitative determination of this ion was of paramount importance. A study of a variety of methods for the estimation of orthophosphate was undertaken.

Analysis involved spectrophotometric measurement of the complex formed between orthophosphate and molybdate ions in acidic solution.

This complex when in the oxidized form has a yellow colour and its greatest absorption maximum at 313 nm, and in the reduced form has a blue colour and a broad absorption maximum from 690 to 735 nm (Figure 2).

A study revealed that the amount and stability of the coloured complex (both oxidized and reduced) was a function of hydrogen ion concentration, molybdate concentration and, in the case of the reduced form, concentration as well as composition of the reducing agent employed. The latter factor was, of course, dependent on the reducing power of the agent. In addition to the above variables, it was found that the hydrogen ion concentration that was optimal at one molybdate concentration was not optimal at another and vice versa. The sensitivity of the various methods studied is recorded in Figure 3.

The method of Fiske and SubbaRow (1925), which utilizes a mixture of α-aminonapthosulphonic acid, sodium bisulphite and sodium sulphite as a reducing agent, was found to be inadequate since the assay did not offer sufficient sensitivity. In the ATPase activity studies, the changes in the amounts of phosphate of the order of 0.010 μ moles were to be detected so that a method that would give a measure of reliability greater than the 0.100 μ moles provided by the Fiske and SubbaRow procedure was obviously required. In addition to the above inadequacy, it was felt that a procedure that gave conditions conducive to non-enzymic hydrolysis of ATP (long incubations in the presence of strong acid) was not desirable.

Procedures designed to avoid the strongly acidic conditions

Figure 2. The absorbance spectra of oxidized (A) and reduced (B) phosphomolybdate complex. The oxidized form was obtained by extraction in an organic phase. The reduced form was obtained according to the method of Fiske and SubbaRow.

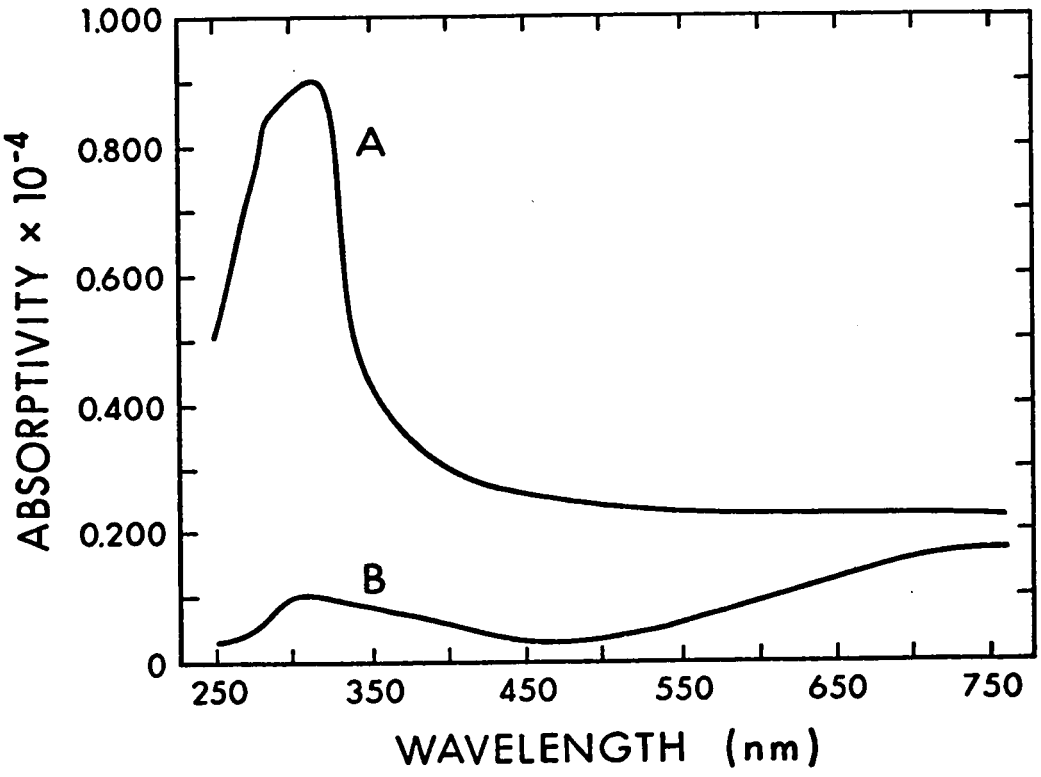
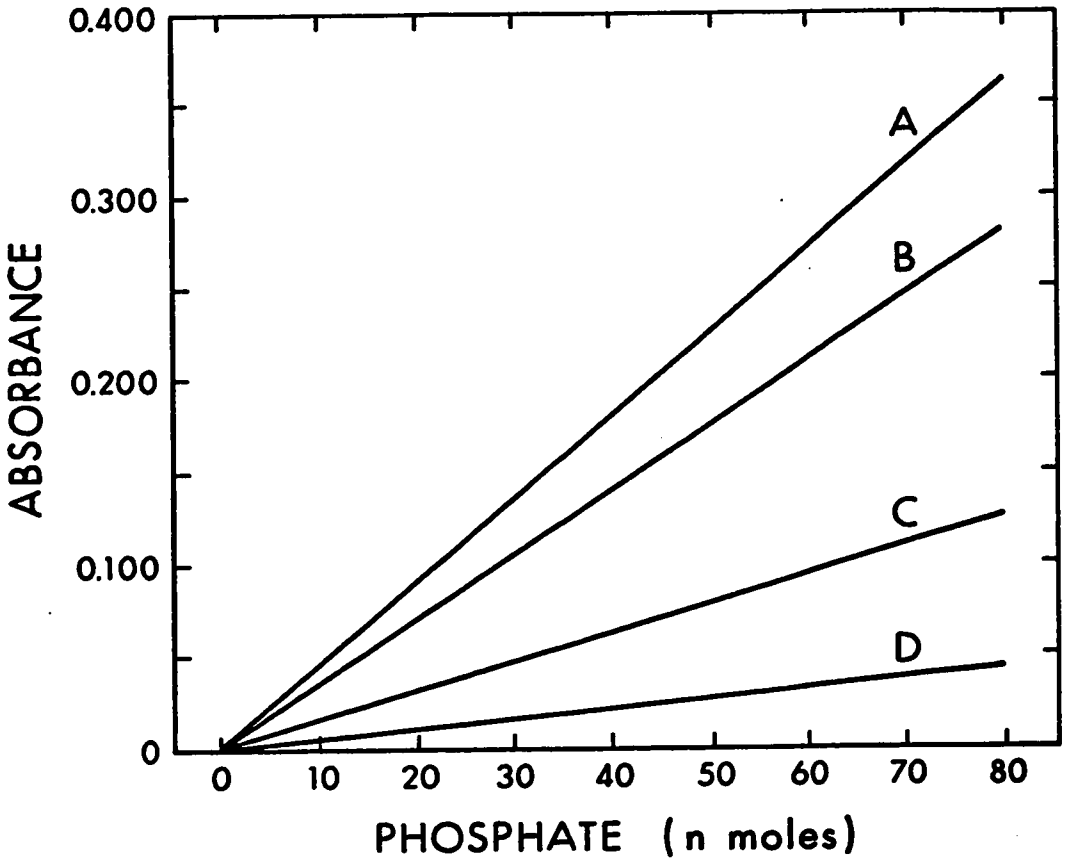

1.000
0.800
0.600
0.400
0.200
0
ABSORPTIVITY × 10⁻⁴
A
B
250
350
450
550
650
750
WAVELENGTH (nm)

Figure 3. Standard curves for phosphate determinations as according to the organic phase extraction technique (oxidized form of phosphomolybdate) (A), the reduction of complex by stannous chloride (B), reduction of complex by ferrous sulphate (C), and use of Fiske and SubbaRow technique (D). The amount of phosphate is expressed as that amount added to a total volume of 1.0 ml of solution as would be found in ATPase activity assays. No corrections have been made for aliquot size or dilution effects.


0.400
0.300
0.200
0.100
0
ABSORBANCE
A
B
C
D
0
10
20
30
40
50
60
70
80
PHOSPHATE (n moles)

were investigated. The first of these attempts utilized an acetate buffer (Rathbun et al. 1968) that was added immediately after the protein precipitation step. The addition of the acetate resulted in increasing the pH of the solution from 1.2 to 3.4. It was found that this increase in pH greatly reduced the chemical hydrolysis of ATP at 25°. (It should be pointed out that acid hydrolysis of ATP was negligible during the protein precipitation step that is conducted at 0°; it is during the incubation period for colour development of the reduced form of the complex that was of concern). This method also employed a stronger reducing agent (stannous chloride) that resulted in an increase of sensitivity over the method of Fiske and SubbaRow. Use of this method was found to be quite reproducible but, however, was quite time consuming if one has many samples to be assayed.

The second method that alleviated the strong acidic conditions of long incubation for colour development utilized sodium lauryl sulphate as an ATPase reaction terminator in place of perchloric or trichloracetic acid (Mann, 1968). (Triton X-100 (octylphenoxypolyethoxy ethanol) which is routinely used as a surface-active agent was found not to inhibit the ATPase reaction.) The use of stannous chloride as a reducing agent in this case, was found to lead to formation of a cloudy suspension. However, use of ferrous sulphate was observed to result in an adequate test system. Sensitivity of this system was found to be acceptable if longer incubation periods of the ATPase reaction were used. The procedure was the most rapid of any studied since it did not require a protein precipitation step. However, it was observed that the presence of protein did interfere with the

determination (Section B.2.).

The final procedure for the determination of orthophosphate, that will be discussed, involved the spectrophotometric determination of the oxidized form of the phosphomolybdate complex. This procedure was a modification from that of Mozersky et al. (1966) and was found to be as sensitive and reproducible as, but less time consuming than, reported by them. The chief advantages of this assay procedure over those involving the reduced form of the complex are: (a) higher degree of sensitivity, (b) high reproducibility and (c) immediate separation of the coloured complex from the aqueous solution containing residual ATP so that non-enzymic hydrolysis of ATP is not observed. The modifications of the original procedure were not major changes but did allow for a more rapid determination of inorganic phosphate. These changes involved (a) the use of a mixture of molybdate, sulphuric acid, sodium perchlorate and hydrochloric acid instead of two separate solutions requiring an additional pipetting operation, (b) a short (45 sec) centrifugation step for separation of the organic and aqueous phases rather than the 20 min step used by the originators and (c) the use of test tubes with slip-off caps rather than Teflon lined screw capped test tubes.

2. Factors Affecting the Quantitative Extraction of Inorganic Phosphate

(i) Presence of Mannitol

Techniques employed in the isolation of mitochondria from tissue must involve the use of an osmotic stabilizer so that the sub-cellular particle may be isolated in its native state. It has been

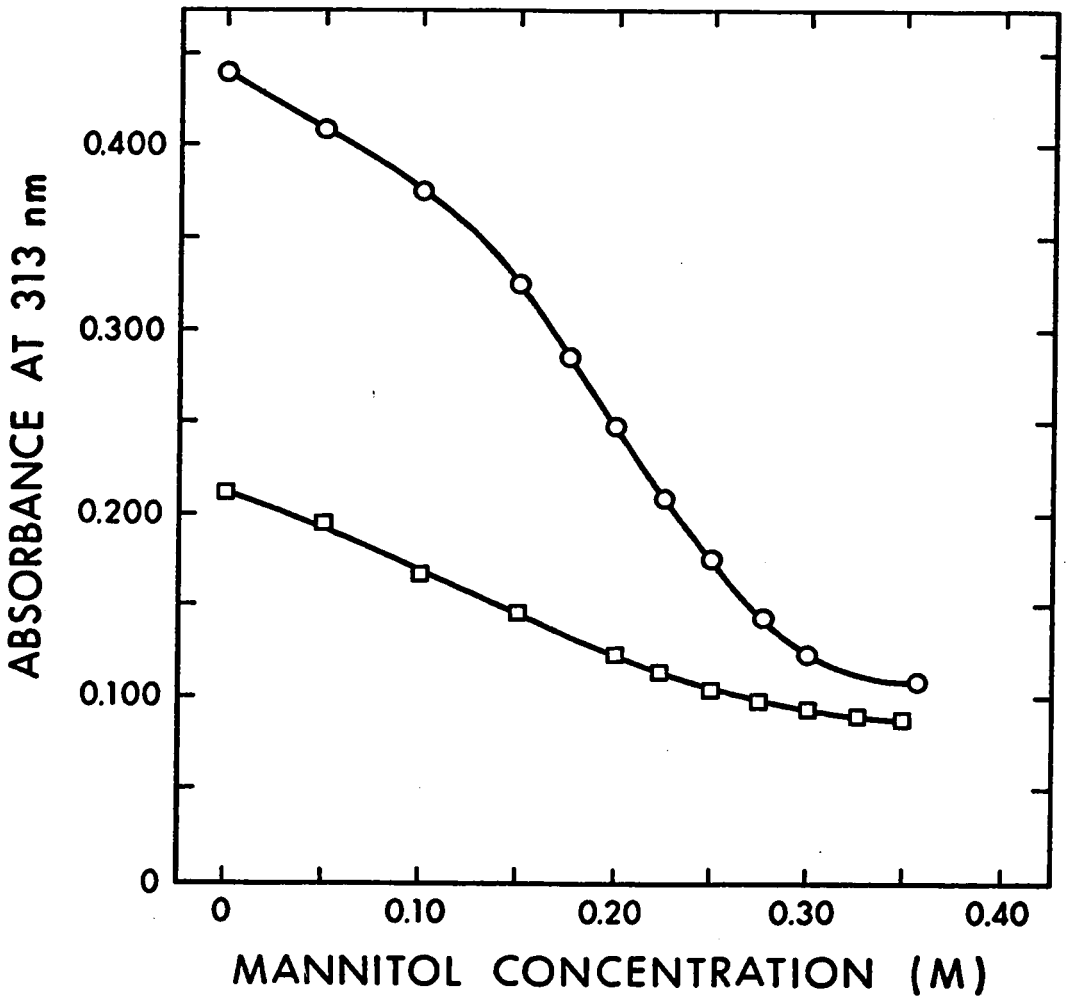
recognized that the sugar alcohol mannitol is superior to sucrose for these purposes since mitochondria exhibit a 'sucrose space' and not a 'mannitol space' and hence the osmotic pressure is more easily controlled.

Initial experiments involved in this study employed mannitol in the ATPase assay medium. It was detected however, that the sensitivity of the phosphate analysis procedure was greatly affected by the presence of mannitol. Upon further investigation, it was observed that at the concentration of mannitol being used (0.3 M), the test system was only about 20% as sensitive as it was in the presence of water (Figure 4). The use of mannitol in the ATPase assay was immediately abandoned. Mixtures that involved the use of sucrose as osmotic stabilizer were investigated and it was observed that sucrose had no deleterious effect on the phosphate analysis system. Despite the disadvantages of the presence of sucrose it was felt that this was the only way to circumvent the problem.

Upon further investigation, it was observed that sugars that exhibit an open chain structure (D-mannitol, D-sorbitol) have a deleterious effect on the phosphate analysis system whereas sugars exhibiting a pyranose or furanose structure (D-glucose, sucrose, meso-inositol) do not exhibit this interference. It has been suggested (Honnelaitre, 1925) that molybdate is irreversibly complexed with the readily available hydroxyl groups of the open chain compounds.

In order to reach a compromise between the use of mannitol vs. sucrose, it was decided that in the procedures involving the separation of mitochondria from starch granules (as suggested by Bonner, 1965),

Figure 4. Effect of mannitol on the sensitivity of the phosphate determination procedure utilizing the organic phase extraction technique. The amount of phosphate added to a total volume of 1.0 ml of the various mannitol concentrations was either 0 (□—□) or 50 n moles (○—○).


ABSORBANCE AT 313 nm
0.400
0.300
0.200
0.100
0
0
0.10
0.20
0.30
0.40
MANNITOL CONCENTRATION (M)

mannitol would be used and the procedures involving the suspending of the mitochondria and ATPase activity measurements would employ sucrose.

(ii) Presence of Protein

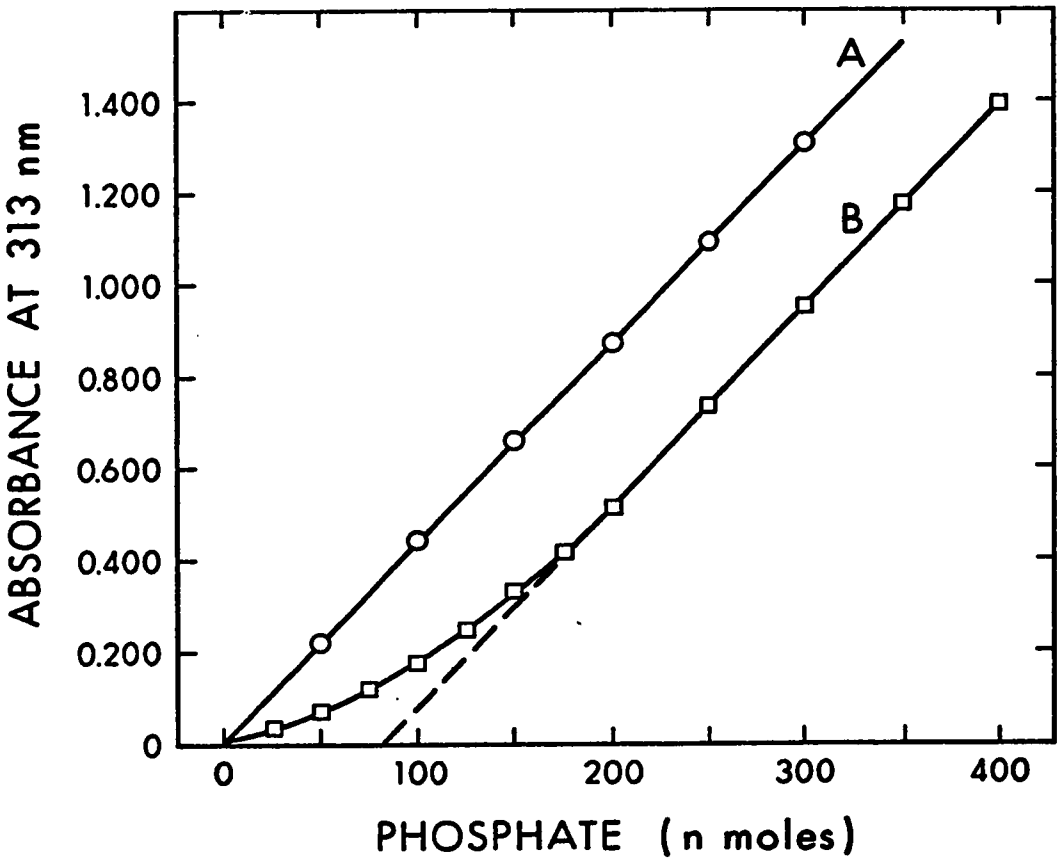
During the evaluation of the phosphate analysis procedures, it was noted that the procedure of Martin and Doty (1963) employs analysis without the precipitation of protein. This modification would allow for a more rapid determination of phosphate by the organic extraction procedure. However, upon investigation, it was observed (Figure 5) that the presence of protein altered the standard phosphate curve. From the shape of the curve, it may be concluded that the protein was able to complex a specific amount of phosphate (from the difference between the parallel portions of the curve, it is estimated that the complexing ability of the mitochondrial protein was about 0.087 μ moles P_i per mg). If one could be certain that blank samples contained phosphate in excess of this level, then the procedure would be reliable. The author never reached this point of absolute certainty and therefore did not use this technique.

B. Evaluation of ADP Analysis Procedures

From the specific activities of the enzymes involved in the coupled enzyme assay procedure, concentrations were selected (Chapter II, Section F) that would give a virtually complete reaction in 3 to 5 min.

It was noted that complete removal of perchlorate after the precipitation of protein was absolutely essential. This removal was

Figure 5. Effect of protein on the phosphate determination procedure that utilized the organic phase extraction of the oxidized phosphomolybdate complex. Addition of 0 (A) or 1.0 mg mitochondrial protein (B) per 1.0 ml.


A
B
1.400
1.200
1.000
0.800
0.600
0.400
0.200
0
0
100
200
300
400
ABSORBANCE AT 313 nm
PHOSPHATE (n moles)

virtually complete if an excess of potassium ion was added and the precipitation of the insoluble potassium perchlorate was conducted in ice. Furthermore, sodium lauryl sulphate could not be used as an ATPase reaction terminator as it also inhibited the coupled enzyme system.

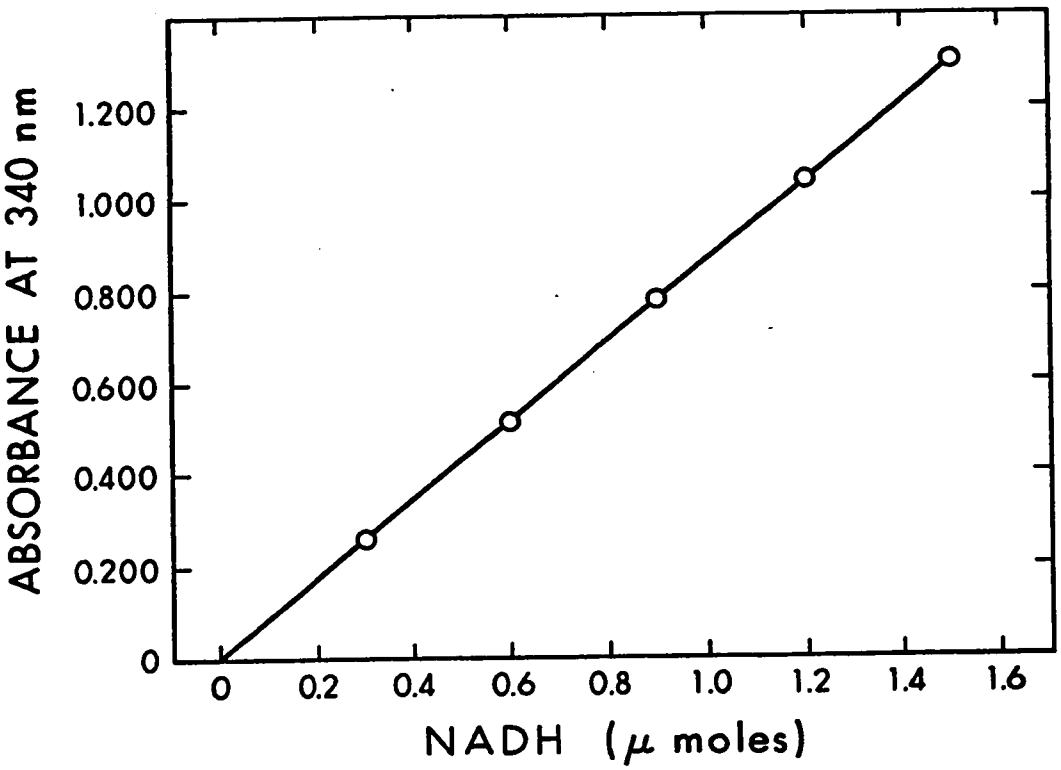
From Figure 6, an initial concentration of NADH was selected so that a large enough excess was present to give an observable optical density on the spectrophotometer. This concentration was 1.1 μ moles of NADH in the final reaction volume of 3.0 ml, and gave an absorbance at 340 nm of about 1.0. The change of absorbance upon the addition of ADP to the complete assay mixture was found to be linear with respect to the concentration of ADP (Figure 7).

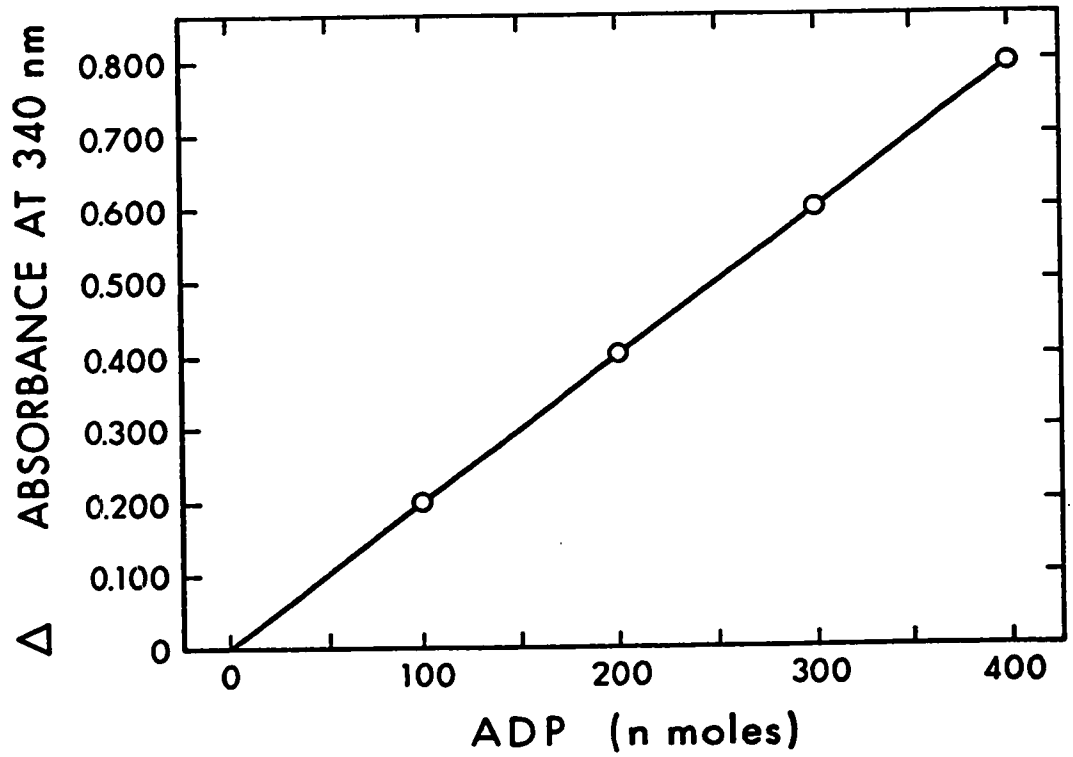
The ADP assay procedure was found not to have the same degree of reliability among replicates of a sample as the phosphate analysis procedures had. (Absorbance changes were 0.200 ± 0.010 for the addition of 0.10 μ moles of ADP whereas analysis for phosphate gave a reliability of 0.235 ± 0.005 for the addition of 0.05 μ moles). It was also observed that the sensitivity of the ADP assay was not nearly as great as the orthophosphate determination.

Since ADP determinations were based on the reduction of pyruvate formed on the addition of phosphoenopyruvate (PEP), trace quantities of pyruvate in the PEP solution would give erroneous results. Examination of this possibility in the absence of ADP resulted in a decrease in $A_{340_{nm}}$ of 0.030 upon the addition of 0.1 ml PEP to a 2.9 ml reaction mixture. However, this effect was found to be directly attributable to a dilution effect since the addition of an equivalent

Figure 6. Standard curve of NADH addition up to a total volume of 3.0 mls of ADP assay mixture.

Figure 7. Standard curve for the observed change in Absorbance (340 nm) upon additon of ADP to 5.4 µg pyruvatekinase, 80 µg lactate dehydrogenase, 1.1 µ mole NADH, 4.5 µmole PEP, 30 µ mole $MgCl_2$ and 150 µ mole KCl in a total volume of 3.0 ml.


ABSORBANCE AT 340 nm
1.200
1.000
0.800
0.600
0.400
0.200
0
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
NADH (μ moles)


Δ ABSORBANCE AT 340 nm
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0
0
100
200
300
400
ADP (n moles)

amount of water resulted in a similar decrease so that the PEP solution had a negligible pyruvate content.

C. Comparison of P_i and ADP Determination Methods

ATPase activity studies were performed on different aliquots from the same mitochondrial suspension sample under identical conditions. From Table I, it was observed that in both experiments studied, the amount of ADP released actually exceeded the amount of phosphate released. However, with the realization that the ADP determination is only half as reliable and half as sensitive as the phosphate determination, it was felt that within the limits of experimental error, the amount of phosphate and amount of ADP released were equal. This comparison almost rules out the possibility of the presence of apyrase activity which has been observed to be present in plant tissue (Brown, 1968).

D. Choice of Mitochondrial Isolation Methodology

Procedures involved in the separation of mitochondria from cellular debris (vacuolar contents) should be accomplished in as rapid a manner as possible. This rapidity of separation is known to be very critical if highly active mitochondria are to be obtained from bean cotyledons (Malhotra and Spencer, 1970a). The loss of respiratory activity of bean cotyledon mitochondria *in vitro* may result from the high phospholipase activity of the preparations as well as loss of soluble respiratory chain components (cytochrome c and NAD^+) (Malhotra and Spencer, 1970b). Pea cotyledon mitochondria, however, were shown

Table 1. Comparison of ATPase activity as estimated by either production of phosphate or ADP.

	ATPase activity (product released/min/mg protein)	
	n moles P_i	n moles ADP
Experiment I	6.60	7.05
	6.67	6.90
	6.63	6.87
Average	6.63	6.94
Experiment II	7.08	6.97
	7.10	7.21
	7.13	7.18
Average	7.10	7.12

The phosphate analysis employed the use of the organic phase extraction technique. The ADP analysis utilized the oxidation of NADH in the coupled enzyme (PK-LDH) system. Both assay conditions were identical and run simultaneously.

to exhibit a high degree of *in vitro* stability (Chapter III, Section B).

The rapid procedure (involving two short centrifugation steps) for the isolation of mitochondria from bean cotyledons used in this laboratory, was employed for isolation of mitochondria from pea cotyledons. However, since there was some possibility of contamination by other cellular inclusions of the mitochondria obtained by this short procedure, a study of the effect of washing the mitochondria thus obtained, was undertaken. The results of this study (Table II) indicated that the washing of the mitochondria vastly improved the respiratory parameters of the mitochondria.

The presence of the loose material around the mitochondrial pellet from the short procedure was found (by addition of a small amount of it during evaluation of the respiratory parameters of washed mitochondria) to have a large amount of State IV but an insignificant amount of State III respiration. As a consequence, it was thought that by washing the mitochondria one was able to remove this component and thus observe such an increase in respiratory parameters. By removing this extraneous protein, the oxygen consumption per mg protein during State III and the respiratory control ratio increased.

It should be noted that the presence of bovine serum albumin (BSA) in the wash media was essential if tightly coupled mitochondria with a high degree of respiratory control were to be obtained.

Further mitochondrial purification procedures were undertaken that involved the use of sucrose density gradient centrifugation. With the use of this technique the mitochondrial suspension obtained by

Table II. Effect of washing on mitochondrial respiratory parameters.

	Respiratory Parameters		
	O_2 Utilization (n moles O_2/min/ml/mg protein)	ADP/O	RCR
Unwashed	6.25	3.28	3.5
Washed	20.5	3.40	11.0

The rate of O_2 consumption noted was during the second cycle of ADP addition. ADP/O values were measured from the polarographic tracing from the ratio of ADP esterified to the n atoms of oxygen consumed. RCR values were calculated from the ratio of O_2 consumption in State III to O_2 consumption in State IV. α-ketoglutarate was used as substrate.

differential centrifugation was subdivided into 3 separate bands. The 3 bands occurred at the interface of the four sucrose solutions used (see Chapter II, Section B). The uppermost band (between 1.11 M and 1.31 M sucrose) was the only band to display any oxygen consumption and response to ADP addition. This uppermost band was therefore termed the mitochondrial band.

A comparison of the respiratory parameters of washed and sucrose gradient purified mitochondria (Table III) indicated that the latter purification step excluded extraneous non-oxygen consuming protein from the washed mitochondria (increase of oxygen consumption) but did not significantly improve ADP:O ratio or RCR values (since these values are independent of protein).

Since the mitochondrial band was by far the largest, it was therefore necessary to determine if, in fact, it contained the ATPase activity observable in a washed mitochondrial preparation.

As shown in Table IV, the mitochondrial band displayed the majority of ATPase activity observable in a washed mitochondrial preparation. The high specific activity recorded in the third band was thought to be subject to a large source of error inherent in its calculation. The protein concentration of this band was extremely low and the optical density change in the phosphate analysis was only 0.010 units, which is in the same order of magnitude as the reliability of the assay.

In consideration of the fact that the majority of the total ATPase activity was in the mitochondrial band and that the respiratory parameters of washed and SDG purified mitochondria were similar, it

Table III. Comparison of respiratory parameters of mitochondria obtained by washing and sucrose density gradient purification procedures.

	Respiratory Parameters		
	O_2 Utilization (n moles O_2/min/ml/mg protein)	ADP/O	RCR
Washed	18.4	3.21	13.0
SDG Purified	25.5	3.15	18.2

Polarographic analysis was conducted in an assay media of the following composition: 0.3 M mannitol, 25 mM TES (KOH) pH 7.2 at 25°, 4 mM $MgCl_2$, 0.075% (w/v) BSA 5 mM H_3PO_4, 8 mM α-keto-glutatate, 5 mM malonate and 0.07 mM thiamine pyrophosphate.

Table IV. ATPase activity of the various fractions obtained by sucrose density gradient centrifugation of a mitochondrial suspension

Band No.	Specific Activity (n moles P_i/min/mg protein)	Total Protein mg	Total Activity n moles P_i/min	%
1	11.10	6.30	69.80	88.0
2	9.66	0.66	6.38	8.1
3	21.08	0.14	2.95	3.9

The sucrose density step gradient was prepared in a 5 ml cellulose nitrate tube as follows: 0.5 ml 1.75 M, 1.4 ml 1.40 M, 1.7 ml 1.31 M, and 1.4 ml 1.11 M sucrose. About 100 to 150 µl of the mitochondrial suspension was layered on to the top of the prepared tubes and the separation into bands was accomplished using an SW 50.1 rotor in a Spinco L2 65B ultracentrifuge set at 42,000 rpm for 210 min at 4°. The fractions with a high protein content (bands) were separated, centrifuged and suspended. Assay conditions were 0.3 M sucrose, 3 mM $MgCl_2$ 3 mM Tris-ATP and 25 mM TES (Tris) pH 7.2 at 25°.

was felt that the time consuming procedures involved in purification by SDG were not warranted. The respiratory parameters of SDG purified mitochondria were analyzed about 12 hours after the initial grinding of the cotyledons.

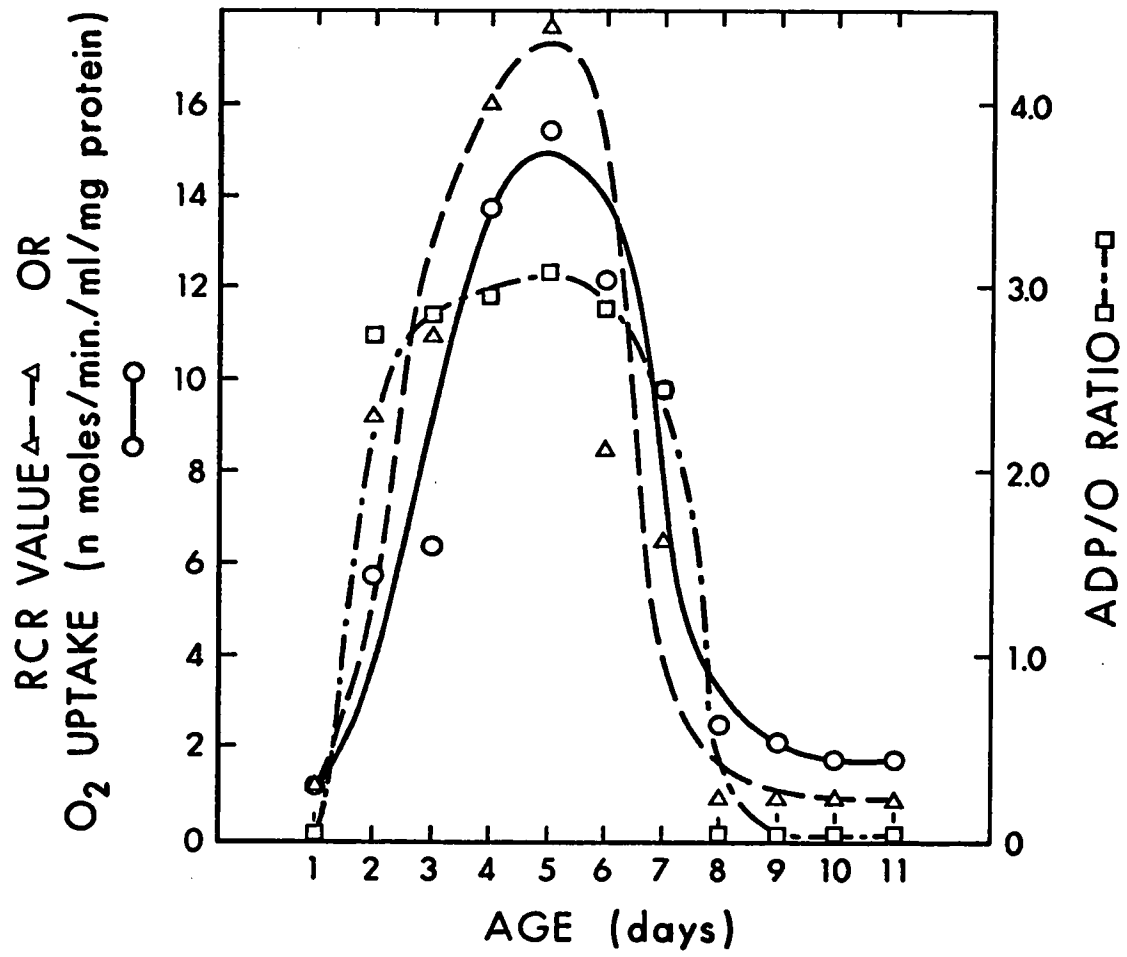
The enzymatic activity of each of the three bands obtained by SDG is presently under investigation in this laboratory. It has been found that the mitochondrial band exhibits high activities of pyruvate dehydrogenase, succinate dehydrogenase and cytochrome oxidase. Electron micrography has confirmed that peroxisomes were found only in bands 2 and 3 that were also rich in peroxidase and catalase activities (Malhotra and Solomos, personal communication).

E. Selection of Tissue Age

After initiation of germination, cotyledons of epigeally growing plants begin to senesce. However, prior to the point of continual degradation, there must be a state after induction of germination wherein the cell is working maximally. It was during this particular period that mitochondria from that tissue would be the most mature and fully developed, show the highest degree of integrity, and thus would be the most desirable for this study.

Mitochondria were isolated from pea cotyledons, harvested at daily intervals after the initiation of germination. It was observed (Figure 8) that mitochondria isolated from 5 day old tissue exhibited maximal respiratory parameters when α-ketoglutarate was used as substrate. By nine days after germination, the mitochondria exhibited a low rate of oxygen utilization as well as characteristics of

Figure 8. Change of respiratory parameters of pea cotyledon mitochondria with increasing tissue age. The tissue was 1 day old, 24 hr after the addition of water to the seeds. Parameters were measured in a polarograph utilizing a Clark oxygen electrode at a temperature of 25°. The substrate utilized was α-ketoglutarate (8 mM). ADP/O and respiratory control ratio (RCR) values were measured by the addition of 300 n moles of ADP.


RCR VALUE Δ—Δ OR
O2 UPTAKE (n moles/min./ml/mg protein) O—O
ADP/O RATIO □--□
0
2
4
6
8
10
12
14
16
0
1.0
2.0
3.0
4.0
1 2 3 4 5 6 7 8 9 10 11
AGE (days)

uncoupled mitochondria. This bell shaped curve of respiratory parameters has also been shown to exist in pea cotyledons by Kolloffel and Sluip (1970). Mitochondria from tissue exhibiting hypogeal growth characteristics (bean) have been described as displaying an inverted bell-shaped curve of ADP:O ratios with increasing age of tissue (Stinson, 1968). However, the rate of oxygen consumption of these mitochondria was found to be maximal 4 to 5 days after germination.

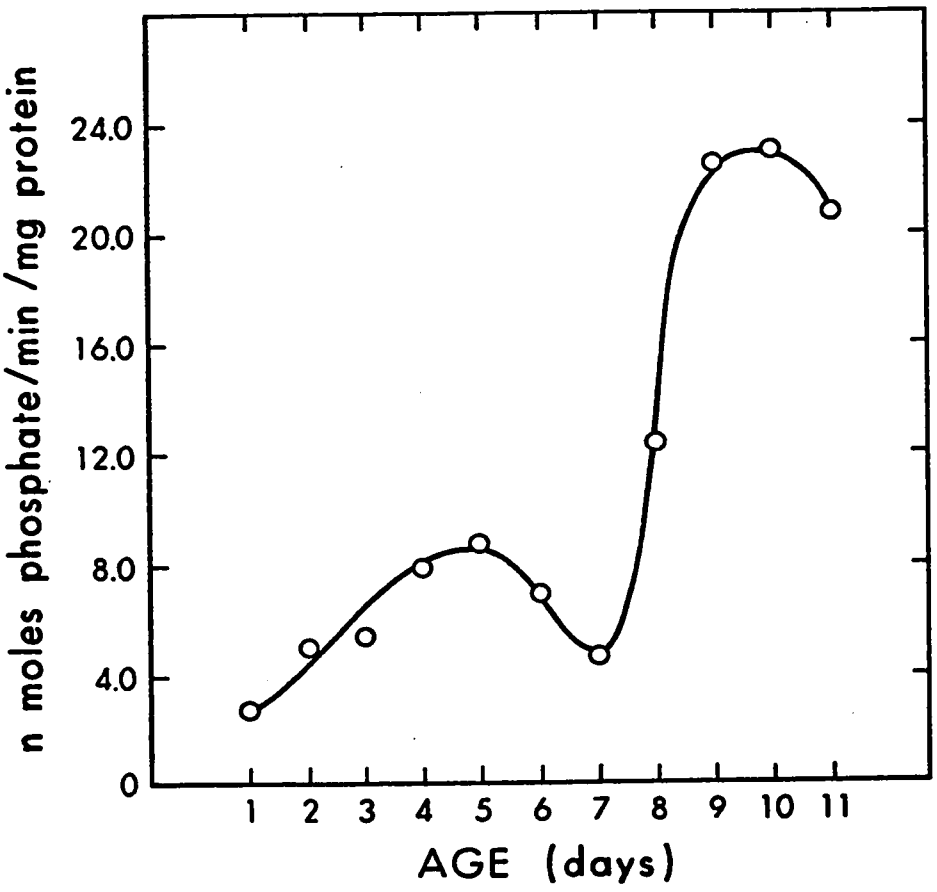
Experiments that involve maceration of tissue, are highly dependent on the methods employed. It may be entirely possible, by altering the procedures of extraction and components present, that mitochondria that exhibit a high degree of respiratory control can be isolated from 9 day old pea cotyledons. Thus it cannot be assumed because the mitochondria extracted from 9 day old tissue using this particular extraction technique were uncoupled, that the mitochondria in the unmacerated tissue were in this state. There was no study made of different tissue maceration procedures during the present work. The methods employed were thought to be as gentle as possible so as to minimize mechanical damage to the cellular components. It was found that disregard of gentle use of the pestle during maceration inevitably led to uncoupled mitochondria. During the grinding of the tissue in the present study, the angle and place that the pestle made contact with the mortar was recognized as being of importance with respect to reproducibility of quality of isolated mitochondria.

While the basic aim of this study was to undertake characterization of the ATPase activity of intact mitochondria, it was also of interest to observe the ATPase activity - age profile. From Figure 9,

it was observed that the ATPase activity - age profile initially followed the respiratory parameters, reaching a maximal value when the respiratory parameters were maximal. However, the activity was found to increase several fold when the respiratory control ratio of the same mitochondria indicated the existence of an uncoupled state.

The increase in ATPase activity displayed in uncoupled mitochondria would be expected since the rate of enzymatic activity would be independent of the rate of reversed electron transport through the electron transport chain (ETC). The fact that the ATPase activity initially increased concomitantly with the respiratory parameters, may be indicative that during this period, the mitochondrion has not attained its full complement of electron carriers, that the components of the chain are not in a fully organized state for efficient transfer of reducing equivalents or that the increase in activity could be a result of de novo synthesis of ATPase. (The increase of the mitochondrial activity in the first period of imbibition of castor bean seeds involves activation rather than de novo synthesis of enzymes (Lade, 1966). Alternatively, certain components of the membranes of the mitochondria could be lacking and might restrict the passage of substrate as well as ATP. However, the fact that the ADP:O ratio increases concomitantly with oxygen consumption would tend to negate this latter theory. The ADP:O ratio would remain unaltered if respiratory substrate were limiting. These hypotheses would hence imply that the mitochondria of ungerminated seeds are not 'true mitochondria'. Studies of the biogenesis of mitochondria are currently underway by various researchers. However, it has not been determined whether or

Figure 9. Variation of ATPase activity of pea mitochondria with the age of tissue. Assay conditions were 0.3 M mannitol, 25 mM TES (Tris) pH 7.2 at 25°, 3 mM $MgCl_2$, 3 mM Tris-ATP, and about 0.5 mg mitochondrial protein in a total volume of 1.0 ml. Incubation was at 25° for 15 min.


n moles phosphate/min /mg protein
0
4.0
8.0
12.0
16.0
20.0
24.0
1 2 3 4 5 6 7 8 9 10 11
AGE (days)

not mitochondria are synthesized *per se* or are formed from pre-existing structures.

The possibility that the grinding procedures used for the young tissue enhanced the loss of soluble ETC components (NAD+ and cytochrome c) from these mitochondria was ruled out because addition of these components to the polarographic assay mixture did not enhance the respiratory parameters of the mitochondria. This loss is known to occur for bean cotyledon mitochondria (Malhotra and Spencer, 1970).

CHAPTER IV

CHARACTERIZATION OF RESPIRATORY PARAMETERS

A. Reproducibility of Polarographic Analysis Procedures

1. Among Similar Samples

To be able to discuss changes in respiratory parameters with any degree of certainty, one must be aware of the variability among different samples from the same mitochondrial suspension. In order to minimize the possibility of variation in the dissolved oxygen concentration, respiratory assay mixtures were stored frozen, thawed and incubated in the constant temperature water bath from which the water circulated to maintain the constant temperature of the respiratory assay mix during the analysis. Since extreme frothing resulted from purging the solution with air (due to presence of BSA), air saturation of the solution was achieved by flushing the volume above the solution in the bottle with air, stoppering the bottle and shaking. This procedure was arbitrarily done a minimum of three times since it was found that after the second such treatment there was no recordable change in the percent oxygen saturation when introduced into the biological oxygen monitor apparatus. Without these treatments it was observed that the oxygen monitor would record different levels of saturation for each sample that was introduced.

The 3.0 ml respiratory substrate solution was introduced into the reaction well of the monitor with the use of a 5.0 ml graduated pipet. Addition of 100 µl of mitochondria suspension was made by using a 250 µl syringe (Hamilton) and addition of 25 µl of ADP solution

with a 50 µl syringe. As shown in Table V, when 3 separate determinations were made on one sample of mitochondria, there was some variation. For comparison purposes, the second cycle of ADP addition was used because of the variation experienced within one sample determination, as will be discussed later (Section A 2.). Most of the variation noted here was felt to be ascribable to the actual measuring of the rates of oxygen consumption after the determination. The width of the pen tracing would account for large errors especially if the rate of oxygen utilization was extremely rapid. The gain control of the recorder was adjusted so as to give a short response time, but not give vibrations. However, this led to very short jumps of the pen so that, in essence, when determining rates, one was drawing a straight line through a sinusoidal tracing. Despite these obvious drawbacks and as a result of repeated trials it was thought that each trace was accurate to within $\pm$ 5%. Any effects that were observed due to addition of any compound were considered significant only if the difference was greater than 5%.

2. Within One Determination

The respiratory control ratio (RCR) of washed mitochondria from pea cotyledons isolated during this study was found to be extremely high in comparison to values obtained from other plant tissue. Values observed for bean mitochondria from this laboratory have been reported between 2 and 4, which is in the normal range for plant tissue. The RCR values observed for pea mitochondria were at least greater than 10 for the second cycle and frequently approached infinity for the

Table V. Variation of respiratory parameters among similar samples of one mitochondrial suspension

	Respiratory Parameters			
	O_2 Utilization (n moles O_2/min/ml/mg protein)		ADP/O	RCR
	State III	State IV		
Sample 1	21.0	2.2	3.30	9.15
2	19.0	1.9	3.26	10.0
3	23.0	2.0	3.18	11.5

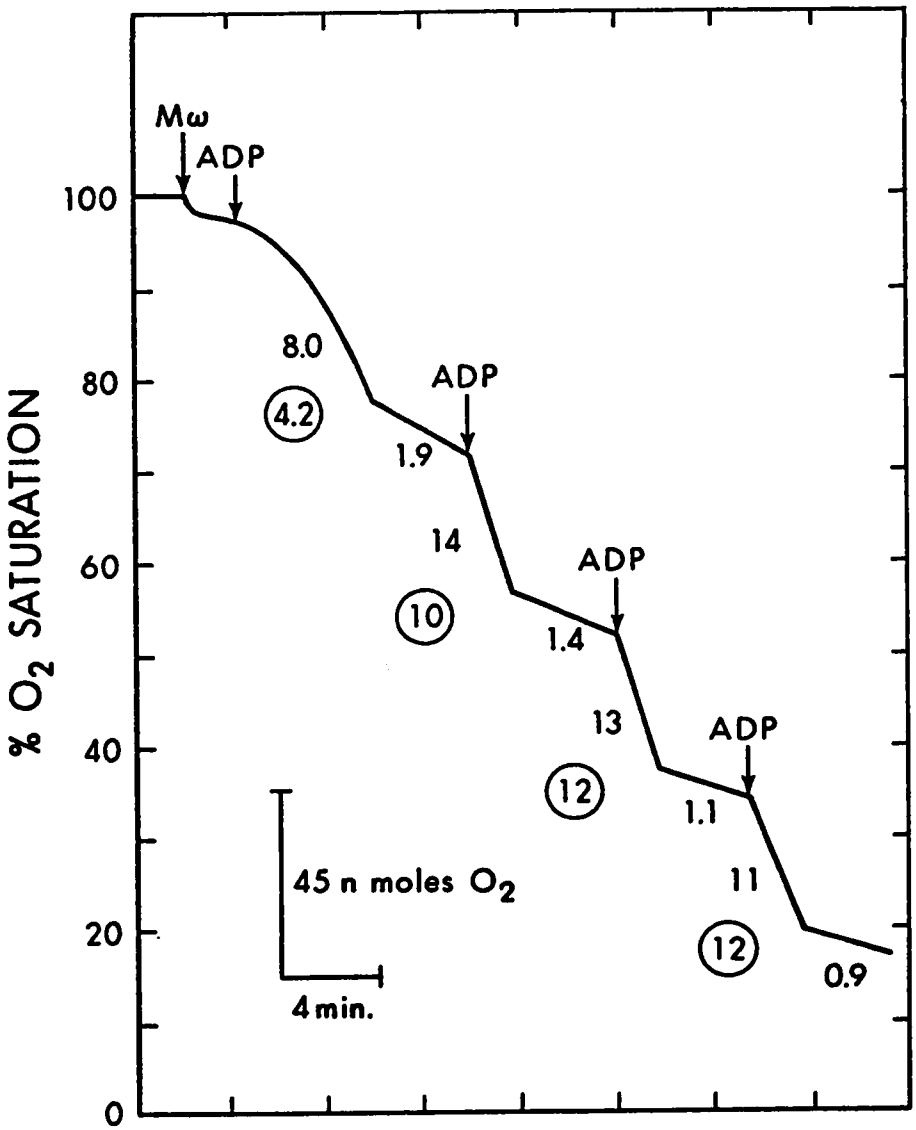
The respiratory parameters noted are for the second cycle of ADP addition. State III respiration is in the presence of, and State IV in the absence of, ADP. Values were obtained from the polarographic tracings. α-ketoglutarate was used as substrate.

fourth cycle of ADP addition. RCR values as high as these have not been observed in the literature for plant mitochondria except for NADH oxidation (Jones et al. 1964). As a criterion for further studies of the mitochondria, only preparations exhibiting this high degree of respiratory control were utilized. It was observed during the study that the isolated mitochondria either exhibited this high degree of control or none whatsoever (they were either very good or very bad, without intermediate degrees).

Figure 10 indicates a typical recorder tracing of oxygen consumption of isolated pea mitochondria with α-ketoglutarate as substrate. The initial decline of oxygen concentration observed immediately after the addition of 1 mg of mitochondrial protein was thought to result from the addition of the anaerobic solution (anaerobiosis being a result of oxidation of endogenous substrates). The ADP:O ratio of the first cycle was observed to be consistently low in each preparation made. The rate of oxygen utilization during this cycle slowly increased to a steady-state value; however, this State III value was lower than that of subsequent cycles. These phenomena of the first cycle could possibly be a result of swollen mitochondria undergoing contraction into a tightly-coupled particle, since addition of ATP was found to overcome this lag. The presence of respiratory substrates and ADP or ATP is known to induce contraction of plant mitochondria (Stoner and Hanson, 1966). Wiskich and Bonner (1963) have also recorded a delay in response to the initial addition of ADP that could be overcome by ATP.

Subsequent additions of ADP gave high ADP:O ratios that did not

Figure 10. A typical polarographic trace of oxygen consumption by pea mitochondria extracted from five day old tissue. Composition of assay mixture (3.0 ml) was 0.3 M mannitol, 50 mM TES (KOH) pH 7.2 at 25°, 4 mM $MgCl_2$, 5 mM phosphate, 0.075% (w/v) BSA, 5 mM malonate, 0.07 mM thiamine pyrophosphate and 8 mM α-ketoglutarate. Additon of about 1 mg washed mitochondria (M_w) was made immediately and was followed by additions of ADP (600 n moles in 25 µl). Recordings were made on a Beckman 100 mv potentiometric recorder at a chart speed of 0.5 in per min. Rates of O_2 uptake (n moles O_2/min/ml/mg protein) are noted beside the tracing. Respiratory control ratio values (RCR) are circled for the appropriate cycle of ADP addition.


Mω
ADP
100
80
60
40
20
0
% O2 SATURATION
8.0
4.2
ADP
1.9
14
10
ADP
1.4
13
12
ADP
1.1
11
12
0.9
45 n moles O2
4 min.

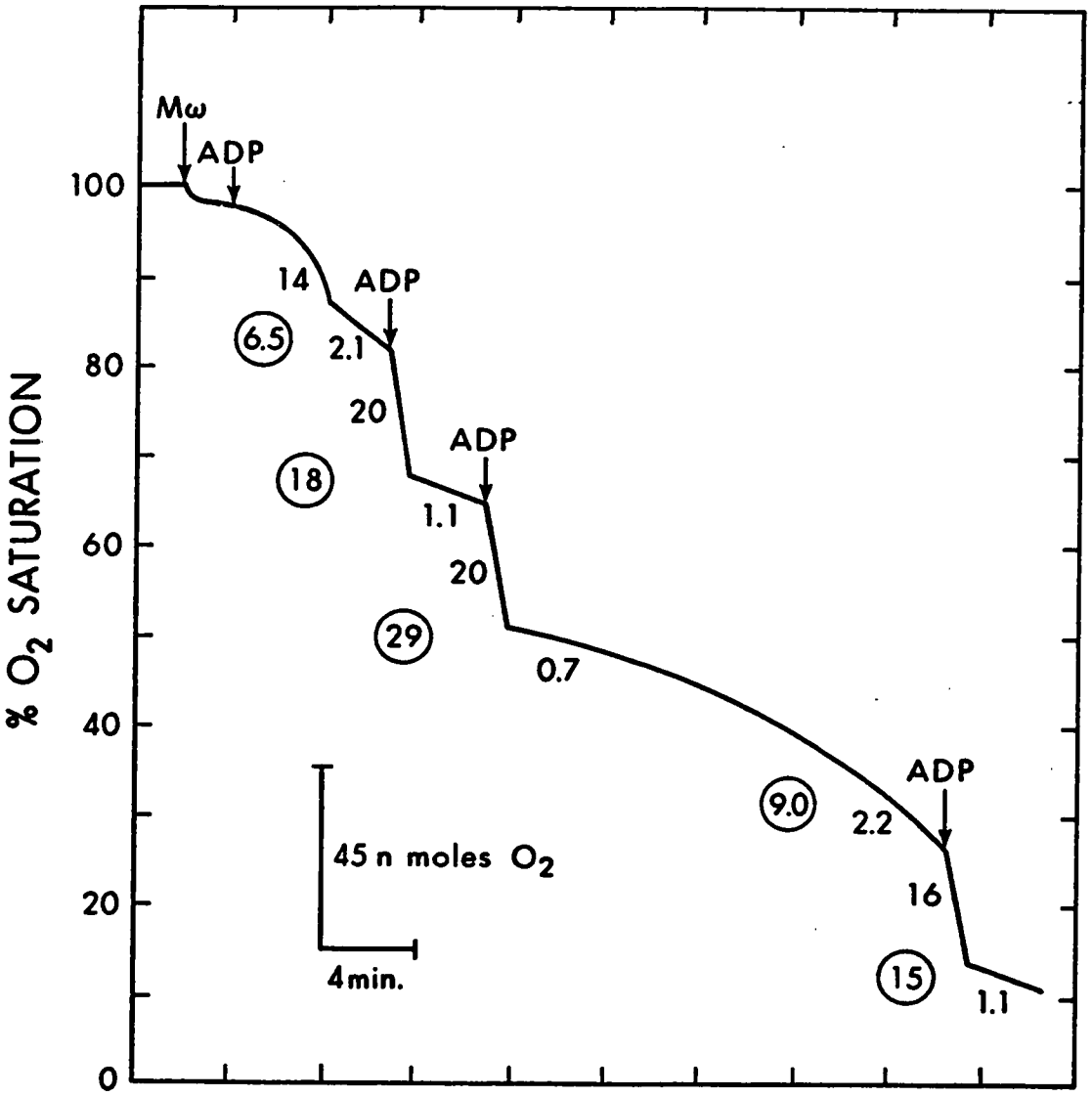
vary appreciably. However, the same did not hold true for RCR values. The respiratory control ratios increased with each cycle and it was observed that this fact resulted from a decline in State IV respiration and not an increase in State III. Since the possibility existed that the accumulation of ATP within the mitochondria was causing this observation, ATP was added at the beginning of the assay. However, an increase of RCR values with each cycle was still observed. Further studies into this response revealed the possibility that the addition of potassium ions which are in the ADP solution (since the ADP solution was adjusted to pH 7.2 by KOH) might be having a deleterious effect on State IV respiration. As will be discussed later (Section E) potassium ions would not account for this.

Additional observations revealed (Figure 11) that the State IV respiration after three consecutive cycles is left undisturbed would gradually increase to a state comparable to State IV respiration after the first cycle. This result would lend itself to the hypothesis that with each cycle the mitochondria contract an additional amount. This state of contraction is unstable and results in the controlled hydrolysis of ATP to ADP thereby increasing respiration. Many investigators have related State IV respiration to ATPase activity of the mitochondria. Alternatively, complete exhaustion of ADP would limit substrate level phosphorylation in theoxidation of α-ketoglutarate (succinyl C_oA synthetase).

B. In Vitro Ageing Characteristics

The mitochondria used in this study were isolated each morning

Figure 11. Polarographic trace of oxygen consumption of mitochondria if left undisturbed after 3 successive additions of 600 n moles of ADP each. Substrate utilized was α-keto-glutarate. O_2 uptake values are noted beside the tracing and RCR values are circled.


Mω
ADP
100
14
ADP
6.5
2.1
80
20
ADP
18
1.1
60
20
29
0.7
40
ADP
9.0
2.2
45 n moles O2
20
16
4min.
15
1.1
0
% O2 SATURATION

and were stored in an ice bath for the remainder of the day. Since various analyses were performed on these preparations over a period of up to 10 hours, it was desirable to know if the mitochondria displayed the same respiratory parameters after such long storage times as they did when freshly isolated. As can be seen in Table VI the respiratory parameters remained relatively constant over a period during which any analysis would be performed. The parameters exhibited by mitochondria 26 hours after isolation were still quite acceptable; however, by 50 hours after isolation the parameters had significantly changed. The data in Table VI were taken for the second cycle of the estimations and it was noted that the first cycle, which was normally slow with a low ADP:O ratio, became increasingly sluggish with still lower ADP:O ratios as in vitro ageing increased. The rates of State III respiration during subsequent cycles did remain relatively constant. Perhaps the mitochondria became increasingly swollen during the storage period and an increasing amount of time was required for them to contract upon the initial ADP addition.

In view of the above study, pea mitochondria exhibited a very high degree of stability upon isolation in comparison to other plant tissue. Because of this stability, it was thought that any analyses performed using the preparation, up to the 12 hour storage times indicated, were performed on intact mitochondria.

C. Respiratory Substrate

As has been reported by other workers (Beevers, 1961) it was found (Table VII) that mitochondria display different rates of oxygen

Table VI. Change in respiratory parameters of pea mitochondria with *in vitro* age at 0°.

Hours after Isolation	O_2 Utilization (n moles/min/ml/mg protein)	ADP/O	RCR
0	22	3.40	15.0
3	20	3.32	11.5
12	23	3.44	12.5
26	19	3.08	9.5
50	14	2.60	3.5

The mitochondria were suspended in 0.3 M sucrose, 3 mM MgCl, 25 mM TES (Tris) pH 7.2 at 25°, at a concentration of 20 mg protein/ml and kept at 0-4° in an ice bath. Respiratory parameters were noted for the second cycle of ADP addition.

utilization in the presence of various substrates. It was observed that with succinate there was a more rapid utilization of oxygen than with α-ketoglutarate. Since both substrates displayed an increase of RCR value with each ADP addition (Figure 12) these aforementioned effects cannot be ascribed to limited uptake of α-ketoglutarate specifically. The observation that the rate of succinate oxidation exceeded that of α-ketoglutarate was opposite to that of Wiskish and Bonner (1963) wherein decreased succinate utilization was thought to be a result of accumulation of inhibitory levels of oxalacetate. A lack of available NAD^+ for α-ketoglutarate oxidation was improbable since exogenous NAD^+ addition did not enhance the rate of oxygen utilization.

Uncoupled succinate oxidation was found to be inhibited by oxalacetate and relieved by subsequent addition of ATP in accord with observations with animal mitochondria (Wojtezak, 1969).

D. Presence of Inhibitors and Uncouplers

In order to further elaborate on the integrity of the mitochondrial preparations, the presence of respiratory inhibitors and uncouplers of oxidative phosphorylation during polarographic analysis was observed. It was found that the mitochondria responsed to the addition of sodium azide and 2,4-dinitrophenol (DNP) in a typical fashion. The inhibitory action of azide and uncoupling action of DNP are shown in Figure 13.

The inhibitory action of azide was observed to occur in the presence of ADP or DNP. According to Zayagilskaya et al. (1969) azide is thought to have multiple effects (See Figure 13). In this

Table VII. Rates of oxygen utilization by pea mitochondria with various substrates.

Substrate	O_2 Utilization (n moles O_2/min/ml/mg protein)
α-ketoglutarate	19.0
succinate	40.0
isocitrate	28.0
malate	15.0
pyruvate	24.0

Each substrate concentration was 8 mM in the polarographic assay mixture. Mannitol and TES (KOH) pH 7.2 at 25° were the osmoticant and buffer system used, respectively.

Figure 12. Effect of respiratory substrate on respiratory parameters of pea mitochondria. Substrates utilized were α-ketoglutarate (8 mM) and succinate (8 mM). ADP additions were 600 n moles for α-ketoglutarate and 300 n moles for succinate. Rates of O_2 uptake were noted beside the tracing and RCR values circled.

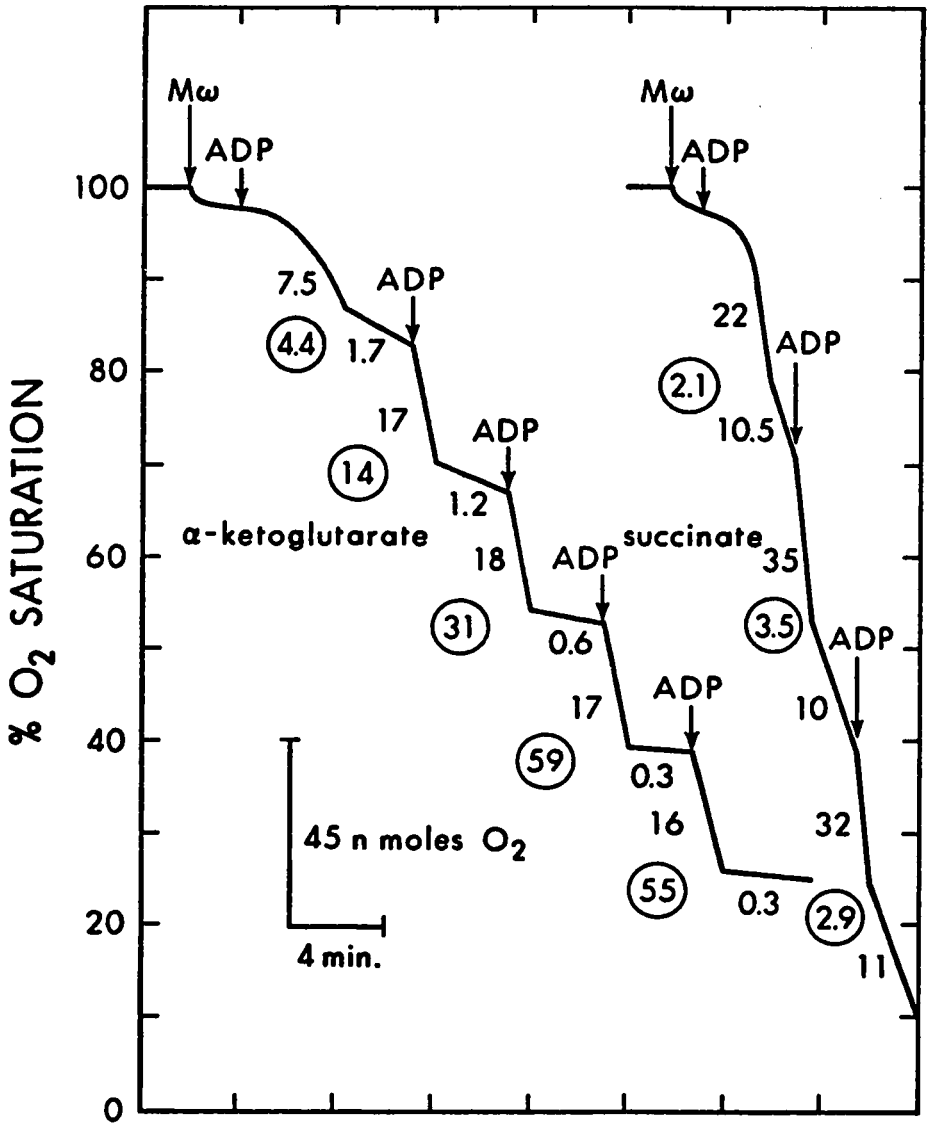
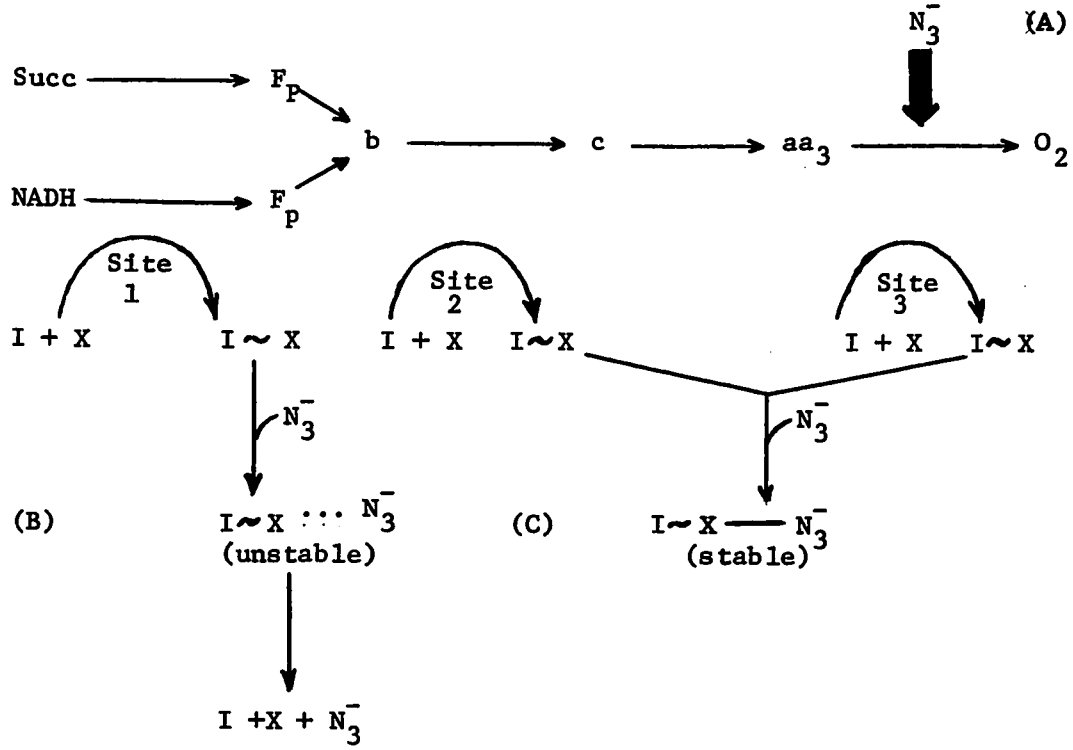

Mω
ADP
100
80
60
40
20
0
% O2 SATURATION
7.5
4.4
1.7
17
14
1.2
α-ketoglutarate
18
31
0.6
17
59
0.3
16
55
0.3
45 n moles O2
4 min.
Mω
ADP
22
2.1
10.5
succinate
35
3.5
10
32
2.9
11

Figure 13. Proposed sites of action of azide and DNP. Respiratory inhibition by azide (upper portion) as described by Zayagilskaya et al. (1969). Cytochrome oxidase is inhibited by azide (A) in the conventionally accepted fashion. From differential effects on succinate oxidation he describes interaction of azide with Site I similar to an uncoupling activity (B) in which the complex of I~X and azide is unstable. In addition, he suggests an interaction of azide with I~X from Sites 2 and 3 to form a stable complex (C) and therefore removes the carriers from participation in oxidative phosphorylation.

The effect of DNP on respiration is thought to be concentration dependent (as outlined by Hanson and Hodges (1968)). At low DNP concentrations the I~X formed during electron transport is cleaved. (The "loose coupling" designation accounts for State IV respiration of mitochondria). At high DNP concentration, the components of the energy transfer reactions are complexed with DNP; this therefore explains the inhibitory activity of high DNP concentrations on respiration.


Succ
NADH
F_p
b
c
aa_3
O_2
N_3^-
(A)
Site 1
Site 2
Site 3
I + X
I ~ X
(B)
(C)
I ~ X ⋯ N_3^-
(unstable)
I ~ X — N_3^-
(stable)
I + X + N_3^-

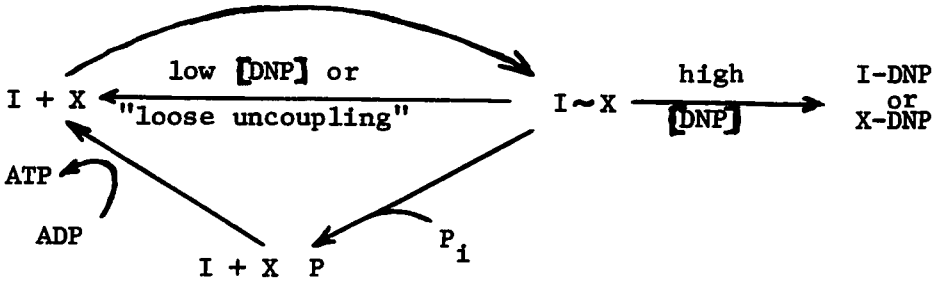

low [DNP] or
"loose uncoupling"
high
[DNP]
I + X
I ~ X
I-DNP
or
X-DNP
ATP
ADP
P_i
I + X P

figure the terminology of Chance and Williams (1956) is used wherein "I" represents a carrier-linked enzyme or reactive group. Azide may act as an inhibitor of electron transport (A), as an uncoupling agent like dinitrophenol at site I by formation of an unstable complex with I~X (B), and as a phosphorylation blocking agent at sites 2 and 3 by formation of a stable complex with I~X (C).

Also shown in this figure is the proposed action of DNP wherein low concentrations promote the hydrolysis of I~X and thereby uncouple oxidative from phosphorylative sequences. At high concentrations, the DNP is proposed to react to produce I-DNP (Chance and Williams, 1956) or X-DNP (Truelove and Hanson, 1966) thus limiting the recycling of I or X and hence limit respiration. The "loose coupling" designation is shown to indicate why most isolated plant mitochondria display high State IV respiration rates and low respiratory control ratios. This spontaneous hydrolysis of I~X was thought to be very low in the preparations made during this study.

E. Effect of Inorganic Cations on Respiration

Inorganic cations have been found to play an increasing role in biochemical reactions as activators of several enzymatic steps (Suelter, 1970). They have been discovered to be allosteric as well as non-allosteric effectors. Their presence in pea mitochondrial ATPase activity studies will be discussed in Chapter V and because of these effects, their addition to polarographic analysis procedures was observed.

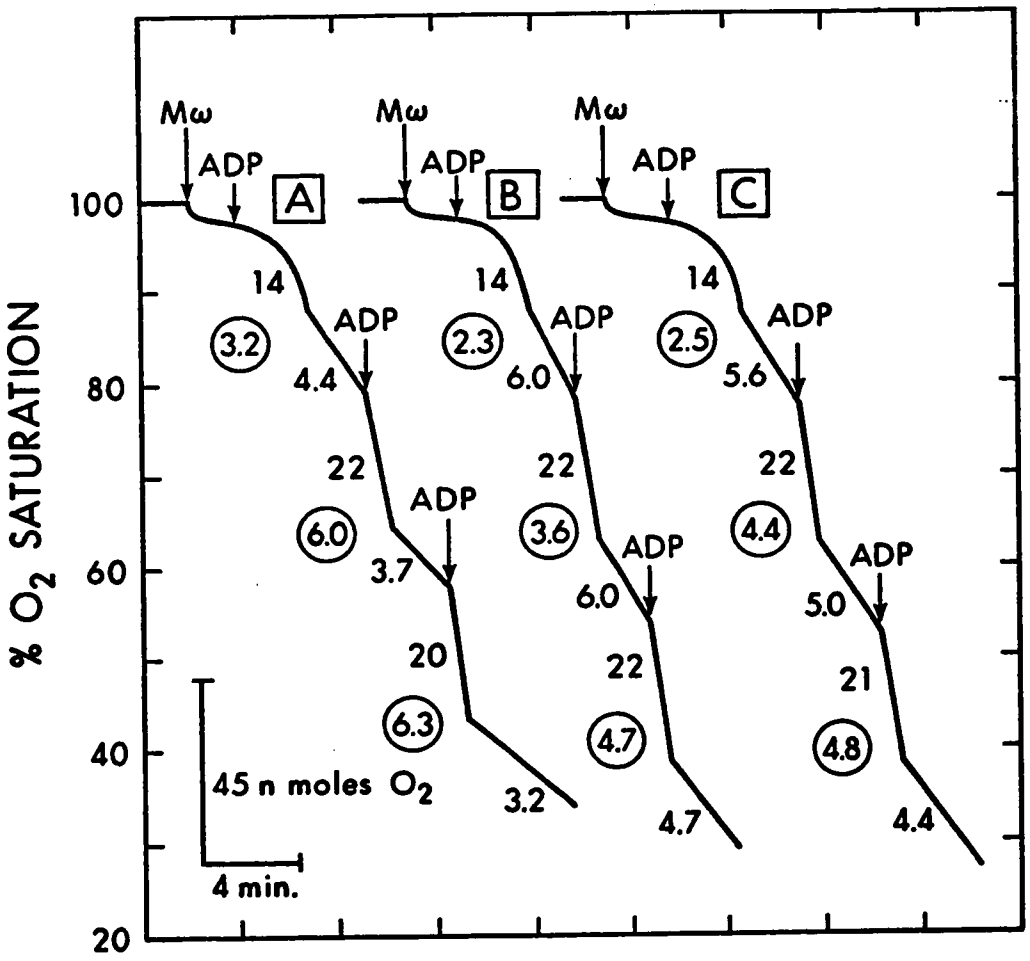
Preliminary investigations revealed that magnesium ion was

essential in mitochondrial preparative procedures in order to obtain mitochondria with a high degree of coupling. Magnesium ions were included in all analysis work.

Although the effect of calcium ions on respiratory parameters was not thoroughly investigated, they were observed to cause a slight (20%) increase in State IV respiration, which would be expected. Calcium ions are known to be accumulated by animal (DeLuca and Engstrom, 1961) and plant (Hodges and Hanson, 1965) mitochondria by an energy-dependent process. It was observed during one of the infrequent experiments in the present study that the Ca^{++}:O consumed was in a ratio of 1.7, which would compare with values of 2.0 reported in the literature (Chance, 1965).

The most thorough investigated cations were the monovalent ions sodium and potassium. As seen in Figure 14, both K^+ and Na^+ were observed to increase State IV respiration resulting in a lowering of the respiratory control ratio. Since only gross changes in rates of respiration can be observed in polarographic analysis, various combinations of Na^+ and K^+ additions to the respiratory assay procedures were not investigated. These additions were found not to affect the ADP:O ratio of the mitochondria so that the increase in respiration was not attributable to a complete uncoupling activity. The increase in respiration may have resulted from an energy dependent accumulation of the ions, with concomitant hydrolysis of ATP and an increase in the steady-state level of ADP (thus stimulating respiration). Alternatively, the ions may have facilitated entry of substrate by causing swelling of the mitochondria (KCl is known to swell mitochondria

Figure 14. Polarographic tracing of O_2 uptake as affected by the presence of monovalent cations. Trace A, α-ketoglutarate as substrate with no addition of cations. Trace B, addition of 90 μ moles KCl was made (to make the solution 30 mM) Trace C - addition of 90 μ moles NaCl was made (to make the solution 30 mM). Monovalent cations were found to stimulate State IV respiration.


% O_2 SATURATION
100
80
60
40
20
Mω
ADP
A
B
C
14
3.2
4.4
22
6.0
3.7
20
6.3
3.2
2.3
6.0
3.6
6.0
22
4.7
2.5
5.6
4.4
5.0
21
4.8
4.4
45 n moles O_2
4 min.

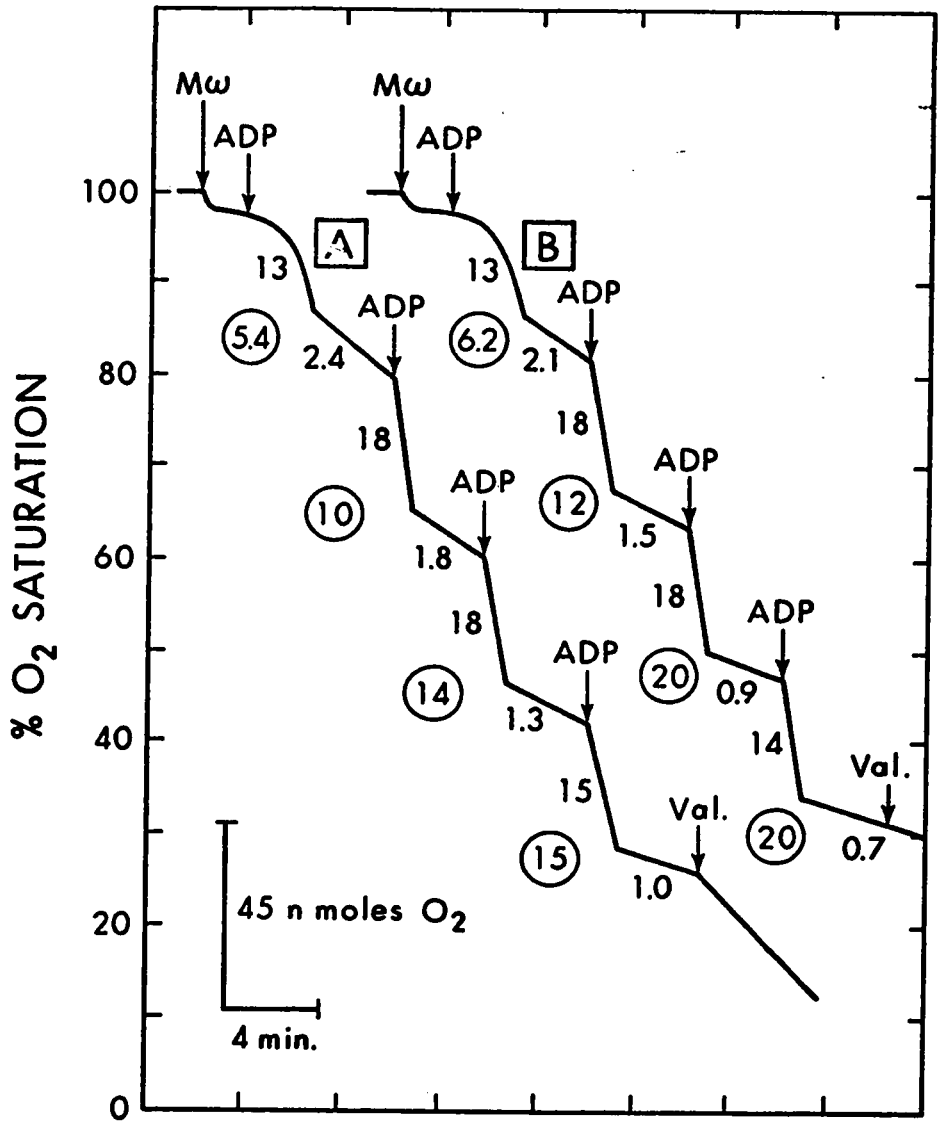
(Hunter and Ford, 1955) and thus increasing the total surface area of the particle. Potassium ions have been shown to increase oxygen utilization of sub-mitochondrial particles, but this response was accompanied by uncoupling activity (Papa et al. 1969). In addition, it is also possible that K^+ may activate an exogenous ATPase activity.

The antibiotic, valinomycin, has been shown to selectively enhance the penetration of K^+ ions through biological membranes (Pressman et al. 1966). The addition of valinomycin after two cycles of ADP addition had been completed in the polarographic analysis with TES (KOH) as buffer was shown to increase respiration whereas no effect was observed if TES (Tris) buffer was used (Figure 15). The small amount of K^+ ions in the TES solution (to make the solution 12.5 mM K^+) did not by itself result in an observable change in State IV respiration. The action of the valinomycin must have been to facilitate entry of these ions in sufficient quantity to result in this observable increase in respiration.

F. Effect of Osmoticant on Respiratory Parameters

As was discussed previously (Chapter III A 2(i)) the presence of mannitol during the analysis of ATPase activity by the phosphate determination procedure was found to be very detrimental to sensitivity. The use of sucrose as an osmotic stabilizer was adopted after this observation. However, when sucrose was used in solutions for polarographic analysis, a considerable decline in the respiratory control ratio was noticed. Typical observations of this type can be seen in Figure 16. This figure includes not only results where both mannitol

Figure 15. Effect of valinomycin addition during polarographic analysis in the presence of assay mixture wherein the buffer TES was neutralized to pH 7.2 with KOH (A) or Tris (B).


% O_2 SATURATION
100
80
60
40
20
0
Mω
ADP
A
B
13
5.4
2.4
18
10
1.8
14
1.3
15
1.0
6.2
2.1
12
1.5
20
0.9
0.7
Val.
45 n moles O_2
4 min.

and sucrose were utilized,but also their use when potassium hydroxide or Tris was used to adjust the buffer TES to pH 7.2.

The presence of K^+ ions in the assay mixtures was not found to significantly affect the level of State IV respiration. As to whether they were in too low a concentration (their concentration would be one-half that of TES or about 12.5 mM) to result in significant increases in respiration or whether, whilst in a complex with the buffer, they were unavailable for uptake by the mitochondria, could not be determined. Tris has been described as being detrimental to integrity of mitochondria from bean cotyledons (Stinson and Spencer, 1969); however this effect was not observed in the present study with pea cotyledon mitochondria.

The fact that State IV respiration was higher in the presence of sucrose than of mannitol may have been because mannitol exerted a more effective osmotic pressure than the sucrose (since part of the sucrose is taken up into the 'sucrose space' of the mitochondria). The mitochondria would then be closer to a contracted state in mannitol than in sucrose. An increase in the sucrose osmolarity to 0.40 M did not decrease the State IV respiration appreciably. The amount of sucrose that enters into the 'sucrose-space', since it is passive transport, would depend on the external sucrose concentrations so that, in effect, by increasing the osmolarity by 0.1 M we are not increasing the effective osmolarity by that amount. The hypothesis of mannitol and sucrose of the same molarity resulting in different states of mitochondrial contraction would leave the mitochondria in the sucrose with the larger surface area and hence, perhaps, greater penetration

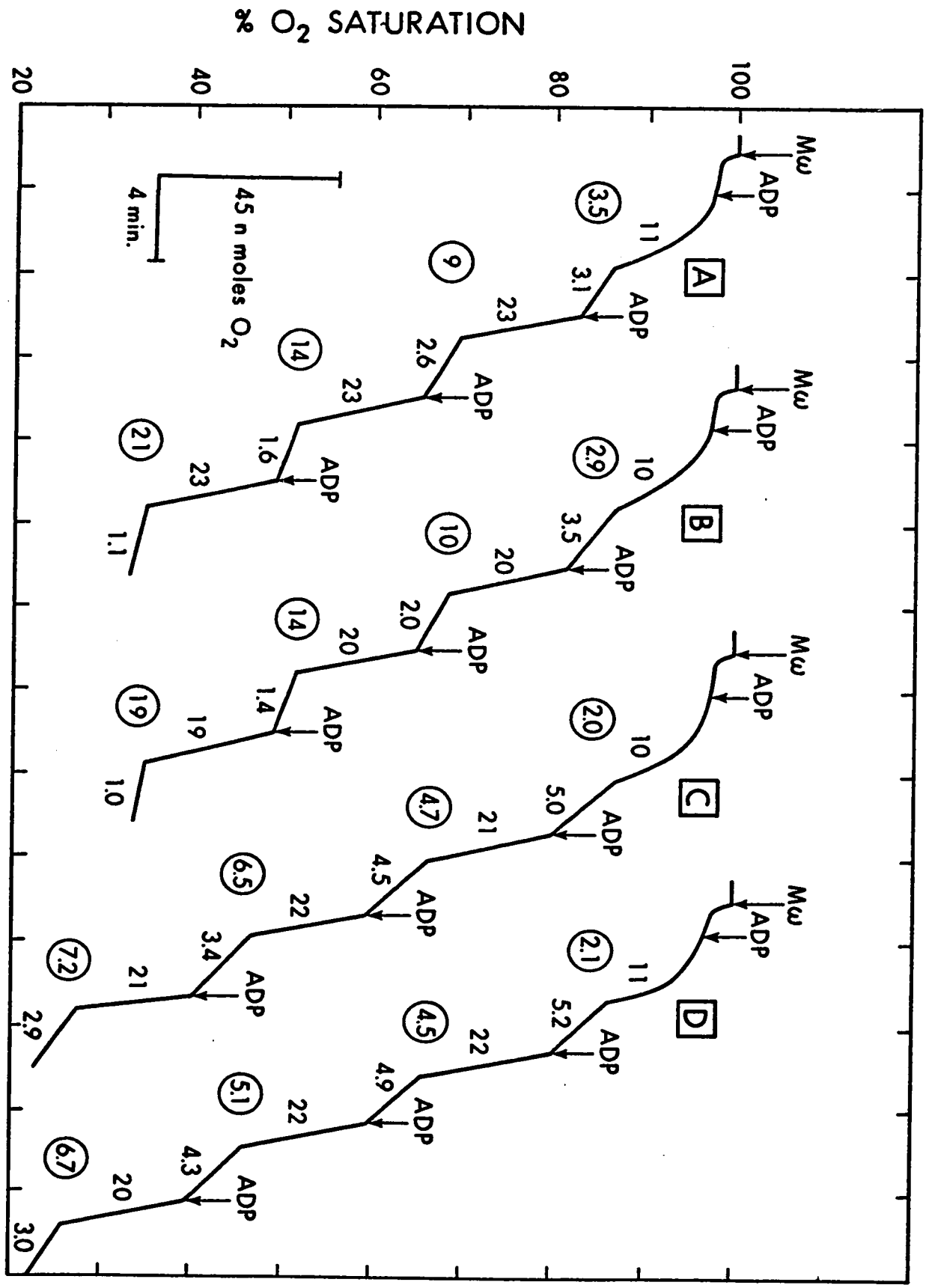
Figure 16. Effect of osmoticant on respiratory parameters of pea mitochondria. Compositions of polarographic analysis solutions were as in Figure 10 except for variation of osmoticant and ion used to adjust the buffer TES to pH 7.2.

A. 0.3 M mannitol and KOH

B. 0.3 M mannitol and Tris

C. 0.3 M sucrose and Tris

D. 0.3 M sucrose and KOH


% O_2 SATURATION
20
40
60
80
100
4 min.
45 n moles O_2
Mω
ADP
A
B
C
D
3.5
11
3.1
9
23
2.6
14
23
1.6
21
23
1.1
2.9
10
3.5
10
2.0
14
20
1.4
19
19
1.0
2.0
10
5.0
4.7
21
4.5
6.5
22
3.4
7.2
21
2.9
2.1
11
5.2
4.5
22
4.9
5.1
22
4.3
6.7
20
3.0

area for substrate and therefore a greater rate of respiration. However, the difference between the use of sucrose or mannitol in State III respiration was negligible so that we would have to assume that the substrate during State III is actively transported and during State IV passively transported. The active transport process would be independent of the surface area of the mitochondria (once some contraction has taken place during the first cycle) and the passive (State IV) transport would be surface area dependent. Alternatively, there is the possibility that mannitol hinders the passive uptake of substrate or sucrose aids the uptake.

CHAPTER V

STUDY OF MITOCHONDRIAL ATPASE ACTIVITY

Extensive studies have been made in recent years of ATPase from mitochondria. This enzyme activity has been that of sub-mitochondrial particles obtained either by the method of Keilin-Hartree (1940), by sonication of mitochondria (Green et al. 1958) or by treatment of mitochondria with detergents (Cooper and Lehninger, 1956). A 'soluble ATPase' isolated from beef heart and rat liver sub-mitochondrial particles (Pullman, Penefsky, Datta and Racker, 1960) has been described as the 'coupling factor' between oxidation and phosphorylation. These 'soluble ATPase' preparations are cold labile, and, since their activity was found to be about ten times that of mitochondrial ATPase stimulated by DNP, they are representative of the latent ATPase of intact mitochondria (Pullman and Penefsky, 1963 and Racker, 1965). It was this latent ATPase activity, according to the terminology of Racker, with which the present study would be concerned. Analyses by previous workers (Reid et al. 1964, and Takeuchi et al. 1969) would indicate extremely low ATPase activity of intact plant mitochondria. The low activity observed in the present study, was found to be significant if methods of analysis are sufficiently sensitive.

There have been relatively few studies of ATPase activity in intact mitochondria and those that have been done are, in general, on mitochondria isolated from animal tissue. Studies on mitochondria isolated from plant tissue have received comparatively little attention, as indicated in the literature review (Chaper I, Section D.2.)

A. Analysis of Assay System

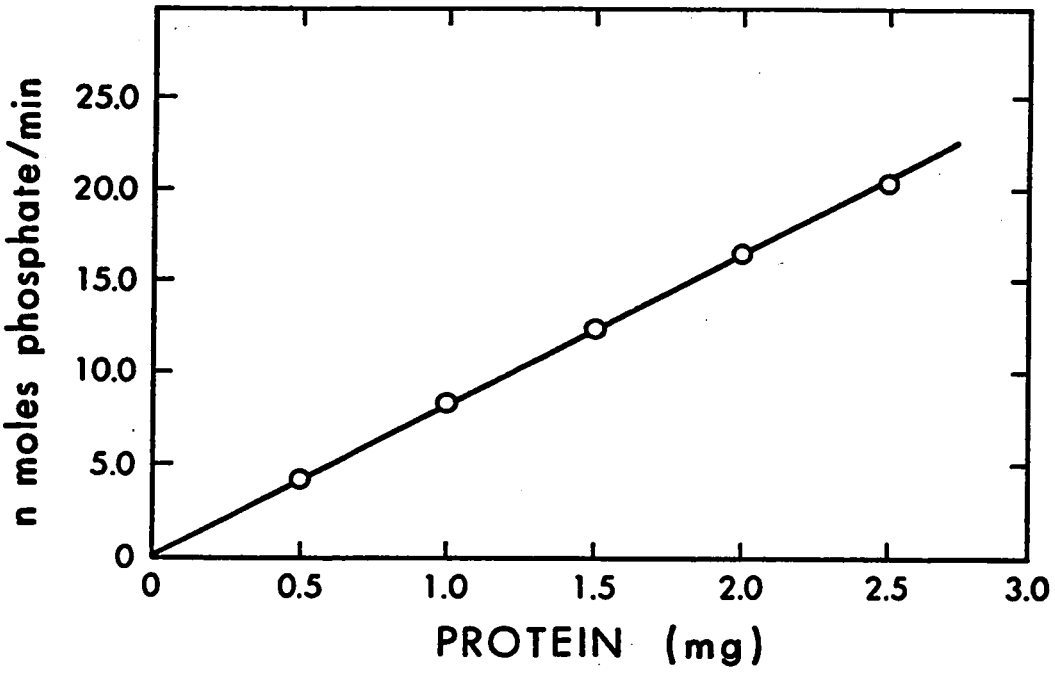
As in the study of any enzymic reaction, preliminary characterization of the test system must be established so as to attain optimum conditions. These studies involved initial velocity studies according to Michaelis-Menten kinetics.

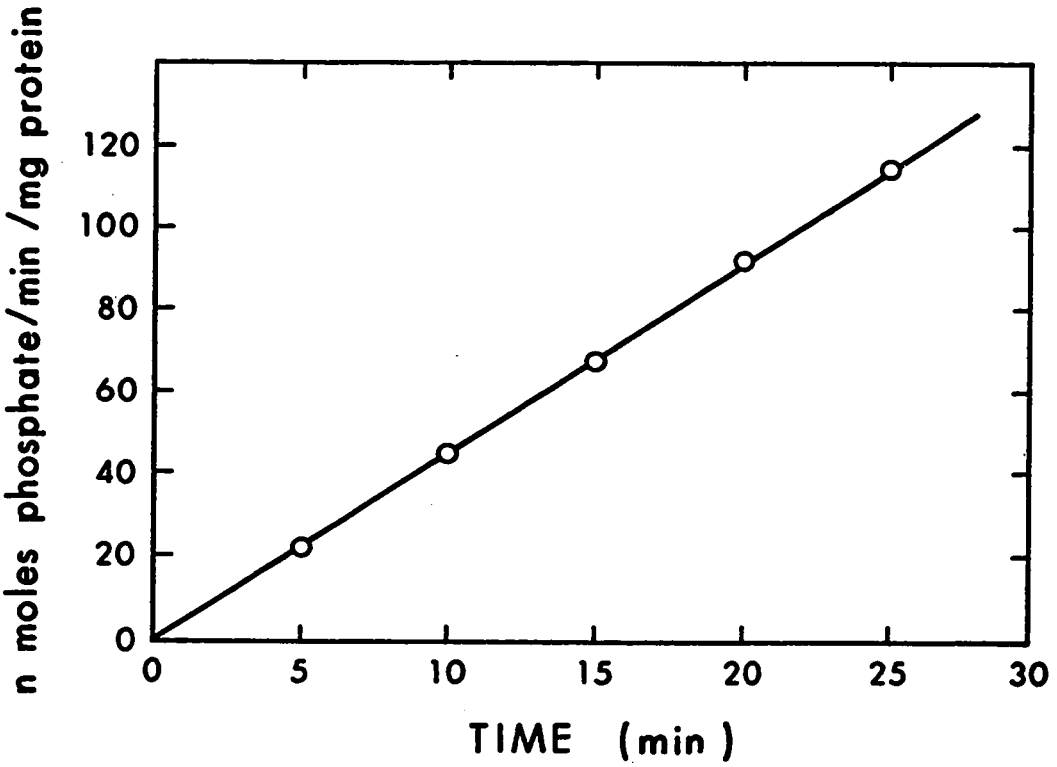
Conditions for these initial reaction rate studies, shown in Figures 17 and 18, indicated linearity with respect to time of incubation and enzyme concentration. As noted previously in Chaper II the studies involved use of about 1.0 mg protein in a reaction volume of 1.0 ml and an incubation time of 15 min. A study of substrate (ATP) saturation (required for M-M kinetics) revealed that activity was dependent on the ratio of magnesium to ATP (Figure 19). This dependence indicated that for utilization of ATP it was essential that it be in the form of Mg ATP since high concentration of ATP (10 mM) in the presence of low concentrations of Mg (3 mM) was inhibitory (ATP alone acting as pseudosubstrate). Such dependency has been shown to exist in the case of membrane ATPase activity (Skou, 1957; Wheeler, 1958; Mann, 1968). Since it was previously shown that mitochondrial isolation procedures necessitated the use of Mg^{++} ions in the solutions used, the activity in the complete absence of Mg^{++} could not be studied. (The total Mg ion content of the mitochondrial suspension resulted in a 0.15 mM Mg^{++} solution in the ATPase assay mixture). However, examination of Figure 19 would indicate that the activity was Mg^{++} dependent.

Activity in the presence of Mg ATP revealed conditions of substrate saturation with an apparent dissociation constant of about

Figure 17. Effect of mitochondrial protein concentration on the rate of hydrolysis of ATP. Assay conditions were 0.3 M sucrose, 25 mM TES (Tris) pH 7.2 at 25°, 3 mM $MgCl_2$, 3 mM Tris-ATP and various amounts of mitochondrial protein in 1.0 ml.

Figure 18. Effect of time of incubation on rate of hydrolysis of ATP. Conditions of assay were 0.3 M sucrose, 25 mM TES (Tris) pH 7.2 at 25°, 3 mM $MgCl_2$, 3 mM Tris-ATP and about 1.0 mg protein.


n moles phosphate/min
25.0
20.0
15.0
10.0
5.0
0
0
0.5
1.0
1.5
2.0
2.5
3.0
PROTEIN (mg)


n moles phosphate/min /mg protein
120
100
80
60
40
20
0
0
5
10
15
20
25
30
TIME (min)

1.0 mM (Figure 20). Hence, 3 mM Mg ions and 3 mM Tris-ATP was routinely employed to give conditions of substrate saturation.

B. Reproducibility

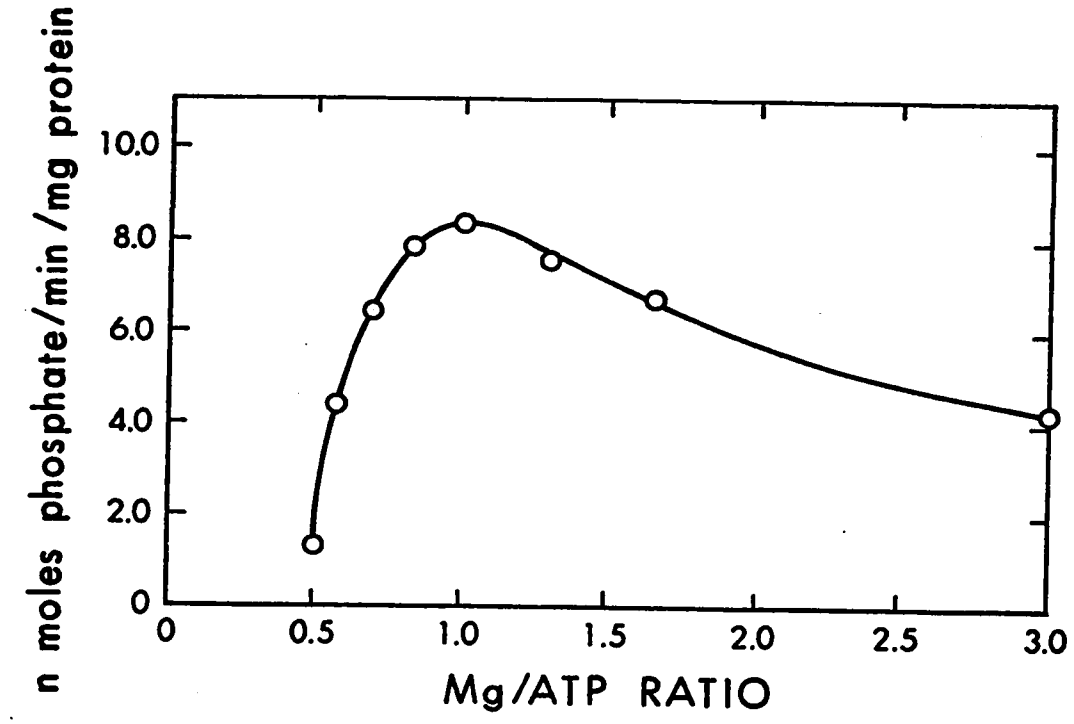
Since this study involved small changes in ATPase activity, it was necessary to have a very sensitive assay system. The detection of the production of phosphate was described in an earlier chapter (Chaper III, Section A.) and was shown to be sensitive to within 10 n moles of P_i. The reproducibility of the test system was found to be dependent on the sensitivity of the P_i analysis procedure since the activity of the enzyme was low. The average of 6 similar determinations revealed an activity of 8.28 n moles P_i/min/mg protein with a standard deviation of $\pm$ 0.03. Each individual assay was performed in triplicate except when a large number of determinations were used (over 30) in which case duplicate determinations were made.

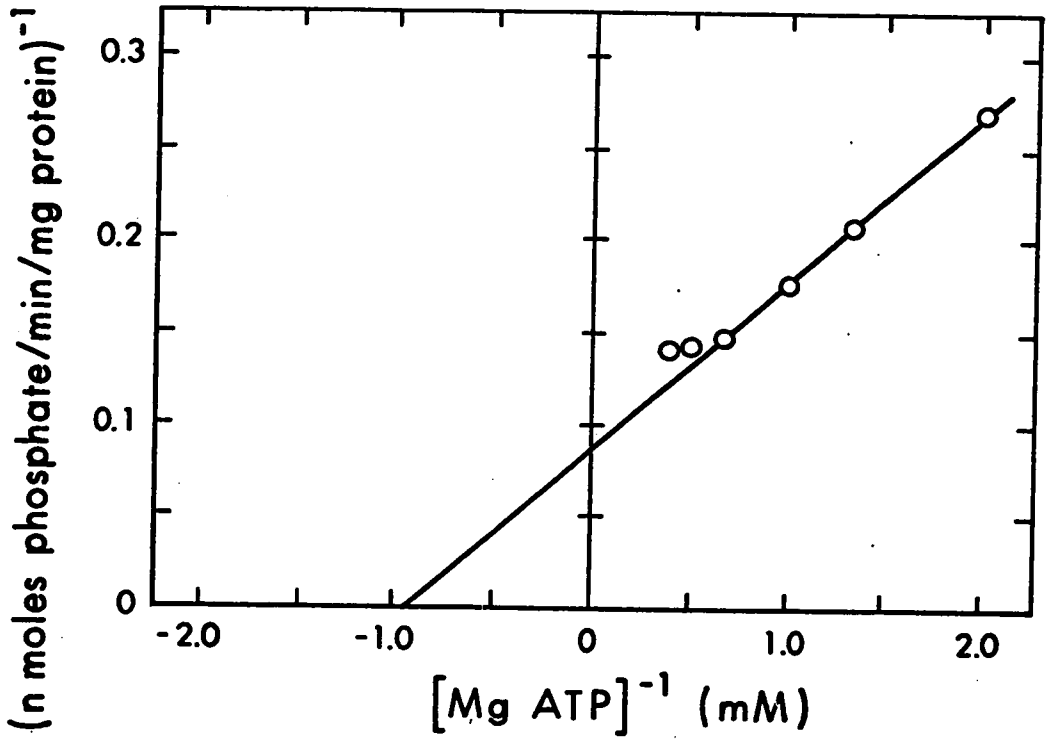
C. Effect of pH

The hydrolysis of ATP results in the liberation of phosphate and hydrogen ions so that the possibility of a drop in pH accompanying this hydrolysis exists. In addition, it has been noted that H^+ extrusion accompanies ion uptake into mitochondria (Pressman, 1967). A change in pH could quite conceivably result in an alteration of enzymic activity. It was found that addition of H_3PO_4 equivalent to twice the amount released from ATP during the incubation period resulted in a negligible pH change, and that the pH at the beginning and end of an assay was unaltered. In addition, the activity of the

Figure 19. Dependence of ATPase activity on the ratio of magnesium to ATP. Incubation mixtures included 0.3 M sucrose, 25 mM TES (Tris) pH 7.2, and 3 mM Tris-ATP with varying Mg^{++} concentrations.

Figure 20. Effect of substrate (Mg ATP) concentration on the rate of hydrolysis of ATP expressed as a double reciprocal plot. Km for Mg ATP is 1.05 mM.


n moles phosphate/min /mg protein
10.0
8.0
6.0
4.0
2.0
0
0
0.5
1.0
1.5
2.0
2.5
3.0
Mg/ATP RATIO


(n moles phosphate/min/mg protein)$^{-1}$
0.3
0.2
0.1
0
-2.0
-1.0
0
1.0
2.0
[Mg ATP]$^{-1}$ (mM)

enzymic hydrolysis was found not to change appreciably over a physiological pH range (Figure 21). The rise in activity at higher pH values has been found to be characteristic of ATPase activity of intact mitochondria and is thought to be a result of destruction of mitochondrial structure (Tekeuchi et al. 1969).

D. Specificity of Measured Activity

1. Unspecific Phosphatase Activity

The unspecific phosphatase activity of the mitochondrial suspension was analyzed according to the methods of Adam (1968) (see Chapter II, Section F.) with the use of p-nitro-phenylphosphate as substrate. This phosphatase activity has been reported to be present in membrane and microsomal preparations and thought to be associated with the 'transport' ATPase of these preparations (Robinson, 1968). This activity is Mg^{++} dependent and K^{+} stimulated. An excess of both ions was present when these assays were performed. It was observed (Figure 22) that the unspecific phosphatase activity of the mitochondrial preparations accounted for about 25% of the ATPase activity. Since the effect of monovalent cations on ATPase activity was investigated, the amount of stimulation of ATPase by K^{+} attributable to unspecific phosphatase cannot be entirely neglected.

It was interesting to observe that the shape of the curve of the unspecific phosphatase activity indicated involvement of a covalent intermediate formation (E.ADP) wherein the release of product ADP was the rate limiting step. The absence of hyperbolic-shaped curve for the ATPase activity cannot, of course, rule out the possibility of

Figure 21. Effect of pH on rate of hydrolysis of ATP. Reaction conditions were 0.3 M Sucrose, 3 mM $MgCl_2$, 3 mM Tris-ATP, and 25 mM TES (Tris) at indicated pH values at 25°.

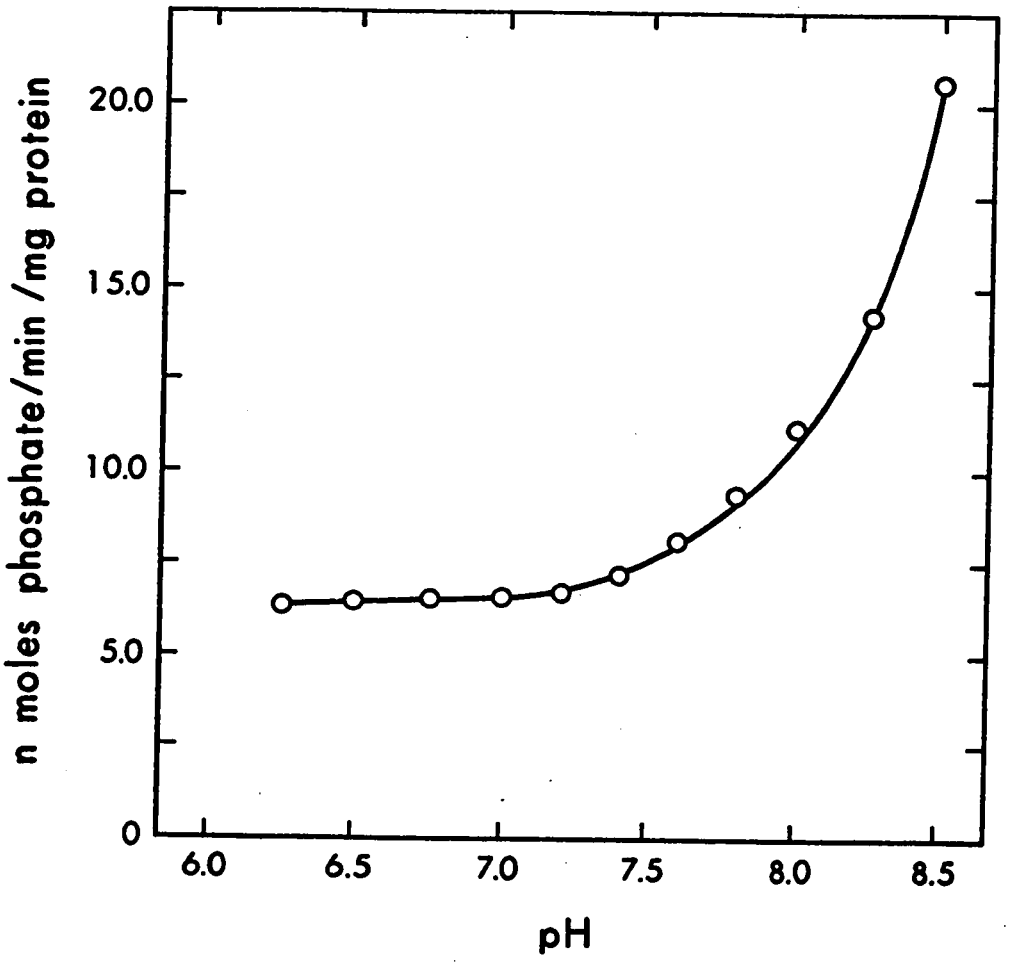
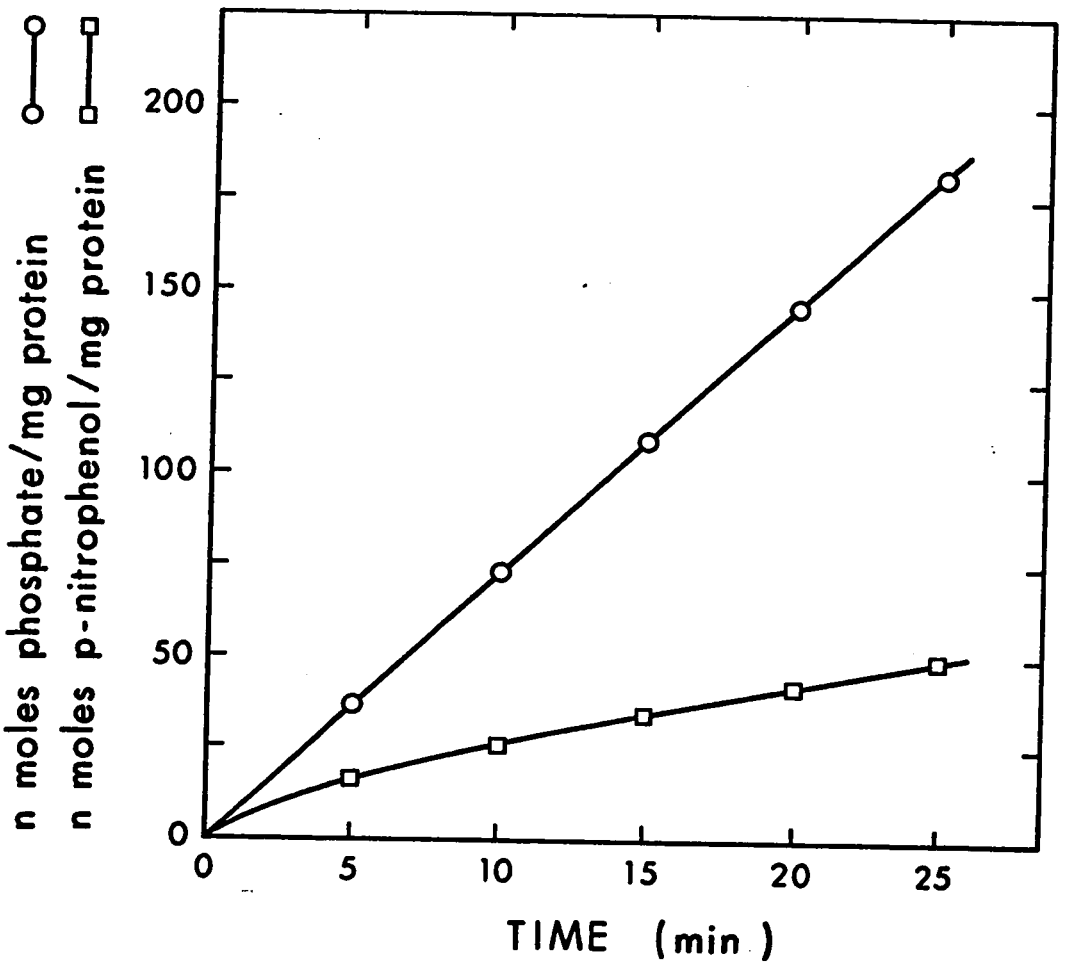

n moles phosphate/min /mg protein
20.0
15.0
10.0
5.0
0
6.0
6.5
7.0
7.5
8.0
8.5
pH

Figure 22. Study of the unspecific phosphatase activity of mitochondrial preparations. Substrates utilized were 3 mM Mg ATP (o——o) or 3 mM p-nitrophenolphosphate (□——□) in the presence of the other assay ingredients.


n moles phosphate/mg protein
n moles p-nitrophenol/mg protein
200
150
100
50
0
0
5
10
15
20
25
TIME (min)

involvement of covalent intermediate formation in this reaction. Recent investigations by Boyer's group (1969) into the isotope exchange reactions of oxidative phosphorylation have indicated the involvement of a concerted reaction (ternary complex formation) with respect to ATP formation from ADP and P_i.

2. Nucleotide Specificity

(i) Adenosine Diphosphate

When ADP was substituted for ATP in an otherwise identical assay system, the amount of ADPase activity (Figure 23) was in the same order of magnitude as the unspecific phosphatase activity and displayed a Km of 2.6 mM.

From the results of competition experiments (Figure 24) it was observed that ADP acts as a non-competitive inhibitor to the ATPase reaction with a K_i of 2.4 mM. The involvement of this inhibition in ATPase activity measurements would be considered negligible (no observed inhibition in the time sequence) since under 10% of the total ATP present was utilized under the assay conditions. These competitive observations indicated that ADP was acting as a pseudosubstrate as well as product inhibitor for the ATPase reaction and do not imply that ADP and ATP react with the different enzyme forms, according to Cleland kinetics (Cleland, 1963).

(ii) Guanosine Triphosphate (GTP)

In order to estimate the specificity for the nucleoside triphosphate, guanosine triphosphate was substituted for ATP under

Figure 23. A double reciprocal plot of phosphatase activity in the presence of Tris-ADP (Δ——Δ) or Tris-ATP (○——○).

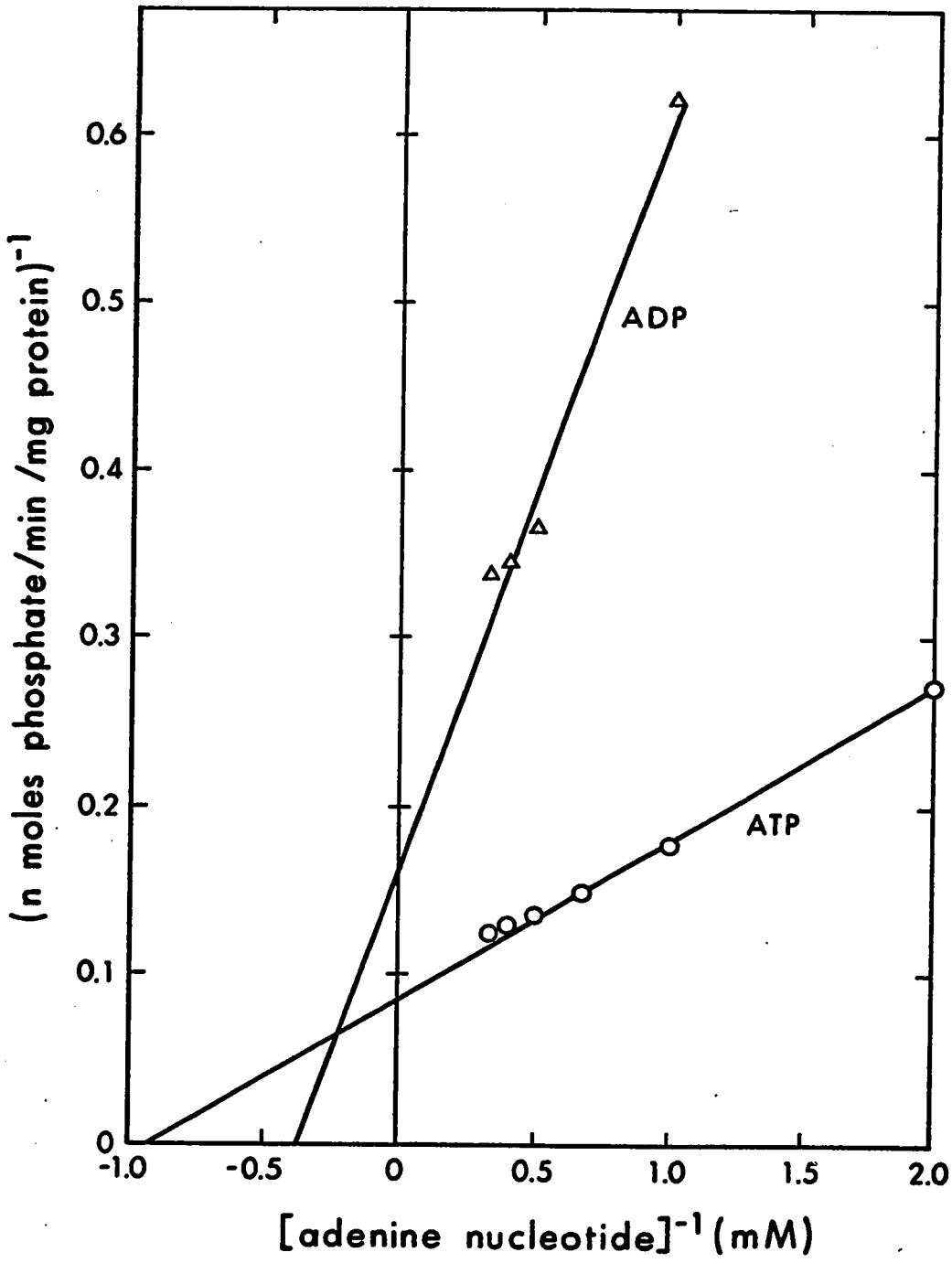
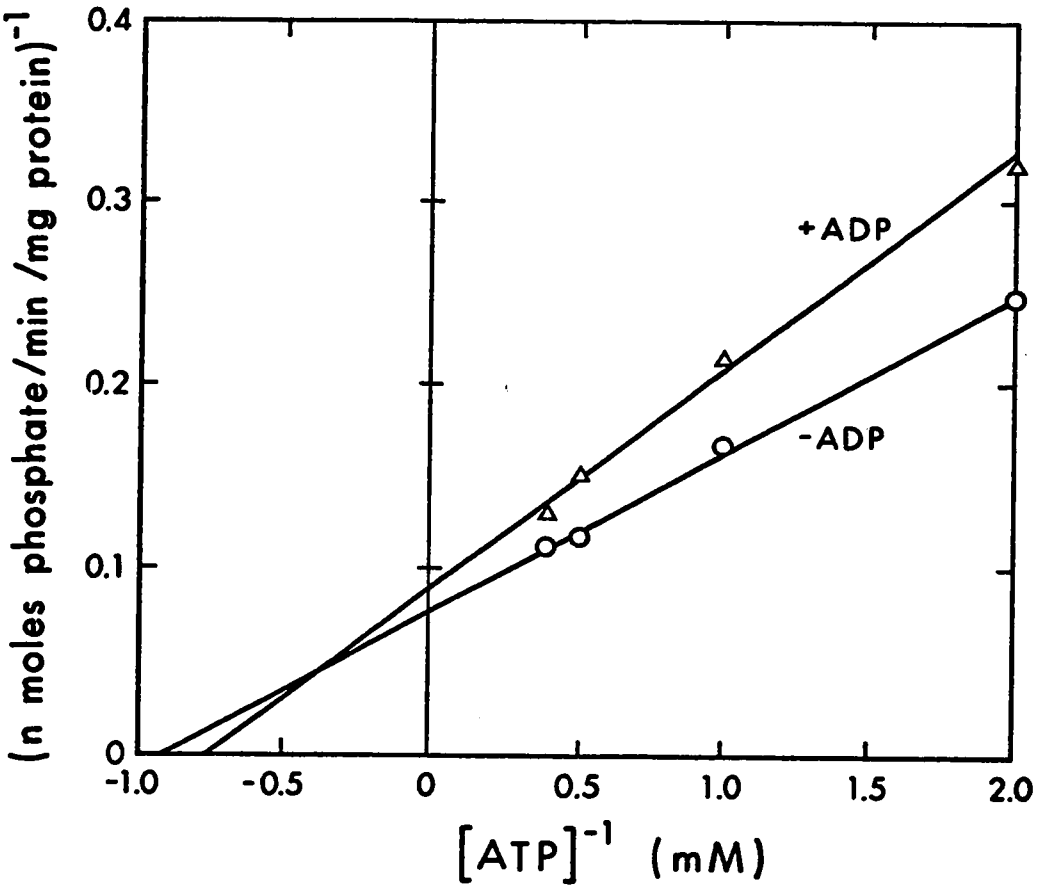

0.6
0.5
0.4
0.3
0.2
0.1
0
-1.0
-0.5
0
0.5
1.0
1.5
2.0
ADP
ATP
(n moles phosphate/min /mg protein)$^{-1}$
[adenine nucleotide]$^{-1}$ (mM)

Figure 24. Double reciprocal plot of ATPase activity at varying Tris-ATP concentrations in the absence (O—O) or presence of a fixed concentration (0.6 mM) of Tris-ADP (Δ—Δ).


0.4
0.3
0.2
0.1
0
-1.0
-0.5
0
0.5
1.0
1.5
2.0
+ADP
-ADP
(n moles phosphate/min /mg protein)$^{-1}$
[ATP]$^{-1}$ (mM)

identical assay conditions. The fact that GTP was not hydrolyzed at a very significant rate (Table VIII) implied that the ATPase reaction was specific for ATP, which is normally observed in oxidative phosphorylation. The low rate of hydrolysis of GTP exceeded that of unspecific phosphatase and may be a result of GTPase activity associated with substrate level phosphorylation of the succinate thiokinase reactions.

E. Respiratory Inhibitors and Uncouplers of Oxidative Phosphorylation

There is to be found in the literature a large volume of data on the various inhibitors of respiration and uncouplers of oxidative phosphorylation (see Chapter I, Section D.). In an effort to establish if the mitochondria examined under the conditions of the ATPase assay were intact mitochondria, (establishment of which was a paramount prerequisite for the entire project) a variety of these compounds was used. If the mitochondria used in this study were intact, the ATPase assay would display inhibition in the presence of respiratory inhibitors (since the ETC would be inoperative), and stimulation in the presence of uncouplers of oxidative phosphorylation (since the ATPase reaction would be operating independently of the ETC).

1. Respiratory Inhibitors

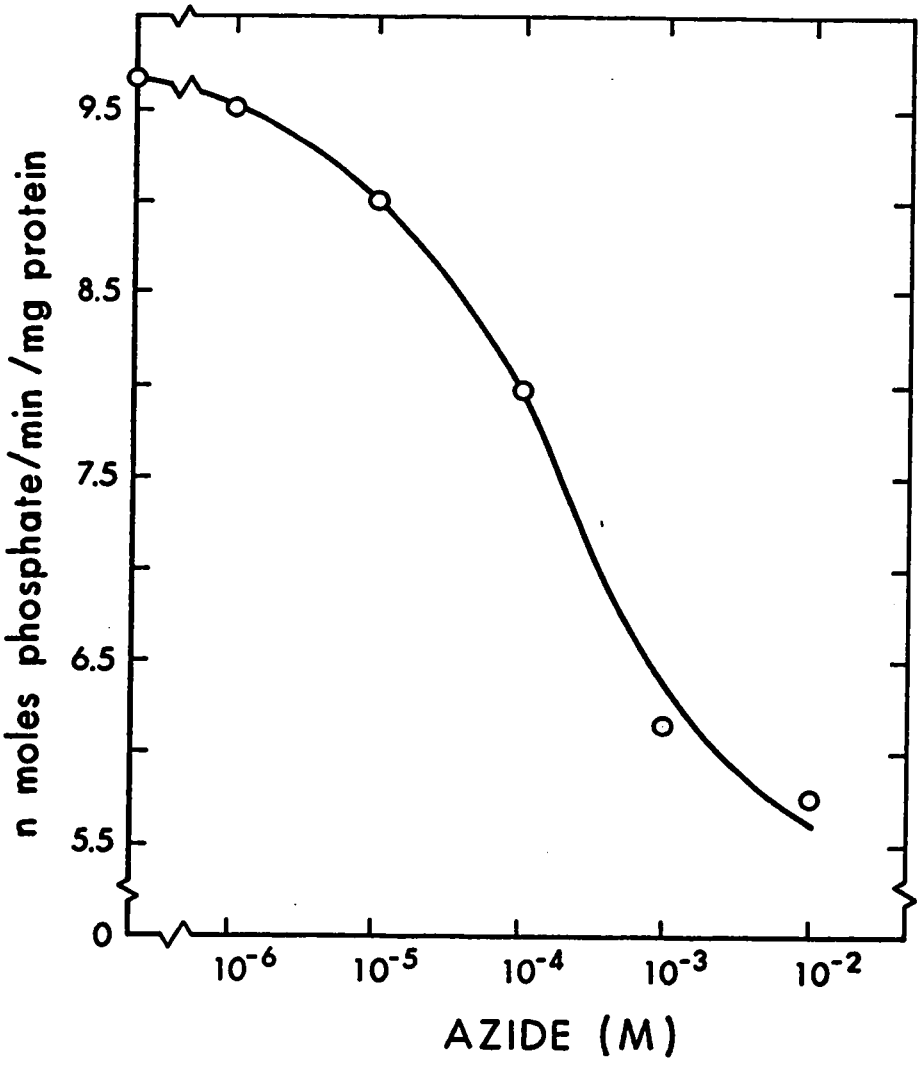
Since any one of a number of respiratory inhibitors known would suffice in this analysis, it was decided to use azide. Azide as was discussed earlier displays a variety of effects on intact mitochondria and was observed to result in about 50% inhibition of the ATPase activity at a concentration of 10.0 mM (Figure 25). It is significant

Table VIII. Hydrolysis of GTP by pea mitochondria.

	n moles P_i/min/mg protein		
	ATP	GTP	% GTPase
Experiment I	8.32	3.6	43
II	7.15	3.4	47

Assay conditions were 0.3 M sucrose, 3 mM $MgCl_2$, 25 mM TES (Tris) pH 7.2 at 25°, either 3 mM Tris-ATP or 3 mM Tris-GTP and about 0.5 mg protein. Incubations were made for 15 min at 25°.

Figure 25. Effect of Azide on the rate of hydrolysis of ATP by pea mitochondria. Assay conditions were 0.3 M sucrose, 25 mM TES (Tris) pH 7.2 at 25°, 3 mM $MgCl_2$, 3 mM Tris-ATP and azide.


n moles phosphate/min /mg protein
9.5
8.5
7.5
6.5
5.5
0
10^{-6}
10^{-5}
10^{-4}
10^{-3}
10^{-2}
AZIDE (M)

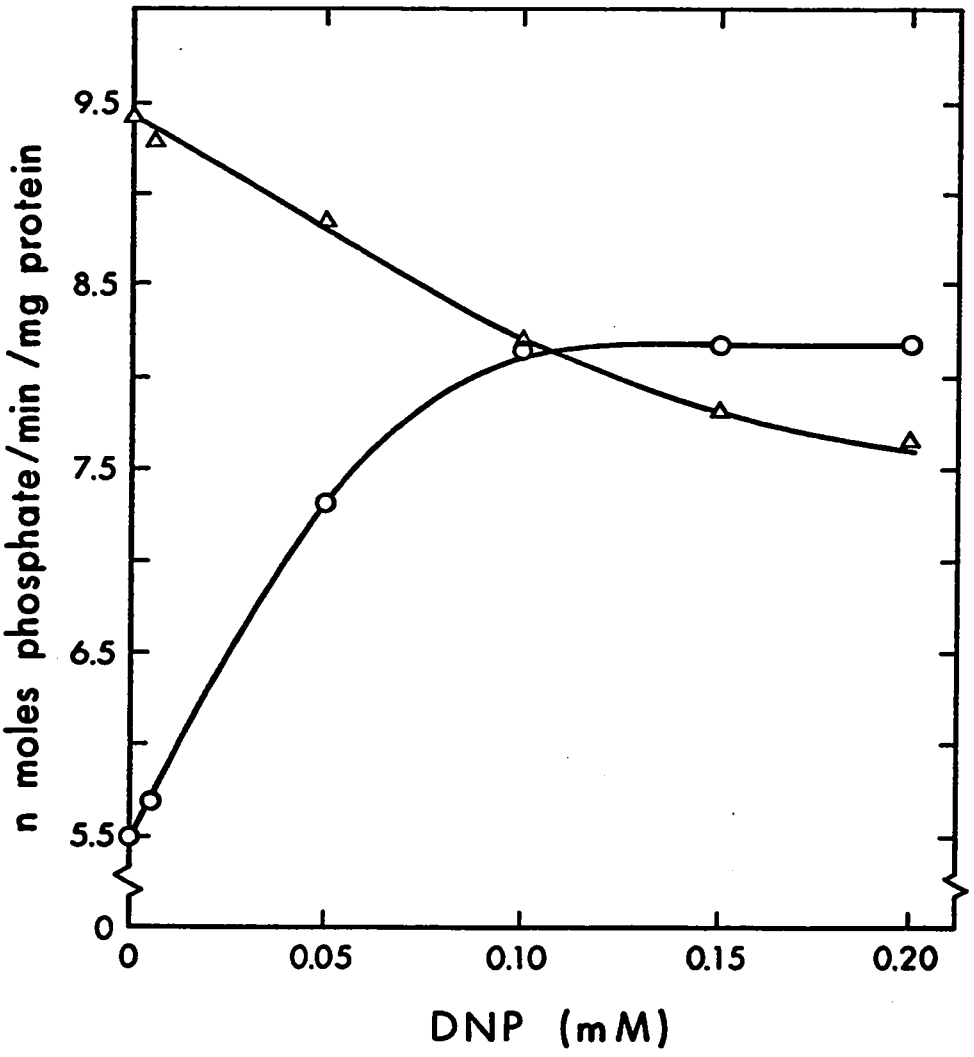
to note that azide has been shown to be inhibitory to isolated 'soluble ATPase' of beef heart mitochondria (Vigers, 1968). These results imply an inhibitory action of azide in energy transferring reactions.

2. Uncouplers of Oxidative Phosphorylation

Among the many uncouplers known, 2,4-dinitrophenol (DNP) is the most widely recognized and was employed in this particular study. It was, however, observed that the DNP stimulation of ATPase activity occurred with the use of mannitol and not sucrose as the osmotic stabilizer (Figure 26). Conflicting reports of stimulation (Forti, 1957; Stoner et al. 1964) inhibition (Reid et al. 1964), or no effect (Wedding and Black, 1962) of DNP on intact plant mitochondrial ATPase activity have occurred in the literature. However, the present author has not observed both stimulatory and inhibitory effects in one report in the literature.

The DNP stimulation of ATPase activity was observed in assay mixtures that contained mannitol. The differences between the base values for mannitol and sucrose ATPase activity are real differences. The ATPase activity measured in sucrose was higher than when it was in the mannitol. This fact may suggest that the lack of DNP stimulation in sucrose was because the mitochondria were already in an uncoupled state. However, as will be shown later, this situation was not likely since under certain conditions DNP stimulation in sucrose solution could be observed. The increase in base level of ATPase activity in sucrose over mannitol was reminiscent of the increase of State IV respiration in sucrose compared to mannitol. As was noted previously

Figure 26. Effect of DNP on the ATPase activity of mitochondria suspended in 0.3 M sucrose (Δ—Δ) or 0.3 M mannitol (○—○), 25 mM TES (Tris) pH 7.2 at 25°, 3 mM $MgCl_2$, 3 mM Tris-ATP and 0.5 mg mitochondrial protein.

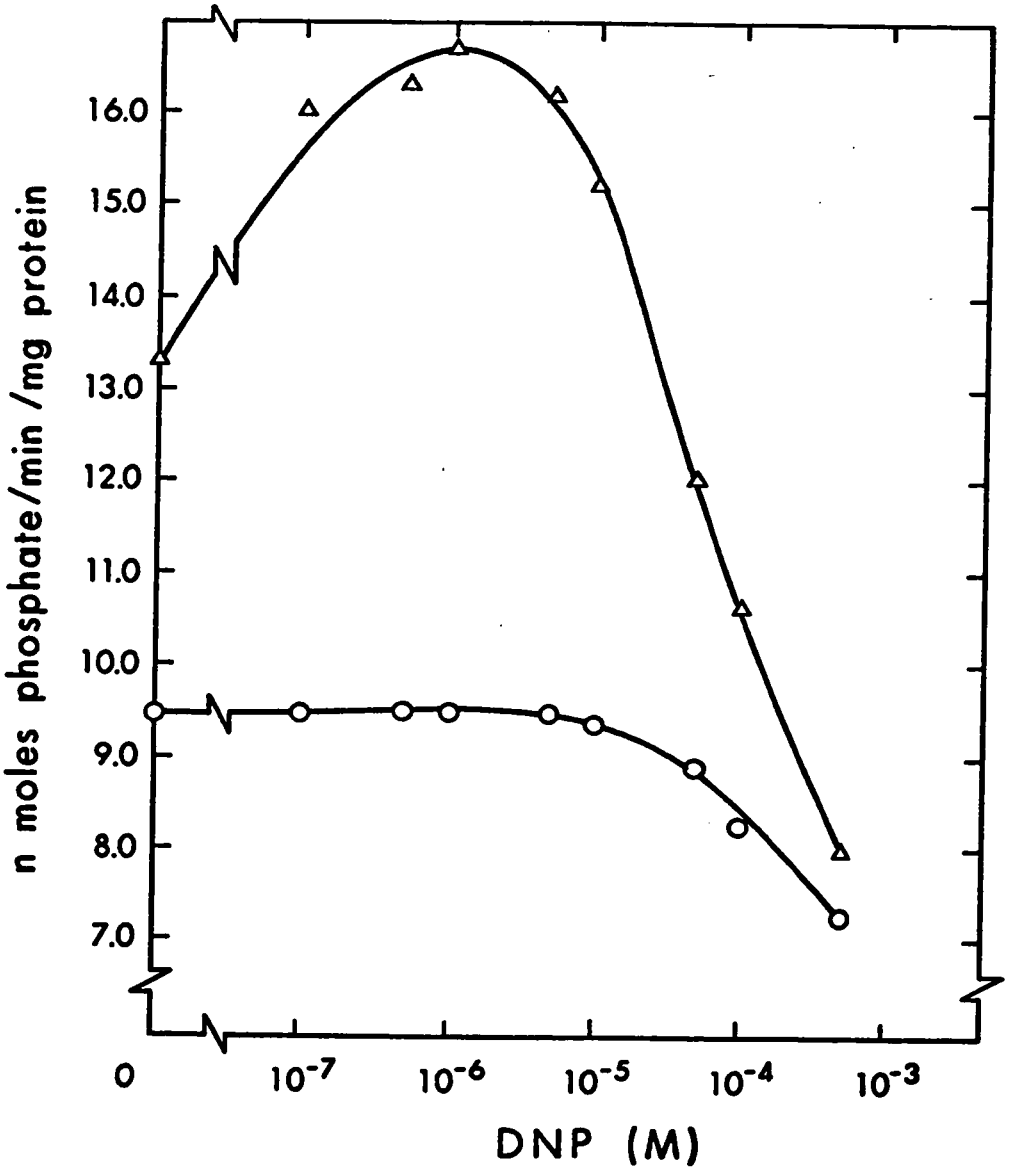

9.5
8.5
7.5
6.5
5.5
0
0
0.05
0.10
0.15
0.20
n moles phosphate/min /mg protein
DNP (mM)

the State IV respiration may account for the ATPase activity and similarly here, the differences between sucrose and mannitol in ATPase activity and State IV respiration may be compared.

From Figure 27, it was observed that the addition of monovalent cations to the sucrose assay mixture would result in DNP stimulation of ATPase. The fact that high concentrations would inhibit the activity in both the presence and absence of monovalent cations was thought to be related to the inhibition of respiration at high DNP concentrations. This inhibition, which is observed in plant as well as animal mitochondria, may be a result of the complexing by DNP of acceptor for the high energy intermediate ("~Y" of Boyer or "~C" of Chance) generated by the ETC. Thus impairment of formation of the complex would lead to inhibition of respiration and, in reverse, ATPase activity. The primary effect of DNP is the destruction of the high energy complex generated by the ETC (X~I or C~Y) (Boyer, 1968).

The involement of monovalent cations in DNP stimulation of ATPase has been recorded in the literature for animal mitochondria (Lardy and Wellman, 1953). Conflicting reports of the effect of DNP on mitochondrial ATPase activity may be a result of the concentration of DNP employed. Undoubtedly, this dependency would also be on the concentration of monovalent cation as well as mitochondria present (ie. the number of DNP molecules per catalytic site). The relationship of the monovalent cation and DNP effect might be attributable to the cations aiding in the penetration or stability of the uncoupler anion. It is thought that the anion of DNP is taken up and is the

Figure 27. Effect of monovalent cations on DNP-ATPase activity in the presence of sucrose. (O—O) represents activity in the presence of 0.3 M sucrose, and (Δ—Δ) represents activity in the presence of 85 mM KCl, 15 mM NaCl and 0.1 M sucrose, in addition to 25 mM TES (Tris) pH 7.2 at 25°, 3 mM $MgCl_2$, 3 mM Tris-ATP and DNP.

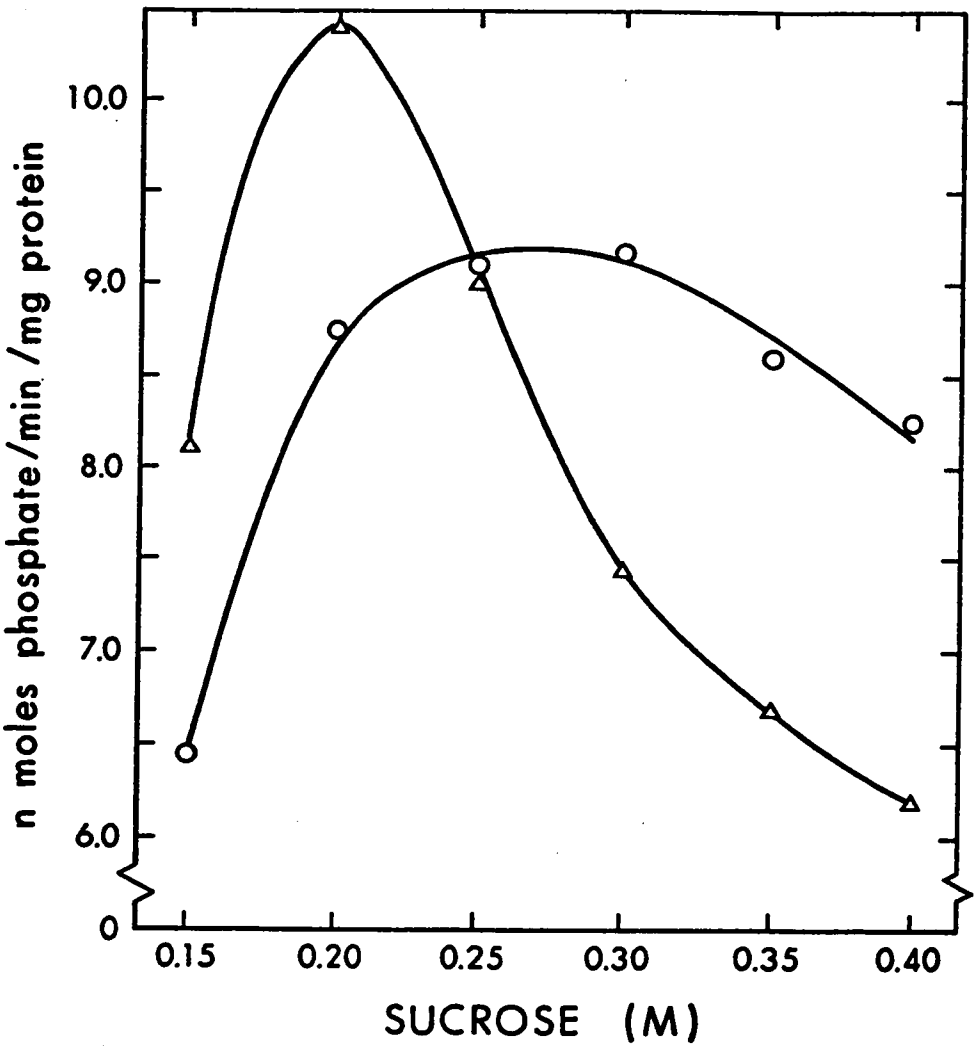

n moles phosphate/min /mg protein
16.0
15.0
14.0
13.0
12.0
11.0
10.0
9.0
8.0
7.0
0
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
DNP (M)

effective moiety (Van Dam and Slater, 1967).

The presence of monovalent cations cannot however account for the stimulation of DNP observed in the presence of mannitol. As was noted previously, State IV respiration is lower when mannitol is used compared to sucrose, and the addition of monovalent cations has a stimulatory effect on State IV respiration. The contamination by monovalent cations of the mannitol and not the sucrose may still be a definite possibility. Atomic absorption measurements of the two solutions were not made. Alternatively, sucrose and mannitol may give different contracted states of the mitochondria because of the 'sucrose-space' of mitochondria. In order to test such a possibility, assays were conducted in various osmolar concentrations of sucrose. From these experiments (Figure 28) it was observed that the ATPase activity does depend on the state of contraction of mitochondria with a concentration of about 0.25 M being optimum, and that the effect of DNP on the activity was similarly dependent on the state of contraction. This experiment thus suggests that the differences in the base level of ATPase in sucrose and mannitol and the dependency of DNP stimulation on sucrose and mannitol may involve the state of contraction of the particles.

It is to be noted that KCl (Solomos, personal communication) and a low sucrose concentration induced swelling of pea mitochondria. However, as observed above, a low sucrose concentration inhibited the ATPase activity and, as observed in the following section (Section F), KCl stimulated the activity. Thus the ATPase activity depended on the particular state of mitochondrial contraction achieved by KCl,

Figure 28. Effect of sucrose molarity on ATPase activity in the absence (O—O) or presence (Δ—Δ) of 0.1 mM DNP.


n moles phosphate/min /mg protein
10.0
9.0
8.0
7.0
6.0
0
0.15
0.20
0.25
0.30
0.35
0.40
SUCROSE (M)

mannitol or sucrose.

Since it had been reported (Takeuchi, 1969) that the lack of stimulation of ATPase activity by DNP in intact mitochondria from castor bean endosperm tissue, was because of a requirement for substrate to be present to give a reduced state of the ETC, a similar experiment was attempted in the present study. However, results in Table IX indicated that when α-ketoglutarate was introduced at the same concentration as it was employed in the respiration assays, it had an inhibitory effect on the ATPase activity (as would be expected since reversed electron transport would be inhibited), α-KG had a similar effect on the DNP induced ATPase activity. There did not seem to be any additive or stimulative effect but the presence of substrate reduced the DNP effect.

F. Stimulation by Monovalent Cations

In experiments designed to evaluate the effect of Na^+ and K^+ on ATPase activity, the osmolarity of the assay medium was kept constant by adjustment of the sucrose concentration. In the interpretation of data such as will be observed, one must remain cognizant of the fact that states of mitochondrial contraction may be intimately involved.

Effect of Individual Cations

The stimulation of ATPase activity in plant mitochondria by monovalent cations has been observed by a few workers in recent reports (Fisher and Hodges, 1969) and no stimulation has been observed by others

Table IX. Effect of the presence of respiratory substrate on mitochondrial ATPase activity.

Treatment	ATPase activity (n moles P_i/min/mg protein)
Control	6.90
DNP	5.17
α-KG	3.76
DNP + α-KG	3.90

The control assay conditions were 0.3 M sucrose, 25 mM TES (Tris) pH 7.2 at 25°, 3 mM $MgCl_2$, 3 mM Tris-ATP and 0.5 mg mitochondrial protein in a volume of 1.0 ml. Treatments involved addition of DNP to a concentration of 0.1 mM and/or α-ketoglutarate to a concentration of 8 mM.

(Reid et al. 1964). This lack of stimulation may be a result of a lack of sensitivity of the analysis since ATPase activity of intact mitochondria is relatively low. In the experiments conducted in this laboratory, the monovalent cations were found to stimulate ATPase activity (Figure 29). This stimulation was dependent on the ion involved as Na^+ and K^+ gave different responses. From the reciprocal plots in Figure 30, it can be observed that the concentration necessary for half stimulation of the activity was also dependent on the ion.

As noted in a previous section (Section C.1) the K^+ stimulated unspecific phosphatase activity of the mitochondria would not entirely account for the stimulation of ATPase as observed in Figure 29.

The activity of the transport ATPase associated with membranes has been found to be dependent on the presence of both Na^+ and K^+ ions whereas in the present case the ions are stimulatory in one another's absence. These results would imply that there is a significant difference between mitochondrial ATPase activity and ATPase activity associated with cell membranes. Since the $Na^+ + K^+$ - ATPase activity associated with cell membranes is for thepurpose of maintaining precise ionic environments (ie. repolarization of nerve tissue etc.) then mitochondria that do not require such exacting conditions may not require such ionic specificity. In addition, the ATPase component of mitochondria is intimately associated with oxidative phosphorylation and may not be primarily concerned with ion transport.

Mitochondria can accumulate both mono and divalent cations in an energy dependent fashion (uptake being inhibited by respiratory inhibition if respiratory substrate is used, or by oligomycin if ATP

Figure 29. Effect of monovalent cations on pea mitochondrial ATPase activity. Concentrations of cations (Na^+ Δ——Δ, K^+, O——O) added as their chloride salts were varied and the concentration of sucrose adjusted to result in a solution of constant osmolarity (equivalent to that of the 0.3 M sucrose).

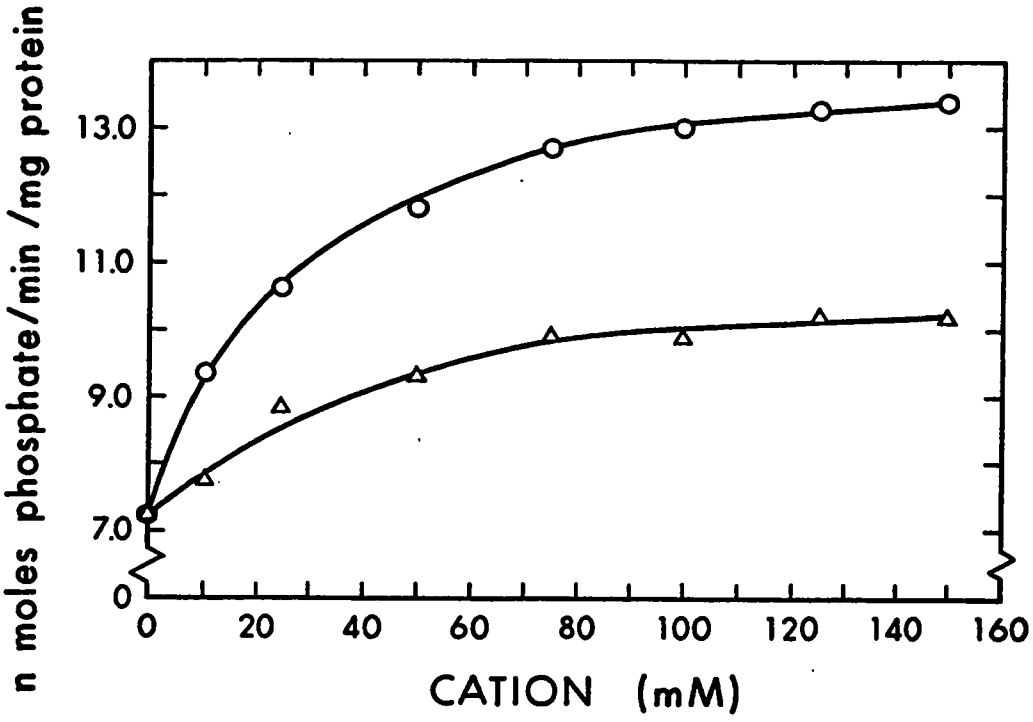
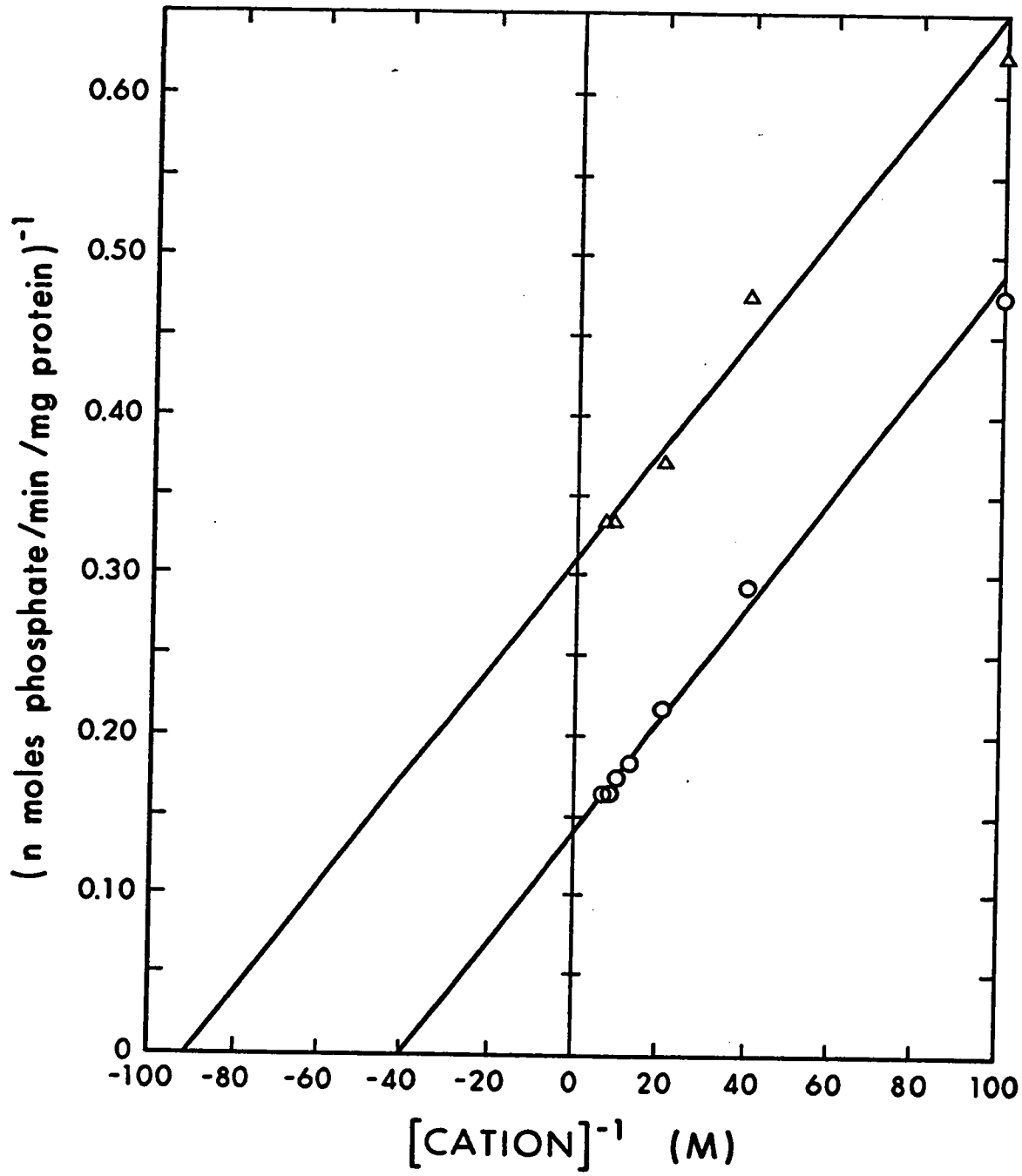

n moles phosphate/min /mg protein
13.0
11.0
9.0
7.0
0
0
20
40
60
80
100
120
140
160
CATION (mM)

Figure 30. Double reciprocal plots of K^+ (o—o) and Na^+ (Δ—Δ) stimulated ATPase activity. The activity in the absence of cation has been subtracted from each cation concentration.


0.60
0.50
0.40
0.30
0.20
0.10
0
(n moles phosphate/min /mg protein)$^{-1}$
-100
-80
-60
-40
-20
0
20
40
60
80
100
[CATION]$^{-1}$ (M)

is supplied). This energy dependent uptake of monovalent cations reported by Pressman et al. (1967), in which up to 7 K^+ ions per ATP molecule are taken up with the concomitant extrusion of H^+ would indeed be relevant to the present investigation. The K^+ induced ATPase reported here could be indicative of an ion transporting system. This ion transporting system may be intimately involved with the maintenance of a structure that adjusts to give a Gibbs-Donnan equilibrium similar to that which exists in red blood cells in order to prevent hemolysis. It has been recognized that the internal concentration of K^+ in mitochondria is extremely high in comparison to Na^+ (Boyer, 1968) (130 mM K^+ and 4 mM Na^+). This differential could not be accounted for by passive diffusion. The ionic composition of mitochondria must be actively maintained. It is recognized, and confirmed in this laboratory (Solomos, personal communication) that the presence of K^+ will cause swelling of pea mitochondria and that addition of ATP (exogenously, or generation by respiratory substrates + ADP) causes a rapid contraction of the mitochondria. The swelling may result from the passive diffusion of K^+ into the vesicle and the contraction from the active extrusion of K^+.

The effects described here are not solely explicable in terms of increased ionic strength of the media since KCl and NaCl would result in similar ionic strengths but they record differential activation of the ATPase. The greater activation by K^+ may be a result of the difference in hydrated radii of the two molecules if both ions are competing for the same site. (The hydrated radii of K^+ is less than that of Na^+).

Effect of Cations in Combination

In order to elucidate as to whether the ions were indeed competing for the same site of uptake, experiments in which the K^+ ion was held constant and the Na^+ varied were performed. From Figure 31, wherein experiments were performed with two different fixed concentrations of K^+, it was observed that sigmoidicity occurred. This sigmoidicity was entirely dependent on the fixed concentration of K^+, with greater sigmoidicity occurring at the high fixed concentrations. This type of result would be expected to occur if the K^+ is preferentially taken up so that addition of Na^+ to the medium would result in decreased activity (since Na^+ does not stimulate as much as K^+). The possibility of this competition was further examined and it was observed that it was complicated by the observation that when the ionic strength was maintained constant and both the Na^+ and K^+ varied, the activity was dependent on the ratio of Na^+ to K^+ (Figure 32). This situation is observed in studies of $Na^+ + K^+$ - ATPase activity of cell membranes and has been accounted for by the proposal of two co-operative binding sites of the ATPase protein (Robinson, 1968). However, the present work would suggest that if the presence of K^+ decreased the binding constant of the enzyme for Na^+, then at low K^+ concentrations, K^+ ions would be preferentially translocated. As the fixed K^+ ion concentration is increased, the binding constant for Na^+ decreases (increase in strength of binding)so that an optimum level would occur wherein the ratio of Na^+ to K^+ is such that maximal stimulation is observed. At relatively low Na^+ and high K^+ concentrations, the enzyme binds Na^+ in preference to K^+ so that the rate would

Figure 31. Effect of monovalent cations on ATPase activity at fixed concentrations of K^+ and varying concentration of Na^+. The fixed concentration of K^+ in A was 10 mM and in B was 25 mM. Total osmolarity was adjusted to 0.3 by addition of sucrose.

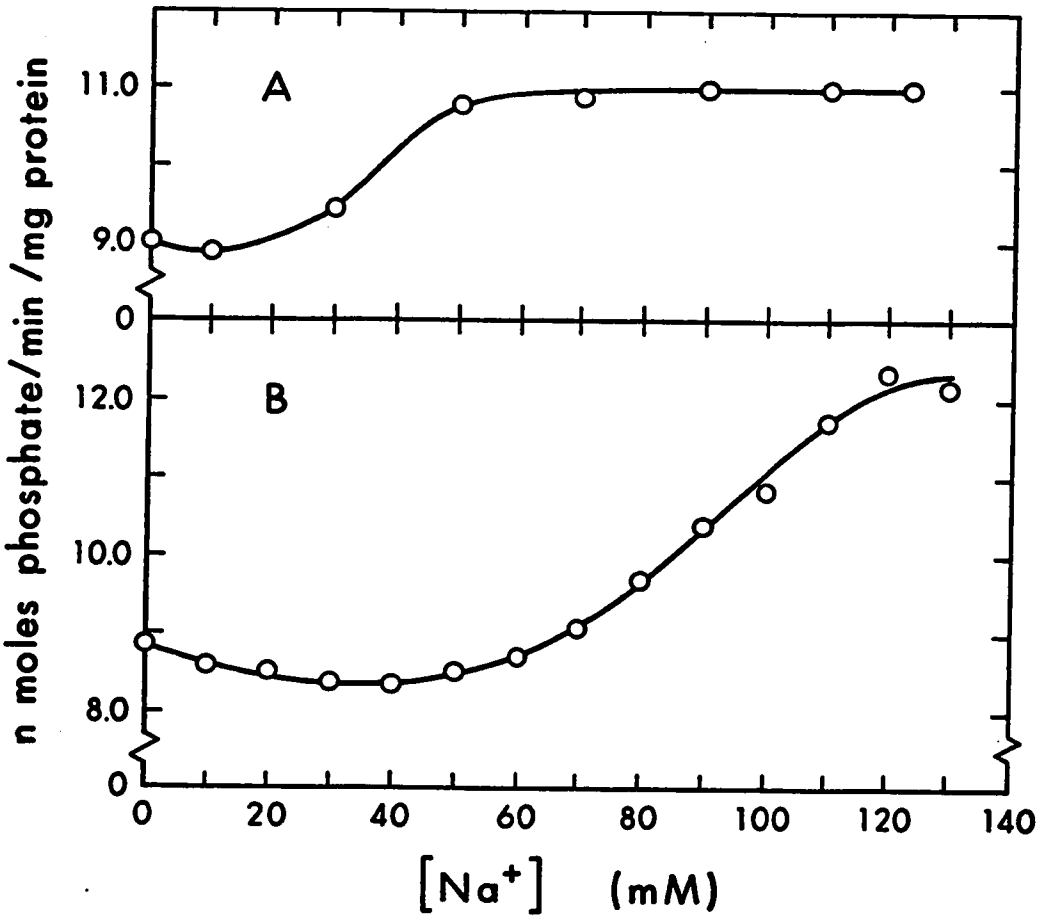
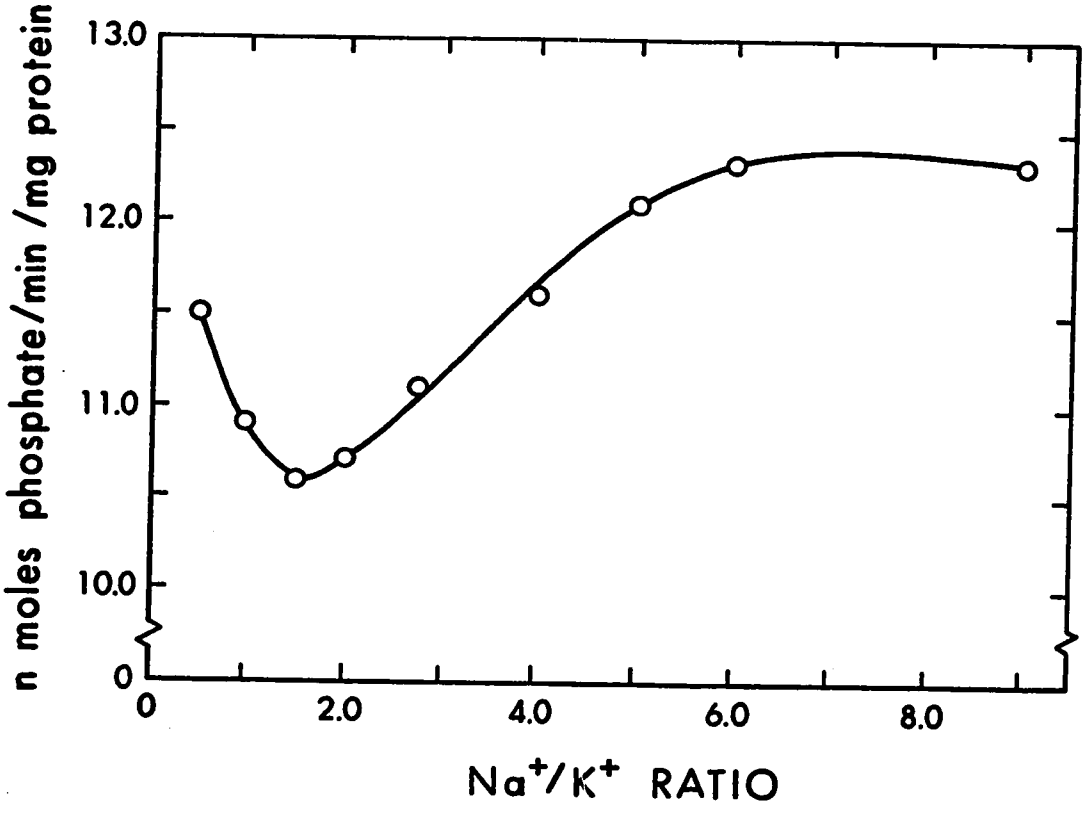

n moles phosphate/min /mg protein
11.0
9.0
0
A
12.0
10.0
8.0
0
B
0
20
40
60
80
100
120
140
[Na+] (mM)

Figure 32. Effect of the Na^+/K^+ ratio on the ATPase activity. The total concentration of cations was kept at 0.15 M.


n moles phosphate/min /mg protein
13.0
12.0
11.0
10.0
0
0
2.0
4.0
6.0
8.0
Na+/K+ RATIO

be low (since Na^+ has a lower Vmax than K^+ (Figure 30)).

G. Effect of Ouabain and DNP on Monovalent Cation Stimulated ATPase

Ouabain is recognized to be a potent inhibitor of membrane $Na^+ + K^+$ - ATPase as it is thought to inhibit the dephosphorylation of the enzyme, the step which is K^+ induced (Skou, 1965). Experiments designed to investigate the involvement of this cardiac glycoside with mitochondrial ATPase are reported in Table X. The role of DNP in this involvement was also recorded. From these experiments it was observed that ouabain did exert some effect in inhibiting the $Na^+ + K^+$ stimulated ATPase (possibly removing the $Na^+ + K^+$ dependent activity and leaving the activity attributable to the K^+ and Na^+ individually). However, ouabain did not inhibit ATPase in the presence of the ions separately. The addition of DNP was observed to inhibit the individual ion-induced activity and to have a synergistic effect on ouabain inhibition of $Na^+ + K^+$ activity. (Ouabain in the presence of DNP was found to give a base activity of the induced ATPase to a point that was not significantly different from the sucrose control level). The possibility of synergism in the ouabain + DNP effect on sucrose and K^+ induced activity was not investigated.

H. Effects of Valinomycin and NAD

Valinomycin is an antibiotic that facilitates K^+ ion transport through biological membranes. Its selectivity for K^+ has been found to be high and it is thought that this circular peptide allows penetration of the ion through its pore. Because of this specificity, the

Table X. Effects of Ouabain and DNP on monovalent cation stimulated ATPase activity of pea mitochondria.

Addition	ATPase activity (n moles P_i/min/mg protein)			
	0.3 M Sucrose	0.2 M Sucrose + 0.05 M KCl	0.2 M Sucrose + 0.05 M NaCl	0.2 M Sucrose + 0.01 M KCl + 0.04 M NaCl
Control	6.20	9.52	9.10	10.00
Ouabain	6.22	9.47	9.16	8.83
DNP	4.50	7.15	6.35	6.95
Ouabain + DNP	4.10	6.34	6.37	6.40

Assay conditions were 0.3 M osmoticant (as noted), 3 mM $MgCl_2$ 3 mM Tris-ATP and 25 mM TES (Tris) pH 7.2 at 25°. Ouabain was added to a concentration of 0.5 mM and DNP to a concentration of 0.1 mM.

effect of this compound on mitochondrial ATPase activity was investigated. In conjunction with these experiments, the effect of nicotinamide adenine dinucleotide (NAD^+) on the ATPase activity was investigated in order to estimate if this acceptor of reducing equivalents from the ETC would be rate limiting to the ATPase activity measurements.

Results recorded in Table XI indicated that valinomycin and NAD^+ had negligible effects on Mg-ATPase activity but both were observed to be effective on monovalent cation-stimulated activity. Valinomycin was observed to give stimulation of K^+ - ATPase activity up to a point comparable to $Na^+ + K^+$ activity and to have a negligible effect on the Na^+ - ATPase thereby showing discriminatory powers with respect to the two cations. The fact that valinomycin stimulated the K^+ activity to a point equivalent to $Na^+ + K^+$ activity, may indicate that this latter activity is the maximum possible and that K^+ stimulated activity is restricted because of K^+ entry into the mitochondria. This hypothesis would further advance the theory that the presence of one ion decreases the binding constant for the second ion and results in an increase of Vmax.

NAD^+ at the concentration used was observed to be inhibitory to ion-stimulated activity. This inhibition may in part result from competition for the monovalent ion carrier system. Competition experiments might elucidate the mechanism further.

I. Effects of Azide and Oligomycin

As outlined in a previous section (Section D.1.) azide was

Table XI. Effects of Valinomycin and NAD^+ on monovalent cation stimulated ATPase activity of pea mitochondria

Addition	ATPase activity (n moles P_i/min/mg protein)			
	0.3 M Sucrose	0.1 M Sucrose + 0.1 M KCl	0.1 M Sucrose + 0.1 M NaCl	0.1 M Sucrose + 0.015 KCl + 0.085 M NaCl
Control	5.77	9.78	7.57	10.26
Valinomycin	5.70	10.30	7.63	10.24
NAD^+	5.76	8.42	6.90	8.53

Assay conditions were 0.3 M osmoticant (as noted), 3 mM $MgCl_2$ 3 mM Tris-ATP and 25 mM TES (Tris) pH 7.2 at 25°. Valinomycin was added to a concentration of 1 µg/ml and NAD^+ to a concentration of 3 mM.

found to inhibit mitochondrial Mg^{++} - ATPase. Since there was the possibility that the addition of ions might have had a deleterious effect on mitochondrial integrity, the effect of azide during monovalent cation-stimulated activity was studied. From Figure 33, it was observed that azide had the capability of not only inhibiting Mg^{++} - ATPase but also $Na^{+} + K^{+}$ - stimulated activity. It could be concluded that the presence of $Na^{+} + K^{+}$ had not altered the integrity of the mitochondria.

Oligomycin, being an inhibitor of mitochondrial phosphorylation reactions would be expected to inhibit mitochondrial ATPase activity. In experiments designed to show this effect, it was observed (Figure 34) that oligomycin inhibited Mg^{++} - ATPase activity but did not inhibit monovalent ion stimulated activity. This insensitivity to oligomycin and sensitivity to azide would imply that the dephosphorylation steps involved in Mg^{++} and $Na^{+} + K^{+}$ were different. Oligomycin is thought to exhibit its inhibitory powers of ATPase at a point where a high energy intermediate is phosphorylated (Ernster et al. 1967). Since the ATPase reaction for $K^{+} + Na^{+}$ is insensitive to oligomycin an alternative pathway which is solely involved with the transporting of monovalent cations may be operative. A recent report of Hodges and Fisher (1969) has shown similar results of insensitivity of oligomycin to monovalent ion stimulated ATPase activity from oat roots. The authors of this report suggest the existence of two separate enzymes, one requiring Mg^{++} and sensitive to oligomycin andtthe other requiring both Mg^{++} and K^{+} and being insensitive to oligomycin.

Figure 33. Effect of azide on cation stimulated ATPase activity. o——o represents activity in 0.3 M sucrose and Δ——Δ activity in 85 mM KCl, 15 mM NaCl and 0.1 M sucrose.

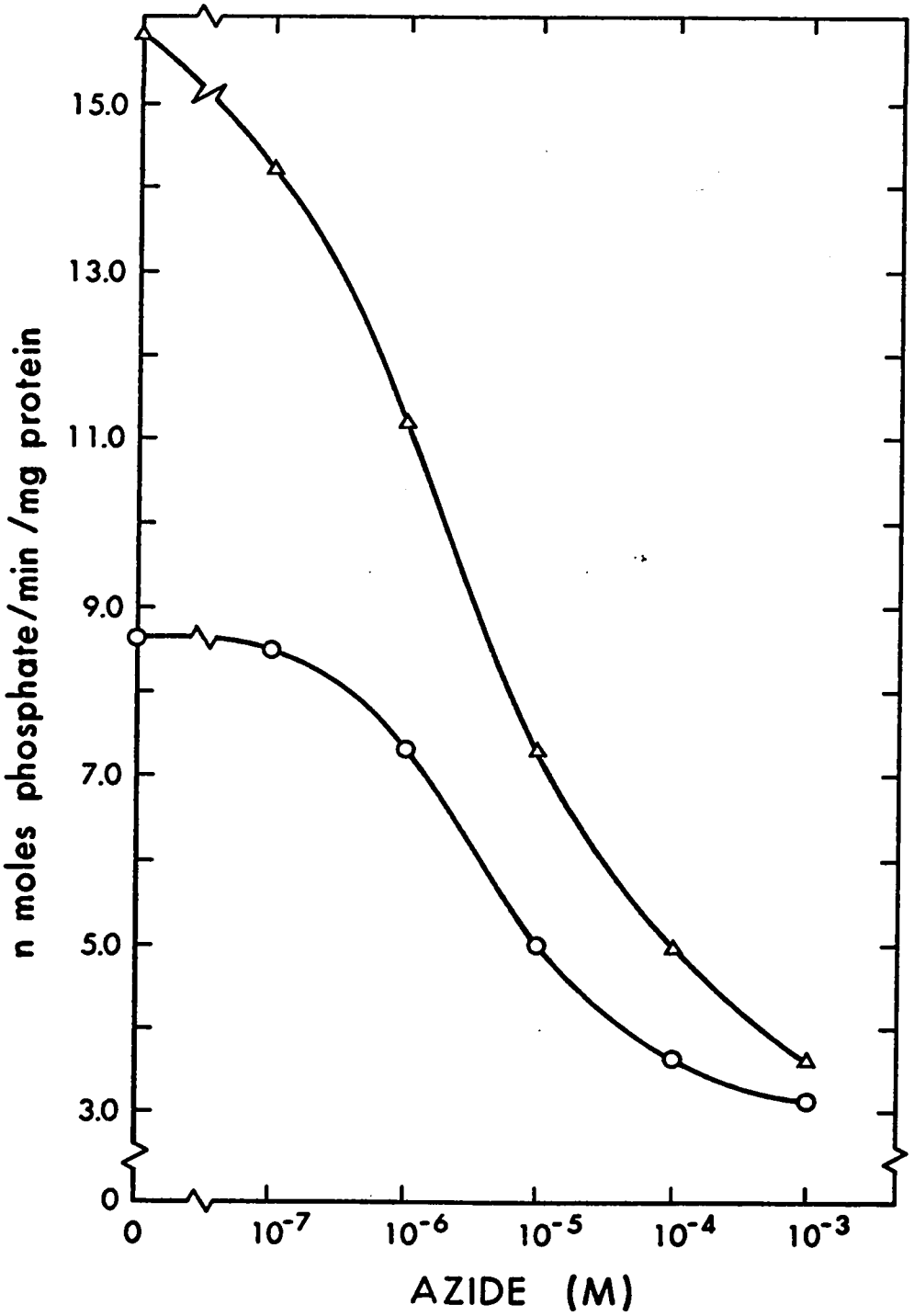
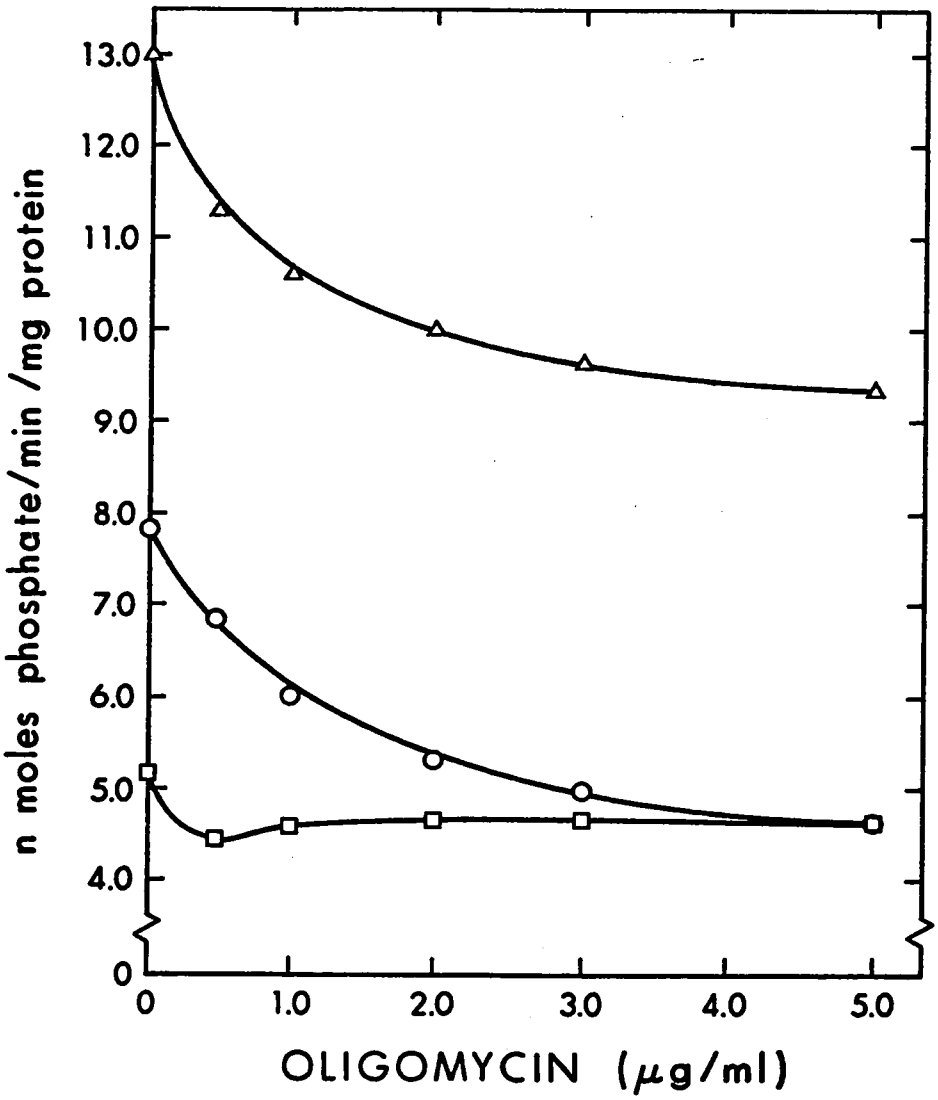

15.0
13.0
11.0
9.0
7.0
5.0
3.0
0
n moles phosphate/min /mg protein
0
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
AZIDE (M)

Figure 34. Effect of oligomycin on mitochondrial ATPase in the presence of 0.3 M sucrose (o——o) or 85 mM KCl, 15 mM NaCl and 0.1 M sucrose (Δ——Δ). The difference between the activity (□——□) was also plotted.


13.0
12.0
11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
0
n moles phosphate/min /mg protein
0
1.0
2.0
3.0
4.0
5.0
OLIGOMYCIN (μg/ml)

CHAPTER VI

INTERACTION OF ETHYLENE WITH MITOCHONDRIAL SYSTEMS

There have been many recent reviews (Burg, 1969; Pratt and Goeschl, 1969 and Spencer, 1969) that have described the various physiological effects of ethylene on plant tissue metabolism. However, to date there are no known effector entities that can be correlated with the variety of responses of plant tissue to ethylene.

The physiological activity of ethylene and mitochondrial activity are intimately related. Treatment of plants with this gas has been observed to induce the climacteric rise in respiration of fruit (Pratt and Workman, 1962; Burg and Burg, 1962). Ethylene is associated with increases in respiration of primary bean leaves (Knight, 1970) and bean cotyledons (Stinson, 1968), and has been found to increase the rate of mitochondrial volume changes (Lyons and Pratt, 1964; Olson, 1967). (Mitochondria have been observed to swell during ripening of pear tissue (Bain and Mercer, 1966) and thus increase the area of penetration for mitochondrial substrates and hence increase respiration (Crane, 1961)).

Ethylene was at one time used as an anaesthetic and would therefore imply that it may have some effect on nerve impulse transmission. $Na^+ + K^+$ - ATPase activity is intimately involved in nerve transmission (Skou, 1965). Anaesthetic activity is also thought to be related to the lipid:air partition coefficient of the compounds (ethylene being fourteen times more soluble in lipid than water) (Egar and Larson, 1964).

As can be seen, many lines of evidence point to an effect of ethylene on mitochondria. Besides these observations, one observes a great variety of different responses of plant tissue to ethylene treatment, so that a basic process of the cell may be the target. Since the mitochondrion is the 'powerhouse' of the cell (Lehninger, 1964) and has a high lipid content, it could possibly be involved in the mode of action of ethylene.

A. Effect on Mitochondrial Respiration

In order to establish if ethylene had any effect on the respiration of mitochondria, these particles were initially suspended in 'suspend media' which had been equilibrated with a 100 ppm ethylene-air mixture. Upon examination of the respiratory parameters of mitochondria treated in this way, it was noted that there was no observable difference in the parameters from that of the controls. These mitochondria were subsequently treated with ethylene again by purging the actual mitochondrial suspension with the 100 ppm ethylene-air mixture for 15 sec. Again, there was no observable change in the respiratory parameters of the mitochondria.

Since the incubations in the ethylene containing media were only for a short period of time (about 1/2 hr), and the addition of ethylene to respiratory assay mixtures would interfere with the analysis, (thus the ethylene treated mitochondrial suspension is greatly diluted (30 fold) on addition to the polarograph assay mixture) the lack of ethylene effect on respiratory parameters observed here cannot negate such an effect.

B. Ethylene and Adenosine Triphosphatase Activity

Previous reports from this laboratory (Olson, 1967) indicated that ethylene was able to affect the rate of mitochondrial contraction in the presence of ATP as well as stimulate the hydrolysis of ATP by intact mitochondria. Upon purification of the ATPase component of mitochondria according to a modification of the Penefsky method (Penefsky, Pullman, Datta and Racker, 1960), he found that ethylene had a negligible effect on the enzyme activity. It was concluded by this worker that the ATPase may require a lipid environment, (as it has in mitochondrial membranes) for observation of an ethylene stimulation. Investigations in the present studies involved the use of intact mitochondria with the membranes having their full complement of protein and phospholipids.

1. Equilibration of an Ethylene:Air:Water Mixture

The length of time required for a solution to absorb its maximum concentration of ethylene from an ethylene:air mixture was analyzed by purging a solution in a vessel with the gas mixture for specific lengths of time. After treating samples and analyzing for the concentration of ethylene in the air space above the solution, it was found that, after 3 min of treating a solution at a flow rate of 50 cc/min, the solution had attained a constant level for this particular mixture. In order to estimate the actual amount of ethylene in this solution, an aliquot of the solution was taken and introduced into another tube which was promptly stoppered. After allowing sufficient time (4 hr) for equilibration of the ethylene in the solution with the air

(ethylene-free) in the tube, a sample of the air mixture was withdrawn and injected into the gas chromatograph (see Appendix for GC response and calibration). From this measurement, it was mathematically calculated that the partition coefficient of ethylene water:ethylene air was 0.090. This coefficient was in close agreement with that reported in the literature of 0.115 at 25° and 760 mm Hg (McAuliffe, 1966).

A 100 ppm ethylene mixture was routinely used since the concentration resulting in the solution would be about 10 ppm and hence would be in a physiological range.

2. Reproducibility of Test System

The test systems involving ethylene stimulation of ATPase activity were found to be not as reproducible as those in the absence of ethylene. The variations recorded (Table XII) for the monovalent cation stimulation seemed to be greater than for Mg^{++} - induced activity alone. However, after observing the difference in response of the two systems to ethylene, the greater variations were understandable. The difference in activity between non-treated and air-treated samples was thought to be attributable to a slight amount of evaporation of the aqueous solution during the treatment. This evaporation would result in a higher enzyme concentration in the sample.

3. Effect of Ethylene on Mg^{++} - ATPase Activity

The treatment of mitochondria with the 100 ppm ethylene mixture

Table XII. Variability of ATPase activity induced by ethylene.

Treatment	ATPase activity (n moles P_i/min/mg protein)					
	0.3 M Sucrose			0.1 M Sucrose + 0.085 M NaCl + 0.015 M KCl		
Non-treated	7.78	7.80		11.50	11.45	
	7.84	7.83		11.46	11.49	
	7.80	7.79		11.43	11.46	
Ave.	7.81 ± 0.03		Ave.	11.46 ± 0.04		
Air-treated	7.94	7.93		11.50	11.57	
	7.97	7.96		11.59	11.65	
	8.03	7.89		11.63	11.52	
Ave.	7.95 ± 0.08		Ave.	11.58 ± 0.07		
Ethylene-treated	10.92	10.97		18.63	18.53	
	10.80	10.79		18.40	18.43	
	10.83	10.85		18.36	18.47	
Ave.	10.87 ± 0.10		Ave.	18.47 ± 0.15		

Assay conditions were 0.3 M osmoticant (as noted), 3 mM $MgCl_2$ 3 mM Tris-ATP and 25 mM TES (Tris) pH 7.2 at 25°. Ethylene treated samples obtained by purging the assay mixture (prior to addition of mitochondria and Tris-ATP) with a 100 ppm ethylene-air mixture for 3 min at a flow rate of 50 cc/min. Air treated samples were similarly prepared by using ethylene-free air.

was found to stimulate the Mg^{++} - ATPase activity by as much as 40%. Since this activity is dependent on a Mg:ATP ratio of unity and substrate was used at saturating concentrations (3 mM Mg ATP with a Km of 1.0 mM), this stimulation could not be a result of an increase of substrate concentration. Ethylene may however be acting as an allosteric heterotropic modifier (Monod et al. 1963) wherein it has structurally modified the enzyme(s) so as to enhance recognition of the substrate by the enzyme(s). Alternatively, this effect could be a result of an increase in permeability of the mitochondrial membrane to ATP translocation.

4. Effect of Ethylene on Cation Stimulated ATPase Activity

The involvement of ethylene with the monovalent cation-stimulated activity was studied. It was observed that ethylene would not stimulate K^+ - ATPase (Figure 35) but did however have an allosteric heterotropic effect on the Na^+ - stimulated ATPase activity (Figures 36 and 37). The validity of subtracting Mg^{++} activity from $Na^+ + K^+ + Mg^{++}$ activity (since there is a possibility that there are two enzymes operating at the same time) is questionable (Robertson, 1967). This stimulation by ethylene may similarly be accountable if the stimulation by Na^+ is dependent on Na^+ transport and if ethylene has increased the rate of transport by altering membrane permeability. The possibility that the ethylene induced Mg^{++} - ATPase activity was a result of increased sensitization of the Na^+ activity in the presence of trace amounts of Na^+ cannot be ruled out.

Stimulation by ethylene of the $Na^+ + K^+$ induced activity was

Figure 35. Effect of ethylene on K^+ stimulated ATPase activity of pea mitochondria. Ethylene treatments (□——□) involved the purging of the assay mixture with a 100 ppm ethylene-air mixture (at a rate of 50 cc/min for 3 min) prior to the addition of mitochondria and Tris-ATP. Solutions were treated at 0° in 100 x 15 mm culture tubes that were immediately stoppered with rubber vial septa. Control tubes (○——○) were treated with ethylene-air and treated similarly.

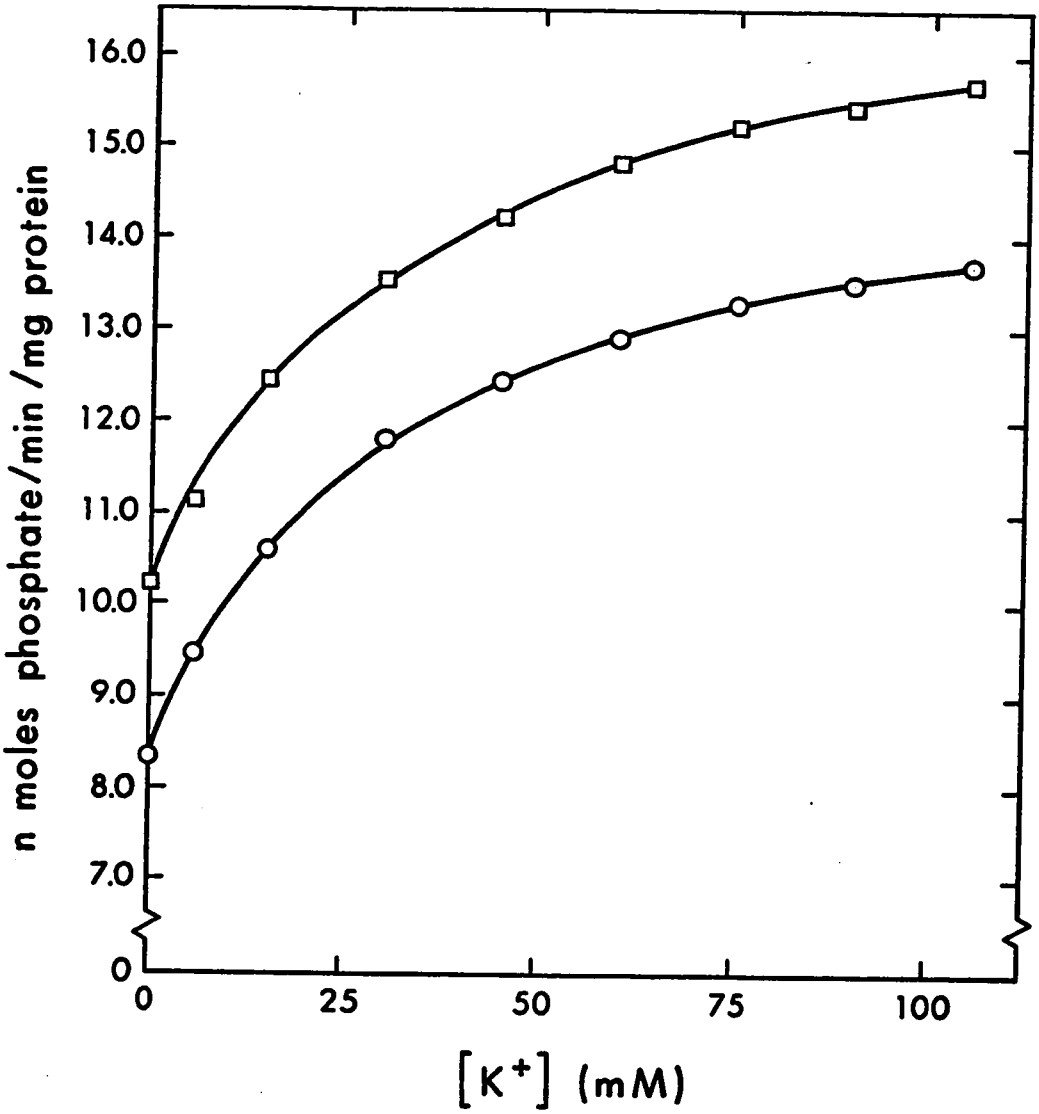

16.0
15.0
14.0
13.0
12.0
11.0
10.0
9.0
8.0
7.0
0
0
25
50
75
100
n moles phosphate/min /mg protein
[K+] (mM)

Figure 36. Effect of ethylene on Na^+ stimulated ATPase activity of pea mitochondria. Ethylene treatments (□——□) are as outlined in Figure 36. Control tubes (○——○) were treated with ethylene-free air.

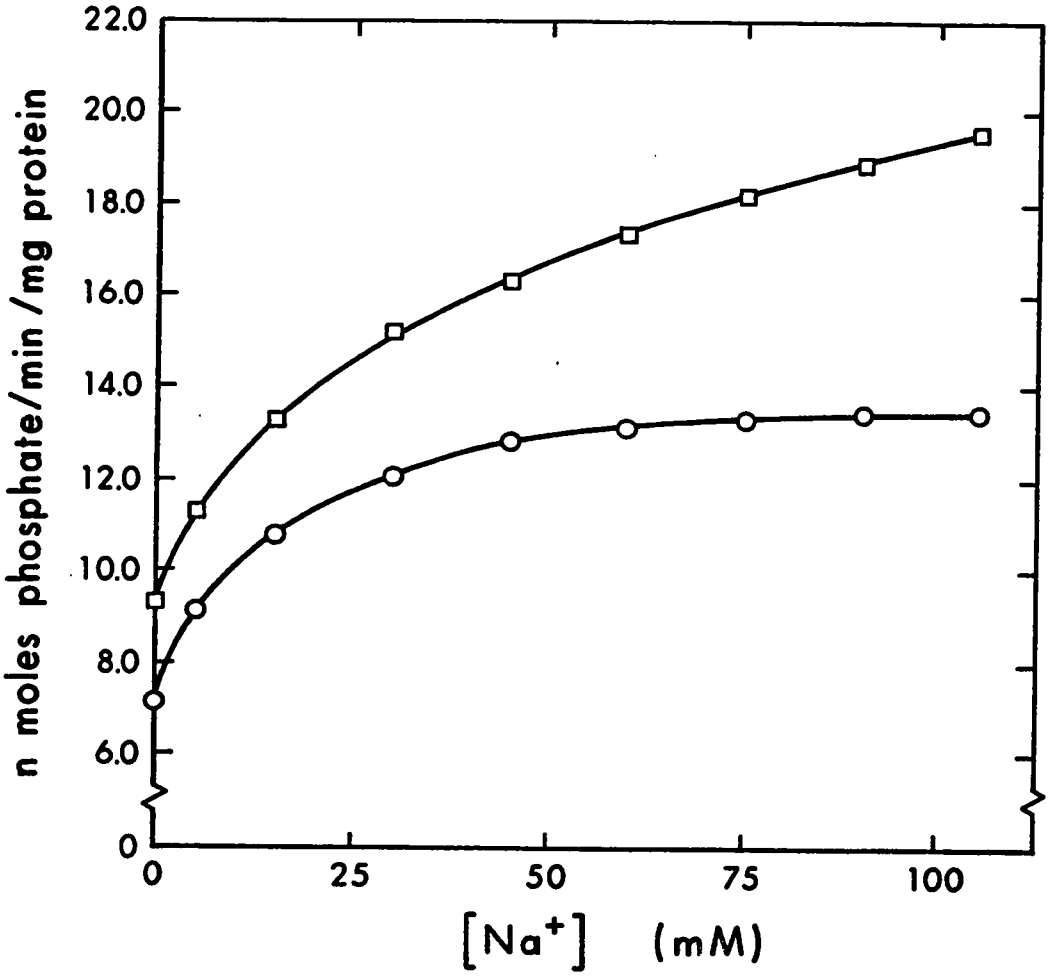
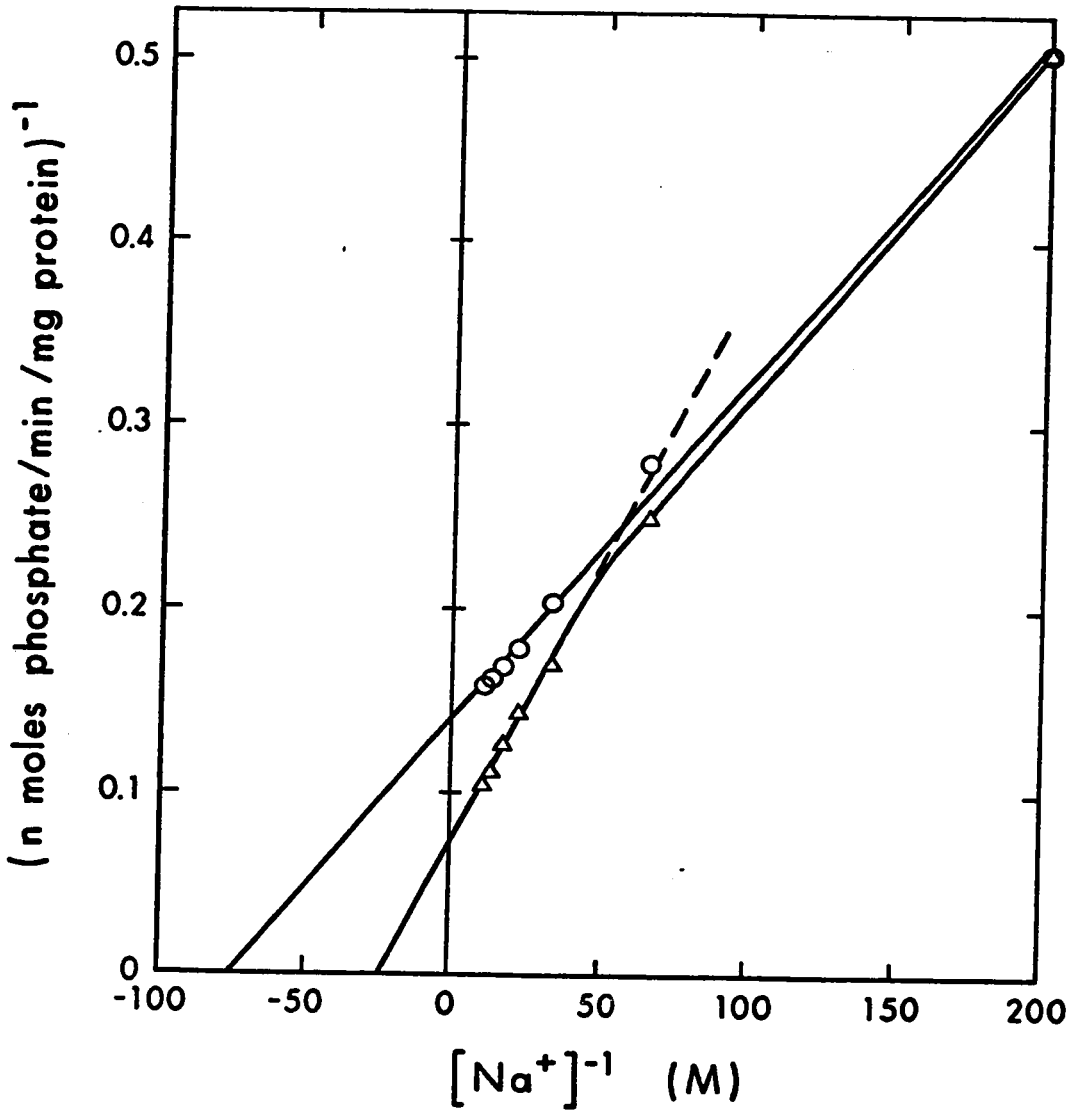

22.0
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
0
n moles phosphate/min /mg protein
0
25
50
75
100
[Na+] (mM)

Figure 37. Double reciprocal plot of the effect of ethylene on Na^+ stimulated activity. The amount of stimulation by ethylene at 0 mM Na has been subtracted from the various values of ethylene stimulated activity. Samples were treated with ethylene (Δ—Δ) or ethylene-free air (○—○) as outlined in Figure 35.


0.5
0.4
0.3
0.2
0.1
0
-100
-50
0
50
100
150
200
(n moles phosphate/min/mg protein)$^{-1}$
$[Na^+]^{-1}$ (M)

also observed (Figure 38). The sigmoidal nature of the curve was still maintained so that it was thought that ethylene instead of enhancing penetration or translocation of the ions was in fact enhancing the ability of the enzyme(s) to recognize Na^+. It may, therefore, act as heterotropic allosteric modifier of the enzyme towards Na^+. If the ethylene were acting as an unspecific modifier of ion transport it would be expected to stimulate ATPase at low concentrations of K^+.

In order to further investigate the effect of ethylene on the Mg^{++} as well as $Mg^{++} + Na^+ + K^+$ - ATPase activity, the concentration dependency of the activity was observed (Figure 39). It can be seen that ethylene offers a hyperbolic relationship with respect to Mg^{++} - ATPase but a linear function with respect to $Na^+ + K^+$ - ATPase activity. Since ethylene was found to stimulate the Na^+ - ATPase components, this may be the effect which is being observed and not, indeed, an effect on the $Na^+ + K^+$ activity, since the sigmoidicity increases with increasing Na^+ concentration.

5. Ethylene and Azide Inhibition

Since the possibility always exists of a change in the character of the mitochondria during studies involving ethylene, an evaluation of this change in character was studied with respect to the action of azide on ethylene stimulated activity. From Figure 40, it was observed that the increased activity due to ethylene still succumbed to the inhibitory activity of azide. As a result of these experiments it was thought that the morphology of the mitochondria had not been altered and that the mitochondria being studied after treatment with

Figure 38. Effect of ethylene on monovalent cation stimulated ATPase activity. The concentration of K^+ was constant at 15 mM. The base level of Mg^{++} - ATPase was 8.30 n moles P_i/min/mg protein. The osmolarity of solutions were adjusted to 0.3 by the addition of sucrose. Ethylene (□—□) and air (○—○) samples were prepared as outlined in Figure 35.

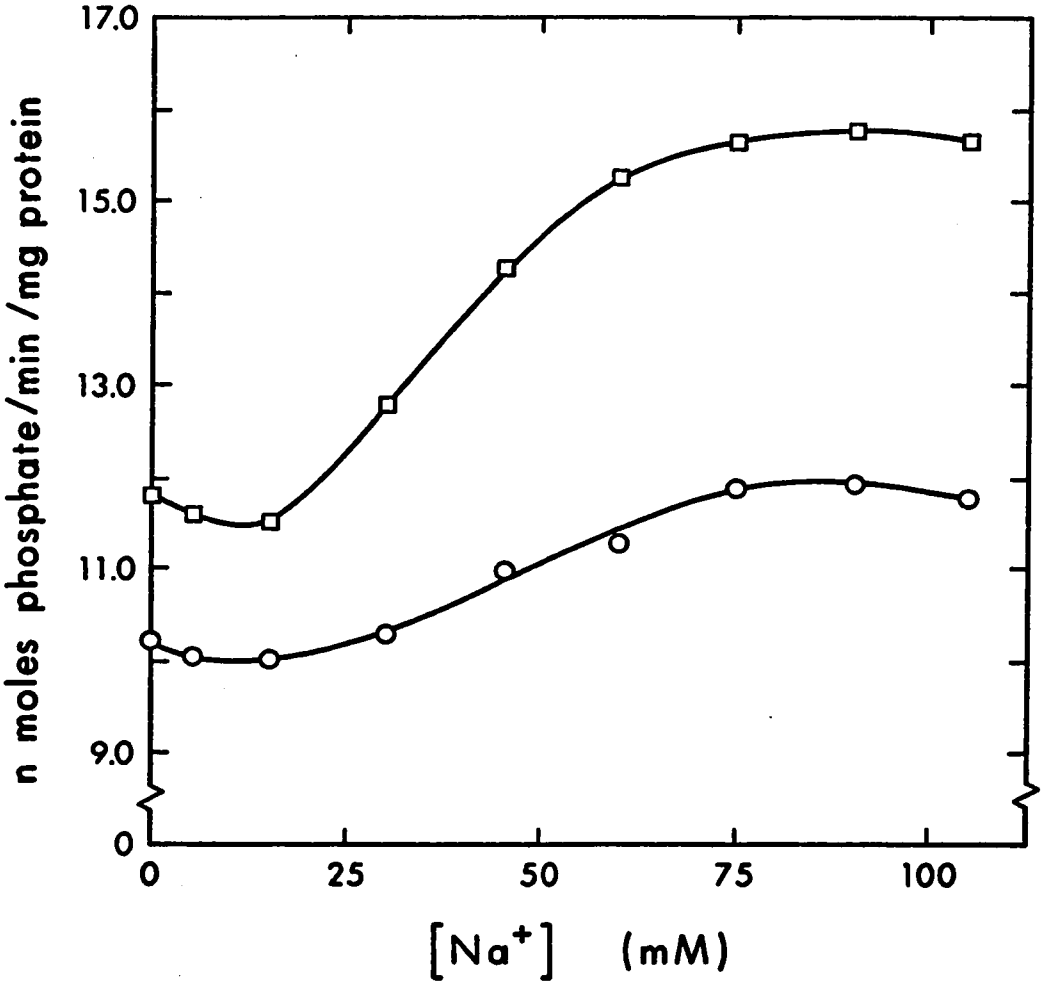

17.0
15.0
13.0
11.0
9.0
0
0
25
50
75
100
n moles phosphate/min /mg protein
[Na+] (mM)

Figure 39. Effect of ethylene concentration on the ATPase activity of pea mitochondria. Assay conditions in the presence of 0.3 M sucrose (o—o) or 0.1 M sucrose 85 mM NaCl, 15 mM KCl (□—□), 25 mM TES (Tris) pH 7.2 at 25°, 3 mM $MgCl_2$, and 3 mM Tris-ATP. The difference between $Na^+ + K^+ + Mg^{++}$ and Mg^{++} activity (Δ—Δ) is shown. Ethylene treated samples were prepared as outlined in Figure 35.

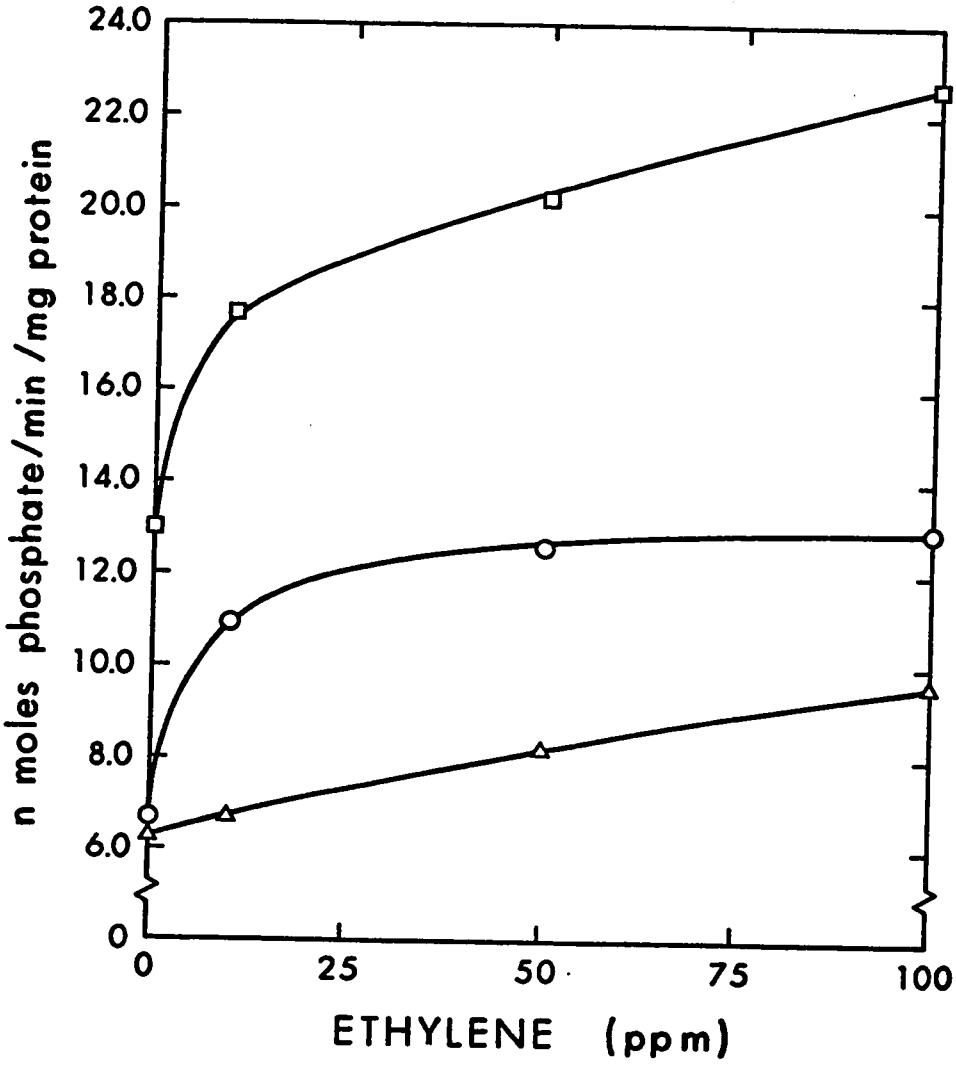
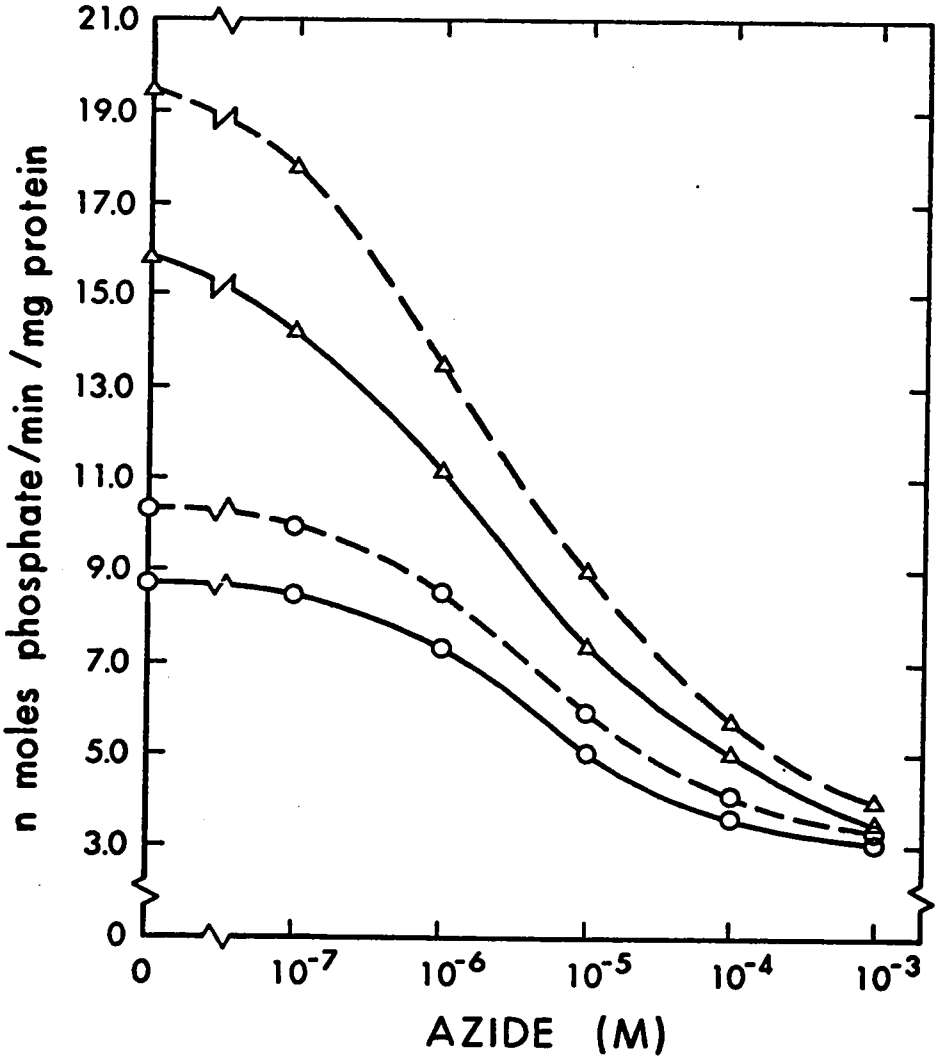

24.0
22.0
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
0
n moles phosphate/min /mg protein
0
25
50
75
100
ETHYLENE (ppm)

Figure 40. Effect of azide on ethylene-stimulated ATPase activity of pea mitochondria - Mg^{++} - ATPase (○) and, $Na^{+} + K^{+} + Mg^{++}$ - activity (▲) in the absence (——) or presence (— —) of ethylene. Assay conditions were either 0.3 M sucrose or 0.1 M sucrose, 85 mM NaCl 15 mM KCl and 3 mM $MgCl_2$, 25 mM TES (Tris) pH 7.2 at 25° and 3 mM Tris-ATP. Ethylene treatment procedure were as outlined in Figure 35.


21.0
19.0
17.0
15.0
13.0
11.0
9.0
7.0
5.0
3.0
0
n moles phosphate/min /mg protein
0
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
AZIDE (M)

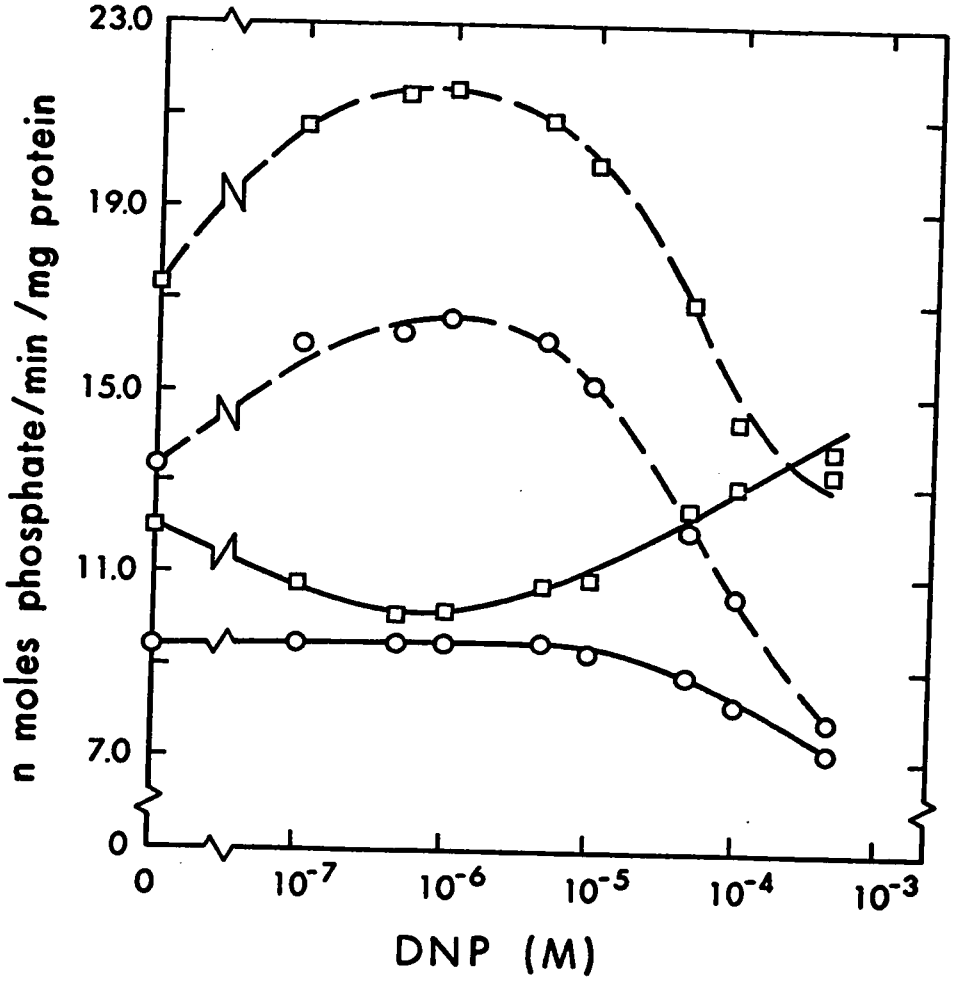
air or with ethylene were one and the same. These results would, therefore, seem to complement those wherein it was observed that ethylene treatment had not altered the respiratory parameters of the mitochondria.

6. Interaction of Ethylene and DNP

During a study of the effects of ethylene on DNP activity of Mg^{++} - ATPase, it was observed that DNP had an inhibitory effect on Mg^{++} - ATPase activity at high concentrations and no effect at low concentrations. However, when ethylene was present in the reaction mixture, DNP was observed (Figure 41) to result in an inhibition of Mg^{++} + ethylene ATPase activity at low concentrations and to subsequently give some stimulation of activity at the normally inhibitory high concentrations. As to whether still higher concentrations of DNP would further stimulate or inhibit Mg^{++} + ethylene - ATPase activity was not determined. Stimulation of $Na^{+} + K^{+}$ - ATPase activity by DNP was not altered by ethylene apart from an apparent constant stimulation.

In order to explain the inhibition of Mg^{++} - ATPase activity at low DNP concentrations and stimulation at high DNP concentrations in the presence of ethylene, the following proposals are made. Throughout the present study it has been observed that the properties of the mitochondria (respiratory parameters and ATPase activity) have been altered when the suspending solution composition was altered. Perhaps the addition of DNP is sufficient at the higher concentrations to alter mitochondrial structure through an osmoregulatory effect. (The addition of cations by changing ionic strength has possibly

Figure 41. Effect of DNP on ethylene-stimulated ATPase activity of pea mitochondria. Mg^{++} - ATPase (—) and Na^{+} + K^{+} + Mg^{++} - activity (— —) in the presence (□) or absence (○) of ethylene. Assay conditions are as outlined in Figure 40. Ethylene treatment procedures were as outlined in Figure 35.


23.0
19.0
15.0
11.0
7.0
0
n moles phosphate/min /mg protein
0
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
DNP (M)

changed mitochondrial protein conformational states and has thus facilitated activation by DNP). Thus, from this change in state, ethylene is now able to activate the changed reaction site. Alternatively, an interaction of ethylene (in one of its resonance forms) and dinitrophenol (possibly the resonance form of partial positive charge on the nitrogen and negative charge on the enolate oxygen) in a lipid phase may result in effective removal of ethylene by complex formation and thus negate its presence at low DNP concentrations. At high DNP concentrations, complex formation in the presence of excess DNP may have allowed conformational changes in the protein of the membrane to occur and subsequently enhance ATP translocation.

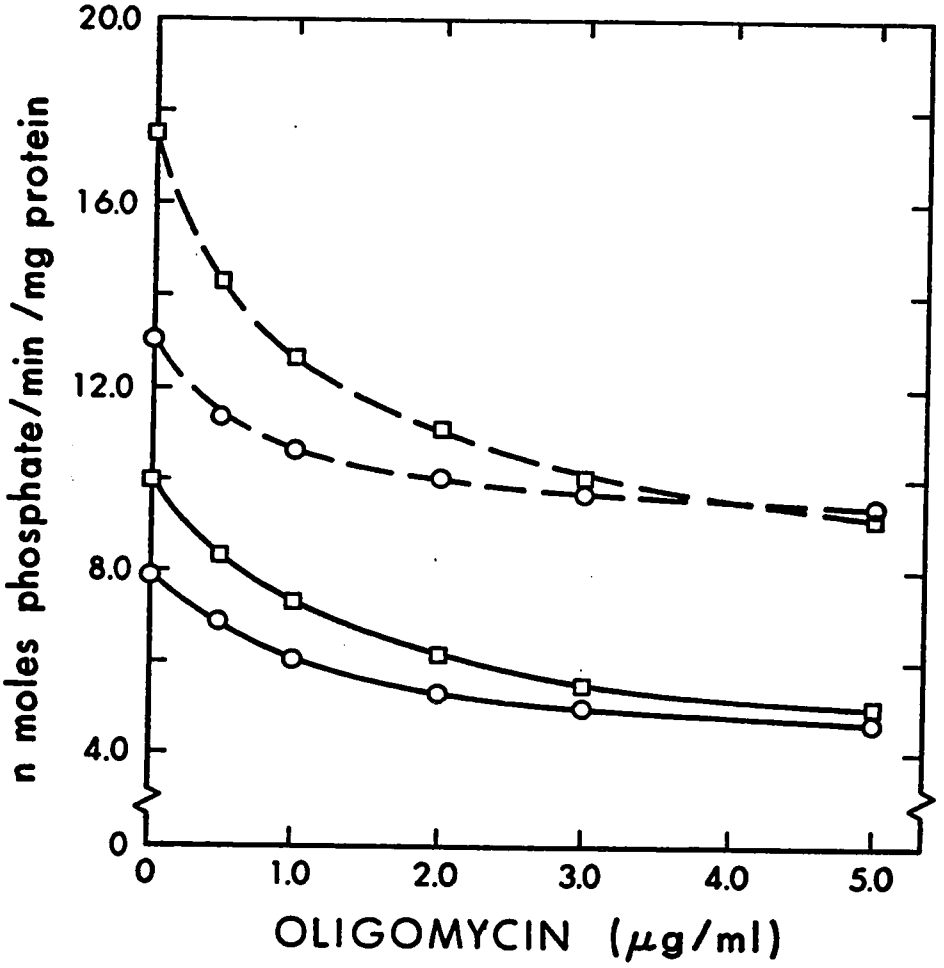
7. Interaction of Ethylene and Oligomycin

An inhibitory action of oligomycin (as recorded in Chapter IV, Section H) on monovalent cation stimulated ATPase activity was not observed. However, the ethylene stimulated activity of the $Na^+ + K^+$ - ATPase was observed to be inhibited by oligomycin (Figure 42). This fact would indeed imply the existence of two separate enzymes, one that is Mg^{++} dependent, Na^+ activated, ethylene activated, and oligomycin sensitive and another that is Mg^{++} dependent, $Na^+ + K^+$ activated, ethylene activated and oligomycin insensitive.

8. Effect of Surface-Active Agents

In order to clarify if ethylene was exerting an effect on altering membrane permeability in a unspecific fashion, experiments were devised that would shed some light on this hypothesis. Triton

Figure 42. Effect of oligomycin on ethylene-stimulated ATPase activity of pea mitochondria. Observations of Mg^{++} - ATPase (—) and $Na^{+} + K^{+} + Mg^{++}$ - ATPase (— —) were recorded in the presence (□) and absence (○) of ethylene. Assay conditions are as outlined in Figure 40. Ethylene treatment procedures were as outlined in Figure 35.

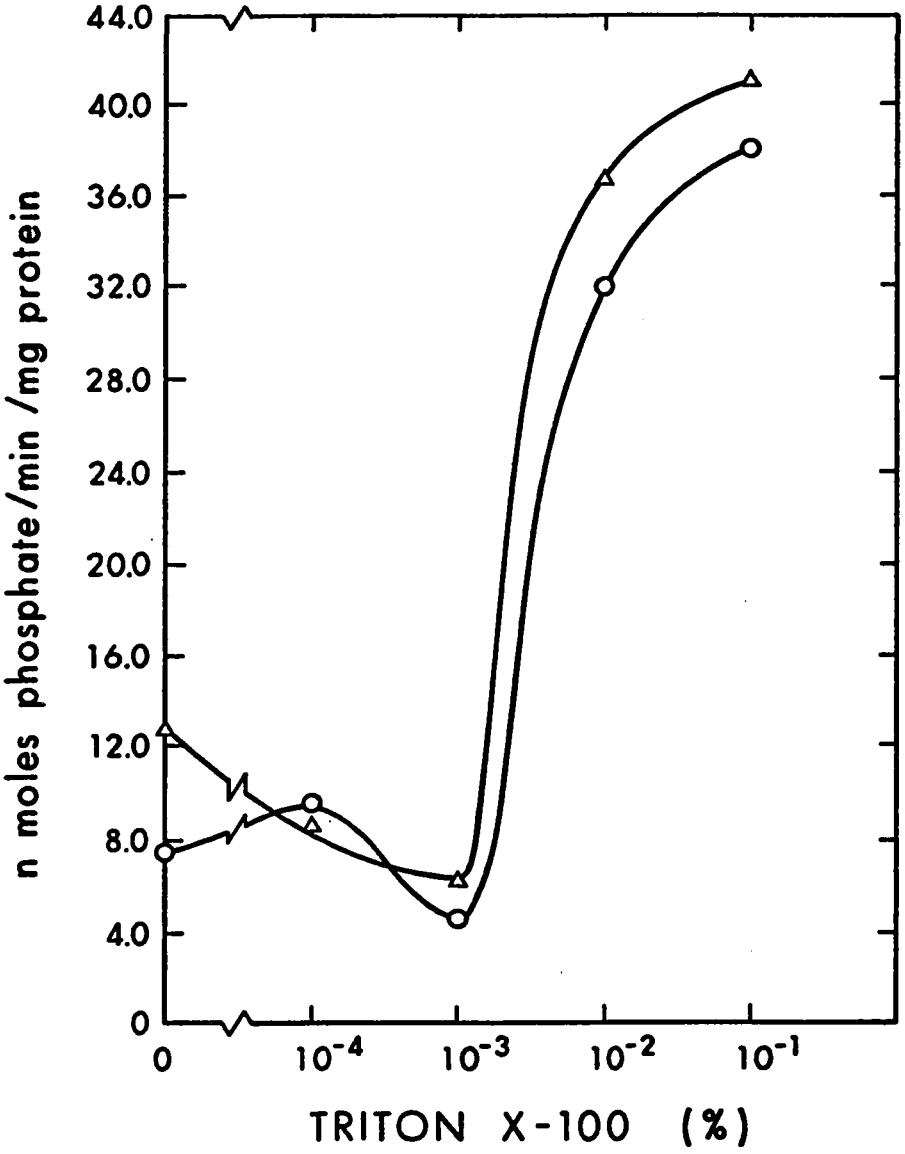

20.0
16.0
12.0
8.0
4.0
0
n moles phosphate/min /mg protein
0
1.0
2.0
3.0
4.0
5.0
OLIGOMYCIN (μg/ml)

X-100 (a non-ionic surfactant) additions to mitochondrial suspensions were made in the presence of ethylene. This surface-active agent is routinely used at high concentration to solubilize mitochondrial protein and to alter membrane configuration at low concentrations. If ethylene and Triton X-100 were having dissimilar effects on mitochondrial membranes, the ATPase activity of the preparations would not be governed solely by the presence of surfactant.

However, when experiments were performed with mitochondria suspended in increasing concentrations of Triton X-100, it was observed (Figure 43) that the effect of ethylene on the activity was, in general, governed by the presence of Triton X-100.

The results of the above experiments as well as in a comparison of the effect of Triton X-100 and ethylene (Figure 38) at various concentrations alone, it could be suggested that if ethylene were altering membrane structure of the mitochondria, it was doing so in a specific fashion.

Figure 43. Effect of Triton X-100 on ethylene stimulated ATPase activity of pea mitochondria. Assays were conducted in the absence (o—o) and presence (Δ—Δ) of ethylene on Mg^{++} - ATPase.


44.0
40.0
36.0
32.0
28.0
24.0
20.0
16.0
12.0
8.0
4.0
0
n moles phosphate/min /mg protein
0
10-4
10-3
10-2
10-1
TRITON X-100 (%)

CHAPTER VII

GENERAL DISCUSSION AND CONCLUSIONS

Ethylene evolution and respiration of plant tissue (climacteric fruit, bean cotyledons and leaves) are known to be intimately related. In addition, previous theses from this laboratory have shown the effect of ethylene on the sub-cellular site of respiration, the mitochondria. The above studies included observations on the rate of volume changes and the rate of ATP hydrolysis as altered by ethylene treatment of the mitochondria from bean cotyledons. The present investigation attempted to characterize the respiratory and ATPase activity of pea mitochondria and to study the effect of ethylene on these parameters.

Mitochondria isolated from five day old pea cotyledons, under the isolation conditions employed, displayed respiratory parameters indicative of mitochondria with a high degree of integrity. These respiratory parameters were observed to change within one polarographic analysis. The first cycle of ADP addition had a very low ADP/O value and a low respiratory control ratio (RCR) compared to subsequent ADP additions. The RCRs were observed to be greater if mannitol instead of sucrose was employed as osmoticant. Pea mitochondria readily oxidized all Krebs cycle acids and gave large RCR values with the substrate α-ketoglutarate (frequently approaching infinity). The effect of uncouplers and inhibitors of respiration was found to be stimulatory (at low concentrations) and inhibitory to oxygen utilization, respectively.

The simultaneous occurrence of maximal respiratory parameters of mitochondria from five day old tissue with a maximal ATPase activity value may, indeed, be very pertinent to the understanding of the development of mitochondria in germinating seeds. This simultaneity may be attributed to increasing amounts or degrees of organization of electron carriers and/or coupling factors of the mitochondria. Alternatively, the '*de novo*' synthesis or activation of these components during the initial growth stages may be suggested.

Preliminary experimentation involved a study of various preparative procedures for mitochondria. A procedure that involved the washing of sedimented mitochondria was found to vastly improve the respiratory parameters of these sub-cellular particles. This washing step removed extraneous protein that had a residual amount of State IV respiration. Sucrose density gradient centrifugation achieved a higher amount of State III respiration of the particles thus obtained however, the ADP/O and RCR values had not changed appreciably. (It was also shown that the mitochondria obtained by SDG procedures, contained the majority of the ATPase activity of the preparation).

The '*in vitro*' stability of the mitochondrial preparations was found to be high; the respiratory parameters of the mitochondria after 26 hr of storage at 0°, were unaltered from the parameters of freshly isolated particles.

In a study of the ATPase activity of these mitochondria, the establishment of a sensitive, reproducible procedure for the detection of the inorganic phosphate produced was imperative. Achievement of this sensitivity was possible by using sucrose as osmoticant

and the spectrophotometric estimation of the oxidized phosphomolybdate complex in an organic phase. By the duplicate estimation of ADP released from hydrolysis of ATP, it was shown that apyrase activity was negligible.

The lack of pH optimum for the ATPase activity of plant mitochondria has also been observed by other workers (Yoshida et al. 1968). The increase of activity at higher pH values may be attributable to loss of membrane structure and/or an uncoupled state.

The partial characterization of the ATPase activity indicated the absolute requirement for the divalent cation, magnesium. Optimum activity was obtained when the ratio of Mg to ATP was unity. An identical requisite has been observed for the ATPase activity of animal red cell membranes (Mann, 1968). The Km of the activity for Mg ATP was found to be about 1 mM which again is in the same order of magnitude as that observed for animal membrane tissue. Since there has been very little experimentation in this area with mitochondria it was impossible to corroborate these findings.

The specificity of the ATPase reaction for ATP was found to be high with ADP, GTP, and with p-nitrophenol phosphate showing limited hydrolysis. ADP was found to non-competitively inhibit the ATPase activity. However, this observation was complex since ADP itself was known to be slowly hydrolyzed.

The use of different osmoticants in the present study indicated a specificity that may resolve conflicting observations reported in the literature. These reports describe the effect of the uncoupler, DNP, on mitochondrial ATPase as that of either having no effect

(Wedding and Black, 1962), being stimulatory (Forti, 1957; Stoner et al. 1964) or inhibitory (Reid et al. 1964). As was observed in this dissertation, each of these possibilities can arise and be altered by judicious choice of osmoticant (composition and strength) and concentration of DNP. DNP will stimulate mitochondrial ATPase activity if mannitol or sucrose plus monovalent cations are employed. A low concentration of DNP in the presence of sucrose alone has a negligible effect whereas a high DNP concentration with sucrose was found to be inhibitory. Thus all combinations of effects of DNP on mitochondrial ATPase activity are possible.

Observations of the effects of monovalent cations on the ATPase activity indicated a specificity that was indeed revolutionary with respect to mitochondria. Not only was it shown that the ions potassium and sodium differentially stimulate the activity but also when used in combination display sigmoidal activation that is dependent on the fixed concentration of potassium ion. The individual activation of the ATPase activity by potassium exceeded that of sodium at identical concentrations as was observed by Lardy and Wellman (1953). Combination of the two ions in an approximate ratio of 5 Na^+ to 1 K^+ was observed to give an activation of the ATPase activity that exceeded the individual ion-induced stimulation at an equivalent osmolarity. These observations are indeed relevant in the light of recent investigations of the ATPase activity of red cell ghosts and nerve tissue. The similarity of results between the ATPase activity of these specialized cells and pea mitochondria are very relevant to the understanding of the ionic composition of vesicular structures

and the polarity of their membranes. Although the characteristics of the mitochondrial ATPase activity observed here are not in complete accord with the well defined characteristics of cellular membrane ATPase activity, the $Na^+ + K^+$ induced activity is significant. The sensitivity of the $Na^+ + K^+$ induced activity to the cardiac glycoside, ouabain, is much greater for the activity of specialized cell membranes than for the mitochondrial activity. However, there may be present in plant tissue an entirely different inhibitor which has greater specificity than ouabain. (Ethancrynic acid is a more potent inhibitor of the Na^+ pump of the toad oocyte than ouabain (Bittar, 1970)).

The antibiotic valinomycin was observed to specifically stimulate K^+ - ATPase activity with a negligible effect on the Na^+ - induced activity. This stimulation by valinomycin of the K^+ - ATPase activity corresponded to the activity induced by $Na^+ + K^+$ in combination. Thus, it may be possible that the presence of sodium ion induced the site of ATP hydrolysis to become more sensitive to potassium ion. Alternatively, since valinomycin is known to facilitate K^+ transport in mitochondria, the reason for $Na^+ + K^+$ activity approximating K^+ + valinomycin activity may be a result of Na^+ enhancing K^+ transport. However, the observation at a fixed K^+ ion concentration and varying Na^+ ion concentration indicated an inhibitory effect of Na^+ ion on the K^+ activity alone with maximal inhibition at a Na^+ to K^+ ratio of unity. Wenner and Hackney (1969) have observed on inhibition by Na^+ of K^+ induced H^+ ion release or light-scattering changes mediated by ATP in mouse-liver mitochondria.

In order to further characterize this ion induced ATPase activity, it was observed that the respiratory inhibitor azide would inhibit the stimulated activity but oligomycin was found to be ineffectual on this activity. This oligomycin insensitivity would lead one to suggest an altered pathway apart from the reverse of oxidative phosphorylation when ATP is utilized in ion translocating processes as has also been suggested by Fisher and Hodges (1969). This pathway may involve the work function ($\sim$ W) of Lardy as described by Ernster et al. (1967).

The present investigation also observed the interaction of ethylene (a plant hormone) with mitochondrial ATPase activity. The intimate relationship of ethylene evolution with respiration of tissue is well known and the effects of ethylene on the sub-cellular site of respiration have been previously studied in this laboratory (Olson, 1967). In accord with this worker, it was observed that ethylene would stimulate mitochondrial ATPase activity. Ethylene was also shown to stimulate Na^+ - induced activity and to have no effect on K^+ - induced activity. The sigmoidicity of the activity in the presence of $Na^+ + K^+$ was found to be altered by ethylene in such a way so as to indicate increased sensitization of the activity for Na^+ ions. This observation was felt to be of significance with respect to not only the concomitant increase of respiration and ethylene evolution of plant tissue (increase of phosphate acceptor) but also the observations of altered rates of mitochondrial volume (osmoregulatory) changes induced by ethylene (that would be related to ion movements across membranes). In addition, it was shown that

the relationship between the effect of ethylene and DNP on mitochondrial ATPase activity was, indeed, complex. In the presence of Mg alone, DNP would inhibit ethylene stimulation at low DNP concentrations and stimulate this activity at high concentrations. This may be of major significance in the understanding of the different states of mitochondrial contraction induced by the various osmoticants employed in research work.

These effects of ethylene were shown to be entirely independent of a membrane disruptive process since the effects induced by Triton X-100, a surfactant, were unrelated to those of ethylene.

All of the above results may lead one to speculate on the possibility that ethylene from its autocatalytic rate of evolution from ageing tissue (Spencer, 1969) may enhance respiration by increasing ion flux and hence the turnover of the phosphate acceptor moiety. Alternatively, with increases in ethylene content, plant cells and mitochondria may find it increasingly difficult to maintain a homeostatic condition because of the more rapid flux of ions (cation ATPase activity was found to increase with ethylene concentration) and that the integrity of the mitochondria eventually comes into question. It has been reported that with senescence of plant tissue, the mitochondria is the first structure which shows signs of disintegration. Another interesting possibility is that with increasing age of tissue, the Na^+ to K^+ ratio of the cytosol may change (a condition which is technically difficult to measure because of the large vacuole of plant cells) and may therefore decrease or increase the sensitization of the mitochondria to ethylene. This could be implicated with

the autocatalytic nature of ethylene production (if ethylene is not serving a useful purpose in older tissue because of the change of the cation ratio, it may be evolved; if it is serving an increasing role of inducing cation flux, this involvement may trigger production and hence evolution of it in order tissue).

The present dissertation has shown a characterization of mitochondrial ATPase activity that has been relatively unexplored. It was of major significance to observe the similarity between the vesicular mitochondria of plants and the specialized cells of animal origin with respect to ATPase activity. A maintenance of homeostatic conditions with changing environments may be possible by the operation of an ATPase activity that is stimulated by cations and ethylene.

REFERENCES

ABELES, F.B. 1966. Ethylene production from linolenic acid. Nature 210: 23-35.

ADAM, H. 1965. Adenosine-5'-diphosphate and adenosine-5'-monophosphate. In:Methods of Enzymatic Analysis. H.U. Bergmeyer, Ed. Academic Press, New York. p. 573.

ANDREOLI, T.E., M. TIEFFENBERG and D.C. TOSTESON. 1967. The effect of valinomycin on the ionic permeability of thin lipid membranes. J. Gen. Physiol. 50:2527.

ASKARI, A. and D. KOVAL. 1968. Different oligomycin sensitivities of the $Na^+ + K^+$ - activated adenosine triphosphatase and its partial reactions. Biochem. Biophys. Res. Commun. 32:227.

AZZI, A. and G.F. AZZONE. 1966. Swelling and shrinkage phenomena in liver mitochondria IV. Reversible swelling changes linked to transport of monovalent cations stimulated by valinomycin. Biochim. Biophys. Acta 113: 445-456.

BAIN, J. and F.V. MERCER. 1966. Subcellular organization of the cotyledons in germinating seeds and seedlings of Pisum sativum L. Aust. J. Biol. Sci. 19:69-84,

BEEVERS, H. 1961. Respiratory metabolism in plants. Harper and Row, New York.

BIELAWSKI, J., T.E. THOMPSON and A.L. LEHNINGER. 1966. The effect of 2,4-dinitrophenol on the electrical resistance of phospholipid bilayer membranes. Biochem. Biophys. Res. Commun. 24:948-953.

BITTAR, E.E. 1970. The action of $Na^+ - K^+$ - ATPase inhibitors on sodium efflux from the toad oocyte. Life Sciences 9: 773-779.

BLAIR, P.V. and F.A. STOLLAR. 1967. Contraction of hypotonically swollen mitochondria by oxidizable substrates. Biochem. Biophys. Res. Commun. 27:202-208.

BONNER, W.D. 1965. Mitochondria and electron transport. In Plant Biochemistry. J. Bonner and J.E. Varner, Eds., 2nd ed. Academic Press, New York. p. 89-123.

BOYER, P.D. 1965. Carboxyl activation as a possible common reaction in substrate-level and oxidative phosphorylation and in muscle contraction. In:Oxidases and related redox systems. T.E. King, H.S. Mason and M. Morrison, Eds. John Wiley and Sons, New York. p. 994-1017.

BOYER, P.D. 1968. Oxidative phosphorylation. In:Biological Oxidations. T.P. Singer, Ed. Interscience Publishers, New York. p. 193-233.

BROWN, H.D., N.J. NEUCERE, A.M. ALTSCHUL and W.J. EVANS. 1965. Activity patterns of purified ATPase from Arachis hypogaea. Life Sciences 4:1439-1447.

BURG, S.P. and E.A. BURG. 1962. Role of ethylene in fruit ripening. Plant Physiol. 37:179-189.

BURG, S.P. 1968. Ethylene, plant senescence and abscission. Plant Physiol. 43:1503-1511.

CARAFOLI, E., C.S. ROSSI and P. GAZZOTTI. 1969. The effects of uncoupling agents and detergents on the movement of monovalent cations between mitochondria and medium. Arch. Biochem. Biophys. 131:527-537.

CASWELL, A.M. 1969. Proton permeability and the regulation of potassium permeability in mitochondria by uncoupling agents. J. Membrane Biol. 1:53-78.

CHANCE, B. and G.R. WILLIAMS. 1955. Simple and rapid assay of oxidative phosphorylation. Nature 175:1120-1121.

CHANCE, B. and G.R. WILLIAMS. 1956. The respiratory chain and oxidative phosphorylation. Advan. Enzymol. 17: 65-134.

CHANCE, B. and T. YOSHIOKA. 1966. External Ca concentrations associated with membrane alkalinization in mitochondria. Biochemistry 5:3224-3231.

CHANDRA, G.R. and M. SPENCER. 1963. Ethylene production by subcellular particles from rat liver, rat intestinal mucosa and Penicillium digitatum. Nature (London) 197: 366-367.

CHAPPELL, J.B. 1964. The effects of 2,4-dinitrophenol on mitochondrial oxidations. Biochem. J. 90:237-248.

CHAPPELL, J.B. and K.N. HAARHOFF. 1967. The penetration of the mitochondrial membrane by anions and cations. In: Biochemistry of mitochondria. E.C. Slater, Z. Kanuiga and L. Wojtczak, Eds. Academic Press, New York, p. 75-91.

CHAPPELL, J.B. and B.H. ROBINSON. 1968. Penetration of the mitochondrial membrane by tricarboxylic acid anions. In: Metabolic Roles of Citrate. T.W. Goodwin, Ed. Academic Press, New York. p. 123-134.

CLELAND, W.W. 1963. The kinetics of enzyme catalyzed reactions with two or more substrates or products. Biochim. Biophys. Acta 67:104-137.

COCKRELL, R.S., E.J. HARRIS and B.C. PRESSMAN. 1966. Energetics of potassium transport in mitochondria induced by valinomycin. Biochemistry 5:2326-2335.

COCKRELL, R.S. and E. RACKER. 1969. Respiratory control and K^+ transport in submitochondrial particles. Biochem. Biophys. Res. Commun. 35:414-419.

COOPER, C. and A.L. LEHNINGER. 1956. Oxidative phosphorylation by an enzyme complex from extracts of mitochondria. J. Biol. Chem. 219:519-529.

CRANE, F.L. 1961. Structure and function of mitochondria. Ann. Rev. Plant Physiol. 12:13-24.

CROSS, R.L., B.A. CROSS and J.H. WANG. 1970. Detection of a phosphorylated intermediate in mitochondrial oxidative phosphorylation. Biochem. Biophys. Res. Commun. 40:1155-1161.

DE LUCA, H.F. and G.W. ENGSTROM. 1961. Calcium uptake by rat kidney mitochondria. Proc. Natl. Acad. Sci. U.S. 47:1744-1750.

EARNSHAW, M.J. and B. TRUELOVE. 1968. Swelling and contraction of Phaseolus hypocotyl mitochondria. Plant Physiol. 43:121-129.

EGAR, E.I. and C.P. LARSON. 1964. Anaesthetic solubility in blood tissues:Values and significance. Brit. J. Anaesthesia 36:140-149.

ERNSTER, L. and C.P. LEE. 1964. Biological oxidoreductions. Ann. Rev. Biochem. 33:729-788.

ERNSTER, L., C.P. LEE and S. JANDA. 1967. The reaction sequence in oxidative phosphorylation. In:Biochemistry of mitochondria. E.C. Slater, Z. Kanuiga and L. Wojtczak, Eds. Academic Press, New York. p. 29-51.

FISHER, J. and T.K. HODGES. 1969. Monovalent ion stimulated adenosine triphosphatase from oat roots. Plant Physiol. 44:385-395.

FISKE, C.M. and Y. SABBAROW. 1925. The colorimetric determination of phosphorus. J. Biol. Chem. 66:375-400.

FORTI, G. 1957. Adenosine triphosphatase activity of pea mitochondria. Physiol. Plantarum. 10:898-909.

GHOSH, A.K. 1970. Studies on the effect of permeant anions on the reversal of Ca-induced mitochondrial alkalinity at different pH values. Ann. New York Acad. Sci. 147:849-855.

GIBSON, M.S. 1963. Production of ethylene by beef heart mitochondria. Biochim. Biophys. Acta 78:528-530.

GOMEZ-PUYOU, A., E. GIJON and M. TUENA. 1965. Studies on the potassium-dependent activation of rat liver mitochondrial adenosine triphosphatase H^+ ejection. Arch. Biochem. Biophys. 110:109-116.

GRAVEN, S.N., S. ESTRADA-O and H.A. LARDY. 1966. Alkali metal cation release and respiratory inhibition induced by nigericin in rat liver mitochondria. Proc. Natl. Acad. Sci. U.S. 56:654.

GREEN, D.E., S. MU and P.M. KOHOUT. 1955. Studies on the terminal electron transport system. I. Succinic dehydrogenase. J. Biol. Chem. 217:551-567.

GREVILLE, G.D. 1969. An evaluation of the chemiosmotic hypothesis. In:Current topics in Bioenergetics. D.R. Sanadi, Ed. Academic Press, New York. Vol. 3 p. 1-78.

HAARD, N.H. and H.O. HULTIN. 1968. An improved technique for the isolation of mitochondria from plant tissue. Anal. Biochem. 24:299.

HANSON, J.B. and T.K. HODGES. 1967. Energy-linked reactions of plant mitochondria. In:Current topics in Biochemistry. D.R. Sanadi, Ed. Academic Press, New York. Vol. 2. p. 65-98.

HARRIS, E.J., M.P. HOFER and B.C. PRESSMAN. 1967. Stimulation of mitochondrial respiration and phosphorylation by transport-inducing antibiotics. Biochemistry 6:1348-1360.

HEMKER, H.C. 1963. Inhibition of adenosine triphosphatase and respiration of rat liver mitochondria by dinitrophenols. Biochim. Biophys. Acta 81:1.

HODGES, T.K. and J.B. HANSON. 1965. Calcium accumulation in maize mitochondria. Plant Physiol. 40:101-108.

HONNELAITRE, A. 1925. The phosphomolybdate complex. Ann. Chim. 3:5-11.

HOPFER, U., A.L. LEHNINGER and T.E. THOMPSON. 1968. Protonic conductance across phospholipid bilayer membranes induced by uncoupling agents for oxidative phosphorylation. Proc. Natl. Acad. Sci. U.S. 50:484-491.

HORTON, R. and D.J. OSBORNE. 1967. Senescence, abscission and cellulase activity in Phaseolus vulgaris. Nature. 214:1086-1088.

HUNTER, F.E. and L. FORD. 1955. Inactivation of oxidative and phosphorylative systems in mitochondria by perincubation with phosphate and other ions. J. Biol. Chem. 216:357.

ISHIDA, M. and S. MIZUSHIMA. 1969. Membrane ATPase of Bacillus megaterium. I Properties of membrane ATPase and its solubilized form. Jour. Biochem. 66:33-43.

JACKSON, J.B., A.R. CROFTS and L.V. VON STEDINGK. 1968. Ion transport induced by light and antibiotics in chromatophores from Rhodospirillum rubrum. European Jour. Biochem. 6:41-52.

JACOBSEN, D.W. and C.H. WANG. 1968. The biogenesis of ethylene in Penicillium digitatum. Plant Physiol. 43:1959-1966.

JAGENDORF, A.T. and G. HIND. 1963. Studies on the mechanism of photophosphorylation. In:Photosynthetic mechanisms of green plants. B. Kok, Ed. Natl. Acad. Sci., Washington, D.C. p. 599-610.

JAGENDORF, A.T. 1967. Acid-base transitions and phosphorylation by chloroplasts. Fed. Proc. 26:1361.

JARDETSKY, O. 1966. Simple allosteric model for membrane pumps. Nature 211:969.

JONES, D.H. and P.D. BOYER. 1969. The apparent absolute requirement of adenosine diphosphate for the inorganic phosphate water exchange of oxidative phosphorylation. J. Biol. Chem. 244:5767-5772.

JONES, J.D., A.C. HULME and L.S.C. WOOLTORTON. 1964. Mitochondrial preparations from the fruit of the apple. II Oxidative phosphorylation. Phytochemistry 3:201.

JUDAH, J.D., A.E.M. McLEAN, K. AHMED and G.S. CHRISTIE. 1965. Active transport of potassium by mitochondria. II Effect of substrates and inhibitors. Biochim. Biophys. Acta 94:441-448.

KENEFICK, D. and J.B. HANSON. 1966a. The site of oligomycin action in corn mitochondria. Biochem. Biophys. Res. Commun. 24:899-902.

KENEFICK, D.G. and J.B. HANSON. 1966b. Contracted state as an energy source for Ca binding and Ca + inorganic phosphate accumulation by corn mitochondria. Plant Physiol. 41: 1601-1609.

KEILIN, D. and E.F. HARTREE. 1949. Activity of the succinic dehydrogenase-cytochrome system in different tissue preparations. Biochem. J. 43:205-218.

KENNEDY, E.P. and A.L. LEHNINGER. 1949. Oxidation of fatty acids and tricarboxylic acid cycle intermediates by isolated rat liver mitochondria. J. Biol. Chem. 179:957-972.

KLINGENBERG, M. and E. PFAFF. 1968. Metabolic control in mitochondria by adenine nucleotide translocation. In:Metabolic roles of citrate. T.W. Goodwin, Ed. Academic Press, New York. p. 105.

KNIGHT, R.M. 1969. Ethylene production and uracil metabolism in ageing bean leaves. Ph.D. Dissert. Univ. of Alberta, Edmonton.

KOLLOFFEL, C. and J.V. SLUIP. 1970. Mitochondrial activity of pea cotyledons during germination. Acta Bot. Neerl. 19:503-508.

LADO, P. 1966. Riattivazione di enzimi mitochondriali nel primo periodo della germinazone di semi di Ricino. Giorn. Bot. Ital. 73:37-40.

LAM, T.H. and M. SHAW. 1970. Removal of phenolics from plant extracts by grinding with anion exchange resin. Biochem. Biophys. Res. Commun. 39:965.

LANG, A. 1967. Plant growth regulation. Science 157:589.

LARDY, H.A. and H. WELLMAN. 1953. Oxidative phosphorylations and the role of sodium and potassium. J. Biol. Chem. 201:357-368.

LARDY, H.A. and S.M. FERGUSON. 1969. Oxidative phosphorylation in mitochondria. Ann. Rev. Biochem. 38:991-1034.

LEHNINGER, A.L. 1955. Oxidative phosphorylation. Harvey Lectures. XLIX:176-204.

LEHNINGER, A.L. 1964. The Mitochondrion. Benjamin Press, New York.

LEHNINGER, A.L., E. CAROFOLI and C.S. ROSSI. 1967. Energy-linked ion movements in mitochondrial systems. Advan. Enzymol. 29:259-320.

LEHNINGER, A.L. 1970. Mitochondria and calcium in transport. Biochem. J. 119:129-138.

LEV, A.A. and E.P. BUZKINSKY. 1967. Cation specificity of model bimolecular phospholipid membranes with incorporated valinomycin. Cytology 9:102-107.

LIEBERMAN, M., A.T. KUNISHI, L.W. MAPSON and D.A. WARDALE. 1966. Stimulation of ethylene production in apple tissue slices by methionine. Plant Physiol. 41:376-382.

LINHARDT, K. and K. WALTER. 1965. Phosphatases. In:Methods of Enzymatic Analysis. H.U. Bergmeyer, Ed. Academic Press, New York. p. 783.

LOWRY, O.H., N.J. ROSEBOROUGH, A.L. FARR and R.J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

LYONS, J.M. and H.K. PRATT. 1964. An effect of ethylene on swelling of isolated mitochondria. Arch. Biochem. Biophys. 104:318-324.

MALHOTRA, S.A. and M.S. SPENCER. 1970a. Changes in the respiratory enzymatic and swelling and contraction properties of mitochondria from cotyledons of Phaseolus vulgaris L. during germination. Plant Physiol. 46:40-44.

MALHOTRA, S.S. and M. SPENCER. 1970b. Properties of mitochondria prepared by two different isolation procedures from the cotyledons of germinating bean seedlings. J. Expt'l. Bot. (In Press).

MANN, J. 1968. Some properties of the membrane ATPase system of the human erythrocyte. Ph.D. Dissert. Univ. Alberta, Edmonton.

MAPSON, L.W., R. SELF and D.A. WARDALE. 1969. Biosynthesis of ethylene. Methanesulfonic acid as a cofactor in the enzymic formation of ethylene methional. Biochem. J. 111:413-418.

MCAULIFFE, C. 1966. Solubility in water of paraffin, cycloparaffin, olefin, acetylene, cycloolefin and aromatic hydrocarbons. J. Phys. Chem. 70:1267-1271.

MCGLASSEN, W.B. and H.K. PRATT. 1964. Effects of wounding on respiration and ethylene production by cantaloupe fruit tissue. Plant Physiol. 39:120-127.

MCMURRAY, A.L. and C.H. MILLAR. 1968. Cucumber sex expression modified by 2-chloroethanephosphonic acid. Science 162:1396-1397.

MEHERIUK, M. 1965. Some biochemical studies on the production of ethylene by a particulate fraction from ripening tomatoes. Ph.D. Dissert. Univ. Alberta, Edmonton.

MILLERD, A., J. BONNER, B.A. AXELROD AND R.S. BANDURSKI. 1951. Oxidative and phosphorylative activity of plant mitochondria. Proc. Natl. Acad. Sci. U.S. 37:855.

MITCHELL, P. 1961. Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. Nature (London). 191:144-148.

MITCHELL, P. 1967. Proton translocation and phosphorylation in mitochondria, chloroplasts and bacteria:Natural fuel cells and solar cells. Fed. Proc. 26:1370.

MITCHELL, P. 1968. Chemiosmotic coupling and energy-transduction. Glyna Research, Bodmin., Cornwall.

MITCHELL, P. and J. MOYLE. 1965a. Stoichiometry of proton translocation through the respiratory chain and adenosine triphosphatase system of rat liver mitochondria. Nature (London) 208:147-151.

MITCHELL, P. and J. MOYLE. 1965b. Evidence discriminating between the chemical and the chemiosmotic mechanisms of electron transport phosphorylation. Nature (London) 208:1205-1206.

MITCHELL, P. and J. MOYLE. 1967. Acid-base titration across the membrane system of rat liver mitochondria. Biochem. J. 104:588-593.

MONOD, J., J. WYMAN and J.P. CHANGEUX. 1965. On the nature of allosteric transitions:A plausible model. J. Mol. Biol. 12:88.

MONTAL, M., B. CHANCE and C.P. LEE. 1970. Ion transport and energy conservation in submitochondrial particles. J. Membrane Biol. 2:201-234.

MOORE, C. and B.C. PRESSMAN. 1964. Mechanism of action of valinomycin on mitochondria. Biochem. Biophys. Res. Commun. 15:562-571.

MORGAN, P.W. and W.C. HALL. 1964. Accelerated release of ethylene by cotton following application of indolyl-3-acetic acid. Nature. 201:99-101.

MOZERSKY, S.M., J.D. PETTANTI and S.D. KOLMAN. 1966. An improved method for the determination of orthophosphate suitable for assay of adenosine triphosphatase activity. Analyt. Chem. 38:1182-1187.

OHNISHI, M. and D.W. URRY. 1970. Solution conformation of valinomycin-potassium ion complex. Science 168:1091-1092.

OLSON, A.O. 1967. Investigations on the effects of ethylene on mitochondrial systems. Ph.D. Dissert. Univ. Alberta, Edmonton.

OPIT, L.J. and J.S. CHARNOCK. 1965. A molecular model for a sodium pump. Nature. 208:471.

PACKER, L., S. MURAKAMI and C.W. MEHARD. 1970. Ion transport in chloroplasts and plant mitochondria. Ann. Rev. Plant Physiol. 21:271-304.

PAPA, S., J.M. TAGER, F. GIARRURI, E. QUAGLIARIELLO. 1969. Effect of monovalent cations on oxidative phosphorylation in sub-mitochondrial particles. Biochim. Biophys. Acta 172:184-186.

PARKER, V.H. 1958. Effect of nitrophenols and halogenophenols on the enzymic activity of rat liver mitochondria. Biochem. J. 69:306.

PFAFF, E. and M. KLINGENBERG. 1968. Adenine nucleotide translocation of mitochondria. I. Specificity and control. European Jour. Biochem. 6:66-79.

PHAN, C.T. 1962. Contribution a l'étude de la production d'éthylène par le Penicillium digitatum. Sacc. Revenue Generale de Botanique. 69:505-543.

PHAN, C.T. 1963. Etude de la teneur en éthylène de l' atmosphère interne des fruits par chromatographie en phase gazeuse. Rev. Gén. Botanique 70:679-687.

PHAN, C.T. 1969. L'origine metabolique de l'éthylène. Annee Biologique VIII: 229-248.

PRATT, H.K. 1944. Studies on the physiology of Penicillium digitatum saccardo. Ph.D. Dissert. Univ. California.

PRATT, H.K. and J.B. BIALE. 1944. Relation of the production of an active emanation to respiration in the Avocado fruit. Plant Physiol. 19:519-528.

PRATT, H.K. and J.D. GOESCHL. 1969. Physiological roles of ethylene in plants. Ann. Rev. Plant Physiol. 20:541-584.

PRATT, H.K. and M. WORKMAN. 1962. Studies on the physiology of tomato fruits. III. The effect of ethylene on respiration and ripening behaviour of fruits stored at 20° C. after harvest. Proc. Am. Soc. Hort. Sci. 81:467-478.

PRESSMAN, B.C., E.J. HARRIS, W.S. JAGGER and J.H. JOHNSON. 1967. Antibiotic-mediated transport of alkali ions across lipid barriers. Proc. Nat. Acad. Sci. U.S. 58:1949-1954.

PULLMAN, M.E., H.S. PENEFSKY, A. DATTA and E. RACKER. 1960. Partial resolution of the enzymes catalyzing oxidative phosphorylation. J. Biol. Chem. 235:3322-3329.

PULLMAN, M.E. and H.S. PENEFSKY. 1963. Preparation and assay of phosphorylating submitochondrial systems. In:Methods of Enzymology. S.P. Colowick and N.O. Kaplan, Eds. Academic Press, New York. VI:277-284.

PULLMAN, M.E. and G. SHANTZ. 1967. Mitochondrial oxidations and energy-coupling. Ann. Ev. Biochem. 36:539-610.

RACKER, E. 1965. Mechanisms in bioenergetics. Academic Press, New York.

RACKER, E. 1967. Resolution and reconstitution of the inner mitochondrial membrane. Fed. Proc. 26:1335-1341.

RATHBUN, W.B. and M.V. BETLACH. 1968. Estimation of enzymically produced orthophosphate in the presence of cysteine and adenosine triphosphate. Anal. Biochem. 28:436-446.

REID, H.B. JR., A.C. GENTILE and R.M. KLEIN. 1964. Adenosine triphosphatase activity of cauliflower mitochondria. Plant Physiol. 39:1020-1023.

ROBINSON, J.D. 1967. Kinetic studies on a brain microsomal adenosine triphosphatase:Evidence suggesting conformational change. Biochemistry 6:3250-3258.

ROBINSON, J.D. 1968. Allosteric nature of ATPase. Nature 220:1325-1326.

ROBINSON, J.D. 1970. Reaction sequence of the K^+ - dependent phosphatase. Biochim. Biophys. Acta 212:509.

ROSSI, E. and G.F. AZZONE. 1969. Ion transport in liver mitochondria. Energy barrier and stoichemetry of aerobic K^+ translocation. European J. Biochem. 7:418-426.

SATO, S. 1968. Enzymatic maceration of plant tissue. Physiologia Planta. 21:1067.

SKOU, J.C. 1965. Enzymatic basis for active transport of Na^+ and K^+ across cell membrane. Physiol. Rev. 45:596-617.

SKULOCHEV, V.P., A.A. SHARAF and E.A. LIBERMAN. 1967. Proton conductors in the respiratory chain and artificial membranes. Nature 216:718-723.

SLATER, E.C. 1953. Mechanism of phosphorylation in the respiratory chain. Nature 172:975-982.

SLATER, E.C. 1958. The mechanism of oxidative phosphorylation. Revs. Pure and Appl. Chem. (Australia) 8:221-238.

SLATER, E.C. 1965. The respiratory chain and oxidative phosphorylation: Some unsolved problems. In:Biochemistry of Mitochondria. E.C. Slater, Z. Kaniuga and L. Wojtczak, Eds. Academic Press, London. p. 1-19.

SLATER, E.C. 1967. An evaluation of the Mitchell hypothesis of chemiosmotic coupling in oxidative and photosynthetic phosphorylation. European J. Biochem. 1:317-326.

SMITH, W.H., D.F. MEIGH and J.C. PARKER. 1964. Effect of damage and fungal infection on production of ethylene by carnations. Nature (London) 204:92-93.

SPENCER, M.S. 1959. Ethylene metabolism in tomato fruit. III Effect of 2,4-dinitrophenol on respiration, ethylene evolution, and ripening. Can. J. Biochem. Physiol. 37:53-59.

SPENCER, M.S. 1965. Fruit ripening. In:Plant Biochem. J. Bonner and J.E. Varner, Eds. Academic Press, New York. p. 536.

SPENCER, M.S. 1969. Ethylene in nature. In:Fortschritte d. Chem. org. Naturst. XXVII:31-80.

SPENCER, M.S. and A.O. OLSON. 1965. Ethylene production and lipid mobilization during germination of castor beans. Nature 215:699-700.

STINSON, R.A. and M. SPENCER. 1967. An evaluation of the effects of five buffers on respiratory parameters for isolated mitochondria. Can. J. Biochem. 46:43-50.

STONER, C.D. and J.B. HANSON. 1966. Swelling and contraction of corn mitochondria. Plant Physiol. 41:255-266.

STONER, C.D., T.K. HODGES and J.B. HANSON. 1964. Chloramphenical as an inhibitor of energy-linked processes in maize mitochondria. Nature. 203:258-264.

STOREY, B.T. 1970. Chemical hypothesis for energy conservation in the mitochondrial respiratory chain. J. Theor. Biol. 28:233-259.

SUELTER, C.H. 1970. Enzymes activated by monovalent cations. Science. 168:789-795.

SZENT-GYORGYI, A. 1957. Bioenergetics. Academic Press, New York.

TAKIUCHI, Y., K. YOSHIDA and S. SATO. 1969. Electron transport ddependent adenosine triphosphatase activity in castor bean mitochondria. Plant Cell Physiol. 10:733-741.

TEDESCHI, H. 1965. Some observations on the permeability of mitochondria to sucrose. J. Cell. Biol. 25:229-242.

THOMPSON, J.E. 1966. Preparation and properties of enzyme powders for ethylene synthesis. Ph.D. Dissert. Univ. Alberta, Edmonton.

THOMPSON, J.E. and M. SPENCER. 1967. Ethylene production from β-alanine by an enzyme powder. Can. J. Biochem. 45:563-571.

TRUELOVE, B. and J.B. HANSON. 1966. Calcium activated phosphate uptake in contracting corn mitochondria. Plant Physiol. 41:1004-1013.

VAN DAM, K. and E.C. SLATER. 1967. A suggested mechanism of respiratory chain phosphorylation. Proc. Natl. Acad. Sci. U.S. 58:2015-2022.

VIGERS, G.A. and F.D. ZIEGLER. 1968. Azide inhibition of mitochondrial ATPase. Ph.D. Dissert. McMaster Univ. Hamilton.

WEDDING, R.T. and M.K. BLACK. 1962. Response of oxidation and coupled phosphorylation in plant mitochondria to 2,4-dichlorophenoxyacetic acid. Plant Physiol. 37:364.

WEIL-MALHERBE, H. and R.H. GREEN. 1951. The catalytic effect of molybdate on the hydrolysis of organic phosphorus bonds. Biochem. J. 49:286-292.

WEINBACH, E.C. and J. GARBUS. 1966. The rapid restoration of respiratory control to uncoupled mitochondria. J. Biol. Chem. 241:3708-3713.

WEINBACH, E.C. and J. GARBUS. 1969. Mechanism of action of reagents that uncouple oxidative phosphorylation. Nature 221:1016-1018.

WENNER, C.E. and J.H. HACKNEY. 1969. Mitochondrial energy flux and ion-induced adenosine triphosphatase activity and light-scattering changes mediated by gramicidin. Biochem. 8:930-938.

WHEELER, K.P. and R. WHITTAM. 1962. Some properties of a kidney adenosine triphosphatase relevant to active cation transport. Biochem. J. 85:495.

WILSON, D.F. and R.D. MERZ. 1967. Inhibition of mitochondrial respiration by uncouplers of oxidative phosphorylation. Arch. Biochem. Biophys. 119:470-481.

WILSON, R.H. and J.B. HANSON. 1969. The effect of respiratory inhibitors on NADH, succinate and malate oxidation in corn mitochondria. Plant Physiol. 44:1335-1341.

WILSON, R.H., J.B. HANSON and H.H. MOLLENHOUSER. 1969. Active swelling and acetate uptake in corn mitochondria. Biochemistry 8:1202-1213.

WINKLER, L.W. 1914. Uber die Bestimmung des im Wasser gelosten Sauerstoffs. Z. Anal. Chem. 53:665-672.

WISKICH, J.T. and W.D. BONNER. 1963. Preparation and properties of sweet potato mitochondria. Plant Physiol. 39:594-604.

WOJTCZAK, A.B. 1969. Inhibitory action of oxaloacetate on succinate oxidation in rat liver mitochondria and the mechanism of its reversal. Biochim. Biophys. Acta. 172:52-65.

YOSHIDA, K. 1968a. Swelling and contraction of isolated plant mitochondria. II. Reversible metabolism-dependent contraction. J. Fac. Sci. Univ. Tokyo 10:63-82.

YOSHIDA, K. 1968b. Swelling and contraction of isolated plant mitochondria. III. Reversible metabolism dependent swelling. J. Fac. Sci. Univ. Tokyo. 10:83-96.

YOSHIDA, K. and S. SATO. 1968. Swelling and contraction of isolated plant mitochondria. I. Passive swelling in sugar and electrolyte solutions. J. Fac. Sci. Univ. Tokyo. 10:49-62.

YOSHIDA, K. and Y. TAKEUCHI. 1970. Properties of a soluble ATPase from castor bean endosperm mitochondria. Plant and Cell Physiol. 11:403-409.

YOUNG, R.E. 1965. Effect of ionizing radiation on respiration and ethylene production of Avocado fruit. Nature (London) 205:1113-1114.

ZAYAGILSKAYA, R.A., K. BORUCKA and L. WOJTCZAK. 1969. Accumulation of azide in mitochondria and the effect of azide on energy metabolism. Acta. Biochemica Polonica. 16:163.

APPENDIX

Figure A-1. Standard curve for protein estimation. The method of Lowry et al. (1951) was employed with human serum albumin as a standard. Protein was expressed as the µg per 1.0 ml of a diluted mitochondrial suspension.

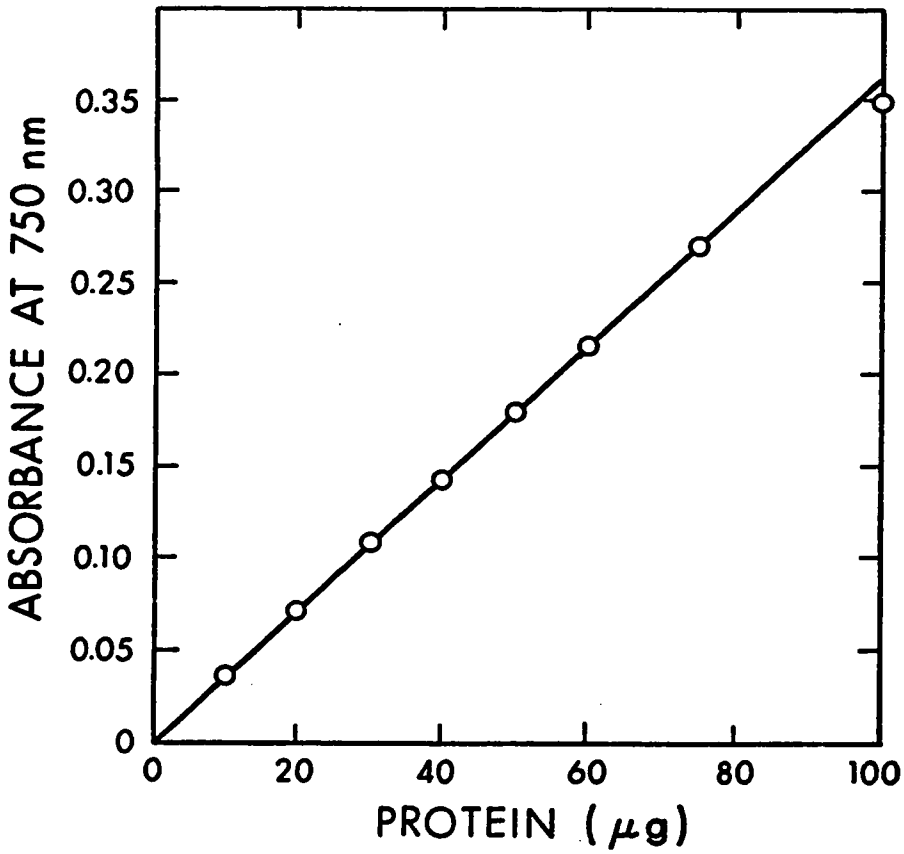

ABSORBANCE AT 750 nm
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
0
20
40
60
80
100
PROTEIN (μg)

Figure A-2. Gas chromatograph recorder response curve for ethylene determination. The peaks are as follows: 1, air + unknown; 2, ethane; 3, ethylene; 4, unknown.

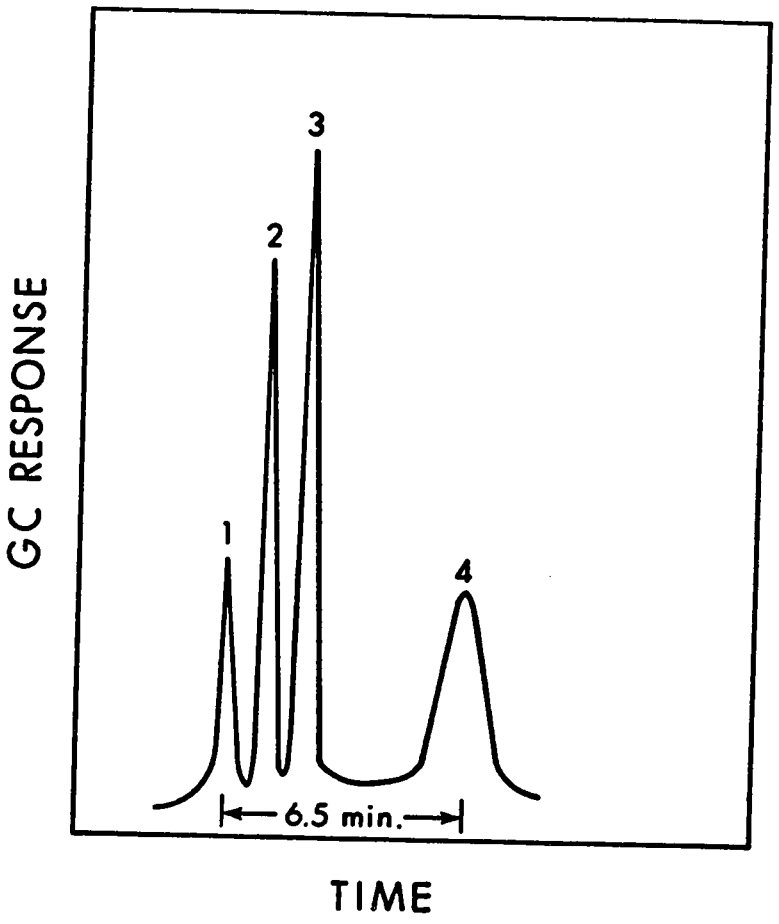
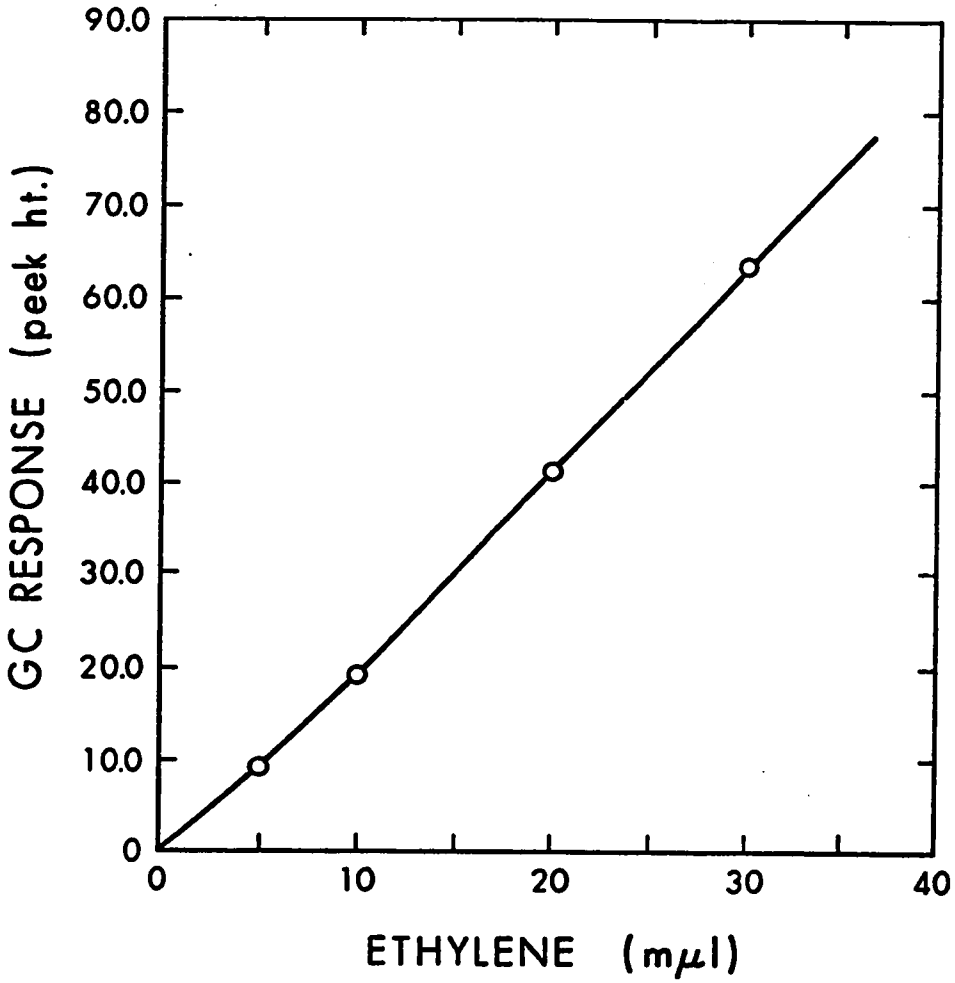

GC RESPONSE
1
2
3
4
6.5 min.
TIME

Figure A-3. Standard curve for ethylene estimation. The gas was analysed by gas chromatography and was resolved by a activated alumina column (with 2-1/2% silicone 550). The column temperature was room temperature and the detector temperature was 125°.


90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0
GC RESPONSE (peek ht.)
0
10
20
30
40
ETHYLENE (mμl)