ION UPTAKE BY BEETROOT MITOCHONDRIA

A thesis submitted to the University of Adelaide as a requirement for the degree of Doctor of Philosophy by

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August 1967

CONTENTS

SUMMARY

Mitochondria were isolated from the root tissue of red beetroot (Beta Vulgaris L.) and oxidation of Kebs cycle intermediates was studied. Succinate, malate, citrate, pyruvate, α-ketoglutorate and ascorbate (plus TMPD) were oxidized most rapidly in the presence of phosphate acceptor (ADP) and inorganic phosphate. On exhaustion of the phosphate acceptor the oxidation rate decreased indicating respiratory control of oxidation by coupled phosphorylation. State 4 oxidation rates and respiratory control ratios indicated that substrate-level phosphorylation was most strongly coupled to oxidation and phosphorylation at site 1 in the electron transfer chain was also strongly coupled whereas site 3 phosphorylation (ascorbate) was very loosely coupled. Respiratory control did not reflect capacity for phosphorylation and each site in the electron transfer chain phosphorylated ADP efficiently. The theoretical ADP/O ratios were measured for each substrate (3 with malate, 2 with succinate and 1 with ascorbate).

Magnesium or calcium and phosphate were taken up by beetroot mitochondria by a substrate-dependent process. Succinate-dependent ion uptake was inhibited by inhibitors (cyanide, antimycin A) and uncouplers (DNP, CCP) indicating association with oxidative phosphorylation. Oligomycin did not affect ion uptake indicating that ATP as such was not required. Ion uptake was completely inhibited under conditions allowing continuous

ATP formation and oligomycin inhibited oxidative phosphorylation and permitted ion uptake to proceed. The ratios of magnesium or calcium/phosphate taken up were close to 1.5.

ATP did not support magnesium or calcium and phosphate uptake although low concentrations (0.5 mM) of ATP stimulated substrate-dependent ion uptake by an oligomycin-insensitive process. BSA and cytochrome c also stimulated ion uptake and these effects were attributed to stabilization of membrane structure or of a precipitated ion complex. High concentrations of ATP (3 mM) inhibited substrate-dependent ion uptake and oligomycin did not relieve inhibition. EDTA also inhibited ion uptake and these effects were related to the chelating properties of these molecules.

Calcium and magnesium competed for membrane binding sites and for substrate-dependent uptake with phosphate. Calcium was the favoured cation and 4-5 times more calcium was taken up by both processes. High calcium and magnesium concentrations had little effect on oxidative phosphorylation although P/O ratios were highest without added $MgCl_2$ or $CaCl_2$. Substrate oxidation was stimulated by low cation concentrations but did not return to the slower rate either with or without phosphate. This result and the low Mg^{++}/O ratios measured were attributed to the high permeability of beetroot mitochondrial membranes to ions.

Monovalent cations inhibited divalent cation and phosphate uptake more strongly than monovalent anions indicating that cations competed

for the uptake mechanism. Monovalent cations were taken up in a substrate-dependent manner and uptake was inhibited by DNP but not by cyanide or oligomycin.

Succinate and ascorbate supported massive magnesium uptake but malate supported very little ion uptake. The capacity for ion uptake was directly proportional to the state 4 oxidation rates and indirectly to the ADP/O and respiratory control ratios.

ATP was synthesized and magnesium and phosphate were taken up when a pH differential was applied briefly across beetroot mitochondrial membranes.

These result were compared with those obtained from similar investigations with other plant and animal mitochondria and were discussed in relation to the current hypotheses of oxidative phosphorylation and ion uptake.

The investigations described in this thesis were carried out in the Botany Department, University of Adelaide, from March 1964 to May 1967. The thesis contains accounts of oxidative phosphorylation and ion uptake by beetroot mitochondria. Two papers (Proc. Natl. Acad. Sci. U.S. (1964), 52, 996, and Plant Physiol. (1965), 40, 1129) were written in collaboration with Dr. J. T. Wiskich and Professor R. N. Robertson and reprints are enclosed (Appendix).

This thesis contains no material which has been presented previously for a degree or diploma at any University or which has been previously written or published by another person except where reference is made in the text.

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August 1967.

ACKNOWLEDGEMENTS

The author is greatly indebted to Dr. J. T. Wiskich and Professor R.N. Robertson (Botany Department, University of Adelaide) for supervision and guidance throughout the course of this work. The author also thanks Mr. K. West for the electron microscopy involved in this thesis.

Acknowledgements are made to Professor R.N. Robertson (Botany Department, University of Adelaide) in whose laboratories this work was carried out. The author acknowledges the support of a C.S.I.R.O. senior studentship during the course of this work.

ABBREVIATIONS.

AcAc	Acetoacetate
ADP	adenosine diphosphate
α- Kg	α - Ketoglutarate
AMP	adenosine monophosphate
Amytal	5-ethyl-5-isoamylbarbiturate
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
CCP	carbonyl cyanide m-chloro-phenylhydrazone
CoA	coenzyme A
CoQ	coenzyme Q (ubiquinone)
cyt.	cytochrome
DNP	2,4-dinitrophenol
EDTA	ethylenediaminetetraacetate
EGTA	ethyleneglycolbis(aminoethyl)tetra acetate
Fp	NADH or succinate dehydrogenase
gl-1-P	glycerol-1-phosphate
HOQNO	2-heptyl-4-hydroxyquinoline-N-oxide
NAD, NAD^+	Nicotinamide adenine dinucleotide (oxidised)
NADH	Nicotinamide adenine dinucleotide (reduced)
NADP, $NADP^+$	Nicotinamide adenine dinucleotide phosphate (oxidised)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced)
OAA	oxaloacetate
TMPD	NNN'N-tetramethyl-p-phenylenediamine
TPP	thiamine pyrophosphate
Tris	tris(hydroxymethyl)aminomethane
u	micron (micro) is designated in the text by u.

CHAPTER ONE

INTRODUCTION

A. RESPIRATION.

It has been well established that plant and animal mitochondria are the sites of aerobic respiration. The respiratory processes and related reactions of plant mitochondria have been reviewed by Hackett (1959, 1963, 1964), Beevers (1961), Bonner (1961), Lundegårdh (1960), Goddard and Bonner (1960), Crane (1961), Zelitch (1964), Ducet and Rosenberg (1962) and Lieberman and Baker (1965) and general plant and animal mitochondrial properties have been reviewed by Ernster and Lee (1964), Lehninger (1964) and Racker (1965). The isolation from many plant tissues of a particulate fraction which showed both succinate and cytochrome oxidase activity was described by Bhagvat and Hill in 1951. Millerd et al (1951) established that isolated mung bean mitochondria could oxidise most of the acid intermediates of the Krebs cycle and this was confirmed by Brummond and Burris (1953) who found that C^{14} from labelled pyruvate fed to lupine mitochondria could be detected in every acid intermediate of the Krebs cycle. Mitochondria isolated from many different plant species and tissues have been shown to be capable of Krebs cycle activity (Goddard and Stafford 1954, Freebairn and Remmert 1956). Millerd et al (1951) also established that ATP was formed during mitochondrial oxidation. Investigations following these reports have been reviewed by Hackett (1959).

Investigations into the components of the electron transfer chain have been carried out more often using animal tissues than plant and the evidence has been reviewed by Chance and Williams (1956), Slater (1958a), Schneider (1959), Green (1959), and Lehninger (1959). The oxidation and reduction of cytochromes in intact tissues of plants have been observed by Keilin and co-workers (1925, 1947), Bhagvat and Hill (1951), Yocum and Hackett (1957), and Lundegårdh (1954), and these and subsequent findings have been discussed by Hill and Hartree (1953), Hartree (1957), James (1957), Smith and Chance (1958), Chance (1958), Hackett (1959) and Lieberman and Baker (1965). The general properties of the cytochrome pigments have been reviewed by Morton (1958). It appears that mitochondria isolated from many plant tissues contain an electron transfer pathway similar to that of animal mitochondria but the status of cytochrome c_1 and b-type cytochromes is in doubt (Lundegårdh 1958, Martin and Morton 1956, 1957, Bonner 1959). The cytochrome components of certain plant tissues showing respiratory activity which is insensitive to classical respiratory inhibitors (cyanide and carbon monoxide) have been investigated by Hackett (1957), Bendall and Hill (1957), Hackett and Haas (1958) and Chance and Hackett (1959), and the situation has been reviewed by Smith and Chance (1958) and Hackett (1959).

B. ISOLATION OF PLANT MITOCHONDRIA.

In plant tissues, investigations into the respiratory electron transport chain and related mitochondrial reactions have been difficult and progress slower than in animal tissues because of the low concentration of

respiratory enzymes (Hill and Hartree 1953, Bonner 1961), and the presence of a wide range of endogenous inhibitory and potentially inhibitory substances present in the cytoplasm and vacuoles of plant cells (Lieberman 1961, Jones and Hulme 1961, Hulme and Jones 1964). These inhibitors are released or formed during the homogenizing and extracting procedures and inactivate the mitochondrial preparations either physically by attachment or adsorption, or chemically by interaction and denaturation. Procedures designed to counteract these inhibitors concentrate on the homogenizing medium where the mitochondria first come into close contact with these inactivators on disruption of the cell and release of the vacuolar and cytoplasmic contents. Acid tissues, such as apple fruit and beetroot require an initially alkaline isolation medium (pH 9 - 10) for demonstration of optimum mitochondrial activity (Lieberman 1958, Honda et al 1958).

The inclusion of EDTA, an effective metal-chelating agent, in the isolation medium can markedly stimulate and stabilise the oxidative capacity of isolated mitochondria (Tager 1954, Lieberman and Biale 1955, Honda et al 1958). The isolation of beetroot mitochondria in EDTA solutions was found to decrease the content of mitochondrial calcium and to increase the rate of oxidation of succinate, malate and α-ketoglutarate and accompanying phosphorylations (Honda and Robertson 1956), and the inclusion of EDTA in the isolating medium stimulated the phosphorylative activity of heart-muscle mitochondria (Slater and Cheland 1952). However, it is possible that the beneficial chelating effect of EDTA may mask some harmful effect such as the chelation of metals required for respiratory processes (e.g. Mg^{++}) (Slater 1957). EDTA, although required during the initial homogenizing procedure, can be

omitted during the subsequent stages of mitochondria isolation without deleterious effects (Lieberman and Biale 1955). Stimulative effects of phosphate in the isolating medium have been reported (Beaudreau and Remmert 1955, Biale et al 1957, Ducet et al 1958, Switzer and Smith 1957).

Reducing agents (e.g. cysteine)have been added to prevent oxidative reactions (Wiskich and Bonner 1963) and in potato homogenates they inhibited a soluble NADH-oxidizing system (Hackett 1958). A method for carrying out isolation under anaerobic conditions has been described (Cohen et al 1956). The deleterious effect of lecithinase may be prevented by fluoride (Goodwin and Waygood 1954). Polyvinyl pyrrolidone has also been added to the isolating medium (Jones and Hulme 1961, Hulme and Jones 1963, Wiskich 1966) to prevent the inhibitory action of tannins.

Other techniques such as differential centrifuging to separate starch and aggregated material from mitochondria have been used to obtain preparations of more active mitochondria (Wiskich and Bonner 1963, Wiskich et al 1964). The co-precipitation of mitochondria and chloroplast fragments (Leech and Ellis 1961) occurs during preparation of mitochondria from green tissues and the chloroplast fraction may make up most of the mitochondrial pellet (Pierpoint 1959). The chloroplast fragments may be separated from the mitochondria by centrifuging through suitable sucrose density gradients (James and Das 1957, Pierpoint 1962, Jagendorf 1955). Dalgarno and Birt (1963) suggested that fatty acids released during homogenizing acted as uncouplers of carrot mitochondria and that the presence of 1% bovine serum albumin in the homogenizing medium prevented uncoupling. Lehninger and Remmert (1959) and Hulsmann et al (1960) have shown that unsaturated fatty acids uncouple oxidative phosphorylation, and Tappel and Zalkin (1959) have suggested that lipid peroxidation may cause damage by producing free radicals which inactivate

enzymes of the mitochondrial phosphorylating system. Methods for producing active plant mitochondria have been reviewed by Hackett (1959) and Lieberman and Baker (1965).

C. REACTIONS OF MITOCHONDRIA.

Isolated plant mitochondria from a great number of plant tissues have been shown to esterify inorganic phosphate during oxidation of substrates via the electron transfer chain (Hackett 1959). Although it has been claimed (James 1957) that P/O ratios higher than one with plant mitochondria were rare due to ATPase activity, a great number of reports has shown that the phosphorylative efficiency of plant mitochondria can be equal to that of animal mitochondria (Hackett 1959) during the oxidation of Krebs cycle acids. Values approaching the theoretical maxima for the P/O ratios have frequently been obtained; i.e. greater than 3.0 for α-ketoglutarate (Akazawa and Beevers 1957, Conn and Young 1957, Freebairn and Remmert 1957, Hackett and Haas 1958, Lieberman and Biale 1956, Wiskich et al and Biale 1964), greater than 2 for citrate, malate and pyruvate (Akazawa and Beevers 1957, Smillie 1955, Wiskich and Bonner 1963, Wiskich et al 1964, Hanson et al 1965), and greater than 1.0 for succinate (Conn and Young 1957, Freebairn and Remmert 1957, Laties 1953, Wiskich et al 1960, Wiskich and Bonner 1963 and many others).

The ability of mitochondria to carry out oxidative phosphorylation with P/O ratios approaching the theoretical maxima was the criterion of intactness of mitochondria in the earlier investigations into the phosphorylative abilities of isolated mitochondria (Laties 1953). However, after the work of Chance and Williams (1956), the degree of ADP or P_i control

over mitochondrial respiration has been considered as a more important criterion of mitochondrial intactness. The respiratory control (R.C.) ratio of mitochondria was defined by Chance and Williams (1956) as the state 3 rate of oxidation divided by the state 4 rate of oxidation where state 3 rate was the rate of oxidation in the presence of substrate, ADP, P_i and oxygen and state 4 was the rate of oxidation obtained when all ADP was converted to ATP and ADP was the rate limiting factor (see Table 1 and Fig. 1, Appendix). Respiratory control is not demonstrated by stimulation of respiratory rate by ADP, (Hackett et al 1960, Tamaoki et al 1960), unless ADP is shown to be limiting.

High respiratory control ratios have been observed in animal mitochondria using a polarographic technique (Chance and Williams 1956, Chance 1956). However, reports of plant mitochondria showing respiratory control have only recently been submitted (Wiskich and Bonner 1963, Jones et al 1964, Wiskich et al 1964, Childress and Stein 1965, Hanson et al 1965), although plant mitochondria have long been shown to couple phosphorylation of ADP to oxidation of substrates with P/O ratios approaching the theoretical maxima. Wiskich and Bonner (1963) using an oxygen electrode have reported R.C. ratios as high as 10 with sweet potato mitochondria oxidizing malate and an R.C. ratio of 1.5 with mitochondria of white potato mitochondria prepared with equal care. R.C. ratios of 16 with NADH were reported in apple mitochondria (Jones et al 1964). Using polarographic techniques ADP/O and R.C. values can be measured over short time periods and changes in R.C. and oxidative phosphorylation can be followed with time. Wiskich et al (1964) have reported a declining respiratory control with

time in avocado mitochondria and they discussed this with relation to factors (e.g. ATPase) which may mask respiratory control.

ADP/O ratios can be calculated using polarographic techniques as described by Chance and Williams (1955) and the ratios so obtained with plant mitochondria are close to the P/O ratios obtained using classical manometric techniques employing a hexokinase-glucose trap for ATP formed. However, ADP/O ratios are usually greater than P/O ratios obtained using the same preparation of mitochondria and substrate (Wiskich et al 1964). Explanations of these differences have implicated shaking mechanisms, penetration factors or the presence of phosphatases (Stickland 1960, Wiskich et al 1964). The ADP/O ratios obtained with plant mitochondria have been shown to be as high as those obtained with animal mitochondria using the same substrate (Wiskich and Bonner 1963), Wiskich et al 1964, Hanson 1965), indicating again that the phosphorylative mechanism is as efficient in plant mitochondria as in animal mitochondria. However, the R.C. ratios for plant mitochondria are usually lower than those obtained for animal mitochondria using the same substrates (Wiskich and Bonner 1963, Wiskich et al 1964, Chance and Williams 1956). This probably reflects the difficulties associated with isolating intact and active plant mitochondria rather than any major inherent differences between the two types of mitochondria.

It is generally considered that there are three sites of ATP formation along the mitochondrial electron-transfer chain (Hackett 1959). The three sites are associated in some way with the oxidation of reduced pyridine nucleotide, cytochrome b and cytochrome c (Slater 1958b). These three sites satisfy (a) the P/O ratios obtained with partial reactions of the respiratory

chain and (b) the thermodynamic distribution of redox potentials in the respiratory chain (Slater 1958b) and (c) the 'cross-over' points observed by Chance and Williams (1956).

Use of extra-mitochondrial NADH as substrate in plant mitochondria which do oxidize NADH with respiratory control have yielded P/O ratios approaching 3 (Wiskich and Bonner 1963), and substrates with NADH-linked dehydrogenases, (e.g. malate) also give P/O ratios approaching 3 (Wiskich and Bonner 1963). Fritz and Nayler (1956), using mung bean particles localized one phosphorylative step between cytochrome c and oxygen and another between succinate and the point where ferricyanide intercepts the respiratory chain:- presumably near the cytochrome c level. Kmetec and Newcomb (1955) observed phosphorylation during the oxidation of reduced cytochrome c by cauliflower mitochondria but were unable to detect it in the preceding steps. These results may be compared with the observations that the phosphorylative step between cytochromes b and c is the most labile in animal mitochondria (Lehninger et al 1958). Phosphorylation occurs between the site of action of ascorbate through TMPD (possibly cytochrome c) and oxygen in animal mitochondria (Racker et al 1963, Howland 1963b), giving an ADP/O ratio approaching one.

Studies with the oxygen electrode have indidated that the first phosphorylation step between NADH and cytochrome b is more strongly coupled to oxidation, (Wiskich and Bonner 1963) than subsequent phosphorylation steps involved in the oxidation of succinate (Wiskich and Bonner 1963, Childress and Stein 1965), or in the oxidation of ascorbate (Packer et al 1963). The strength of coupling of the phosphorylation steps are reflected

in the respiratory control ratios obtained from the same preparation of mitochondria with different substrates. The substrate-level phosphorylation site associated with ∝-ketoglutarate oxidation has been reported to be even more strongly coupled to oxidation (Wiskich et al 1963).

Beevers and Walker (1966) and Walker and Beevers (1956) demonstrated the requirement of all associated co-factors by isolated castor bean mitochondria for the oxidation of various Krebs cycle acids. A similar requirement of added co-factors was reported by Lieberman and Biale (1956b) for mitochondria isolated from sweet potatoes although a requirement of lipoic acid for pyruvate and ∝-ketoglutarate oxidation was not demonstrated. Arsenite inhibited the oxidation of both ∝-keto acids indicating that a dithiol such as lipoate (Reed 1957) was involved. Plant mitochondria have been isolated recently which do not require addition of a full complement of cofactors for maximum oxidative activity (Wiskich and Bonner 1963, Wiskich et al 1964, Childress and Stein 1965), although requirement of some cofactors is still demonstrated. Addition of horse heart cytochrome c has been commonly shown to increase the oxidative capacity of isolated plant and animal mitochondria (Wiskich et al 1960), although this does not always occur (Childress and Stein 1965).

D. ACTION OF INHIBITORS AND UNCOUPLERS

The use of inhibitors and uncouplers of the electron transfer chain and associated phosphorylations has helped in identifying the components of the chain in their correct sequence and the sites of phosphorylation. The sites of action of various inhibitors and uncouplers have been reviewed recently in detail by Ernster and Lee (1964) and by Lieberman and Baker (1956).

The respiratory activity of many plant mitochondria has been shown to be inhibited by cyanide and carbon monoxide (Hackett 1959), indicating the involvement in the electron transfer chain of the terminal cytochrome oxidase, although the respiration of many plant tissues is not easily inhibited by cyanide and carbon monoxide (Hartree 1959, James 1953, Smith and Chance 1958). Cyanide has been shown to exert multiple effects on electron transport and phosphorylation depending on the concentration used (Hackett et al 1960).

Amytal inhibits mitochondrial respiration supported by pyridine nucleotide-linked substrates but does not inhibit respiration associated with succinate oxidation (Ernster et al 1955). High concentrations of amytal have been found to partially inhibit aerobic oxidation of succinate in both tightly coupled and DNP-uncoupled rat liver mitochondria (Pumphrey and Redfearn 1962, 1963). Amytal can also reduce P/O ratios (Greengard et al 1959, Azzone et al 1961). Chance and co-workers (1962, 1963) have concluded that amytal combines an inhibition of electron transfer between flavoprotein and cytochrome b with an inhibition of energy transfer at the NADH and flavoprotein site.

Antimycin A has been shown to inhbit electron transfer between cytochromes b and c in animal mitochondria (Potter and Reif 1952, Chance 1952, 1958, Chance and Williams 1956), and in plant mitochondria (Martin and Morton 1957). Estabrook (1958) demonstrated that in guinea pig liver mitochondria antimycin A caused a reduction of cytochrome b but did not interfere with the oxidation of cytochrome c, and Estabrook (1962) concluded that the carrier with which antimycin A interacts probably is not cytochrome b, but the oxidized carrier subsequent to cytochrome b (possibly) non-heme iron). Estabrook (1962) also found that the antimycin A titer of the mitochondria was equal under phosphorylating and non-phosphorylating

(uncoupled) conditions although antimycin A has been reported to exhibit effects on energy transfer (Myers and Slater 1957, Siekevitz et al 1958, Low et al 1958).

HOQNO (Lightbown and Jackson 1956, Jackson and Lightbown 1958) inhibits electron transfer at the same site as antimycin A:- between cytochromes b and c. Howland (1963a) reported that the inhibition of succinate oxidation in rat liver mitochondria by HOQNO was partially reversed by low concentrations of DNP and he concluded that HOQNO acts on both energy transfer and electron transfer reactions.

The reaction and site of action of the classical uncoupling agent DNP has been reviewed by Slater (1961) who considers that DNP (and CCP) act by uncoupling or discharging a non-phosphorylated "high energy" intermediate of oxidative phosphorylation (Borst and Slater 1961). The stimulation of ATPase activity by DNP is assumed to result from partial reversal of oxidative phosphorylation (Racker 1961) and this action is inhibited by ADP or oligomycin. Wadkins and Lehninger (1959) demonstrated that rates of ATPase activity were maximal when the respiratory carriers were in the oxidized state and minimal when the carriers were in the reduced state. Hemker (1962) found that the activity of uncoupling phenols was related to the amount dissolved in the mitochondrial lipid, which depended on pH and other factors. Weinbach and Garbus (1964) have demonstrated that uncoupling phenols bind to proteins within mitochondria after traversing a lipid layer. The uncoupling of oxidative phosphorylation in plant mitochondria by DNP has been well established (Hackett 1959).

The antibiotic oligomycin was introduced by Lardy et al (1958) as an inhibitor of oxidative phosphorylation. Oligomycin inhibits substrate oxidation in tightly coupled mitochondria and this inhibition is reversed by DNP (Lardy and McMurray 1958). Oligomycin blocks ATP-phosphate exchange, phosphate-water

O^{18} exchange, and inhibits ATPase activity in the presence of DNP, indicating that oligomycin inhibits near the site of incorporation of inorganic phosphate (Lardy and Connelly 1961). The uncoupling action of DNP would then occur before the site of phosphate incorporation and the site of oligomycin action.

Arsenate uncouples phosphorylation by forming a high-energy intermediate complex which is easily dissociated by arsenolysis. ADP is required for maximal rate of arsenate-stimulated respiration (Estabrook 1962). The inhibitory effect of oligomycin in tightly coupled mitochondria is not relieved by arsenate (Estabrook 1961, Huijing and Slater 1961), indicating that the uncoupling action of arsenate differs from the uncoupling action of DNP. Phosphate inihibits arsenate-stimulated respiration in the absence of ADP (Crane and Lipmann 1953, Ter Welle and Slater 1964), and ADP or DNP reverses this inhibition. Arsenate and P_i appear then to compete for a site in the respiratory chain phosphorylation mechanism, with P_i the more successful competitor, and Slater (1966) concluded that arsenate uncouples by reacting with a non-phosphorylated high-energy compound preceding P_i in the phosphorylation sequence, but P_i competes so successfully with arsenate that in the absence of ADP it inhibits the arsenate-stimulated respiration. Slater (1966) considers that these data indicate the existence of a phosphorylated High-energy intermediate but an alternative mechanism for the action of arsenate has been proposed (Lardy et al 1964, Chappell 1966).

Atractyloside is another inhibitor of oxidative phosphorylation which appeared to have a site of action similar to that of oligomycin in blocking phosphorylation (Bruni et al 1962, 1965, Vignais et al 1962, Bruni and Azzone 1964). However, atractyloside appears to act at the surface of the

mitochondrial membrane as a competitive inhibitor of adenine nucleatide penetration (Bruni 1966, Kemp and Slater 1964, Heldt et al 1965, Chappell and Crofts 1965b, Brierley and Green 1965). Atractyloside may inhibit phosphorylation of endogenous ADP (Bruni 1966). The inhibitory action of atractyloside depends on the maintenance of mitochondrial membrane structure.

The sites of action of these and other inhibitors and uncouplers and their effects on electron transfer and phosphorylation reactions in mitochondria and sub-mitochondrial particles have been reviewed by Ernster and Lee (1964) and Lieberman and Baker (1965), and discussed by Slater (1966), Bruni (1966) and Chappell and Crofts (1966).

E. HYPOTHESES OF OXIDATIVE PHOSPHORYLATION.

1. Chemical.

Oxidative phosphorylation consists of the flow of reducing equivalents along the electron transfer chain and concurrent coupling to phosphorylation of ADP to form ATP. Many hypotheses have been proposed for the coupling action and the actual formation of ATP. Most of these hypotheses are variations of a scheme of chemical coupling between the electron transfer chain and ATP formation depending on existence of "energy rich" chemical intermediates common to both electron transfer and phosphorylation, (Slater 1953, 1958b, Chance and Williams 1956, Myers and Slater 1957, Lehninger et al 1958, Racker 1961, Green and Fleischer 1962). The chemical coupling hypothesis has been reviewed recently by Ernster and Lee (1964), Sanadi (1965), Racker (1965), Slater (1958b), Lehninger (1962), and Lehninger and Wadkins (1962), and will be referred to as the "classical" hypothesis of

oxidative phosphorylation. This hypothesis is based on analogy with substrate-level phosphorylation where a common intermediate is present between electron transport and ATP formation and two different mechanisms can be identified (Lehninger 1964).

a. Type 1 Mechanism:- A type 1 mechanism first proposes that one substance (not P_i) combines with the electron carrier during coupled oxido-reduction to form a high energy intermediate shown as $A \sim C$. This intermediate, which is common to the oxido-reduction pathway and the ATP-forming reaction, then reacts with P_i and ATP to form ATP (Slater 1953, Lehninger 1955), as follows:-

$$AH_2 + B + C \longrightarrow A \sim C + BH_2 \quad \ldots\ldots 1$$

$$A \sim C + P_i + ADP \longrightarrow A + C + ATP \quad \ldots\ldots 2$$

$$\text{Sum} \quad AH_2 + B + P_i + ADP \longrightarrow A + BH_2 + ATP \quad \ldots\ldots 3$$

The finding that arsenate, unlike DNP, does not relieve the inhibition of respiration by oligomycin led Estabrook (1961) and Huijing and Slater (1961), to conclude that $A \sim C$ does not react directly with P_i and that a second high-energy intermediate is involved.

$$A \sim C + D \longrightarrow A + C \sim D \quad \ldots\ldots 4$$

$$C \sim D + P_i + ADP \Rightarrow C + D + ATP \quad \ldots\ldots 5$$

$$\text{Sum} \quad A \sim C + P_i + ADP \Rightarrow A + C + ATP \quad \ldots\ldots 6$$

An alternative mechanism (Chappell 1966, Lardy et al 1964), suggests that arsenate could form a stable $C \sim As$ compound which reacts with ADP in an oligomycin-sensitive reaction.

Reaction 5 is generally considered to proceed via an energy-rich phosphate compound and can be split into two different equations:-

$$C \sim D + P_i \longrightarrow C + D \sim P \quad \text{.....7}$$

$$D \sim P + ADP \longrightarrow D + ATP \quad \text{.....8}$$

$$\text{Sum} \quad C \sim D + P_i + ADP \rightarrow C + D + ATP \quad \text{.....9}$$

The reaction has also been considered as a concerted mechanism without a $\sim P$ compound being formed as intermediate (Kulka and Cooper 1962). The finding that phosphate inhibits the arsenate-stimulated respiration in the absence of ADP and that DNP or ADP reverses the inhibition suggests the presence of the $\sim P$ intermediate shown in equation 7 above (Crane and Lipmann 1953, Ter Welle and Slater 1964).

b. Type 2 Mechanism:- The second mechanism involving high-energy intermediates postulates the reaction of phosphate with the electron carrier molecule either before or during oxido-reduction thus:-

$$A\,H_2 + B + P_i \longrightarrow A \sim P + B\,H_2 \quad \text{.....10}$$

$$A \sim P + ADP \longrightarrow A + ATP \quad \text{.....11}$$

$$AH_2 + B + P_i + ADP \longrightarrow A + BH_2 + ATP \quad \text{.....12}$$

Various chemical models involving quinol phosphate have been suggested (Clark et al 1960, Vilkas and Lederer 1962) on the basis of entry of phosphate prior to oxidation. However, there is little evidence in favour of this second mechanism and most biochemical evidence suggests that phosphate enters after oxidation (Racker 1965). Evidence in favour of a non-phosphorylated high-energy intermediate has come from studies of the energy driven reduction

of NAD by succinate. When succinate oxidation is used to provide necessary energy the reduction of NAD takes place in the presence of oligomycin in mitochondria depleted of phosphate (Ernster 1963, Snoswell 1962). It has been shown that neither phosphate nor trans-phosphorylative enzymes are required for the formation of a non-phosphorylated high-energy intermediate or for the trans-hydrogenation step (Racker and Monroy 1964). Oligomycin markedly stimulates the rate of NADP reduction presumably by inhibiting the breakdown of a high-energy intermediate (Racker 1965). Oligomycin has been shown to still exert its stimulatory effect on an energy-linked trans-hydrogenase in the virtual absence of phosphate, and phosphate did not enhance the stimulating effect, indicating that P_i is not needed for the action of oligomycin (Ter Welle 1966). The action of dinitrophenol in stimulating oxidation in the absence of P_i indicates that the respiratory carriers interact with each other in the absence of P_i (Lehninger 1964). However, it could be postulated that oxidation in the presence of dinitrophenol occurred through replacement of P_i by water rather than by formation of an actual non-phosphorylated high-energy intermediate (Racker 1965). There appears to be more support for the type 1 mechanism.

c. General:- It is thermodynamically possible for the energized form of the electron carrier in equation 1 ($A\sim C$) to be in either the oxidized or reduced form. The equations 1-12 have indicated the energized form of the electron carrier to be in the oxidized state although an alternative mechanism has been proposed by Chance and Williams (1956), where the energized form of the electron carrier is in the reduced form as follows:-

$$AH_2 + B + C \longrightarrow A + BH_2 \sim C \quad .....13$$

$$BH_2 \sim C + P_i + ADP \longrightarrow BH_2 + ATP + C \quad .....14$$

$$AH_2 + B + P_i + ADP \longrightarrow A + BH_2 + ATP \quad .....15$$

A mechanism has been recently proposed by Estabrook et al (1963) based on observations of fluorescent enhancement when NADH was added to mitochondria. They suggest that a charge-transfer complex is formed between NADH and mitochondrial imidazole groups whereby the hydrogen of the imidazole nitrogen is labilized and removed by the respiratory chain. Simultaneously a lone pair electron from the pyridine nitrogen is added to the imidazole ring which then makes a nucleophilic attack on the phosphate. This variant of a former view (Grabe 1958) accounts for observations of a phosphorylated histidine in a mitochondrial protein (Boyer 1963), and includes proposals on the function of imidazoles in electron transport (Theorell 1941, Urry and Eyring 1963).

The proposed site of action for oligomycin in the chemical coupling hypothesis theory has already been discussed. The action of DNP is considered to occur at the siteof the non-phosphorylated high-energy intermediate, hydrolyzing the high-energy compound and allowing the continued passage of reducing equivalents along the electron transfer chain without incorporation of the $\sim$ from $A \sim C$ into ATP (Lardy and Elvehjem 1945, Slater 1966).

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

$$\text{thus} \quad A \sim C \xrightarrow{\text{DNP}} A + C \quad .....16$$

$$\text{Sum} \quad AH_2 + B \xrightarrow{\text{DNP}} A + BH_2 \quad .....17$$

Experiments have been presented, however, which suggest the hydrolysis of the C $\sim$ D (equation 4) intermediate (Chance and Williams 1956, Wadkins and Lehninger 1959), or D $\sim$ P (equation 7) compound (phosphorylated high-energy intermediate) Drysdale and Cohn 1958, Racker 1965). A proposal that DNP affects structural relationships rather than catalysing of the hydrolysis of an intermediate has also been put forward (Racker 1965).

The DNP-stimulated ATPase activity of mitochondria can be explained by the classical hypothesis as a partial reversal of the coupling steps from ATP to the site of action of DNP (Myers and Slater 1957, Hemker and Hulsmann 1961, Racker 1965).

thus $ATP + A + C \longrightarrow A \sim C + P_i + ADP$ 18

$A \sim C \xrightarrow{DNP} A + C$ 19

Sum $ATP \xrightarrow{DNP} P_i + ADP$ 20

Oligomycin would then inhibit DNP-induced ATPase activity by inhibiting the reaction between ATP, A and C.,

i.e. $ATP + A + C \xrightarrow{\text{oligomycin}} A \sim C + P_i + ADP$ 21

The isolation and purification of many coupling factors from mitochondria and submitochondrial particles has been described (Pinchot 1953, 1957, Racker 1963, 1964, 1965, Wadkins and Lehninger 1963, Green et al 1963). This subject has been reviewed by Lieberman and Baker (1965), Ernster and Lee (1964) and Racker (1965). The various soluble "coupling factors" which have been isolated do not appear to affect the primary energy-conserving reaction, and it has been demonstrated (Lee et al 1964) that non-phosphorylating respiratory chain preparations can be made to phosphorylate by the addition

of "coupling factors". Moreover these preparations are still able, in the absence of coupling factors, to provide energy which can be used for the energy linked transhydrogenase reaction and for reversal of the respiratory chain. It appears that coupling factors "promote only energy transfer rather than energy coupling in the true sense" (Ernster and Lee 1964).

Reconstitution of the respiratory chain has also been attempted with complexes (Hatefi et al 1961, 1962, Green and Wharton 1963), and with purified enzymes (Keilin and King 1960, King 1962, 1963). However, the absolute structural and functional reconstruction of the respiratory chain may not be possible as reconstitution requires elements of mitochondrial membranes (Green and Fleischer 1963), which require a matrix of structural protein, phospholipids and other lipids (Das and Crane 1964), to provide attachment positions for the purified carriers. Reconstitution cannot be considered as a reassembly of components to form the actual multi-enzyme respiratory chain "until oxidative phosphorylation, reversed electron transfer active ion transport and other functions of the respiratory chain have also been reconstituted" (Lieberman and Baker 1965).

2. Boyer's Hypothesis.

Another hypothesis of oxidative phosphorylation (Boyer 1965) proposes that energy made available by an oxido-reduction reaction is conserved in a conformational change in the protein of the respiratory carrier. This can be shown as:

$$AH_2 + B \longrightarrow A^* + BH_2 \qquad \text{.....22}$$

$$A^* + X \longrightarrow A \sim X \qquad \text{.....23}$$

$$\text{Sum} \quad AH_2 + B + X \longrightarrow A \sim X + BH_2 \qquad \text{.....24}$$

where A$\sim$X is suggested as being a thiol ester (Boyer 1965). A* cannot be reduced by the reaction which normally results in reduction of A to AH_2 and uncouplers result in the transformation of A* to A. Thus the A$\sim$C of equation 1 of the "classical" hypothesis is replaced by A*. Boyer (1965) has also suggested that only one phosphorylation site might exists for the entire phosphorylation process and oxidation-reduction reactions in different portions of an organized unit could lead to deformities transmitted to one locus. Thermodynamic calculations (Veeger 1965) show that this theory is feasible but the search for the A* or A$\sim$C and other intermediates still remains.

3. Summary.

The hypotheses described have in common the chemical coupling between the oxido-reduction pathway and the phosphorylation pathway, involving a high-energy intermediate of some form or other. The basic chemical theory can be summarised in Fig. I1 and most mechanisms are variations on this theme, depending on the number of high-energy intermediates postulated, the existence of the high-energy phosphorylated intermediate, and the presence of A$\sim$C (Slater 1966), A* (Boyer 1965), or A$\sim$I (Chance and Williams 1956), where I represents inhibition of respiration in the absence of ADP and P_i. The cyclic oxidation of the carrier AH is not represented in Fig. I1 because A$\sim$C may represent the reduced (Chance and Williams 1956), or the oxidized (Racker et al 1965, Slater 1966) form of the component A of the electron transfer chain. The energy-rich bond may be considered as a potential site for occupation by water or for hydrolysis (Mitchell 1966), and so high-energy bonds flowing in one direction from the chain to ATP are equivalent to water flowing in the opposite direction.

Slater has stated recently (1966) that "attempts to isolate compounds with the properties expected of a high-energy intermediate have met with conspicuous non-success" and the current position of claims of isolated high-energy intermediates were discussed (Slater 1966). However, the failure to isolate ~ compounds may only mean that they are very labile or stable only in a lipid phase, and not that they do not exist.

4. Chemiosmotic Hypothesis

Another hypothesis has been proposed for oxidative phosphorylation by Mitchell (1961) based on the suggestion by Lundegardh (1945), from the work of Lund (1928) and Stiehler and Flexner (1938), that if oxido-reduction through the cytochrome system were anisotropically organised across a membrane, H^+ would be produced on one side and consumed on the other. This idea of the conversion of energy of electron transport to osmotic potential of the H^+ concentration difference across a proton-impermeable membrane was used by Robertson and Wilkins (1948) to explain salt accumulation by plant tissues, and by Robertson (1960) and Davies and Ogston (1950) to explain acid secretion by the gastric mucosa.

The idea of separation of H^+ and OH^- across a membrane has been developed by Mitchell (1966) as a mechanism of oxidative phosphorylation whereby the separation of charges by the oxido-reduction system is coupled to a reversible anisotropic ATPase system. Thus if the active centre of a membrane-located ATPase were specifically accessible to H^+ from one side only, to OH^- from the other side and to water as H_2O from neither side, ATP hydrolysis could be reversibly coupled to the translocation of OH^- groups or ions across the system (i.e. equivalent to protons translocated in the

FIGURE I.1

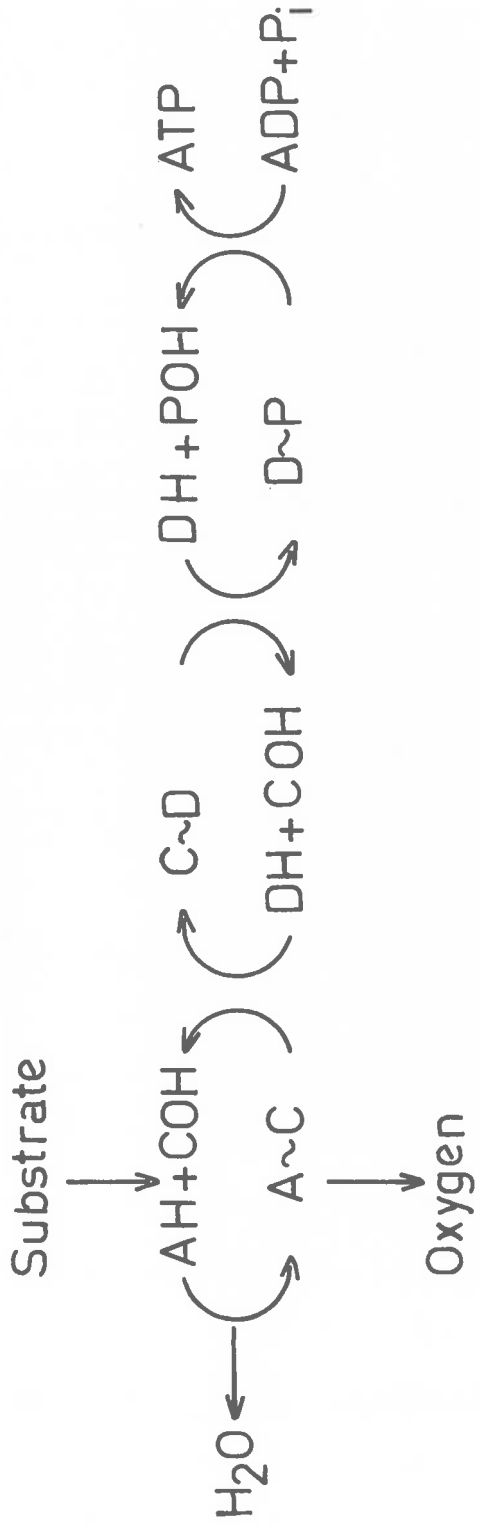
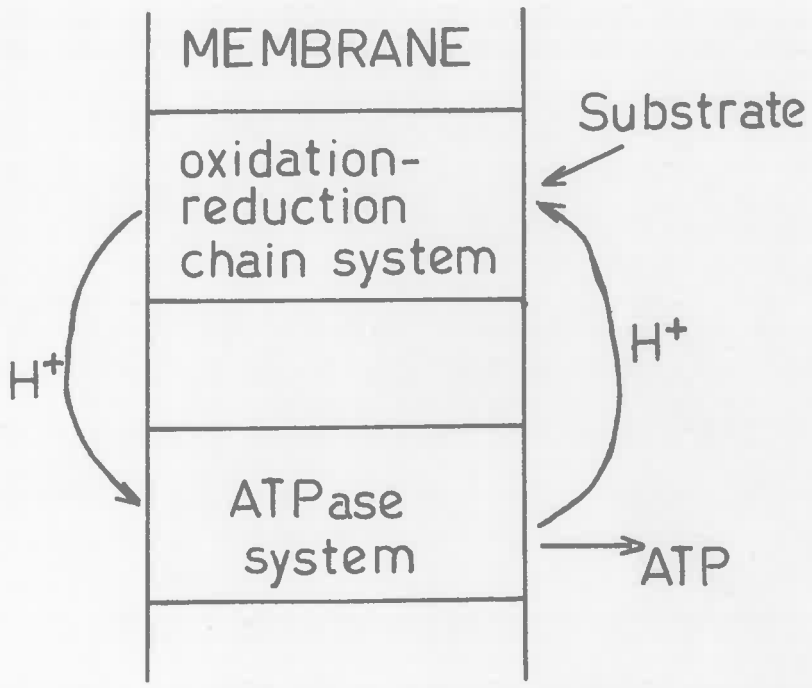


FIGURE I.2.
(Mitchell 1966)



opposite direction). Thus the reversed ATPase system could couple ATP synthesis to flow back across the membrane of protons which were produced by the oxido-reduction system.

This hypothesis has been termed the chemosmotic hypothesis (Mitchell 1961a) and is shown simply in Fig. I2. ATP hydrolysis and substrate oxidation would each generate a difference in proton concentration in the same direction across a proton-impermeable membrane such that electron transport through the oxido-reduction system would reverse ATP hydrolysis to give ATP synthesis if the difference in proton concentration were large enough and ATP hydrolysis would exert a back force on electron transport. The high pH differential required for the operation of this system was decreased by the postulation (Mitchell 1961a) of a coupling membrane which has a low ion permeability, in general and not only to protons, such that the electron transport and ATPase systems would be coupled not just by a pH differential but by the sum of the osmotic pressure difference and the electrical pressure difference (i.e. the electrochemical potential difference, (P.M.F.), (Mitchell 1966).

The low permeability of the mitochondrial cristae membrane to non-electrolytes containing 50 H groups or more has been established (Lehninger 1962) and early suggestions that the membrane has a low permeability to monovalent cations (MacFarlane and Spencer 1963, Bartley 1961), to monovalent anions (Robertson et al 1955) and to anions of higher valency (Bartley and Enser 1964) have been extended by Chappell and Crofts (1966). However, calculations of permeability coefficients have rarely been made (Robertson et al 1955) and relative impermeabilities have only been estimated qualitatively

FIGURE I.3.
(Mitchell 1966)

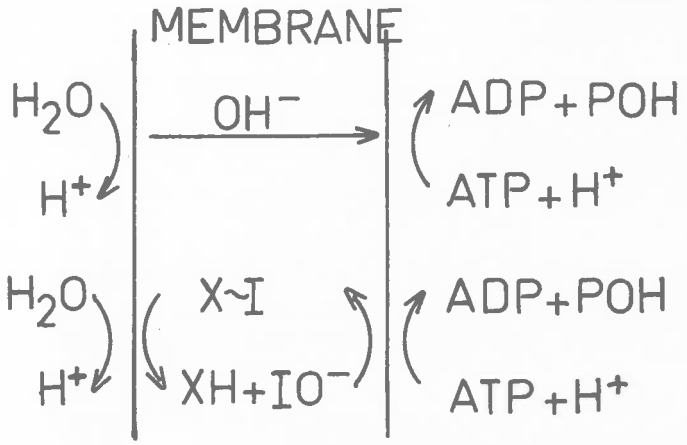
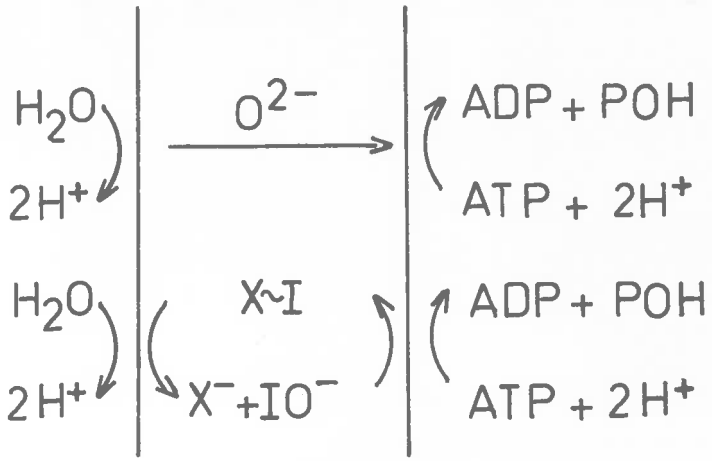


FIGURE I.4.
(Mitchell 1966)



(Chappell and Crofts 1966). Cristae membranes in rat liver mitochondria have been shown to have a low permeability to protons (Mitchell 1961b, 1963, Mitchell and Moyle 1965).

In intact mitochondria from rat liver it has been shown that protons are translocated outwards during substrate oxidation and during ATP hydrolysis (Mitchell 1962a, Mitchell and Moyle 1965a) and this appears to be the direction of proton translocation in certain bacteria (Mitchell 1962b). However, it has been observed that protons appear to pass in through the grana membranes of fragmented spinach chloroplasts during electron transport (Jagendorf and Hind 1963, Neumann and Jagendorf 1964a, Jagendorf and Neumann 1965), and there is evidence that ATP synthesis is coupled to the outward flow of protons through the grana membranes (Jagendorf and Uribe 1966). Thus the anisotropic ATPase of the chemosmotic theory and the respiratory chain systems would appear to be orientated such that protons are translocated inwards through the chloroplast grana membranes or lamellae, and outwards through the plasma membrane of certain bacteria and through the cristae membranes of intact mitochondria (Mitchell 1966).

a. ATPase System:- The reversible ATPase portion of the oxido-reduction/hydro-dehydration couple has been formulated in two ways. An ATPase system of type I (Fig. I3) would require an electrochemical potential difference of 420 mV or -7 pH units to poise the ATP/ADP couple centrally and an ATPase system of type II (Fig. I4) would require a force of 210mV or -3.5 pH units (Mitchell 1966). The hydro-dehydration system (ATPase) can be written as a pair of mono-electronic half reactions connected by the flow of OH^- (Fig. I3), or as a pair of di-electrogenic half reactions connected by

FIGURE I.5.
(Mitchell 1966)

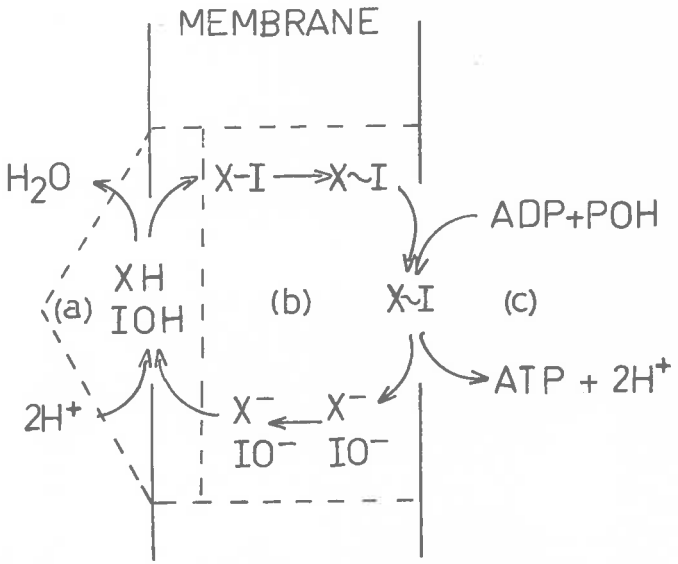
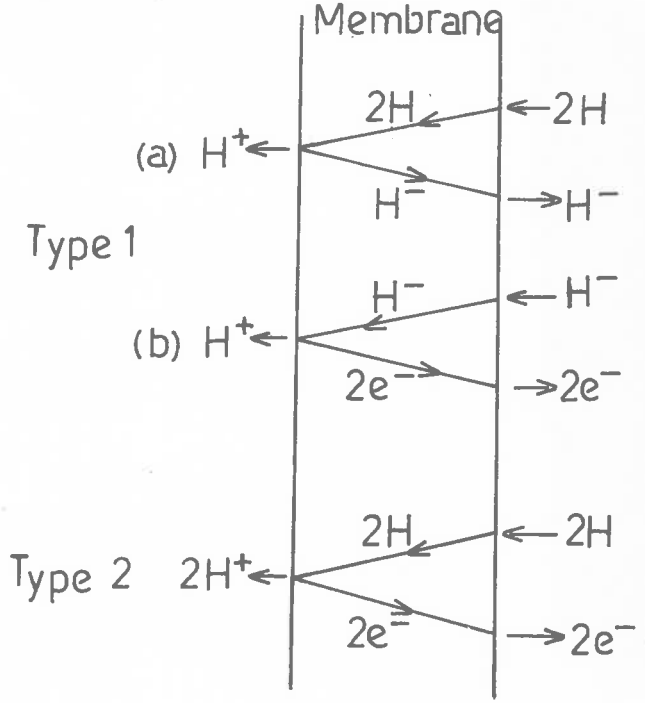


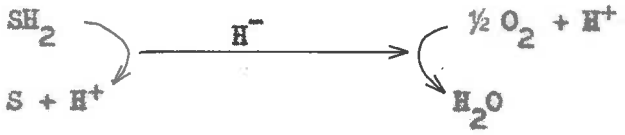
FIGURE I.6.
(Mitchell 1966)



the flow of O^{2-} groups (Fig. I4), just as the oxido-reduction reaction

$$SH_2 + \tfrac{1}{2} O_2 \longrightarrow S + H_2O \qquad \text{.....25}$$

can be written vectorically in two different ways, by the flow of hydride ions between two half reactions,



.....26

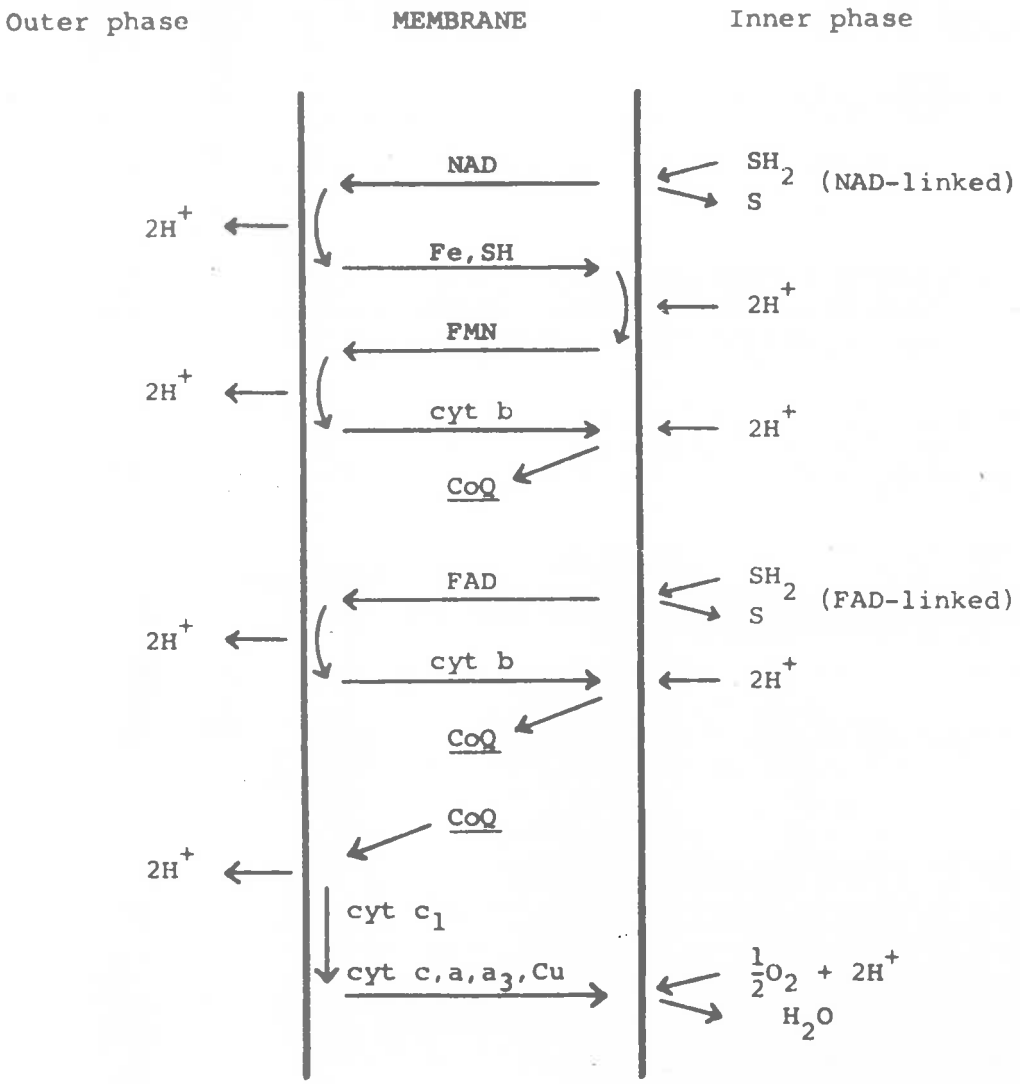
or by the flow of pairs of electrons.

.....27

The ATPase of type II has been considered in more detail by Mitchell (1966) who postulates the existence of three functional regions involving the formation of an anhydride (X - I) :- X - I hydrolase (a), X - I translocase (b) and X - I synthetase (c) (Fig. I5). The movement of X - I from left to right and the transformation to the "high energy" form X~I is considered to be due to the lowering of the ground state energy for X - I hydrolysis in going from phase L to R (Mitchell 1966).

The ATPase system of mitochondria and chloroplasts is considered to being coupled to the oxido-reduction system or respiratory chain by means of the proton current or electrochemical potential difference, and no chemical link exists between the two systems. Thus if an electrochemical potential difference of 200 to 250 mV, or -3 to -4 pH units were artificially imposed across the coupling membrane for a short time there should theoretically be some synthesis of ATP, and the maximum amount of ATP synthesis would be equal to half the number of protons titrating across the membrane during the

FIGURE I 6A
(Mitchell 1966)



electrochemical or pH differential decay. Hind and Jagendorf (1965) observed that when the pH values of the energy-accepting (light) stage and the ATP and P_i-requiring (dark) stage in spinach chloroplasts were 4.6 and 8.0 respectively, controls which had not been illuminated in the first stage synthesized a considerable amount of ATP in the second stage. Thus ATP was apparently synthesized by the application of a pH differential established for a short time across the membrane (Jagendorf 1966). ATP synthesis induced by a pH gradiant across the coupling membrane of mitochondria has been measured recently by Reid et al (1966).

The site of action of oligomycin is considered to be located in the X — I translocase or X — I hydrolase functional regions of the ATPase II system (Mitchell 1966). The reconstituted system of oxidative phosphorylation involving a combination of coupling factors (Racker 1964, 1965), and its reactions with oligomycin have been interpreted by Mitchell (1966) as being consistent with the chemiosmotic hypothesis.

b. Oxido-reduction System:- The oxido-reduction system or electron transfer chain can be considered (Mitchell 1966) as a series of oxido-reduction loops with a transition between the different currencies of oxido-reduction at the junction of the two arms of the loop as shown in Fig. I6. Two main types of systems are distinguished in Fig. I6:- the first type translocating one proton in the 2 electron equivalent oxido-reduction process, and the second type translocating 2 protons (Mitchell 1966). A complete scheme for the oxido-reduction pathway of Type II is shown in Fig. I6A. (Mitchell 1966), where respiratory chains are shown for oxidation of NAD-linked and succinate-linked substrates in mitochondria, with the chains branching at Co Q. The components of the chain are shown in the order usually

presented for the "classical" hypothesis of oxidative phosphorylation. Although the position of Co Q is usually considered to be on the substrate side of cytochrome b (Massey and Veeger 1963, Ernster and Lee 1964, Sanadi 1965), the original suggestion of Hatefi (1959) and Moret et al (1961) placed Co Q at the position shown in Fig. 16A. in respect to cytochrome b. The observed stoichiometry between oxygen reduced and ATP produced (i.e. P/O ratios) can be accounted for by a scheme of this type and it has been shown (Mitchell and Moyle 1965a) that the oxidation of succinate and β-hydroxybutyrate by intact rat liver mitochondria is accompanied by the translocation of approximately 4 protons and 6 protons respectively, outward through the coupling membrane, and nearly 2 protons are translocated outwards per ATP hydrolyzed. These data suggest the operation of an ATPase system of Type II.

The folding of the oxido-reduction chain as shown in Fig.16A. is suggested(Mitchell (1966) as representing structural configuration of the chain component molecules. A tendency to short circuit would be expected in a system of this type with the functional activity very sensitive to physical displacement of the spatially related carriers and the structural components of the lipoprotein membrane. "Non phosphorylating" mitochondrial preparations are considered to differ from intact "phosphorylating" mitochondria in that the respiratory chain in the former has been damaged so that both cytochrome b and Co Q have become partially dislocated (Chance 1958, Redfearn and Pumphrey 1960), and short circuiting across this region would consequently impair the coupling between oxido-reduction and phosphorylation (Mitchell 1966). The classical hypothesis of oxidative phosphorylation considers the "phosphorylating" electron transfer chain as to be a different pathway from the

"non-phosphorylating" chain.

c. Action of Inhibitors and Uncouplers:- The action of the respiratory chain inhibitors, antimycin A, HOQNO, amytal and others are explained as in the "classical" hypothesis by reaction with the oxido-reduction components, blocking the flow of reducing equivalents. The electron and hydrogen transfer function in the Loop 1 (Fig.I6A). or NAD-FMN coupling region is extremely labile toward reagents which dislocate the structural integration of the system such as amytal, rotenone and sub-lytic concentrations of Triton X-100 (Chappell 1964, Redfearn and King 1964, Burgos and Redfearn 1965, Chance and Hollunger 1963a).

The supposed specificity of guanidine and its derivatives for the A$\sim$C intermediates of the "classical" hypothesis (Chance and Hollunger 1963b, Chappell 1963, Pressman 1963a,b) has been used as an argument against the chemiosmotic hypothesis on the basis that "3 different ATPases would be required to account for the site specificity of the guanidines" (Slater 1966). However, Mitchell (1966) has discussed the action of guanidines in relation to the observation (Pressman and Park 1963) that guanidine competes with Mg^{++} for entry into mitochondria and enhances uptake of P_i. The inhibitory action of the guanidine group could thus be explained on the basis of cation uptake along an electro-chemical gradient and accumulation of these lipid-soluble compounds in the mitochondria, leading to inhibition of the oxido-reduction chain at the susceptible sites I and II (Fig.I6A) or in one case at site III. The action of uncouplers in reversing guanidine group inhibition is accounted for by postulating breakdown of the membrane potential by uncouplers followed by removal of the accumulated inhibitors (Pressman 1963b, Schafer 1964, Guillory and Slater 1965, Mitchell 1966).

The chemiosmotic hypothesis bases the uncoupling action of DNP on the observation that DNP and other uncouplers hasten acid-base equilibrium across the membranes of certain bacteria and rat liver mitochondria (Mitchell 1961b, 1963, Mitchell and Moyle 1965a, Chappell and Crofts 1966), and chloroplasts (Jagendorf and Neumann 1965). The uncouplers DNP and CCP are considered to be specific proton conductors. The production of a pH differential across the membrane of rat liver mitochondria by the addition of HCl or alkali in an anaerobic KCl medium was followed by only a limited rapid phase of pH equilibration on addition of DNP or CCP (Mitchell 1966). The creation of a pH differential by respiratory or ATPase activity was, however, followed, on addition of uncouplers, by a rapid and complete collapse of the pH differential (Mitchell 1966). This would be expected if the uncouplers were specific proton conductors and the membrane was relatively impermeable to K^+ and Cl^-. The membrane potential developed on transfer of H^+ alone would prevent complete equilibration. If, however, the membrane was made specifically permeable to K^+ by valinomycin then migration of K^+ (from inside) would collapse the membrane potential and the equilibration of the acid-produced pH differential could go to completion in the presence of the uncoupler.

The antibiotic polypeptides, valinomycin and gramicidin have been found to release respiratory control and simultaneously cause uptake of K^+ and output of H^+ (Pressman 1963a, Moore and Pressman 1964, Pressman 1965). Chappell and Crofts (1965a, 1966) showed that gramcidin and valinomycin confer upon natural lipid membranes a specific permeability to certain cations, and that these antibiotics had similar effects on artificial and natural lipid membranes suggesting that the specific ion-conducting property existed in the polypeptide molecules themselves. Thus valinomycin and gramicidin should be

membrane potential-collapsing agents and the effects of these compounds on stoichiometric proton translocating measurements (Mitchell and Moyle 1966) suggests that the greater part of the PMF produced during operation of the oxidoreduction chain consists of membrane potential. The action of uncouplers in pH equilibration across a membrane, where a pH differential has been developed by addition of acid or alkali or by the operation of the oxidoreduction chain, and the action of valinomycin on this pH equilibration confirms this suggestion (Mitchell 1966).

d. Respiratory Control:- The start of oxido-reduction through the respiratory chain is accompanied in mitochondria and bacteria by net output of protons (Mitchell and Moyle 1965a, Mitchell 1963), and in chloroplasts and chromatophores by a net intake of protons (Neumann and Jagendorf 1964a, Baltscheffsky and von Stedingk 1966). However, a steady state is soon reached in which there is little or no net acid production or consumption based on the rate of return of protons across the membrane. During oxidation of succinate by rat liver mitochondria in the absence of phosphate acceptor the return flow of protons produced by the oxido-reduction system would be restricted due to the absence of phosphate acceptor, the PMF would be high and respiration would be slowed to the controlled rate of oxidation (state 4). The addition of phosphate acceptor would allow phosphorylation with coupled flow of protons through the reversed ATPase system and the decrease in PMF would allow a new steady state value $\approx$(state 3 rate of oxidation). During state 4/state 3 transitions large changes of external pH would not be expected (Mitchell 1966), although a significant change in PMF (the greater part existing as membrane potential) would be expected.

Low concentrations of divalent cations produce an uncoupling effect on mitochondrial oxidation while the divalent cation is taken up by the mitochondria (Chance 1965b, Chappell and Crofts 1966). According to the chemiosmotic hypothesis this effect would be due to collapse of the membrane potential as the cations moved into the mitochondria along the electrochemical gradient. Oxidation rate would increase due to this collapse, as the membrane potential is a major part of the PMF which controls the oxido-reduction state of the respiratory carriers. Oxidation rate would decrease on uptake of all the divalent cations. Further addition of divalent cation in the absence of phosphate would repeat this effect until respiratory stimulation accompanying membrane potential collapse produced sufficient protons and OH^- (or pH differential) to produce a PMF large enough to oppose movement of reducing equivalents along the oxido-reduction chain. The system would then return to the controlled state of oxidation. Thus in the absence of a penetrating anion (e.g. phosphate, acetate), (Chappell and Crofts 1966) the cation-induced inhibited oxidation rate (state 6, Chance 1964, 1965) would be a result of increased alkalinity within the mitochondria in the absence of an exchangeable anion for OH^-. Breakdown of the mitochondrial membrane with Triton X-100 after uptake of divalent cation alone, did cause pH neutralization (Chappell et al 1962, 1963, Brierley et al 1964). Addition of penetrating anion (phosphate) would allow exchange for OH^- and continued cation uptake and stimulated respiration.

e. Exchange Diffusion:- Mitchell (1966) postulates the existence of exchange diffusion systems as an integral part of the chemosmotic hypothesis whereby diffusion of ions (other than protons or OH^-) ions down the

electro-chemical gradient across the coupling membrane, and their accumulation in osmotically disruptive concentrations in the internal phase, are counter-balanced by an exchange diffusion for protons or OH^- (Mitchell 1961a). The entry of substrates against the electrical gradient would be facilitated by exchange diffusion for H^+ or OH^- (Mitchell 1962a). A specific translocation system appears to be operating in rat liver mitochondria for the entry of inorganic phosphate (Chappell and Crofts 1965a), citrate, malate (Chappell 1964) and other Krebs cycle acids (Gamble 1965).

The chemiosmotic hypothesis attempts to explain the coupling between oxido-reduction and phosphorylation without assuming the existence of chemical intermediates common to both pathways. The translocation of protons in the membrane-located oxido-reduction system is by means of oxido-redution loops each consisting of one hydrogen and one electron carrier in series. The translocation of protons also occurs across a membrane-located reversible ATPase, probably of type II (Fig.I5) in mitochondria, which translocates two protons per ATP hydrolysed. The operation of both systems in an ion-impermeable membrane would create both pH differential and membrane potential, together forming a proton motive force (PMF). The presence of exchange diffusion systems would regulate the internal pH and osmotically active ions and would enhance the membrane potential component at the expense of the pH differential. This chemosmotic hypothesis as modified by Mitchell (1966) still involves a "high energy" or an anhydro intermediate in the reversible ATPase system. This intermediate is not common to both oxido-reduction and phosphorylation systems as proposed in the "classical" hypothesis. The problems of isolating this intermediate are the same as for the "classical"

FIGURE I.7.
(Ernster & Lee 1964)

high-energy intermediates. This ATPase intermediate may not exist in a "stable" form (Mitchell 1966).

F. ENERGY-LINKED REACTIONS OF MITOCHONDRIA

1. Reversed Electron Transfer.

Mitochondria and submitochondrial particles from a variety of tissues have been shown to catalyze an energy-dependent reversal of electron transport through the respiratory chain. The energy for the reaction can be supplied either by added ATP or by energy generated in some other portion of the respiratory chain. When the energy is supplied by ATP the reaction (by the classical hypothesis) involves an actual reversal of oxidative phosphorylation and it appears that electron transport through all carriers of the respiratory chain from NAD^+ to cytochrome oxidase can be reversed in this oligomycin-sensitive manner (Ernster and Lee 1964). When energy is supplied by operation of some portion of the respiratory chain to supply endogenous high-energy intermediates by the classical hypothesis, the reversal of electron transport proceeds at the expense of these high-energy intermediates without involvement of the actual phosphorylating system in an oligomycin-insensitive manner. The relation of these two types of energy dependent reversal of electron transport to oxidative phosphorylation by the classical hypothesis are shown in Fig. I7. This subject has recently been reviewed by Ernster and Lee (1964) and Lieberman and Baker (1965).

The chemiosmotic hypothesis can also explain reversed electron transfer. ATPase activity through the membrane-located ATPase system would produce protons on the outside of the membrane (similarly oxido-reduction reactions through part of the respiratory chain would produce protons), increase the PMF, and the oxido-reduction reaction through one or more of the oxido-

reduction loops would be reversed if the PMF exceeded the mid-point potential span across that loop system. The normal direction of oxido-reduction has been reversed (through the part of the respiratory chain between succinate dehydrogenase and a point on the oxygen side of the antimycin A-sensitive site - probably at cytochrome c), in intact beef heart mitochondria by reducing internal fumarate with an externally added artificial electron donor (Mitchell and Moyle 1965b). The reaction was sensitive to antimycin A and caused translocation of protons inwards instead of outwards across the mitochondrial membrane system (Mitchell 1966).

2. Swelling.

When isolated mitochondria are exposed to hypotonic solutions the swelling produced has been shown to be related to membrane permeability (Tedeschi 1959). The total volume increases as well as the volume of the intercristal space and this swelling is related to the appearance of certain enzyme activities not present in the intact mitochondrion such as NADH oxidation abilities (Green 1959c, Bendall and de Duve 1960). Conductivity studies indicate that swollen mitochondria tend to equilibrate with the external medium whereas shrunken mitochondria tend to maintain a constant ionic composition regardless of changes in external ionic strength (Pauly et al 1960, Pauly and Packer 1960).

Swelling and contraction of mitochondria is also related to the process of oxidative phosphorylation. It was found (Harmon and Fiegelson 1952) that loss of mitochondrial integrity paralleled swelling, and subsequently it was found that ATP was lost during swelling, and that addition of ATP and magnesium ions would reverse degradative swelling of

mitochondria (Price et al 1956). The various factors that induce swelling in rat liver mitochondria have been outlined by Lehninger (1959) and swelling induced by most of the agents was reversed by ATP, magnesium ions, serum albumin and EDTA in various combinations. Hunter et al (1959) have suggested that an oxidation - reduction process at one or more points in the electron transfer chain is associated with the swelling process whether or not phosphate is present. Chappell and Greville (1960) have concluded that swelling induced by substrate oxidation in the presence of phosphate does not involve coupled phosphorylation. This conclusion is based on the inhibitory effects of oligomycin, amytal and antimycin A on swelling induced by partial reactions of the electron transport chain.

Agents that cause swelling in mitochondria do not always increase ATPase activity (Maley and Johnson 1957). However, a group of compounds (calcium, thyroxin, fatty acids and cysteine) cause swelling and an increase in ATPase activity. DNP stimulates ATPase but inhibits swelling (Chappell & Greville 1960). Calcium has been shown to stimulate ATPase activity as well as swelling in pea mitochondria (Forti 1957). In corn mitochondria calcium had no effect on ATPase activity (except at high concentrations in the presence of magnesium when it inhibited), and calcium stimulated ATP+ magnesium-induced contractions of isolated corn mitochondria (Hanson et al 1965). The swelling associated with energy-dependent cation uptake has been studied in detail in rat liver mitochondria by Chappell and Crofts (1965a, b and c), Crofts and Chappell (1965), and Chance (1965), and in corn mitochondria by Hanson (1965). Swelling and contraction of isolated mitochondria has been reviewed by Chappell and Greville (1963a), Lehninger

(1962) and Crane (1961).

Slater (1966) distinguishes three types of mitochondrial swelling:-

(1) that caused by the osmolarity of the suspending medium being lower than that of the mitochondrial contents when water enters the mitochondrion (Kölliker 1888, Cleland 1952, and Tedeschi 1955),

(2) that caused by energy-dependent uptake of cations followed by uptake of anions, resulting in a higher osmolarity of the mitochondrial contents than the suspending medium and thus water enters the mitochondria (Chappell and Crofts 1965c, 1966, Chance 1965, Crofts and Chappell 1965, Hanson et al 1965) and

(3) that caused by damage to the mitochondrial membrane causing increased permeability to the solutes of the suspending medium. High permeability of the membrane to a certain combination of cation and anion will also cause swelling.

Ammonium phosphate or acetate caused swelling in rat liver mitochondria whereas NH_4Cl, KCl, potassium phosphate or potassium acetate did not produce swelling (Chappell and Crofts 1966). Corn mitochondria, showed substantial spontaneous swelling in KCl alone but did not in sucrose (Stoner and Hanson 1966), indicating that corn mitochondria may be more permeable to these ions than rat liver mitochondria (Chappell and Crofts 1966). Contraction in corn mitochondria was obtained after spontaneous swelling by the addition of substrate alone in contrast to results obtained with animal mitochondria (Chappell and Greville 1963, Lehninger 1962). Phosphate reduced contraction produced on addition of ATP and Mg^{++}, or succinate to corn mitochondria

(Stoner and Hanson 1966). Substrate-induced contraction was inhibited by respiratory inhibitors and ATP contraction was inhibited by oligomycin, while both were inhibited by CCP (Stoner and Hanson 1966). The different volume changes of plant and animal mitochondria in response to the same treatments may reflect different permeabilities in the mitochondrial membranes (either present originally or produced by isolation techniques), rather than differences in swelling and contraction mechanisms.

3. Ion Uptake.

Another energy-linked activity of mitochondria which has recently been studied in detail is ion uptake and accumulation by isolated mitochondria. The uptake of ions by plant cells has been under investigation for many years in the study of plant-soil relationships and has been the subject of many monographs (Briggs et al 1961, Steward and Sutcliffe 1961, Jennings 1963) and reviews (Lundegardh 1951, Epstein 1956, Briggs and Robertson 1957, Laties 1959, Robertson 1960, Brouwer 1965).

Robertson (1951) suggested that mitochondria could act as temporary sites of ion accumulation in cells and serve as carriers in the intracellular translocations of ions through the cytoplasm from the external medium or cell wall to the vacuole. Hertz and Levitt (1961) have subsequently repeated this postulation. Isolated plant mitochondria have been shown to accumulate ions against a concentration gradient (Robertson et al 1955), and Jackson et al (1962) have implicated mitochondria as the particulate sites of phosphate absorption in barley roots. Robertson (1960) discussed the separation of H^+ and electrons or OH^- during electron transfer in the respiratory chain situated in the matrix of the mitochondrial membrane, which

acts as a non-conducting lipoidal insulating layer. He suggested that this separation of charges could cause the movement of an ion down an electrochemical gradient into the mitochondria, the respiratory carriers serving as ion carriers in a non-aqueous medium. The active uptake of ions into mitochondria has been reviewed by Lehninger (1962).

a. Divalent Cation and Phosphate Uptake: Recent studies have indicated that isolated mitochondria can accumulate high concentrations of divalent cations and phosphate by an energy-dependent process (Vasington and Murphy 1961, 1962, Vasington 1963, De Luca and co-workers 1961, 1962, Sallis et al 1963, Brierley and co-workers 1962, 1963, 1963b, 1964, 1964b, Brierley 1963, Chappell et al 1962, 1963, 1965c, Lehninger et al 1963, Rossi and Lehninger 1963a, 1963b, 1964, Carafoli 1965a, 1965b and co-workers 1964, 1965a, 1965b, Hodges and Hanson et al 1965). Mitochondria from a variety of animal and plant tissues are capable of taking up large amounts of Ca (Vasington and Murphy 1961, 1962, De Luca et al 1961, 1962, Brierley et al 1963, 1964b, Rossi and Lehninger 1963a, 1963b, Hodges and Hanson 1965), Mg (Brierley et al 1962, 1963b), Mn (Chappell et al 1962, 1963) and Sr (Carafoli 1965a, 1965b, et al 1965b), in the presence of phosphate and added substrate. In the absence of phosphate (Pi), Ca (Rossi and Lehninger 1964, Chance 1965), Mg (Brierley et al 1964a) and Mn (Chappell 1963) was taken up to a limited extent by a substrate-dependent process. This P_i-independent cation uptake has been interpreted as indicating that the cation is taken up by an active process and is followed passively by P_i during divalent cation and phosphate cation and phosphate uptake (Chappell et al 1963, Chance 1965).

FIGURE I.8.
(Brierley 1963)

(1) Requirements for Uptake:- The massive accumulation of divalent metal ions and phosphate, and the lesser uptake of cations in the absence of phosphate, has been shown to be dependent on the presence of substrate and aerobic conditions (Brierley et al 1962, 1964b, Vasington and Murphy 1962, Rossi and Lehninger 1963b, Chappell et al 1963). Incubation at 0°C. or in the presence of uncoupling agents such as DNP and CCP, or of respiratory inhibitors such as cyanide, amytal, HOQNO or antimycin A completely inhibits the substrate-dependent ion uptake (Brierley et al 1962, Vasington and Murphy 1962, 1963a, Chappell et al 1963). Oligomycin, however, does not inhibit substrate-dependent ion uptake suggesting that ATP as such is not involved in the process (Brierley et al 1962, Chappell et al 1963). Massive cation and phosphate accumulation by mitochondria is supported by a large number of respiratory substrates (Brierley et al 1962, Vasington 1963). Operation of only a portion of the respiration chain supports ion uptake e.g. ascorbate - TMPD - cytochrome c - O_2 (Brierley and Murer 1964) and succinate - ferricyanide (Chappell 1965c).

The requirement for substrate and for passage of reducing equivalents along whole or part of the respiratory chain, and the action of inhibitors and uncouplers has been attributed to the requirement for a high-energy non-phosphorylated (Chance 1965, Brierley et al 1962), or phosphorylated (Hodges and Hanson 1965) intermediate to energise ion uptake. Fig I8. summarises the proposed mechanisms for ion uptake based on the classical hypothesis of oxidative phosphorylation (Brierley et al 1962, 1964b, Hodges and Hanson 1965). Substrate-dependent ion uptake based on the chemiosmotic hypothesis of oxidative phosphorylation has already been discussed and, in summary, involves the movement of cations into the mitochondria down an electro-chemical gradient

set up by the operation of the oxido-reduction pathway which produces H^+ on the outside of the mitochondria.

Respiratory-chain inhibitors would inhibit ion uptake by preventing either formation of high-energy intermediates by the classical view, or the PMF (pH differential and membrane potential)responsible for the movement of ions by the chemiosmotic view. The uncouplers DNP and CCP would inhibit ion uptake in the classical view (Brierley et al 1962, 1964b, Chance 1965) by breakdown of the high-energy non-phosphorylated intermediate (equation 16) responsible for energising ion uptake. The high-energy phosphorylated intermediate (equation 7) has also been implicated (Hodges and Hanson 1965, Hanson et al 1965) with DNP inhibiting by mass action. DNP and CCP would, by the chemiosmotic hypothesis, allow the passage of the protons produced by the oxido-reduction system back through the membrane, collapsing the membrane PMF potential and thus preventing ion uptake.

(2) Effect of Oxidative Phosphorylation:- The presence of ADP, or of ADP plus glucose and hexokinase, i.e. conditions allowing oxidative phosphorylation to occur with formation of ATP or glucose-6-phosphate, completely inhibited substrate-dependent Mg and Pi uptake by beef heart mitochondria (Brierley 1962).

The concentrations of Ca used in Ca and Pi accumulation experiments were reported to completely uncouple oxidative phosphorylation in animal and plant mitochondria (Vasington and Murphy 1962, Hanson 1965). Although the presence of ADP and a hexokinase trap was reported to completely inhibit Ca and Pi uptake in rat liver mitochondria (Carafoli et al 1964) this was probably due to a complete elimination of ATP required for substrate-dependent Ca and

Pi uptake, rather than to any inhibitory effect of an operating oxidative phosphorylation process.

Brierley et al (1962) interpreted the inhibitory effect of oxidative phosphorylation on ion uptake as a competition between ion uptake and oxidative phosphorylation for the high-energy intermediates formed on operation of the respiratory chain (Fig.I8). The competition favoured oxidative phosphorylation to occur (Brierley et al 1962, 1963b). The chemiosmotic view, with a reversible ATPase system synthesizing ATP and returning protons, formed by operation of the oxido-reduction pathway, to the other side of the coupling membrane, also explains this competitive effect between ion uptake and ATP formation. The return of the protons via the reversible ATPase system synthesizing ATP must offer less resistance to PMF collapse than the movement of cations through the membrane down an electro-chemical gradient. Brierley et al (1963b) found that oligomycin, by inhibiting oxidative phosphorylation, promoted ion uptake under these dual conditions. This action of oligomycin can be simply explained by either hypothesis (Fig.I8).

(3) ATP requirement:- The massive accumulation of Ca and Pi (Vasington and Murphy 1962, Rossi and Lehninger 1963a,b), but not of Mg (Brierley et al 1962), Mn (Chappell et al 1963) or Sr (Carafoli et al 1965), was found to require the presence of ATP in addition to substrate. This oligomycin-insensitive effect of ATP has been attributed either to the chelating properties of ATP in binding excess Ca and thus reducing mitochondrial-membrane damage, Ca leakage and Ca-induced swelling (Chappell et al 1963), or to a specific role of ATP in stabilizing the calcium phosphate salt presumed to precipitate within the mitochondria (Carafoli et al 1965).

The Ca and Pi-dependent adenine nucleotide uptake by rat liver mitochondria has been studied by Carafoli and Lehninger (1964) and Carafoli (1965a), and from investigations with atractyl-oxide they have suggested that uptake of ATP or ADP is a pre-requisite for Ca and Pi accumulation. This could be due to stabilization of the precipitated salt or to phosphorylation of an active site (by an oligomycin-insensitive reaction) which provides a nucleus for salt precipitation. They concluded from the action of atractyloside on ion accumulation and on ADP/ATP accumulation ratios that at high concentrations of Ca (when mitochondrial membranes were damaged) both ATP and ADP were bound. At low concentrations of Ca (when the membranes were presumably intact and atractyloside-sensitive) the predominant or only nucleotide bound was ADP.

The substrate-dependent uptake of Ca and Pi by beef heart mitochondria did occur in the absence of ATP (Brierley et al 1964b) but the accumulated ions leaked out again after a very short time (20 seconds), whereas in the presence of ATP accumulation continued for 20 minutes or more without loss of the accumulated ions. Substrate-dependent uptake of Ca and Pi occurred in corn mitochondria (Hodges and Hanson 1965) in the absence of ATP but uptake was increased by addition of ATP. However, ATP inhibited substrate-dependent Ca and Pi uptake in soybean mitochondria and massive uptake occurred in the absence of ATP. (Hanson 1965).

ATP inhibited substrate-dependent Mg uptake in beef heart mitochondria (Brierley et al 1963b), and this was related to ATPase activity providing ADP for the competitive oxidative phosphorylation reaction. Oligomycin did reduce the inhibitory effect of ATP on the uptake of Mg and Pi but complete recovery was not observed (Brierley et al 1963b).

(4.) ATP-Supported Uptake:- ATP was able to replace substrate oxidation (although usually to a limited extent) in supporting massive ion accumulation. This was been demonstrated for Ca by Rossi and Greenawalt (1963, when 15 mM ATP was required), Brierley et al, (1963a, 1964b), Hanson and Hodges (1965), Hanson (1965), for Mg (Brierley et al 1963a) and for Sr (Carafoli et al 1965b). ATP-supported ion uptake was oligomycin-sensitive, cyanide and antimycin A-insensitive and was not greatly suppressed by omission of Pi, due presumably to ATPase activity providing Pi for massive cation phosphate precipitation. Under the conditions for massive Ca and Mg uptake, substrate-supported cation uptake was not detected in the absence of Pi in many investigations (Vasington and Murphy 1962, Brierley et al 1962, but c.f. Rossi and Lehninger 1964, and Brierley et al 1964a).

ATP-supported ion uptake has been explained by the classical hypothesis of oxidative phosphorylation (Brierley et al 1964a, 1964b, Hodges and Hanson 1965) by a partial reversal of oxidative phosphorylation producing the high-energy intermediate required for ion uptake (Fig.I8). The chemiosmotic hypothesis also provides a mechanism for ATP-supported ion uptake by the membrane-located ATPase system producing protons on the outside of the coupling membrane, i.e. a PMF is set up in the same manner and direction as that produced under the action of the oxido-reduction system. Ion uptake would thus be supported by either ATP or substrate oxidation, but the former process would be sensitive to oligomycin and the latter insensitive. The inefficient ATP support of ion uptake compared with substrate support (Brierley et al 1964b, Hodges and Hanson 1965) could result from the phosphorylation system being more labile than the oxido-reduction system, or, in the absence of an

operating respiratory chain, from breakdown of membrane structure and increased "leakiness" or proton permeability.

(5) Pi-independent Cation Uptake:- Chappell et al (1963) reported that a small quantity of Mn could be accumulated by rat liver mitochondria in the absence of phosphate in a respiration-dependent, DNP-sensitive and oligomycin-insensitive manner. This small amount of Mn exceeded the endogenous Pi in the mitochondrion by 10-20 fold. The Pi-independent Mn uptake was inhibited and reversed by addition of DNP, whereas Mn accumulated in the presence of Pi was inhibited, but not reversed, by DNP. If Pi was added immediately after Mn was accumulated from a Pi-free medium then the mitochondria took up Pi by a respiration-independent process.

Chappell et al (1963) concluded from these results that the primary respiration-dependent process is the accumulation of divalent cation, that uptake of phosphate occurs passively along the electro-chemical gradient and that phosphate precipitates with the cation inside the mitochondria. Subsequently it has been demonstrated that Ca and Mg are accumulated by mitochondria under suitable conditions (low Ca concentration), in the absence of Pi (Rossi and Lehninger 1964, Chance 1965, Brierley et al 1964a).

(6) Precipitation of Ions:- Electron micrographs have demonstrated electron dense deposits in mitochondria incubated under conditions allowing massive divalent cation and phosphate accumulation. Mitochondria incubated without the divalent cation, or mitochondria at 0°C. did not show these deposits (Brierley and Slauterback 1964, Peachey 1964, Lehninger et al 1963, Greenawalt and Carafoli 1966, Greenawalt et al 1964). Deposits have been clearly demonstrated in mitochondria under conditions allowing massive Ca or

Sr and Pi accumulation, whereas conditions allowing Mg or Mn and Pi accumulation did not yield visible electron dense deposits. This was probably due to technical difficulties associated with preserving and observing the low density and more soluble Mg and Mn phosphates (Brierley et al 1964).

The electron dense deposits, micro-incineration analyses on these deposits and accumulation of divalent cations with phosphate ratios (Brierley et al 1962, Chappell et al 1963) have suggested the precipitation of cation phosphate salts within the mitochondria either as the $Mg_3(PO_4)_2$, $Mn_3(PO_4)_2$ and $Ca_3(PO_4)_2$ salts (Brierley et al 1962, Chappell 1963, Thomas and Greenawalt 1964) or as $(Ca_3(PO_4)_2)_3.Ca(OH)_2$ (Vasington and Murphy 1962, Rossi and Lehninger 1963 a,b.).

On the basis of measurements of the enhancement of polar relaxation rates, Chappell et al (1963) suggested the existence of 3 stages in Mn uptake by rat liver mitochondria:- (a) surface binding to the mitochondria by a rapid DNP-sensitive, Ca and Mg-competitive reaction, (b) respiration-dependent accumulation in the absence of Pi which is both inhibited and reversed by DNP and respiratory inhibitors, and (c) precipitation of the accumulated Mn as $Mn_3(PO_4)_2$ in the presence of phosphate, by passive entry of the phosphate following active uptake of Mn^{++}.

The production of H^+ in the external medium during the accumulation of Mg and Pi (Brierley et al 1962) and Mn and Pi (Chappell et al 1963) was considered to be a result of the precipitation of $M_3(PO_4)_2$ within the mitochondria. The M^{++} : H^+ ratio was approx. 1:1. However, H^+ was also produced during the uptake of Mn in the absence of phosphate in a ration Mn^{++} : H^{++} = 0.9 (Chappell et al 1963) suggesting that the movement of H^+ out

of the mitochondria is one of the primary considerations in cation uptake (Mitchell 1966, Chance 1965).

(7) Ion Competition:- The effects of various cations on the uptake of one particular cation were generally inhibitory to varying degrees. NaCl and Tris were reported to inhibit Mg uptake (Brierley et al 1963b) at relatively low concentrations, whereas high concentrations of monovalent cations were required to inhibit Ca uptake (Vasington and Murphy 1962, Carafoli et al 1964). Calcium inhibited Mg uptake (Brierley et al 1962) although Ca has been reported to induce Mg uptake when no Mg uptake occurred in the absence of Ca (Carafoli et al 1964).. In most cases Mg was required for Ca in uptake in mitochondria (Vasington and Murphy 1964b, Brierley et al and Carafoli et al 1964), whereas in submitochondrial particles Mg inhibited Ca uptake (Carafoli et al 1964). Calcium inhibited the respiration-linked accumulation of Sr by rat liver mitochondria (Carafoli 1965a).

These effects indicate a general competition between all cations for an uptake mechanism although competition between divalent cations is most marked (Brierley et al 1962, Carafoli 1965a). The affinity of the available binding sites in the mitochondrial membrane for different cations could produce the apparent selectivity of the substrate-dependent uptake mechanism for different cations. In the presence of phosphate, different solubilities of the cation phosphate salt could also affect the apparent selectivity of substrate-dependent cation uptake.

(8) Hormone Effects:- De Luca et al (1961, 1962), and Sallis et al (1963, 1965), have investigated the effects of parathyroid hormone and vitamin D on mitochondrial ion movements. Liver mitochondria from parathyroidectomized

rats were unable to accumulate Mg and Pi unless parathyroid hormone was added (Sallis et al 1963). Parathyroid hormone may be involved in substrate-dependent accumulation of Mg and Pi by the mitochondria, whereas vitamin D acts directly or indirectly by promoting release of accumulated Ca.

(9) Ion Effects on Respiration, H^+ Production and Volume:-

Respiratory Stimulation:- Chappell et al (1963) and Chappell and Greville (1963b) observed the effect of low concentrations of Mn on the respiratory rate of isolated rat liver mitochondria during substrate-dependent uptake of Mn. The oxidation rate in the presence of substrate and Pi but without phosphate acceptor was increased markedly by the addition of low concentrations of Mn and this was followed by an inhibited oxidation rate. Mn accumulation was measured and the Mn^{++}:O ratio was 5.4 with glutamate, the Mn^{++}:H^+ value was 1.1 with phosphate and 0.9 in the absence of phosphate, and the Mn^{++}:Pi ratio was 1.5 (Chappell et al 1963).

The effects of low concentrations of calcium on the oxidation rates of respiring mitochondria were observed in 1955 by Chance, who found that the reaction of low concentrations of calcium with phosphorylating mitochondria led to a short-term activation of respiration, followed by a restitution of respiratory control, similar to the effect of ADP on the mitochondria. Calcium activation of respiration was observed in 1953 by Potter et al and Siekevitz and Potter (1955) who reported a 3-fold stimulation of respiration on addition of 500mM calcium to rat liver mitochondria. These results were confirmed by Lindberg and Ernster (1954). However, the respiratory activation was not followed by a restitution of respiratory control and calcium was considered (Lehninger 1949, 1956) to uncouple respiration in DNP-like fashion.

Chance (1956, 1963), however, found that the reaction of calcium with mitochondria resembled that of ADP and phosphate in that a definite calcium: oxygen stoichiometry was observed, and cycles of oxidation-reduction changes of pyridine nucleotide and other respiratory components accompanied the calcium-activated respiration. Subsequently the reactions of low concentrations of calcium on the oxidation rates of animal mitochondria have been investigated by Chance (1964, 1965), Rasmussen et al (1965), Rossi and Lehninger (1964), and Saris (1963) and by Hanson et al (1965) in corn mitochondria. Respiratory effects of low concentrations of strontium have been studied by Carafoli (1965b).

Calcium and manganese accumulation has been shown to be independent of added phosphate (Chance 1963, Rossi and Lehninger 1964, Chappell et al 1963). The reaction of calcium with mitochondria in the absence of phosphate leads to a new steady state of the respiratory carriers in pigeon-heart and rat liver mitochondria (highly oxidized) and this effect is reversed on addition of phosphate (Chance 1964, 1965). Acetate can replace phosphate (Rasmussen et al 1965), and arsenate can also replace phosphate in supporting cation uptake (Brierley et al 1963a, b, Chappell et al 1963, 1966).

Hydrogen Ion Production:- An increase in external H^+ concentration has also been reported on the addition of calcium and manganese to respiring mitochondria and this change has been followed with a glass electrode (Saris 1963, Chappell et al 1963), or with a glass electrode associated with a spectrophotometric indicator technique (based on bromothymol blue) (Chance 1965). The rapid H^+ increase is closely synchronized with the interval of stimulated respiration produced by calcium or manganese addition (Chance 1965).

The various stoichiometries reported by Chance (1965) for calcium: oxygen uptake or: H^+ production were:- Ca^{++}: O_2= 5 - 6 with succinate, in the presence or absence of phosphate; Ca^{++}: ADP = 2; Ca^{++}: H^+ = 1.0 in the presence of phosphate and this ratio varied between 0.9 in the presence of high potassium and 2.5 in the absence of potassium; Ca^{++}: H^+ = 1.7 in the absence of phosphate; and Ca^{++}: H^+ = 2.3 in the presence of acetate. Rossi and Lehninger (1964) found that Ca^{++}:O = 4 with succinate, 8 with α-keto-glutarate and 6 with β-hydroxybutyrate in the absence of phosphate although these ratios were based on the "extra" oxygen uptake produced by the calcium addition. Chance (1965), however, calculated Ca^{++}: O ratios by the procedure for calculation of ADP/O ratios (Chance and Williams 1955). Saris (1963) found that Ca^{++}: H^+ = 1.2 in the presence of phosphate. Chappell et al (1963) recorded Mn^{++}: H^+ = 1.1 with phosphate, 0.9 without phosphate, and Mn^{++}: O = 5.4 with glutamate and phosphate, calculated from "extra" oxygen uptake. Carafoli (1965b) measured Sr^{++}: O ratios of 3.8 with succinate and 5.5 with β-hydroxy-butyrate in the presence of phosphate and the calculations were based on "extra" O uptake after strontium addition. In the absence of phosphate, respiration continued at the stimulated rate indefinitely when concentrations of strontium greater than 400-500mM were used.

Measurements of H^+ produced during cation uptake in the presence of phosphate (Brierley et al 1964b, Chappell et al 1963, Chance 1965) need not be related to the primary role of H^+ produced during uptake in the absence of phosphate. With phosphate present precipitation of a cation-phosphate salt and concurrent release of H^+ would alter the M^+: H^+ ratios (Brierley et al 1964b, Chappell et al 1963). Changes in H^+ concentration due to ATPase

activity induced by calcium mustalso be considered (Brierley et al 1964b).

Volume Changes:- In addition to the mitochondrial changes in respiratory activity and H^+ production on addition of low concentrations of divalent cation, changes in the light scattering properties of the mitochondria have been recorded (Chance 1965, Chappell et al 1965c, 1966, Stoner and Hanson 1965). These changes have been interpreted as volume changes in the mitochondria on cation addition and uptake.

Swelling and contractions observed in mitochondria on the addition of divalent cations with various anions has been discussed by Chappell and Crofts (1966) and the effects of inhibitors and uncouplers on both the accumulated ions and the volume changes of the mitochondria were studied. In summary, swelling was observed on accumulation of divalent cations if the anion present formed either a soluble or insoluble complex with the cation (Chappell and Crofts 1966). The swelling observed as a consequence of calcium and acetate accumulation differed markedly from calcium and phosphate-induced swelling. The former occurred immediately on addition of calcium and was reversed by addition of calcium and was reverded by addition of inhibitors or by anaerobic conditions, whereas the latter occurred only after substantial calcium accumulation and was enhanced and not reversed by respiratory inhibitors, uncouplers and anaerobic conditions (Chappell and Crofts 1965c, Crofts and Chappell 1965). Chappell and Crofts (1966) observed that 1 u mole of calcium, when added to mitochondria suspended in the presence of succinate and rotenone, produced a small burst of respiration followed by a decreased rate of oxidation (Chappell et al 1963, Chance 1963, 1965), which was, however, higher than the rate before calcium addition. Swelling did

not occur at this stage but addition of acetate caused marked swelling and a stimulated respiration rate, followed by a decline when less than 10% calcium was free outside the mitochondria. Further addition of calcium increased the respiration rate and produced more swelling, but addition of phosphate, while stimulating respiration rate, produced rapid mitochondrial contraction. These results were interpreted (Chappell and Crofts 1966) as the replacement of the soluble calcium acetate salt by the insoluble hydroxyapatite $(Ca_3(PO_4)_2)_3$. $Ca(OH)_2$ complex. Marked swelling of the mitochondria was then observed when the suspension became anaerobic, a characteristic of calcium and phosphate-treated mitochondria. Chappell and Crofts (1966) suggested that in the case of calcium acetate-induced swelling the mitochondrial volume change occurred as a result of increased internal osmotic pressure due to the soluble calcium acetate salt. The swelling which occurred when calcium and phosphate (or arsenate) was accumulated was attributed to some property of the hydroxyapatite accumulated, which has been shown to act as an ion-exchange resin (Armstrong 1952), and not to an effect of the calcium itself (Chappell 1966).

Calcium can produce swelling in the absence of phosphate (Slater and Cleland 1953, Tapley 1965), but this swelling is most probably due to release of mitochondrial fatty acids and damage to the mitochondrial membrane. This action of calcium is prevented by addition of serum albumin (Lehninger and Remmert 1959, Wojtczak and Lehninger 1961) which does not affect calcium and phosphate-induced swelling (Chappell and Crofts 1965c).

The effects of calcium on the volume changes of corn mitochondria were investigated by Hanson et al (1965) who found that calcium reduced the extent

of spontaneous swelling in KCl and enhanced the contraction produced by addition of ATP and magnesium, or substrate, to swollen mitochondria. This calcium-induced contraction was particularly marked in the presence of phosphate. These results agree with the suggestion of Chappell and Crofts (1966) that formation of an insoluble calcium-phosphate complex can cause contraction of swollen mitochondria. Plant mitochondria appears from studies of spontaneous swelling in KCl to be more permeable to some ions than animal mitochondria (Hanson et al 1965, Stoner and Hanson 1966).

Summary:- The effects of low concentrations of divalent cations on respiring mitochondria can be summarized by the observations of Chance (1965) in relation to the reaction of calcium or ADP with mitochondrial suspensions. On the addition of these substances to phosphate-supplemented mitochondria, four distinct phenomena occurred simultaneously.

(I) the steady state of the respiratory carriers increased to a new and characteristic oxidation-reduction level (state 4 to state 3 transition),

(II) electron transport was stimulated (state 3 oxidation rate on ADP addition),

(III) light scattering changes occurred, and

(IV) in the case of ADP phosphorylation to ATP H^+ accumulation occurred, and in the case of calcium accumulation H^+ extrusion occurred.

The changes produced on addition of low concentrations of divalent cations to plant mitochondria have not been extensively investigated, but studies with corn mitochondria indicate that similar changes can occur in plant mitochondria in response to divalent cations but the changes from one

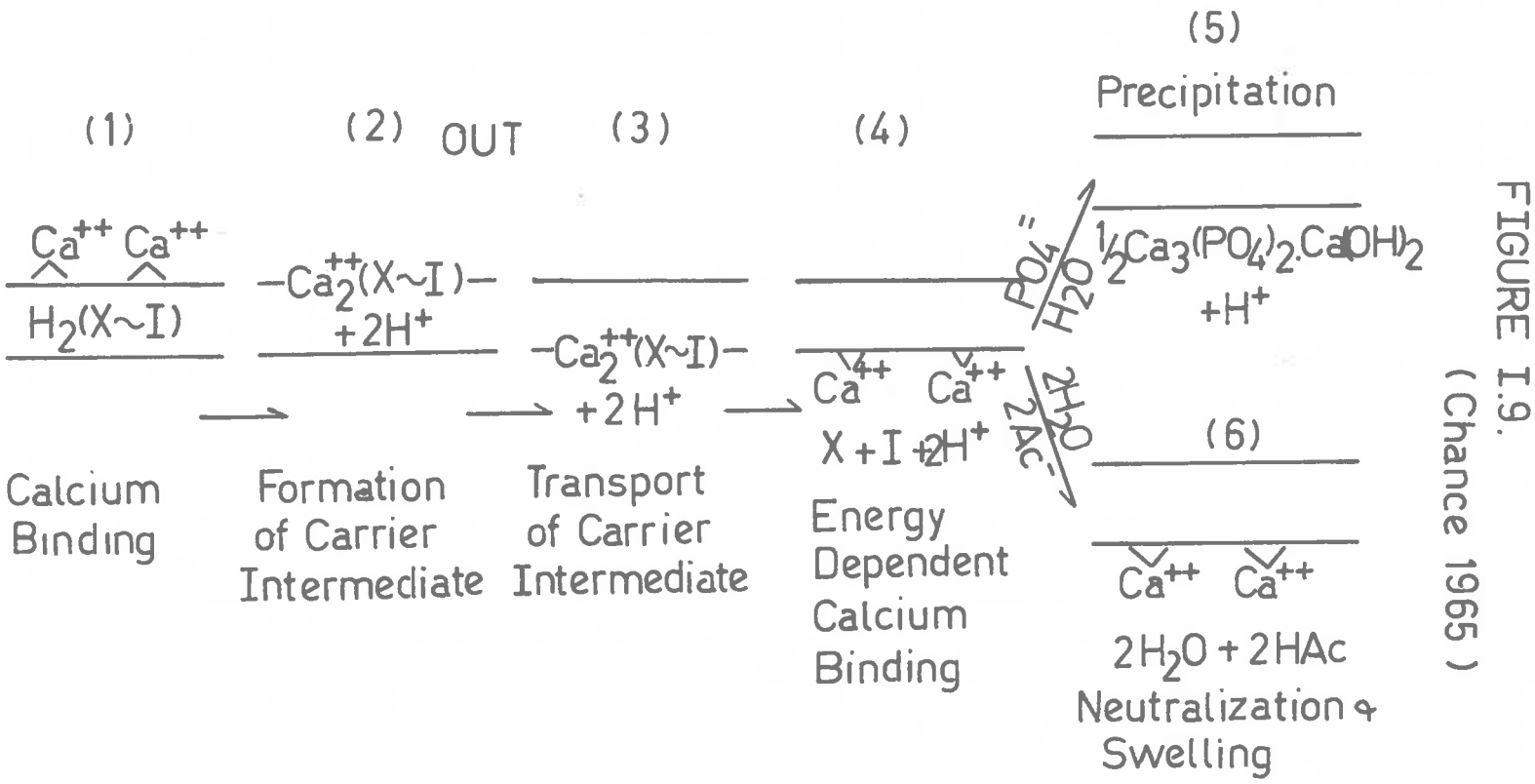


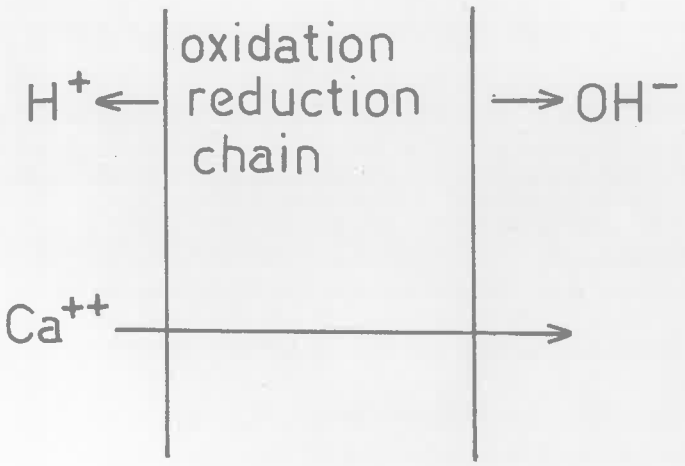
FIGURE I.9. (Chance 1965)

state to another are not as marked (Hanson et al 1965). This could result from differences in membrane permeabilities between plant and animal mitochondria.

(10) Mechanisms for Divalent Cation and Phosphate Uptake:-

Classical Chemical:- Studies of divalent cation uptake by isolated mitochondria have produced various mechanisms for the substrate-dependent uptake of ions. Most have been based on the classical view of oxidative phosphorylation and the high-energy non-phosphorylated or phosphorylated intermediates have been implicated as the energy source for substrate-dependent ion uptake (Brierley et al 1962, 1963b, 1964, Chance 1965, Hanson et al 1965). (Fig.I8). The involvement of a high-energy phosphorylated intermediate (Brierley et al 1962, Hanson et al 1965) appears to be less likely than that of a non-phosphorylated intermediate from results obtained with arsenate and acetate in place of phosphate (Chappell et al 1963, Brierley et al 1963b, Chance 1965, Rasmussen et al 1965, Chappell and Crofts 1966), and from the action of oligomycin and DNP on substrate and ATP-supported ion uptake. A mechanism for divalent cation uptake based on the classical chemical hypothesis has been put forward by Chance (1965) and is summarized in Fig.I9. Membrane-bound calcium reacts with a compound ($H_2(X \sim I)$), is transferred across the membrane and is bound by an energy-dependent process. Penetrating anions may cause precipitation and H^+ ejection (phosphate) or internal neutralization and swelling (acetate). This mechanism produces the observed stoichiometries of calcium : oxygen =2 (calcium : 2e = 2), and Ca^{++}: H^+ = 1 at low concentrations of calcium in the absence of phosphate (Chance 1965). Step 5b (Fig.I9) postulates the reaction in the presence of acetate where the H^+ released in the previous step is neutralized and swelling

FIGURE I.10.



occurs due to the osmotic activity of calcium acetate, whereas in step 5a precipitation of calcium phosphate produces H^+ and swelling does not occur (Rasmussen et al 1965, Chance 1965).

Chemiosmotic:- The chemiosmotic hypothesis of oxidative phosphorylation can account for divalent cation uptake in the presence or absence of phosphate or acetate, and the observed stoichiometries, as discussed previously. In summary (Fig.I10), the membrane potential (PMF) set up by the operation of the oxido-reduction pathway allows the movement of cations down an electro-chemical gradient into the mitochondria. Massive accumulation of the cation occurs in the presence of a permeable anion which may move into the mitochondria either in association with the divalent cation (2+) or in an exchange reaction with OH^- (Mitchell 1966, Chappell and Crofts 1966). This mechanism of ion uptake provides the observed stoichiometry of $Ca^{++}:H^+ = 1$ in the presence or absence of phosphate, and $Ca:2H^+$ ($2e^-$ or $\frac{1}{2} O_2$ reduced) = 2. Movement of the OH^- in response to calcium and phosphate movement is also possible. The presence of acetate as the permeable anion would provide the observed result of little or no external H^+ production either by exchange of acetate for OH^- (Mitchell 1966), or by penetration of the unionised acetate and ionization within the mitochondria (Chappell and Crofts 1966).

A mechanism proposed by Chappell (1966) and Chappell and Crofts (1966) to account for ion uptake by mitochondria comprises a H^+ pump exchanging protons for cations in a similar manner to the chemiosmotic scheme. However, this mechanism differs basically from the chemiosmotic scheme in the origin of the H^+ which is considered to be produced in some way from the $X \sim I$ or $C \sim D$ (non-phosphorylated high-energy intermediate) formed during classical

oxidative phosphorylation (Chappell 1966). The chemiosmotic hypothesis provides H^+ directly from the oxido-reduction reaction without involvement of phosphorylation or related reactions (Mitchell 1966). The actual mechanism for production of H^+ from $C\sim D$ or $X\sim I$ was not postulated by Chappell and Crofts (1966), but one $\sim$ produced one H^+ whereas Chance (1965) postulated one $\sim$ producing $2H^+$ (Fig.I9).

Discussion:- Both the classical and chemiosmotic hypotheses can adequately explain the results obtained from ion uptake studies and the stoichiometries measured. The chemiosmotic hypothesis has the advantage of being a more straightforward exchange of H^+ for cations, without the involvement of an unknown compound ($H_2(X\sim I)$, Chance 1965). The production of OH^- within the mitochondria by this hypothesis permits some freedom in considering the exchange of anions, cations, H^+ and OH^- and the origin of stoichiometries of cation: H^+, cation: O and cation: anion measured.

The reversible stimulation of the oxidation rate in rat liver mitochondria by low concentrations of calcium in the absence of phosphate, and the simultaneious change in oxidation-reduction states of the respiratory components to highly oxidized states have been discussed by Chance (1965) in terms of a chemical mechanism (Fig. I9). "these data suggest that oxidation-reduction states of the respiratory carriers unrelated to the electron transfer activity can be obtained in the phosphate-depleted system. The small burst of respiration observed on the addition of calcium in the absence of added phosphate represents the utilization of an endogenous material. The fact that a second, later addition of calcium causes a second burst of respiration suggests that not all the material is depleted by the first addition of calcium".

The endogenous substance refers to the available energy-dependent binding sites for calcium (Chance 1965, Fig.I9) or $\sim X$ or $X \sim I$ (Rasmussen et al 1965). Chance (1965) suggested that the stimulation of oxidation and the return of cytochrome b and pyridine nucleotide to their initial reduced states on phosphate addition was due to relief of the available energy-dependent calcium binding sites (or X) by precipitation of the calcium as a calcium phosphate complex (Fig.I9). These calcium-binding sites could also be released by addition of acetate which neutralized the H^+ produced but did not form an insoluble complex with calcium (Chance 1965), Rasmussen et al 1965).

Rossi and Lehninger (1964) observed transient stimulations in oxidation by addition of low concentrations of calcium to rat liver mitochondria in the absence of phosphate similar to those reported by Chance (1965). Addition of higher than 800uM calcium no longer stimulated oxidation and it was suggested that the "respiratory chain is saturated with calcium". Phosphate did not change the Ca^{++}/O ratio but prevented the marked return to the inhibited state of oxidation shown in the absence of phosphate. Increasing concentrations of phosphate reduced the point of inflection and at concentrations of phosphate greater than 2mM stimulation of oxidation continued indefinitely, but ATP or oligomycin prevented this effect.

The oxidized state of the respiratory carriers (Chance 1965) on the addition of calcium to mitochondria in the absence of phosphate, and subsequent relief of the oxidized states and inhibited oxidation by phosphate addition can be considered in terms of the chemiosmotic hypothesis. Production of H^+ in the external medium by the oxido-reduction pathway

leads, in the absence of phosphate acceptor, to PMF build up which exerts a back pressure on the oxidation-reduction pathway producing a steady state in the respiratory carriers (State 4). Addition of calcium allows breakdown of membrane potential by movement of calcium along the electrochemical gradient into the mitochondria and this produces stimulation of oxidation and oxidation of respiratory carriers, followed by a return to previous states and rate of oxidation on complete uptake of calcium. Further addition of calcium (or an initial high concentration) after initial breakdown of membrane potential with some uptake of calcium, produced an increased pH differential-portion of the PMF which leads to a highly alkaline mitochondrial interior, the inhibited oxidation rate and the highly oxidized respiratory carriers. Introduction of a permeable anion (phosphate or acetate) which can exchange with OH^-, reduces the pH differential and allows further oxido-reduction activity i.e. stimulated oxidation and uptake of calcium (Chappell and Crofts 1966), Mitchell 1966).

The action of calcium in releasing fatty acids and producing increased mitochondrial permeability and swelling (Chappell and Crofts 1966) could affect this view of divalent cation and phosphate uptake. Precipitation of a calcium phosphate complex occurs after and apart from the initial cation uptake process but this could subsequently affect the initial uptake process by production of H^+. A precipitated calcium phosphate complex could also eventually alter the volume and permeability of the mitochondria (Chappell and Crofts 1966). The continuous stimulation of oxidation produced by high phosphate concentrations after calcium binding by rat liver mitochondria (Rossi and Lehninger 1964) could be a result of permeability changes. ATP (or oligomycin) could prevent this effect

by stabilizing the precipitated calcium phosphate complex (Carafoli et al 1965a) or membrane structure.

Summary of Ion Uptake Mechanisms:- The three stages of ion uptake proposed by Chappell et al (1963) (see p.44), the 3 or 4 stages proposed in the mechanism of Chance (1965) and Rasmussen et al (1965) for calcium uptake (Fig.I9) and the 4 steps of ion uptake recognized by Rossi and Lehninger (1964) are consistent with the chemiosmotic scheme (Mitchell 1966).

Surface binding of the cations to the surface of the mitochondria by a passive process, as proposed by all mechanisms (Chance 1965, Chappell 1963, Rossi and Lehninger 1964), could be necessary for the entry of the particular cation into the field of influence of the PMF produced by the coupling membrane. In plant mitochondria in particular, which are more loosely "coupled" to phosphorylation and more permeable to ions in general than animal mitochondria, the cations would need to be adjacent to the site of PMF production for their uptake. Cation uptake does occur in plant mitochondria although cation uptake was not measured in the absence of phosphate (Hodges and Hanson 1965, Hanson et al 1965, Hanson 1965). Initial passive binding of cations to the mitochondrial membranes could play a major part in determining the selectivity of the substrate-dependent cation uptake subsequently measured. The solubilities of divalent cation phosphate (or other anion) complexes formed could also produce apparently selective uptake of particular ions. These effects could account for the observed preference of mitochondria for calcium uptake over magnesium uptake (Carafoli et al 1964, Brierley et al 1962, Hanson 1965, Hanson et al 1965).

The second stage of ion uptake proposed by Chappell et al (1963), Chance (1965), Rasmussen et al (1965) and Rossi and Lehninger (1964) and by the chemiosmotic hypothesis comprises the energy-dependent step with production of H^+. The mechanisms based on the classical hypothesis of oxidative phosphorylation differ from the chemiosmotic view (Mitchell 1966) in either the H^+ production mechanism alone (Chappell 1966), or in the mechanism of H^+ production and actual uptake process of the cations (Chance 1965, Rasmussen et al 1965).

The last steps of ion uptake:- the entry of anions and formation of calcium complexes with precipitation in some cases, are basically the same although the chemiosmotic mechanism also postulates exchange of OH^- for anions.

b. Movovalent Ion Uptake:- The uptake of monovalent ions into isolated mitochondria has been studied in detail in recent years, particularly in relation to the effects of the antibiotics valinomycin and gramicidin (Pressman 1963, Chappell and Crofts 1966, 1965a). Robertson et al (1955) measured accumulation of NaCl against a concentration gradient by beetroot mitochondria, and potassium binding by mitochondria has been recorded by Bartley and Davies (1954), Ulrich (1959), Gamble (1962), and Christie et al (1961). Uptake of potassium by rat liver mitochondria has been demonstrated from a sucrose medium containing succinate, ATP and KCl (Solomon 1964, Rottenberg and Solomon 1965). This uptake was partly inhibited by DNP and completely inhibited by the respiratory inhibitors, antimycin A and cyanide which caused potassium leakage from the mitochondria. Oligomycin had no effect, or stimulated potassium uptake, suggesting that the

mechanism for this potassium uptake could be similar to that proposed for divalent cation uptake.

(1) Valinomycin and Gramicidin Effects:- The unusual effect of valinomycin and gramicidin in stimulating movement of alkali metal ions into or out of mitochondria, and the associated changes in H^+ concentration and light-scattering measurements, have been investigated by Pressman (1963, 1965), Moore and Pressman (1964), Harris et al (1966), Chappell and Crofts (1965a, 1966) and Neubert and Lehninger (1962).

Valinomycin was reported to uncouple oxidative phosphorylation in 1959 (McMurray and Begg), but Pressman (1963) showed that its action on mitochondria differed markedly from that of other uncoupling agents such as DNP. His investigation into valinomycin-stimulated mitochondrial ATPase showed that more hydrogen ions were produced in the external medium than those liberated by ATPase action. When H^+ production was measured in an ATP-free medium containing KCl, H^+ was expelled rapidly from the mitochondria on addition of valinomycin, followed by a phase in which some H^+ returned to the mitochondria, followed by a third phase during which no further changes took place. DNP added at this point returned the external pH to the original value.

Subsequent studies (Pressman 1965, Harris et al 1966, Chappell and Crofts 1966), have indicated that valinomycin-induced H^+ production is accompanied by potassium uptake and respiratory stimulation. Rubidium and caesium will substitute for potassium but sodium and sithium will not, and phosphate or arsenate are required for valinomycin-stimulated respiration (Moore and Pressman 1964, Pressman 1965). Hydrogen ion ejection

and potassium uptake does not require the presence of phosphate (Moore and Pressman 1964), although phosphate and acetate increased the rate of potassium uptake by mitochondria over that observed in chloride medium (Harris et al 1966).

Low concentrations of KCl produced ejection of H^+ and uptake of potassium on addition of valinomycin and these changes were reversed by DNP, antimycin A and anaerobiosis. Higher concentrations of KCl produced transient ion changes and the pH returned to the original after a short time (Pressman 1963). The ratio of H^+: potassium was 0.9 at 1mM KCl. These results were interpreted as indicating that valinomycin induced an energy-dependent potassium uptake by the mitochondria, leading to the ejection of an equivalent amount of H^+ (Moore and Pressman 1964), and that valinomycin increased the permeability of the mitochondrial membrane to potassium (Harris et al 1966). Magnesium decreased the induced potassium uptake slightly while calcium caused movement of potassium out of the mitochondria (Harris et al 1966).

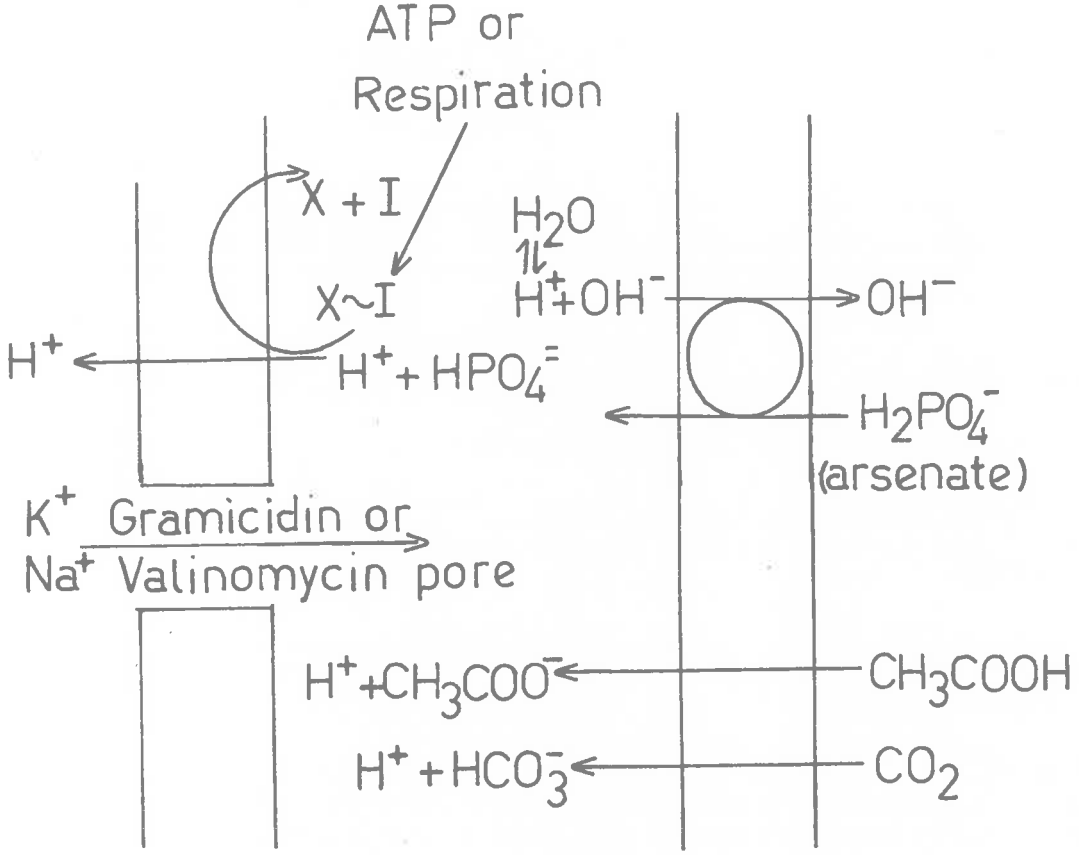
The action of gramicidin has been shown to be similar to that of valinomycin (Chappell and Crofts 1965a, Pressman 1965), although gramicidin acts with any alkali metal ion (K, Li, Cs, Rb or Na) and not specifically with K, Rb and Cs. Chappell and Crofts (1965a, 1966) have confirmed the observations that valinomycin and gramicidin, in the presence of an oxidizable substrate and an alkali metal ion, cause the appearance of hydrogen ions in the suspending medium, uptake of the alkali metal ions by the mitochondria and, when phosphate is also

present, stimulation of respiration and mitochondrial swelling. Inhibitors (cyanide and antimycin A), uncoupling agents (DNP and CCP) and anaerobiosis inhibited and reversed the pH changes, potassium changes and mitochondrial swelling. These effects are similar to those described for manganese accumulation by rat liver mitochondria in the absence of phosphate (Chappell et al 1963).

ATP can support ion accumulation induced by gramicidin and valinomycin, and ATPase activity has been shown to be stimulated in mitochondria by gramicidin (Lehninger et al 1959). ATP caused marked mitochondrial swelling associated with potassium accumulation induced by valinomycin and gramicidin in the presence of phosphate (Chappell and Crofts 1966). Oligomycin caused potassium efflux and contraction of the mitochondria. Swelling of mitochondria by valinomycin-induced potassium uptake was dependent on the presence of phosphate, arsenate, carbon dioxide (not HCO_3^-), or acetate (Chappell and Crofts 1965a, 1966). Addition of NH_4^+ to mitochondria which had undergone gramicidin-induced ATP-dependent swelling in the presence of potassium and phosphate caused a very rapid contraction of the mitochondria (Chappell and Crofts 1966), parallel to the effect of NH_4^+ on respiration-dependent swelling (Chappell and Crofts 1965a).

Chappell and Crofts (1965a, 1966) have proposed a mechanism for the action of gramicidin and valinomycin. They have suggested that normally

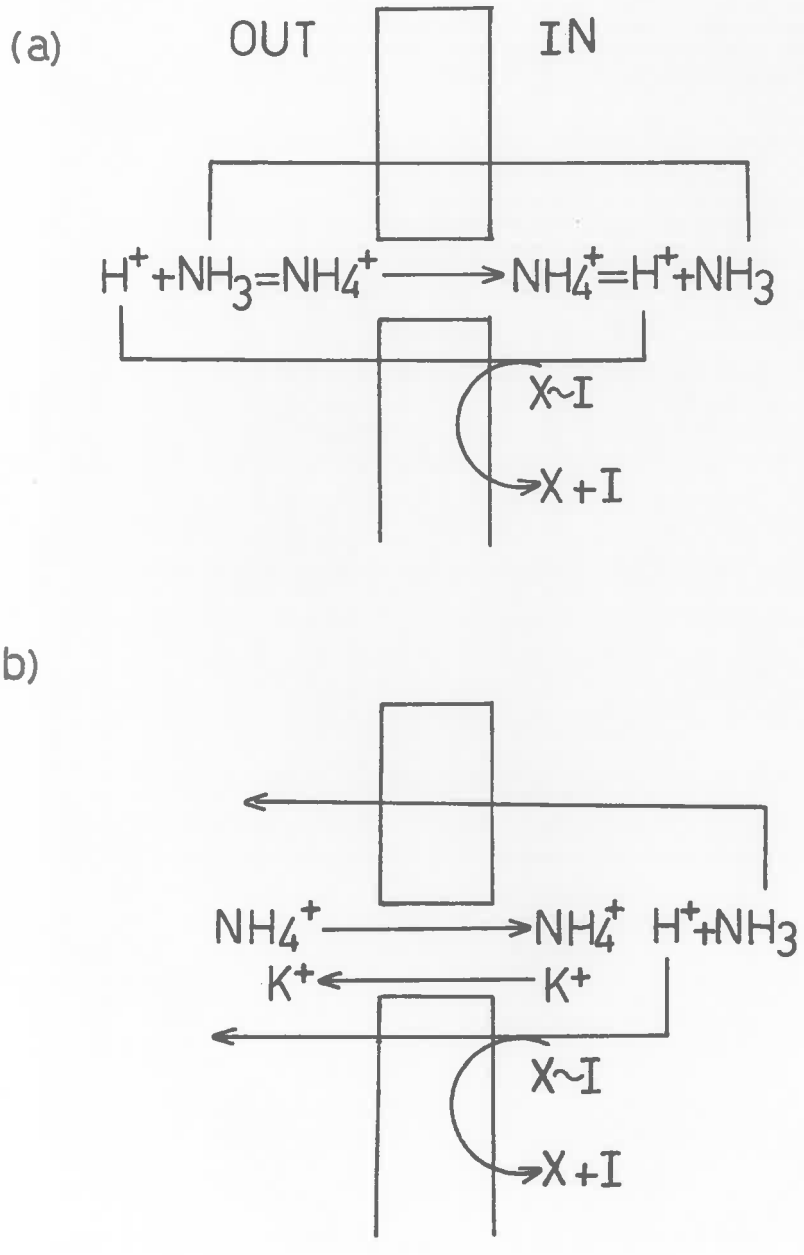
FIGURE I.11.
(Chappell & Crofts 1966)



the mitochondrial membrane is relatively impermeable to potassium and chloride ions, but permeable to NH_4^+, phosphate, acetate, arsenate and carbon dioxide, that an energy-dependent (respiration or ATP-dependent) H^+ pump ejects H^+ from the mitochondria, and that gramicidin and valinomycin increase the permeability of the mitochondrial membrane to monovalent cations. The basic mechanism is shown in Fig. I.11, and the postulated action of NH_4^+ is shown in Fig. I.12a. NH_4^+ can enter via the gramicidin pore like the other alkali metal ions and then dissociate in the mitochondria to produce NH_3 and H^+, the H^+ exchanging for more NH_4^+. Stimulation of respiration by NH_4^+ would thus be observed in the absence of arsenate or phosphate. The NH_4^+ caused contraction and potassium efflux from potassium - phosphate - gramicidin - induced swollen mitochondria and this mechanism is shown in Fig. I.12b.

Chappell and Crofts (1965a, 1966) suggest that the H^+ pump is energised by $X \sim I$ (or $C \sim D$), the H^+ being derived from inside the mitochondria (Fig. I.11). The exchange of H^+ for potassium which enters through a valinomycin or gramicidin pore requires the maintenance of H^+ production within the mitochondria for the stimulation of respiration and continued entry of potassium. Potassium entry would thus be limited by this mechanism unless water could continue to break down in the alkaline interior of the mitochondria to produce further H^+ and OH^-. Phosphate if present could, however, exchange for OH^- and stimulate respiration, and further dissociation of phosphate could occur within the mitochondria, providing more H^+ for potassium uptake. Accumulation of potassium and

FIGURE I.12.
(Chappell & Crofts 1966)



phosphate leads to increase in mitochondria osmolarity and swelling. Entry of unionized acetate and carbon dioxide could also produce ionized forms within the mitochondrion, providing H^+ for the pump and leading to potassium acetate accumulation and swelling (Chappell and Crofts 1966).

A similar mechanism based on the chemiosmotic scheme accounts for the observed effects of gramicidin and valinomycin on monovalent cation uptake. The origin of the H^+ would be the oxido-reduction pathway rather than an $\mathbb{X} \sim \mathbb{I}$-energised H^+ pump. The monovalent cation would enter the mitochondria along the electro-chemical gradient through a membrane made permeable to these cations by valinomycin or gramicidin. In the absence of a permeant anion stimulation of respiration would not be observed (although a transient effect similar to that produced by calcium in the absence of phosphate would be expected at low concentrations of potassium), and H^+ production and potassium uptake would reach a maximum due to build up of OH^- within the mitochondria. In the presence of phosphate, or other permeant anion which could exchange for OH^-, respiratory stimulation and H^+ production would continue and potassium and phosphate would accumulate. Acetate and arsenate could also exchange for OH^- as ionized forms. The production of H^+ by further ionization of phosphate and arsenate is not essential for the maintenance of H^+ production by this scheme. However, the dissociation of the unionized forms of acetate and carbon dioxide within the mitochondria would result in effective neutralization of the OH^-. The net result would be potassium and anion uptake by the mitochondria, increased respiration and swelling. Measurement of stoichiometries must consider

the rates of both influx and efflux of potassium and other ions. Valinomycin and gramicidin activity as membrane-potential collapsing reagents has already been discussed (~~Chapter 1~~ p.28.) in relation to the action of uncouplers (DNP) (Mitchell 1966).

The mechanism of NH_4^+ action by the chemiosmotic hypothesis would be similar to that proposed by Chappell and Crofts (1966) (Fig. I.12), although the origin and mechanism of H^+ extrusion is different. Thus valinomycin-induced NH_4^+ uptake could, by internal dissociation to H^+ and NH_3, completely collapse PMF and stimulate respiration in the absence of a permeant anion. NH_4^+ added to mitochondria swollen in the presence of gramicidin, potassium and phosphate would lead to exchange of NH_4^+ for K^+, extrusion of phosphate on dissociation of NH_4^+, neutralization of OH^- and shrinkage of the mitochondria. The action of ATP in producing H^+ by the membrane-located ATPase system has been discussed in relation to divalent cation uptake and the same mechanism applies to ATP-supported valinomycin-induced monovalent cation uptake. The action of uncouplers, inhibitors, anaerobiosis, oligomycin and atractyloside on substrate and ATP-supported divalent cation uptake also applies to their action on monovalent cation uptake. Inhibitors, by stopping H^+ production, and uncouplers, by allowing rapid return of H^+, would lead to removal of accumulated potassium and any associated anion, contraction, and return to the original pH value. The reversal of divalent cation uptake by DNP was observed by Chappell et al (1963) when manganese was accumulated without phosphate.

Chappell and Crofts (1966) found that gramicidin and valinomycin had similar effects on both artificial and natural lipid membranes indicating

that the specific ion-conducting property is resident in the polypeptide molecule. Ogata and Rasmussen (1966) have observed that valinomycin permits potassium to compete with calcium in rat liver mitochondria, and conclude that valinomycin acts by making the membrane permeable to potassium ions. Azzi and Azzone (1966) arrived at a similar conclusion from swelling and concentration studies of mitochondria. The mechanism by which valinomycin and gramicidin increase the permeability of membranes to alkali metal ions (Pressman 1965), or effectively produce monovalent cation "pores" (Chappell 1966), is not known.

G. MITOCHONDRIAL STRUCTURE

Mitochondria have been shown to consist of an inner almost structureless matrix bordered by two membranes, the inner of which is periodically invaginated to form infoldings or cristae (Palade 1953, Sjostrand 1953). The space enclosed by these infoldings (intracristal space) is often seen to communicate with the spaces within the inner and outer membranes where these membranes run parallel, and these connected spaces constitute the external compartment (Whittaker 1966). The structure of mitochondria and its relation to function has been reviewed by Green (1958, 1959b), Green and Crane (1958), Crane (1961), and Lehninger (1964). The two membranes of the mitochondrion have been considered to be "unit" membranes (Robertson et al 1963) where the membranes consist of a lipid bilayer with outwardly directed polar groups to which stabilizing layers of aproteins are fixed. Thus the external compartment is banded by hydrophilic surfaces and is unlikely to be filled with lipid. The infolding of the inner membrane presumably provides

access to the respiratory enzymes and could also provide additional membrane surface to accommodate these. The matrix of mitochondria from cells with high oxygen uptake such as those of the flight muscles of insects is almost entirely occupied by tightly-packed highly-ordered cristae, and the cristae also have invaginations (Smith 1963). Mitochondria of the almost anaerobic liver fluke have very few cristae (Bjorkman and Thorsell 1962). Electron microscopy indicates that only one organelle exists within the matrix:- the dark granules discussed by Peachey (1964) which may be sites for precipitation of divalent metal salts (Greenawalt and Carafoli 1966).

Investigations into the structure of mitochondrial membranes have revealed the presence of spherical knobs protruding from membranous material released from the mitochondria by hypotonic treatment, sonication, or exposure of the mitochondria to negative stain without prior fixation (Fernandez-Moran 1962, Whittaker and Home 1962, Whittaker 1963, Stoekenius 1963, Greville et al 1965, Chance and Parsons 1963). These spherical knobs (90A°) are attached by stalks (40-50A°) and are directed outwards on ribbons, parallel membranes or vesicles, and are thus considered as inner membrane sub-units. Whittaker (1966) considers that this material could be derived from the pleating in of the cristal membranes. These particles have been considered to be aggregates of respiratory enzymes (Fernandez-Moran et al 1964), while Lehninger (1964) has suggested that the respiratory assemblies might be arranged in hexagonal arrays on the main mitochondrial surface to account for the required particle weight of a complete respiratory assembly. Chance et al (1964) and Stasny and Crane (1964), using sonicated mitochondria,

have stripped the particles from the smooth membranes and have found that the latter still contains cytochromes a, b, c_1 and c. Particle-studded membranes have been isolated which lack cytochromes a and c_1, or are low in cytochrome c (Chance and Parsons 1963). It appears then that the particles cannot be respiratory assemblies. The particles have been considered as "F_1 coupling factor (cold-labile ATPase)" by Racker et al (1964) based on the morphology of preparations of cold-labile ATPase. They have also been considered as pre-existing structural components without cytochrome or ATPase activity (Williams and Parsons 1964), or as structures produced as a result of disruption (Bangham and Horne 1964).

The addition of lysolecithin to lecithin caused the laminated structure to break up into particles of 70-80A° in diameter, closely resembling the mitochondrial particles (Bangham and Horne 1964). Sonication or disruption of mitochondria in hypotonic media could be accompanied by formation of lysolecithin from lecithin, producing micellization of part of the inner mitochondrial membrane. There is, however, little evidence that the stalked particles exist as such in intact mitochondria although granularity of inner mitochondrial membranes has been demonstrated by freeze etching in fractured, unfixed mitochondria within frozen cells (Moor et al 1964), and in other membranes (Horne and Whittaker 1962).

H. SUB-MITOCHONDRIAL PARTICLES

Sub-mitochondrial particles of mitochondria have recently been isolated by various treatments and their reactions studied. Sonically disrupted mitochondria catalyse oxidative phosphorylation (Racker 1961),

energy-linked transhydrogenation (Lee and Ernster 1966), and other reversed-electron transfer reactions (Low et al 1963). Electron microscopy has shown that sonic particles are vesicular and the stalked spherical bodies seen on the inside of the cristae membranes of negatively stained mitochondria (Fernandez-Moran et al 1964) appear to be present on the outside of the vesicular sonic particles (Stanby and Crane 1964, Greville et al 1965). It could thus be considered that the membranes of sonic particles are the osmotically-functional coupling membranes of mitochondria and that these membranes are inside out (Mitchell 1966). Mitchell and Moyle (1965b) have shown that sonic particles from beef heart mitochondria do possess an osmotically functional membrane, and they ~~have~~ considered that the membrane was inside out from the direction of proton translocation during oxidation of succinate or NADH and during hydrolysis of ATP.

Similar measurements from particles isolated by digitonin treatment ~~treatment~~ (Wadkins and Lehninger 1963) have indicated that the polarity is the same as that of intact mitochondria. Chance and Fugmann (1961) and Lee (1963), during studies on ATP-driven reversed electron and hydrogen transfer in particles from pigeon heart mitochondria found that digitonin particles oxidised exoganous cytochome c but reduced only endogenous NAD, whereas sonic particles reduced exogenous NAD but oxidised only endogenous cytochrome c. The atractyloside-sensitive ATP and ADP transfer reaction is absent in sonic particles (Low et al 1963) but is partially intact in digitonin particles (Vignais et al 1962). Furthermore, Vasington (1963) has shown that digitonin particles take up calcium by an energy-linked

process as do intact mitochondria, whereas sonic particles do not take up calcium. The inhibitory effects of hexyl guanidine on respiration in intact mitochondria do not occur in sonic motochondrial particles (Guillory and Slater 1965).

Electron micrographs show that the stalked particles similar in appearance to those on inner mitochondrial membranes exist on the external surface of the chloroplast grana (Parsons et al 1965). These results could indicate the existence of "sides" in the mitochondrial inner membrane and chloroplast grana membrane.

I. THE PRESENT INVESTIGATION

Plant mitochondria isolated from many tissues have been shown to esterify inorganic phosphate with high efficiency during substrate oxidation (Hackett 1959). The ADP or P_i control over mitochondrial respiration is, however, considered to be a more important criterion of mitochondrial intactness (Chance and Williams 1956). Reports of plant mitochondria showing respiratory control have only recently been submitted (Wiskich and Bonner 1963, Wiskich et al 1964, Jones et al 1964). Wiskich et al (1960) described the isolation of beetroot mitochondria and investigated their oxidative and phosphorylative capacity. An investigation was made into the degree of ADP control over substrate oxidation in beetroot mitochondria using the recently developed oxygen electrode (Chapter 3).

Isolated plant mitochondria have been shown to accumulate ions against a concentration gradient (Robertson et al 1955), and Robertson (1951) suggested that mitochondria could act as temporary sites of ion accumulation in cells and serve as carriers of ions from the external medium to the

vacuole. Recent studies have indicated that isolated mitochondria can accumulate high concentrations of divalent cations and phosphate by an energy-dependent process (Vasington and Murphy 1961), (Brierley et al 1962, Chappell et al 1963, Hanson et al 1965). Monovalent cations and other anions can also be taken up, and changes in H^+ concentration, oxidation rate and volume are associated with ion movements into and out of mitochondria (Pressman 1963, Chappell et al 1963, 1966, Chance 1965).

Following a preliminary study of substrate-dependent phosphate uptake by beetroot mitochondria (Millard 1963), a detailed investigation was made into the reactions of divalent and monovalent cations and phosphate with these mitochondria (Chapters 4 - 6). Competition between ions was recorded and has been related to a general substrate-dependent cation uptake mechanism (Chapter 7). The efficiencies of various substrates in supporting magnesium and phosphate uptake by beetroot mitochondria differed from those reported for animal mitochondria and this aspect was studied in detail (Chapter 8).

Current hypotheses of oxidative phosphorylation and related mechanisms for ion uptake have been discussed in Chapter 1. The effects of inhibitors and uncouplers of oxidative phosphorylation on substrate-dependent magnesium and phosphate uptake by beetroot mitochondria will be compared with results of similar investigations with animal mitochondria (Brierley et al 1963, Chance 1965) and correlated with ion uptake hypotheses (Chapters 4 - 6). ATP formation and ion uptake in beetroot mitochondria supported by acid-base titrations (Reid et al 1966) are reported (Chapter 9).

Thus the work to be described consists of an investigation into the control of oxidation by phosphorylation in beetroot mitochondria (Chapter 3) and a detailed study of ion uptake by these mitochondria (Chapter 4 - 9).

CHAPTER TWO

MATERIALS AND METHODS

A. PREPARATION OF BEETROOT MITOCHONDRIA.

The isolation of active mitochondria from red beetroot (Beta vulgaris L.) tissue has been described by Wiskich et al (1960). This tissue yields active mitochondria relatively easily in comparison with other plant tissues (Hackett 1959) in which starch, fats, tannins and chlorophylls often interfere with the isolation of biochemically active mitochondria (Chapter 1). Wiskich et al (1960) added Tris to neutralize the acid contents of the vacuole released during blending in 200 ml of 0.4M sucrose, and EDTA (5mM) was also included during blending to remove Ca^{++} and other interfering cations by chelation (Honda and Robertson 1956). The water-soluble red pigment was found to be easily removed by washing the mitochondria at least once by re-suspension in 0.4M sucrose.

The initial preparations of beetroot mitochondria used in experiments described in this thesis were isolated by the method of Wiskich et al (1960). Sufficient 1M Tris was added to the blending medium initially to maintain an alkaline brei of pH 7.2 or more during disruption of the tissue. Acid conditions (pH 6.8) resulted in lower oxidation rates and more important, in the abolition of respiratory control. Similar deleterious effects of acidity have been reported by Lieberman (1960) and Wiskich and Bonner (1963). Lieberman (1960) has

suggested that a high pH prevents adsorption of extraneous protein which inactivates the mitochondria. This may be associated with tannin formation (Hulme and Jones 1963). Increasing the pH of the brei above 7.8 during the isolation of beetroot mitochondria also reduced the oxidation rates. The most active beetroot mitochondria with regard to oxidation rates, respiratory control and ion accumulation were obtained when the pH of the brei was between 7.2 and 7.6. Oxaloacetate is more stable at a higher pH and may be associated with the lower succinate and malate oxidation rates obtained when the pH was above 7.8.

The first centrifuging was at 2,000 x g for 10 minutes and the second and subsequent centrifugings were at 10,000 x g for 15 minutes. All centrifuging was carried out at $0^{o}C$ in a refrigerated centrifuge. The mitochondria, after precipitation by high speed centrifuging, were washed twice by resuspending in 0.4M sucrose with a Teflon-based Potter-Elvehjem hand homogenizer and recentrifuging. The final mitochondrial precipitate was resuspended with the aid of a hand homogenizer in 7-14ml 0.4M sucrose and kept at $0^{o}C$. When a high speed centrifuge (Sorvall RC-2) became available it was possible to layer the mitochondria (suspended in 0.4M sucrose) over 1M sucrose during a final centrifuging at 18,000 x g for 20 minutes. This proved beneficial for ion uptake studies but not necessarily for succinate oxidation, ADP/O ratios or respiratory control ratios. Fig. II3 shows that the layering technique produced an increase in Mg^{++} and P_i accumulated /mg mitochondrial nitrogen(N). A purer preparation of intact mitochondria was obtained by layering as some

Table II. 1. Activity of Mitochondria Isolated with a Waring Blendor or a Braun Juice Extractor.

Beetroot mitochondria were isolated using a Waring Blendor in Expt. 1, and a Braun juice extractor in Expt. 2 as described in the text. Mitochondria were incubated at 25°C in the standard oxygen electrode cell medium (9 mM potassium phosphate) with 4 uM cytochrome c, 8 mM Tris succinate and 116 ug mitochondrial N/ml (Expt. 1) or 106 ug mitochondrial N/ml (Expt. 2). The state 3 oxidation rates were produced by addition of 0.15 mM ADP and the state 4 rates were measured after phosphorylation of this ADP. Respiratory control ratios and ADP/O ratios were calculated by the procedure of Chance and Williams (1955).

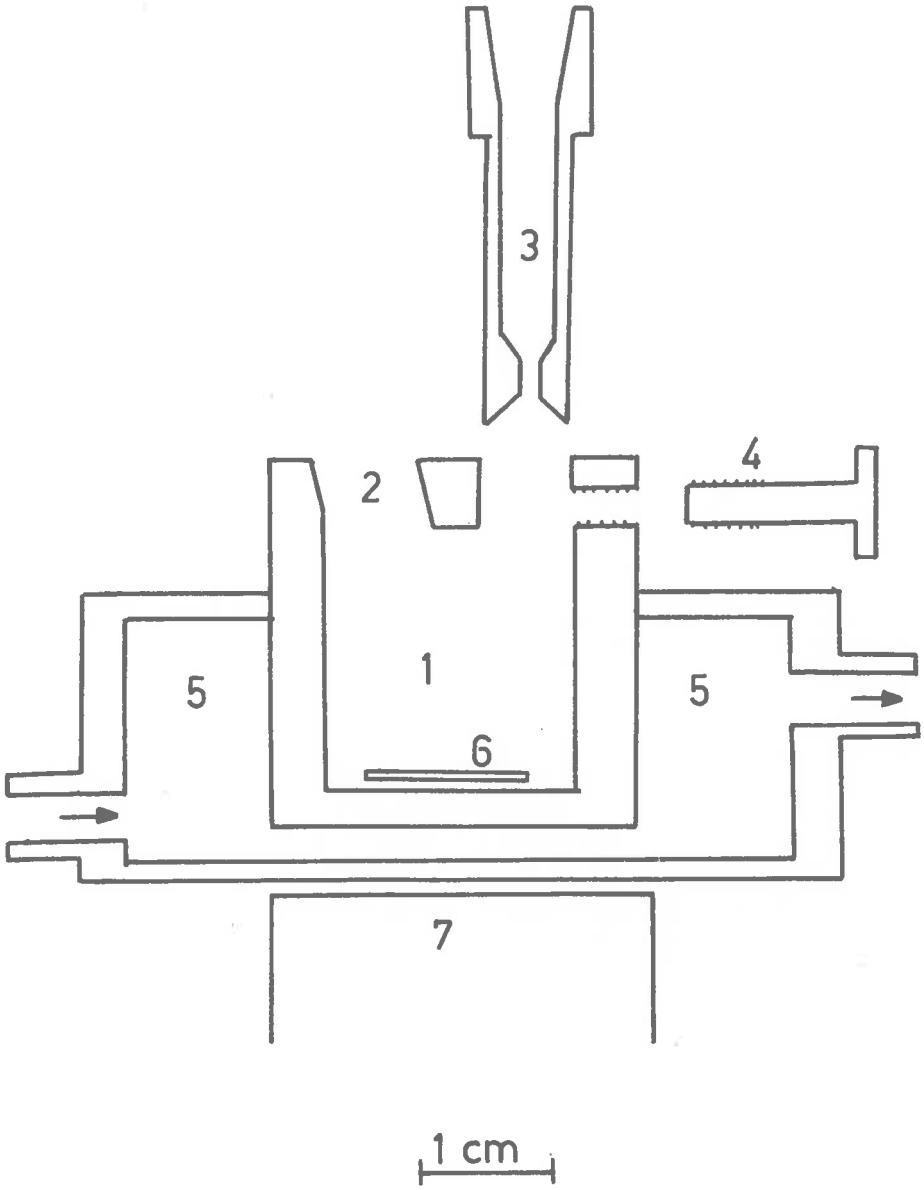
	Expt. 1			Expt. 2		
Oxidation rates (mumoles O_2/min)	1	2	3	1	2	3
State 3	174	197	189	195	365	380
State 4	113	119		129	140	
Respiratory control ratio	1.5	1.7		1.5	2.6	
ADP/O ratio	1.3	1.3		1.5	1.5	

broken mitochondria and non-mitochondrial material did not precipitate through the 1M sucrose and did not contribute to the total nitrogen analysis. Better removal of the red pigment was also obtained.

The use of a "Braun blendor-mix" juice extractor instead of a Waring blendor made it possible to extract ~~the~~ mitochondria from more fresh beetroot tissue in a smaller volume of isolation medium, and allowed the rapid separation of beetroot juice from the cell debris. Although a larger yield of mitochondria was obtained per isolation procedure the mg mitochondrial $\frac{N}{\text{extracted}}$ $\frac{\text{per g}}{\text{fresh weight}}$ of beetroot tissue did not change. The mitochondria were, however, more "active" in oxidizing succinate and malate in the presence of ADP and phosphate (Table II 1). The second and third state 3 succinate oxidation rates increased by approximately 50% and the ADP/O ratios also increased when the mitochondria were isolated using the Braun juice extractor. Ion uptake/mg N was also increased. This general increase in activity was probably due to the rapid separation of the beetroot juice containing the mitochondria from the cell debris and unbroken beet tissue. No preliminary separation of juice from the debris by filtering through muslin or calico was required with this juice extractor. Very little debris in the form of cell walls or nuclei was precipitated during the low-speed centrifuging in contrast to the large precipitate formed when the Waring blendor was used. The supernatant from this first centrifuging was, however, passed through washed unbleached calico to separate the thin layer of froth at the surface of the supernatant. Enough 1M Tris to

Fig. II. 1. Cross section of the apparatus used for polarographic measurements of mitochondrial reactions.

1. Oxygen electrode cell of 3.0 - 3.5 ml volume.
2. Oxygen electrode inlet
3. Plunger
4. Screw holding plunger
5. Water bath
6. Glass or teflon covered iron rod
7. Magnetic stirrer


3
2
4
1
5
5
6
7
1 cm

produce a final juice pH of approximately 7.4 was added (with EDTA) to the empty juice receptacle. The initial high pH when the juice volume was low did not appear to harm the mitochondria if the final pH, reached within 2-3 minutes, was below 7.8. If Tris was added after juice extraction, the short exposure (2-3 minutes) of the mitochondria to the cell contents at pH 5-6 completely inactivated the isolated mitochondria.

The mitochondrial suspension was kept in an ice bath during the course of the experiments and the experiment was completed within two hours of isolation unless otherwise specified.

B. MEASUREMENT OF MITOCHONDRIAL ACTIVITIES:

1. Oxidative Phosphorylation.

Oxidation of various Krebs cycle intermediates by isolated beetroot mitochondria was studied. Oxygen uptake was measured polarographically in a sealed perspex vessel (with a circulating water bath at 25°C) of 3.3 ml volume enclosing a Clark oxygen electrode (Yellow Springs Instrument Company, Cleveland, Ohio) connected to a 1mv. recorder (either Varian G14, Varian Associates, Palo Alto, California, or Honeywell-Brown, Middlesex, England). Fig. II.1 shows the cross-section of the oxygen electrode cell. The oxygen electrode tip was sealed with a polyethylene membrane. Additions were made by micropipettes through a small pore in a perspex plunger at the top of the cell and mixing was achieved by moving the plunger vertically. A virtual seal from atmospheric oxygen was obtained by placing the small pore (1.5 mm diam. x 1 mm in length) of the plunger below the surface of the cell contents.

Diffusion of oxygen through the pore into the cell liquid was negligible during the usual time period of an experiment (2-5 minutes). The cell contents were stirred by a glass- or Teflon-covered iron flea, rotated by a magnetic stirrer below the cell.

When oxygen uptake was measured with this apparatus 2.8 ml of medium, prewarmed to 25°C, containing 250 mM sucrose, 6 mM $MgCl_2$, 4 mM potassium or Tris-phosphate buffer (pH 7.2) and 10 mM Tris-HCl buffer (pH 7.2) was added and the recorder adjusted to 100%. The concentration of oxygen in this air-saturated medium was 240 uM at 25°C calculated by the procedure of Chappell (1964). Generally 0.5ml mitochondrial suspension was added, or a volume to provide between 200 - 500 ug mitochondrial N. When the mitochondrial suspension at 0°C was added to the reaction medium at 25°C, there was an abrupt deflection of the tracing (Fig. III 1). This was caused by dilution of the oxygen in the reaction medium by the anaerobic cold mitochondria. Further small additions (15 ul - 50 ul) were made through the pore in the plunger and the rate of oxygen uptake was calculated from the slope of the trace. The recorder chart speed was 1 inch/minute. The linearity of the electrode response over the range of oxygen concentrations from 250 uM to 0 uM was tested by measuring the constant respiration rate of yeast. Less than 10% variation from linearity was found over this range of oxygen concentration (Chance and Williams 1955).

Substrate oxidation and coupled phosphorylation by isolated beetroot mitochondria were also determined by chemical and manometric

techniques (Hunter 1955, Wiskich et al 1960), with air at the gas phase. The manometer vessel contained 0.25M sucrose, 10 mM Tris-HCl buffer (pH 7.2), 10 mM potassium or Tris-phosphate buffer (pH 7.2), 6 mM $MgCl_2$, 20 mM glucose, excess hexokinase, 0.5 mM ADP, 8 mM Tris succinate, 3uM cytochrome c and between 150 and 500 ug mitochondrial N in a total volume of 3.5 ml. The centre well contained 0.2 ml of 20% KOH and oxygen uptake was measured at 25°C for 40-50 minutes. The reaction was stopped with 3% $HClO_4$ (final concentration) and the glucose-6-PO_4 formed enzymically was estimated spectrophotometrically after neutralization of the perchloric acid with KOH, and removal of precipitated potassium perchlorate and mitochondria by centrifuging (Kornberg 1950). The reaction was started by glucose-6-PO_4 dehydrogenase addition, and buffered with 0.04M glycyl-glycine buffer (pH 7.5). Reduction of NADP was measured at 340 mu using a Shimadzu spectrophotometer or a Beckman DB spectrophotometer connected to a Beckman recorder.

2. Ion Uptake.

Reaction mixtures for ion uptake studies were placed in 25 ml conical flasks in a standard Warburg bath and shaken at 25°C during the incubation period. The standard reaction medium contained 0.25M sucrose, 10 mM Tris-HCl buffer (pH 7.2), 3 mM potassium or Tris-phosphate buffer (pH 7.2), 4 uM cytochrome c, 15 mM $MgCl_2$ or 2 mM $CaCl_2$, 7 mM Tris succinate or other substrate and between 100 ug and 200 ug mitochondrial N/ml. The total volume varied between 3.5 and 6.5 ml. When $CaCl_2$ was present 2 mM $MgCl_2$ was also included.

Separation of the mitochondria from the incubation medium was achieved by taking 1.0 ml samples and either (1) layering over 3 ml of

Table II. 2. Effect of centrifuging and filtering on Ion Uptake by Mitochondria.

Beetroot mitochondria were incubated at 25°C in the standard ion uptake medium (potassium phosphate) with 5 mM Tris succinate and 137 ug mitochondrial N/ml. In Expt. 2, 1 ug/ml oligomycin was also present. Mitochondria were separated from the medium by sucking onto 1.2 u Millipore filters, or by centrifuging at 0°C through 3 ml of 1 M sucrose for 6 minutes at 25,000 x g. The results have been corrected for the uptake of ions in the absence of substrate.

	Mg^{++} umoles/mg N		Pi umoles/mg N	
Minutes	Filtered	Centrifuged	Filtered	Centrifuged
Expt. 1				
3	0.8	0.2	0.5	0.3
14	2.3	1.5	1.3	0.7
Expt. 2				
3	2.3	0.8	1.1	0.9
14	3.1	1.9	1.9	1.3

1M sucrose and centrifuging at 25,000 x g for 5-7 minutes at 0°C in a refrigerated International Centrifuge, or (2) pipetting onto Millipore filters (1.2u mean pore size, Millipore Filter Corporation, Bedford, Massachusetts) and separating the mitochondria by filtration under suction for 15 seconds. The supernatant from (1) was removed by suction and the centrifuge tube carefully rinsed with 3 ml of cold 1M sucrose. The mitochondrial pellet in (1) and the Millipore filters from (2) were suspended in 2-3 ml of 5.5% $HClO_4$ and the extract was analyzed.

Two conditions are essential for studying ion uptake by isolated mitochondria. Firstly the mitochondria must be collected rapidly without loss of internal contents and accumulated ions. This could occur during centrifuging through a sucrose layer and then washing the pellet with 1M sucrose as described in method (1). Secondly the collected mitochondria must be free from the reaction medium to avoid large correction factors for the extra-mitochondrial ions. Table II2 compares the earlier technique of centrifuging through 1M sucrose with the later filtration method. Substantial loss of accumulated ions occurred during centrifuging through 1M sucrose at 0°C for 5-7 mins. This was also demonstrated by Brierley et al (1963b). Ion uptake in Table II2 was in each case corrected for the uptake of ions in the absence of added substrate, thus correcting for any error caused by the small retention of magnesium and phosphate within the Millipore filters (see Chapter 4).

Initially, high blanks were recorded with the filtration technique due to the retention of medium in the filter, particularly for

Fig. II. 2. Mg^{++} and Pi uptake by isolated beetroot mitochondria incubated in a medium containing 250 mM sucrose, 10mM Tris-HCl buffer pH 7.2, 3.3mM Tris-phosphate buffer pH 7.2, 5 uM cytochrome c, 13mM $MgCl_2$, 1 ug/ml oligomycin and 78 ug mitochondrial N/ml.

Mitochondria separated by filtration through 1.2 u Millipore filters:-

● -- ●, with 8mM Tris succinate as substrate.

o -- o, without substrate.

Mitochondria separated by filtration with 2 ml cold 1 M sucrose through 1.2 u Millipore filters:-

■ – ■, with 8 mM Tris succinate as substrate.

□ – □, without substrate.

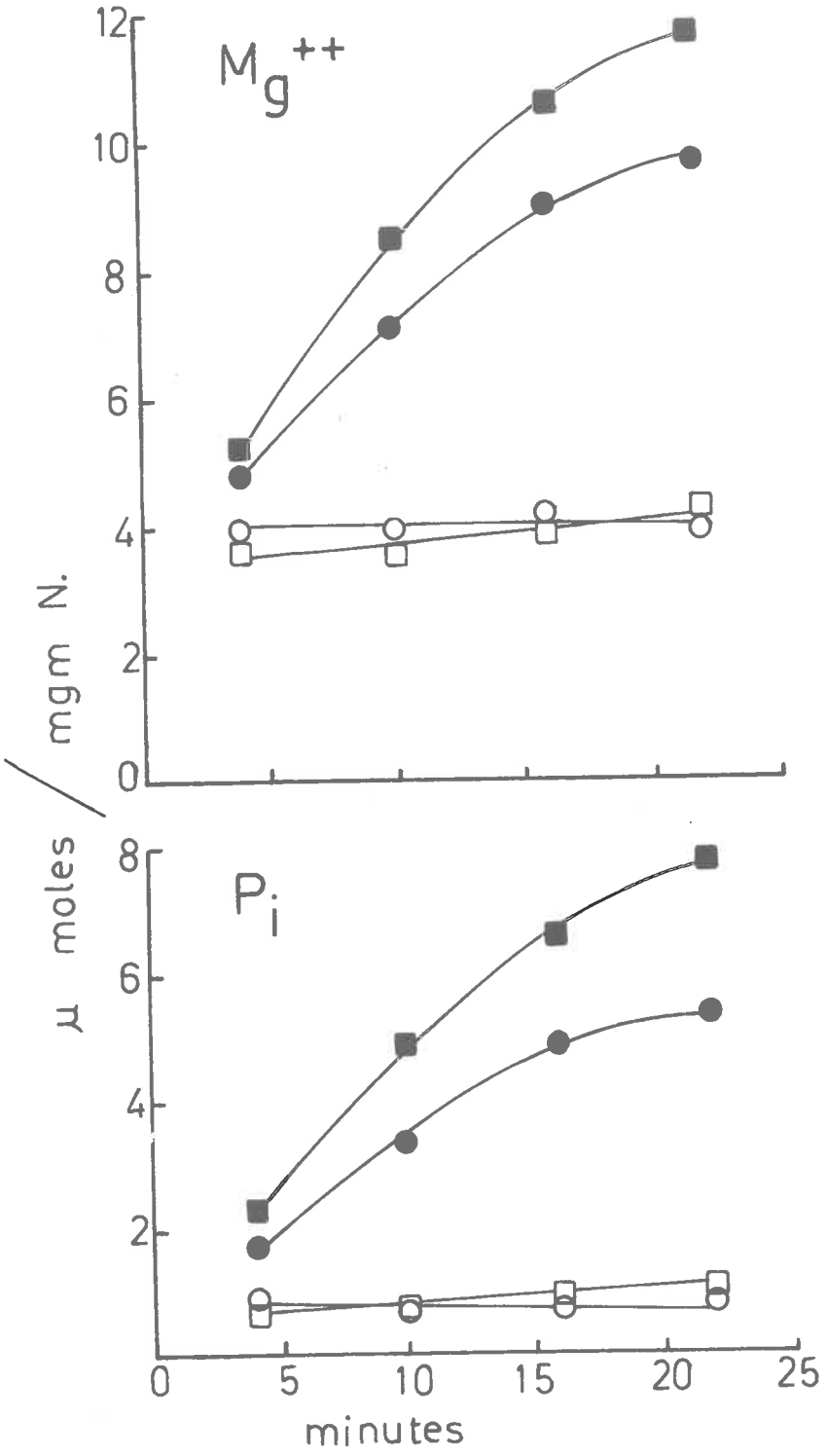

Mg^{++}
mgm N.
P_i
μ moles
minutes
0
2
4
6
8
10
12
5
15
20
25

Fig. II. 3. Mg^{++} and Pi uptake by isolated beetroot mitochondria incubated in a medium containing 250 mM sucrose, 10 mM Tris-HCl buffer pH 7.2, 5 uM cytochrome c, 3.2 mM Tris-phosphate buffer pH 7.2, 13 mM $MgCl_2$, 1 ug oligomycin/ml and 153 ug mitochondria N/ml.

Mitochondria prepared by standard method described in text:-

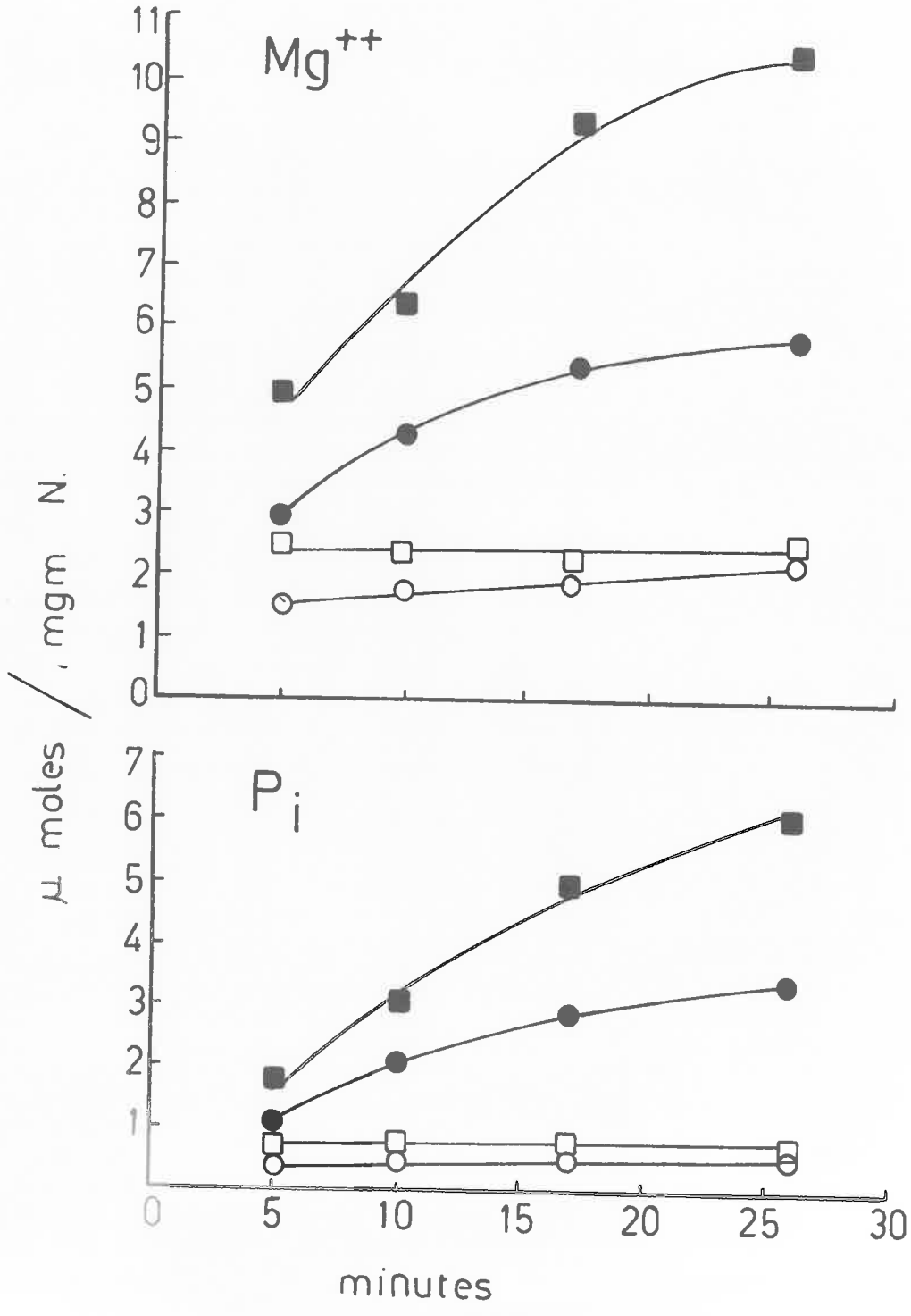
● — ●, with 10 mM Tris succinate as substrate.

o -- o, without substrate,

Mitochondria prepared by layering over 1M sucrose for the last centrifuging step:-

■ - ■, with 10 mM Tris succinate as substrate.

□ - □, without substrate.


Mg++
Pi
μ moles / , mgm N.
minutes
0
1
2
3
4
5
6
7
8
9
10
11
5
10
15
20
25
30

magnesium which was present at a high concentration (15 mM) in the reaction medium. This correction factor was large in proportion to the actual substrate-dependent magnesium uptake and reduced the accuracy of measuring substrate-dependent ion uptake. To overcome this problem 2 ml of cold 1M sucrose were placed on each filter and the 1 ml samples were pipetted onto the sucrose and suction applied immediately. This proved beneficial in two ways. Firstly the larger liquid volume allowed a more even deposition of mitochondria on the filter resulting in more rapid separation (2-3 seconds compared with 5 seconds). Secondly, lower and more consistent correction factors were required and, unexpectedly, higher rates of uptake were recorded (Fig. II2). The cold 1M sucrose would itself be beneficial in maintaining the internal contents of the mitochondria while the faster separation would prevent some loss of accumulated ions. In all experiments reported where the mitochondria were separated from the medium by Millipore filtration, 2 ml of cold 1M sucrose were present on the filters unless otherwise indicated.

Both intact and disrupted mitochondria could contribute to oxidative studies but presumably only intact beetroot mitochondria can maintain accumulated ions. In the last step of the mitochondrial preparation centrifuging through 1M sucrose was introduced in an effort to eliminate mitochondrial fragments from the final suspension (Section II A). The effectiveness of this procedure is demonstrated in Fig. II.3, where uptake of magnesium and phosphate is shown on a total nitrogen basis. Increase in ion uptake was due mainly to removal of non-

contributing nitrogenous material but greater removal of soluble extraneous material (e.g. the red pigment) may have aided in preserving the mitochondrial structure and contents during the experiment.

The mitochondrial pellet or the Millipore filter was treated with 5.5% $HClO_4$. Three hours were sufficient to extract accumulated magnesium and phosphate but the extraction was usually extended for 12 hours before final analysis. Mitochondria extracted in H_2O for 12 hours did not release all accumulated magnesium and phosphate although all monovalent ions were released during this H_2O extraction.

Inorganic phosphate was determined from these $HClO_4$ samples spectrophotometrically by the method of Marsh (1959).

Magnesium was estimated at first by a spectrophotometric method (Vogel 1961a) and subsequently by atomic absorption spectrophotometry when an Atomic Absorption Spectrophotometer (Techtron AA3, Melbourne) became available. The latter method increased sensitivity of magnesium determinations by approximately 50% over the range 0.02 u moles to 0.3 u moles Mg. The standard Mg solution was $MgCl_2$, standardised against EDTA (Vogel 1961b). Phosphate did not interfere with Mg determination by the atomic absorption technique (David 1958).

Calcium was determined by atomic absorption spectrophotometry (David 1959), and interference by phosphate was masked by addition of 25 mM $SrCl_2$ (Willis 1960). The standard Ca solution was $CaCl_2$, which was standardised against EDTA (Vogel 1961b). $CaH_4(PO_4)_2.H_2O$ and $CaH.PO_4$ were also tested as suitable standards and in the presence of 25 mM $SrCl_2$ gave the same standard curves as $CaCl_2$ plus 25 mM $SrCl_2$. Increasing the $SrCl_2$

concentration to 50 mM $SrCl_2$ reduced sensitivity by 10 - 20%.

Monovalent ion uptake was measured from the standard ion uptake reaction medium containing 20 mM NaCl or KCl, and 1 mM $MgCl_2$ instead of 15 mM $MgCl_2$. H_2O extraction for 12 hours after separation of mitochondria by Millipore filtration removed all accumulated monovalent ions from the mitochondria, although 1% $HClO_4$ was generally used for extraction. Sodium and potassium were estimated with an EEL flame photometer (Evans Electroselenium Ltd. Essex, England). In some early experiments when chloride movement was measured in association with magnesium and phosphate uptake into isolated mitochondria, chloride was estimated spectrophotometrically (Vogel 1961c). In later experiments when chloride movement was measured in association with sodium or potassium uptake into mitochondria, chloride was analysed by the method of Furman and Low (1935).

3. ATPase Activity.

ATPase activity of isolated beetroot mitochondria was estimated by measuring the release of inorganic phosphate from added ATP by the method of Marsh (1959). The reaction medium contained 0.25M sucrose, 10 mM Tris-HCl buffer (pH 7.2), 3 uM cytochrome c, 3 mM Na_2ATP, 6 mM $MgCl_2$ and 300-400 ug mitochondrial N in a total volume of 3.5 ml. These reaction mixtures were shaken at 25°C in conical flasks in a conventional Warburg apparatus and 1 ml samples were taken at intervals and pipetted into 3% $HClO_4$ at 0°C in an ice bath. Water and butanol were added and the P_i released from ATP was measured immediately.

C. ESTIMATION OF NITROGEN:

The total nitrogen content of the mitochondrial suspension was determined by the method of McKenzie and Wallace (1954), by steam distillation and titration after digestion in sulphuric acid using mercury as catalyst.

D. MATERIALS AND CHEMICALS:

1. Beetroot Tissue.

Beetroot tissue was obtained at weekly intervals from one market garden and freshly pulled beetroot was transferred to vermiculite in a glasshouse and kept moist by regular watering. Seasonal variation in the biochemical activity of isolated mitochondria and in the pH of the cell contents was observed. Mitochondria isolated from beetroot tissue obtained in January and February were relatively inactive, probably from a combination of the very hot weather and insufficient watering at the market garden for maintenance of biochemical activity. This phenomenon was not studied in detail. Seasonal variations in mitochondria from beetroot tissue was observed by Wiskich et al (1960) while similar seasonal variations in mitochondria from silver beet have been reported (Martin and Morton 1956a).

2. Chemicals.

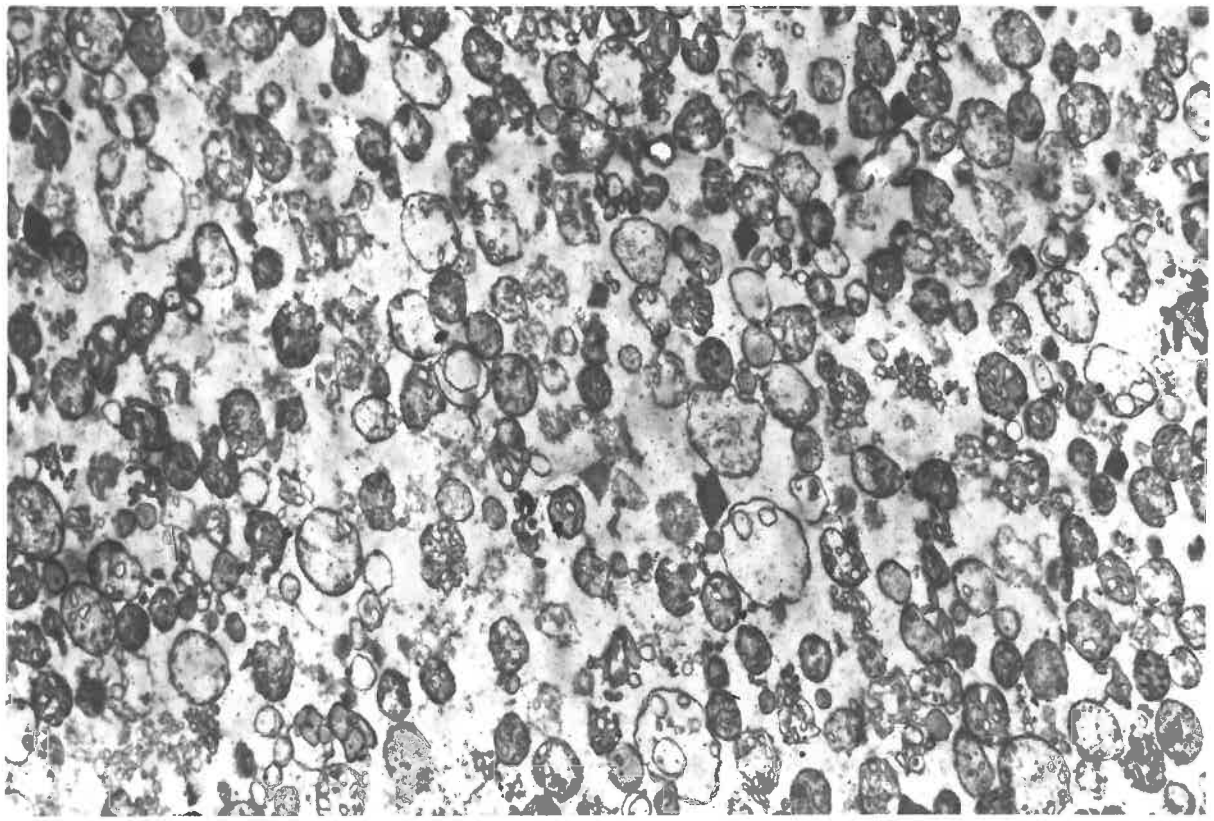
Cytochrome c, HOQNO, hexokinase (type IV), glucose-6-phosphate dehydrogenase, NAD, NADH and NADP were obtained from Sigma Chemical Company, St. Louis. Antimycin A was obtained from Kyowa Hakko Kogyo Company Ltd. Tokyo, Japan. ADP, ATP and AMP were obtained from P.L.

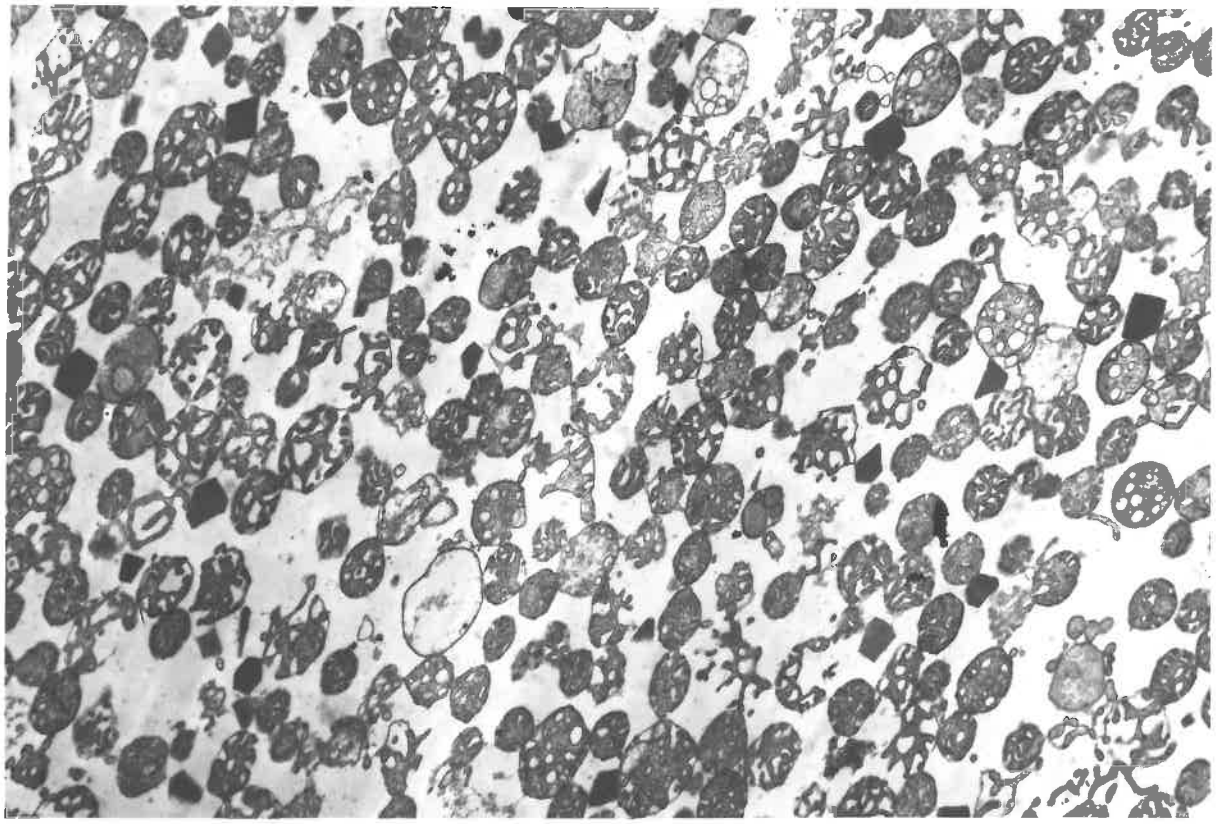
Fig. II 4. Electron micrographs of beetroot mitochondria

Magnification x 12,100.

Top, from the upper part of a freshly prepared pellet.

Bottom, from the bottom of the pellet.





Biochemicals, Wisconsin. Gifts of oligomycin (Professor Elizabeth McCoy, University of Wisconsin, U.S.A.) and CCP (Dr. Heytler, E.I. Du Pont de Nemours and Co. (Inc.) Delaware, U.S.A.) are gratefully acknowledged. Analytical-grade reagents were used wherever possible. Sodium pyruvate was prepared from commercial pyruvic acid (Lardy (1959) and re-crystallized from 80% ethyl alcohol.

ADP concentrations were determined after phosphorylation with phospho-enol pyruvate and pyruvate kinase, by measuring the oxidation of NADH on reduction of the pyruvate formed with lactic dehydrogenase. Reagents for the assay of ADP were obtained from C.F. Boehringer and Soehne, Mannheim, Germany, or the enzymes were purchased individually from Sigma Chemical Company, St. Louis.

E. STRUCTURE OF BEETROOT MITOCHONDRIA:

The structure of isolated beetroot mitochondria was investigated by electron microscopy. Freshly prepared mitochondrial pellet was fixed for 45 minutes at $0^{\circ}C$ in sodium veronal-sodium acetate buffer (pH 7.4) containing 2% $KMnO_4$. The fixed pellet was washed, dehydrated by passage through an ethanol series and embedded in araldite. The araldite was polymerised at $60^{\circ}C$ for two days. Sections were cut with a "Si-ro-flex" Ultramicrotome using glass knives and examined and photographed with a Siemens, Elmiskop 1, electron microscope. Fig. II 4 shows that the beetroot mitochondria prepared by the isolation technique described were in most cases intact with a dense matrix and well defined cristae. There was little contamination by non-mitochondrial matter. Some swollen mitochondria and broken mitochondria were present (mainly at the top of the

pellet) but their proportion was small in comparison with the intact mitochondria of general size 1u x 0.5u to 2u x 1u.

Samples of mitochondria were also observed by electron microscopy after incubation in a standard ion uptake reaction medium containing 15mM $MgCl_2$ or 2 mM $CaCl_2$, 3 mM P_i and succinate at 25°C for 15-20 minutes. These mitochondria were compared by electron microscopy with mitochondria incubated in the same medium but in the absence of substrate. No deposits of electron dense material were observed in mitochondria incubated with Mg and P_i or Ca and P_i in the presence of substrate, although duplicate samples taken after the same incubation period indicated that these mitochondria had taken up about 8 u moles Ca or Mg /mg N. Beef heart mitochondria accumulated massive amounts of Mg and P_i but electron dense deposits were not observed in these mitochondria (Brierley and Slautterback 1964) due presumably to the low electron density of Mg and the removal of precipitated phosphate by the fixation procedure in unbuffered osmium tetroxide. Deposits were not expected in beetroot mitochondria after incubation in Mg and P_i. Electron dense deposits were expected but not observed in beetroot mitochondria after accumulation of Ca and P_i. Electron dense calcium phosphate deposits have been observed in animal mitochondria (Brierley and Slautterback 1964, Greenawalt et al 1964, Peachey 1964). Modification and refinement of the fixation technique could, however, reveal electron dense deposits in beetroot mitochondria incubated with Ca and P_i in the presence of substrate. The absence of these deposits in

electron micrographs need not indicate that deposits of Ca or Mg phosphate complexes are not formed within the beetroot mitochondria after massive uptake of these ions.

CHAPTER THREE

SUBSTRATE OXIDATION AND ASSOCIATED PHOSPHORYLATION

A. INTRODUCTION.

Mitochondria isolated from a large number of plant species and tissues have been shown to oxidize Krebs cycle intermediates and to couple this oxidation to esterification of inorganic phosphate (Hackett 1959). Reports have indicated that the phosphorylative efficiency of plant mitochondria is as high as that of animal mitochondria and P/O ratios approaching the theoretical maxima have frequently been reported (Hackett 1959, Lieberman and Baker 1965).

However, reports of isolated plant mitochondria exhibiting respiratory control have only recently been submitted where oxidation has been measured polarographically, and ADP/O ratios and respiratory control ratios (R.C.) have been calculated by the procedure of Chance and Williams (1955, 1956). Mitochondria showing respiratory control have been isolated from sweet and white potatoes (Wiskich and Bonner 1963, Baker 1963), cauliflower bud (Bonner and Voss 1961), apple (Johes et al 1964), avocado (Wiskich et al 1964), pea root (Childress and Stein 1955), and corn shoots (Hanson et al 1965). The substrate providing maximum respiratory control varied between mitochondria from different sources but ADP/O ratios approached the theoretical maxima for each substrate.

The proposed sites of action of the inhibitors and uncouplers used in this study of oxidative and phosphorylative abilities of beetroot mitochondria have been discussed previously (Chapter 1). These and other agents and their sites of action have recently been reviewed by Ernster and Lee (1964).

Fig. III. 1. The oxidation of Tris succinate by beetroot mitochondria measured polarographically at 25°C in a medium containing 250 mM sucrose, 10 mM Tris-HCl buffer, pH 7.2, 4 mM Tris-phosphate buffer pH 7.2, 5 mM $MgCl_2$ and 3 uM cytochrome c. 324 ug mitochondrial N was added at Mw. Other additions are as shown.

Rates of oxygen uptake are expressed as mu moles O_2/minute. The washed mitochondria were isolated from a brei of pH 7.3.

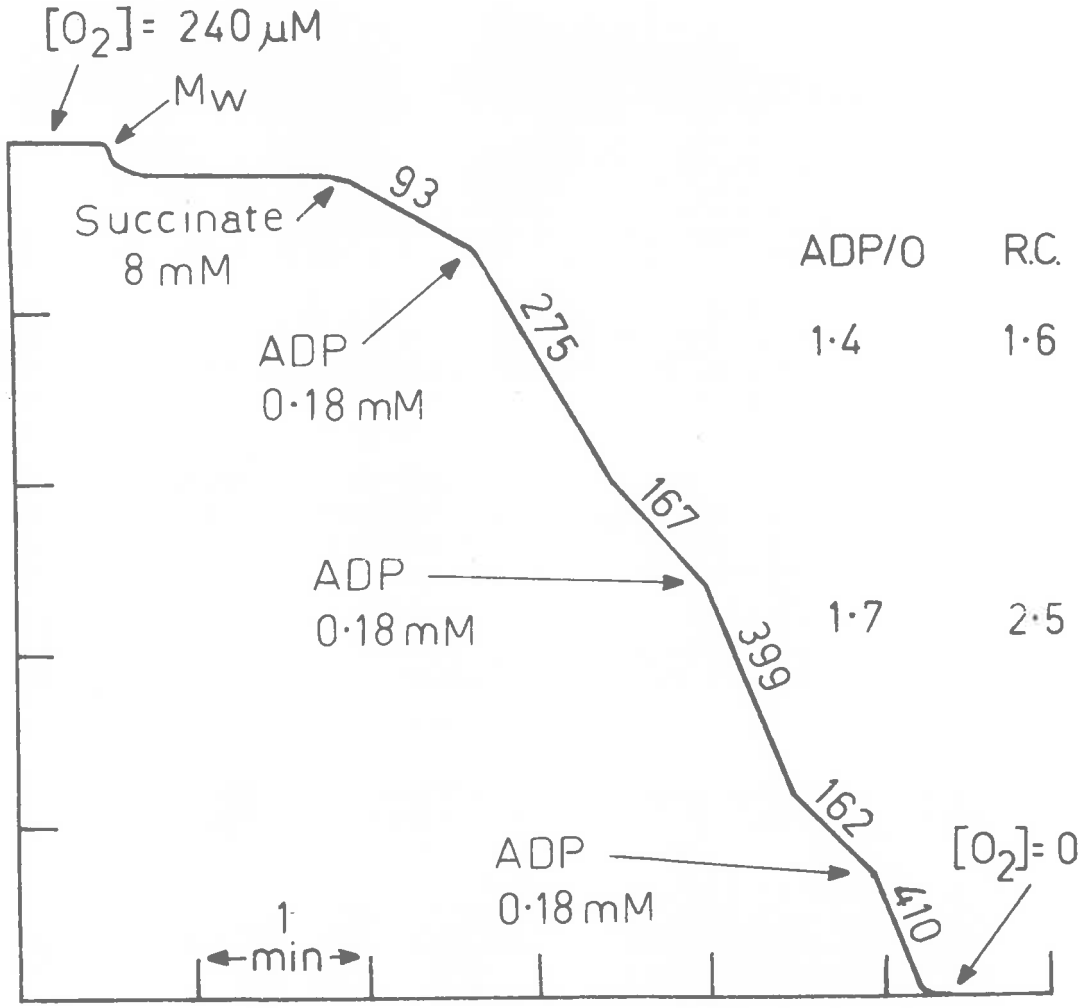
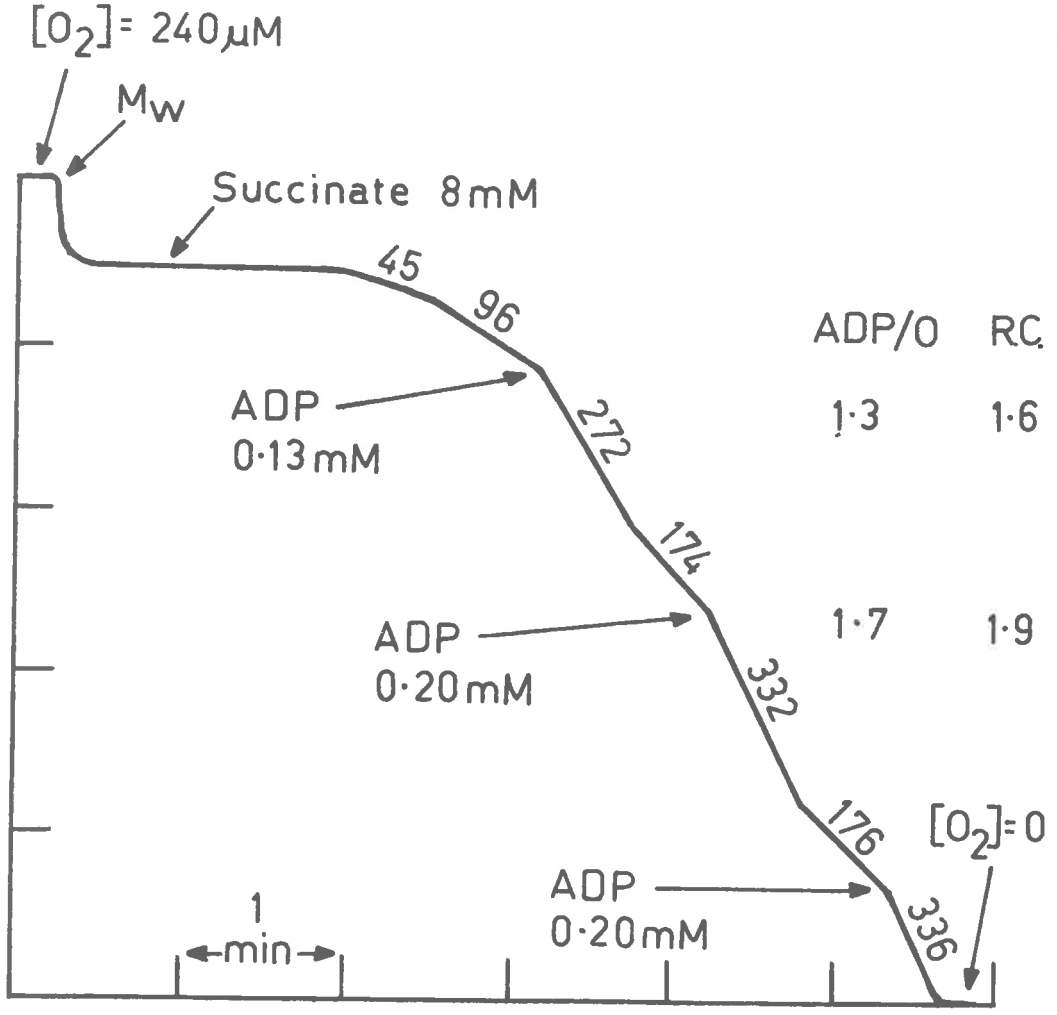

[O2] = 240 µM
Mw
Succinate
8 mM
93
ADP
0·18 mM
275
167
ADP
0·18 mM
399
162
ADP
0·18 mM
410
[O2] = 0
1
min
ADP/O
R.C.
1·4
1·6
1·7
2·5

Fig. III. 2. Polarographic tracing of oxygen uptake by beetroot mitochondria with Tris succinate as substrate. The medium contained 250 mM sucrose, 10 mM Tris-HCl buffer pH 7.2, 4 mM Tris-phosphate buffer pH 7.2, 6 mM $MgCl_2$ and 4 uM cytochrome c. 272 ug mitochondrial N was added at Mw. Other additionm are as shown. Rates of oxygen uptake are expressed as mu moles O_2/minute. The washed mitochondria were isolated from a brei of pH 7.8.


[O2] = 240 µM
Mw
Succinate 8 mM
45
96
ADP
0·13 mM
272
174
ADP
0·20 mM
332
176
ADP
0·20 mM
336
[O2] = 0
1
min
ADP/O
RC.
1·3
1·6
1·7
1·9

This chapter will describe the oxidation of tricarboxylic acid cycle intermediates by beetroot mitochondria and the control of ADP phosphorylation over this oxidation. Oxidative and phosphorylative capacities will be compared with those reported for other plant mitochondria.

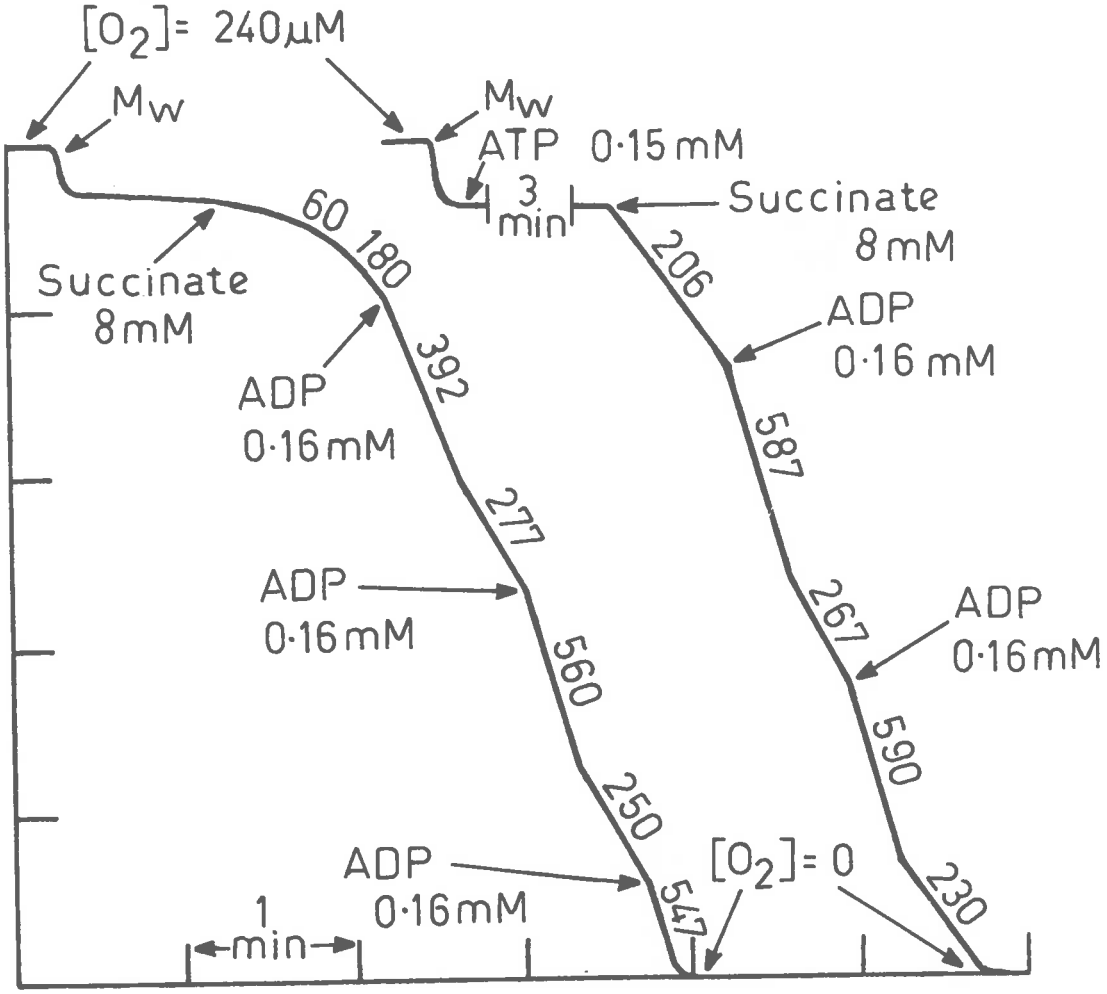
B. RESULTS.

1. Succinate.

Fig. III 1 shows the oxidation of succinate by beetroot mitochondria measured polarographically. The initial rate of oxidation was stimulated by the addition of ADP and the rate of oxidation decreased when the ADP was phosphorylated. Further additions of ADP stimulated the oxidation rate. The ADP-stimulated rate is referred to as state 3 in accordance with Chance and Williams (1955a), and the subsequent slower rate of oxidation after the added ADP is phosphorylated, as state 4 (Table 1, Fig. 1, Appendix). The ratio of state 3 / state 4 (the respiratory control ratio) as defined by Chance and Baltscheffsky (1958) gives a measure of the degree of coupling of phosphorylation to oxidation, and in this experiment the ratios were 1.6 and 2.5. The ADP/O ratios (1.4 and 1.7) calculated according to Chance and Williams (1955) were close to the theoretical ADP/O ratio of 2 for succinate.

In Fig. III 1 the initial rate of succinate oxidation was slower than subsequent state 4 rates and the first addition of ADP did not produce the maximum state 3 oxidation rate. However, the second and third additions of ADP produced rapid maximum rates of oxidation. Fig. III 2 shows a similar pattern of succinate oxidation but with a more

Fig. III. 3. Polarographic tracings of oxygen uptake by beetroot mitochondria with Tris succinate as substrate. The medium contained 250 mM sucrose, 10 mM Tris-HCl buffer pH 7.2, 4 mM Tris-phosphate buffer pH 7.2, 6 mM $MgCl_2$ and 4 uM cytochrome c. 492 ug mitochondrial N was added at Mw. Other additions are as shown. Rates of oxygen uptake are expressed as mu moles O_2/minute. The washed mitochondria were isolated from a brei of pH 7.6.


[O2]= 240μM
Mw
Mw
ATP 0·15 mM
3 min
Succinate 8 mM
Succinate 8 mM
60
180
392
277
560
250
547
206
587
267
590
230
ADP 0·16 mM
ADP 0·16 mM
ADP 0·16 mM
ADP 0·16 mM
ADP 0·16 mM
[O2]= 0
1 min

marked initial inhibition of oxidation. This trace was obtained using mitochondria isolated from a more alkaline (pH 7.8) medium than normal (pH 7.3 in Fig. III 1). A similar succinate oxidation pattern with initial inhibition of oxidation has been observed with cauliflower mitochondria (Bonner and Voss 1961) and sweet potato mitochondria (Wiskich and Bonner 1963) and it was suggested that oxaloacetate was inhibiting oxidatioh of succinate. It has been shown that oxaloacetate inhibits succinate oxidation in plant mitochondria (Avron and Biale 1957), (Wiskich and Bonner 1963) and in animal mitochondria (Azzone and Ernster 1960, Pardee and Potter 1948). Wiskich et al (1964) reported an inhibition of succinate oxidation by oxaloacetate which became progressively stronger as the pH of the isolation medium was increased from 7 to 8. These results may reflect the greater stability of oxaloacetate under alkaline conditions.

Incubation of beetroot mitochondria with ATP prior to succinate addition produced a rapid response to succinate and a maximum state 3 oxidation rate after the first addition of ADP (Fig. III 3). Mitochondria pre-incubated for three minutes with ATP and oligomycin showed an initial rapid response to succinate addition indicating that ATPase activity was not responsible for the increased initial succinate oxidation rate with ATP. This ATP-effect has been interpreted as relief of oxaloacetate inhibition of succinate oxidation (Wiskich et al 1964).

The respiratory control ratios obtained with beetroot mitochondria oxidizing succinate indicate the degree of coupling between phosphorylation and oxidation. State 4 rates indicate leakage of electrons along the transfer chain without concomitant phosphorylation. However, the

Fig. III. 4. Polarographic tracing of oxygen uptake by beetroot mitochondria with Tris succinate as substrate. The medium contained 250 mM sucrose, 10 mM Tris-HCl buffer pH 7.2, 4 mM Tris-phosphate buffer pH 7.2, 6 mM $MgCl_2$ and 5 uM cytochrome c. 258 ug mitochondrial N was added at Mw. Other additions are as shown. Rates of oxygen consumption are expressed as mu moles O_2/minute.

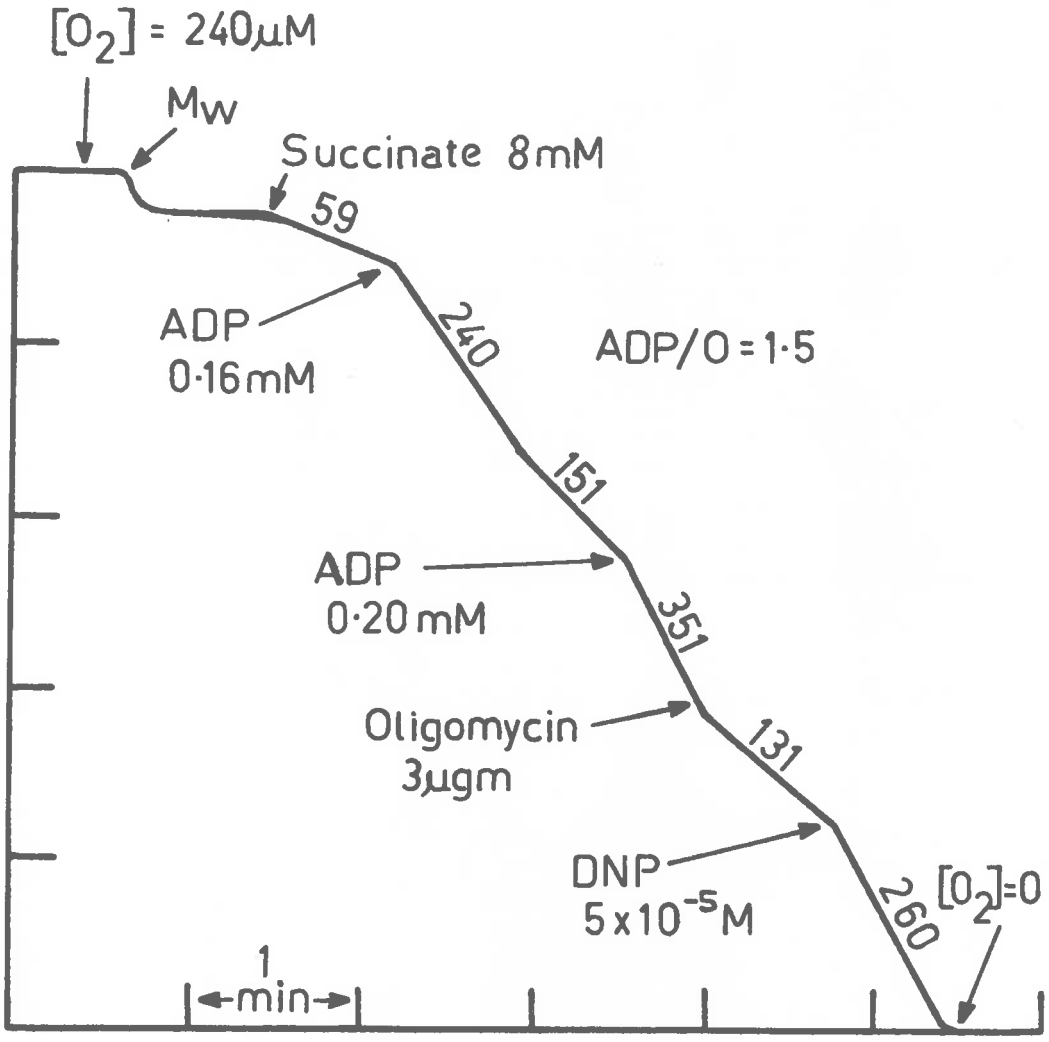

[O2] = 240µM
Mw
Succinate 8mM
59
ADP
0·16mM
240
ADP/O = 1·5
151
ADP
0·20 mM
351
Oligomycin
3µgm
131
DNP
5x10-5M
260
[O2]=0
1
←min→

Fig. III. 5. The oxidation of α-ketoglutarate by beetroot mitochondria incubated in a medium of 3.3 ml containing 250 mM sucrose, 10 mM Tris-HCl buffer pH 7.2, 4 mM Tris-phosphate buffer pH 7.2, 6 mM $MgCl_2$, 5 uM cytochrome c, 30 mM TPP, 5 mM sodium malonate and 356 ug mitochondrial N. Mw indicates the addition of mitochondria and other additions are shown as final concentrations. Rates are expressed as mu moles O_2/minute.

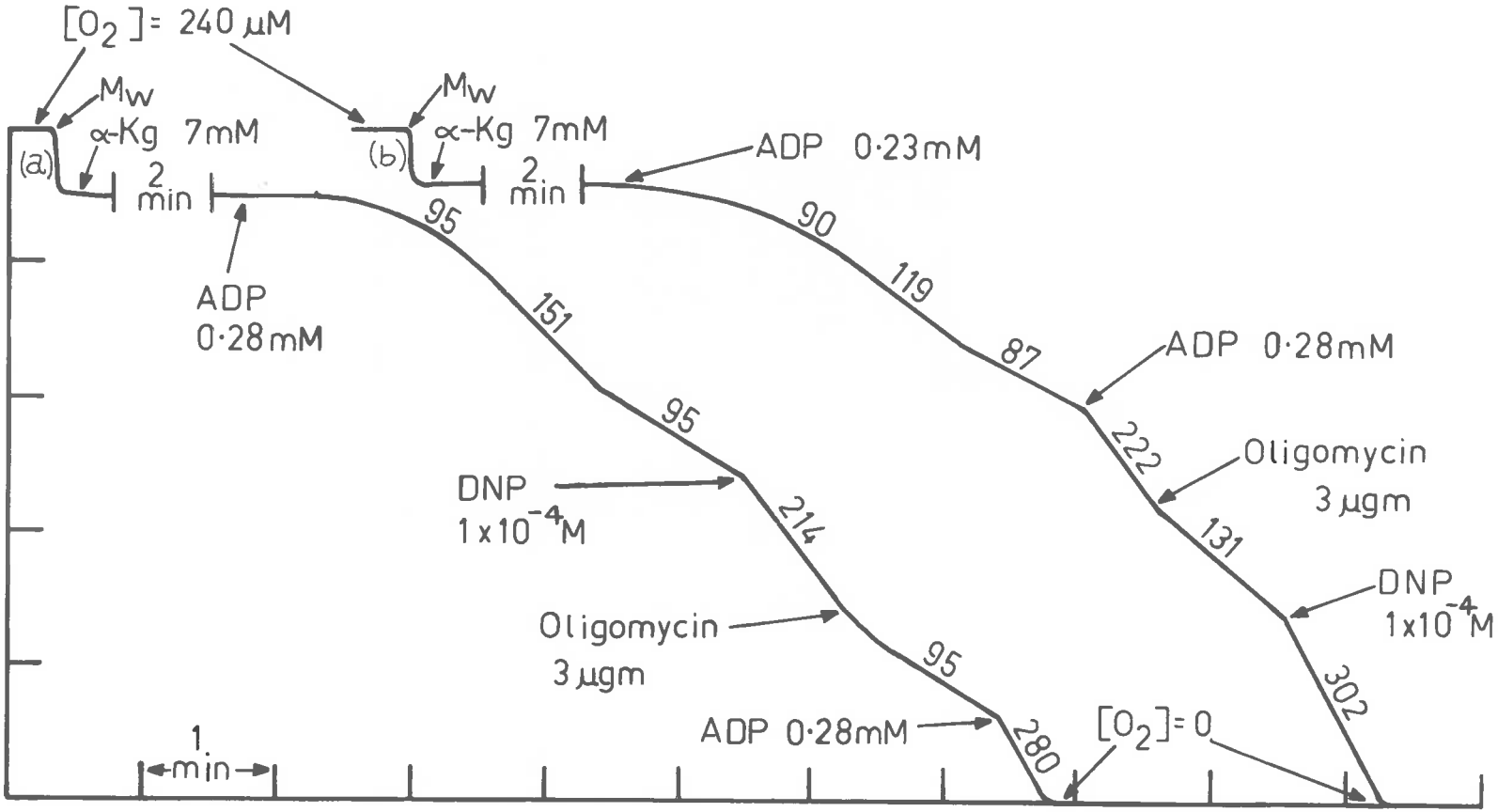
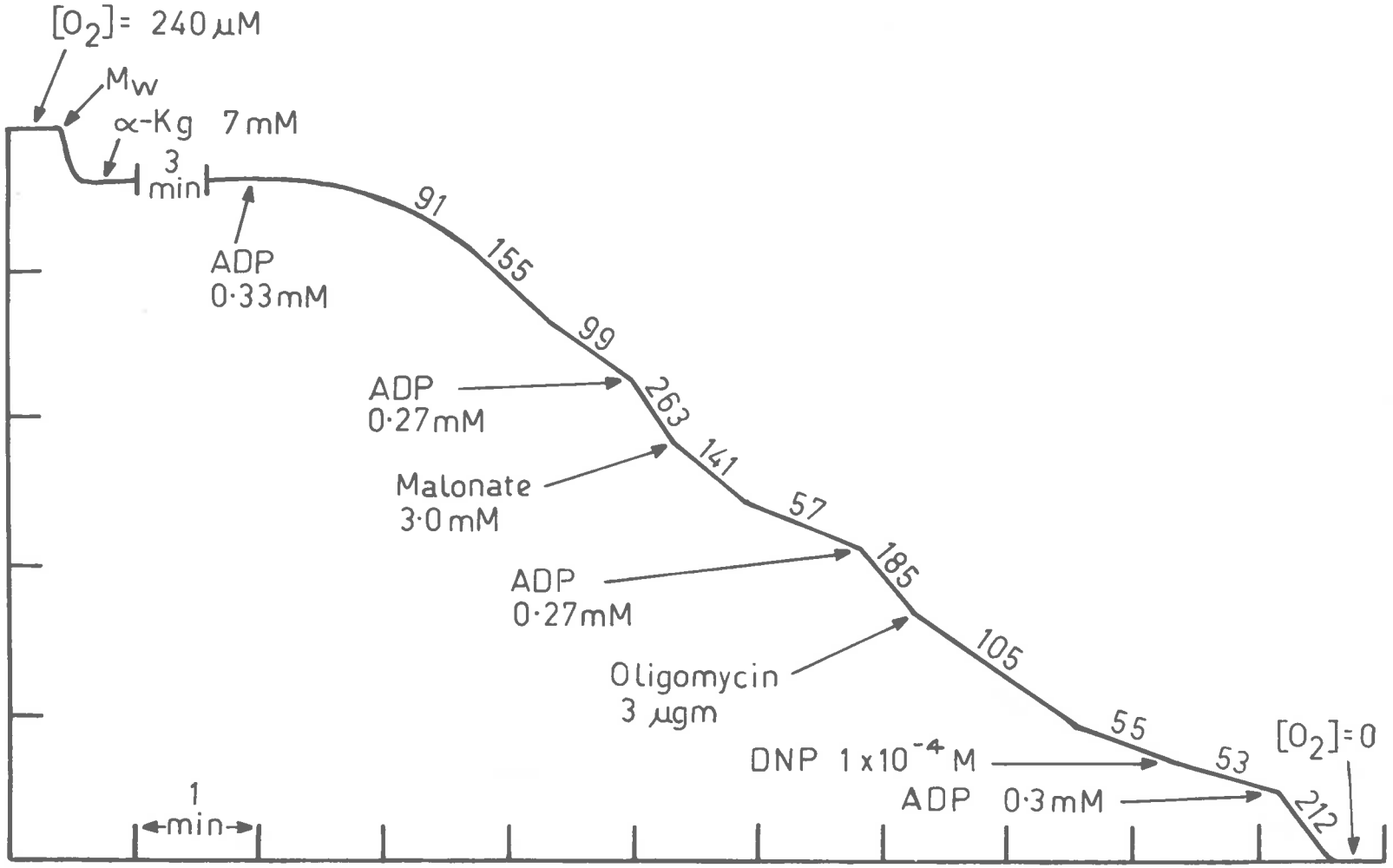

[O2] = 240 µM
Mw
α-Kg 7mM
(a)
2 min
ADP 0·28mM
95
151
95
DNP 1x10-4M
214
Oligomycin 3 µgm
95
ADP 0·28mM
280
[O2] = 0
1 min
Mw
α-Kg 7mM
(b)
2 min
ADP 0·23mM
90
119
87
ADP 0·28mM
222
Oligomycin 3 µgm
131
DNP 1x10-4M
302

Fig. III. 6. The oxidation of α-ketoglutarate by beetroot mitochondria assayed in a medium of 3.3 ml containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 3 mM Tris-phosphate buffer pH 7.2, 5 mM $MgCl_2$, 4 uM cytochrome c, 30 mM TPP, and 380 ug mitochondrial N. Mw indicates the addition of mitochondria and other additions are shown as final concentrations. Rates are expressed as nn moles O_2/minute.


[O2]= 240 µM
Mw
α-Kg 7mM
3 min
ADP 0·33mM
91
155
99
ADP 0·27mM
263
141
Malonate 3·0mM
57
ADP 0·27mM
185
105
Oligomycin 3 µgm
55
DNP 1x10-4 M
53
ADP 0·3mM
212
[O2]=0
1 min

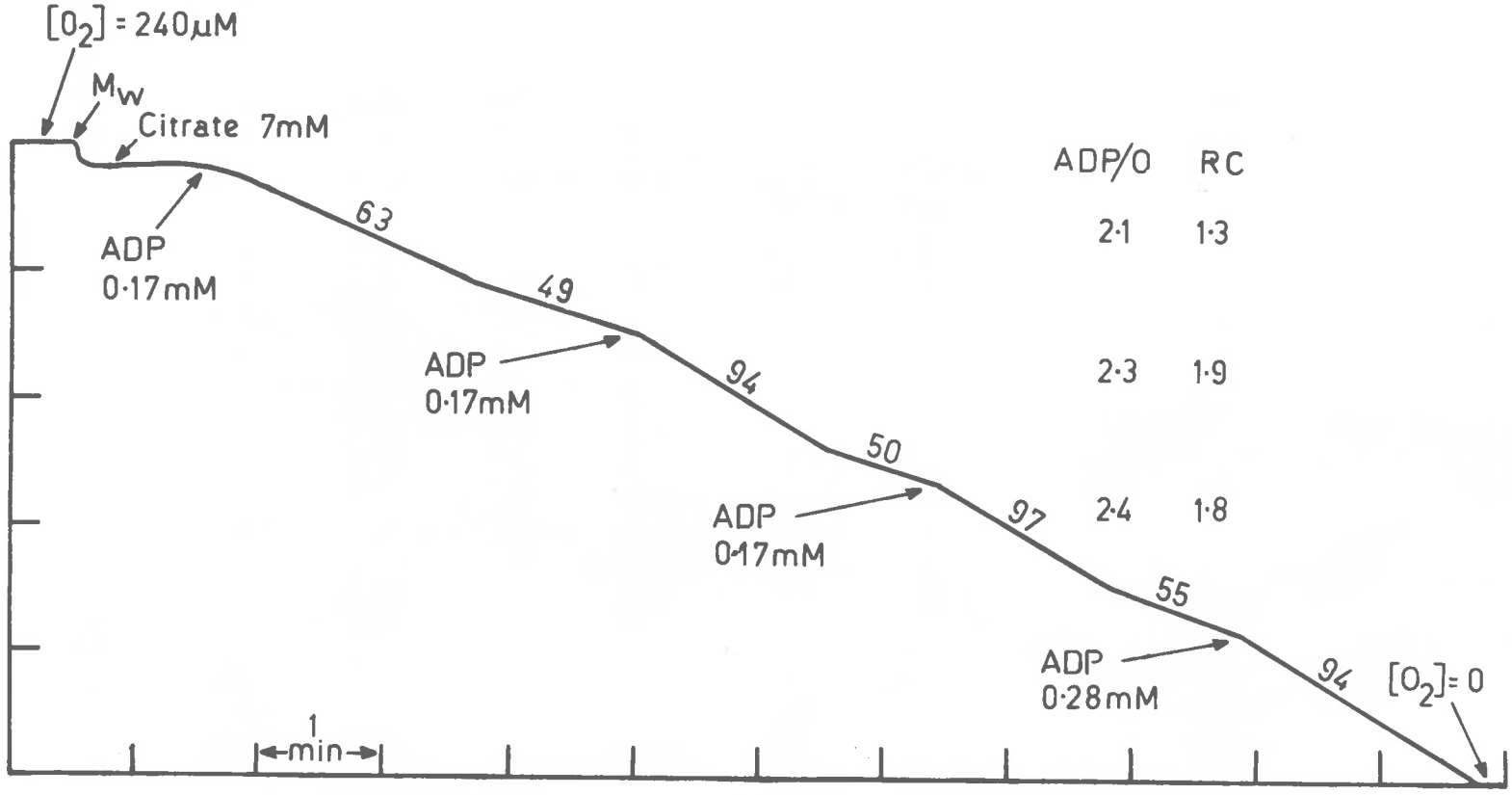
state 4 rate of oxidation would not be a true measure of the non-phosphorylating oxidation rate if ATPase activity allowed ADP to re-cycle. In Fig. III 4 the addition of oligomycin, which inhibits mitochondrial phosphorylation without uncoupling (Lardy et al 1958, Chappell and Greville 1961), and ATPase inhibited succinate oxidation in beetroot mitochondria to a rate 13% less than the previous state 4 oxidation rate. In a normal succinate tracing (Fig. III 1) the variation between state 4 oxidation rates was usually between 5 and 20%. Thus ATPase activity contributed insignificantly to the state 4 oxidation rate. Fig. III 4 indicates that oligomycin inhibited oxidation by inhibiting phosphorylation, an inhibition that was released by addition of the uncoupling agent DNP.

2. <u>α-Ketoglutarate</u>.

Figs. III 5 and 6 show the oxidation pattern of α-ketoglutarate by beetroot mitochondria. TPP was required and α-ketoglutarate was not oxidised until ADP was added whereas some oxidation of succinate occurred in the absence of ADP. This effect, and the high respiratory control ratios observed during α-ketoglutarate oxidation can be attributed to strong coupling between oxidation and phosphorylation at the substrate-level phosphorylation site (Wiskich et al 1964). An induction period was required for maximal α-ketoglutarate oxidation (Figs. III 5 and 6) as observed by Chance and Baltscheffsky (1958). Malonate was included in Fig. III 5 to inhibit the oxidation of succinate formed from α-ketoglutarate oxidation (Wiskich et al 1964).

Figs. III 5 and 6 show that substrate-level phosphorylation was rate limiting during state 4 oxidation. Fig. III 5b indicates that oligomycin did not reduce the ADP-stimulated state 3 oxidation rate to the level of the previous state 4 rate, although oxidation was inhibited. Thus electron transfer chain phosphorylations, which are inhibited by oligomycin (Lardy et al 1958), cannot be responsible for the very low state 4 oxidation rate. Oligomycin does not inhibit substrate-level phosphorylation (Chappell and Greville 1961). DNP recovered the oligomycin-inhibited oxidation rate in Fig. III 5b when excess ADP was present. The stimulation of the state 4 rate by DNP in Fig. III 5a suggests that DNP was making ADP available to the substrate-level site of phosphorylation since DNP does not uncouple substrate-level phosphorylation (Chappell and Greville 1961, Wiskich et al 1964). Wiskich et al (1964) showed that the stimulation by DNP of the state 4 rate of α-ketoglutarate oxidation in avocado mitochondria was due to a stimulation of adenosine triphosphatase activity rather than to a more favourable ATP/ADP ratio at the active site as suggested by Azzone and Ernster (1961a and b). Fig. III 6 repeats these effects with beetroot mitochondria. Oligomycin, when added during the state 3 rate of α-ketoglutarate oxidation, inhibited oxidation by 40% due to inhibition of electron transfer chain phosphorylations. When ADP became rate-limiting the coupling of the substrate-level phosphorylation induced the very low state 4 rate (Fig. III 6). DNP did not stimulate this state 4 oxidation rate in the presence of oligomycin, indicating that

Fig. III. 7. The oxidation of sodium citrate by beetroot mitochondria assayed in a medium of 3.3 ml containing 250 mM sucrose, 10 mM Tris-HCl buffer pH 7.2, 4 mM Tris-phosphate buffer pH 7.2, 6 mM $MgCl_2$, 4 uM cytochrome c and 260 ug mitochondrial N. Rates are expressed as mu moles O_2/minute.


[O2] = 240µM
Mw
Citrate 7mM
ADP
0·17mM
63
49
ADP
0·17mM
94
50
ADP
0·17mM
97
55
ADP
0·28mM
94
[O2]= 0
1
min
ADP/O
RC
2·1
1·3
2·3
1·9
2·4
1·8

DNP stimulation of state 4 oxidation in Fig. III 5a was due to stimulation of ATPase activity. Addition of oligomycin (which would inhibit both electron transfer chain phosphorylations and ATPase activity) in Fig. III 5a inhibited the DNP-stimulated oxidation rate to the former state 4 oxidation rate. The DNP-stimulated oxidation rate in Fig. III 5a was much greater than the previous state 3 oxidation rate due to the induction period required for maximum α-ketoglutarate oxidation.

Sodium arsenite at a concentration of 1×10^{-3}M inhibited α-ketoglutarate oxidation in the presence of malonate by 90%. Arsenite is a fairly specific inhibitor of enzymes containing active disulphydryl groups such as lipoic acid (Peters 1949, Lieberman and Biale 1956b), indicating that such a substance was involved in α-ketoglutarate oxidation by beetroot mitochondria.

3. Citrate.

The oxidation pattern of citrate by beetroot mitochondria is shown in Fig. III 7. ADP/O ratios of 2.2 to 2.6 and respiratory control ratios of 1.8 were obtained. Neither NAD nor NADP were required for maximum citrate state 3 oxidation rates, respiratory control ratios, or ADP/O ratios (Beaudreau and Remmert 1955).

4. NADH.

NADH was oxidized very slowly by intact beetroot mitochondria without respiratory control in comparison with results obtained with sweet potato mitochondria (Wiskich and Bonner 1963). Addition of

Fig. III. 8. Polarographic tracing showing the oxidation of sodium pyruvate by beetroot mitochondria. Assayed in a 3.3 ml medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 3 mM Tris-phosphate buffer pH 7.2, 5 mM $MgCl_2$, 0.3 mM Tris malate, 4 uM cytochrome c, 3 ug/ml NAD, 30 mM TPP, 0.3 M coenzyme A and 465 ug mitochondrial N. Mw indicates the addition of mitochondria and other additions are shown as final concentrations. Rates are expressed as mu moles O_2/minute.

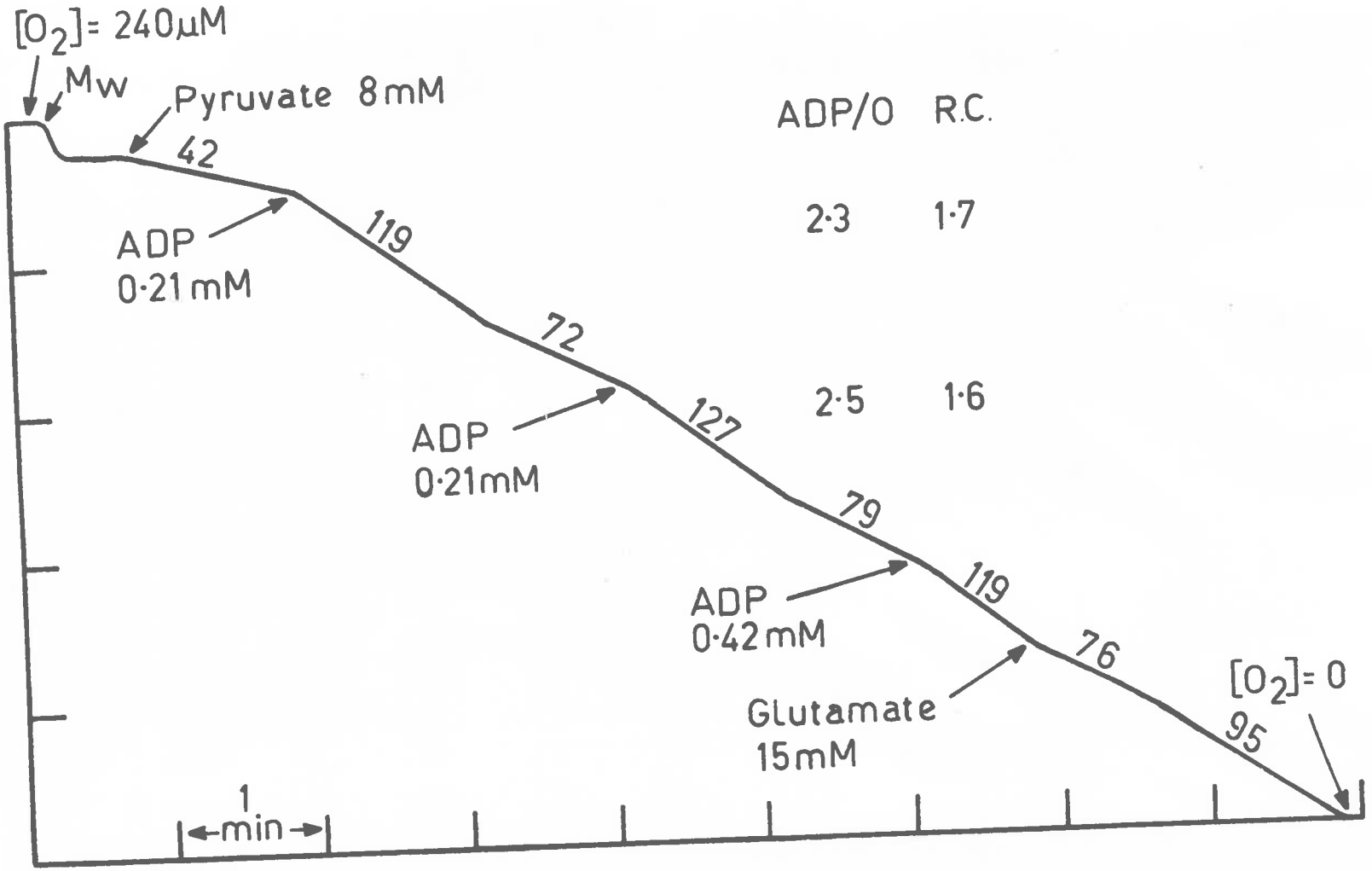
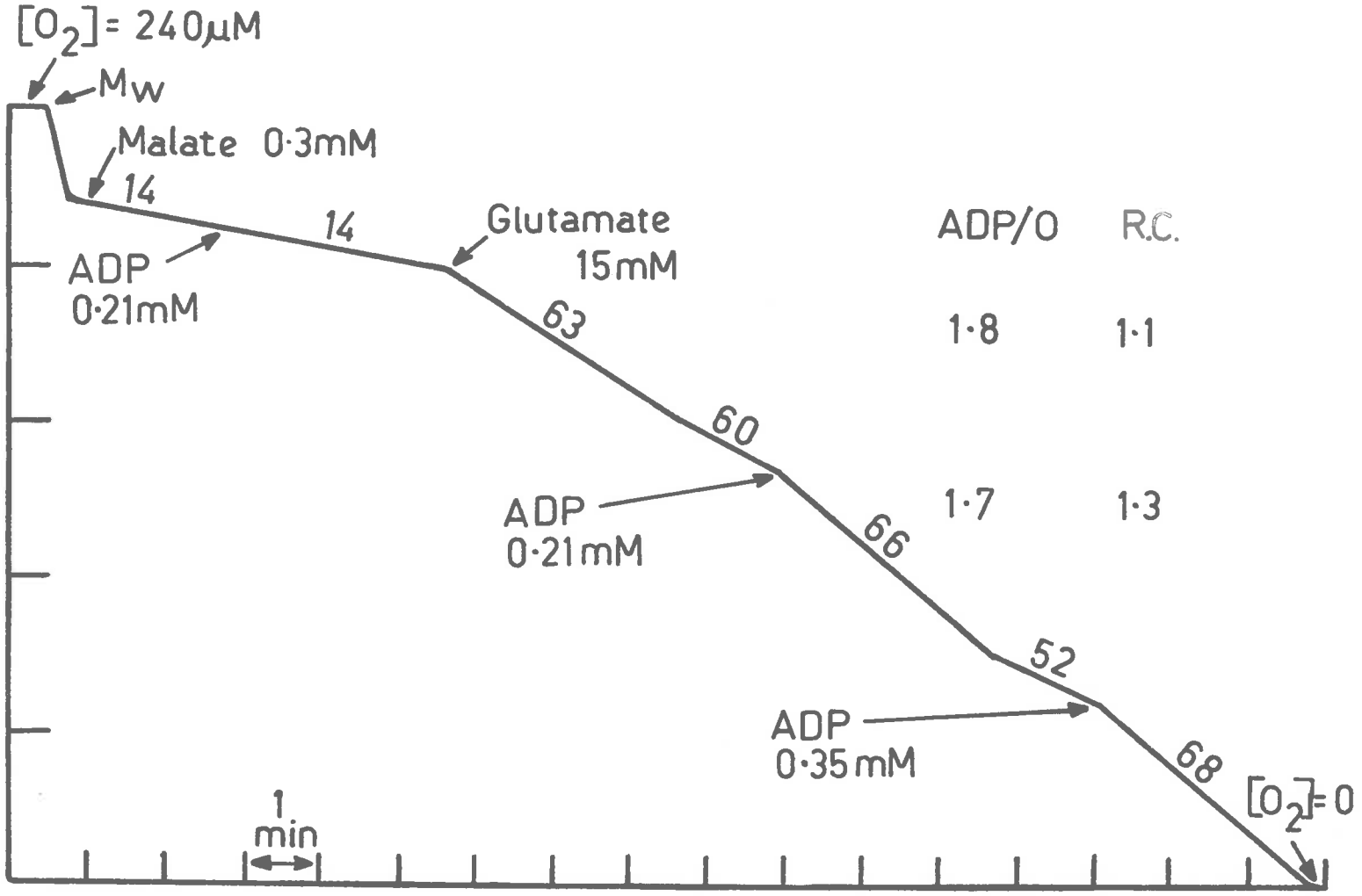

[O2]= 240µM
Mw
Pyruvate 8mM
42
ADP
0·21 mM
119
72
ADP
0·21mM
127
79
ADP
0·42mM
119
76
Glutamate
15mM
[O2]= 0
95
1
←min→
ADP/O R.C.
2·3 1·7
2·5 1·6

Table III. 1. Effect of Cofactors on the Oxidation of Pyruvate and Malate.

Beetroot mitochondria was incubated at 25°C in the standard oxygen electrode cell medium (Tris phosphate) with 5 uM cytochrome c, 92 ug mitochondrial N/ml, 0.25 mM ADP, and where indicated 3 ug NAD/ml, 30 mM TPP and 0.2 M coenzyme A.

Cofactors	Sodium Pyruvate mM	Tris Malate mM	Oxidation Rate (mumoles O_2/min).
NAD	-	0.3	10
-	8.0	-	0
TPP	8.0	-	10
TPP + CoA	8.0	-	20
TPP + CoA + NAD	8.0	-	23
NAD	8.0	0.3	10
NAD + TPP	8.0	0.3	84
TPP + CoA + NAD	8.0	0.3	120

Fig. III. 9. The oxidation of a low concentration of Tris malate by beetroot mitochondria. Assayed in 3.3 ml of medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 3mM Tris-phosphate buffer pH 7.2, 5 mM $MgCl_2$, 4 uM cytochrome c, 3 ug/ml NAD and 465 ug mitochondrial N. Rates are expressed as mu moles O_2/minute.


[O2] = 240µM
Mw
Malate 0·3mM
14
14
Glutamate
15mM
ADP
0·21mM
63
60
ADP
0·21mM
66
52
ADP
0·35mM
68
[O2] = 0
1
min
ADP/O
R.C.
1·8
1·1
1·7
1·3

cytochrome c greatly increased the rate of NADH oxidation while HOQNO added at a concentration of 4×10^{-6}M completely inhibited oxidation. Mitochondria prepared under acid conditions (pH of brei = 6.5 - 7.1) oxidized NADH more rapidly than those prepared under normal or alkaline conditions (pH of brei = 7.2 - 8.0).

5. Pyruvate.

Sodium pyruvate was oxidized by beetroot mitochondria in the presence of a primer acid (0.3 mM Tris malate) (Fig. III 8). TPP, coenzyme A, and NAD were required for maximum pyruvate oxidation rates (Lieberman and Biale 1956, Walker and Beevers 1956). Addition of ADP increased the oxidation rate and this state 3 rate was replaced by the state 4 oxidation rate after the ADP was phosphorylated. Subsequent additions of ADP gave further state 3 → state 4 transitions and the ADP/O ratios calculated were 2.3 and 2.5. The respiratory control ratios were 1.7 and 1.6.

In the absence of the low concentration of malate, pyruvate was oxidized very slowly with no respiratory control (Table III 1). Table III 1 also shows the effects of various cofactors on pyruvate oxidation.

Fig. III 9 shows that the low concentration of Tris malate used as a primer acid for pyruvate oxidation was oxidized very slowly with no respiratory control in the absence of pyruvate. Glutamate, which could remove accumulated oxaloacetate by transamination, increased the malate state 3 oxidation rate and transition to state 4 rate occurred

Fig. III. 10. The effect of sodium arsenite and Tris glutamate on the oxidation of sodium pyruvate by beetroot mitochondria. Assayed in a 3.3 ml medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 3 mM Tris-phosphate buffer pH 7.2, 5 mM $MgCl_2$, 4 uM cytochrome c, 0.3 mM Tris malate, 3 ug/ml NAD, 30 mM TPP, 0.2 M coenzyme A and 430 ug mitochondrial N. Rates are expressed as mu moles O_2/min.

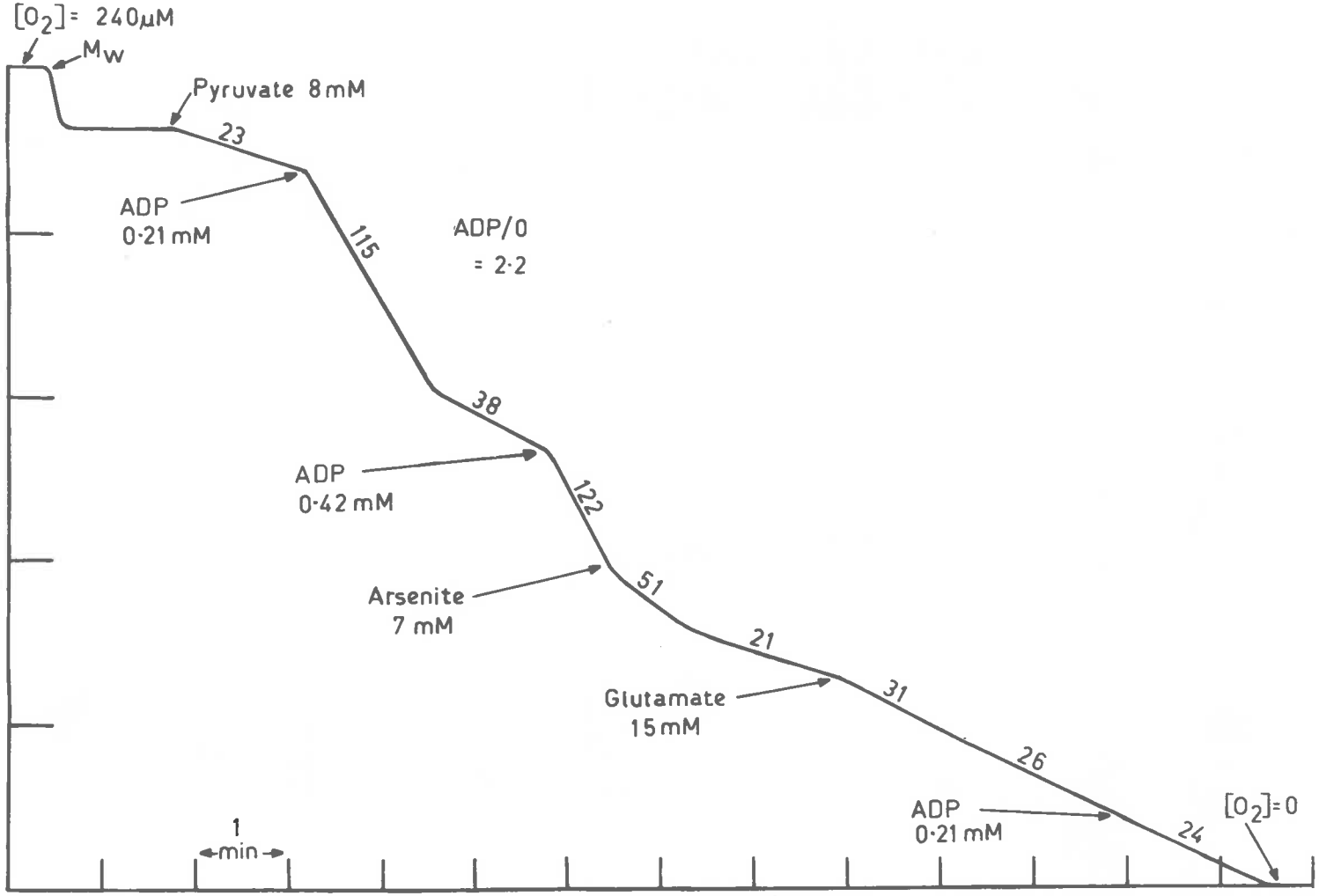
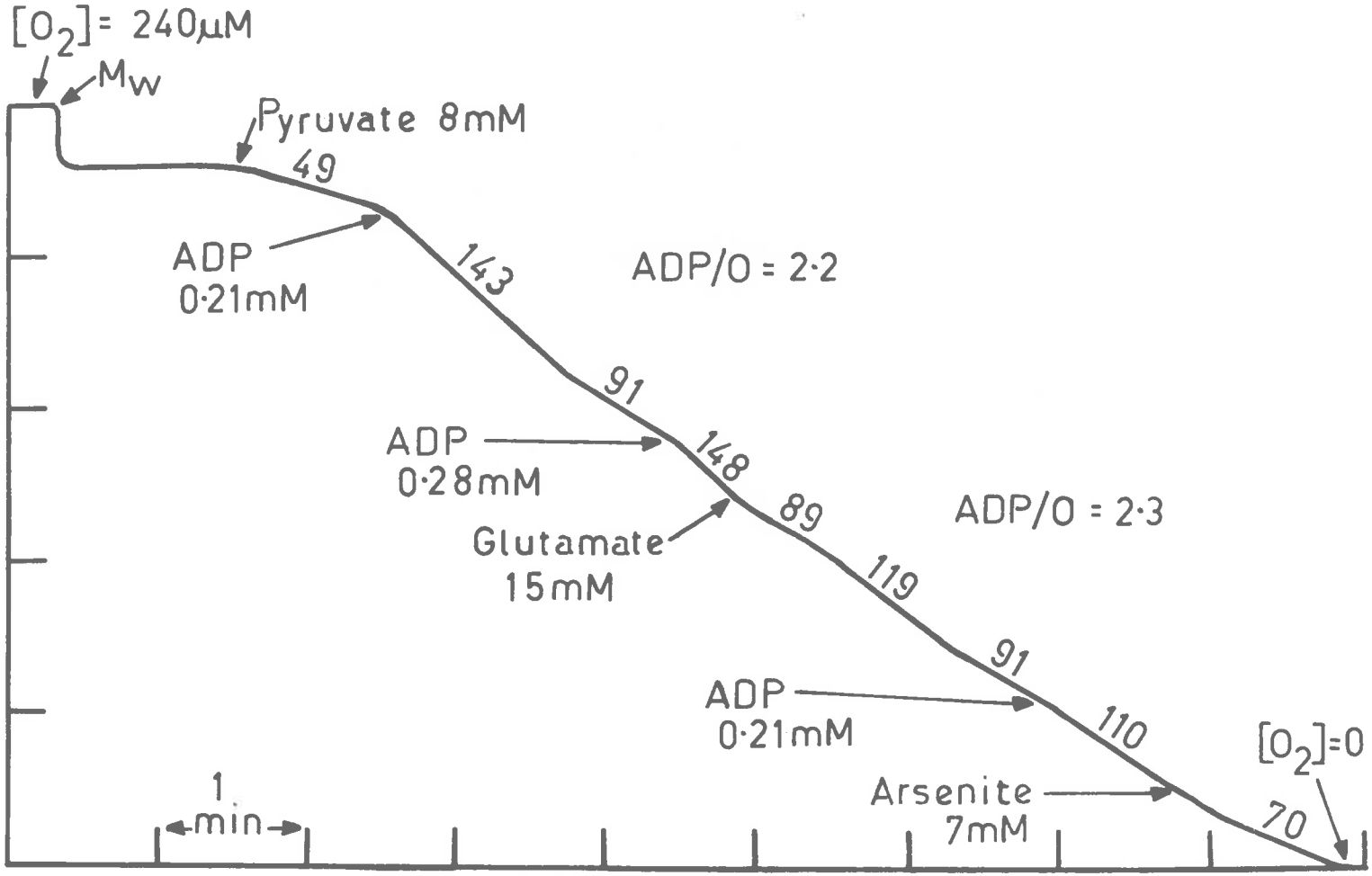

[O2]= 240μM
Mw
Pyruvate 8mM
23
ADP
0·21 mM
115
ADP/O
= 2·2
38
ADP
0·42 mM
122
Arsenite
7 mM
51
21
Glutamate
15mM
31
26
ADP
0·21 mM
24
[O2]=0
1
min

Fig.III. 11. The effect of Tris glutamate and sodium arsenite on the oxidation of sodium pyruvate by beetroot mitochondria. Assayed in a 3.3 ml medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 3 mM Tris-phosphate buffer pH 7.2, 5 mM $MgCl_2$, 4 uM cytochrome c, 0.3 mM Tris malate, 3 ug/ml NAD, 30 mM TPP, 0.2 M coenzyme A and 465 mitochondrial N. Rates are expressed as mu moles O_2/minute.


[O2]= 240µM
Mw
Pyruvate 8mM
49
ADP
0·21mM
143
ADP/O = 2·2
91
ADP
0·28mM
148
89
Glutamate
15mM
ADP/O = 2·3
119
91
ADP
0·21mM
110
[O2]=0
Arsenite
7mM
70
1
min

on phosphorylation of the added ADP (Fig. III 9). The respiratory control ratios were very low and the ADP/O ratios of 1.8 and 1.7 were also much lower than the theoretical ratio of 3 usually measured during malate oxidation in the presence of glutamate (Fig. III 14). Glutamate is not oxidized as such by beetroot mitochondria (Millard 1963).

Lipoic acid had no effect on the oxidation of pyruvate and malate by sweet potato mitochondria (Lieberman and Biale 1956b). Pyruvate plus malate oxidation by beetroot mitochondria was also not affected by addition of lipoic acid. Arsenite inhibited by 83% the oxidation of pyruvate plus malate by beetroot mitochondria (Fig. III 10), indicating that a lipoic acid-like substance was involved (Peters 1949, Lieberman and Biale 1956b). The low residual oxidation rate in Fig. III 10 was probably due to direct oxidation of malate (Fig. III 9) which was not inhibited by arsenite. Addition of glutamate (Fig. III 10) after inhibition of pyruvate oxidation by arsenite stimulated the rate of this residual malate oxidation, probably by removal of oxaloacetate, but the oxidation rate gradually decreased after the initial stimulation.

Addition of glutamate before arsenite in Fig. III 11 decreased the state 3 pyruvate plus malate oxidation rate. This may have resulted from competition between pyruvate and glutamate for oxaloacetate formed from malate oxidation. Arsenite addition decreased the oxidation rate still further after a lag phase (Fig. III 11). The residual oxidation rate would be due entirely to malate oxidation.

Fig. III. 12. Polarographic tracing showing the oxidation of Tris ascorbate by beetroot mitochondria. Assayed in 3.3 ml of medium containing 250 mM sucrose, 10 mM Tris-HCl buffer pH 7.2, 4 mM Tris-phosphate buffer pH 7.2, 6 mM $MgCl_2$, 2 uM HOQNO, 380 uM TMPD and 65 ug mitochondrial N. Mw indicates the addition of mitochondria and additions are shown as final concentrations. Rates are expressed as mu moles O_2/minute.

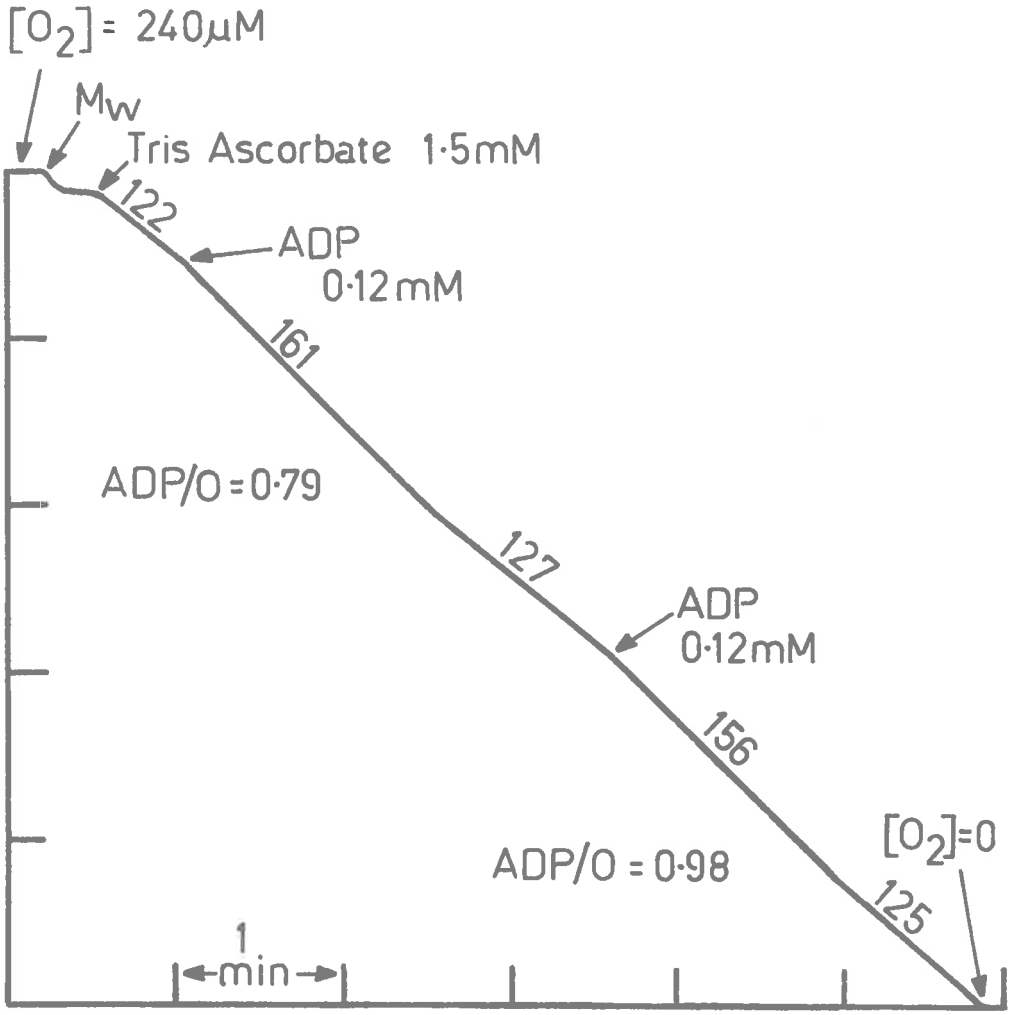

[O2] = 240μM
Mw
Tris Ascorbate 1·5mM
122
ADP
0·12mM
161
ADP/O = 0·79
127
ADP
0·12mM
156
[O2]=0
ADP/O = 0·98
125
1
min

Fig. III. 13. The effects of oligomycin and DNP on the oxidation of Tris ascorbate by beetroot mitochondria. Assayed in a 3.3 ml medium containing 250 mM sucrose, 10 mM Tris-HCl buffer pH 7.2, 4 mM Tris-phosphate buffer pH 7.2, 6 mM $MgCl_2$, 3 uM cytochrome c, 2 uM HOQNO, 380 uM TMPD and 55 ug mitochondrial N. Rates are expressed as mu moles O_2/minute.

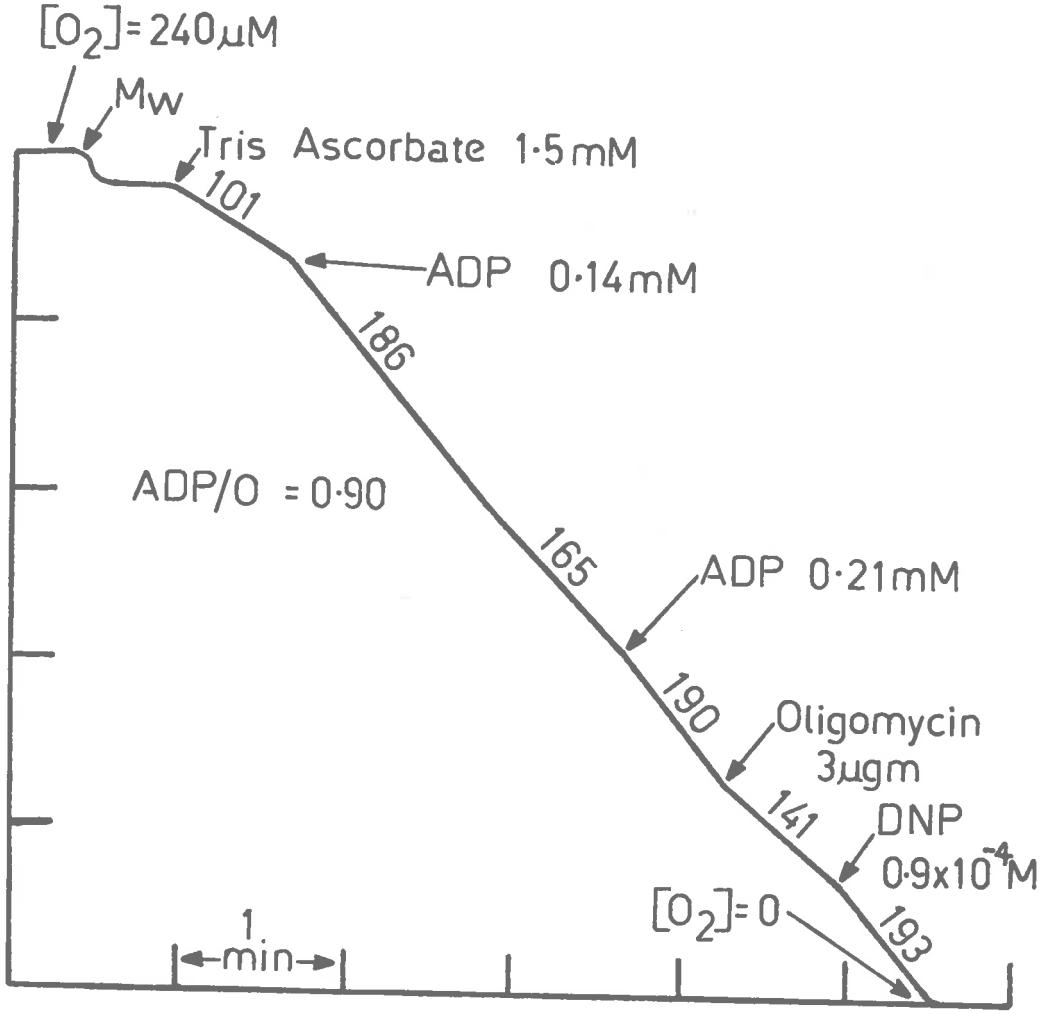

[O2]=240μM
Mw
Tris Ascorbate 1·5mM
101
ADP 0·14mM
186
ADP/O = 0·90
165
ADP 0·21mM
190
Oligomycin
3μgm
141
DNP
0·9x10⁻⁴M
[O2]=0
193
1
min

Table III. 2. Effect of TMPD and Antimycin A on Ascorbate Respiratory Control and ADP/O Ratios.

Beetroot mitochondria were incubated in the oxygen electrode cell medium (Tris phosphate) with 1.5 mM Tris ascorbate and 20 ug mitochondrial N/ml. State 3 oxidation rates were produced by 0.07 mM ADP.

TMPD uM	Antimycin A (1 ug/ml)	Respiratory Control Ratio		ADP/O Ratio	
		1	2	1	2
76	-	1.09	-	0.50	-
228	-	1.05	1.19	0.50	0.53
380	-	1.12	1.22	0.53	0.51
380	1	1.10	-	0.52	-
380	2	1.09	-	0.49	-

Table III. 3. Effect of Cytochrome c on Ascorbate Oxidation and Respiratory Control and ADP/O ratios.

Beetroot mitochondria were incubated in the O_2 electrode cell medium (Tris phosphate) with 1.5 mM Tris ascorbate and 27 ug mitochondrial N/ml (Expt. 1), or 15 mM Tris ascorbate and 20 ug mitochondrial N/ml (Expt. 2).

Addition	Oxidation Rate (mumoles O_2/min)	R.C. Ratio	ADP/O Ratio
Expt. 1, A			
380 uM TMPD	172		
0.61 mM ADP	236		
	206 state 4	1.14	0.68
6 uM cytochrome c	245		
0.30 mM ADP	265		
	238 state 4	1.13	0.75
Expt. 1, B			
380 uM TMPD	196		
6 uM cytochrome c	230		
0.61 mM ADP	294		
	265 state 4	1.12	0.70
0.30 mM ADP	296		
	270 state 4	1.09	0.76
Expt. 2			
3.8 mM TMPD	575		
0.61 mM ADP	585		
	530 state 4	1.10	0.53
6 uM cytochrome c	615		
0.30 mM ADP	650		
	570 state 4	1.14	0.51

6. TMPD-Ascorbate.

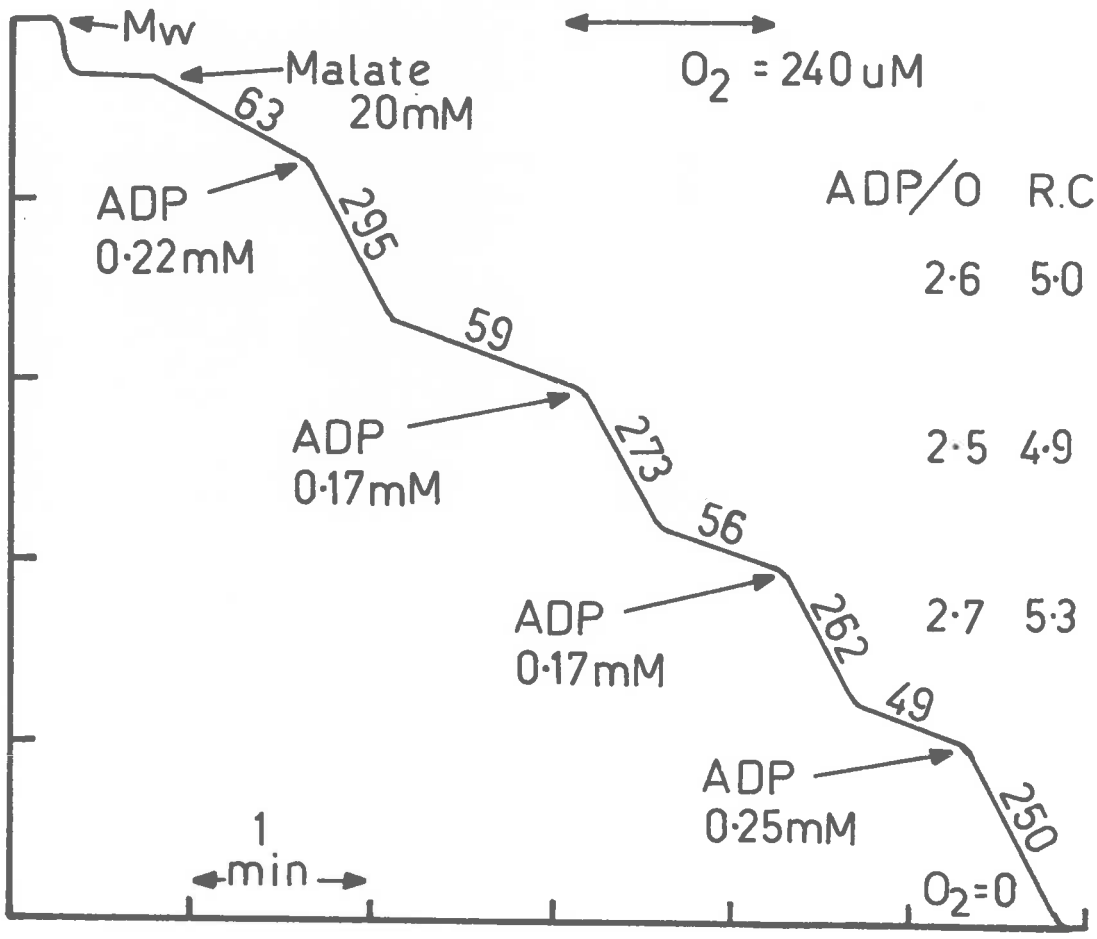
Beetroot mitochondria coupled the oxidation of Tris ascorbate, in the presence of catalytic amounts of TMPD, to the phosphorylation of ADP (Jacobs 1960). Fig. III 12 indicates that the respiratory control ratios were low (1.3 and 1.2) but the ADP/O ratios were 0.77 and 0.99 in agreement with the theoretical ratio of 1.0 and with results obtained using rat liver mitochondria (Packer et al 1963, Packer and Jacobs 1962) and rat heart mitochondria (Tyler et al 1965, 1966). Oligomycin added in the presence of excess ADP inhibited the ascorbate oxidation rate and this inhibition was relieved by DNP (Fig. III 13) (Packer et al 1963).

The effect of increasing TMPD concentrations (80 uM to 380 uM) on the respiratory control and ADP/O ratios is shown in Table III 2. HOQNO and antimycin A at concentrations which completely inhibited succinate oxidation increased ascorbate oxidation rates by between 5 and 20% when the TMPD concentration was 380 uM, but had no effect on either respiratory control or ADP/O ratios (Table III 2). Addition of cytochrome c produced a 10-30% stimulation in state 3 and state 4 ascorbate oxidation rates independent of the TMPD concentration, but again the ADP/O ratios and R.C. ratios were not affected (Table III 3).

7. Malate.

The oxidation of malate by beetroot mitochondria has been demonstrated manometrically (Wiskich et al 1960) and polarographically (Millard 1963), with P/O and ADP/O ratios approaching the theoretical maximum of 3. Fig. III 14 shows the oxidation of malate by beetroot

Fig. III. 14. Polarographic tracing showing the oxidation of Tris malate by beetroot mitochondria. Assayed in 3.3 ml of medium containing 250 mM sucrose, 10 mM Tris-HCl buffer pH 7.2, 4 mM Tris-phosphate buffer pH 7.2, 6 mM $MgCl_2$, 3 uM cytochrome c, 15 mM Tris glutamate and 310 ug mitochondrial N added at Mw. Rates of oxygen uptake are expressed as mu moles O_2/minute.


Mw
Malate 20mM
63
ADP 0·22mM
295
59
ADP 0·17mM
273
56
ADP 0·17mM
262
49
ADP 0·25mM
250
1 min
O2 = 240 uM
O2=0
ADP/O R.C
2·6 5·0
2·5 4·9
2·7 5·3

mitochondria measured polarographically in the presence of 15 mM glutamate. ADP/O ratios of 2.5 - 2.7 and R.C. ratios of 4.9 - 5.3 were recorded. Malate oxidation measured in the presence of glucose, hexokinase and ADP has been found to progressively decrease with time (Wiskich and Bonner 1963, Wiskich et al 1964) and this was interpreted as oxaloacetate accumulation and inhibition of malate dehydrogenase. Glutamate prevented the progressive decrease in malate oxidation presumably by removing oxaloacetate by transamination (Krebs and Bellamy 1960, Wiskich et al 1964). The 15 mM glutamate included in Fig. III 14 did not entirely prevent the decrease of state 3 and state 4 malate oxidation rates with time.

8. Action of Inhibitors.

Potassium cyanide at a concentration of 0.9×10^{-4}M inhibited succinate and ascorbate plus TMPD oxidation by 98% in beetroot mitochondria.

HOQNO inhibited NADH and succinate oxidation by 100% at a concentration of 5×10^{-6}M.

Antimycin A inhibited succinate and malate oxidation by 100% at a concentration of 0.3 ug/ml.

Sodium malonate at 4.5 mM inhibited succinate oxidation by 90% but did not affect malate or NADH oxidation.

C. DISCUSSION:

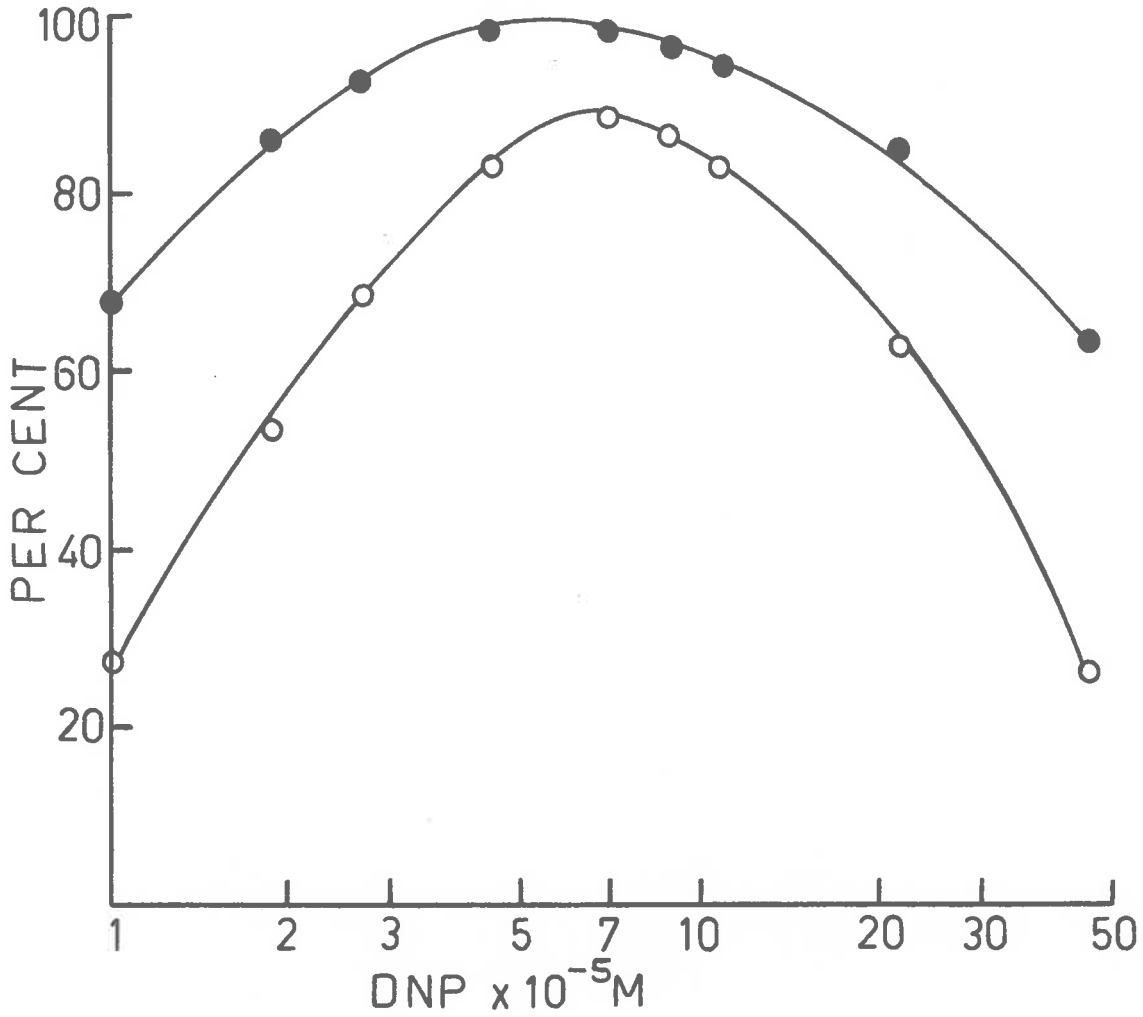
1. Respiratory Control.

Beetroot mitochondria were shown to oxidize succinate, malate,

Fig. III. 15. A semilogarithmic plot showing the effect of DNP on the rate of succinate oxidation by beetroot mitochondria. The mitochondria were incubated at 25°C in an oxygen electrode cell in a medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 3 mM Tris phosphate buffer pH 7.2, 5 mM $MgCl_2$ 3 uM cytochrome c, 8 mM Tris succinate and 184 ug mitochondrial N/ml. State 3 oxidation rates were produced by addition of 0.14 mM ADP.

● -- ●, DNP rate expressed as a percentage of the previous (second) state 3 oxidation rate (100%).

o -- o, DNP rate expressed as a percentage stimulation of the state 4 rate $\frac{\text{(DNP rate - state 4 rate)}}{\text{state 4 rate}} \times 100$


100
80
60
40
20
PER CENT
1
2
3
5
7
10
20
30
50
DNP x 10⁻⁵M

α-ketoglutarate, citrate, pyruvate and ascorbate plus TMPD. Maximum oxidation for each of these substrates did not occur unless a suitable phosphate acceptor and inorganic phosphate was present. On exhaustion of the phosphate acceptor the oxidation rate decreased, indicating respiratory control with coupling of oxidation to phosphorylation. A stimulation of mitochondrial substrate oxidation following ADP addition does not represent respiratory control unless it is established that ADP is rate limiting. Absence of respiratory control does not, however, reflect the capacity of mitochondrial phosphorylation and may be due in part to action of ATPase (Wiskich et al 1964). ATPase activity can be demonstrated with oligomycin which inhibits ATPase activity and oxidative phosphorylation without uncoupling (Lardy et al 1958). The effectiveness of oligomycin in inhibiting the ADP-stimulated succinate oxidation rate of beetroot mitochondria was shown in Fig. III 4. The oligomycin-inhibited oxidation rate was approximately 10% below the previous state 4 oxidation rate indicating that ATPase activity contributed insignificantly to the state 4 succinate oxidation rate (see Chapter 8). Oligomycin at this concentration (1 ug/ml) inhibited ATP formation and ATPase by 80-90% (Chapter 4). However, oligomycin did not inhibit the substrate-level phosphorylation associated with α-ketoglutarate oxidation (Fig. III 5.)

2. Action of DNP.

DNP stimulated state 4 or oligomycin-inhibited substrate oxidation of beetroot mitochondria (Fig. III 4). Fig. III 15 summarises a

series of experiments in which the DNP-stimulated oxidation rates were measured at different DNP concentrations. Succinate was the substrate and two ADP additions were made, providing two state 3 and two state 4 oxidation rates before DNP was added. The second state 4 oxidation rate was recorded for at least one minute before DNP addition. DNP-oxidation rates are expressed in Fig. III 15 both as the percentage of the previous (second) state 3 oxidation rate (100%) and as the percentage stimulation of the previous (second) state 4 oxidation rate, $\left(\frac{\text{DNP rate - state 4 rate}}{\text{state 4 rate}} \times 100\right)$.

In three separate experiments the optimum DNP concentration for maximum stimulation of oxidation was between 50 and 80uM. At low concentrations of DNP, additions of ADP further increased the rate of oxidation whereas at higher DNP concentrations, ADP additions had no effect. Thus at low concentrations of DNP the stimulation of oxidation was small due to incomplete coupling but at high concentrations the inhibitory effects of DNP on oxidation became evident. High levels of DNP were necessary for complete uncoupling of oxidative phosphorylation. A concentration of 100 uM DNP was required for minimal ATP formation by beetroot mitochondria oxidizing succinate in the presence of glucose, hexokinase and ADP. Thus DNP inhibits oxidation in beetroot mitochondria at effective uncoupling concentrations (Wiskich and Bonner 1963). This result could indicate why there have been few reports of DNP-stimulated oxidation in plant mitochondria (Hackett 1959). DNP rates of oxidation greater than state 3 oxidation rates cannot be expected unless phosphory-

lation of ADP is rate limiting, a condition which has not been demonstrated in plant mitochondria (Wiskich and Bonner 1963).

CCP uncoupled phosphorylation associated with succinate oxidation by beetroot mitochondria at a concentration of 1 uM.

3. Inhibition of Succinate and Malate Oxidation.

Beetroot mitochondria oxidized succinate with an initial inhibition of the oxidation rate. This initial inhibition has been observed previously in both plant (Wiskich and Bonner 1963, Bonner and Voss 1961), and animal mitochondria (Azzone and Ernster 1960, Chappell 1961, Pardee and Potter 1948). The initial inhibition of succinate oxidation in beetroot mitochondria was overcome by pre-incubation with ATP. Wiskich and Bonner (1963) and Chappell (1961) have suggested that succinate inhibition in potato and rat liver mitochondria was due to exaloacetate inhibition of succinate dehydrogenase. Oxaloacetate is a competitive inhibitor of succinate dehydrogenase (Pardee and Potter 1948). ATP may either dissociate an oxaloacetate-succinate dehydrogenase complex, or remove oxaloacetate completely by phosphoenol pyruvate carboxykinase activity (Pardee and Potter 1948, Chappell 1961). It has been shown (Mazelis and Vennesland 1957) that phosphoenol pyruvate carboxykinase is widely distributed in plant tissues and that the plant enzyme is specific for ATP. Wiskich and Bonner (1963) found that only adenine nucleotides were effective in overcoming oxaloacetate inhibition in sweet potato mitochondria, but in rat liver mitochondria inosine triphosphate was as effective as ATP (Chappell 1961). In rat liver

homogenates oxaloacetate inhibition of succinate oxidation was removed by incubation with glutamate (Krebs and Bellamy 1960). This was not observed in beetroot mitochondria or in potato mitochondria (Wiskich and Bonner 1963). The action of oxaloacetate in inhibiting succinate oxidation has been recently discussed by Greville (1966) and Klingenberg (1966) and arguments against the action of oxaloacetate as a competitive inhibitor of succinate dehydrogenase have been presented.

The progressive decrease in malate oxidation rate with time has also been attributed to inhibition caused by oxaloacetate accumulation (Wiskich and Bonner 1963, Wiskich et al 1964). Glutamate largely prevented this inhibition of malate oxidation in beetroot mitochondria (Fig. III 14). Very little of the recovered malate oxidation could have been due to glutamate oxidation which was not oxidized as such by beetroot mitochondria (Millard 1963), and the prevention of inhibition must have resulted from removal of oxaloacetate by transamination (Krebs and Bellamy 1960, Wiskich and Bonner 1963).

4. α-ketoglutarate Oxidation.

Substrate-level phosphorylation associated with α-ketoglutarate oxidation by beetroot mitochondria was shown to be rate-limiting during oxidation in the absence of ADP. The effects of oligomycin and DNP on α-ketoglutarate oxidation by avocado mitochondria were reported by Wiskich et al (1964) and similar experiments with beetroot mitochondria have been discussed. The DNP stimulation of α-ketoglutarate state 4 oxidation rate was shown to be due to the stimulation of adenosine tri-

phosphatase activity which could be inhibited by oligomycin.

5. NADH Oxidation.

NADH was oxidized slowly without respiratory control by beetroot mitochondria prepared under standard conditions from a brei of pH 7.2 - 7.6. Mitochondria prepared under acid conditions (brei pH of less than 7.0) oxidized NADH more rapidly (no respiratory control) while oxidizing succinate slowly with poor respiratory control. Intact animal mitochondria are generally found to be impermeable to externally-added NADH (Kaplan 1960), and exposure of these mitochondria to conditions increasing permeability allows entrance and oxidation of external NADH (Maley 1957). However, plant mitochondria can usually oxidize externally-added NADH and high respiratory control ratios with NADH have been reported for sweet and white potato mitochondria (Wiskich and Bonner 1963) and apple mitochondria (Jones et al 1964).

6. Ascorbate plus TMPD Oxidation.

The coupled oxidation of ascorbate plus 50 uM TMPD yielded P/O ratios significantly greater than 1.0 with rat liver mitochondria (Howland 1963, Tyler et al 1966) and P/O ratios of 1.0 or less with rat heart mitochondria (Tyler et al 1966). Addition of antimycin A, HOQNO, rotenone, or an increase in TMPD concentration to 300 uM was found to reduce the P/O ratios obtained with liver mitochondria oxidizing ascorbate to values close to 1.0, but had no significant effect on the P/O ratios obtained with heart mitochondria (Tyler et al 1966). Tyler et al (1966) concluded that the true value of the P/O ratio supported by the

ascorbate plus TMPD substrate system was close to and no greater than 1.0, and that P/O ratios in excess of 1.0 observed during ascorbate plus 50 uM TMPD oxidation in liver mitochondria by themselves and Howland (1963) were due to the simultaneous coupled oxidation of NAD-linked endogenous substrates.

The oxidation of ascorbate plus 50 uM TMPD by beetroot mitochondria was coupled to phosphorylation of ADP producing ADP/O ratios of 0.99 or less. Increasing the concentration of TMPD to 400 uM did not affect these ratios. HOQNO and antimycin A did not reduce the ADP/O ratios obtained by oxidation of ascorbate plus 400 uM TMPD by beetroot mitochondria. Oxidation (Fig. III 3) and ion uptake (Chapter 4) studies indicate that isolated beetroot mitochondria contain very little endogenous substrate.

7. Pyruvate Oxidation.

Pyruvate was oxidized by beetroot mitochondria only in the presence of a full complement of cofactors, including coenzyme A, TPP, and NAD, and in the presence of a primer acid (malate). Lieberman and Biale (1956b) showed that sweet potato mitochondria required TPP, NAD, AMP and a primer acid, but coenzyme A was not required for the low oxidation rates obtained. Walker and Beevers (1956) using castor bean mitochondria demonstrated pyruvate oxidation in the presence of NAD, ATP, coenzyme A and TPP with a number of different primer acids. The primer acid provides a condensing partner for acetyl-coenzyme A such that the two-carbon moiety resulting from the oxidative decarboxylation

of pyruvate can enter the Krebs cycle. Guinea-pig heart sarcosomes oxidized pyruvate very rapidly initially and the rate of oxidation decreased with time in the absence of added cofactors and without added primer acid (Davis 1965). Malate and α-ketoglutarate prevented the decline in pyruvate oxidation rate or restored the oxidation rate after it had declined to almost zero (Davis 1965). This effect was interpreted as indicating an initial depletion of acetyl-coenzyme A acceptor and replacement of this acceptor by addition of malate or some other Krets cycle intermediate (Davis 1965).

Plant mitochondria (e.g. beetroot, castor bean, Walker and Beevers 1956, and sweet potato, Lieberman and Biale 1956b) required a full complement of cofactors for pyruvate oxidation. This requirement may be due to removal of cofactors by solubilization or extraction during isolation of the mitochondria without altering the total capacity for pyruvate oxidation. In contrast animal mitochondria did not require added cofactors for maximum pyruvate oxidation rates (Davis 1965).

8. Cytochrome c Effect.

Cytochrome c has been shown to be extracted during isolation of beetroot mitochondria (Wiskich et al 1960). Additions of horse heart cytochrome c in final concentrations of 2-5uM to beetroot mitochondria sometimes increased, but often had no effect on succinate oxidation rates although added cytochrome c always increased ascorbate plus TMPD oxidation rates (Chapter 8). Cytochrome c at this concentration had no effect on respiratory control ratios or ADP/O ratios with any substrate

and cytochrome c was routinely added in all experiments unless specifically omitted.

D. CONCLUSIONS:

Isolated beetroot mitochondria oxidized α-ketoglutarate, malate, citrate, pyruvate, NADH, succinate and ascorbate plus TMPD. Respiratory control with coupling of oxidation to ADP phosphorylation was demonstrated for all substrates except NADH. The ADP/O ratios calculated for these substrates approached the theoretical maximal ratios (Chapter 1). Respiratory control ratios and state 4 oxidation rates indicate the strength of coupling between phosphorylation and oxidation. In beetroot mitochondria substrate-level phosphorylation associated with α-ketoglutarate oxidation was most strongly coupled and the electron transfer chain site 1 phosphorylation (malate oxidation) was also strongly coupled to oxidation. Phosphorylation at site 3 (ascorbate or cytochrome c oxidation) was very loosely coupled to oxidation. The phosphorylation efficiency was high, however, and the ADP/O ratios approached 1.0 with ascorbate.

CHAPTER FOUR.

MAGNESIUM AND PHOSPHATE UPTAKE

A. INTRODUCTION.

Brierley et al (1962) studied the uptake of phosphate and magnesium by beef heart mitochondria. Ion uptake depended either directly or indirectly on oxidative phosphorylation, requiring substrate and oxygen and being inhibited by inhibitors and uncouplers such as antimycin A, cyanide, 2-4 dinitrophenol and dicoumarol. However, oligomycin did not inhibit ion uptake,, Conditions allowing oxidative phosphorylation to proceed, (ADP, hexokinase and glucose) inhibited uptake, but the addition of oligomycin inhibited ATP formation and allowed ion uptake to proceed. ATP could not replace substrate in supporting ion uptake. Brierley et al (1962) suggested that $Mg_3(PO_4)_2$ was precipitated within the mitochondria and that H^+ released during the precipitation of this salt accounted for the H^+ increase in the external medium. They concluded that an intermediate of oxidative phosphorylation was involved in the active uptake of both phosphate and magnesium (Fig.18.)

Further investigation (Brierley et al 1963, 1963b, Brierley 1963) revealed that magnesium and phosphate accumulation in beef heart mitochondria could be supported by ATP in the presence of antimycin A or cyanide. This ATP-supported ion uptake was inhibited by oligomycin. Brierley et al (1964a) reported that small amounts of magnesium were bound

Table IV. 1. Effect of Centrifuging and Filtering on Substrate-Independent Ion Uptake by Beetroot Mitochondria.

Mitochondria were incubated at 25°C in the standard ion uptake medium (potassium phosphate) with 137 ug mitochondrial N/ml. Mitochondria were collected by sucking onto 1.2 u Millipore filters or by centrifuging at 0°C through 3 ml of 1 M sucrose for 6 minutes at 25,000 x g.

	Mg^{++} (mumoles/mg N)		Pi (mumoles/mg N)	
Minutes	Filtered	Centrifuged	Filtered	Centrifuged
3	2,810	1,030	400	110
9	3,250	1,030	420	110
14	3,250	1,030	420	110
22	3,500	1,300	430	130

by beef heart mitochondria by a substrate-dependent process in the absence of added phosphate and that H^+ was produced at the same time in the external medium. Similar investigations into manganese and phosphate uptake by rat liver mitochondria (Chappell et al 1963) have been discussed in Chapter 1. Magnesium and phosphate uptake by beetroot mitochondria will be described in this chapter and related to magnesium and phosphate uptake by beef heart mitochondria (Brierley et al 1962, 1963, 1963b 1964).

B. RESULTS.

1. Endogenous Levels and Substrate-Independent Uptake of Mg and Pi:-

Isolated beetroot mitochondria contained between 400 and 800 mu moles Mg^{++}/mg N and between 20 and 150 mu moles Pi/mg N. Very little of this was removed by washing the mitochondria by centrifuging through 1M sucrose as described in Chapter 2.

When beetroot mitochondria were suspended in a medium containing $MgCl_2$ or Pi but no substrate the Mg^{++} or Pi content of the mitochondria increased depending on the external condentration of the added ion (Figs IV 1 and 2). Table IV 1 shows the magnesium and phosphate contents of beetroot mitochondria after incubation in a medium containing 15 mM $MgCl_2$ and 3mM Pi in the absence of added substrate. The Mg^{++} and Pi contents of mitochondria separated from the medium by Millipore filtration were compared with the contents of mitochondria separatéd by centrifuging through 1M sucrose. Some of the magnesium and phosphate which entered passively was removed by washing the mitochondria, but much magnesium and

Table IV. 2. Substrate-Independent Ion Uptake by Mitochondria Incubated in 0 mM or 10 mM $MgCl_2$ or Potassium Phosphate.

Beetroot mitochondria were incubated at 25°C in a medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 4 uM cytochrome c and 345, 265 or 245 ug mitochondrial N/ml in Expts. 1, 2 and 3 respectively. 10 mM potassium phosphate buffer (pH 7.2) or 10 mM $MgCl_2$ were added where indicated. Mitochondria were separated by centrifuging.

	Minutes	Pi (mumoles/mg N)		Mg^{++} (mumoles/mg N)	
		+KPi	-KPi	$+MgCl_2$	$-MgCl_2$
Expt.1	6	105	17	1,730	640
	20	112	20	1,770	750
Expt. 2	5	286	89	1,650	605
	15	250	89	1,500	550
Expt. 3	5	244	108	2,000	412
	20	282	132	2,100	480

a little phosphate remained bound to the mitochondria. The mitochondria on the filters were washed with 2ml of cold 1M sucrose (Chapter 2.) but some medium was probably retained in the filter. Thus the differences between Mg^{++} and Pi contents of filtered and centrifuged mitochondria would not be due to washing losses along. A more precise idea of the extent of ion losses during washing was given in Table II 2 where the Mg^{++} and Pi contents of mitochondria separated by filtering and centrifuging were compared after incubation in the presence of substrate, 15mM $MgCl_2$ and 3mM Pi. The results were corrected for uptake in the absence of substrate thus eliminating the error of medium contaminating the filtered mitochondria and giving a more exact indication of ions lost by centrifuging. However, the ion loss in Table II 2. represents loss of ions taken up by a substrate-dependent process and not of passively moved ions as shown in Table IV 1.

Table IV 1. indicates that the passive movement of these ions into the mitochondria reached equilibrium before 3 minutes. Other experiments have shown that equilibrium was usually reached before 1 minute at this (15mM) concentration of $MgCl_2$ (Chapter 7).

Passive uptake of magnesium and phosphate is also shown in Table IV 2. The phosphate retained in washed mitochondria after incubation with 10mM phosphate was 2-6 times the endogenous Pi content. Magnesium levels also increased 2-5 times above the endogenous level when mitochondria were incubated in 10mM $MgCl_2$. However, the actual increase of passively moved Pi (200 mu moles/mg N) was small in comparison with the actual increase in magnesium content (1,500mu moles/mg N) although the mitochondria were incubated in 10mM of each ion.

Table IV. 3. Mg^{++} and Pi uptake by Beetroot Mitochondria Incubated with Phosphate but without Magnesium.

Mitochondria were incubated in a medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 3.2 mM Tris-phosphate buffer pH 7.2, 4 uM cytochrome c, 440 and 109 ug mitochondrial N/ml in Expts. 1 and 2, and where indicated 9 mM Tris succinate. Mitochondria were collected by centrifuging in Expt. 1, and by filtering in Expt. 2. Mg^{++}/Pi ratios were calculated after correcting for ion uptake in the absence of substrate.

Minutes	Mg^{++} (mumoles/mg N) + Substrate	Mg^{++} (mumoles/mg N) -Substrate	Pi (mumoles/mg N) +Substrate	Pi (mumoles/mg N) -Substrate	Mg^{++}/Pi
Expt. 1					
8	930	660	275	120	1.1
26	900	720	302	122	1.0
Expt. 2					
7	651	550	632	522	0.91
16	720	500	715	495	1.0
25	710	500	685	455	0.92

2. Substrate-Dependent Phosphate Uptake in the Absence of Magnesium:-

Phosphate was taken up in a substrate-dependent manner by beet-root mitochondria when no $MgCl_2$ was added. Table IV 3 shows that this phosphate uptake was extremely small and was accompanied by magnesium, also taken up by a substrate-dependent process. The magnesium content of the mitochondria in the absence of substrate was 500-700 mu moles/mg N (the usual endogenous level). Additional magnesium taken up (or bound) by the substrate-dependent process could have arisen from bound magnesium released by swollen or broken mitochondria. The Mg^{++}/Pi ratios varied between 0.9 and 1.1 after substrate-dependent ion uptake was corrected for uptake in the absence of substrate (Table IV 3.). These ratios indicate that one molecule of magnesium was taken up for each molecule of phosphate taken up in a substrate-dependent manner. The results do not indicate whether both ions were taken up simultaneously or whether one was moved in a substrate-dependent manner and the other ion followed passively. However, substrate-dependent phosphate uptake did not proceed beyond the point where the proportion of phosphate to magnesium taken up was approximately 1 : 1, although 3.2mM Pi and no $MgCO_2$ was present in the medium.

The substrate-independent phosphate level in Experiment 2 of Table IV 3 was much higher than that in Experiment 1 due to the different techniques of separating the mitochondria. However, the endogenous magnesium contents were approximately the same in both experiments in spite of the mitochondrial wash in Experiment 1, indicating that endogenous

Table IV. 4. Mg^{++} and Pi Uptake by Beetroot Mitochondria Incubated with Magnesium but without Phosphate.

Mitochondria were incubated in a medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 4 uM cytochrome c, 15 mM $MgCl_2$, 245, 255 and 355 ug mitochondrial N/ml in Expts. 1, 2 and 3 respectively and 11 mM Tris succinate where indicated. Mitochondria were collected by centrifuging and Mg^{++}/Pi ratios were calculated after correcting for ion uptake in the absence of substrate.

Minutes	Mg^{++} (mumoles/mg N) +Substrate	-Substrate	Pi (mumoles/mg N) +Substrate	-Substrate	Mg^{++}/Pi
Expt. 1					
6	1,950	1,789	127	98	5.9
20	2,280	2,100	134	110	7.4
Expt. 2					
6	2,120	1,880	122	89	7.3
20	2,400	2,180	120	89	7.1
Expt. 3					
6	2,090	1,250	55	17	22
20	1,810	1,030	63	20	18

Fig. IV. 1. The effect of $MgCl_2$ concentration on Mg^{++} and Pi uptake by beetroot mitochondria. 443 ug mitochondrial N/ml were incubated for 8 minutes in a medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 3.2 mM potassium phosphate buffer pH 7.2 and 4 uM cytochrome c. The mitochondria were collected by centrifuging through 3 ml of 1 M sucrose for 6 mins at 25,000 x g at 0°C.

With 11 mM Tris succinate:-

o — o Mg^{++} uptake

Δ — Δ Pi uptake

Without substrate:-

● — ● Mg^{++} uptake

▲ — ▲ Pi uptake.

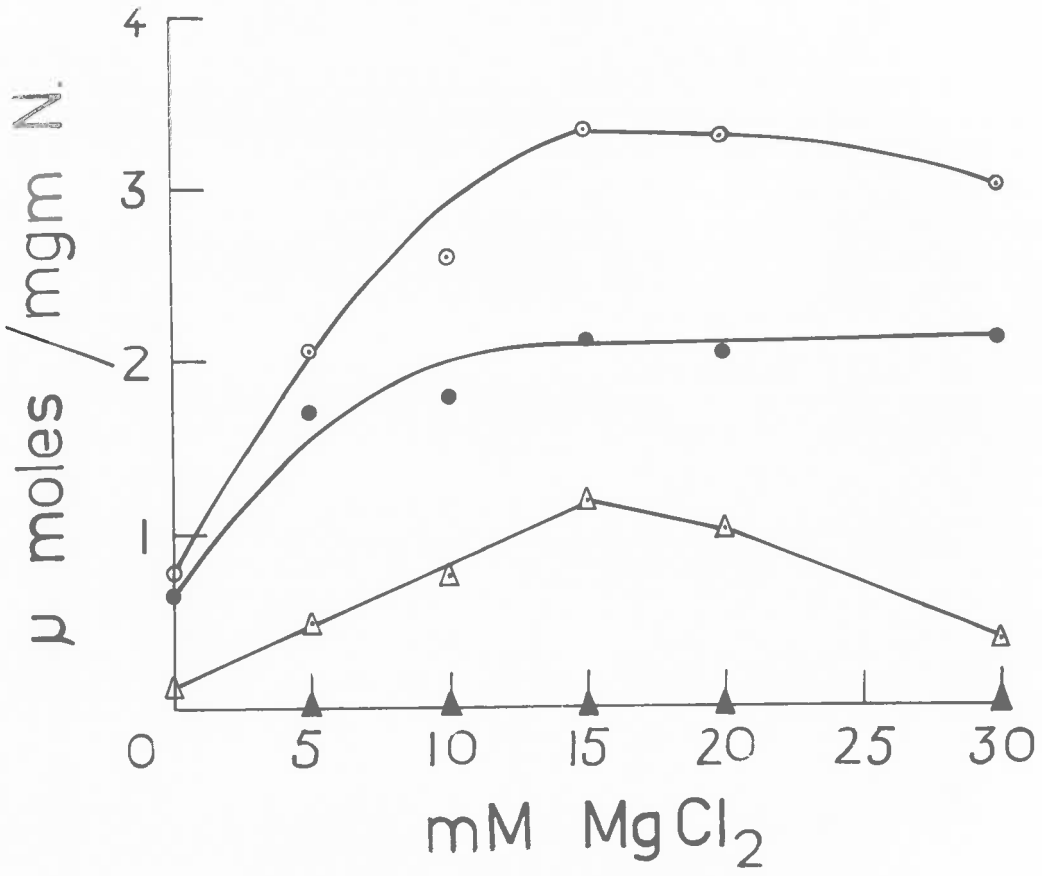

μ moles /mgm N.
4
3
2
1
0
5
10
15
20
25
30
mM MgCl2

Fig. IV. 2. The effect of $MgCl_2$ concentration on Mg^{++} and Pi uptake by beetroot mitochondria in a medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 3.1 mM Tris-phosphate buffer pH 7.2, 4 uM cytochrome c and 100 ug mitochondrial N/ml. The mitochondria were separated from the medium by suction onto 1.2 u Millipore filters.

With 7 mM Tris succinate as substrate;

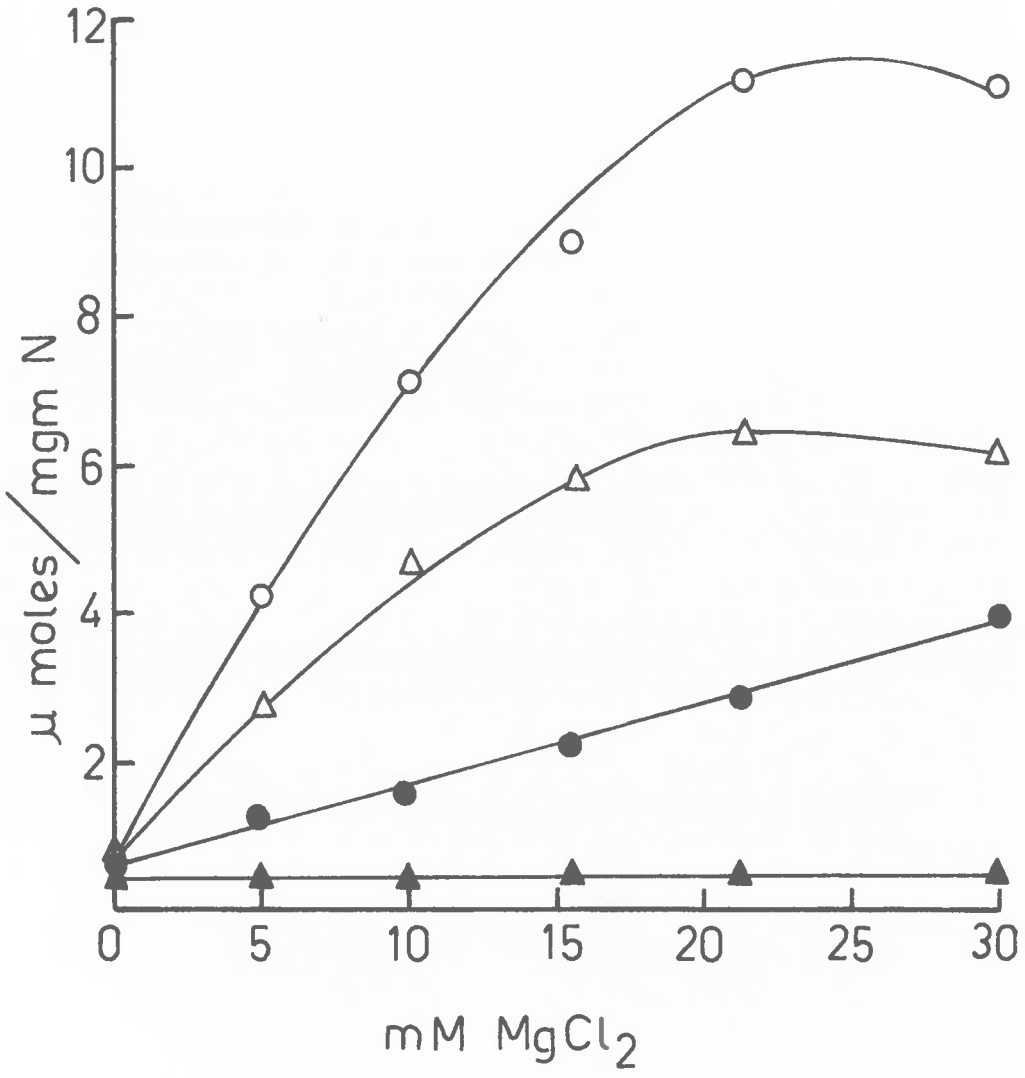
o -- o Mg^{++} uptake

Δ— Δ Pi uptake

Without substrate:

● -- ● Mg^{++} uptake

▲ — ▲ Pi uptake


12
10
8
6
4
2
µ moles / mgm N
0
5
10
15
20
25
30
mM MgCl2

magnesium was present in a firmly bound form.

3. Substrate-Dependent Magnesium Uptake in the Absence of Phosphate:-

Magnesium was taken up by a substrate-dependent process when no Pi was present in the medium. Table IV 4 indicates magnesium and phosphate levels of washed mitochondria after incubation with 15mM $MgCl_2$ but no added phosphate. The phosphate contents represent the usual endogenous levels of washed beetroot mitochondria. Very little phosphate, but a larger quantity of magnesium was taken up in a substrate-dependent manner (Table IV 4). The Mg^{++}/Pi ratios were calculated from corrected substrate-dependent magnesium and phosphate uptake and ranged from 5.9 to 22. These ratios indicate that magnesium taken up by a substrate-dependent process was not accompanied by phosphate in a 1 : 1 ratio unless phosphate was being recycled by some mechanism. Magnesium was taken up independently or accompanied by another anion (e.g. succinate or chloride).

4. Substrate-Dependent Magnesium and Phosphate Uptake:-

Massive uptake of magnesium and phosphate by beetroot mitochondria was obtained only in the presence of an oxidizable substrate and with both magnesium and phosphate in the incubation medium.

a. Effect of External Magnesium Concentration:- Figs IV 1 and 2 show the effect of $MgCl_2$ concentration on magnesium and phosphate uptake. Magnesium uptake in the absence of substrate increased linearly with increasing $MgCl_2$ concentration in Fig IV 2. In figure IV 1 substrate-independent magnesium uptake increased greatly as external $MgCl_2$ increased from 0 to 5mM but the uptake rate decreased as $MgCl_2$ was further increased. The mitochondria were separated from the medium by Millipore filtration

Fig. IV. 3. The effect of $MgCl_2$ concentration on substrate-dependent Mg^{++} and Pi uptake by beetroot mitochondria.

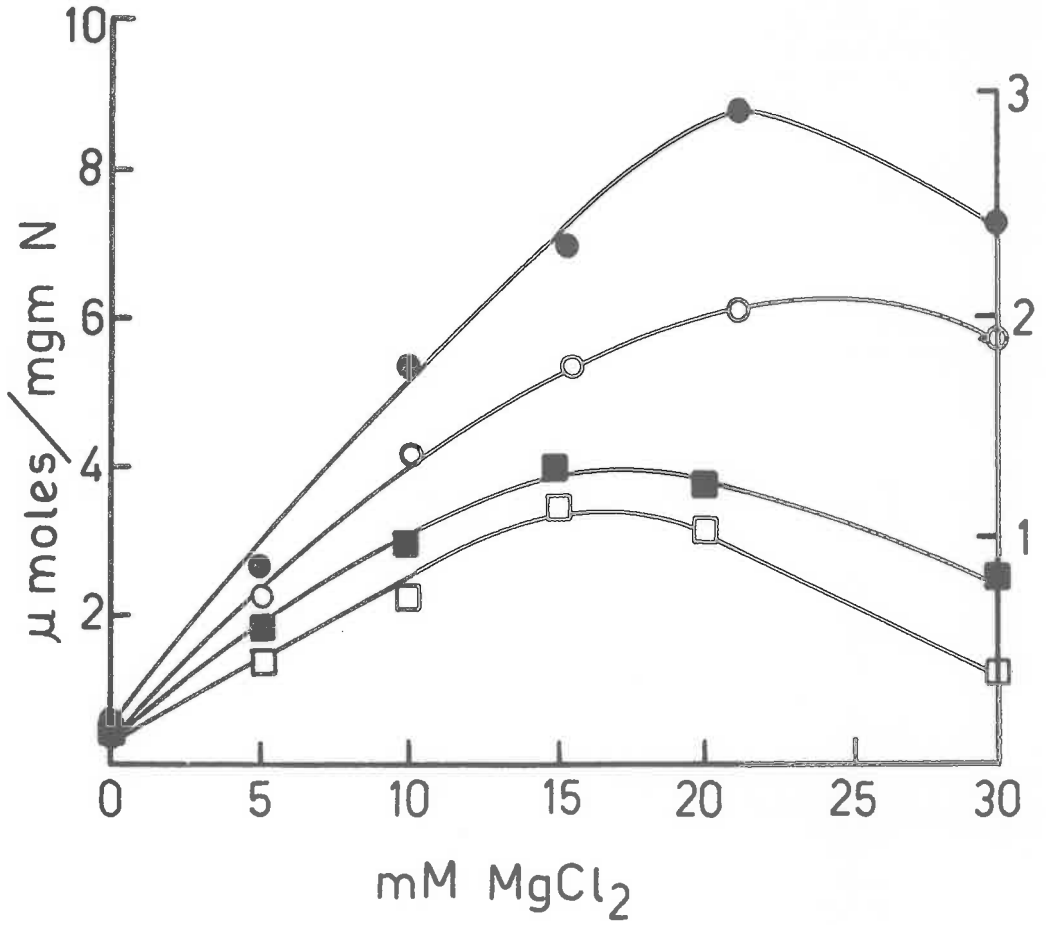
The mitochondria were incubated under the conditions described in Figs. IV. 2 and 1, and the Mg^{++} and Pi uptake in the presence of Tris succinate in these experiments has been corrected for uptake in the absence of substrate.

Experiment 1, where the mitochondria were separated by centrifuging through 3 ml of 1 M sucrose at 25,000 g for 6 mins:-

■ — ■ Mg^{++} uptake)
) using right axis
□ — □ Pi uptake)

Experiment 2, where the mitochondria were separated by suction onto 1.2 u Millipore filters.

● -- ● Mg^{++} uptake)
) using left axis.
o -- o Pi uptake)


10
8
6
4
2
μ moles/mgm N
3
2
1
0
5
10
15
20
25
30
mM MgCl2

in Fig IV 2, whereas in Fig IV 1 separation was achieved by layering the mitochondria and medium over 1M sucrose and centrifuging for 7 minutes at 0°C. Some magnesium would have been lost during centrifuging (Table IV 1) but the linear increase in magnesium uptake with external $MgCl_2$ concentration in Fig.IV 2 was probably due to retention of medium within the pores of the washed Millipore filter. Fig.IV 1 would represent magnesium binding with increasing $MgCl_2$ and most binding sites were saturated at 5 mM $MgCl_2$. Further $MgCl_2$ increase had little effect on amount of bound magnesium.

The substrate-independent phosphate levels in Figs. IV 1 and 2 were low after incubation in 3 mM phosphate and these phosphate levels were not affected by external $MgCl_2$ concentration.

Massive uptake of both magnesium and phosphate occured in the presence of an oxidizable substrate (Figs. IV 1 and 2). Errors connected with separating techniques, from retention of medium or removal of diffusable ions by washing, were eliminated by subtracting substrate-independent uptake from substrate-dependent uptake. This correction was applied to Figs. IV 1 and 2 and the results are shown in Fig.IV 3. Variation in the external $MgCl_2$ produced similar effects on substrate-dependent magnesium and phosphate uptake independent of the technique of separating the mitochondria from the medium. Maximum magnesium uptake occurred at 15-25mM external $MgCl_2$ and maximum phosphate uptake occurred at 15 mM $MgCl_2$ in Expt. 1 and at 20-30 mM $MgCl_2$ in Expt. 2 of Fig. IV 3. In both experiments ion uptake was inhibited by high $MgCl_2$ concentrations

(greater than 20mM). This inhibitory effect of high $MgCl_2$ could have been due to either the concentration of chloride ions competing with phosphate, or the high magnesium concentration which has a deleterious effect on oxidative phosphorylation and integrity of mitochondria (Purvis and Slater 1959). These effects will be discussed later (Chapters 6 and 7).

b. Magnesium/Phosphate Ratios:- The ratios of magnesium / phosphate obtained from substrate-dependent uptake by beetroot mitochondria varied between 1.1 and 1.6 and could indicate precipitation of $Mg_3(PO_4)_2$ or a similar salt within the mitochondria. The deposition of a salt within the mitochondria after substrate-dependent uptake of magnesium followed by passive entry of phosphate would reduce the concentration of free or bound magnesium in the mitochondria. Thus substrate-dependent uptake of magnesium need not imply uptake against an electrical or concentration gradient.

c. Substrate Requirement and Effect of Incubation Time:- Massive uptake of magnesium and phosphate by isolated beetroot mitochondria was dependent on the presence of an oxidizable substrate (Figs. IV 1 and 2). The substrate most commonly used was succinate, as either the sodium or Tris salt, but other substrates which were oxidized with coupled ADP phosphorylation by beetroot mitochondria were also tested for their ability to support ion uptake (see Chapter 8). The level of endogenous substrate in isolated beetroot mitochondria was usually low. In most experiments the substrate-independent levels of magnesium and phosphate

Fig. IV. 4. Mg^{++} and Pi uptake by beetroot mitochondria incubated in a medium containing 250 mM sucrose, 10 mM Tris-HCl buffer pH 7.2, 3.3 mM potassium phosphate buffer pH 7.2, 15 mM $MgCl_2$, 5 uM cytochrome c, 206 ug mitochondrial N/ml in Experiment 1 and 186 ug mitochondrial N/ml in Experiment 2. The mitochondria were separated from the medium in both experiments by centrifuging through 3 ml of 1 M sucrose at 25,000 x g for 6 mins at 0°C. With 8mM Tris succinate:-

● -- ● Experiment 1

Δ — Δ Experiment 2

Without substrate:-

o -- o Experiment 1

▲ — ▲ Experiment 2

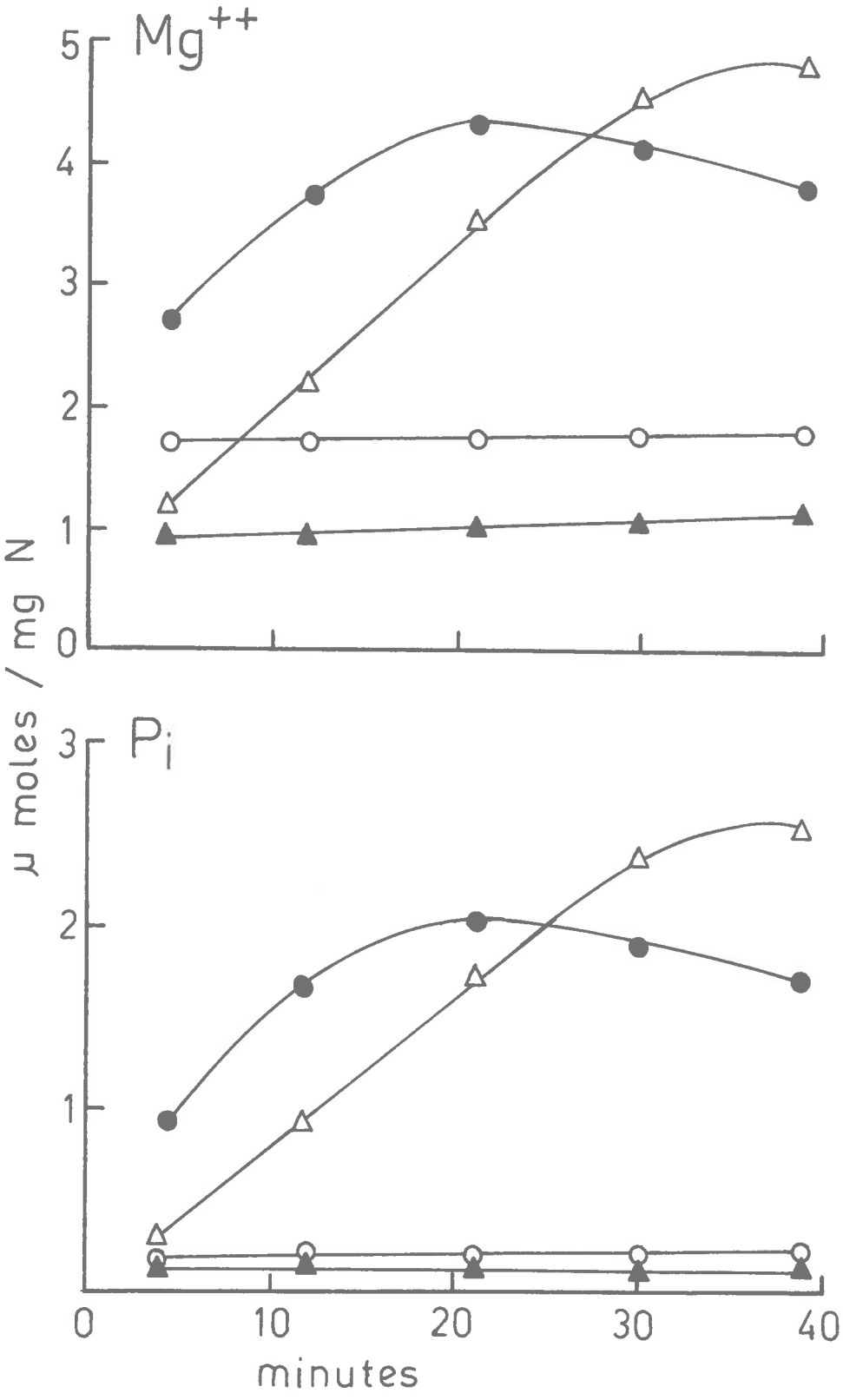

Mg++
Pi
μ moles / mg N
minutes
0
1
2
3
4
5
10
20
30
40

Table IV. 5. Effect of Uncouplers and Inhibitors on Mg^{++} and Pi Uptake.

Beetroot mitochondria were incubated for 18 mins. in the standard ion uptake medium (10 mM potassium phosphate) with 340 ug mitochondrial N/ml and 11 mM sodium succinate. Mitochondria were separated from the medium by centrifuging.

	Mg^{++}		Pi	
	mumoles/ mg N	% Inhibition	mumoles/ mg N	% Inhibition
Control	3,280	-	980	-
No Substrate	2,160	34	106	89
+ Antimycin A (ug/ml)	1,800	45	92	91
+ HOQNO (21.5 uM)	2,010	39	114	88
+ CCP (1.3 um)	2,010	39	107	89
+ DNP (1×10^{-4} M)	1,910	42	97	90

Table IV. 6. Effect of Cyanide on Mg^{++} and Pi Uptake.

Beetroot mitochondria were incubated for 15 mins. in the ion uptake medium (10 mM potassium phosphate) with 305 ug mitochondria N/ml and 11 mM sodium succinate. Mitochondria were separated from the medium by centrifuging.

	Mg^{++}		Pi	
	mumoles/ mg N	% Inhibition	mumoles/ mg N	% Inhibition
Control	3,200	-	580	-
Minus substrate	2,200	31	70	88
Plus KCN_4 (1×10^{-4} M)	2,050	36	75	87

were the same whether or not an inhibitor or uncoupler of oxidative phosphorylation was included. However, in a few experiments (Table IV 5) where the mitochondria were inefficiently washed during isolation some endogenous substrate was present and inhibitors and uncouplers reduced the magnesium and phosphate levels below the substrate-independent levels.

Figure IV 4 shows the effect of a 40 min. incubation period on ion uptake by two preparations of beetroot mitochondria. The mitochondria were isolated 5 days apart and used within 1 hour of isolation. The results emphasise the different magnitudes but similar overall effects obtained by treating two different mitochondrial preparations identically. Substrate-dependent ion uptake usually increased almost linearly for 15-20 minutes, after which the ion content changed very little for another 10-20 minutes. Longer periods of incubation usually resulted in a slow loss of ions already taken up. Incubation periods were generally of 10-25 minutes duration.

Substrate-dependent ion uptake by beetroot mitochondria has been shown to be inhibited by anaerobic conditions (Millard 1963).

d. Effect of Inhibitors and Uncouplers:- Substrate-dependent magnesium and phosphate uptake was inhibited by uncouplers of oxidative phosphorylation (DNP and CCP) and by inhibitors of electron transfer (antimycin A, HOQNO and cyanide) (Tables IV 5 and 6). The concentrations of these substances required to inhibit ion uptake were approximately the same as those required to inhibit substrate-oxidation or uncouple

Table IV. 7. Effect of Oligomycin on Ion Uptake and Glucose-6-Phosphate Formation.

Expt. 1. Beetroot mitochondria were incubated in the ion uptake medium (10 mM Tris phosphate) with 8 mM Tris succinate and 115 ug mitochondrial N/ml. The results have been corrected for uptake of ions in the absence of substrate.

Expt. 2. As for Expt. 1 with 13 mM glucose, 0.66 mM ADP and excess hexokinase.

Expt. 1	Mg^{++} mumoles/mg N 6 min.	21 min.	Pi mumoles/mg N 6 min.	21 min.
Control	3.3	9.2	2.5	7.5
Plus oligomycin (1 ug/ml)	3.3	9.9	2.6	7.7

Expt. 2	Glucose - 6 - Phosphate mumoles/mg N 6 min.	24 min.
Control	10.4	31.4
Minus Substrate	3.8	5.3
Plus oligomycin (1 ug/ml)	3.8	4.9

Table IV. 8. Effect of Oligomycin on Mg^{++} and Pi Uptake by Beetroot Mitochondria.

Mitochondria were incubated in an ion uptake medium (10 mM potassium phosphate) with 11 mM sodium succinate and 246 ug mitochondrial N/ml. The results have been corrected for uptake of ions in the absence of succinate.

	Mg^{++} mumoles/mg N		Pi mumoles/mg N	
	6 min.	20 min.	6 min.	20 min.
Control	270	930	210	910
Plus Oligomycin (1 ug/ml)	460	2000	400	1400

Fig. IV. 5. Mg^{++} and Pi uptake and glucose-6-phosphate formation by beetroot mitochondria incubated in a medium containing 250 mM sucrose, 10 mM Tris-HCl buffer pH 7.2, 5 mM potassium phosphate buffer pH 7.2, 15 mM $MgCl_2$, 5 uM cytochrome c, 20 mM glucose and 173 ug mitochondria N/ml. Mitochondria were separated from the medium by suction onto 1.2 u Millipore filters for ion uptake studies.

With 6 mM Tris succinate;

● -- ● Mg^{+} uptake

■ – ■ Pi uptake

x -- x glucose6-PO_4 formation.

With 6 mM Tris succinate plus 0.6 mM ADP and excess hexokinase;

◒ -- ◒ Mg^{++} uptake

◘ – ◘ Pi uptake

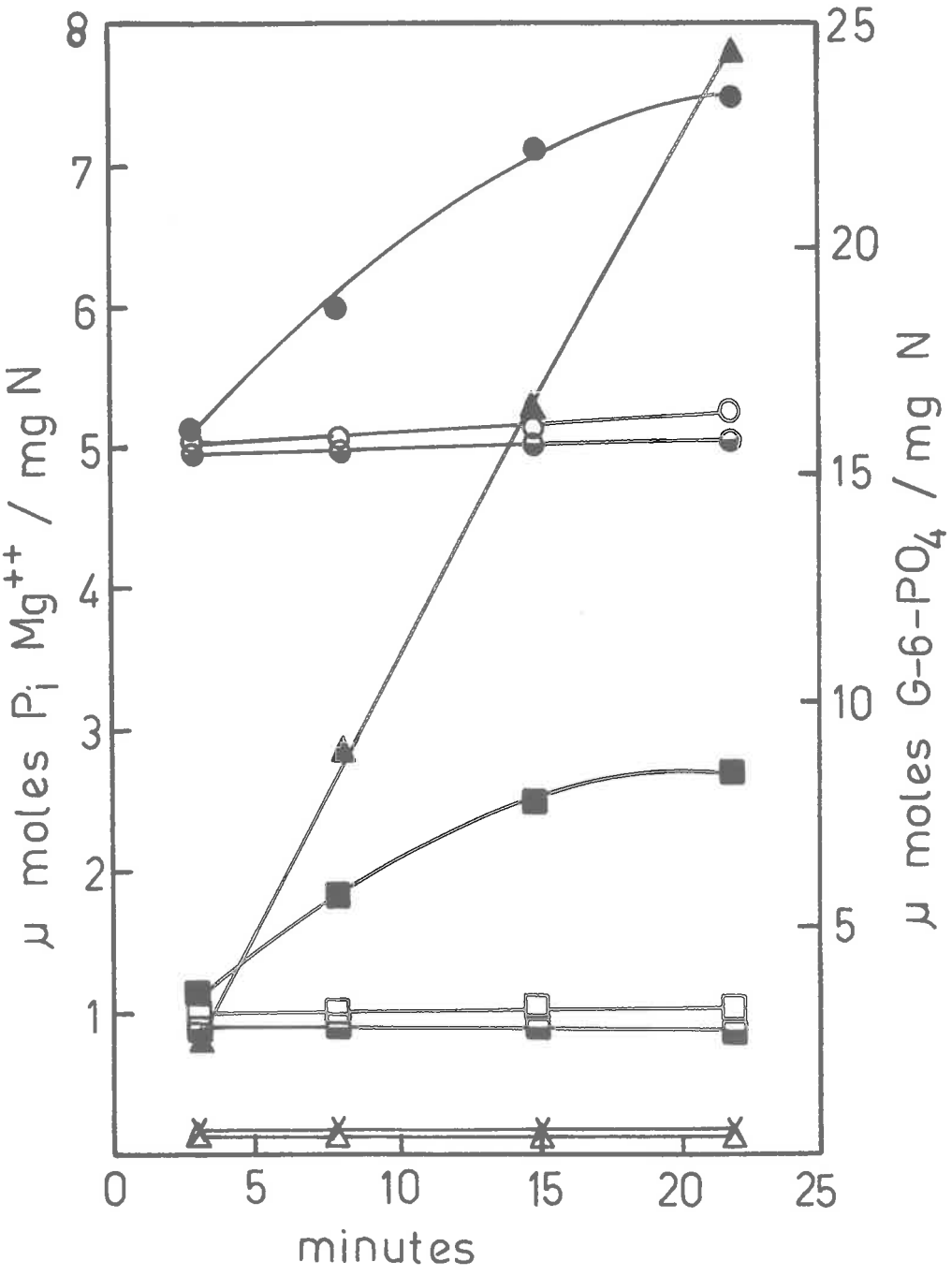
▲ – ▲ glucose-6-PO_4 formation

Without succinate:

o -- o Mg^{++} uptake

□ – □ Pi uptake

Δ — Δ glucose-6-PO_4 formation.


8
7
6
5
4
3
2
1
25
20
15
10
5
0
5
10
15
20
25
minutes
μ moles P_i Mg^{++} / mg N
μ moles G-6-PO_4 / mg N

oxidative phosphorylation (Chapter 3). Substrate-independent ion uptake was not corrected for in the calculations of percentage inhibition in Tables IV 5 and 6. The inhibitors and uncouplers were more effective in preventing magnesium uptake than the lack of oxidizable substrate in Table IV 5. This suggests the presence of some endogenous substrate.

Massive uptake of magnesium and phosphate by beetroot mitochondria was dependent on substrate and aerobic conditions, and inhibited by electron transfer inhibitors. Thus uptake was directly or indirectly dependent on the movement of reducing equivalents along the electron transfer chain. The inhibitory action of uncouplers suggests that oxidative phosphorylation is required for ion uptake.

e. Oligomycin Effect:- Oligomycin, a potent inhibitor of oxidative phosphorylation (Lardy et al 1958, Chapter 3) had no effect on, or stimulated, magnesium and phosphate uptake by beetroot mitochondria. Table IV 7 shows that oligomycin had no effect on magnesium and phosphate uptake while the same concentration completely inhibited glucose-6-phosphate formation. Since oligomycin prevents ATP (and glucose-6-phosphate) formation it appears that ATP as such is not directly involved in the mechanism of magnesium and phosphate uptake. In many experiments oligomycin stimulated magnesium and phosphate uptake by 20-100% (Table IV 8). The reaction of ion uptake to oligomycin depended on the mitochondrial preparation.

f. Effects of Oxidative Phosphorylation:- Fig. IV 5 shows that ADP in the presence of glucose and hexokinase allowed glucose-6-phosphate

Table IV. 9. Effect of Oligomycin on Mg^{++} and Pi Uptake Under Conditions for ATP formation.

Beetroot mitochondria were incubated at 25°C for 20 minutes in an ion uptake medium (10 mM Tris phosphate) with 7 mM Tris succinate, 38 ug mitochondrial N/ml and where indicated 0.7 mM ADP, 15 mM glucose, 14 ug dialysed hexokinase/ml. Oligomycin (1 ug/ml) was added in the (+) treatments. The results have been corrected for ion uptake and glucose-6-phosphate formation in the absence of substrate and ADP.

	Mg^{++} umoles/mg N		Pi umoles/mg N		Glucose-6-phosphate umoles/mg N	
	-	+	-	+	-	+
Control	10.5	10.4	8.3	8.0	0	0
+ ADP, Glucose, Hexokinase	0.7	11.6	0.5	8.8	53.4	11.5

Table IV. 10. Effect of Arsenate on Mg^{++} and Pi Uptake by Beetroot Mitochondria.

Mitochondria were incubated for 15 minutes in an ion uptake medium (minus phosphate) with 7 mM Tris succinate and 52 ug mitochondrial N/ml. Tris phosphate and disodium arsenate were added where indicated. The results have been corrected for the uptake of ions in the absence of substrate.

	Mg^{++} umoles/mg N		Pi + Arsenate umoles/mg N	
Treatment	Uptake	% Inhibition	Uptake	% Inhibition
+2.9 mM Phosphate	6.94	-	5.40	-
+2.9 mM Phosphate +4.9 mM Arsenate	1.90	73	1.66	69
+2.9 mM Phosphate +9.8 mM NaCl	3.60	48	2.85	47
+4.9 mM Arsenate	0	100	0.08	99

Table IV. 11. Effects of Arsenate on Mg^{++} and Pi Uptake and Glucose-6-Phosphate Formation.

Beetroot mitochondria were incubated for 15 minutes in an ion uptake medium (Tris phosphate) with 14 mM glucose, 0.7 mM ADP, excess dialysed hexokinase, 7 mM Tris succinate and 58 ug mitochondrial N/ml. Arsenate (disodium) and 1 ug/ml oligomycin were added where indicated. The results have been corrected for ion uptake and glucose-6-phosphate formation in the absence of substrate.

Treatment	Mg^{++} umoles/mg N	Pi umoles/mg N	Glucose-6-phosphate umoles/mg N
Control	0.30	0.23	3.93
+ Oligomycin	7.42	5.73	0.80
+4.9 mM Arsenate	0.40	0.22	2.17
+4.9 mM Arsenate + Oligomycin	2.20	2.00	0.60
+9.7 mM Arsenate	1.0	0.70	1.70

formation to occur but completely inhibited magnesium and phosphate uptake. These results suggest that magnesium and phosphate uptake competes with ATP formation and that under conditions allowing both to occur, oxidative phosphorylation (ATP formation) is the favoured process. Addition of oligomycin, under conditions suitable for both processes, inhibited ATP formation andallowed magnesium and phosphate uptake to occur (Table IV 9).

5. Arsenate Effects.

a. Ion Uptake:- Arsenate was analysed by the same method as phosphate (Marsh 1959) and thus arsenate uptake was not separable from phosphate uptake. Table IV 10 shows that 4.9 mM disodium arsenate inhibited magnesium and phosphate uptake by ~70% and 9.8mM sodium chloride inhibited ion uptake by 50%. Sodium inhibition of ion uptake will be discussed in Chapter 7. The extra inhibition must have been due to arsenate. Replacing phosphate by 4.9mM arsenate completely inhibited ion uptake (Table IV 10).

b. Glucose-6-phosphate Formation:- Table IV 11 shows the inhibition of ion uptake by conditions for ATP formation and the relief of ion uptake by oligomycin. Arsenate (4.9mM) inhibited glucose-6-phosphate formation by 45% but no ions were taken up. Oligomycin (with 4.9mM arsenate) relieved ion uptake to the arsenate-70%-inhibited level (Tables IV 10 and 11). Arsenate (9.7mM) inhibited glucose-6-phosphate formation by 55% and relieved ion uptake slightly (Table IV 11).

Table IV. 12. Effect of ATP on Substrate-Independent Mg^{++} and Pi Uptake by Beetroot Mitochondria.

Mitochondria were incubated for 10 minutes in an ion uptake medium (Tris phosphate) with 66, 136 and 101 ug mitochondrial N/ml in Expts. 1, 2 and 3 respectively and 1 ug/ml oligomycin and 14 mM Tris succinate where indicated. Disodium ATP (pH 6.8) was added in Expt. 1 and Tris ATP (pH 7.0) in Expts. 2 and 3. The mitochondria were collected by filtering.

	Mg^{++}		Pi	
	umoles/mg N	% Change	umoles/mg N	% Change
Expt. 1				
Control	3.32	-	0.64	-
+ Succinate	7.45	+124	3.77	+490
+ 2.9 mM ATP	3.26	-2	1.27	+98
+ 5.7 mM ATP	2.95	-11	1.55	+142
+ 5.7 mM ATP + Oligomycin	2.90	-13	1.48	+131
Expt. 2				
Control	2.60	-	0.55	-
+ Succinate	8.43	+224	5.91	+970
+ 6.4 mM ATP	1.62	-38	0.91	+64
Expt. 3				
Control	2.50	-	0.45	-
+ Succinate	6.30	+152	3.35	+650
+ 0.6 mM ATP	2.66	+6	0.71	+58

Table IV. 13. Effect of Tris ATP, Tris ADP and Tris HCl on Substrate-Independent Mg^{++} and Pi Uptake.

Beetroot mitochondria were incubated for 13 minutes in an ion uptake medium (Tris phosphate) with 87 ug mitochondrial N/ml, and 8 mM Tris succinate and 1 ug/ml oligomycin were added where indicated. ATP and ADP were added as Tris salts (pH 7.0). The mitochondria were separated from the medium by filtering.

	Mg^{++}		Pi	
	umoles/ mg N	% Change	umoles/ mg N	% Change
Control	3.56	-	0.86	-
+ Succinate	9.65	+171	5.70	+560
+3.0 mM Tris-HCl	2.59	-27	0.81	-5
+2.7 mM ATP	2.70	-24	1.15	+34
+2.7 mM ATP + Oligomycin	2.59	-27	1.20	+40
+3.0 mM ADP	2.47	-31	0.86	0
+3.0 mM ADP + Oligomycin	2.59	-27	0.96	+12

6. Adenine Nucelotide Effects on Ion Uptake.

a. Substrate Absent:- ATP did not support magnesium and phosphate uptake by beetroot mitochondria although significant changes occurred in the levels of mitochondrial magnesium and phosphate (Tables IV 12 and 13). These ATP effects have been compared with substrate-supported magnesium and phosphate uptake measured in the same experiments.

ATP markedly increased the phosphate contents of the mitochondria depending on the external ATP concentration and the nature of the ATP salt (Tables IV 12 and 13). Disodium ATP produced larger phosphate increases than the same concentration of Tris ATP. The ATP-induced phosphate levels were reached within 2 minutes and remained constant during the incubation period (Table IV 13). This suggests diffusion or binding (cf. Mg binding, Tables IV 1 and 2). ATP-supported phosphate uptake by beetroot mitochondria was not affected by oligomycin (Tables IV 12 and 13), in contrast to ATP-supported phosphate and magnesium uptake by beef heart mitochondria (Brierley et al 1963). Tris ADP with or without oligomycin did not affect substrate-independent phosphate levels in beetroot mitochondria (Table IV 13).

ATP may have diffused into or been bound by the mitochondria, and the terminal phosphate group may have been liberated on mitochondrial extraction in 5.5% $HClO_4$ in the presence of magnesium. The ADP phosphate group is less susceptible to acid attack. Alternatively ATP may have increased mitochondrial affinity for phosphate, or reduced or prevented phosphate leakage from the mitochondria by an oligomycin-insensitive

Fig. IV. 6 The effect of Tris ATP at a low concentration on Mg^{++} and Pi uptake by beetroot mitochondria incubated in a medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 3.1 mM Tris-phosphate buffer pH 7.2, 15.7 mM $MgCl_2$, 4 uM cytochrome c and 130 ug mitochondrial N/ml.

o -- o no addition

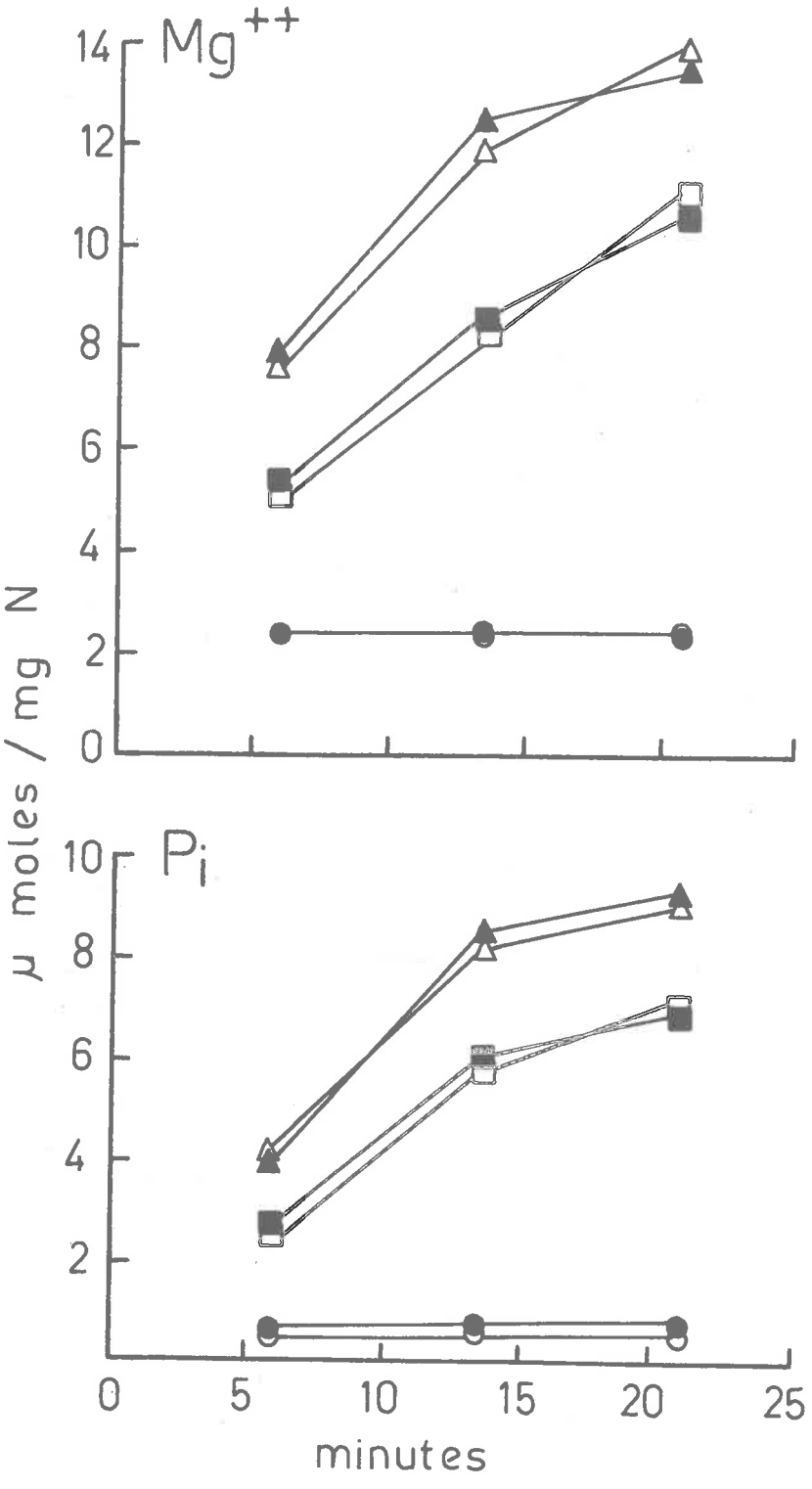
● -- ● with 0.6 mM Tris ATP

□ – □ with 7 mM Tris succinate

■ – ■ with 7 mM Tris succinate plus 1 ug/ml oligomycin

△ – △ with 7 mM Tris succinate and 0.6 mM Tris ATP

▲ – ▲ with 7 mm Tris succinate, 0.6 mM Tris ATP and 1 ug/ml oligomycin.


Mg++
Pi
μ moles / mg N
minutes
0
2
4
6
8
10
12
14
0
5
10
15
20
25

process. Oligomycin-insensitive adenine nucleotide binding associated with calcium and phosphate uptake has been reported for rat liver mitochondria (Carafoli et al 1965a, Carafoli and Lehninger 1964). Differences in the binding properties of disodium and Tris ATP could account for different effects on phosphate levels.

The ATP-induced increases in phosphate levels were accompanied by decreases in magnesium levels (Tables IV 12 and 13). Tris ATP inhibited substrate-independent magnesium levels to the same extent as Tris ADP and to a greater extent than disodium ATP (at the same concentrations). Oligomycin had no effect on these inhibitions (Tables IV 12 and 13). Tris HCl (3mM) inhibited the substrate-independent magnesium content of beetroot mitochondria to the same extent as 3mM Tris ATP and 3mM Tris ADP (Table IV 13). The inhibitions could be due to Tris replacing or displacing magnesium at the cation binding sites of the mitochondrial membranes. Sodium did not compete successfully with magnesium for the cation binding sites. Table VII (1)compares the effects of Tris - HCl and NaCl on substrate-independent magnesium uptake by beetroot mitochondria.

b. Substrate Present:-

(1) Low Concentrations of ADP and ATP:- Substrate-dependent magnesium and phosphate uptake was greatly stimulated by a low ATP concentration (0.6mM) and this increase was not affected by addition of oligomycin (Fig. IV 6). The stimulation cannot be explained by ATP-induced substrate-independent phosphate uptake (Fig. IV 6). This

Fig. IV. 7. The effect of Tris ATP and Tris ADP in low concentrations on Mg^{++} and Pi uptake by beetroot mitochondria incubated in a medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 3.1 mM Tris-phosphate buffer pH 7.2, 15.7 mM $MgCl_2$, 4 uM cytochrome c and 130 ug mitochondrial N/ml.

x -- x no addition

△ — △ with 7 mM Tris succinate

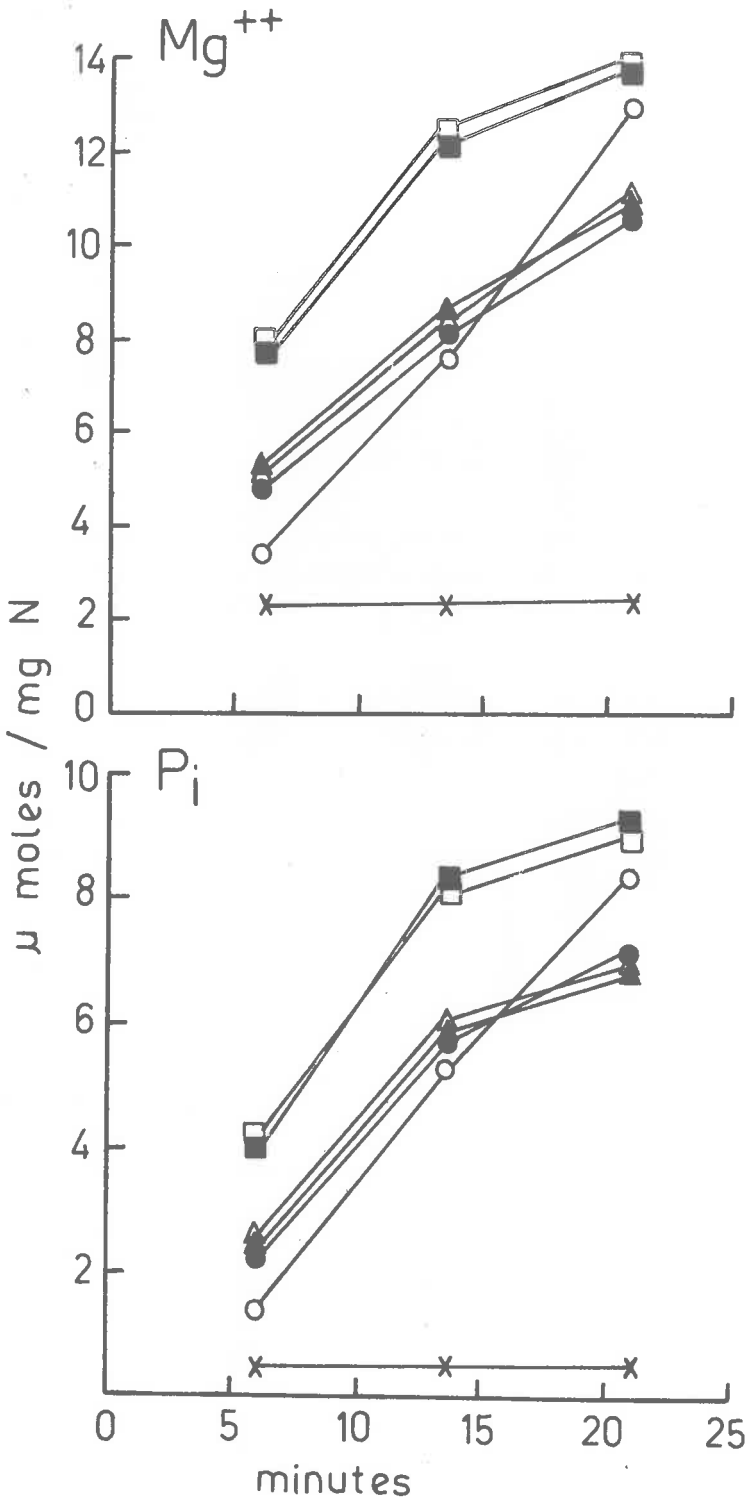
▲ — ▲ with 7 mM Tris succinate and 1 ug/ml oligomycin

□ — □ with 7 mM Tris succinate and 0.6 mM Tris ATP

■ — ■ with 7 mM Tris succinate, 0.6 mM Tris ATP and 1 ug/ml oligomycin

o -- o with 7 mM Tris succinate and 0.6 mM Tris ADP

● -- ● with 7 mM Tris succinate, 0.6 mM Tris ADP and 1 ug/ml oligomycin


Mg++
Pi
μ moles / mg N
minutes
0
2
4
6
8
10
12
14
0
5
10
15
20
25

concentration of Tris ATP could have stabilized mitochondrial structure and prevented leakage of ions after uptake, or stabilized a magnesium and phosphate precipitated salt, or stimulated the actual ion uptake rate. Any of these processes would produce the increased net ion uptake measured. An ATP-induced structural change need not be related to ATP-induced oligomycin-sensitive contraction of mitochondria (Neubert and Lehninger 1962). ATP binding or oligomycin-insensitive enzymic phosphorylation of a protein or lipid could induce a mitochondria membrane structural change. Carafoli et al (1965a) postulated such a process after investigating the oligomycin-insensitive ATP requirement for massive substrate-dependent calcium and phosphate uptake by rat liver mitochondria. At low concentrations of calcium (when the mitochondrial membrane was presumably intact) atractyloside inhibited uptake of both calcium and ATP and Carafoli et al (1965a) suggested that calcium uptake was inhibited because ATP uptake failed. Investigation into the effect of atractyloside on the ATP stimulation of substrate-dependent magnesium and phosphate uptake by beetroot mitochondria might indicate the site of this ATP action (surface or internal) although the site of action and mechanism of atractyloside is not definitely known (Chapter 1.). ATP was not requried for massive substrate-dependent magnesium and phosphate uptake by beetroot mitochondria in contrast to the situation with calcium and phosphate uptake by rat liver mitochondria (Carafoli et al 1965a).

Fig. IV 7 shows the effect of low concentrations of Tris ADP on substrate-dependent magnesium and phosphate uptake by beetroot mito-

Fig. IV. 8. The effect of Tris ATP at different concentrations on Mg^{++} and Pi uptake by beetroot mitochondria incubated in a medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 3 mM Tris-phosphate buffer pH 7.2, 15.7 mM $MgCl_2$, 4 uM cytochrome c and 130 ug mitochondrial N/ml.

x — x no addition

V — V with 6.4 mM Tris ATP

△ — △ with 7 mM Tris succinate

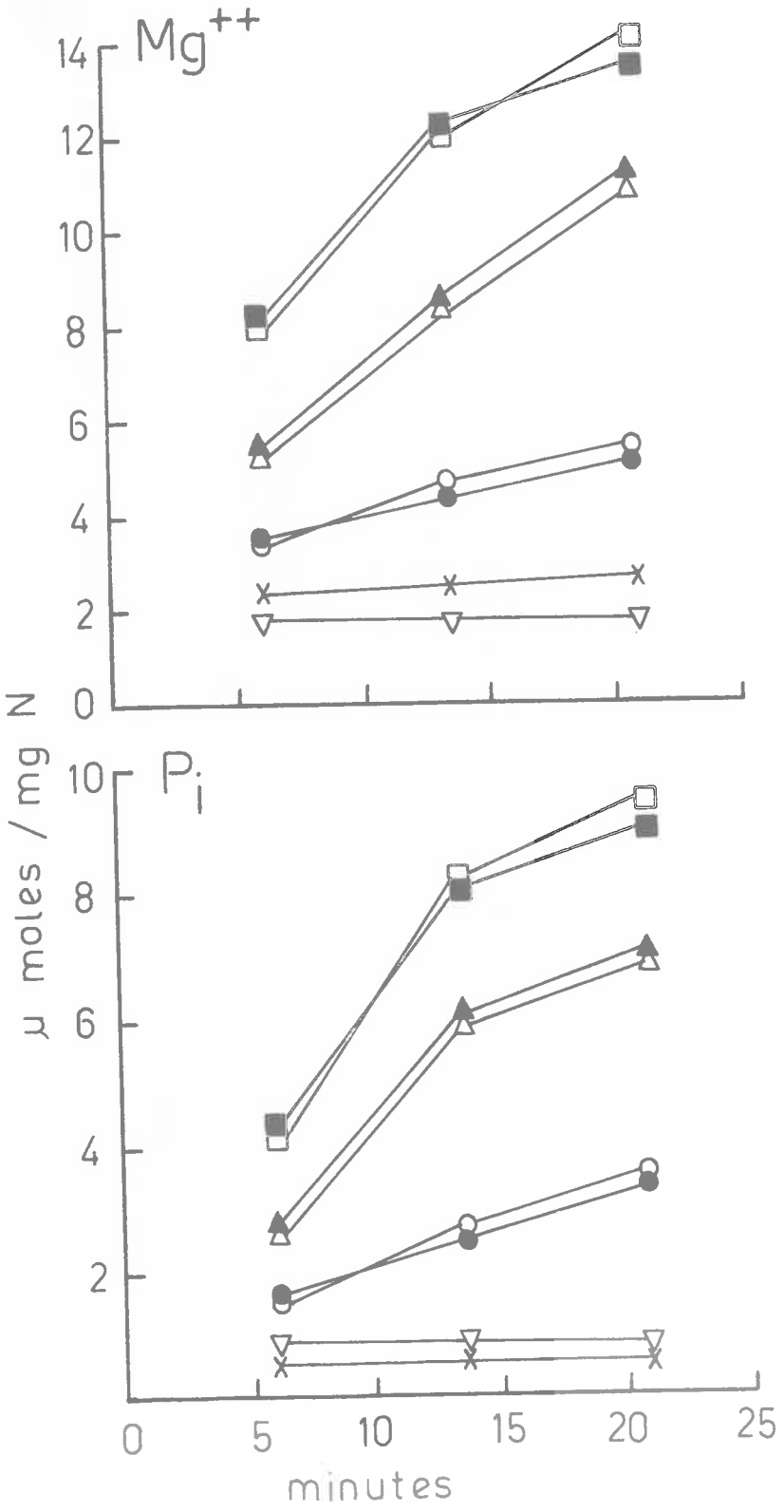
▲ — ▲ with 7 mM Tris succinate and 1 ug/ml oligomycin

□ — □ with 7 mM Tris succinate and 0.6 mM Tris ATP

■ — ■ with 7 mM Tris succinate, 0.6 mM Tris ATP and 1 ug/ml oligomycin

o — o with 7 mM Tris succinate and 6.4 mM Tris ATP

● — ● with 7 mM Tris succinate, 6.4 mM Tris ATP and 1 ug/ml oligomycin


Mg++
14
12
10
8
6
4
2
0
N
Pi
10
8
6
4
2
μ moles / mg
0
5
10
15
20
25
minutes

chondria. The inhibitory effect of ADP (with glucose and hexokinase) on ion uptake has already been reported (Fig. IV 5, Table IV 9). The succinate state 3 oxidation rate was measured polarographically (Chapter 3) and indicated that ATP formation would continue for approximately 3 minutes with the concentrations of ADP and mitochondria present in Fig. IV 7. The first sampling time was at 6 minutes in Fig IV 7 and some ADP-inhibition of magnesium and phosphate uptake was evident. Oligomycin, as expected (Table IV 9), prevented this initial ADP-inhibition of ion uptake. The rate of magnesium and phosphate uptake with ADP from 6 to 21 minutes was approximately the same as the ATP-stimulated rate of ion uptake (Fig. IV 7). Although oligomycin prevented the initial ADP-inhibition of ion uptake, it also prevented the subsequent ADP-stimulation of ion uptake (Fig IV 7). This suggests that the delayed ADP-stimulation of ion uptake was due to the ATP produced by the initial phase of oxidative phosphorylation.

(2) <u>High Concentrations of ADP and ATP (2-6 mM)</u>:- High concentrations of ADP and ATP strongly inhibited substrate-dependent magnesium and phosphate uptake by beetroot mitochondria. Fig. IV 8 shows that 6mM Tris ATP inhibited ion uptake by 55% at 13.30 minutes while 0.6mM Tris ATP stimulated uptake by 50% at the same time. The inhibition increased as ATP concentration increased. The ATP-inhibition of ion uptake was not affected by oligomycin (Fig IV 8.).

Brierley et al (1963, 1963b) reported a similar ATP-inhibition of magnesium and phosphate uptake by beef heart mitochondria. The inhibition was largely abolished by oligomycin and they concluded that the

Table IV. 14. Effect of Tris ATP, Tris ADP and Tris-HCl on Mg^{++} and Pi Uptake.

Beetroot mitochondria were incubated for 13 minutes in an ion uptake medium (Tris Phosphate) with 87 ug mitochondrial N/ml and 8 mM Tris succinate. 1 ug/ml oligomycin was added where indicated. The mitochondria were separated from the medium by filtering. Results were corrected for substrate independent ion uptake.

System	Mg^{++} umoles/mg N		Pi umoles/mg N	
	-Oligo mycin	+Oligo-mycin	-Oligo mycin	+Oligo mycin
Control	6.4	6.5	4.8	5.0
+3mM Tris-HCl	6.2	-	4.6	-
+3mM Tris ATP	0.8	4.3	0.3	2.9
+3mM Tris ATP	4.6	4.6	3.1	3.0

Table IV. 15. Effect of Tris EDTA on Mg^{++} and Pi Uptake.

Beetroot mitochondria were incubated in an ion uptake medium (Tris phosphate) with 7.9 ug mitochondrial N/ml and where indicated 7 mM Tris succinate. The mitochondria were separated from the medium by filtering.

	Mg^{++} umoles/mg N		Pi umoles/mg N	
	12 mins	19 mins	12 mins	19 mins
Control	3.70	3.64	0.82	0.70
+ 3 mM Tris EDTA	3.90	4.01	0.78	0.95
+ Tris Succinate	10.9	14.5	5.60	7.00
+ Tris Succinate + 3 mM Tris EDTA	4.85	5.0	1.50	1.77

ATP-inhibition was a result ATPase activity supplying ADP for the favoured oxidative phosphorylation process (Brierley et al 1963b). However, oligomycin did not completely abolish ATP-inhibition of ion uptake (Brierley et al 1963b, Fig.6.) and UTP inhibited uptake by an oligomycin-insensitive process. This suggests a more general nucleotide effect on ion uptake by beefheart mitochondria and can be related to the oligomycin-insensitive ATP-inhibition of substrate-dependent magnesium and phosphate uptake by beetroot mitochondria.

Table IV 14 shows that a high concentration (3mM) of Tris ADP completely inhibited magnesium and phosphate uptake. ATP formation would continue for approximately 20 minutes at this concentration of ADP. Oligomycin restored ion uptake to the inhibited level of uptake measured in the presence of 3mM ATP (Table IV 14). Tris-HCl (3mM) inhibited substrate-dependent ion uptake slightly (Table IV 14) but this did not account for the large adenine nucleotide-inhibition. Ion uptake in Table IV 14 was corrected for substrate-independent uptake and the effects of Tris on magnesium binding and ATP on phosphate levels (Table IV 13) were thus removed.

7. EDTA Effects

Table IV 15 shows that 3mM Tris-EDTA greatly inhibited (85%) substrate-dependent magnesium and phosphate uptake but did not affect substrate-independent ion levels. EDTA inhibition could be due to chelation of magnesium making it unavailable for uptake. Tris-EDTA (3mM) would remove one fifth of the added 15mM $MgCl_2$ by forming a 1:1

Table IV. 16. Effect of Bovine Serum Albumin, Cytochrome c, ADP and ATP on Mg^{++} and Pi Uptake.

Beetroot mitochondria were incubated for 10 minutes in an ion uptake medium (Tris phosphate) with 7.2 mM Tris succinate, 130 ug mitochondrial N/ml and where indicated 5 uM cytochrome c, 0.01% or 0.04% BSA, 0.7 mM Tris ADP and 0.6 mM Tris ATP. The results have been corrected for the uptake of ions in the absence of substrate.

	Mg^{++} umoles/mg N	Pi umoles/mg N
Control	5.3	4.7
+ Cytochrome c	6.1	5.3
+ BSA (0.01%)	6.3	5.4
+ BSA (0.04%)	5.4	4.7
+ Cytochrome c + BSA (0.01%)	6.7	5.7
+ Cytochrome c + ADP	7.6	6.6
+ Cytochrome c + ADP + BSA (0.01%)	7.5	6.4
+ Cytochrome c + ATP	10.1	7.5
+ Cytochrome c + ATP + BSA (0.01%)	9.9	7.3

chelation complex and could be expected to inhibit substrate-dependent ion uptake by 20-30% (Fig.IV 3). Chelation of EDTA to membrane-bound magnesium could also inhibit ion uptake by preventing uptake of this magnesium into the mitochondria. Mitochondrial membrane permeability could also be affected by EDTA. Brierley et al (1963b) reported that phosphate uptake by beef heart mitochondria was inhibited 25% by 1mM EDTA and 95% by 10mM EDTA.

The inhibitory effects of high ATP and ADP (+ oligomycin) on ion uptake can be related to the chelating properties of these compounds. The 30% inhibition by 3mM Tris ATP (Table IV 14) can be related to the 20-30% inhibition of ion uptake by a one fifth reduction in external $MgCl_2$ concentration (Fig. IV 3).

8. Effects of BSA, Cytochrome c and Dialysed Hexokinase.

Bovine serum albumin has been added to media for isolating plant mitochondria to prevent uncoupling caused by fatty acids released during homogenizing (Dalgarno and Birt 1963) and it has also been used to stabilize plant preparations (Crane 1957, Price and Thimann 1954, Throneberry 1961, Wiskich and Bonner 1963). Bovine serum albumin (0.1%) was used by Chappell et al (1963) to stabilize the system whereby manganese was taken up by rat liver mitochondria in the absence of added phosphate. Table IV 16 shows the effect of low BSA concentrations on the uptake of magnesium and phosphate by beetroot mitochondria. 0.01% BSA stimulated substrate-dependent ion uptake by 20% whereas 0.04% BSA had no effect on uptake.

Fig. IV. 9. Effects of Bovine serum albumin, dialysed hexokinase and 0.5 mM ADP on Mg^{++} and Pi uptake by beetroot mitochondria incubated in a medium containing 250 mM sucrose, 8.6 mM Tris-HCl buffer pH 7.2, 3 uM cytochrome c, 2.9 mM Tris-phosphate buffer pH 7.2, 12.6 mM $MgCl_2$ and 76 ug mitochondrial N/ml. Mitochondria were separated from the medium by Millipore filtration.

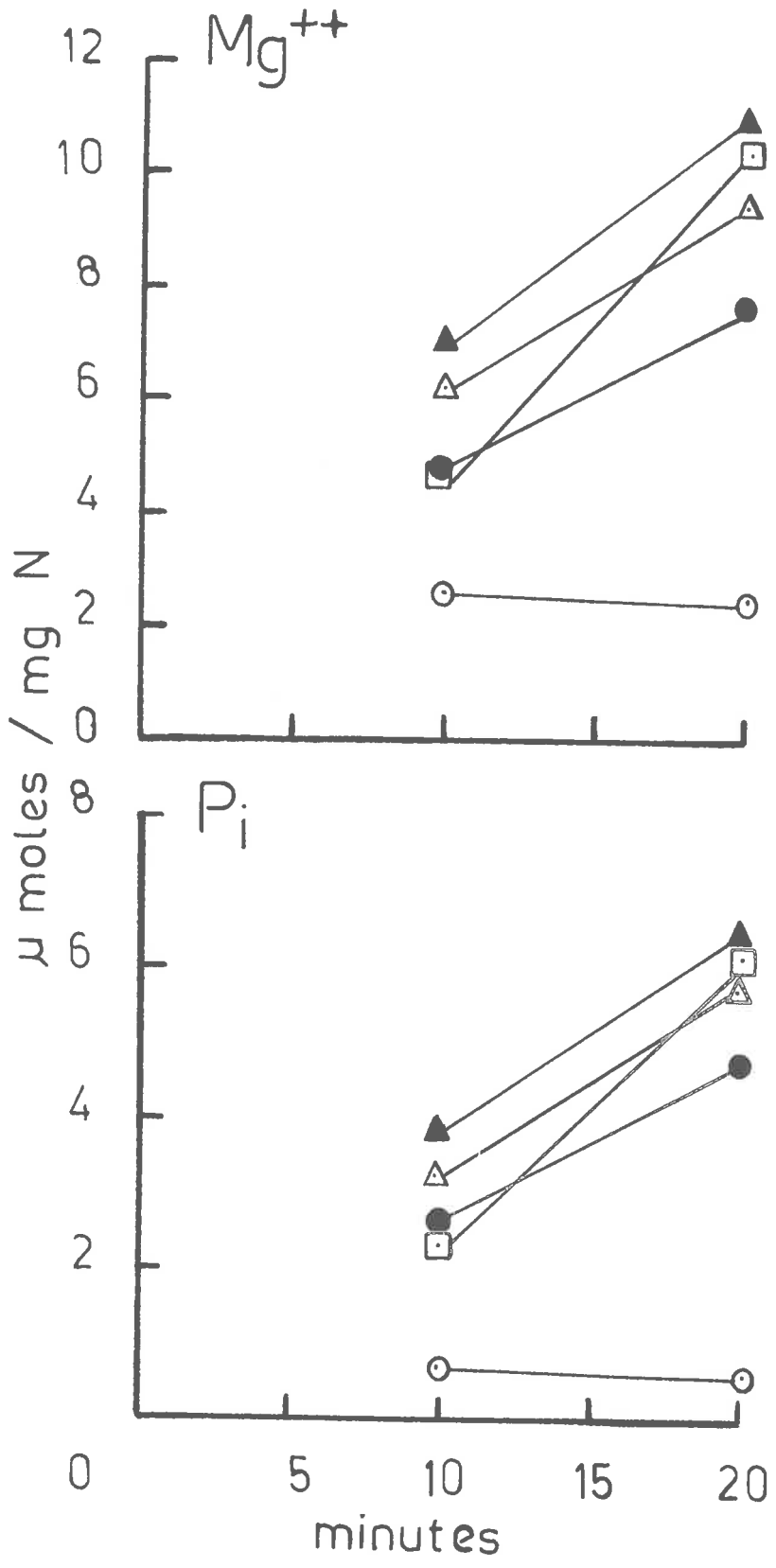
o -- o no addition

● — ● with 7 mM Tris succinate

△ — △ with 7 mM Tris succinate and 0.01% B.S.A.

▲ — ▲ with 7 mM Tris succinate and 14 ug dialysed hexokinase/ml in 0.01% B.S.A.

□ — □ with 7 mM Tris succinate and 0.5 mM $^{di}_{A}$ sodium ADP


Mg++
12
10
8
6
4
2
0
Pi
8
6
4
2
0
μ moles / mg N
5
10
15
20
minutes

Cytochrome c either slightly stimulated or had no effect on succinate oxidation by beetroot mitochondria and had no effect on RC ratios and ADP/O ratios (Chapter 3). Table IV 16 compares the effects of cytochrome c, BSA, 0.6mM ADP and 0.6mM ATP separately and together on the uptake of magnesium and phosphate by beetroot mitochondria. Dialysed hexokinase containing 0.03% glucose and 0.01% BSA stimulated magnesium and phosphate uptake by beetroot mitochondria (Fig IV 9). The effects of 0.01% BSA and 0.5mM ADP on uptake are also shown. Although BSA would have contributed to the stimulation produced by dialysed hexokinase, the additional stimulation must have been due to the hexokinase itself or other proteins added with this preparation (Sigma Type IV).

The stimulation of ion uptake by low concentrations of BSA, dialysed hexokinase and cytochrome c could be due to stabilization of mitochondrial membrane structure by protein. The occasional stimulation of succinate oxidation by cytochrome c (see Chapter 3), could be related to its effect on ion uptake. However, cytochrome c - stimulation of succinate oxidation was not always obtained whereas cytochrome c consistently produced a small stimulation of ion uptake. This stimulation was probably due to the addition of a stabilizing protein molecule rather than to any lack of cytochrome c as a constituent of the electron transfer chain. BSA and dialysed hexokinase did not stimuate succinate oxidation.

C. DISCUSSION.

1. Summary of Magnesium and Phosphate Uptake.

The general characteristics of magnesium and phosphate uptake by isolated beetroot mitochondria were.

(1) Magnesium was bound in the absence of substrate and most of this bound magnesium was retained during washing of the mitochondria. Phosphate also entered the mitochondria passively but was almost completely removed by washing.

(2) Substrate-dependent phosphate uptake occurred in the absence of added magnesium but this phosphate was taken up in a 1:1 ratio with contaminating magnesium.

(3) Substrate-dependent magnesium uptake occurred in the absence of added phosphate and the ratio magnesium : phosphate was usually between 5 and 10.

(4) Massive magnesium and phosphate uptake was dependent on substrate oxidation.

(5) Massive magnesium and phosphate uptake required the presence of both ions and maximum uptake was recorded with external $MgCl_2$ and phosphate concentrations of approximately 15mM and 3mM respectively.

(6) High concentrations of $MgCl_2$ depressed ion uptake.

(7) Magnesium and phosphate uptake was completely inhibited by inhibitors of the electron transfer chain e.g. antimycin A, KCN, HOQNO.

(8) Magnesium and phosphate uptake was completely inhibited

by uncouplers of oxidative phosphorylation e.g. DNP, CCP.

(9) Magnesium and phosphate uptake was not inhibited by oligomycin and was often stimulated by 10 - 100%.

(10) Massive magnesium and phosphate uptake produced magnesium/phosphate ratios of approximately 1.5.

(11) Conditions allowing ATP formation by oxidative phosphorylation completely inhibited magnesium and phosphate uptake.

(12) Oligomycin inhibited ATP formation and relieved the inhibition of magnesium and phosphate uptake.

(13) ATP did not support magnesium and phosphate uptake. Tris ATP inhibited substrate-independent magnesium binding and both Tris and disodium ATP increased substrate-independent phosphate levels. Tris ADP repeated the inhibition of magnesium content but did not stimulate phosphate levels. Oligomycin did not alter these effects.

(14) Low concentrations of ATP stimulated substrate-dependent magnesium and phosphate uptake. Oligomycin did not affect this stimulation.

(15) Low concentrations of ADP stimulated substrate-dependent magnesium and phosphate uptake after an initial inhibition. Oligomycin abolished both the initial inhibition and the subsequent stimulation of uptake by ADP.

(16) High concentrations of ATP inhibited substrate-dependent magnesium and phosphate uptake. Inhibition increased with increasing ATP concentration and was not affected by oligomycin.

(17) High concentrations of ADP completely inhibited ion uptake as established previously (11). Oligomycin partially relieved this inhibition.

(18) Arsenate did not replace phosphate in supporting substrate-dependent magnesium uptake and arsenate inhibited magnesium and phosphate uptake.

2. Endogenous Ion Levels.

The endogenous magnesium content of isolated beetroot mitochondria (500-700 mu moles/mg N) was higher than that reported for beef heart mitochondria (250 mu moles/mg N, Brierley et al 1964a). These levels could reflect the isolating procedures, the binding capacities of the mitochondria or the amount of magnesium available in beetroot and beef heart cells. Endogenous magnesium was firmly bound to the beetroot mitochondria and was not removed by centrifuging through 1M sucrose for 6 minutes at $0^{\circ}C$ and then rinsing several times with cold 1M sucrose. The endogenous magnesium of beef heart mitochondria was also firmly bound and not removed by repeated sucrose washings (Brierley et al 1964a). Approximately 1,000 - 2,000 mu moles magnesium/mg N were retained in washed beetroot mitochondria after incubation in 10-15mM $MgCl_2$ whereas washed beef heart mitochondria retained 60 mu moles magnesium/mg protein (380 mu moles/mg N) after incubation in 15-17mM $MgCl_2$. Again this could reflect different magnesium binding abilities of the two mitochondrial types.

3. Magnesium or Phosphate Uptake.

Substrate-supported uptake of both magnesium and phosphate took place when beetroot mitochondria were incubated with phosphate but without magnesium and the Mg/Pi ratios were close to 1. Substrate-supported

magnesium uptake occurred in mitochondria incubated with magnesium but without phosphate. Some phosphate was also taken up but the magnesium / phosphate ratios were high (between 5 and 20). These results indicate that magnesium taken up in a substrate-dependent manner was not accompanied by phosphate in a 1:1 ratio. Phosphate could have been recycled or another anion (succinate or chloride) taken up with the magnesium.

Brierley et al (1964c) reported that magnesium was taken up by a substrate-dependent process by beef heart mitochondria incubated with $MgCl_2$ but without phosphate. Chappell et al (1963) found that respiration-dependent manganese uptake by rat liver mitochondria occurred in the absence of phosphate and that respiration-independent uptake of phosphate could be induced after manganese uptake. They concluded that cation uptake was the first step in divalent cation and phosphate uptake. (Chappell et al (1963) and Brierley et al (1964c) demonstrated H^+ production during substrate-dependent uptake of manganese or magnesium in the absence of phosphate. Both H^+ production and cation uptake were inhibited by antimycin A and DNP but were not affected by oligomycin. Manganese uptake and pH changes were reversed by DNP in the absence of phosphate (Chappell et al (1963). If cation uptake is the first step in ion uptake then H^+ production must be either the cause or result of cation uptake.

4. Massive Magnesium and Phosphate Uptake.

Massive magnesium and phosphate uptake by beetroot mitochondria required the presence of both ions and a substrate in the incubation

medium. Anaerobic conditions and respiratory chain inhibitors (HOQNO, KCN and antimycin A) inhibited ion uptake, indicating that passage of electrons along the respiratory chain was essential for ion uptake. Electron movement along the entire chain was not required and ascorbate in the presence of TMPD supported massive magnesium and phosphate uptake by beetroot mitochondria (see Chapter 8) and by beef heart mitochondria (Brierley and Murer 1964).

Uncouplers of oxidative phosphorylation (DNP and CCP) inhibited ion uptake in beetroot and beef heart mitochondria and Brierley et al (1962) suggested that an intermediate of oxidative phosphorylation energised ion uptake (Fig.I 8). Oligomycin did not inhibit substrate-supported ion uptake indicating that ATP as such was not involved in the uptake process.

Magnesium and phosphate uptake in beetroot mitochondria was completely inhibited under conditions allowing ATP formation (ADP + glucose + hexokinase) and oligomycin inhibited oxidative phosphorylation and permitted ion uptake to proceed. Brierley et al (1962, 1963b) reported similar effects with beef heart mitochondria and suggested that ATP formation and ion uptake competed for the same high-energy intermediate of oxidative phosphorylation (Fig.I 8). ATP formation was the favoured process under competitive conditions.

ATP could not replace substrate in supporting magnesium and phosphate uptake by beetroot mitochondria. Brierley et al (1963b) reported that magnesium and phosphate uptake by beef heart mitochondria was

supported by substrate or less effectively byATP. Substrate-supported uptake was sensitive to antimycin A and cyanide and insensitive to oligomycin while ATP-supported uptake was insensitive to antimycin A and cyanide and sensitive to oligomycin. The failure of ATP to support magnesium and phosphate uptake in beetroot mitochondria could be due to a labile or inefficient ATP-breakdown system which produces "energy" for ion uptake. ATP breakdown by beetroot mitochondria was very slow under conditions for ion uptake (Chapter 6).

5. $Mg_3(PO_4)_2$ Precipitation.

Brierley et al (1962) suggested that $Mg_3(PO_4)_2$ was precipitated within beef heart mitochondria during massive substrate-dependent magnesium and phosphate uptake. They measured magnesium/phosphate ratios of 1.5 and reported production of H^+ in the external medium and rise in alkalinity within the mitochondria. These results were consistent with $Mg_3(PO_4)_2$ precipitation. Magnesium/phosphate ratios of approximately 1.5 were measured in beetroot mitochondria and could indicate precipitation of $Mg_3(PO_4)_2$ or a similar salt within the mitochondria following massive ion uptake.

Electron micrographs of animal mitochondria incubated with calcium, strontium or barium and phosphate have shown electron dense deposits within the mitochondria (Lehninger et al 1963, Brierley and Slautterback 1964, Peachey 1964, Greenawalt and Carafoli 1966). These deposits suggest precipitation of divalent cation-phosphate complexes within the mitochondria.

Electron dense deposits were not observed after massive uptake of magnesium or manganese and phosphate (Brierley and Slautterback 1964, Peachey 1964). Brierley and Slautterback (1964) calculated that 80% of accumulated magnesium and phosphate in beef heart mitochondria was lost from the mitochondria during fixation and Peachey (1964) suggested that magnesium and manganese were not dense enough to be distinguished against the dark background of the mitochondrial matrix and membranes. Electron dense deposits were not observed in beetroot mitochondria after massive uptake of phosphate with either calcium or magnesium (Chapter 2).

6. Arsenate.

Arsenate could be expected to replace phosphate in supporting divalent cation uptake by both the classical and chemiosmotic mechanisms (Chapter 1). Chappell et al (1963) showed that phosphate was taken up passively after substrate-dependent manganese uptake into rat liver mitochondria. Arsenate could be expected to replace phosphate in passive movement into the mitochondria or exchange for OH^- (Chappell and Crofts 1966) and in subsequent precipitation with the cation. Chappell et al (1963) reported that arsenate could replace phosphate in supporting manganese uptake by rat liver mitochondria. However, arsenate produced rapid swelling with calcium and no ion uptake occurred (Chappell et al 1963). Arsenate could not replace phosphate in supporting magnesium uptake by beef heart mitochondria (Brierley et al 1963b) or by beetroot mitochondria. Furthermore, arsenate inhibited magnesium and phosphate uptake by beef heart (Brierley et al 1963b) and beetroot mitochondria.

These effects could be related to permeability, structural and volume changes brought about by the action of arsenate on these mitochondria.

D. CONCLUSIONS.

Magnesium and phosphate were taken up by beetroot mitochondria by a substrate-dependent process closely associated with oxidative phosphorylation. This ion uptake was similar in magnitude and properties to the massive magnesium and phosphate uptake by beef heart mitochondria (Brierley et al 1962, 1963b). The characteristics of magnesium and phosphate uptake by beetroot mitochondria were consistent with ion uptake mechanisms based on the classical chemical and chemiosmotic hypotheses of oxidative phosphorylation. (Brierley et al 1962, 1963, Chance 1965, Chappell 1966, Mitchell 1966). These mechanisms were discussed in detail in Chapter 1.

CHAPTER FIVE

CALCIUM AND PHOSPHATE UPTAKE.

A INTRODUCTION.

1. High Calcium Concentrations.

Most investigations into uptake of divalent cations and phosphate by mitochondria have been concerned with calcium. De Luca et al (1961, 1962), Vasington and Murphy (1962), Lehninger et al (1963), Carafoli et al (1964), and Brierley et al (1964b) have described the general requirements for massive uptake of calcium and phosphate by isolated animal mitochondria. The calcium concentrations (2-4mM) used in these studies completely uncoupled oxidative phosphorylation and massive calcium uptake required substrate, ATP, phosphate and magnesium. ADP could replace ATP in part (Vasington and Murphy 1962), but Carafoli et al (1964) reported that ATP in the presence of a hexokinase trap completely inhibited calcium uptake. Calcium and phosphate uptake was inhibited by cyanide, azide, DNP and EDTA, but not by oligomycin. In general, high concentrations of monovalent cations had relatively little effect on massive calcium and phosphate uptake (Vasington and Murphy 1962, Carafoli et al 1964) in contrast to their strong inhibition of magnesium and phosphate uptake.

Lehninger et al (1963) reported that calcium and phosphate were taken up in the absence of substrate when the ATP concentration was increased to 15mM. Oligomycin inhibited this ATP-supported uptake.

Brierley et al (1963, 1964b) reported that calcium and phosphate uptakeby fresh beef heart mitochondria was supported by ATP in an oligomycin-sensitive process or by substrate plus ATP. Calcium was taken up with substrate alone but was retained for very short periods in the absence of ATP. Carafoli et al (1964) and Brierley et al (1964b) reported that H^+ was released into the medium during calcium and phosphate uptake and attributed this to precipitation of a complex within the mitochondria and to ATPase activity.

The anion deficit measured during calcium and phosphate uptake by rat liver mitochondria (Carafoli et al 1964) was suggested by Carafoli and Lehninger (1964) and Carafoli et al (1965a) to be a result of adenine nucleotide binding during ion uptake. Adenine nucleotide binding was dependent on calcium uptake and was inhibited by DNP and CCP but not by oligomycin. Carafoli et al (1965a) suggested that the ATP (or ADP)-requirement of calcium and phosphate uptake was due to stabilization of the precipitated calcium phosphate complex by bound adenine nucleotide. Calcium causes enzymic formation of free fatty acids in mitochondria during calcium-induced swelling, and ATP produces contraction of calcium-swollen mitochondria and disappearance of free fatty acids (Wojtczak and Lehninger 1961). Chappell et al (1963) suggested that the requirement for ATP during respiration-dependent calcium uptake was related to the prevention of calcium-induced mitochondrial swelling by ATP chelation of free calcium. However, other chelating nucleotides did not support calcium uptake (Carafoli et al 1965a).

Vasington (1963) has compared calcium uptake by rat liver mitochondria with uptake by digitonin fragments of these mitochondria. The fragments (which were capable of carrying out oxidative phosphorylation) took up calcium by a substrate-dependent, phosphate-requiring process which was inhibited by magnesium, ATP and ADP. Inhibitors and uncouplers inhibited, but oligomycin had no effect on calcium uptake. Monavalent cations had greater inhibitory effects on calcium uptake by fragments than on uptake by intact mitochondria. The calcium/oxygen ratios were 1-2 in fragments and 4 in intact mitochondria (β-OH-butyrate) (Vasington and Murphy 1962). The structure of digitonin fragments appears to be vesicular in electron micrographs (Siekevitz and Watson 1957). Phosphorylating sonic particles of rat liver mitochondria did not accumulate calcium (Vasington 1962).

During massive calcium and phosphate uptake by mitochondria the ratios of calcium/phosphate were approximately 1.6 (Brierley et al 1964b) or 1.8 (Lehninger et al 1963, Carafoli et al 1964). These ratios were consistent with precipitation within themitochondria of either $Ca_3(PO_4)_2$ or $(Ca_3(PO_4)_2)_3.Ca(OH)_2$. Electron microscopy has shown electron dense particles in mitochondria which have taken up calcium and phosphate (Brierley and Slautterback 1964, Lehninger et al 1963, Greenawalt et al 1964, Peachey 1964). These deposits may be associated with the cristae membranes (Brierley and Slautterback 1964, Vasington and Greenawalt 1964), or with growth of granules already present in the mitochondrial matrix (Peachey 1964). Deposits have been shown in the mitochondria of

toad bladder cells in vitro after incubating in a high calcium and phosphate medium (Peachey 1964). Incineration techniques (Weinbach and Von Brand 1965, Thomas and Greenawalt 1964) have confirmed that the electron dense particles are calcium precipitates and have indicated that adenine nucleotides may be associated with the precipitated calcium complex.

2. Low Calcium Concentrations.

Calcium and phosphate uptake was investigated using low calcium concentrations which did not uncouple oxidative phosphorylation. Calcium was taken up by mitochondria in the absence of adenine nucleotides and phosphate (Rossi and Lehninger 1964, Chance 1965). Rossi and Lehninger (1964), Saris (1963), Rasmussen et al (1965) and Chance (1965) have investigated the effects of low calcium concentrations on respiration, swelling, H^+ production and respiratory carrier states in animal mitochondria. These effects were discussed in Chapter 1.

3. Plant Mitochondria.

Studies with corn mitochondria (Hodges and Hanson 1965, Hanson 1965) have indicated that plant mitochondria can take up calcium and phosphate in a substrate or ATP-dependent manner from a medium containing low calcium concentrations. In the presence of both substrate and ATP a non additive stimulation of uptake occurred. Phosphate was required, although ATPase activity produced phosphate sufficient for 30% of the maximum uptake. Magnesium was required for ATP-supported uptake and high ATPase activity but not for substrate-supported uptake. Oligomycin inhibited uptake by the former but not the latter process. DNP inhibited

Table V. 1. Effect of $MgCl_2$ and Incubation Time on the Ca^{++} and Mg^{++} Contents of Beetroot Mitochondria.

Mitochondria were incubated in a medium containing 250 mM sucrose, 4 mM Tris-HCl buffer pH 7.2, 4 uM cytochrome c, 3 mM Tris-phosphate buffer pH 7.2, and 79, 108 and 80 ug mitochondrial N/ml in Expts. A, B and C respectively. Mitochondria were separated from the medium by filtering.

	External Conc. mM		Ca^{++} umoles/mg N			Mg^{++} umoles/mg N		
Expt.	$CaCl_2$	$MgCl_2$	5 min.	12 min.	18 min.	5 min.	12 min.	18 min.
A	0	15	0.44	0.10	0	3.54	3.60	3.50
B	0	2.9	0.42	0.36	0.30	0.80	0.79	0.77
B	0.2	2.9	0.55	0.45	0.44	0.66	0.59	0.61
B	0.4	2.2	0.61	0.61	0.64	0.65	0.59	0.58
C	2	0	3.1	3.4	3.4	0.48	0.48	0.48

Table V. 2. Effect of $CaCl_2$ Concentration on Ca^{++} and Pi Contents of Beetroot Mitochondria.

Mitochondria were incubated for 11 minutes in a medium containing 250 mM sucrose, 8.6 mM Tris-HCl buffer pH 7.2, 3 uM cytochrome c, 2.9 mM $MgCl_2$, 88 and 74 ug mitochondrial N/ml in Expts. A and B and 2.9 mM Tris-phosphate buffer pH 7.2 in Expt. B. The mitochondria were collected by filtering.

		Ca^{++} umoles/mg N			Pi umoles/mg N		
External Conc. $CaCl_2$ mM		+ mitochondria	- mitochondria	Ca^{++} bound	+ mitochondria	- mitochondria	Pi content
Expt. A.	4	1.48			0.07		
Expt. B.	0	0.41	0.50	-0.10	0.81	0.60	0.21
	0.4	0.61	0.60	0.01	0.83	0.58	0.25
	1	1.20	0.53	0.67	0.89	0.55	0.34
	2	3.45	2.03	1.42	1.72	1.40	0.32
	3	6.40	4.90	1.50	3.65	3.21	0.44
	5	12.50	11.50	1.00	8.13	7.80	0.33
	7	18.20	17.90	0.30	12.20	12.00	0.20

substrate-supported uptake completely but only partially inhibited ATP-supported uptake. The ratio calcium + magnesium/phosphate was 1.0 calculated from total phosphate content and not acid-extractable phosphate. Massive calcium uptake occurred at 2.5mM calcium when oxidative phosphorylation was uncoupled.

In contrast to these results with corn mitochondria massive calcium and phosphate uptake by soybean mitochondria was supported by substrate but not by ATP. Furthermore ATP inhibited substrate-supported calcium uptake by soybean mitochondria (Hanson 1965).

B RESULTS.

1. Substrate-Independent Uptake.

Isolated beetroot mitochondria contained between 300-450 mu moles calcium/mgN. This calcium content decreased slowly during incubation of the mitochondria in the absence of substrate and calcium. High magnesium concentrations increased the rate of calcium loss during incubation (Table V 1).

a. Effect of $CaCl_2$ Concentrations:- The substrate-independent calcium content of beetroot mitochondria increased as the external $CaCl_2$ concentration increased (Table V 2). Table V 2 indicates that calcium was retained in the Millipore filters when mitochondria were omitted from the medium. The difference in calcium contents between the treatments with and without mitochondria would represent actual calcium binding by the mitochondria at different $CaCl_2$ concentrations (Table V 2). Retention of calcium in the filter at high $CaCl_2$ concentrations

Table V. 3. The Effect of $CaCl_2$ Concentration on Mg^{++} Bound by Beetroot Mitochondria.

The mitochondria and conditions were the same as in Experiment B, Table V. 2.

	Mg^{++} umoles/mg N		
External Conc. $CaCl_2$ mM	+ mito chondria	- mito chondria	Mg^{++} Bound
0	0.87	0.45	0.42
0.4	0.70	0.45	0.25
1	0.68	0.43	0.25
2	0.70	0.45	0.25
3	0.81	0.49	0.32
5	1.14	0.95	0.19
7	1.05	0.90	0.15

Table V. 4. Effect of $MgCl_2$ Concentration on Substrate-Independent Ion Uptake.

Beetroot mitochondria were incubated for 19 minutes in a medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 2.9 mM Tris phosphate buffer pH 7.2, 3 uM cytochrome c, 2 mM $CaCl_2$ and 79 ug mitochondrial N/ml. The mitochondria were collected by filtering.

External $MgCl_2$ mM	External Mg^{++}/Ca^{++}	Pi umoles/ mg N	Ca^{++} umoles/ mg N	Ca^{++} % of total Cations	Mg^{++} umoles/ mg/N	Mg^{++} % of total Cations
0	0	2.24	3.40	88	0.48	12
2	1	1.97	2.72	70	1.16	30
5	2.5	1.57	1.87	51	1.77	49
10	5	1.22	1.36	34	2.66	66
15	7.5	1.02	1.53	21	3.88	79

was probably due to calcium phosphate precipitating in the medium. This was indicated by the corresponding high phosphate-retention in the filter which increased as the $CaCl_2$ concentration increased. Precipitation did not occur when phosphate was omitted from the medium (Table V 2). When mitochondria were omitted the ratios of calcium/phosphate retained in the filter increased from 0.8 at 0 mM $CaCl_2$ to 1.5 at 2-7 mM $CaCl_2$. This suggests formation of a salt of the composition $Ca_3(PO_4)_2$ at the higher $CaCl_2$ concentrations. The decrease in actual calcium binding at 5 and 7 mM $CaCl_2$ (Table V 2) could have been due to removal of available calcium by precipitation or to changes in the structural and binding properties of the mitochondria. No precipitate was visible during these experiments. Medium pH was 7.0 - 7.2.

Table V 3 shows the changes in magnesium binding as external $CaCl_2$ concentration was increased. Magnesium retention in the filter was low at this $MgCl_2$ concentration (2.9 mM) in contrast to calcium retention at 2.9 mM $CaCl_2$ (Table V 2). Corrected magnesium binding decreased as external $CaCl_2$ increased. This decrease was probably due to calcium replacing magnesium at membrane binding sites. At high $CaCl_2$ concentrations magnesium retention by the filter increased (Table V 3). This could be due to formation of an insoluble magnesium-calcium-phosphate complex.

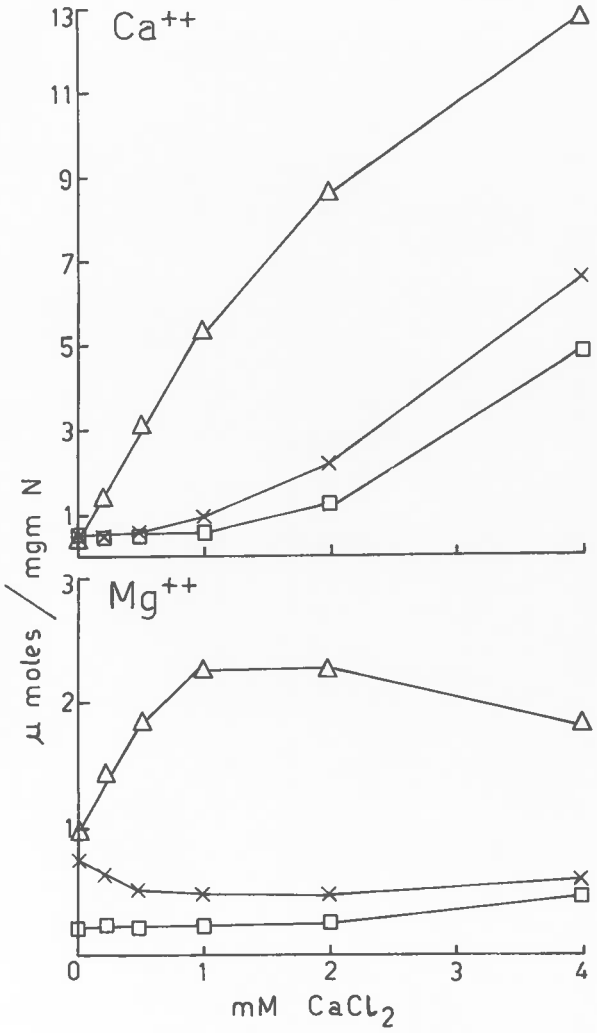
b. Effect of $MgCl_2$ Concentration:- Table V 4 shows the effect of external $MgCl_2$ concentration on substrate-independent calcium, magnesium and phosphate uptake by beetroot mitochondria. Calcium uptake decreased as $MgCl_2$ increased due to replacement of bound calcium by

Fig. V. 1. Effect of $CaCl_2$ concentration on Ca^{++}, Mg^{++} and Pi uptake by beetroot mitochondria incubated for 17 minutes in a medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 2.9 mM Tris-phosphate buffer pH 7.2, 3 uM cytochrome c, 2.9 mM $MgCl_2$ and 108 ug mitochondrial N/ml.

x -- x no addition.

Δ — Δ with 14 mM Tris. succinate

□ — □ with 14 mM Tris succinate, and 0.4M sucrose in place of mitochondria.


Ca++
Mg++
μ moles / mgm N
mM CaCl2

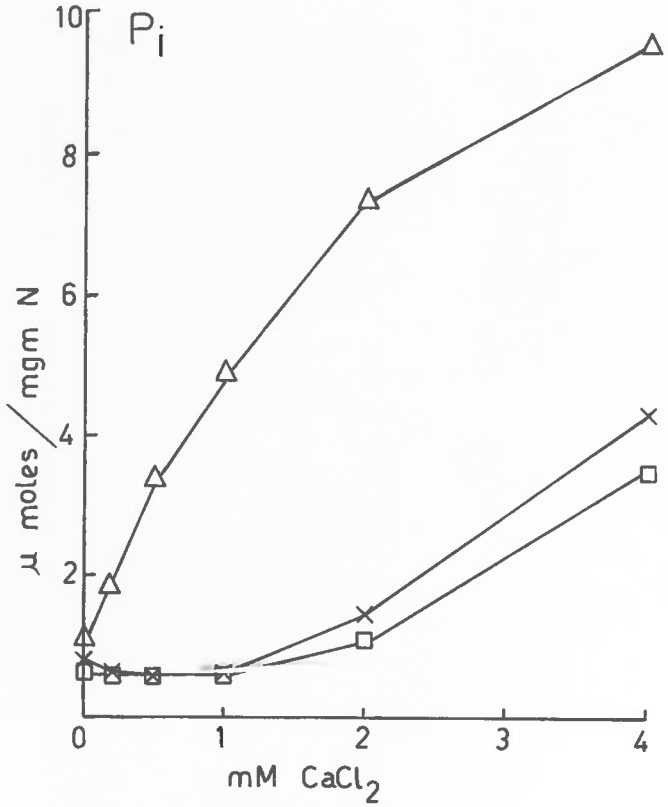
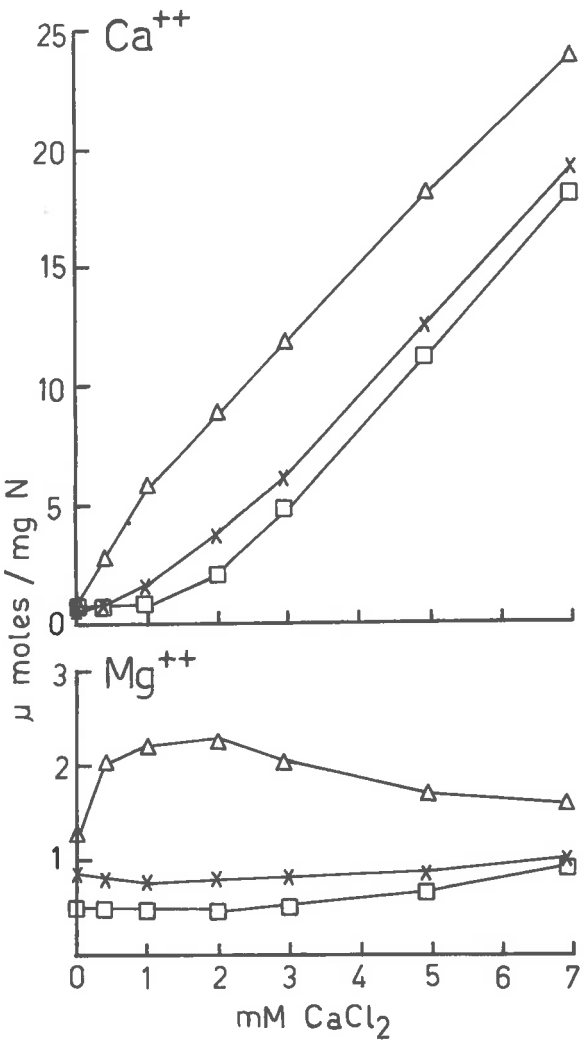

Pi
μ moles / mgm N
mM CaCl2

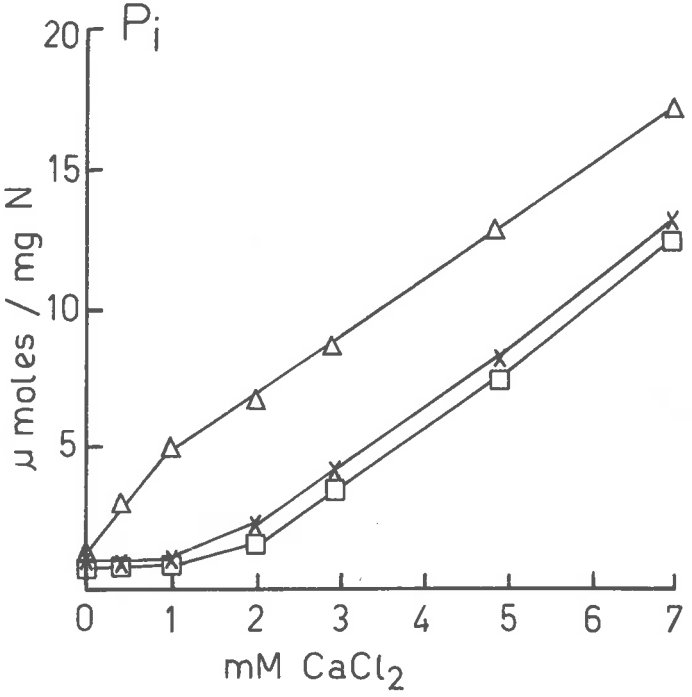
Fig. V. 2. Effect of $CaCl_2$ concentration on Ca^{++}, Mg^{++} and Pi uptake by beetroot mitochondria incubated for 17 minutes in a medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 2.9 mM Tris-phosphate buffer pH 7.2, 3 uM cytochrome c, 2.9 mM $MgCl_2$ and 74 ug mitochondrial N/ml.

x -- x No addition

Δ — Δ with 14 mM Tris succinate

□ — □ with 14 mM Tris succinate, and 0.4 M sucrose in place of mitochondria.


Ca++
Mg++
μ moles / mg N
mM CaCl2


Pi
μ moles / mg N
mM CaCl2

magnesium and to reduced formation and filter retention of calcium phosphate. Phosphate uptake followed the calcium uptake response to $MgCl_2$ concentration. Magnesium content increased with $MgCl_2$ concentration from increased binding and filter retention.

2. Substrate-Dependent Uptake.

Massive calcium and phosphate uptake by beetroot mitochondria was dependent on substrate oxidation. Both calcium and phosphate were required for massive ion uptake and maximum uptake occurred in the presence of 1-2 mM $MgCl_2$. Inhibitors (cyanide, antimycin A) and uncouplers (DNP) of oxidative phosphorylation inhibited massive calcium and phosphate uptake by beetroot mitochondria but oligomycin had no effect on uptake (Fig. V 4).

a. Effect of $CaCl_2$ Concentration:- Figs. V 1 and 2 show the effect of increasing external $CaCl_2$ on substrate-dependent calcium and phosphate uptake. Filter retention of ions is shown in the treatment omitting mitochondria. Maximum substrate-dependent calcium and phosphate uptake occurred when external $CaCl_2$ was 2-5 mM (Figs. V 1 and 2). Substrate-dependent magnesium uptake was low at the $MgCl_2$ concentration present (2 mM, cf Figs. IV 1 and 2). The enhancement of substrate-dependent magnesium uptake by low $CaCl_2$ concentrations (0.2 - 1 mM) could have been due to structural improvement (after mitochondrial isolation in EDTA) or to formation of a calcium-magnesium-phosphate complex within the mitochondria less soluble than the magnesium phosphate salt formed. The decrease in substrate-dependent magnesium uptake at higher $CaCl_2$ concentrations (Figs. V 1 and 2) could have resulted from calcium and

Table V. 5. Effect of $CaCl_2$ Concentration on Cation/Phosphate Ratios.

Beetroot mitochondria were incubated for 11 minutes in a medium containing 250 mM sucrose, 8.6 mM Tris-HCl buffer pH 7.2, 2.9 mM $MgCl_2$, 2.9 mM Tris-phosphate buffer pH 7.2, 3 uM cytochrome c, 14 mM Tris succinate and 108 ug mitochondrial N/ml. Cation/phosphate ratios were calculated after correcting for ion uptake in the absence of substrate.

External $CaCl_2$ (mM)	Mg^{++}/ Pi	Ca^{++}/ Pi	Mg^{++} + Ca^{++}/Pi
0	1.5	-	1.5
0.2	0.69	0.90	1.59
0.5	0.48	1.02	1.5
1	0.38	1.08	1.46
2	0.30	1.15	1.45
4	0.22	1.13	1.45

Table V. 6. Effect of $CaCl_2$ Concentration on Ion Uptake with 15 mM $MgCl_2$.

Beetroot mitochondria were incubated for 19 minutes in a medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 2.9 mM Tris-phosphate buffer pH 7.2, 3 uM cytochrome c, 15 mM $MgCl_2$, 79 ug mitochondrial N/ml, and 14 mM Tris succinate in B, where the results have been corrected for uptake in the absence of substrate (A).

External		Pi	Ca^{++}		Mg^{++}	
$CaCl_2$ mM	Mg^{++}/Ca^{++}	umoles/ mg N	umoles/ mg N	% of total cations	umoles/ mgN	% of total cations
A.						
0		0.68	0	0	3.13	100
2	7.5	1.02	1.53	28	3.88	72
5	3	2.58	3.68	47	4.08	53
10	1.5	9.25	12.92	45	5.90	55
B.						
0		6.26	0.34	3	10.4	97
2	7.5	12.10	4.9	31	11.2	69
5	3	14.92	10.8	51	10.5	49
10	1.5	11.10	12.4	72	4.8	28

magnesium competition for uptake or for phosphate, or from calcium damage to the mitochondria.

Figs. V 1 and 2 indicate that substrate-dependent calcium uptake was 4-5 times greater than magnesium uptake at the same concentrations of $MgCl_2$ and $CaCl_2$ (2.9 mM). This preferential calcium uptake could have been due to a selective uptake process, to different magnesium and calcium binding affinities, or to different magnesium and calcium phosphate complex solubilities. Substrate-independent calcium binding (corrected for filter retention) was 4-6 times greater than substrate-independent magnesium binding when the $MgCl_2$ and $CaCl_2$ concentrations were both 2.9 mM (Tables V 1 and 2). Chappell et al (1963) and Chance (1965) suggested that substrate-dependent uptake of bound divalent cation was the first step in ion uptake. Cation and phosphate uptake would then reflect the ratio of bound cations until the external concentration limited membrane binding of the preferred cation.

Calcium/phosphate ratios of 0.9 - 1.2 were measured at different $CaCl_2$ concentrations (Table V 5). However, calcium + magnesium/phosphate ratios of 1.5 were obtained when the contribution of substrate-dependent magnesium uptake was considered. As $CaCl_2$ concentration increased the calcium/phosphate ratios increased and the magnesium/phosphate ratios decreased but the total cation/phosphate ratios remained constant at 1.5-1.6 (Table V 5). Thus any precipitated salt would probably have the form of $M_3(PO_4)_2$.

Table V 6 shows the effect of increasing $CaCl_2$ concentration on

Table V. 7. Effect of $MgCl_2$ Concentration on Ion Uptake.

Beetroot mitochondria were incubated for 19 minutes in a medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 2.9 mM Tris-phosphate buffer pH 7.2, 3 uM cytochrome c, 2 mM $CaCl_2$, 14 mM Tris succinate and 79 ug mitochondrial N/ml. The results have been corrected for ion uptake in the absence of substrate.

		Pi	Ca^{++}		Mg^{++}	
External		umoles/ mg N	umoles/ mg N	% of total Cat-ions	umoles/ mg N	% of total Cat-ions
$MgCl_2$ mM	Mg^{++}/Ca^{++}					
0	0	3.76	4.60	96	0.20	4
2	1	10.13	9.58	72	3.80	28
5	2.5	11.94	9.33	53	7.96	47
10	5	11.78	6.64	41	9.34	59
15	7.5	12.10	4.90	30	11.22	70

ion uptake when the external $MgCl_2$ concentration was 15 mM (cf Chapter 4). The effects were similar to those observed when external $MgCl_2$ was 2.9 mM (Tables V 2 and 3, Figs. V 1 and 2). Substrate-dependent magnesium uptake was again increased by low $CaCl_2$ concentrations (Table V 6, Fig. V 1). The calcium + magnesium/phosphate ratios varied between 1.3 and 1.6. The reduction in total ion uptake at 10 mM $CaCl_2$ may have been due to mitochondria damage by calcium or to limited availability of phosphate. Substrate-dependent calcium uptake was greater than magnesium uptake when the external magnesium/calcium ratio fell below 3 (Table V 6).

b. Effect of $MgCl_2$ Concentration:- Table V 7 shows the effect of external $MgCl_2$ concentration on substrate-dependent ion uptake. The total cation uptake remained constant from 5-15 mM $MgCl_2$ although the calcium and magnesium proportions of this uptake varied. Magnesium + calcium/phosphate ratios were constant throughout at 1.3 - 1.4. Substrate-dependent calcium and phosphate uptake was low at 0 mM $MgCl_2$ (Table V 7) and magnesium could have been required for oxidative processes although the endogenous magnesium content was 500 mu moles/mg N (Table V 4). Maximum calcium uptake was measured at 2 mM $MgCl_2$. At equal concentrations of $MgCl_2$ and $CaCl_2$ (2 mM) calcium contributed to 72% of the total substrate-dependent cation uptake. Magnesium uptake became greater than calcium uptake when the external magnesium/calcium ratio increased beyond 3.

3. Effects of ATP and ADP.

a. Substrate Absent:- Calcium and phosphate uptake by beetroot

Table V. 8. Effect of ATP on Substrate-Independent Ion Uptake.

Beetroot mitochondria were incubated in a medium containing 250 mM sucrose, 9 mm Tris-HCl buffer pH 7.2, 3 uM cytochrome c and 1 ug/ml oligomycin where indicated. Expt. A contained 2.9 mM $CaCl_2$, 2.9 mM $MgCl_2$, 2.9 mM Tris-phosphate buffer pH 7.2 and 140 ug mitochondrial N/ml. Expt. B contained 2.0 mM $CaCl_2$, 2.0 mM $MgCl_2$, 2.0 mM Tris-phosphate buffer pH 7.2 and 100 ug mitochondrial N/ml. The mitochondria were separated by filtering.

	Pi umoles/mg N	Ca^{++} umoles/mg N	Mg^{++} umoles/mg N
Expt. A.			
Control	1.80	2.90	0.49
+ 3 mM ATP(Na)	1.12	1.20	0.40
+ 3 mM ATP(Na) + Oligomycin	1.10	1.21	0.39
+ 6 mM ATP(Na)	0.83	0.85	0.29
Expt. B.			
Control	0.59	1.24	0.45
+ 0.6 mM ATP(Tris)	0.70	1.01	0.40
+ 2.6 mM ATP(Tris)	0.79	0.71	0.35
+ 2.6 mM ATP(Tris) + Oligomycin	0.78	0.70	0.33

Fig. V. 3. Effect of Tris ATP at a low concentration on uptake of Ca^{++}, Mg^{++} and Pi by beetroot mitochondria incubated in a medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 2 mM Tris-phosphate buffer pH 7.2, 3 uM cytochrome c and 101 ug mitochondrial N/ml.

o -- o no addition

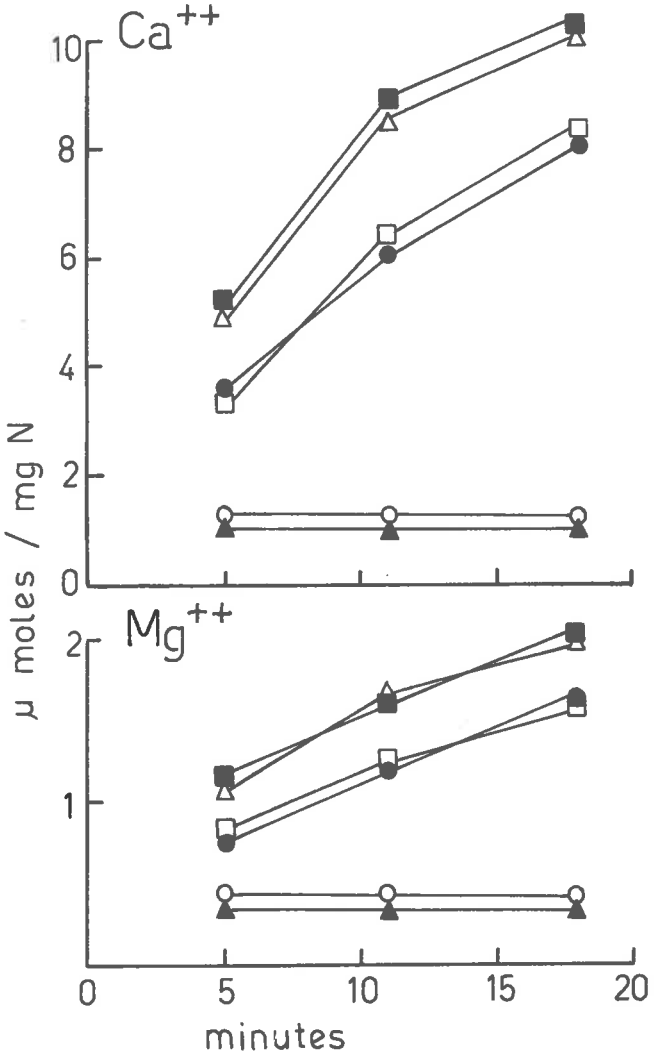
● -- ● with 7 mM Tris-succinate

□ — □ with 7 mM Tris succinate and 1 ug/ml oligomycin

▲ — ▲ with 0.64 mM Tris ATP

Δ — Δ with 7 mM Tris succinate and 0.64mM Tris ATP

■ — ■ with 7 mM Tris succinate, 0.64 mM Tris ATP and 1 ug/ml oligomycin.


Ca++
Mg++
μ moles / mg N
minutes
0
5
10
15
20

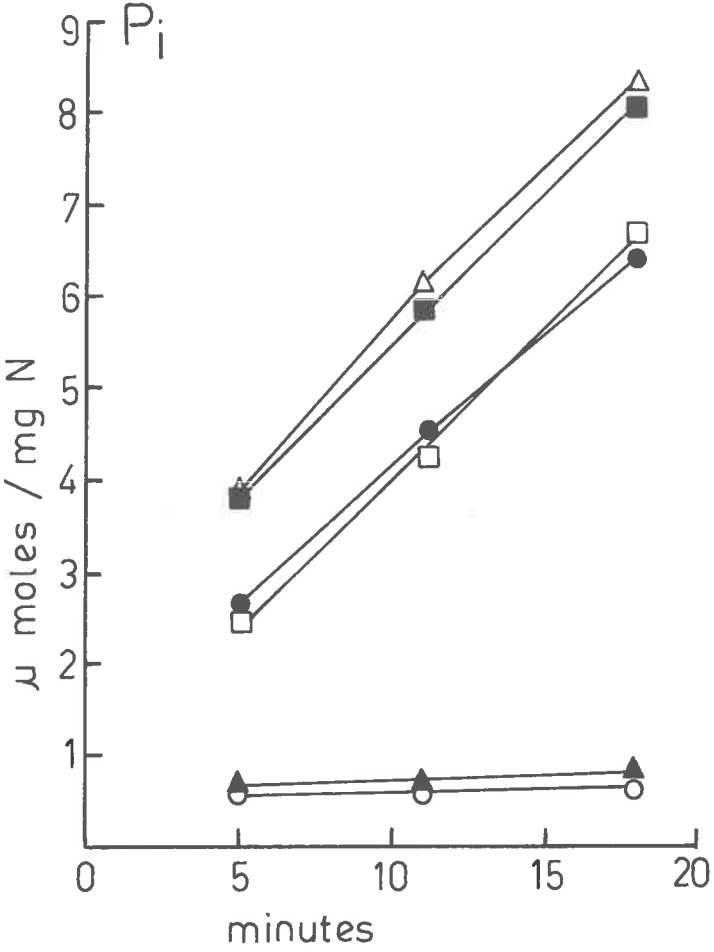

Pi
μ moles / mg N
minutes
0
5
10
15
20

Fig. V 4. Effect of ATP at a high concentration on Ca^{++}, Mg^{++} and Pi uptake by beetroot mitochondria incubated in a medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 2.0 mM $CaCl_2$, 2.0 mM $MgCl_2$, 2.0 mM Tris-phosphate buffer pH 7.2, 3 uM cytochrome c and 104 ug mitochondrial N/ml

x -- x no addition

Δ -- Δ with 2.6 mM Tris ATP

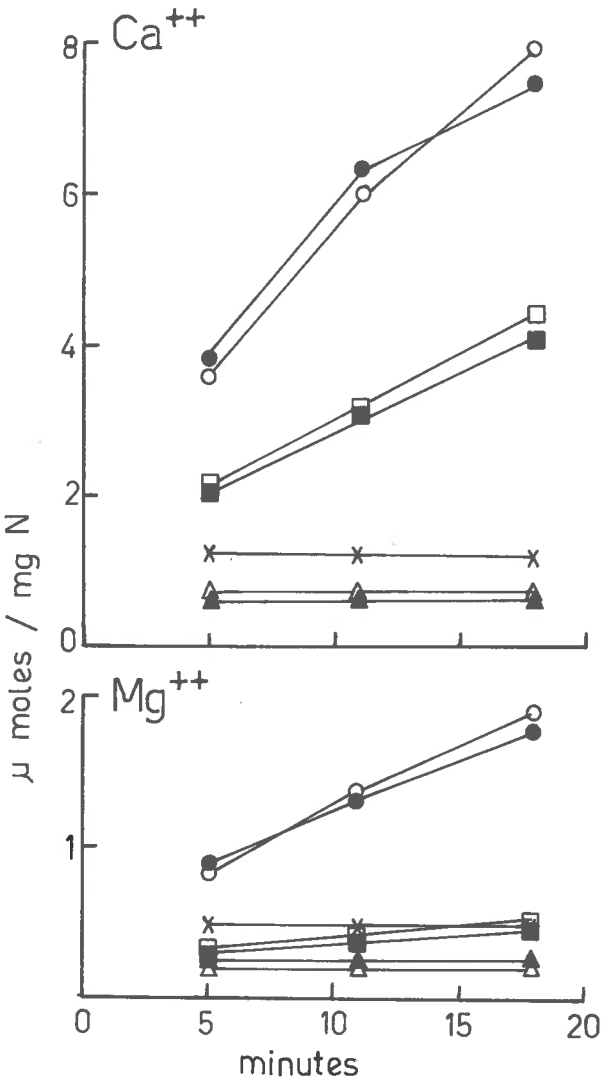
▲ -- ▲ with 2.6 mM Tris ATP and 1 ug/ml oligomycin

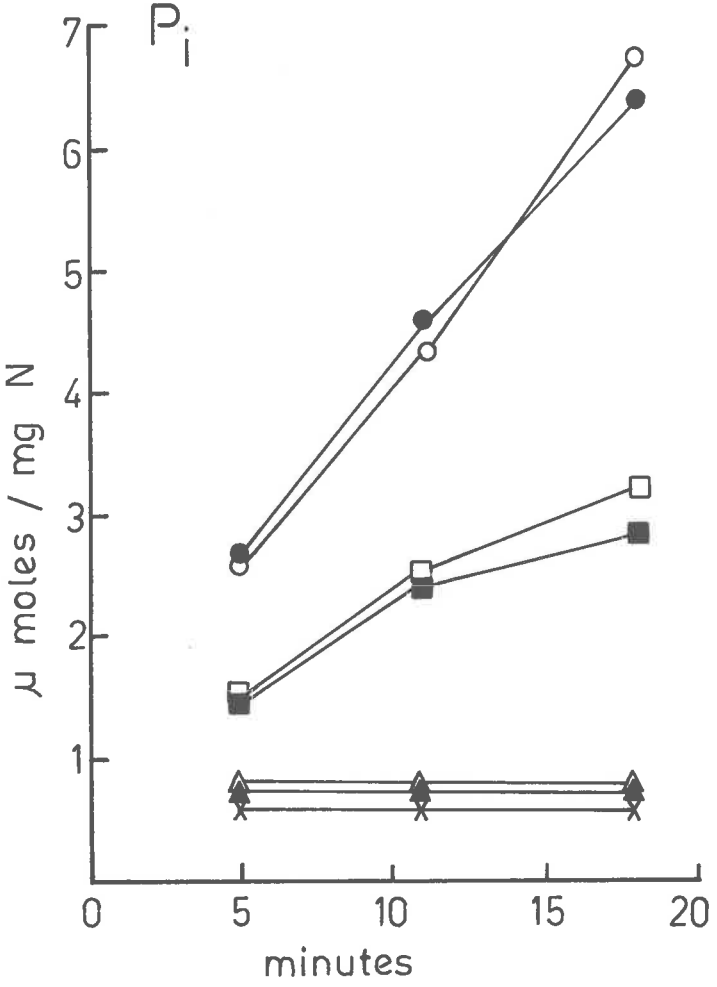
o -- o with 18 mM Tris succinate

● -- ● with 18 mM Tris succinate and 1 ug/ml oligomycin

□ -- □ with 18 mM Tris succinate and 2.6 mM Tris ATP

■ -- ■ with 18 mM Tris succinate, 2.6 mM Tris ATP and 1 ug/ml oligomycin.


Ca++
Mg++
μ moles / mg N
minutes
0
5
10
15
20
1
2
4
6
8


Pi
μ moles / mg N
minutes
0
5
10
15
20
1
2
3
4
5
6
7

mitochondria was not supported by Tris or disodium ATP in the absence of substrate (Table V 8). Tris ATP stimulated but disodium ATP inhibited phosphate contents (Table V 8). Both Tris and disodium ATP stimulated phosphate levels when calcium was not present in the medium (Table IV 12). The inhibition of phosphate content could be related to the large inhibition of calcium levels by disodium ATP (Table V 8). Chelation of free calcium by disodium ATP would reduce retention of both calcium and phosphate in the filter. Tris ATP also inhibited calcium levels (Table V 8). Both disodium and Tris ATP inhibited magnesium substrate-independent uptake by beetroot mitochondria (Table V 8). Tris could replace bound magnesium (Chapter 4) and ATP could chelate much of the magnesium added (2.0 or 2.9 mM). Oligomycin did not change these ATP effects on ion levels which were constant over the incubation periods suggesting physical adjustment rather than adjustment by an energy-dependent process.

b. Substrate Present:- ATP was not required for massive substrate-dependent calcium and phosphate uptake by beetroot mitochondria from a medium containing high concentrations of $CaCl_2$ (2-7 mM) in contrast to results reported for animal mitochondria (Brierley et al 1964b, Lehninger et al 1963, Vasington and Murphy 1962).

(1) Low ATP concentrations:- A low concentration (0.6 mM) of Tris ATP stimulated massive calcium and phosphate uptake by beetroot mitochondria (Fig. V 3). Oligomycin had no effect on this stimulation of ion uptake (Fig. V 3).

(2) High ATP and ADP Concentrations:- Fig. V 4 shows that substrate-dependent calcium, phosphate and magnesium uptake was inhibited

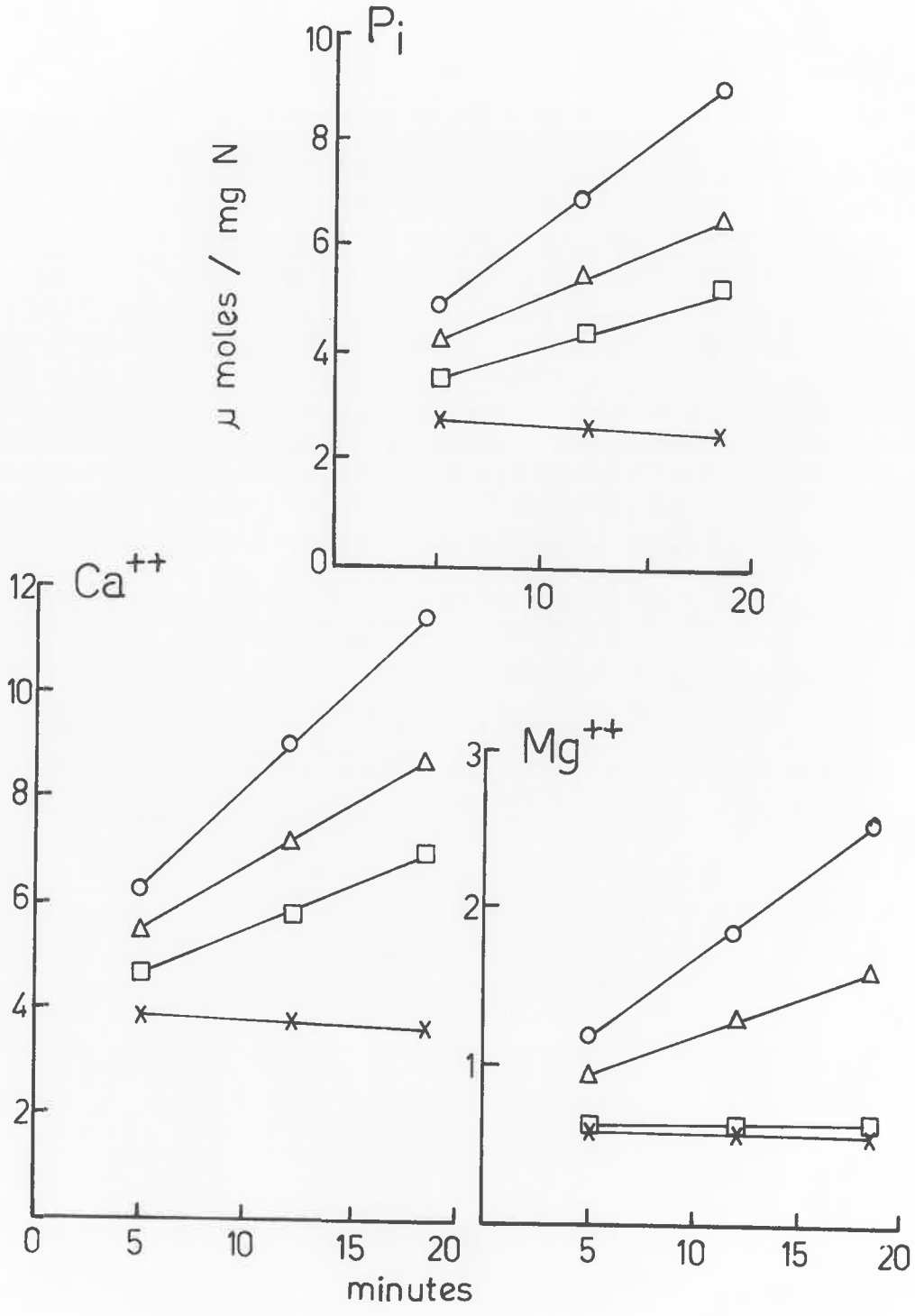
Fig. V. 5. The effect of NaCl and Na ATP on uptake of Ca^{++}, Mg^{++} and Pi by beetroot mitochondria incubated in a medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 2.9 mM Tris-phosphate buffer pH 7.2, 2.9 mM $MgCl_2$, 2.9 mM $CaCl_2$, 3 uM cytochrome c and 115 ug mitochondrial N/ml.

x -- x no addition

o -- o with 14 mM Tris succinate

Δ -- Δ with 14 mM Tris succinate and 6 mM NaCl

□ -- □ with 14 mM Tris succinate and 2.9 mM disodium ATP


Pi
μ moles / mg N
Ca++
Mg++
minutes

by 2.6 mM Tris ATP and that oligomycin had no effect on this inhibition. Magnesium uptake was completely inhibited whereas calcium and phosphate uptake were only partially inhibited.

Fig. V 5 shows the effect of disodium ATP and NaCl on calcium, magnesium and phosphate uptake. NaCl inhibited ion uptake (see Chapter 7) but ATP containing the same concentration of sodium inhibited ion uptake more severely. This was particularly apparent with magnesium uptake which was inhibited 40% by NaCl but completely inhibited by ATP. The high concentration of ATP (a chelator) present in comparison with the total cation concentration would make availability of the cations for uptake an important consideration.

ADP in high concentrations completely inhibited calcium, magnesium and phosphate uptake. Oxidative phosphorylation was still operative at the concentrations of $CaCl_2$ (2-3 mM) used in these experiments (Chapter 6). Oligomycin restored the ADP-inhibited calcium, magnesium and phosphate uptake to the ATP-inhibited rate.

C. DISCUSSION.

1. Summary of Calcium and Phosphate Uptake:-

The general characteristics of calcium and phosphate (and magnesium) uptake by beetroot mitochondria were:-

(1) Endogenous calcium contents of beetroot mitochondria were lower than endogenous magnesium contents. High $MgCl_2$ concentrations hastened the loss of endogenous calcium during incubation.

(2) Calcium was bound in the absence of substrate but high

~~high~~ magnesium concentrations reduced bound calcium. Calcium and phosphate were retained in the Millipore filters.

(3) Massive calcium and phosphate uptake was dependent on substrate oxidation.

(4) Calcium and phosphate uptake reached a maximum at 4-5 mM $CaCl_2$. Higher $CaCl_2$ concentrations depressed ion uptake.

(5) Calcium and phosphate uptake required magnesium (1-2 mM) for maximum uptake. High $MgCl_2$ concentrations decreased calcium uptake.

(6) Magnesium was taken up by a substrate-dependent process in the presence of calcium and phosphate, but calcium uptake was 4-5 times greater than magnesium uptaken when $CaCl_2$ and $MgCl_2$ concentrations were equal. When the external Mg/Ca ratio increased above 3, magnesium uptake became greater than calcium uptake.

(7) Calcium (1 mM) was required for maximum substrate-dependent magnesium uptake. High $CaCl_2$ concentrations depressed magnesium uptake.

(8) Calcium/phosphate ratios of 1.2 - 1.4 were measured during massive calcium and phosphate uptake in the absence of magnesium. Total cation (magnesium + calcium)/phosphate ratios were between 1.3 and 1.6 when magnesium was included.

(9) Calcium and phosphate (and magnesium) uptake was inhibited by inhibitors of the electron transfer chain and by uncouplers of oxidative phosphorylation. Uptake was not affected by oligomycin.

(10) Conditions allowing oxidative phosphorylation to proceed

(phosphate acceptor present) completely inhibited calcium and phosphate uptake. Oligomycin inhibited oxidative phosphorylation and promoted ion uptake.

(11) ATP did not support calcium and phosphate uptake and inhibited the mitochondrial binding of calcium and magnesium. Oligomycin did not alter these effects.

(12) Low concentrations of ATP stimulated substrate-dependent calcium, phosphate and magnesium uptake. Oligomycin did not affect this stimulation.

(13) High concentrations of ATP inhibited substrate-dependent calcium, phosphate and magnesium uptake. This inhibition was not affected by oligomycin.

This summary of the general characteristics of calcium and phosphate uptake by beetroot mitochondria shows the similarity to massive magnesium and phosphate uptake by these mitochondria.

2. Competition between Calcium and Magnesium:-

Substrate-dependent calcium and phosphate uptake by beetroot mitochondria proceeded most rapidly with a low (1-2 mM) $MgCl_2$ concentration. Magnesium was also taken up in a substrate-dependent manner, but to 20% - 25% of calcium uptake. Magnesium could have been required for maximum activity of oxidative processes (although ADP/O ratios were highest without added magnesium, Chapter 6), or for maintenance of mitochondrial structure. Brierley et al (1964b) found that calcium and phosphate uptake by beef heart mitochondria was accompanied by uptake of magnesium

but the amount of magnesium accumulated was less than 25% of the calcium accumulated. Carafoli et al (1964) reported calcium and phosphate uptake in rat liver mitochondria and this was accompanied by some uptake of magnesium. However, this magnesium uptake was dependent on calcium uptake and may have been due to association of magnesium with a precipitated calcium phosphate complex. Vasington and Murphy (1962) reported that 10 mM $MgCl_2$ greatly enhanced calcium binding and suggested that magnesium was necessary for oxidative phosphorylation.

Calcium (1 mM) enhanced substrate-dependent magnesium uptake by beetroot mitochondria. Calcium was not required for oxidative phosphorylation (although calcium increased oxidation rates, Chapter 6). Calcium could have affected membrane structure, binding or retention properties of mitochondria isolated with EDTA, or formed a magnesium-phosphate-calcium complex less soluble than $Mg_3(PO_4)_2$.

In beetroot mitochondria calcium competed with magnesium for substrate-independent binding sites and for substrate-dependent uptake into the mitochondria. In both cases calcium was the favoured cation and 3-5 times more calcium was bound and taken up than magnesium when both cations were at equal concentrations in the external medium. Calcium was taken up with phosphate in preference to magnesium by animal mitochondria (Carafoli et al 1964, Brierley et al 1964b). This preference could reflect either the ratio of bound cations or a selective uptake mechanism. The presence of phosphate could also produce an apparently selective uptake mechanism by formation of complexes with different

solubilities. A selective substrate-dependent uptake mechanism favouring one of a number of competing cations could thus be produced by physical properties alone.

3. Precipitation.

The calcium/phosphate ratios measured in beetroot mitochondria during massive substrate-dependent ion uptake in the absence of magnesium were 1.2 - 1.4, and in the presence of magnesium the total cation/phosphate ratios were 1.3 - 1.6. These ratios suggest that the form of any precipitated cation phosphate complex within the mitochondria could be $M_3(PO_4)_2$. Brierley et al (1964b) suggested formation of a calcium phosphate salt of this form in beef heart mitochondria while Lehninger et al (1963) and Carafoli et al (1964) have suggested a formation of $\{(Ca_3(PO_4)_2)_3.Ca(OH)_2\}$ in rat liver mitochondria. Electron micrographs have shown electron dense deposits in mitochondria incubated under conditions allowing massive calcium and phosphate uptake in animal mitochondria (Brierley and Slautterback 1964, Lehninger et al 1963, Peachey 1964).

4. Effects of ATP.

Massive calcium and phosphate uptake by beetroot mitochondria occurred in the presence of substrate from a medium containing from 2-7 mM $CaCl_2$. ATP was not required for massive uptake. Massive calcium and phosphate uptake by animal mitochondria from media containing high $CaCl_2$ (1-4 mM) required substrate in the presence of ATP (Vasington and Murphy 1962, Lehninger et al 1963, Brierley et al 1964b). Calcium uptake supported by substrate alone was reported by Brierley et al (1964b) but

uptake was not maintained beyond 40 seconds. The oligomycin-insensitive ATP-requirement for massive substrate-dependent calcium and phosphate uptake has been related to chelating properties of ATP (Chappell et al 1963), or to stabilization of membrane structure or of calcium phosphate precipitates by adenine nucleotide binding (Carafoli et al 1965). Oxidative phosphorylation was uncoupled in animal mitochondria at the calcium contentrations used for uptake studies (Vasington and Murphy 1962, Carafoli et al 1964). Oxidative phosphorylation was functional in beetroot mitochondria at high calcium concentrations (Chpt.6), and structure was unaffected. ATP would thus not be required for stabilization of structure during massive calcium and phosphate uptake.

Brierley et al (1964b) and Hodges and Hanson (1965) reported that either substrate or ATP could support massive calcium and phosphate by beef heart and corn mitochondria using low $CaCl_2$ concentrations. At higher $CaCl_2$ concentrations ATP supported less calcium and phosphate uptake than substrate + ATP (Brierley et al 1964). ATP (15 mM) supported massive calcium uptake in rat liver mitochondria in the absence of substrate (Lehninger et al 1963).

ATP did not support calcium and phosphate uptake by beetroot mitochondria and ATP at high concentrations inhibited substrate-dependent calcium and phosphate uptake. These results can be related to those obtained with soybean mitochondria (Hanson 1965). Substrate-supported calcium and phosphate uptake was greater in soybean mitochondria than in corn mitochondria but ATP did not support uptake in soybean mitochondria.

Substrate-supported calcium uptake by soybean mitochondria was inhibited by ATP but in corn mitochondria was stimulated by ATP (Hodges and Hanson 1965, Hanson 1965). Digitonin fragments prepared from rat liver mitochondria (Vasington 1963) took up calcium and phosphate with substrate but not ATP, and ATP and ADP inhibited substrate-supported calcium and phosphate uptake. These results were in contrast to those reported for intact rat liver mitochondria. ATP-inhibition of substrate-supported calcium and phosphate uptake by beetroot mitochondria was not affected by oligomycin and could not have been due to ATPase activity providing ADP for oxidative phosphorylation. The inhibition could be related to the inhibitory effects of sodium and Tris on divalent cation and phosphate uptake (see Chapter 7) and to the chelating properties of ATP. This latter consideration may be of major importance in these ion uptake studies by beetroot mitochondria where the ATP concentration was equal to the calcium or magnesium concentration in the medium.

The oligomycin-insensitive stimulation of massive substrate-dependent calcium and phosphate uptake by beetroot mitochondria by low ATP concentrations could be attributed due to structure stabilization by adenine nucleotide binding (see Chapter 4).

5. Effect of ADP.

Oxidative phosphorylation occurred in beetroot mitochondria at $CaCl_2$ concentrations of up to 3 mM without reduction in P/O ratios (Chapter 6). The inhibitory effect of high ADP concentrations on calcium and phosphate uptake by beetroot mitochondria can thus be related to the competitive inhibition of ion uptake by oxidative phosphorylation.

Oligomycin relieved uptake but only partially due to the inhibitory chelating effects of adenine nucleotides. Carafoli et al (1964) reported that ADP could partly replace ATP in maintaining substrate-supported calcium and phosphate uptake but that ATP, hexokinase and glucose completely abolished uptake. This was probably due to removal of ATP required for maintenance of massive calcium and phosphate uptake rather than to inhibition by oxidative phosphorylation. The concentration of calcium (4.0 mM) present was reported to completely uncouple oxidative phosphorylation. Hodges and Hanson (1965) reported that conditions for ATP formation inhibited substrate-dependent calcium and phosphate uptake by corn mitochondria and they suggested a competitive effect of oxidative phosphorylation. Oligomycin partially recovered uptake. The high ADP concentration used or ions added with hexokinase (Chapter 7) could have prevented complete recovery.

6. Mechanisms of Calcium and Phosphate Uptake.

The characteristics of massive calcium and phosphate uptake by beetroot mitochondria were similar to those of magnesium and phosphate uptake. Calcium and phosphate uptake by isolated mitochondria can be accounted for by the mechanisms of ion uptake based on classical or chemiosmotic hypotheses of oxidative phosphorylation (Chapter 1). The ATP-requirement for substrate-dependent calcium and phosphate uptake by animal mitochondria and the ability of calcium to uncouple oxidative phosphorylation and affect structure, permeability and volume of mitochondria are additional considerations (Chappell et al 1963, 1966).

Calcium phosphate precipitation also affects mitochondrial volume and structure (Chappell and Crofts 1966).

The failure of ATP to support calcium and phosphate uptake by beetroot mitochondria, soybean mitochondria (Hanson 1965) and digitonin fragments of rat liver mitochondria (Vasington 1963) may be due to a more labile or damaged ATPase system. Damage to the phosphorylating system during digitonin fragment formation from rat liver mitochondria could prevent ATP-supported uptake but need not affect substrate-supported uptake (Vasington 1963). The results can be explained on this basis more readily by the chemiosmotic mechanism for ion uptake in which the two systems for producing PMF are separate whereas the classical mechanism supports ion uptake by a high-energy compound common to both substrate oxidation and ATP breakdown. Chelation of calcium by ATP would reduce cation binding and uptake by ATPase activity as substrate-dependent ion uptake was reduced by high ATP concentrations.

High calcium concentrations (3mM) did not uncouple oxidative phosphorylation in beetroot mitochondria (Chapter 6) and any energy (intermediates or PMF) for ion uptake produced by slow ATPase activity (Chapter 6) would be immediately used by the favoured ATP reformation process. Thus ATP would only be expected to support ion uptake at concentrations of $CaCl_2$ and $MgCl_2$ (Chapter 4) which uncoupled the favoured oxidative phosphorylation process.

CHAPTER SIX

MAGNESIUM AND CALCIUM EFFECTS ON MITOCHONDRIAL REACTIONS

A. INTRODUCTION

1. Low Divalent Cation Concentrations.

The effects of low concentrations of divalent cations on respiring animal mitochondria have been discussed in Chapter 1, (~~Section F 3 a (9)~~ page 46). In summary, addition of a low concentration of divalent cation to phosphate-supplemented mitochondria produced four distinct phenomena simultaneously.

(1) the steady state of the respiratory carriers increased to a new and characteristic oxidation-reduction level,

(2) electron transport was stimulated,

(3) light scattering changes occurred, and

(4) hydrogen ions were extruded.

The divalent cation and phosphate were taken up by the mitochondria and on complete uptake of the cation the steady state of the respiratory carriers returned to their previous level, oxidation returned to the basal rate and H^+ production stopped.

In the absence of phosphate the same phenomena occurred to a limited extent and addition of the divalent cation above a certain concentration produced an inhibited respiration rate and new highly oxidized respiratory carrier levels (state 6) (Chance 1965). Phosphate addition relieved the oxidized carrier levels, stimulated the oxidation rate and

promoted divalent cation uptake.

Modifications must be made to this general summary according to the cation and anion used, precipitation of a cation-anion complex, and the effects of external and accumulated ions on membrane permeability and on volume changes (Chance 1965, Chappell and Crofts 1966, Rossi and Lehninger 1964, Carafoli 1965b).

2. Calcium Effects on Corn Mitochondria.

The uptake of calcium by corn mitochondria from a medium containing low concentrations of calcium (Hanson et al 1965) was accompanied by changes in respiration rate and swelling similar to those observed in animal mitochondria (Chance 1965, Chappell and Crofts 1966). Hanson et al (1965) measured respiratory control in isolated corn mitochondria oxidizing pyruvate and malate and stimulation of the state 4 oxidation rate by calcium. This stimulation continued undiminished for at least 4 min. Calcium did not interfere with subsequent state 3 - state 4 transitions on addition of ADP, and the ADP/O and P/O ratios were reduced only slightly by the calcium concentration added. DNP produced a greater stimulation of respiration than did calcium, and the calcium-stimulated rate was only 42% of the subsequent state 3 rate.

In the absence of phosphate and ATP, a low calcium concentration (0.4 mM) produced an increase in the respiratory rate and then a slow decline, similar to results reported by Rossi and Lehninger (1964) and Chance (1965), but with a less distinct transition between stimulated

Fig. VI. 1. Effect of $MgCl_2$ concentration on rate of succinate oxidation and ADP/O and R.C. ratios. Beetroot mitochondria were incubated in an oxygen electrode cell at 25°C in a medium containing 250 mM sucrose, 3 mM Tris-HCl buffer pH 7.2, 7 mM potassium phosphate buffer pH 7.2, 4 uM cytochrome c, 8 mM Tris succinate and 394 ug mitochondrial N in a total volume of 3 ml. The 1st and 2nd state 3 oxidation rates were produced by additions of 0.11 mM ADP, and the DNP rate was produced by addition of 5×10^{-5} M DNP during the 2nd state 4 oxidation rate.

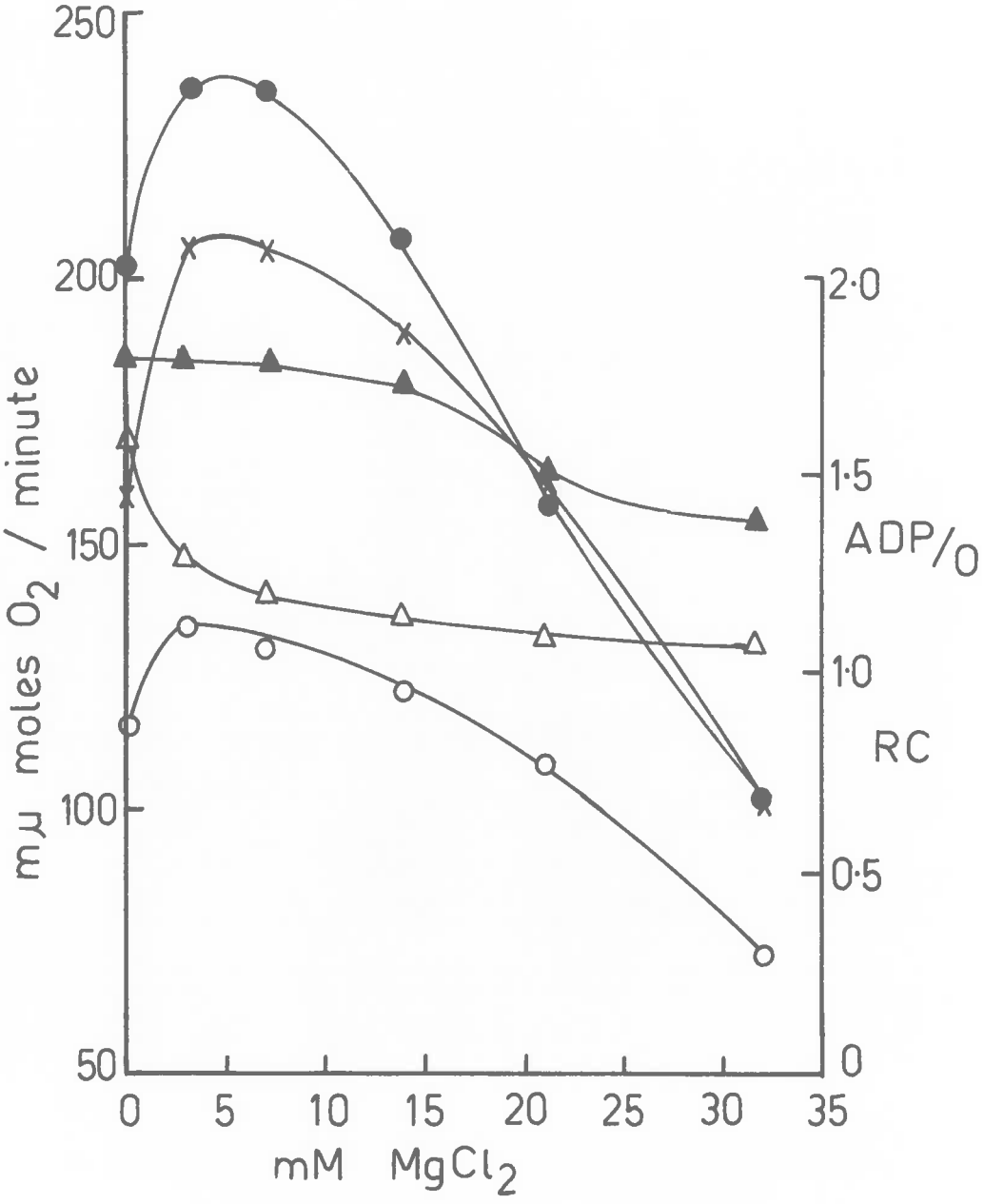
● -- ● 2nd state 3 rate

o -- o 2nd state 4 rate

x -- x DNP-induced oxidation rate

▲ -- ▲ Respiratory control ratios

Δ -- Δ ADP/O ratios.


250
200
150
100
50
mμ moles O_2 / minute
2·0
1·5
1·0
0·5
0
ADP/O
RC
0
5
10
15
20
25
30
35
mM $MgCl_2$

and inhibited respiration rates. Addition of phosphate produced a slight rise in oxidation rate which was not reversed. Lower concentrations of calcium did not have any marked effect on oxidation rates and subsequent phosphate addition did not increase the oxidation rate. The exposure of the corn mitochondria to calcium prior to phosphate addition resulted in loss of respiratory control and a weak response to ADP and DNP, although these concentrations of calcium did not affect P/O ratios (Hanson et al 1965).

B. RESULTS

1. Effect of Magnesium.

a. Oxidation, ADP/O Ratios and Respiratory Control Ratios:- The effect of magnesium on various reactions of beetroot mitochondria was investigated (ATPase activity, substrate oxidation rates and ADP/O ratios), and these were related to magnesium and phosphate uptake by beetroot mitochondria (Chapter 4).

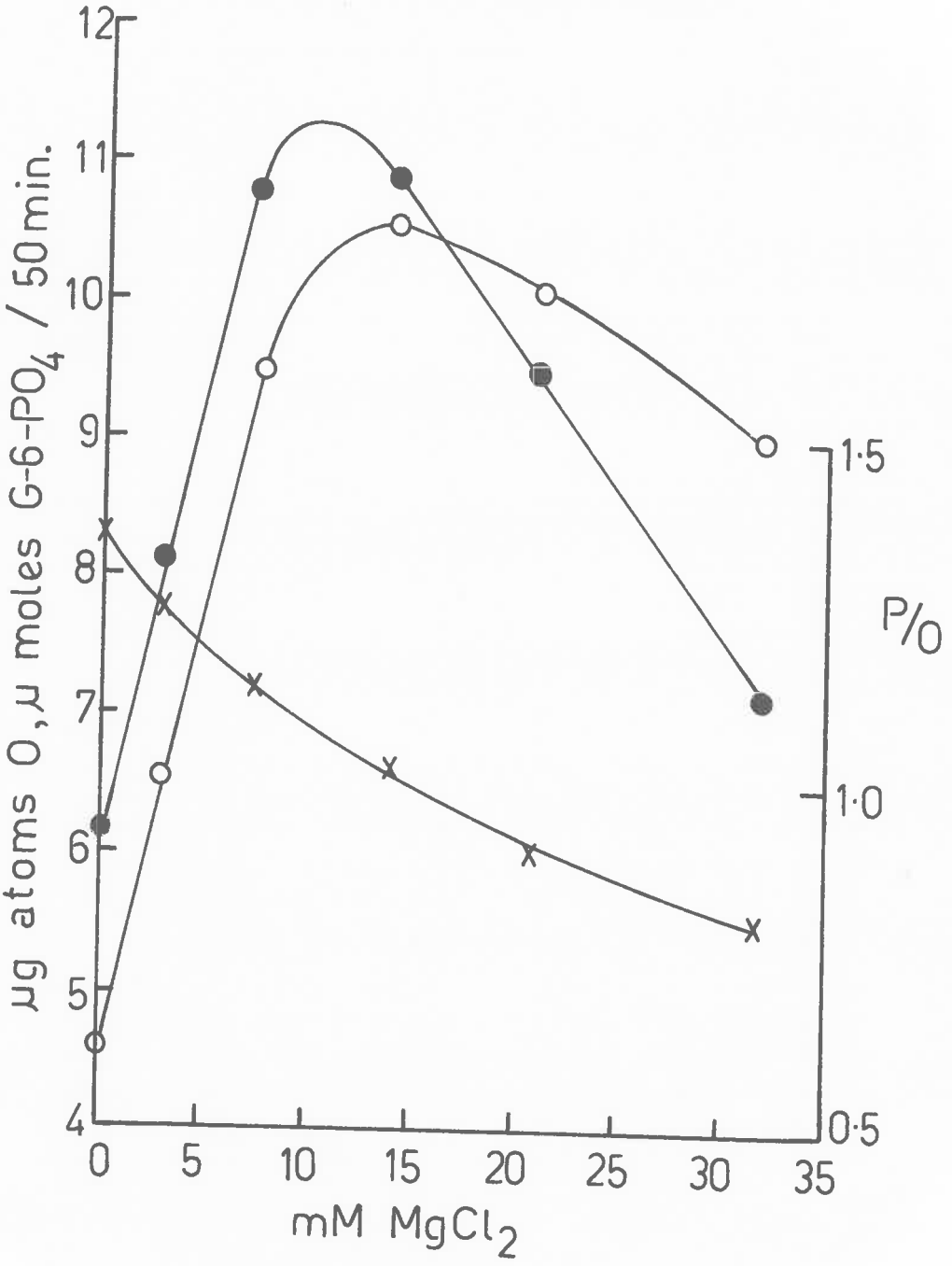
Fig. VI 1 shows the effect of $MgCl_2$ concentration on succinate oxidation rates and ADP/O ratios. An oxygen electrode was used and ADP/θ ratios were calculated by the procedure of Chance and Williams (1955). The state 3 and state 4 rates of oxidation increased with external $MgCl_2$ concentration to a maximum at 2 - 7 mM. Further $MgCl_2$ increase depressed the oxidation rate. Although the first succinate state 3 rate was lower than the second (oxaloacetate inhibition, Chapter 3), the response to a particular magnesium concentration was identical for all state 3 rates. Fig. VI 1 shows second state 3 rates. The ADP/O ratios decreased from 1.6 at 0 mM $MgCl_2$ to 1.1 at 21 - 32 mM $MgCl_2$.

Fig. VI. 2. Effect of $MgCl_2$ concentration on rate of succinate oxidation and P/O ratios, measured manometrically. Beetroot mitochondria were incubated at 25°C in a manometer vessel in a medium containing 250 mM sucrose, 8 mM Tris-HCl buffer pH 7.2, 8 mM potassium phosphate buffer pH 7.2, 3 uM cytochrome c, 10 mM glucose, 0.5 mM ADP, excess hexokinase, 8 mM Tris succinate and 492 ug mitochondrial N in a volume of 3.2 ml.

o -- o rate of succinate oxidation

● -- ● glucose-6-phosphate formation

x -- x P/O ratios


12
11
10
9
8
7
6
5
4
µg atoms O, µ moles G-6-PO4 / 50 min.
1·5
1·0
0·5
P/O
0
5
10
15
20
25
30
35
mM MgCl2

Maximum ADP/O ratios were always obtained in the absence of added $MgCl_2$. The R.C. ratios were 1.8 at 0-7 mM $MgCl_2$ and decreased as $MgCl_2$ increased further.

Fig. VI 1 shows that DNP (5×10^{-5}M) added during the second state 4 oxidation rate produced oxidation rates 80-100% of the second ADP-stimulated state 3 rates, as expected (Fig. III 15). Uncoupling of oxidation by DNP at high $MgCl_2$ concentrations (20-30 mM), did not, however, recover the oxidation rate to the state 3 oxidation rate in the presence of 2-7 mM $MgCl_2$. The DNP-rates of oxidation decreased as $MgCl_2$ increased (Fig. VI 1). Thus the oxidation mechanism was inhibited at high $MgCl_2$ concentrations and this inhibition was not relieved by the uncoupling action of DNP.

The effect of $MgCl_2$ concentration on oxidation was also determined manometrically, and P/O ratios were calculated by measuring glucose-6-phosphate formation in the presence of ADP, glucose and hexokinase (Chapter 2). Fig. VI 2 shows that the succinate oxidation rate increased with $MgCl_2$ concentration to a maximum at 10-20 mM $MgCl_2$ and decreased slowly with further $MgCl_2$ increase. Glucose-6-phosphate formation followed oxidation but the P/O ratios were decreased from 1.4 at 0 mM $MgCl_2$ to 0.8 at 31 mM $MgCl_2$, indicating that glucose-6-phosphate formation was inhibited more by high $MgCl_2$ concentrations than oxidation (Fig. VI 2). The same preparation of mitochondria provided the results of Figs. VI 1 and 2.

The maximum state 3 succinate oxidation rate was measured at 3-7

Table VI. 1. Effect of $MgCl_2$ and DNP on ATPase Activity of Fresh and Frozen Beetroot Mitochondria.

Mitochondria were incubated at 25°C in a medium containing 250 mM sucrose, 8 mM Tris-HCl buffer pH 7.2, 3 uM cytochrome c, 2 mM disodium ATP and 75 ug mitochondrial N/ml. Mitochondria were used fresh 1 hour after isolating or stored for 18 hours at 0°C.

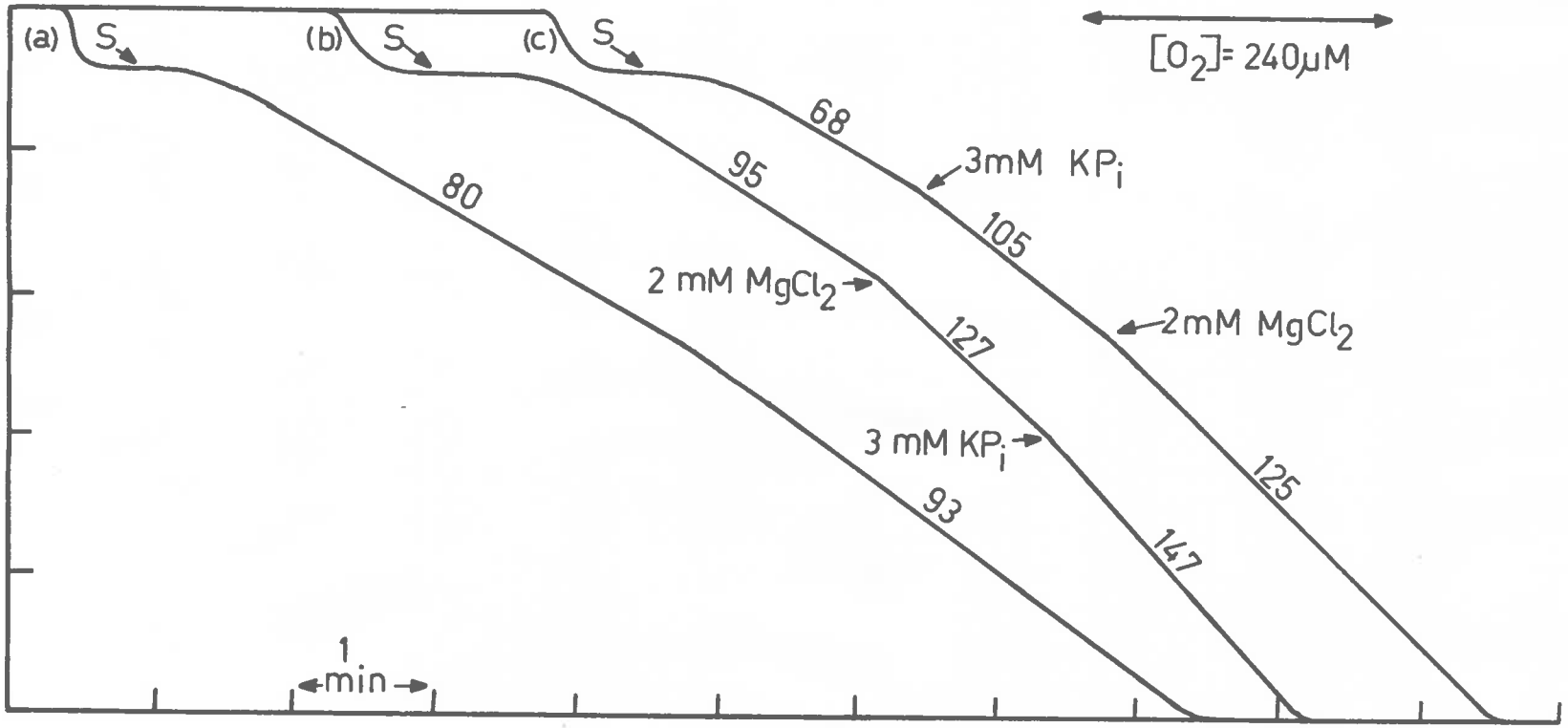
External Conc. mM	ATPase Activity (umoles Pi released/mg N/10 minutes) Fresh	Frozen
Expt. A. $MgCl_2$		
0	0.97	0.30
5	1.00	0.62
9	0.95	0.59
14	0.97	0.50
23	1.00	0.59
30	0.95	0.61
Expt. B. DNP		
5×10^{-3}	0.86	0.34
1×10^{-2}	0.75	0.34
5×10^{-2}	0.49	0.38
1×10^{-1}	0.38	0.40
5×10^{-1}	0.32	0.36
1	0.33	0.36

mM $MgCl_2$ with the oxygen electrode whereas the maximum rate measured manometrically was at 15 mM $MgCl_2$. The inhibition of oxidation rate by high $MgCl_2$ concentrations was more marked in polarographic than in manometric measurements. Similarly, the ADP/O ratios differed from the P/O ratios although both responded in the same way to $MgCl_2$ concentration, decreasing from a maximum value at 0 mM $MgCl_2$ to a minimum at the highest $MgCl_2$ concentration. The maximum ADP/O ratio measured was 1.6 whereas the maximum P/O ratio was 1.4 using the same mitochondrial preparation and concentration. The ADP/O ratios decreased to 1.1 at 21 - 32 mM $MgCl_2$ whereas the P/O ratios decreased to 0.9 at 21 mM $MgCl_2$ and 0.8 at 31 mM $MgCl_2$.

P/O ratios lower than corresponding ADP/O ratios have been measured in other plant mitochondria by Wiskich et al (1964) and Strickland (1960) who implicated shaking mechanisms, penetration factors or the presence of enzymes reducing the glucose-6-phosphate yield. The different magnesium concentrations for maximum oxidation rates (Figs. VI 1 and 2) could also result from the general differences in measurement techniques.

b. ATP-ase Activity:- Table VI 1 shows the effect of $MgCl_2$ concentration on the ATP-ase activity of beetroot mitochondria. Activity was low under the experimental conditions used and 3 - 5 u moles phosphate/mg N/hour were released from 3 mM ATP at 0 mM $MgCl_2$. This rate can be compared with the 20 u moles phosphate/mg N/hour released from 5 mM ATP by corn mitochondria in the presence of 0.2 M KCL (Hanson et al 1965). Increasing the $MgCl_2$ concentration from 0 mM to 30 mM had no

Fig. VI. 3. The effects of $MgCl_2$ and potassium phosphate on succinate oxidation by beetroot mitochondria, measured with an oxygen electrode. Mitochondria were incubated at 25°C in a medium containing 200 mM sucrose, 8 mM Tris-HCl buffer pH 7.2, 3 uM cytochrome c and 360 ug mitochondrial N in 3.1 ml total volume. Phosphate (K Pi) was added as potassium phosphate buffer pH 7.2 and 8 mM sodium succinate was added at S. Rates of oxidation are expressed as mu moles O_2/minutes.


(a)
S
(b)
S
(c)
S
[O2]= 240µM
68
3mM KPi
95
105
80
2 mM MgCl2
127
2mM MgCl2
3 mM KPi
125
93
147
1
min

effect on the ATPase activity of beetroot mitochondria under the conditions of these experiments. Freezing the mitochondria for 18 hours decreased the ATPase activity by 40% (Table VI 1), and again $MgCl_2$ concentration had little effect. Activity at 0 mM $MgCl_2$ was inhibited more by freezing than activity at 5 - 28 mM $MgCl_2$.

The ATPase activity of beetroot mitochondria was inhibited at 80% by oligomycin which inhibited glucose-6-phosphate formation by 80% at the same concentration. Very small amounts of glucose-6-phosphate were formed from endogenous sources of ADP (with succinate, glucose and hexokinase), but the effect of oligomycin in inhibiting this formation by 70-80% could still be measured.

The effect of DNP concentration on beetroot mitochondrial ATPase activity is shown in Table VI 1. Increasing the DNP concentration from 5×10^{-6}M to 1×10^{-3}M in the absence of $MgCl_2$ decreased ATPase activity. Freezing the mitochondria for 18 hours decreased the rate of ATPase activity, and DNP concentration had no effect on activity after freezing.

c. Succinate Oxidation Rates:- The addition of low $MgCl_2$ concentrations to beetroot mitochondria oxidizing succinate stimulated oxidation.

Fig. VI 3 compares the succinate oxidation rate in the absence of phosphate, $MgCl_2$ and ADP (which increased slowly with time) with the rate when 2 mM $MgCl_2$ and 3 mM potassium phosphate were added. Magnesium increased the oxidation rate by 45% and phosphate further stimulated oxidation by 25% (Fig. VI 3b). Similarly (Fig. VI 3c), addition of

Fig. VI. 4. The effects of $MgCl_2$ on rates of succinate oxidation by beetroot mitochondria incubated at 25°C in a 3.2 ml oxygen electrode cell containing 200 mM sucrose, 8 mM Tris-HCl buffer pH 7.2, 3 mM potassium phosphate buffer pH 7.2, 3 uM cytochrome c and 585 ug mitochondrial N. 8 mM Tris succinate was added at S and 0.16 mM ADP at ADP. Rates of oxidation are expressed as mu moles O_2/min. ADP/O ratios were (A) 1.60, (B) 1.6, (C) 1.5, (D) 1.6 and (E) 1.6.

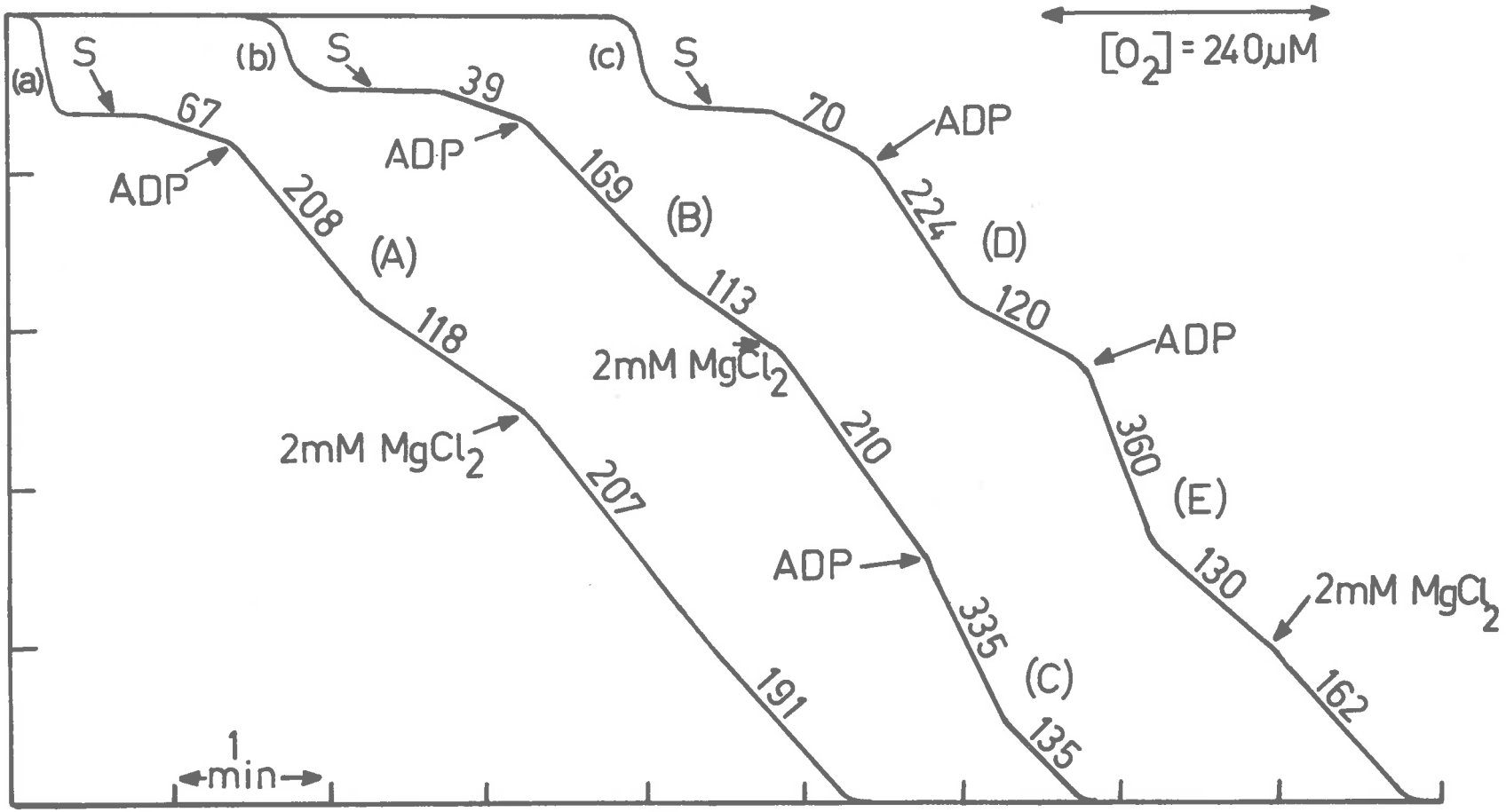
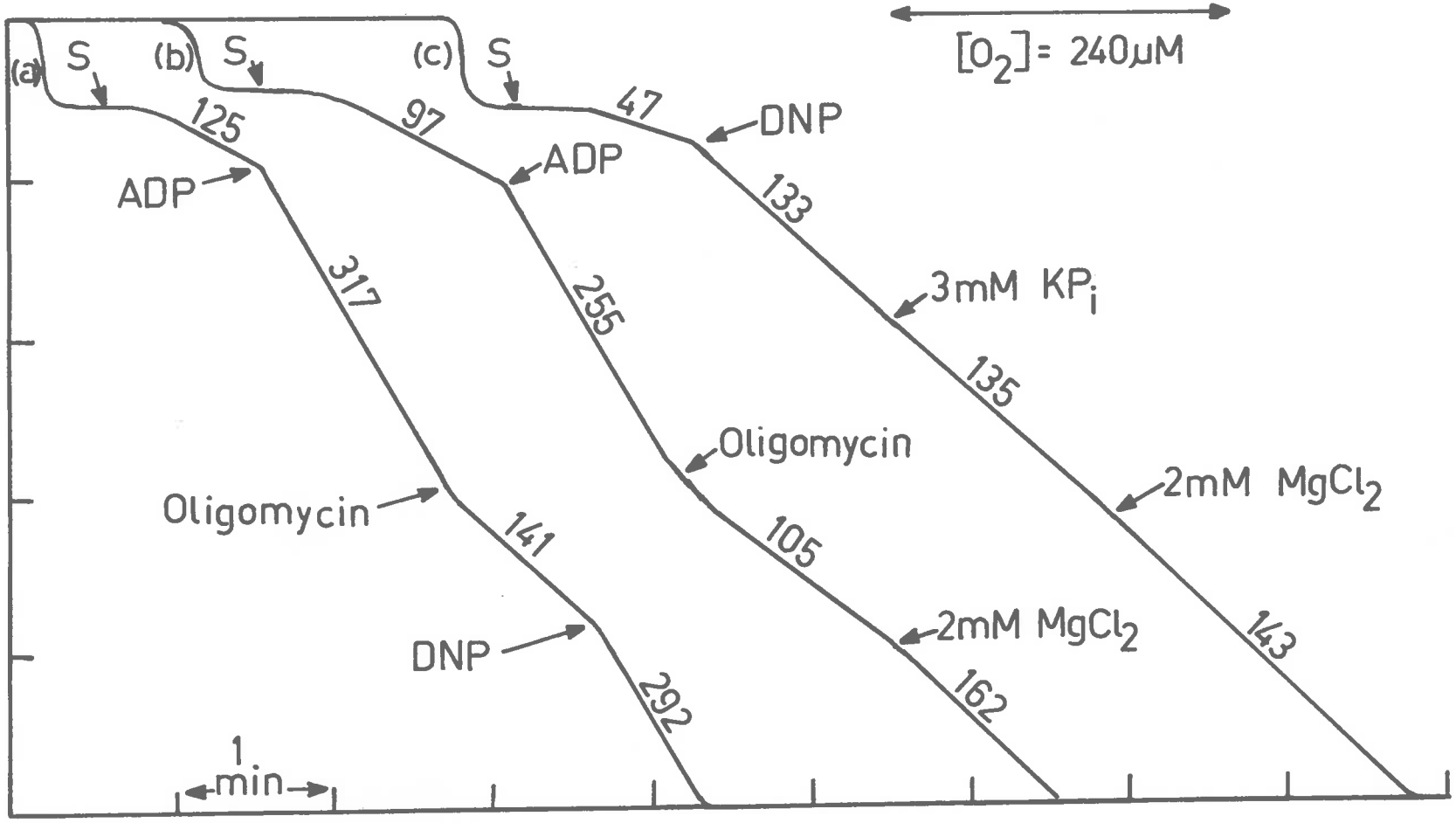

(a)
S
67
ADP
208
(A)
118
2mM MgCl2
207
191
(b)
S
39
ADP
169
(B)
113
2mM MgCl2
210
ADP
335
(C)
135
(c)
S
70
ADP
224
(D)
120
ADP
360
(E)
130
2mM MgCl2
162
[O2] = 240µM
1
min

Fig. VI. 5. The effects of DNP and $MgCl_2$ on the oligomycin-induced rate of succinate oxidation by beetroot mitochondria. Mitochondria were incubated at 25°C in a medium containing 200 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 3 uM cytochrome c and 488 ug mitochondria N in a total volume of 3.1 ml. 7 mM Tris succinate was added at S, 0.70 mM ADP at ADP and the final concentrations of oligomycin and DNP were 1 ug/ml and 9×10^{-5}M. 3 mM potassium phosphate buffer pH 7.2 was present initially in (a) and (b) and 2 mM $MgCl_2$ was present in (a). Rates of oxidation are expressed as mu moles O_2/minute.


(a)
S
(b)
S
(c)
S
$[O_2]$ = 240μM
125
ADP
317
Oligomycin
141
DNP
292
97
ADP
255
Oligomycin
105
2mM $MgCl_2$
162
47
DNP
133
3mM KP_i
135
2mM $MgCl_2$
143
1
min

phosphate first produced a 54% stimulation, and 2 mM $MgCl_2$ produced a further 22% stimulation. The increase in succinate oxidation rate during 7 minutes incubation in the absence of phosphate, $MgCl_2$ and ADP was only 16% (Fig. VI 3a). Addition of $MgCl_2$ in low concentrations (1 - 2 mM) after stimulation of oxidation by one addition of 2 mM $MgCl_2$ did not further stimulate oxidation. A second $MgCl_2$ addition after phosphate-stimulation also had little further effect on oxidation.

$MgCl_2$ (2 mM) added after state 3 to state 4 rate transition with ADP and phosphate, stimulated the state 4 oxidation rate by 60% (Fig. IV 4a and c), and subsequent state 3 - state 4 transitions were not impaired by $MgCl_2$ (Fig. VI 4b). These effects of $MgCl_2$ can be predicted from Fig. VI 1.

Fig. VI 5a shows that 8×10^{-5}M DNP stimulated the oligomycin-inhibited succinate rate by 110% in the presence of 3 mM phosphate and 2 mM $MgCl_2$. $MgCl_2$ (2 mM) produced a 55% stimulation in oligomycin-inhibited rate (Fig. VI 5b) in the same experiment. Fig. VI 5c shows the stimulation of succinate oxidation by DNP in the absence of phosphate and magnesium. Additions of phosphate and magnesium had very little effect on the DNP-stimulated rate of oxidation.

Decreasing the $MgCl_2$ concentration added had little effect on the stimulation of succinate oxidation. Return to the initial or inhibited oxidation rate after a stimulation by $MgCl_2$ was never observed as reported for animal (Chance 1965, Rossi and Lehninger 1964, Chappell et al 1963) and corn mitochondria (Hanson et al 1965). Fig. VI 6 shows the

Fig. VI. 6. Effects of low concentrations of $MgCl_2$ and Tris-phosphate on succinate oxidation by beetroot mitochondria incubated at 25°C in a medium containing 200 mM sucrose, 8 mM Tris-HCl buffer pH 7.2, 3 uM cytochrome c, 0.3 mM Tris ATP, 1 ug/ml oligomycin and 570ug mitochondria N in a total of 3.2 ml. 7 mM Tris succinate was added at 5. Rates are expressed as mu moles O_2/minute.

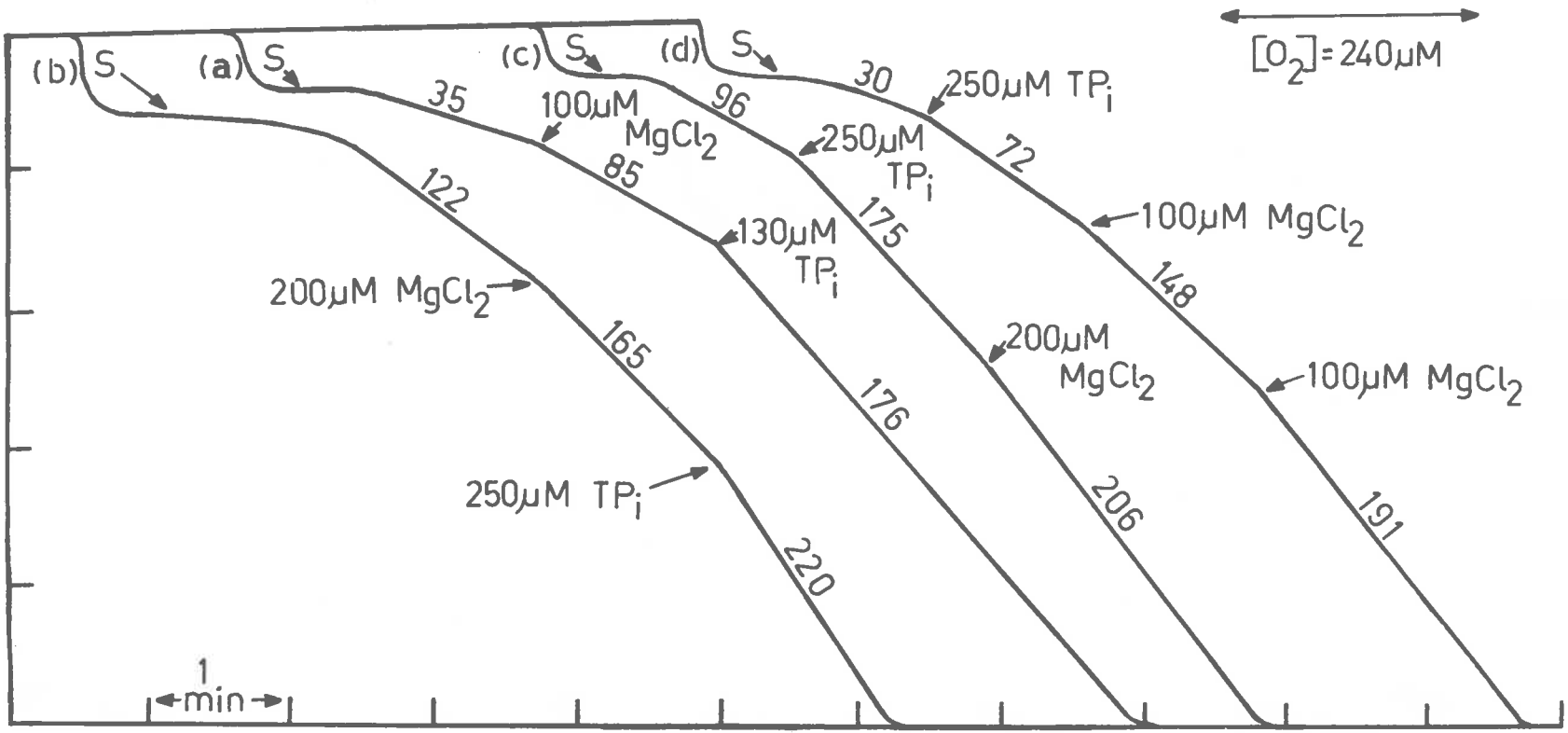

(b) S
(a) S
(c) S
(d) S
[O2] = 240µM
30
250µM TPi
72
100µM MgCl2
148
100µM MgCl2
191
35
100µM MgCl2
85
130µM TPi
175
200µM MgCl2
206
96
250µM TPi
122
200µM MgCl2
165
176
250µM TPi
220
1 min

Table VI. 2. Mg^{++}/O Ratios.

Beetroot mitochondria were incubated in an O_2 electrode cell in an ion uptake medium (Tris phosphate) with 63 ug mitochondrial N/ml and where indicated 8 mM Tris succinate, 15 mM Tris malate (with 10 mM Tris glutamate), 1 ug/ml Oligomycin and 0.90 mM ADP. Mg^{++} uptake and oxygen uptake were corrected for uptake in the absence of substrate. "Extra O" was calculated by subtracting the rate of oxygen uptake in the absence of $MgCl_2$ from the rate of oxygen uptake in the presence of $MgCl_2$.

	O ug atoms/ mg N/5 min.	Mg^{++} umoles/ mg N/5 min.	Mg^{++}/O	Mg^{++}/ "Extra O"
Succinate	6.26	1.89	0.30	3.1
Succinate	6.78	1.69	0.25	2.7
Succinate + Oligomycin	5.64	1.60	0.28	2.9
Succinate + Oligomycin	6.0	1.61	0.27	2.9
Succinate + ADP	6.68	0.06	0.009	-
Succinate + ADP	6.94	0.10	0.014	-
Malate	4.34	0.24	0.06	0.6
Malate	4.30	0.30	0.07	0.7

stimulation of succinate oxidation by 100 uM $MgCl_2$ (Fig. VI 6a), or 200 uM $MgCl_2$ (Fig. VI 6b and c). ATP plus oligomycin were included to prevent the initial inhibition of succinate oxidation and any change in oxidation rate with time (Fig. III 3). Magnesium stimulated oxidation but no subsequent inhibited oxidation rate was observed.

Addition of 130 uM or 250 uM Tris - Pi, before or after $MgCl_2$, stimulated succinate oxidation (Fig. VI 6) and the stimulated oxidation rate continued until all oxygen was utilized. A second addition of 100 uM $MgCl_2$ in the presence of 250 uM phosphate further stimulated succinate oxidation (Fig. VI 6d).

d. Mg^{++}/O ratios:- Substrate-dependent magnesium uptake by beetroot mitochondria was shown to occur to a small extent in the absence of phosphate and massively in the presence of phosphate (Chapter 4). The stimulation of succinate oxidation by low concentrations of $MgCl_2$ should be accompanied by uptake of magnesium but the oxidation rate did not return to the slower rate and an Mg^{++}/O ratio (similar to an ADP/O ratio) could not be calculated. However, incubation mixtures were removed from the oxygen electrode cell after 4-5 minutes incubation in the presence of substrate, 13 mM $MgCl_2$ and 3 mM phosphate and the mitochondria were collected by Millipore filtration and magnesium and phosphate uptake was determined. Mg^{++}/O ratios were calculated and Mg^{++}/"extra O" ratios were also estimated by correcting the succinate oxidation rates after magnesium addition for the rate before addition (Chappell et al 1963).

Table VI 2 shows the Mg^{++}/O ratios calculated after incubating

Fig. VI. 7. Effects of low concentrations of KCl, $MgCl_2$ and Tris-phosphate on succinate oxidation by beetroot mitochondria incubated at 25°C in a medium containing 200 mM sucrose, 8 mM Tris-HCl buffer pH 7.2, 3 uM cytochrome c and 450 ug mitochondrial N in a total volume of 3.0 ml. 7 mM Tris succinate was added at S. Rates are expressed as mu moles O_2/minute.

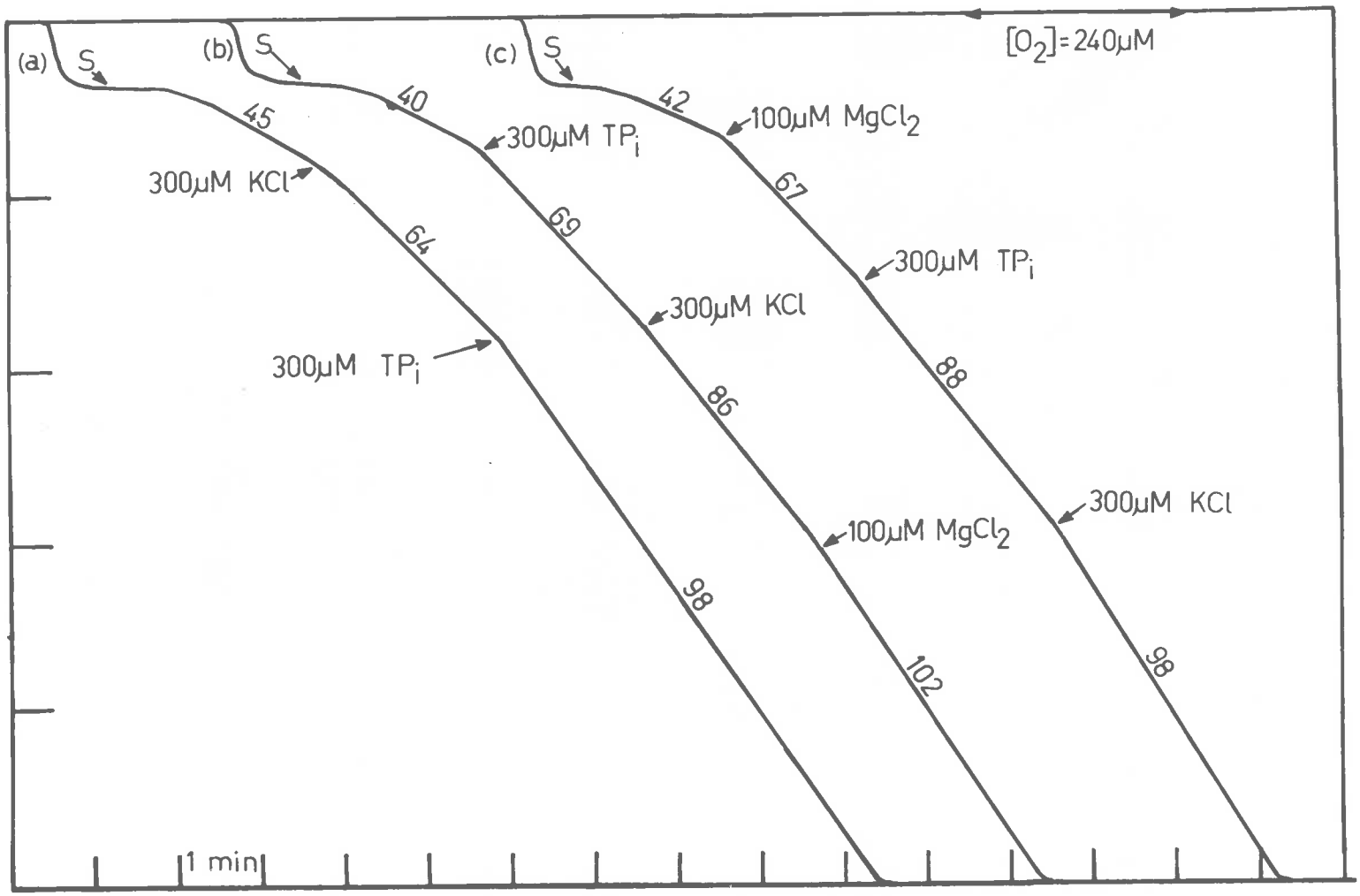

(a)
S
45
300μM KCl
64
300μM TPi
98
(b)
S
40
300μM TPi
69
300μM KCl
86
100μM MgCl2
102
(c)
S
42
100μM MgCl2
67
300μM TPi
88
300μM KCl
98
[O2]=240μM
1 min

Fig. VI. 8. Effect of $CaCl_2$ concentration on malate oxidation, ADP/O and R.C. ratios of beetroot mitochondria incubated in a medium containining 200 mM sucrose, 8 mM Tris-HCl buffer pH 7.2, 3 uM cytochrome c, 4 mM Tris-phosphate buffer pH 7.2, 2 mM $MgCl_2$, 15 mM Tris Malate, 10 mM Tris glutamate and 380 ug mitochondrial N in a volume of 3.3 ml. The state 3 oxidation rate was produced by addition of 0.30 mM ADP and the DNP rate was produced by addition of 5×10^{-5} M DNP during the 1st state 4 oxidation rate.

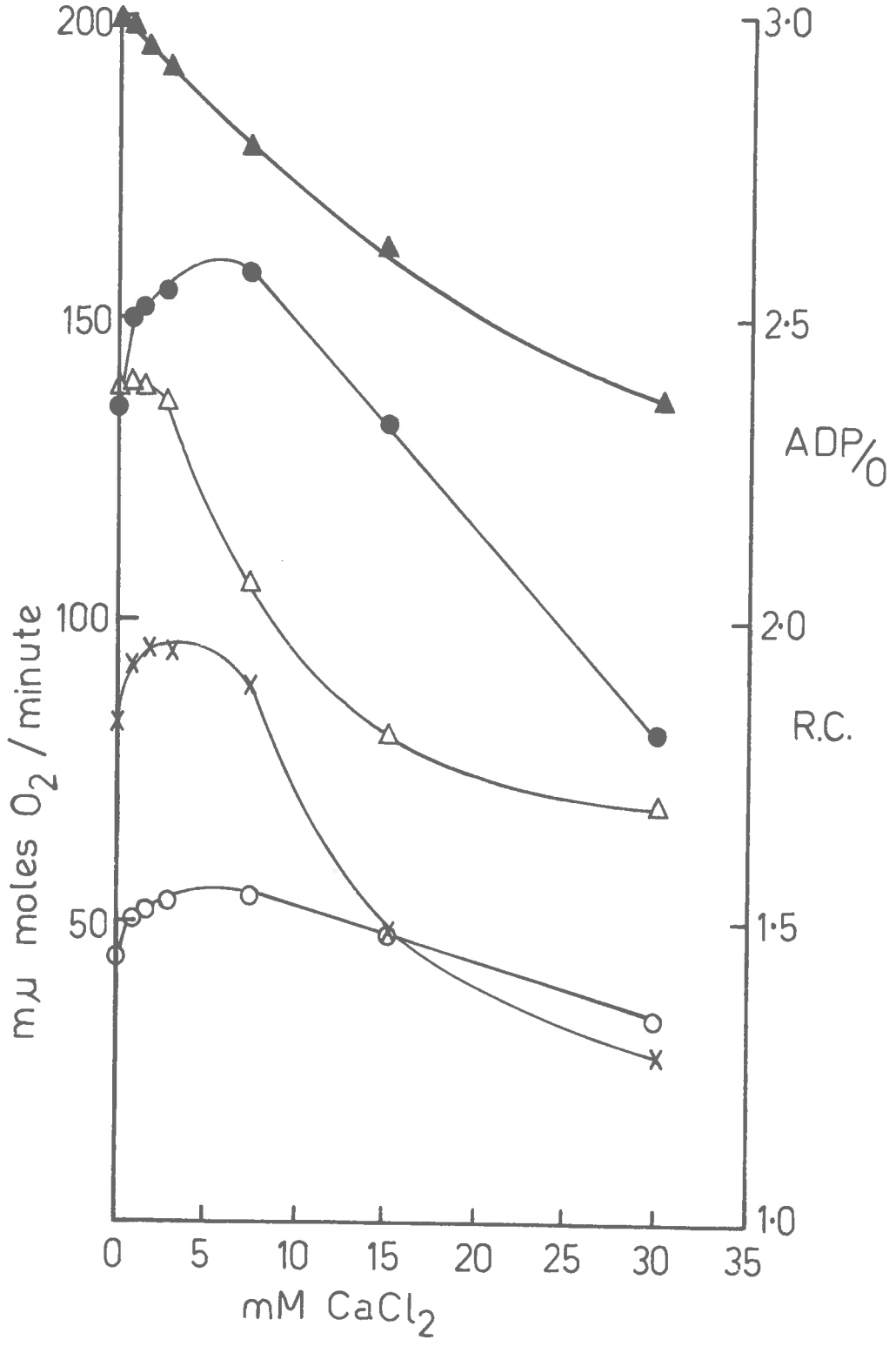
● -- ● 1st state 3 rate of oxidation

o -- o 1st state 4 rate

x -- x DNP-induced oxidation rate

▲ -- ▲ Respiratory control ratios

Δ -- Δ ADP/O ratios.


200
150
100
50
3·0
2·5
2·0
1·5
1·0
ADP/O
R.C.
mμ moles O2 / minute
0
5
10
15
20
25
30
35
mM CaCl2

mitochondria with succinate or malate. The magnesium uptake was corrected for uptake in the absence of substrate when no oxidation was measured. The Mg^{++}/O ratios were low (0.25 - 0.3) with succinate and were approximately zero with malate or in the presence of ADP. The substrate-dependent magnesium uptake rate recorded in Table VI 2 (1-2 u moles/mg N/ 5 min.) was the rate measured in normal ion uptake experiments (Chapter 4). Mg^{++}/"extra O" ratios of 0.6 - 3.0 were calculated (Table VI 2).

2. Effect of NaCl and KCl.

The stimulation of succinate oxidation by low $MgCl_2$ concentrations was repeated by low concentrations of NaCl and KCl. Fig. VI 7 shows that 300 uM KCl stimulated succinate oxidation and $MgCl_2$ and Tris phosphate in low concentrations further stimulated oxidation. Initial addition of 8 mM Tris - HCl had no effect on the rate of succinate oxidation.

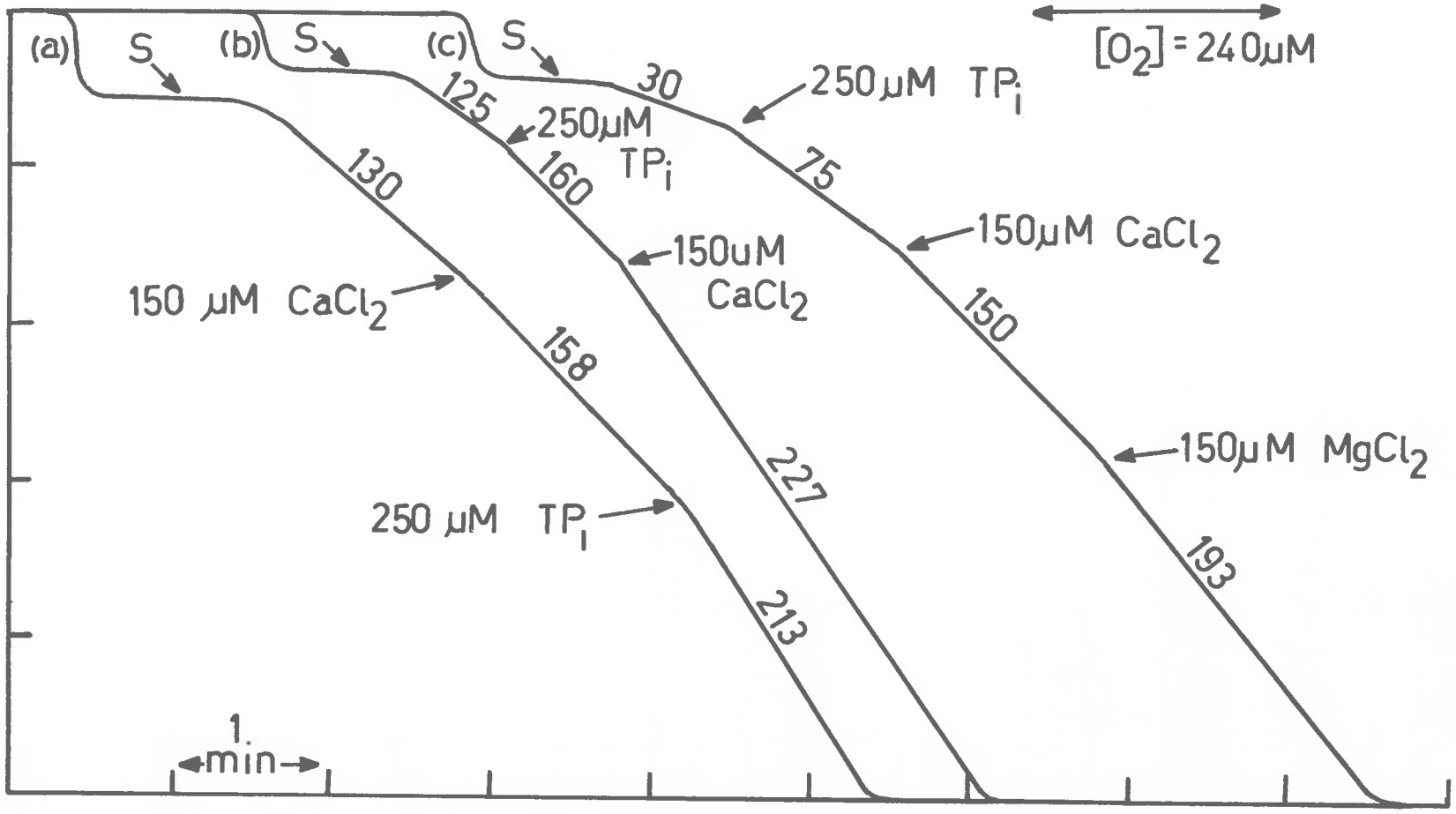
3. Effect of Calcium.

a. Oxidation, ADP/O Ratios and R.C. Ratios:- The uptake of calcium by beetroot mitochondria (Chapter 5) was related to the effects of calcium on other mitochondrial reactions.

Fig. VI 8 shows the effect of $CaCl_2$ concentration on malate oxidation rates and associated ADP/O and R.C. ratios. Calcium effect on succinate oxidation and ADP phosphorylation was also investigated. The state 3 malate oxidation rates increased as $CaCl_2$ concentration increased from 0 mM to 8 mM and were depressed on further $CaCl_2$ increase (Fig. VI 8). The malate ADP/O ratios remained constant at 2.4 from 0-3 mM $CaCl_2$ and then decreased as the $CaCl_2$ concentration increased (Fig. VI 8).

Succinate ADP/O ratios decreased from 1.6 at 0 mM $CaCl_2$ to 1.3 at

Fig. VI. 9. Effects of low concentrations of $CaCl_2$ and Tris-phosphate on succinate oxidation by beetroot mitochondria incubated at 25°C in an oxygen electrode cell of 3.2 ml in a medium containing 200 mM sucrose, 8 mM tris-HCl buffer pH 7.2, 3 uM cytochrome c, and 570 ug mitochondrial N. 7 mM Tris succinate was added at S. Rates are expressed as mu moles O_2/minute.


(a)
S
(b)
S
(c)
S
[O2] = 240µM
30
250µM TPi
125
250µM TPi
160
150µM CaCl2
75
150µM CaCl2
130
150 µM CaCl2
158
227
150
150µM MgCl2
250 µM TPi
213
193
1 min

3 mM $CaCl_2$ and 1.1 at 30 mM $CaCl_2$. Thus the response of the phosphorylating system in beetroot mitochondria to calcium concentration was similar to the response to magnesium concentration. Calcium did not reduce oxidative phosphorylation until relatively high concentrations were present, in contrast to results reported with other mitochondria (Vasington and Murphy 1962, Carafoli et al 1964, Hanson et al 1965). At the $CaCl_2$ concentration (2 - 3 mM) used for ion uptake experiments (Chapter 5), oxidative phosphorylation would not be greatly affected.

State 4 malate oxidation rates followed the state 3 rates in response to $CaCl_2$ concentration but the respiratory control ratios decreased as $CaCl_2$ concentration increased (Fig. VI 8). DNP (5×10^{-5}M), added during state 4, uncoupled oxidation to 60% of the previous state 3 rate at 0-7 mM $CaCl_2$ and to 40% at higher $CaCl_2$ concentrations. At high $CaCl_2$ concentrations DNP did not stimulate the state 4 oxidation rate (Fig. VI 8). These experiments were carried out in the presence of 2 mM $MgCl_2$ and the mitochondria were incubated with the different $CaCl_2$ concentrations for 10 minutes before calculation of the ADP/O ratios.

b. Succinate Oxidation Rates:- Fig. VI 9a and b show that 150 uM $CaCl_2$ stimulated succinate oxidation in the absence of magnesium and phosphate. Addition of 250 uM Tris phosphate produced a further stimulation. When 3 mM $MgCl_2$ was initially present (with or without phosphate) addition of 150 uM or 3 mM $CaCl_2$ or $MgCl_2$ did not affect the succinate oxidation rate. This can be predicted from Figs. VI 1 and 8. When 150 uM $CaCl_2$ was present with 250 uM phosphate addition of 150 uM $MgCl_2$ or $CaCl_2$ stimulated oxidation (Fig. VI 9c).

The stimulation of oxidation by low concentrations of $CaCl_2$ was not followed by return to the original oxidation rate (with or without phosphate), but continued undiminished until oxygen was depleted or another addition was made. Succinate Ca^{++}/O ratios were calculated by the method described for measuring Mg^{++}/O ratios and were approximately the same (0.2 - 0.3). Failure to retain calcium and magnesium taken up without phosphate or to precipitate the cation with phosphate would produce continually stimulated oxidation rates and low Mg^{++} or Ca^{++}/O ratios. Estimation of Mg^{++} and Ca^{++}/O ratios during normal ion uptake experiments in the presence of phosphate confirmed the low ratios of 0.2 - 0.4 calculated in these short-term experiments.

C. DISCUSSION

1. Effect of Magnesium on Oxidative Phosphorylation.

$MgCl_2$ stimulated state 3 and state 4 rates of succinate oxidation by beetroot mitochondria, but ADP/O and P/O ratios were highest in the absence of added $MgCl_2$. Thus endogenous magnesium (500 mu moles/mg N) was sufficient for operation of oxidative phosphorylation at maximum efficiency (cf. Purvis and Slater 1959, Baltscheffsky 1957). The effects of $MgCl_2$ concentration from 0 to 2.5 mM on oxidation by beetroot mitochondria were not investigated.

Succinate oxidation was inhibited by very high $MgCl_2$ concentrations (20 - 30 mM) and DNP did not ~~completely~~ relieve this inhibition. Thus the electron transfer system was inhibited by high $MgCl_2$ concentrations in addition to the phosphorylation system. High $MgCl_2$ concentrations have been reported to inhibit oxidative phosphorylation in digitonin particles

of animal mitochondria (Cooper and Lehninger 1957, Purvis and Slater 1959).

Ion uptake by beetroot mitochondria was investigated using succinate and 15 mM $MgCl_2$. At this concentration of $MgCl_2$ the state 3 and state 4 oxidation rates were stimulated although the R.C. ratios had decreased from 1.8 at 0 mM $MgCl_2$ to 1.6 or 1.7. The ADP/O ratio was 1.1 compared with 1.6 at 0 mM $MgCl_2$. These results were obtained from polarographic measurements. The maximum succinate oxidation rate (with ADP, hexokinase and glucose) was measured manometrically at 10 - 15 mM $MgCl_2$ and the P/O ratio was 1.0 at 15 mM $MgCl_2$ compared with 1.35 at 0 mM $MgCl_2$. Conditions for ion uptake experiments were similar to those for manometry experiments with respect to length of experiment (15-40 minutes) and aeration mechanism (shaking). Polarographic experiments had a short experimental period and the incubation mixtures were stirred by a magnetically controlled bar (Chapter 2).

2. ATPase Activity.

The ATPase activity of fresh beetroot mitochondria was very low in comparison with that reported for corn mitochondria in the absence of $MgCl_2$ (3.5 u moles Pi/hour/mg N compared with 20 u moles Pi/hour/mg N), Hanson et al (1965). ATPase activity is defined as the amount of inorganic phosphate released from ATP in a given time, and does not necessarily imply the existence of a single or specific enzyme. The conditions for measuring ATPase activity of beetroot mitochondria were the same as those for measuring oxidative phosphorylation or ion uptake by these

mitochondria. Thus the contribution of ATPase activity could be estimated in these experiments. Oligomycin action on ATPase activity was also investigated in an effort to account for the massive stimulation of ion uptake by oligomycin in some experiments (Table IV 8). The medium for measuring ATPase activity in beetroot mitochondria contained 0.25 M sucrose and no KCL whereas the ATPase activity of corn mitochondria was measured in a medium containing 0.2 M KCl (Hanson et al 1965). The temperature and pH were similar in both investigations (25°C and 7.4 for beetroot and 28°C and 7.5 for corn mitochondria).

Reid et al (1964) reported that potassium and magnesium stimulated, calcium inhibited and sodium did not affect ATPase activity of cauliflower mitochondria. $MgCl_2$ (1 mM) stimulated the ATPase activity of corn mitochondria by 100% (Hanson et al 1965). The ATPase activity of beetroot mitochondria was not affected by $MgCl_2$ concentrations between 0 and 30 mM. ATPase activity of cauliflower mitochondria was greatly increased in a medium of low sucrose tonicity and the increased activity was inhibited by DNP, while that of suspensions incubated in 0.45 M sucrose was not affected by DNP (Reid et al 1964). DNP increased ATPase activity of pea mitochondria in proportion to DNP concentration between 6×10^{-5}M and 3×10^{-3}M (Forti et al 1964). ATPase activity of beetroot mitochondria was inhibited by increasing DNP concentrations from 5×10^{-6}M to 1×10^{-3}M. Aging of cauliflower mitochondria (Reid et al 1964) for 30 minutes in ice cold 0.45 M sucrose inhibited ATPase activity. Freezing beetroot mitochondria for 18 hours inhibited ATPase activity. The ATPase activities of different plant mitochondria can not, however, be compared unless the

mitochondria have been tested under the same conditions. ATPase activity of one mitochondrial type varied greatly under different conditions and inherent differences in activity between mitochondrial types could only be estimated under identical conditions for both isolation of mitochondria and measurement of activity.

Oligomycin (1 ug/ml) inhibited ATPase activity of beetroot mitochondria by 80%. Very little glucose-6-phosphate was formed in beetroot mitochondria from endogenous ADP but the 80% inhibition by oligomycin was measurable. The stimulation of ion uptake by oligomycin in some experiments (Table IV 8) cannot, however, be accounted for on the basis of inhibition of endogenous ATP formation or ATPase activity. Oligomycin could stabilise membrane structure or a precipitated cation phosphate complex. Rossi and Lehninger (1964) reported that oligomycin and ATP allowed the calcium-stimulated oxidation rate in rat liver mitochondria to return to the inhibited oxidation rate after uncoupling by phosphate. ATP action was suggested to be a result of bound adenine nucleotide stabilising membrane structure or a precipitated complex (Carafoli et al 1965a).

3. Effect of Calcium on Oxidative Phosphorylation.

The effects of calcium on oxidative phosphorylation in beetroot mitochondria were similar to the effects of magnesium. In animal mitochondria (Vasington and Murphy 1962, Brierley et al 1964b) 2-3 mM $CaCl_2$ was reported to completely uncouple oxidative phosphorylation. In corn mitochondria (Hanson et al 1965) 2 mM $CaCl_2$ reduced P/O ratios with pyruvate and malate by approximately 60%. In beetroot mitochondria calcium stimulated state 3 and 4 oxidation rates and the ADP/O ratios

of 2.4 (malate and glutamate) at 0-3 mM $CaCl_2$ decreased to 1.7 at 30 mM $CaCl_2$. Similarly ADP/O ratios with succinate decreased from 1.6 at 0 mM $CaCl_2$ to 1.1 at 30 mM $CaCl_2$. Thus high $CaCl_2$ concentrations did not drastically reduce oxidative phosphorylation in beetroot mitochondria and had almost the same effect as $MgCl_2$ over the same concentration range. At the $CaCl_2$ concentrations used for ion uptake experiments (2-3 mM) the succinate oxidation rate was stimulated and the ADP/O ratio was reduced by only 19%. The failure of calcium to uncouple oxidative phosphorylation more than magnesium in beetroot mitochondria could be related to the isolating procedure (with EDTA) or to membrane structure and calcium binding capacity. Calcium and magnesium effects on different mitochondria cannot be compared unless the mitochondria have been isolated and tested under identical conditions. Isolating procedures and experimental conditions could affect a membrane structure and cation binding capacity and the stability of the oxidative phosphorylation process.

4. Ion Effects on Oxidation.

Low concentrations of $MgCl_2$ and $CaCl_2$ stimulated succinate oxidation (with or without phosphate) but oxidation did not return to the original slower rate. Low concentrations of calcium, strontium ormanganese added to animal mitochondria stimulated oxidation, followed by a return to the original rate (Chance 1965, Rossi and Lehninger 1964, Chappell et al 1963). In corn mitochondria (Hanson et al 1965) calcium stimulation of oxidation was followed by a return to a slower rate, but phosphate irreversibly stimulated oxidation. This phosphate effect was also reported by Rossi and Lehninger (1964) and may be related to swelling or loss of

integrity of mitochondria on formation of a calcium phosphate complex (Chappell and Crofts 1966). ATP or oligomycin prevented this phosphate effect.

The failure of calcium and magnesium-stimulated succinate oxidation rates to return to the original oxidation rate may be related to the permeability of beetroot mitochondria. Plant mitochondria appear to be more permeable to ions (Stoner and Hanson 1966) than animal mitochondria (Chappell and Crofts 1966). Delay in cation-phosphate complex formation and continuous leakage of cations taken up back into the medium could produce continually stimulated oxidation in the presence or absence of phosphate. The ability of low concentrations of NaCl and KCl (but not Tris-HCl) to produce similar stimulations in oxidation suggests a general cat ionic effect or uptake process.

Phosphate (Tris) added before or after divalent cation addition produced further stimulation of oxidation in beetroot mitochondria. More rapid spontaneous breakdown of D∼P than C∼D (classical), or exchange of phosphate for OH^- and partial relief of PMF (chemiosmotic) could produce this phosphate effect.

Mg^{++}/O and Ca^{++}/O ratios of 0.2 - 0.4 were calculated by measuring magnesium or calcium uptake by beetroot mitochondria oxidizing succinate. These ratios were much lower than the ratio of 4 calculated in animal mitochondria for this substrate (Chance 1965) which agrees with the mechanisms of ion uptake based on either the classical or chemiosmotic hypotheses of oxidative phosphorylation (Chapter 1). The low cation/O

ratios measured in beetroot mitochondria could result from poor precipitation of cation-phosphate complex and leakage of the accumulated ions out of the mitochondria.

D. CONCLUSIONS

Low concentrations of $MgCl_2$ and $CaCl_2$ irreversibly stimulated substrate oxidation by beetroot mitochondria in contrast to the situation with animal and corn mitochondria (Chance 1965, Hanson et al 1965). The Mg^{++}/O and Ca^{++}/O succinate ratios of 0.2 - 0.4 in beetroot mitochondria were much lower than those of 4 calculated in animal mitochondria (Chance 1965). These differences could be due to greater permeability of beetroot mitochondrial membranes to ions than animal mitochondrial membranes, poor precipitation of cation-phosphate complex in beetroot mitochondria and continuous recycling of magnesium and calcium taken up producing the stimulated oxidation and low cation/oxygen ratios in these mitochondria.

ADP/O and P/O ratios were highest in beetroot mitochondria in the absence of added $MgCl_2$ or $CaCl_2$. Both substrate oxidation and ADP-phosphorylation were inhibited by high $MgCl_2$ and $CaCl_2$ concentrations and phosphorylation was inhibited more than oxidation. However, at the concentrations of $MgCl_2$ and $CaCl_2$ used for ion uptake studies substrate oxidation was stimulated and ADP/O and P/O ratios were reduced by only 20% compared with the oxidation and ADP/O ratios when no $MgCl_2$ and $CaCl_2$ were present. Calcium did not uncouple oxidative phosphorylation in beetroot mitochondria more than magnesium in contrast to results with animal and corn mitochondria (Vasington and Murphy 1962, Hanson et al 1965). This could be related to the different isolating and experimental techniques

producing changes in oxidative phosphorylation, stability or in membrane structure and binding capacity.

The ATPase activity of beetroot mitochondria was low under the conditions for ion uptake or oxidative phosphorylation studies. Activity could not be compared with that of other mitochondria due to different isolating and test conditions. Different experimental conditions produced very large changes in ATPase activity of one mitochondrial type.

CHAPTER SEVEN

MONOVALENT IONS - UPTAKE AND EFFECT ON DIVALENT ION UPTAKE

A. INTRODUCTION.

1. Monovalent Ion Uptake.

The ability of isolated plant mitochondria to accumulate monovalent ions against a concentration gradient has been demonstrated (Robertson et al 1955, Jackson et al 1962). Monovalent cation uptake by animal mitochondria has been reported (Bartley and Davies 1952, 1954, Gamble 1962, 1965, Ulrich 1959). Solomon (1964) and Rottenberg and Solomon (1965) showed that rat liver mitochondria could accumulate potassium by a respiration-dependent oligomycin-insensitive process. Gamble (1965, 1962) reported that potassium was taken up by mitochondria and held in association with accumulated substrate anions (citrate or malate). Accumulation of citrate and malate was associated with the release of nearly equal amounts of phosphate. Respiration was not required (cyanide had little effect) but DNP inhibited citrate and malate accumulation (Gamble 1962). Bartley and Davies (1952, 1954) demonstrated that phosphate and organic acid anions could be concentrated by mitochondria.

2. Valinomycin and Gramicidin-Induced Ion Movement.

Recent investigations have shown that movement of potassium and other alkali metal ions into and out of mitochondria can be induced by valinomycin and gramicidin (Pressman 1963, Moore and Pressman 1964,

Chappell and Crofts 1965, 1966). Induced potassium uptake required substrate or ATP and was accompanied by H^+ production and by respiratory stimulation with swelling of the mitochondria if a permeable anion (e.g. phosphate, arsenate or acetate) was included (Moore and Pressman 1964, Chappell and Crofts 1965, 1966). These effects and a mechanism to account for them (Chappell and Crofts 1966) have been discussed in Chapter 1.

3. Competitive Ion Effects.

Substrate-dependent divalent cation uptake by mitochondria has been demonstrated in the absence of phosphate and it has been suggested that divalent cation uptake is the first step in massive cation and phosphate uptake (Chappell et al 1963, Brierley et al 1963a, Chance 1965). Unless a specific ion carrier (permeases, (Chappell and Crofts 1965a) or coupled cation and phosphate uptake (Brierley et al 1962, Hanson et al 1965) is proposed then all cations in the medium should be available for the energy-dependent cation uptake mechanism. There is little evidence to support the proposals that cation and phosphate enter simultaneously or that phosphate is accumulated first by energy-dependent involvement in oxidative phosphorylation (Hanson et al 1965). Phosphate may be replaced by acetate or arsenate (Chappell and Crofts 1965c, 1966) or may be omitted during cation uptake (Chappell et al 1963, Chance 1965). A specific ion "permease" (Chappell et al 1963, Chance 1965) appears unlikely from evidence of strong competition between divalent cations for uptake (Brierley et al 1962, Vasington and Murphy 1962).

Energy-dependent uptake of one cation in the presence of others would depend on the rate and strength of passive binding to mitochondrial membranes. Binding may be considered as the first step or as a prerequisite for the uptake mechanism. Concentrations of cations in the medium would affect binding. Permeability of the cation must be considered unless a specific carrier or binding site is postulated (Chance 1965, Chappell and Crofts 1965a). Presence of a penetrating anion (e.g. phosphate or acetate) would also affect cation uptake. Phosphate entry could result in precipitation of one cation (magnesium) but not of another (potassium). The competition between divalent cations and the effects of monovalent ions on divalent cation and phosphate uptake have been discussed in Chapter 1.

Magnesium uptake was inhibited by lower concentrations of monovalent ions (Brierley et al 1963b) than was calcium uptake (Vasington and Murphy 1962). This could reflect a strength of binding to the mitochondrial membranes or the solubility of the cation-phosphate complex formed within the mitochondria. While sodium and potassium inhibited magnesium and phosphate uptake in beef heart mitochondria to the same extent (Brierley et al 1963b), more calcium and phosphate was accumulated by rat liver mitochondria in a sodium medium than in a potassium medium (Carafoli et al 1964). Stimulation of calcium and phosphate uptake by sodium, potassium and lithium in rat kidney mitochondria may reflect membrane permeability or binding capacity changes produced by these monovalent cations (Vasington and Murphy 1962).

4. Permeability.

Investigations were made by Chappell and Crofts (1966) into volume changes of rat liver mitochondria (estimated by light scattering measurements) in KCl, potassium phosphate, NH_4Cl, ammonium phosphate and ammonium acetate with rotenone and EGTA present (Chappell and Greville 1963a). Swelling in such a system would occur only when both anion and cation penetrated the mitochondrion, and occurred only when NH_4^+ was present with phosphate or acetate. Chappell and Crofts (1966) concluded from these studies that the rat liver mitochondria were impermeable to potassium and chloride and permeable to phosphate, acetate and NH_4^+. The phosphate was considered to enter the mitochondria by an exchange diffusion carrier in exchange for OH^- or another anion.

Amoore and Bartley (1958) and Amoore (1960) demonstrated that K^{45} exchanged very poorly with potassium of liver mitochondria and McFarlane and Spencer (1953) suggested that the mitochondrial membranes had a low permeability to monovalent cations. Robertson et al (1955) followed chloride movement in beetroot and carrot mitochondria, and a diffusion coefficient was calculated which was typical for membranes of oriented lipoids stabilised by proteins (Davson and Danielli 1942). Anions of higher valency (Bartley and Enser 1964) have been shown to penetrate the membranes of mitochondria slowly.

Light-scattering changes were followed after suspending corn mitochondria in various media and these changes ~~have~~ were been related to volume changes (Stoner and Hansen 1965). Corn mitochondria did not swell appreciably in sucrose but did swell spontaneously in iso-osmolar KCl.

Table VII. 1. Effect of Tris-HCl and NaCl on Substrate-Independent Mg^{++} and Pi Uptake.

Beetroot mitochondria were incubated at 25°C in an ion uptake medium (Tris phosphate) with 200 ug mitochondrial N/ml. Mitochondria were separated from the medium by centrifuging.

	Mg^{++} umoles/mg N		Pi umoles/mg N	
	5 min.	14 min.	5 min.	14 min.
Control	1.67	1.70	0.14	0.13
+ 10 mM Tris-HCl	1.35	1.37	0.14	0.12
+ 10 mM NaCl	1.65	1.69	0.13	0.14
+ 10 mM KCl	1.67	1.69	0.14	0.14

Lyons et al (1964) recorded similar results with a variety of plant mitochondria. Stoner and Hansen (1965) assumed that swelling was accompanied by penetration of the suspending solute (KCl) and suggested that corn mitochondria were more permeable to ions than most animal mitochondria. Accurate statements concerning membrane permmeabilities can not be made, however, until diffusion coefficients for different ions across mitochondria membranes have been calculated. Only relative permeabilities can be determined by light scattering changes (Chappell and Crofts 1966) if these changes are related to solute entry. Membrane permeability to ions would vary between different preparations of the same mitochondrial type.

B. RESULTS.

1. Effects of Manovalent Ions on Divalent Cation and Phosphate Uptake.

a. Substrate - Independent Uptake:- The effects of 10mM Tris-HCl, KCl and NaCl on the magnesium and phosphate levels of beetroot mitochondria incubated in the absence of substrate are shown in Table VII 1. NaCl and KCl (10mM) had no effect on magnesium content but 10mM Tris-HCl reduced the magnesium level by 20% during incubation period. NaCl, KCl and Tris-HCl had no effect on the substrate-independent phosphate levels. The inhibition of bound magnesium content by Tris has already been discussed (Table IV 13).

b. Substrate - Dependent Uptake:- The effect of sodium and potassium on magnesium and phosphate uptake by beetroot mitochondria was shown by substituting Tris-succinate for sodium succinate and by varying

Fig. VII. 1. The effect of sodium and potassium phosphate concentration on Mg^{++} and Pi uptake by beet-root mitochondria incubated at 25°C in a medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 4 uM cytochrome c, 15 mM $MgCl_2$ and 330 ug mitochondrial N/ml. The results have been corrected for the uptake of ions in the absence of substrate.

x -- x with 11 mM Tris succinate and 3.2 mM potassium phosphate buffer pH 7.2.

o -- o with 11 mM Tris succinate and 10 mM potassium phosphate.

Δ -- Δ with 11 mM sodium succinate and 3.2 mM potassium phosphate.

□ -- □ with 11 mM sodium succinate and 10 mM potassium phosphate.

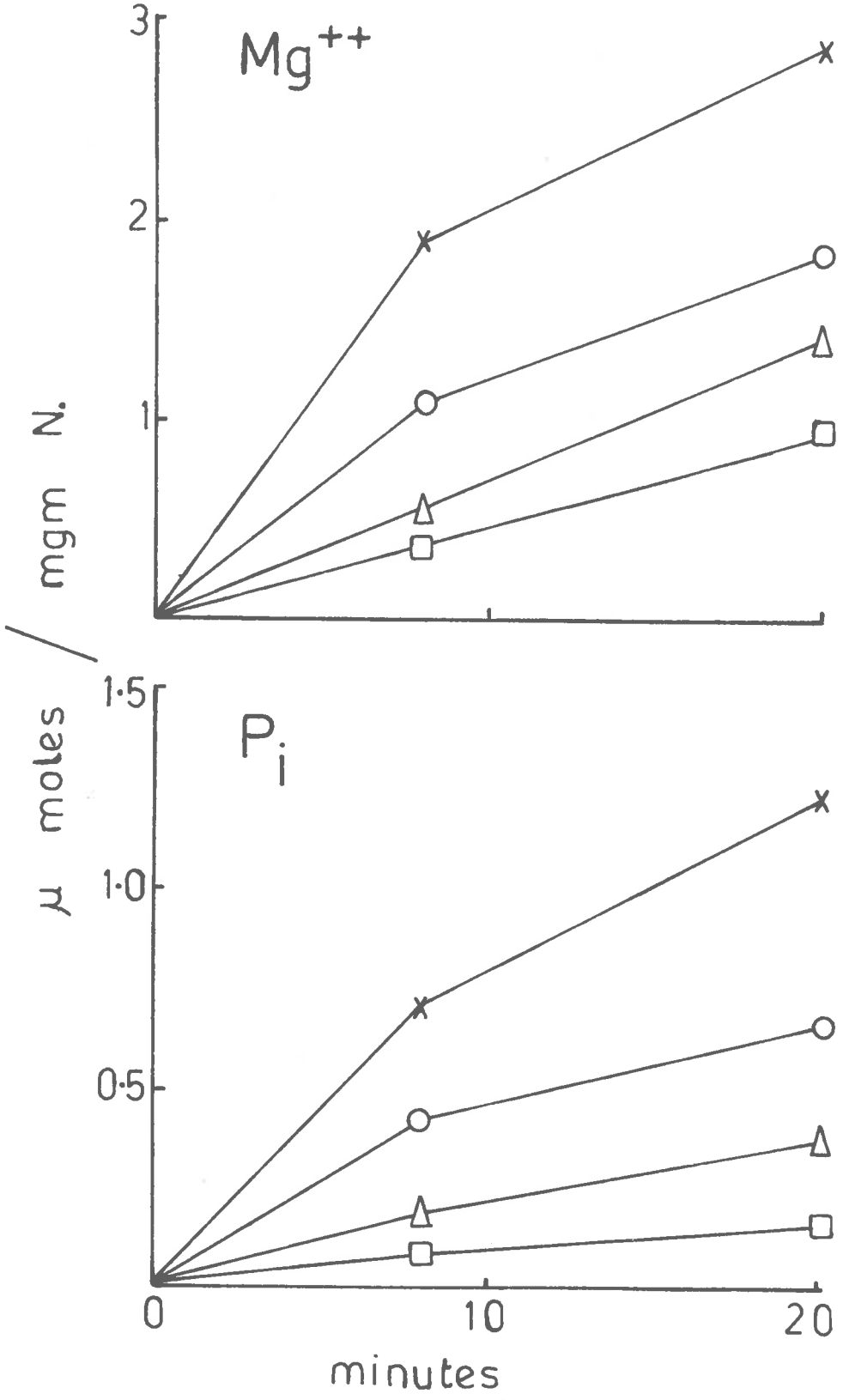

3
2
1
Mg++
mgm N.
1·5
1·0
0·5
Pi
μ moles
0
10
20
minutes

Fig. VII. 2. Effects of sodium, potassium and Tris chlorides and of potassium and Tris phosphates on the uptake of Mg^{++} and Pi by beetroot mitochondria incubated in a medium containing 250 mM sucrose, 15 mM $MgCl_2$, 5 uM cytochrome c, 186 ug mitochondrial N/ml, 3.3 mM Tris-phosphate buffer pH 7.2 and 8 mM Tris succinate.

△ -- △ minus substrate

● -- ● no addition

■ -- ■ with 3.3 mM potassium phosphate buffer pH 7.2 in place of the 3.3 mM Tris - phosphate buffer.

□ -- □ plus 10 mM Tris-HCl buffer pH 7.2.

0 -- 0 plus 10 mM NaCl

x -- x plus 10 mM KCl

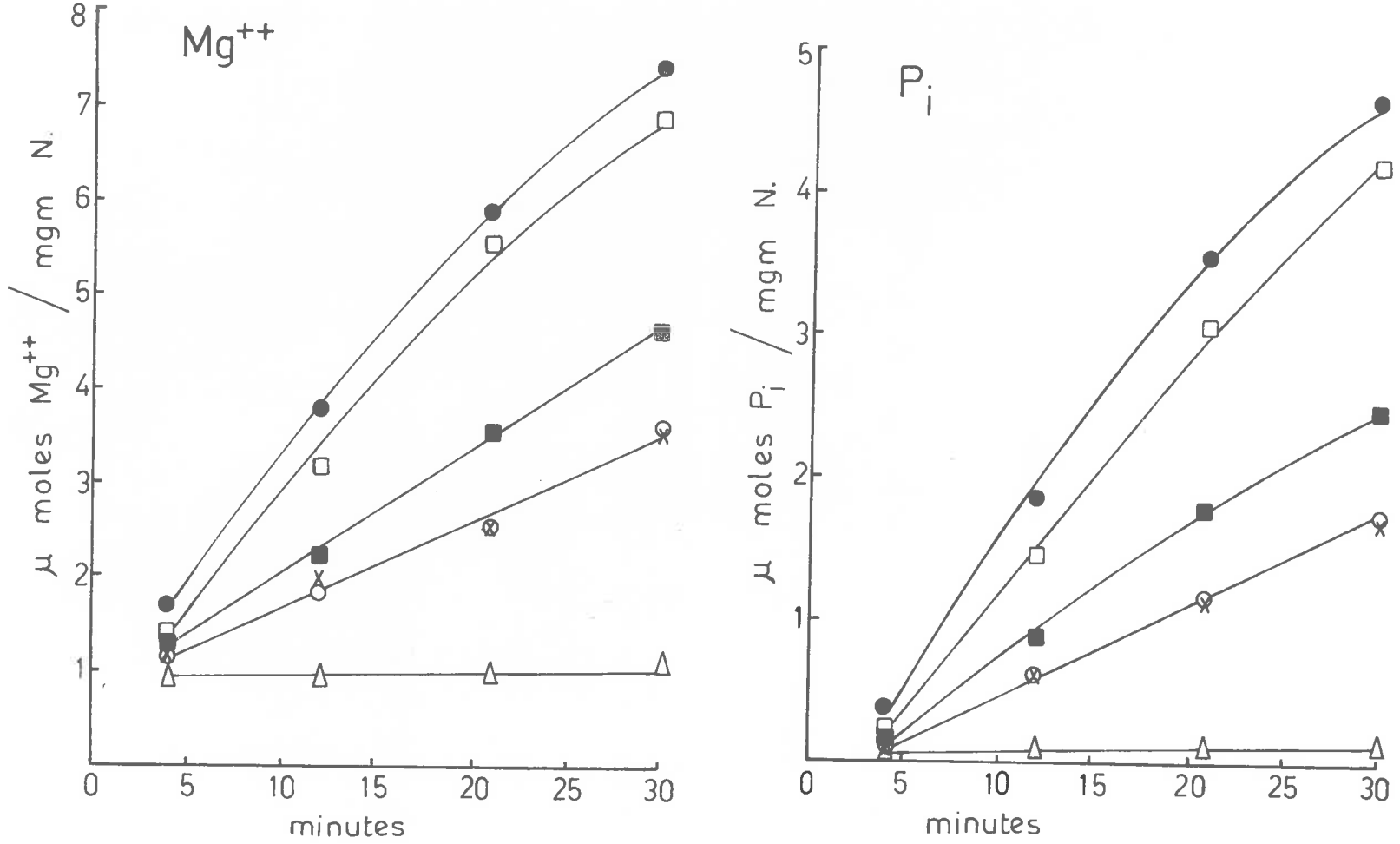

Mg++
μ moles Mg++ / mgm N.
minutes
Pi
μ moles Pi / mgm N.
minutes

Table VII. 2. Effect of NaCl and KCl on Ca^{++} and Pi Uptake by Beetroot Mitochondria.

Mitochondria were incubated for 19 minutes in a medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 3 uM cytochrome c, 2.9 mM $MgCl_2$, 3 mM $CaCl_2$, 3 mM Tris-phosphate buffer pH 7.2, 8 mM Tris succinate and 115 ug mitochondrial N/ml. The results were corrected for the ion uptake in the absence of substrate.

Addition	Ca^{++}		Pi	
	umoles/mg N	% Inhibition	umoles/mg N	% Inhibition
Control	7.9	-	6.6	-
+ 3 mM NaCl	6.2	22	5.2	21
+ 3 mM KCl	5.4	32	4.7	29
+ 6 mM NaCl	4.2	47	3.4	49
+ 6 mM KCl	4.2	47	3.5	47
+ 3 mM NaCl + 3 mM KCl	4.0	49	3.4	49

the concentration of potassium included as phosphate buffer (K_2HPO_4/ KH_2PO_4, pH 7.2) Fig. VII 1. shows that 11mM Tris-succinate was more favourable to magnesium and phosphate uptake than 11mM sodium succinate and that 3.2mM potassium phosphate buffer was more favourable than 10mM potassium phosphate buffer. The inhibitory effects of sodium and potassium were approximately additive. These results could indicate a competition between the sodium, potassium and magnesium for uptake.

Fig.VII 2 shows that replacement of potassium phosphate buffer by Tris-phosphate buffer stimulated substrate-dependent uptake of magnesium and phosphate. Fig.VII 2 also shows that 10mM NaCl or KCl severely inhibited whereas 10mM Tris-HCl buffer (pH 7.2) slightly inhibited ion uptake. The small inhibition observed with Tris-HCl could have been due to either the Tris or the chloride, but the strong inhibition produced by NaCl and KCl (containing the same concentration of chloride as the Tris-HCl) must have been due to sodium and potassium. The data of Figs. VII 1 and 2 suggest that isolated beetroot mitochondria did not discriminate between sodium and potassium but did discriminate between these cations and Tris. Although Tris inhibited passive magnesium binding (Table.VII 1) substrate-dependent magnesium and phosphate uptake was not greatly affected by the slowly penetrating Tris. The more permeable cations sodium and potassium offered more competition to substrate-dependent magnesium and phosphate uptake.

The effect of NaCl and KCl on substrate-dependent calcium and phosphate uptake by beetroot mitochondria is shown in Table.VII 2.

Fig. VII. 3. The effects of Tris-HCl buffer on the retention of Mg^{++} and Pi taken up by beetroot mitochondria incubated in a medium containing 250 mM sucrose, 4 uM cytochrome c, 3.2 mM potassium phosphate buffer pH 7.2, 14 mM $MgCl_2$ and 170 ug mitochondrial N/ml. Mitochondria were separated from the medium by centrifuging at 25,000 x g for 6 minutes through 3 ml of 1 M sucrose at 0°C.

▲ -- ▲ no addition

● -- ● with 8 mM Tris succinate

o -- o with 8 mM Tris succinate and 10 mM Tris-HCl buffer pH 7.2.

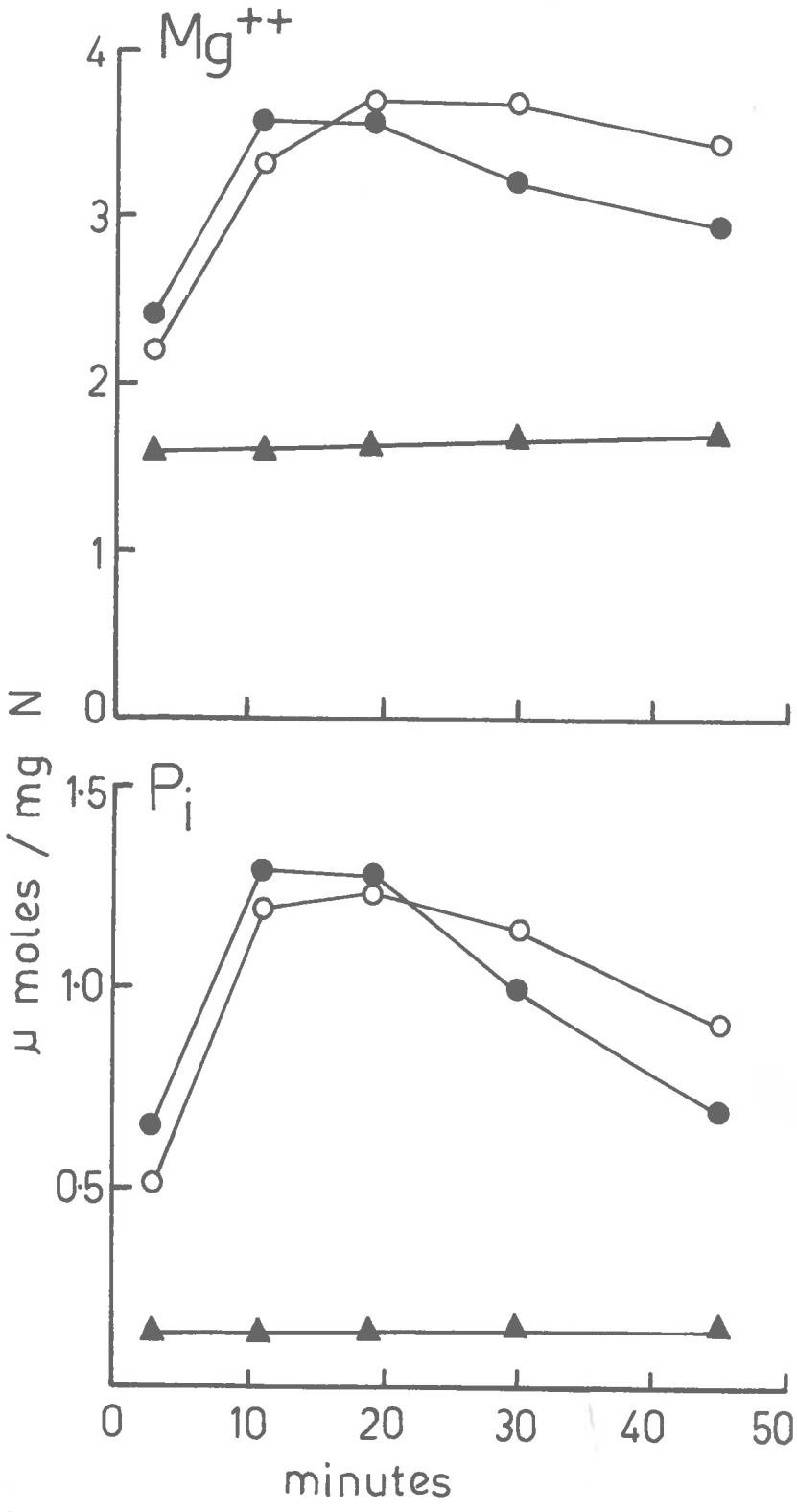

Mg++
4
3
2
1
0
N
Pi
1·5
1·0
0·5
μ moles / mg
0
10
20
30
40
50
minutes

Table VII. 3. Effects of Anion/Phosphate and Cation/Mg^{++} Ratios on Mg^{++} and Pi Uptake.

Beetroot mitochondria were incubated for 20 minutes in a medium containing 250 mM sucrose, 8 mM Tris succinate, 8.6 mM Tris-HCl buffer pH 7.2, 5 uM cytochrome c, 105 ug mitochondrial N/ml and unless otherwise indicated 15 mM $MgCl_2$ and 3.1 mM Tris-phosphate buffer pH 7.2. % inhibitions were calculated after correcting for ion uptake in the absence of substrate.

$MgCl_2$ mM	Tris-Pi mM	Cl^-/Pi	% Inhibition Mg^{++} Uptake	% Inhibition Pi Uptake
15.7	7.1	5.6	0	0
31.4	7.1	10	2	0
15.7	3.1	12.9	3	10
31.4	3.1	23	61	52
NaCl or KCl mM		Na^+ or K^+/Mg^{++}	% Inhibition Mg^{++} Uptake	% Inhibition Pi Uptake
10		$Na^+/Mg^{++} = 0.7$	61	65
10		$K^+/Mg^{++} = 0.7$	65	66
3.3		$K^+/Mg^{++} = 0.2$	45	49

KCl and NaCl (3mM) inhibited ion uptake by 20 - 30% while 6mM KCl, 6mM NaCl or 3mM KCl + 3mM NaCl inhibited uptake by 40 - 50%.

Tris-HCl buffer (pH 7.2) reduced the leakage rate of accumulated magnesium and phosphate from beetroot mitochondria over a long incubation period. Fig VII 3 shows that in the absence of Tris-HCl buffer (pH 7.2) magnesium and phosphate were taken up by a substrate-dependent process for 20 minutes and then uptake stopped and the ions were slowly lost from the mitochondria. In the presence of 10mM Tris-HCl buffer (pH 7.2) the initial uptake of magnesium and phosphate was reduced but the ions were retained in the mitochondria for a longer period. The increase in buffering capacity with 10mM Tris-HCl buffer (pH 7.2) or an increase in membrane-bound Tris could have stabilised the mitochondria structure and prevented leakage of accumulated ions.

c. Cation/Mg^{++} And Anion/Phosphate Ratios:- Substrate-dependent cation uptake was considered to be the first step in the uptake of divalent cations and phosphate (Chappell et al 1963, Chance 1965). Inhibition of substrate-dependent magnesium and phosphate uptake by cations should thus be more severe than inhibition by anions. Table.VII 3 indicates that low ratios of Na^+ or K^+/Mg^{++} severely inhibited magnesium and phosphate uptake but extremely high Cl^-/Pi ratios were required for substantial inhibition of ion uptake. When Na^+ or K^+/Mg^{++} = 0.7, ion uptake was inhibited 60%, but a Cl^-/Pi ratio of 10 had no effect on uptake. Only when Cl^-/Pi = 23 did strong inhibition of ion uptake occur. Inhibition was dependent on the ratio of Cl^-/Pi and not on the absolute

Table VII. 4. Effect of $(NH_4)_2SO_4$ on Mg^{++} and Pi Uptake by Beetroot Mitochondria.

Mitochondria were incubated for 20 minutes in an ion uptake medium (10 mM Tris phosphate) with 7.2 mM Tris succinate, 36 ug mitochondrial N/ml and where indicated 0.7 mM ADP, 13.7 mM glucose, 14 ug hexokinase/ml, 1 ug oligomycin/ml and 2 ug bovine serum albumin/ml. The results were corrected for ion uptake and glucose-6-phosphate formation in the absence of substrate and ADP. Hexokinase (with 0.04% BSA) was dialysed where indicated against 1% glucose for 2 hours at 0°C.

System	Mg^{++} umoles/mg N		Pi umoles/mg N		Glucose-6-phosphate umoles/mg N	
	-Oligo mycin	+Oligo mycin	-Oligo mycin	+Oligo mycin	-Oligo mycin	+Oligo mycin
Control	10.5	10.4	8.3	8.0	0	0
+Hexokinase	4.9	5.2	4.4	4.3	0	0
+Dialysed Hexokinase	17.2	14.9	11.6	9.9	0.1	0
+ADP+Glucose +Hexokinase	0	5.5	0	4.3	56.7	12.1
+ADP+Glucose +Dialysed Hexokinase	0.7	11.6	0.5	8.8	53.4	11.5
+Glucose+BSA	14.2	-	10.3	-	0	-

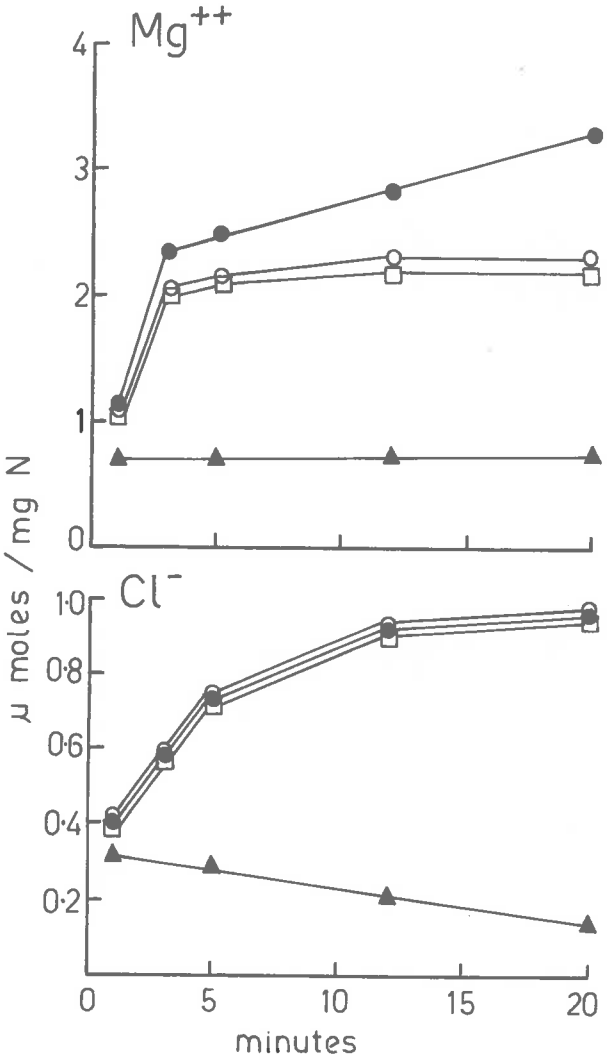
Fig. VII. 4. Mg^{++}, Pi and Cl^{-} uptake by beetroot mitochondria incubated at 25°C in a medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 5 uM cytochrome c and 560 ug mitochondrial N/ml.
The mitochondria were separated from the medium by centrifuging at 25,000 x g for 5 minutes through 3 ml of 1 M sucrose at 0°C.

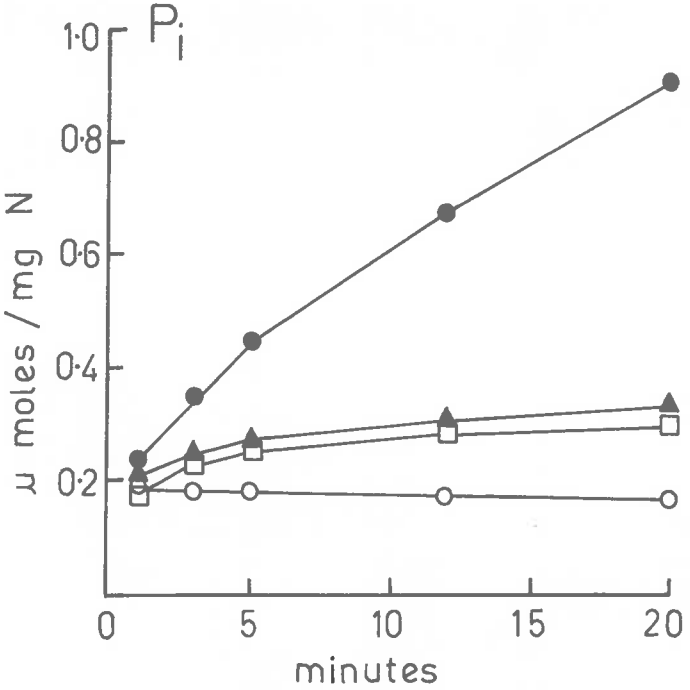
▲ -- ▲ plus 10 mM sodium succinate and 10 mM potassium phosphate buffer pH 7.2.

0 -- 0 plus 10 mM sodium succinate and 52 mM $MgCl_2$.

● -- ● plus 10 mM sodium succinate, 52 mM $MgCl_2$ and 10 mM potassium phosphate buffer pH 7.2.

□ -- □ plus 52 mM $MgCl_2$ and 10 mM potassium phosphate buffer pH 7.2.


Mg++
Cl⁻
μ moles / mg N
minutes


Pi
μ moles / mg N
minutes

concentrations of either (Table.VII 3).

d. Hexokinase Effects:- The inhibition of magnesium and phosphate uptake by monovalent ions could obscure other results. Magnesium and phosphate uptake was completely inhibited by conditions allowing continuous ATP formation with ~~either~~ hexokinase (in $(NH_4)_2SO_4$ suspension) or dialysed hexokinase (Table.VII 4). Glucose-6-phosphate formation was the same in both cases and oligomycin inhibited formation by 80%. However, oligomycin completely recovered ion uptake when dialysed hexokinase was present but only partially recovered uptake with undialysed hexokinase. Table.VII 4 shows that undialysed hexokinase (without ADP and glucose) inhibited magnesium and phosphate uptake whereas dialysed hexokinase stimulated ion uptake. The stimulation can be related to the stimulation by BSA (Table.VII 4) and has been discussed in Chapter 4. The inhibition of ion uptake by the $(NH_4)_2SO_4$ included with the undialysed hexokinase masked the recovery effect of oligomycin.

2. Monovalent Ion Uptake.

Competition between divalent cations and monovalent cations for uptake has been indicated by the inhibition of divalent cation and phosphate uptake by NaCl and KCl in beetroot mitochondria but this does not demonstrate actual monavalent cation uptake.

a. Chloride Movements:- The movement of chloride was followed in early investigations into magnesium and phosphate uptake by beetroot mitochondria. Fig.VII 4 shows chloride, magnesium and phosphate uptake by beetroot mitochondria over 20 minutes. Magnesium and phosphate were taken up by a substrate-dependent process but substrate did not alter the chloride

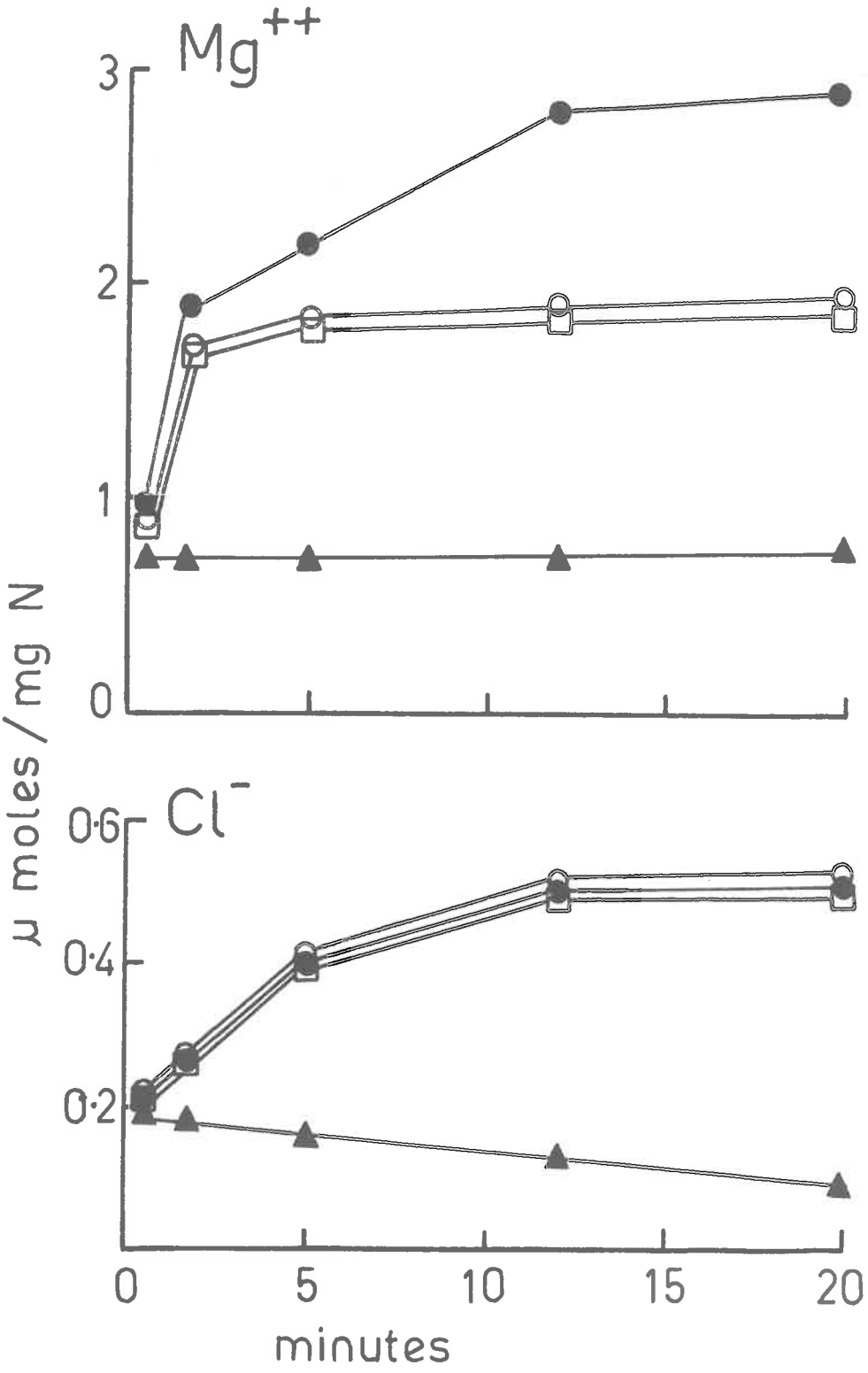
Fig. VII. 5. Mg^{++} and Cl^{-} uptake by beetroot mitochondria incubated in a medium containing 250 mM sucrose, 10 mM Tris-HCl buffer pH 7.2, 4 uM cytochrome c and 371 ug mitochondria N/ml. The mitochondria were separated from the medium by centrifuging at 25,000 x g. for 5 minutes through 3 ml of 1 M sucrose at $0^{o}C$.

▲ -- ▲ plus 10 mM sodium succinate and 10 mM potassium phosphate buffer pH 7.2.

o -- o plus 10 mM sodium succinate and 18 mM $MgCl_2$.

● -- ● plus 10 mM sodium succinate, 18 mM $MgCl_2$ and 10 mM potassium phosphate buffer pH 7.2.

□ -- □ plus 18 mM $MgCl_2$ and 10 mM potassium phosphate buffer pH 7.2.


Mg++
3
2
1
0
Cl⁻
0·6
0·4
0·2
0
5
10
15
20
minutes
μ moles / mg N

content of the mitochondria. The endogenous magnesium content (700 mu moles / mg N) was the level usually found in isolated beetroot mitochondria (Chapter 4). Incubation in 52mM $MgCl_2$ increased substrate-independent magnesium uptake to a constant level after 3 minutes. This would be a result of magnesium binding (see Fig.IV 1). When phosphate was omitted magnesium was bound to the same extent but a very small amount was taken up by a substrate-dependent process (Fig.VII 4).

The endogenous phosphate content of 180 mu moles / mg N remained constant throughout the incubation period (Fig.VII 4). The substrate-independent phosphate level was higher with 10mM phosphate present and increased slowly during the incubation period. This may have been due to diffusion or to some endogenous substrate or magnesium.

Fig.VII 4 indicates that chloride movement was not dependent on the presence of substrate or phosphate. The endogenous chloride content of the washed mitochondria (300 mu moles / mg N) decreased slowly during the incubation period. When 52 mM $MgCl_2$ was present the chloride level reached equilibrium in 5 to 12 minutes. This chloride movement can be interpreted as diffusion into the mitochondria.

Fig.VII 5 shows that magnesium was taken up by a substrate-and phosphate-dependent process, while chloride movement was independent of substrate and phosphate. The constant substrate-independent magnesium level (Fig.VII 5, 18mM $MgCl_2$), was reached within 2 minutes and was only 16% less than the level with 52 mM $MgCl_2$ (Fig.VII 4). The equilibration level of chloride was more than 40% lower with 18mM $MgCl_2$ (Fig.VII 5) than with 52mM (Fig.VII 4), indicating that chloride moved into the

Fig. VII. 6. K^+ and Pi uptake by beetroot mitochondria incubated for 4 minutes in a medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 3 uM cytochrome c, 0.9 mM $MgCl_2$, 2.9 mM Tris-phosphate buffer pH 7.2, 94 ug mitochondria N/ml and different concentrations of KCl. Mitochondria were separated from the medium by Millipore filtration.

o — o no addition

● — ● with 7 mM Tris succinate.

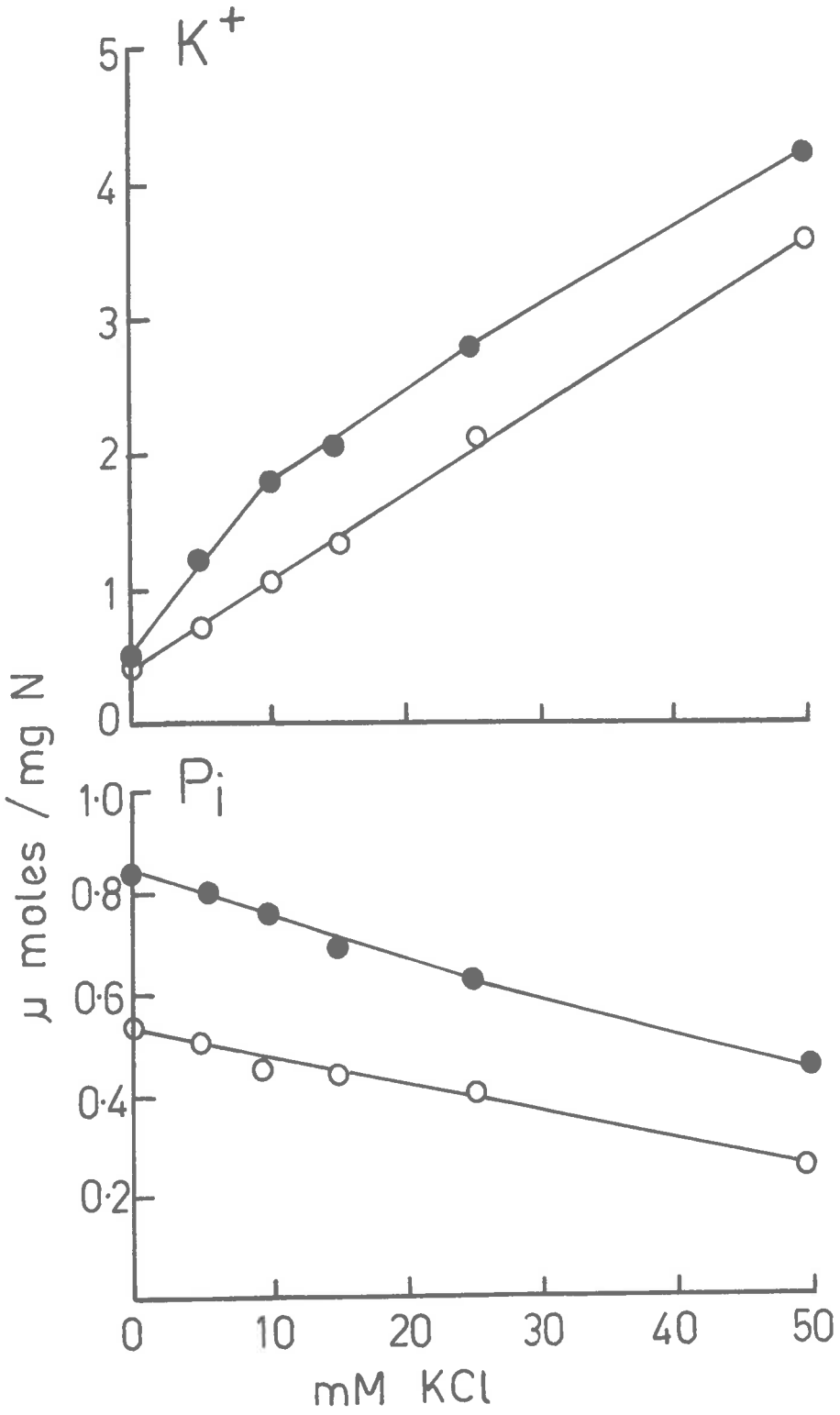

K+
5
4
3
2
1
0
Pi
1·0
0·8
0·6
0·4
0·2
µ moles / mg N
0
10
20
30
40
50
mM KCl

Fig. VII. 7. K^+ and Pi uptake with tissue by beetroot mitochondria incubated in a medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 3 uM cytochrome c, 0.9 mM $MgCl_2$, 2.9 mM Tris-phosphate buffer pH 7.2, 7 mM Tris succinate and 94 ug mitochondria N/ml.

The results have been corrected for uptake of ions in the absence of substrate.

● -- ● + 0 mM KCl

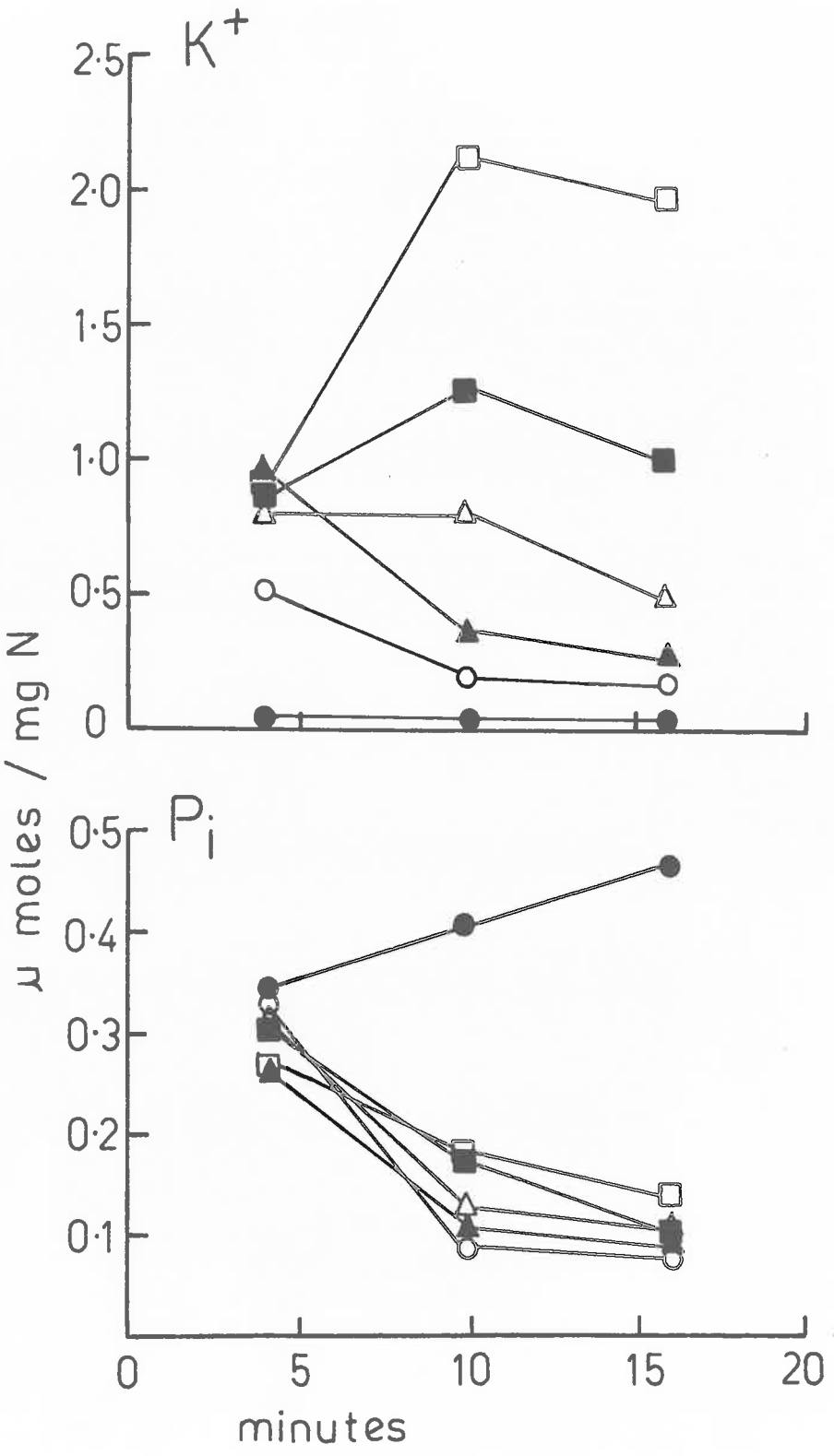
o -- o + 5 mM KCl

▲ -- ▲ + 10 mM KCl

△ -- △ + 15 mM KCl

■ -- ■ + 25 mM KCl

□ -- □ + 50 mM KCl.


K+
2·5
2·0
1·5
1·0
0·5
0
Pi
0·5
0·4
0·3
0·2
0·1
μ moles / mg N
0
5
10
15
20
minutes

mitochondria by diffusion along a concentration gradient. The equilibration time for chloride was again 8 - 10 minutes (Fig.VII 5).

Robertson et al (1955) measured chloride efflux from beetroot mitochondria after accumulation of chloride. The half time of chloride efflux was approx. 5 minutes in comparison with the 3 - 6 minute half time of chloride uptake in Figs.VII 4 & 5. The experiments described here were carried out with early preparations of beetroot mitochondria and were not repeated after modifications were made to the isolation procedure which produced mitochondria with higher respiratory control, less endogenous substrate, a greater potential for ion uptake and possibly a lower permeability to ions (Chapter 2).

b. Sodium, Potassium and Chloride Uptake:- Monovalent cation (sodium and potassium) and chloride uptake was investigated in beetroot mitochondria isolated by the modified method using the Braun juice extractor (Chapter 2). The Millipore filtration technique did not allow the measurements of chloride and cation uptake to be used for calculating permeability coefficients due to errors introduced by ion retention in the filter.

Fig.VII 6 shows the uptake of potassium and phosphate by beetroot mitochondria as the external KCl concentration was increased from 0 - 50mM. Fig.VII 7 indicates the changes in substrate-dependent potassium and phosphate uptake with incubation time in the same experiment. As KCl concentration increased the substrate-independent potassium level increased linearly (FiglVII 6). This would be due to increase in potassium

Table VII. 5. Substrate-Dependent Na^+ and Cl^- Uptake by Beetroot Mitochondria.

Mitochondria was incubated in a medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 3 uM cytochrome c, 0.9 mM $MgCl_2$, 2.9 mM Tris-phosphate buffer pH 7.2, 7 mM Tris succinate and 76 ug mitochondrial N/ml. The ion contents in the absence of substrate were subtracted from the contents in the presence of substrate.

NaCl mM	Na^+ umoles/mg N	Cl^- umoles/mg N
0	0.24	-0.34
5	0.50	-0.62
10	0.99	-0.42
15	1.42	-0.50
25	1.62	-0.32
50	3.5	1.1

diffusion and retention by the filter. The endogenous potassium level was 400 - 450 mu moles / mg N. Potassium was taken up in a substrate-dependent manner and was greatest at 10-50mM KCl. (Fig.VII 6). Substrate-dependent potassium uptake decreased after 4 minutes at KCl concentrations of 0-20mM (Fig.VII 7). Only at 25mM and 50mM KCl did substrate-dependent potassium uptake increase with time.

$MgCl_2$ (0.9mM) was included in these experiments and phosphate was taken up in a substrate-dependent manner (Fig.VII 6). Substrate-independent and dependent phosphate uptake decreased as the external KCl concentration increased (Fig.VII 6). Substrate-dependent phosphate uptake increased with time when KCl was omitted (probably in association with magnesium uptake) but decreased when KCl at any concentration was present (Fig.VII 7).

Thus substrate-dependent potassium uptake occurred at all KCl concentrations but the potassium was soon lost at the lower concentrations. Phosphate uptake was greatest in the absence of KCl. KCl inhibited magnesium and phosphate uptake and magnesium and phosphate taken up was mobilised and lost after ashort time. Some phosphate was retained at 50mM KCl probably in association with potassium. Substrate-dependent potassium uptake does not necessarily mean uptake dependent on substrate oxidation, and may be a result of succinate uptake and accumulation.

Table.VII 5 indicates that sodium was taken up by a substrate-dependent process and uptake increased with external NaCl concentration. Table.VII 5 shows that chloride content was higher in the presence of

Table VII. 6. Effects of Substrate, DNP, KCN and Oligomycin on K^+ and Cl^- Uptake by Beetroot Mitochondria.

Mitochondria were incubated at 25°C in a medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 3 uM cytochrome c, 20 mM KCl, 2 mM Tris-phosphate buffer pH 7.2, 0.9 mM $MgCl_2$ and where indicated 9 mM Tris succinate, 1 ug/ml oligomycin, 1 x 10^{-4} M DNP and 1 x 10^{-4} M KCN. Expt. A contained 79 ug mitochondrial N/ml and Expt. B contained 0.01% BSA, 0.5 mM Tris ATP and 138 ug mitochondrial N/ml. The mitochondria were separated from the medium by filtering.

Treatment	K^+ umoles/mg N	
	3 min.	8 min.
Expt. A.		
Control	2.04	2.10
+ Succinate	2.52	2.79
+ Succinate + Oligomycin	2.46	2.85
+ Succinate + DNP	2.16	2.07
+ Succinate + KCN	2.51	3.06
+ Succinate - TPi	2.25	2.58

	K^+ umoles/mg N		Cl^- umoles/mg N	
	2 min.	6 min.	2 min.	6 min.
Expt. B.				
Control	1.80	1.85	2.60	2.35
+ Succinate	2.23	2.55	2.64	2.72
+ Succinate + DNP	1.90	1.84	2.25	2.20
+ Succinate + KCN	2.47	2.84	-	-

Table VII. 7. Uptake of Na^+ and Endogenous Levels of Na^+ and K^+ in Beetroot Mitochondria.

Mitochondria were incubated in a medium containing 250 mM sucrose, 8.6 mM Tris-HCl buffer pH 7.2, 3 uM cytochrome c, 2.9 mM Tris-phosphate buffer pH 7.2, 1.5 mM $MgCl_2$ and where indicated 10 mM Tris succinate, 0.9×10^{-4} M DNP and 140 ug mitochondrial N/ml. Part A also contained 20 mM NaCl. Mitochondria were collected by filtering.

Treatment	Na^+ umoles/mg N 3 min.	7 min.	11 min.
A.			
Control	5.83	4.80	4.68
+ Succinate	6.20	6.48	7.70
+ Succinate + DNP	5.80	4.90	4.75

	Na^+ umoles/mg N		K^+ umoles/mg N	
	3 min.	11 min.	3 min.	11 min.
B.				
Control + 20 mM KCl	1.95	3.30	2.67	2.85
Control + 20 mM NaCl	5.83	4.68	0.29	0.26

substrate at 50mM NaCl but at lower NaCl concentrations the chloride content was higher in the absence of substrate. Succinate (7mM) may have been taken up with sodium at the lower NaCl concentrations.

6. Action of Inhibitors and Uncouplers:- Table.VII 6 shows that substrate-omission and DNP decreased, but KCN did not affect, the potassium and chloride levels in the presence of substrate. Phosphate omission partially reduced, and oligomycin did not affect, substrate-dependent potassium levels.

Table.VII 7 shows the uptake of sodium by beetroot mitochondria from a medium containing 20mM NaCl. Tris-succinate increased the sodium level above that in the absence of substrate or in the presence of substrate plus DNP. Table.VII 7 also shows that, with 20mM KCl but no NaCl or substrate in the medium, the sodium content was as high as the potassium content. When 20mM NaCl was added to the medium without KCl the sodium content was very high while the potassium content was very low. These results were corrected for the sodium present in the extraction liquid (less than 0.3 u moles / mg N). No potassium was present in the extraction medium. Thus the endogenous level of sodium in the mitochondria was much higher than the potassium level. Higher endogenous sodium levels than potassium were also observed by Robertson et al (1955) in beetroot mitochondria.

C. DISCUSSION.

1. Summary.

Sodium, potassium and chloride reactions with beetroot mitochondria can be summarised as follows:-

(1) Sodium and potassium strongly inhibited substrate-dependent magnesium or calcium and phosphate uptake by beetroot mitochondria, whereas Tris had little inhibitory effect.

(2) The monovalent cations, sodium and potassium, inhibited divalent cation and phosphate uptake more strongly than the monovalent anion, chloride, when the ratios Na^+ or K^+ / Mg^{++} and Cl^- /Pi were equal. High Cl^- /Pi ratios were required before divalent cation and phosphate uptake was inhibited.

(3) Chloride penetrated the mitochondrial membrane and reached equilibrium with a half time of 4 - 5 minutes.

(4) Sodium and potassium were taken up by beetroot mitochondria in a substrate-dependent manner. Substrate-dependent chloride uptake was also recorded during potassium uptake. Substrate-dependent monovalent cation uptake was:-

(a) inhibited by DNP.

(b) partially inhibited by omission of phosphate,

(c) not affected by oligomycin,

(d) not affected, or stimulated by KCN.

(5) The endogenous level of sodium in beetroot mitochondria was much higher than the level of potassium.

2. Monovalent Ion Inhibition of Divalent Cation and Phosphate Uptake.

NaCl and KCl inhibited magnesium and phosphate uptake by beetroot mitochondria with no distinction between the two salts. Magnesium and phosphate uptake by beef heart mitochondria was inhibited to the same

extent by NaCl and KCl. Magnesium and phosphate uptake by beef heart mitochondria was inhibited 45% by 33mM NaCl or KCl whereas 10mM NaCl or KCl inhibited ion uptake by 60% in beetroot mitochondria. These inhibition differences could reflect differences in permeability of the mitochondrial membranes. Magnesium and Pi uptake was not observed in some investigations with animal mitochondria and this could have been due to the high concentration of monovalent ions in the incubation media (Carafoli et al 1964). Sodium and potassium did not inhibit calcium and phosphate uptake as severely as magnesium and phosphate uptake by animal mitochondria (Carafoli et al 1964, Vasington and Murphy 1962, Vasington 1963, Drahota and Lehninger 1965, Brierley et al 1963b). Very high concentrations of monovalent ions were required to inhibit calcium and phosphate uptake (Carafoli et al 1964, Vasington 1963). Sodium and potassium had different effects on calcium and phosphate retention by rat liver mitochondria (Drahota and Lehninger 1965). Calcium and phosphate uptake by beetroot mitochondria was inhibited to the same extent by NaCl and KCl. Increase in mitochondrial membrane permeability to all ions in beetroot mitochondria could mask any differences in penetration of various monavalent ions. Ammonium sulphate included with hexokinase could have inhibited divalent cation and phosphate uptake in beef heart and corn mitochondria and produced the incomplete recovery of ion uptake by oligomycin under conditions for ATP formation (Brierley et al 1963b, Hanson et al 1965).

Monovalent ion inhibition of divalent cation and phosphate uptake suggests that the various ions compete for the uptake process. The

strong inhibition of uptake by monovalent cations in contrast to the small inhibition by monovalent anions supports the suggestion that cation uptake is the primary substrate-dependent act in divalent cation and phosphate uptake (Chappell et al 1963). Inhibition does not, however, demonstrate actual uptake of monovalent cations by the mitochondria.

3. Mönovalent Ion Uptake.

Sodium and potassium were taken up by beetroot mitochondria by a substrate-dependent process. Substrate-dependent uptake of chloride also occurred when 20 mM KCl or 50 mM NaCl were added to the medium. Substrate-dependent sodium and potassium uptake was inhibited by DNP, partly inhibited by omission of phosphate and was not affected by KCN or oligomycin. Phosphate omission would prevent precipitation of $(Mg)_3(PO_4)_2$ within the mitochondria and allow continuous recycling of magnesium and competition with potassium for uptake. Substrate-dependent potassium and sodium uptake did not depend on substrate oxidation.

KCN did not affect potassium uptake by beetroot mitochondria whereas this concentration of KCN (10^{-4}M) completely inhibited substrate-dependent magnesium and phosphate uptake and succinate oxidation. Potassium could be taken up as a result of succinate movement into the mitochondria.

Gamble (1965) reported that uptake of citrate and malate by rabbit liver mitochondria did not require respiration (cyanide did not affect citrate incorporation), but DNP inhibited accumulation. Gamble (1963a,b) showed that potassium and phosphate were released together from aging mitochondria and he suggested that citrate and malate entered a potassium-

containing compartment in the mitochondria and caused displacement of phosphate (Gamble 1965). Inward movement and increase in retention of potassium was recorded in rabbit kidney mitochondria incubated with succinate (Gamble 1962). In rabbit liver mitochondria (Gamble 1965) this result was obtained after incubation with citrate. Citrate accumulation was shown to occur in a potassium-free medium (Gamble 1965). The mechanism of citrate, succinate and malate uptake was not determined but an exchange diffusion mechanism between substrate anions and anions within the mitochondria has been suggested (Gamble 1965, Chappell and Crofts 1965a, Chappell 1964, Mitchell 1966).

Succinate-dependent potassium uptake by beetroot mitochondria could depend on an exchange diffusion process between succinate and some mitochondrial anion in the presence of KCN. DNP addition should not prevent succinate exchange for mitochondrial anions but could prevent potassium uptake by inducing preferential H^+ uptake. Citrate and malate uptake was severely inhibited by DNP (Gamble 1965) and DNP may affect membrane structure and the exchange diffusion mechanism and thus prevent both substrate anion and potassium uptake. Succinate oxidation could support potassium uptake by association of the cation with fumarate or malate. Substrate oxidation would also provide high-energy intermediates or PMF for monovalent cation uptake by the mechanisms for divalent cation uptake (Chapter 1).

Succinate-supported potassium uptake by beetroot mitochondria was often higher in the presence of KCN (Table VII 6). The products of succinate oxidation (fumarate and malate) could move out of the mito-

chondria and some potassium would also be lost. Potassium uptake energised by substrate oxidation would rapidly decrease (depending on membrane permeability) unless the cation was associated with an anion retained in the mitochondria. Oligomycin would have no effect on potassium taken up by succinate accumulation or by succinate oxidation.

D. CONCLUSIONS

Divalent cation and phosphate uptake by beetroot mitochondria was inhibited by NaCl and KCl (but not by Tris-HCl) suggesting competition between monovalent and divalent cations for a common uptake process. Monovalent cations severely inhibited, but monovalent cations had little effect on, divalent cation and phosphate uptake, supporting the proposal that cation uptake is the primary substrate-dependent act in ion uptake (Chappell et al 1963, Chance 1965).

Sodium and potassium were taken up by beetroot mitochondria in a succinate-dependent manner but succinate oxidation was not required. Uptake was inhibited by DNP. Monovalent cation uptake may have been associated with the uptake and accumultation of succinate by exchange diffusion with some mitochondrial anion or by some other process. DNP may have inhibited the exchange diffusion mechanism and thus prevented succinate uptake, or affected membrane structure promoting preferential H^+ uptake and thus inhibited monovalent cation uptake. Substrate oxidation (by production of high-energy intermediate or PMF) could also support monovalent cation uptake by the mechanisms for divalent cation uptake but with no precipitation of cation-phosphate complex the monovalent cations would rapidly leak out of the mitochondria.

CHAPTER EIGHT.

SUBSTRATE SUPPORT OF ION UPTAKE.

A INTRODUCTION.

1. Divalent Cation and Phosphate Uptake.

Brierley et al (1964a) reported that substrate-dependent magnesium uptake by beef heart mitochondria in the absence of phosphate was more efficient with pyruvate plus malate than with succinate. Calcium (10^{-5}M) stimulated succinate-supported magnesium uptake to the level of pyruvate plus malate-supported uptake. Chappell et al (1963) reported that malate plus glutamate supported manganese uptake in the presence and absence of phosphate in rat liver mitochondria.

Massive magnesium and phosphate uptake by beef heart mitochondria was supported by succinate, pyruvate plus malate, β-hydroxy butyrate and artificial electron donors (reduced silicomolybdate) (Brierley et al 1962). With pyruvalte plus malate some of the accumulated ions were lost after 10 minutes incubation and with other substrates accumulation decreased after 30 minutes (Brierley et al 1963b). Pyruvate plus malate in low concentrations was,however, the most efficient phosphate accumulator per oxygen utilized. Massive uptake of magnesium and phosphate by beef heart mitochondria was also supported by ascorbate plus TMPD in the presence of antimycin A (Brierley and Murer 1964).

The substrates most effective in supporting massive calcium and phosphate accumulation by rat kidney mitochondria were isocitrate, citrate and succinate (Vasington and Murphy 1962). Digitonin fragments of rat liver mitochondria accumulated calcium and phosphate in the

presence of β-hydroxy-butyrate and succinate but other substrates did not support calcium uptake (Vasington 1963). Cooper and Lehninger (1956) reported that digitonin fragments oxidized only β-hydroxy-butyrate and succinate effectively. Succinate, isocitrate and citrate supported calcium and phosphate uptake by intact rat liver mitochondria but β-hydroxy-butyrate was not an efficient substrate (Vasington 1963). Calcium and phosphate uptake by animal mitochondria was also supported by ascorbate plus TMPD (Chappell and Crofts 1963c, Rossi and Lehninger 1963).

Calcium and phosphate uptake by corn mitochondria was supported by succinate, pyruvate plus oxaloacetate, pyruvate plus succinate or to a small extent by α-ketoglutarate (Hodges and Hanson 1965). Calcium uptake was not obtained with citrate and this was attributed to chelation of the calcium ion (Hodges and Hanson 1965, Vasington and Murphy 1962). Calcium and phosphate uptake usually continued in corn mitochondria for 10 minutes and then decreased with some loss of accumulated ions (Hodges and Hanson 1965).

Strontium and phosphate accumulation by rat liver mitochondria was supported most effectively by succinate and by β-hydroxy-butyrate, α-ketoglutarate, pyruvate, malate and fumarate. (Carafoli et al 1965).

2. Substrate Oxidation.

The effectiveness of different substrates in supporting uptake of various divalent cations has been related to the rates of oxidation of these substrates (Carafoli et al 1965, Vasington 1963, Hodges and

Hanson 1965, Brierley et al 1963b). Substrate oxidation rates recorded during magnesium and phosphate uptake by beef heart mitochondria (Brierley et al 1962, 1963b) were low and corresponded to state 4 oxidation rates rather than to state 3 rates (Appendix Table 1). Uptake of calcium and phosphate was usually investigated with ATP in the medium and under these conditions when ATPase activity was high the substrate oxidation rate was also rapid (Brierley et al 1964b).

Low concentrations of divalent cations produced reversible stimulations of oxidation when succinate, α-ketoglutarate, β-hydroxybutyrate and malate plus glutamate were used as substrates (Rossi and Lehninger 1964, Chappell 1963, Carafoli 1965b, Chance 1965). The ratios of divalent cation taken up: oxygen utilized during uptake wereapproximately 8 with α-ketoglutarate, 4 with succinate and 6 with β-hydroxybutyrate and related substances (Rossi and Lehninger 1964, Carafoli 1965b, and Chance 1965, and Chappell 1963), indicating that 2 divalent cations were taken up as a pair of electrons traversed each of the three energy-coupling sites of the respiratory chain. Chance (1965) measured calcium/ADP ratios of approximately 2 with succinate and glutamate as substrates and related these to the efficiency of calcium accumulation by mitochondria (Chance et al 1964).

B. RESULTS.

1. Ion Uptake.

The majority of investigations into magnesium and phosphate uptake by beetroot mitochondria were carried out using succinate as substrate (Chapter 4). Other compounds oxidized by beetroot mitochondria with

Table VIII. 1. Mg^{++} and Pi Uptake Supported by Different Substrates.

Mitochondria were incubated at 25°C in an ion uptake medium (Tris phosphate) and where indicated 10 mM Tris succinate, 20 mM Tris malate, and 1 ug oligomycin/ml. With malate as substrate 10 mM Tris glutamate, 3 uM NAD and 5 mM Tris malonate were included. 0.3 mM TMPD and 1 mM Tris ascorbate were present in Expt. 1 initially but 3 mM TMPD and 20 mM Tris ascorbate were added after 7 minutes. In Expt. 2, 3.6 mM TMPD and 14 mM Tris ascorbate (1) or 0.36 mM TMPD and 1.4 mM Tris ascorbate (2) were present. The concentration of Tris ascorbate (2) was maintained by further additions at 5 min. intervals. 53 and 72 ug mitochondrial N/ml were present in Expts. 1 and 2. The results were corrected for ion uptake in the absence of added substrate.

	Mg^{++} (umoles/mg N)		Pi (umoles/mg N)	
Expt. 1	6 min.	25 min.	6 min.	25 min.
Succinate	3.28	11.4	2.5	9.4
Succinate + Oligomycin	3.16	11.2	2.5	8.9
Malate	0.30	2.84	0.34	1.02
Malate + Oligomycin	1.02	5.9	0.79	3.28
Ascorbate	10.6	24.5	8.4	18.4
Ascorbate + Oligomycin	12.6	25.0	10.1	18.5
Expt. 2	5 min.	12 min.	5 min.	12 min.
Succinate	3.21	8.8	1.93	5.27
Ascorbate (1)	5.03	20.8	2.61	12.57
Ascorbate (2)	13.3	36.8	8.49	25.7

Table VIII. 2. ADP/O and Respiratory Control Ratios.

Beetroot mitochondria were incubated in an oxygen electrode cell under the conditions for Table VIII 1, but with 5 mM $MgCl_2$. Expts. 1 and 2 correspond to Expts. 1 and 2 in Table VIII 1. 3.6 mM TMPD and 15mM Tris ascorbate were present in Expt. 2 and 0.4 mM TMPD and 1.5 mM Tris ascorbate in Expt. 3. Succinate and malate state 3 oxidation rates were produced by 0.17 mM ADP and ascorbate state 3 rates by 0.05 mM ADP. Where indicated 1 ug/ml oligomycin was added during the second state 3 rate and the oligomycin R.C. ratio was calculated by dividing the second state 3 rate by the oligomycin-induced oxidation rate. The concentrations of mitochondria (ug N/ml) were; Expt. 1, 40; Expt. 2, 54 (succinate), 11 (ascorbate); Expt. 3, 98 (succinate) 20(ascorbate).

	ADP/O			R.C.		
	1	2	3	1	2	3
Expt. 1.						
Succinate	1.5	1.4	1.5	1.7	2.5	2.7
Succinate	1.5	-		1.7	2.8	(+Oligomycin)
Malate	2.3	2.5	2.5	4.4	4.6	4.6
Malate	2.3	-		4.5	7.7	(+Oligomycin)
Expt. 2						
Succinate	1.3	1.4		1.6	2.2	
Ascorbate	0.34			1.2		
Expt. 3.						
Succinate	1.6	1.7		1.6	2.5	
Ascorbate	0.77	0.95		1.3	1.3	

coupled ADP-phosphorylation were tested for their ability to support ion uptake.

Table.VIII 1. demonstrates the effectiveness of Tris succinate, Tris malate plus Tris glutamate, and Tris ascorbate plus TMPD in supporting magnesium and phosphate uptake by beetroot mitochondria. Succinate supported massive uptake which was not affected by oligomycin. Malate plus glutamate supported very little ion uptake but uptake was greatly stimulated by oligomycin. Ascorbate plus TMPD supported even more massive ion uptake than succinate and oligomycin did not affect uptake.

Table VIII 2 shows the respiratory control and ADP/O ratios obtained with the mitochondria used in Table VIII 1. ADP/O ratios approached 3 with malate, 2 with succinate and 1 with ascorbate. Malate and succinate No. 1 state 3 rates were approximately the same but subsequent malate state 3 rates decreased slowly due to incomplete removal of oxaloacetate by glutamate whereas the second succinate state 3 rate was greatly increased by ATP-removal of inhibiting oxaloacetate (Chapter 3). Ascorbate state 3 rates were higher than succinate rates when the TMPD concentration was greater than 0.2 - 0.3mM. State 4 oxidation rates also increased from a very slow rate with malate, to a faster rate with succinate and to an extremely rapid rate with ascorbate. State 4 rates with one substrate did not change significantly during a series of ADP additions and variation from the mean state 4 rate was between 0 - 15% (Chapter 3) and Table VIII 3). Thus respiratory control ratios with malate did not vary greatly from No 1 to No 3 whereas succinate R.C. ratios increased significantly from No 1 to No 2 (Table

Table VIII. 3. Effect of Oligomycin on State 4 Oxidation Rates.

Beetroot mitochondria were incubated in an oxygen electrode cell under conditions for Table VIII 1 with 5 mM $MgCl_2$, 15 mM glucose, and 0.36 mM TMPD and 1.4 mM Tris ascorbate where indicated. Nos. 1 and 2 state 3 oxidation rates were produced by 0.17 mM ADP (succinate and malate) or 0.05 mM ADP (ascorbate) or by excess hexokinase (15 ug/ml) (No. 2 rates). No. 2 state 4 rates were produced by 1 ug/ml oligomycin with the exception of those marked (*) which were produced by ADP conversion to ATP. The concentrations of mitochondria (ug N/ml) were 66 in Expt. 1, 71 in Expt. 2, 27 in Expt. 3 and 17 in Expt. 4.

Substrate	Expt. No.	No. 2 State 3 Oxidation Rate Produced by	State 4 Oxidation Rate mumoles O_2/min.		% Inhibition
			No. 1	No. 2	
Succinate	1	ADP	145	139*	4
Succinate	1	ADP	151	132	13
Succinate	2	ADP	157	130	17
Succinate	1	Hexokinase	151	107	29
Succinate	2	Hexokinase	150	93	38
Malate	1	ADP	85	80*	6
Malate	1	ADP	88	51	42
Malate	1	Hexokinase	95	43	59
Ascorbate	3	ADP	278	275*	1
Ascorbate	3	ADP	276	246	11
Ascorbate	4	ADP	170	147	14

VIII 2). Ascorbate R.C. ratios were very low and constant.

2. Oligomycin Effect on Substrate Oxidation.

Table VIII 2 shows the malate and succinate respiratory control ratios calculated by dividing the second state 3 oxidation rate (excess ADP) by the oxidation rate induced by oligomycin. The oligomycin-R.C. ratio for succinate was not much higher than the expected R.C. ratio but the oligomycin ratio for malate was far greater than that expected (Table VIII 2). Table VIII 3 shows that oligomycin did not greatly reduce succinate state 4 oxidation rates when the No 2 state 3 rate was produced by ADP. However, oligomycin reduced malate state 4 rates by approximately 40%, producing the increased malate-oligomycin-R.C. ratio in Table VIII 2. Ascorbate state 4 rates were not greatly affected by oligomycin. Oligomycin inhibited ATP formation and ATPase activity by 80-90% (Chapter 4 and 6). Thus ATPase activity contributed to a large proportion of the state 4 malate oxidation rate.

Table VIII 3 also indicates experiments where the No 2 state 3 oxidation rates were produced by addition of hexokinase (+ glucose). The concentration of hexokinase was sufficient to remove any ATP formed as glucose-6-phosphate before it could produce ADP by ATPase or adenylate kinase activity (Purvis and Slater 1959). Oligomycin would inhibit the hexokinase rate by inhibition of ATP formation and this oligomycin-rate would not include contributions from oligomycin-insensitive ATPase or adenylate kinase activity. Oligomycin inhibited the succinate state 4 rate by 30-40% when hexokinase produced the No 2 state 3 oxidation rate and malate state 4 rate was inhibited by 60% (Table VIII 3). These

Table VIII. 4. Mg^{++}/O Ratios with Succinate, Malate and Ascorbate.

Beetroot mitochondria were incubated under the conditions for Table VIII 1 with 1.4 mM, 14 mM and 28 mM Tris ascorbate and 0.36 mM, 3.6 mM and 7.2 mM TMPD in Expt. 2 (1), (2) and (3) respectively. 63 and 53 ug mitochondrial N/ml were present in Expts. 1 and 2. Samples in Expt. 1 were taken after 5-10 mins. incubation in an O_2 electrode cell and Mg^{++} and Pi uptake measured. In Expt. 2 Mg^{++} uptake was measured over a period of 15 mins. while parallel experiments measured O_2 uptake polarographically. Mg^{++} and O uptake is expressed as umoles and ug atoms/mg N/initial 5 mins. of incubation after correcting for uptake in the absence of substrate.

	O ug atoms/ mg N/5 min.	Mg^{++} umoles/ mg N/5 min.	Mg^{++}/O	Mg^{++}/ "Extra O"
Expt. 1				
Succinate	6.26	1.89	0.30	3.1
Succinate	6.78	1.69	0.25	2.7
Succinate + Oligomycin	5.64	1.50	0.27	2.9
Succinate + Oligomycin	6.0	1.61	0.27	2.9
Malate	4.34	0.24	0.06	0.67
Malate	4.56	0.30	0.07	0.71
Expt. 2				
Succinate	5.1	2.7	0.53	
Succinate + Oligomycin	4.9	2.7	0.55	
Malate	2.7	0.25	0.09	
Malate + Oligomycin	2.0	0.87	0.43	
Ascorbate (1)	25.6	8.95	0.35	
Ascorbate (1) + Oligomycin	25.1	10.5	0.42	
Ascorbate (2)	71	5·0	0·07	
Ascorbate (3)	70	1·7	0·02	

results indicate that ATPase activity contributed to a large part (½ - 2/3) of the very slow malate state 4 oxidation rate but to a smaller proportion (1/4 - 1/3) of the more rapid succinate state 4 rate. Succinate and ascorbate state 4 oxidation must have consisted mainly of uncoupled or non-phosphorylative oxidation.

3. Mg^{++}/O ratios.

Mg^{++}/O ratios were calculated for different substrates by measuring ion uptake after oxygen uptake was recorded polarographically (Table VIII 4). Mg^{++}/extra O" ratios were also calculated as described in Chapter 6. Mg^{++} and Pi uptake and oxygen uptake were also measured in parallel experiments and Mg^{++}/O ratios calculated indirectly (Table VIII 4).

Although malate oxidation rates were lower than succinate rates, the Mg^{++}/O ratios calculated directly or indirectly were much lower with malate than with succinate (Table VIII 4). Oligomycin did not affect succinate state 4 rates (Table VIII 3), ion uptake (Table VIII 1) or Mg^{++}/O ratios (Table VIII 4). Oligomycin reduced state 4 malate oxidation (Tables VIII 3), increased ion uptake (Table VIII 1) and increased Mg^{++}/O ratios (Table VIII 4). The Mg^{++}/O ratios obtained using low ascorbate and TMPD concentrations were close to those obtained with succinate. Oligomycin increased the ascorbate Mg^{++}/O ratio in Table VIII 4. Ascorbate oxidation rates increased 150% on ten fold increase of ascorbate and TMPD concentration and Mg^{++} and Pi uptake was inhibited, thus reducing the calculated Mg^{++}/O ratio. A further two fold concentration increase did not affect oxidation but further inhibited ion uptake and the Mg^{++}/O

Table VIII. 5. Effect of TMPD and Ascorbate Concentrations on Ascorbate Oxidation.

Beetroot mitochondria were incubated at 25°C in a medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 5 mM $MgCl_2$, 3 mM Tris-phosphate buffer pH 7.2, 4 uM cytochrome c and 18 and 20 ug mitochondrial N/ml in Expts. 1 and 2. In Expt. 2 the state 3 oxidation rate was produced by 0.07 mM ADP and the state 4 oxidation rate was measured after phosphorylation of this ADP.

		Oxidation Rate (μmoles O_2/min)			
Tris Ascorbate mM	TMPD mM	Expt. 1	Expt. 2		
			State 3	State 4	ADP/O
1.5	0	12			
1.5	0.08	56	44	34	0.52
1.5	0.15	95			
3.0	0.15	95			
3.0	0.23	125	72	60	0.51
3.0	0.30	152			
3.0	0.38	170	121	100	0.50
3.0	3.82	390			
15.0	3.82	400			

Table VIII. 6. Glucose-6-Phosphate Formation and Oxygen Uptake.

Beetroot mitochondria were incubated at 25°C in an ion uptake medium (8 mM Tris phosphate) with 15 mM glucose, 0.7 mM ADP, excess hexokinase (14 ug/ml) and when indicated 7 mM Tris succinate, 20 mM Tris malate, 14 mM Tris ascorbate with 3.6 mM TMPD in Expt. 1 and 1.4 mM Tris ascorbate with 0.36 mM TMPD in Expt. 2. 15 mM Tris glutamate, 3 mM Tris malonate and 4 uM NAD were included with malate. In Expts. 1 and 2 the concentrations of mitochondria were 127 and 88 $\frac{ug}{\wedge}$N/ml. Oxygen uptake was measured polarographically in parallel experiments. Glucose-6-phosphate formation and oxygen uptake were corrected for formation and uptake in the absence of substrate. The ADP/O ratios were measured with the same preparations and concentrations of mitochondria and with 15 mM $MgCl_2$.

	Glucose-6-phosphate (umoles/mg N)		Oxygen Uptake (ug atoms O/ mgN)		P/O		ADP/O
Expt. 1	6 min.	15 min.	6 min.	15 min.	6 min.	15 min.	
Succinate	6.6	16.9	3.5	22	0.78	0.77	1.1
Malate	10.7	25.1	7.7	17	1.4	1.5	2.1
Ascorbate	3.5	7.4	42.7	105	0.08	0.07	
Expt. 2	7 min.	14 min.	7 min.	14 min.	7 min.	14 min.	
Succinate	16.0	33.7	16.3	34	0.98	0.99	1.3
Ascorbate	20.4	42.6	33	67	0.62	0.63	0.75

ratio (Table VIII 4).

Table VIII 5 shows the effects of ascorbate and TMPD concentrations on oxidation. Tris ascorbate was oxidized very slowly in the absence of TMPD and as TMPD increased the ascorbate oxidation rate also increased. Beyond 4 - 8mM TMPD the oxidation rate did not increase further. The maximum rate of ascorbate oxidation and the TMPD concentration for this rate varied from one mitochondrial preparation to another. A low ascorbate concentration produced a maximum rate of oxidation providing that this low concentration was continually replenished.

4. Glucose-6-phosphate Formation.

Glucose-6-phosphate formed with various substrates was measured under the conditions for ion uptake (15mM $MgCl_2$). Oxidation of succinate, malate and ascorbate was measured polarographically in parallel experiments and P/O ratios were calculated indirectly (Table VIII 6). ADP/O ratios were also calculated directly from polarographic measurements with 15mM $MgCl_2$ in the medium. ADP/O ratios were reduced by high concentrations of $MgCl_2$ (Chapter 6) and the low ADP/O ratios of Table VIII 6 were expected under these conditions. The indirectly calculated P/O ratios were lower than ADP/O ratios. This result was reported and discussed in Chapter 6 and was probably a result of shaking, length of experiment or presence of phosphatases.

Table VIII 6 indicates that with high ascorbate and TMPD concentrations (14mM and 3.5mM) very little glucose-6-phosphate was formed, oxidation was rapid and thus the P/O ratios were extremely low. With lower

ascorbate and TMPD concentrations (1.4mM and 0.35mM) the P/O ratios increased and corresponded to the ADP/O ratios measured polarographically.

Mg^{++}/P ratios calculated from the Mg^{++}/O and P/O ratios (Tables VIII 4 and 6) gave values of 0.6 for succinate, 0.07 for malate and 0.8 for low concentrations of ascorbate. Chance (1965) calculated Ca^{++}/ADP ratios from ADP/O and Ca^{++}/O ratios measured directly by stimulation of oxidation by low concentrations of ADP or $CaCl_2$. These Ca^{++}/ADP ratios approached 2 for all substrates. Indirectly calculated Mg^{++}/P ratios should still indicate approximately the ability of substrates to take up ions related to ADP-phosphorylation.

Oligomycin greatly increased malate-supported ion uptake but had little effect on succinate and ascorbate-supported uptake (Tables VIII 1 and 4). Oligomycin inhibited glucose-6-phosphate formation by 80-90% but a hypothetical malate Mg^{++}/P ratio of 0.3 was calculated from the oligomycin Mg^{++}/O ratio.

5. Ascorbate and TMPD Effects.

a. Oxidation:- Increasing TMPD concentrations increased the state 3 and state 4 rates of ascorbate oxidation (Table VIII 5). TMPD from 80uM to 400uM did not affect ascorbate ADP/O ratios or R.C. ratios (Tables VIII 5) and respiratory control was recorded at 4mM TMPD (Table VIII 2). The L-ascorbic acid used to prepare Tris ascorbate, and the TMPD used in these experiments were laboratory grade reagents. The effects of these compounds on ion uptake, glucose-6-phosphate formation and oxidation could be partly or wholly due to impurities present.

Table VIII. 7. Effect of TMPD and Ascorbate Concentrations on Glucose-6-phosphate Formation.

Beetroot mitochondria were incubated 14 min. at 25°C in a medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 13 mM $MgCl_2$, 10 mM Tris-phosphate buffer pH 7.2, 17 mM glucose, 0.7 mM ADP, excess hexokinase, 4 uM cytochrome c, 89 ug mitochondrial N/ml and where indicated 14 mM Tris succinate. The results were corrected for glucose-6-phosphate formation in the absence of added substrate. The concentration of ascorbate in (1) and (2) was maintained by additions of 1.4 mM Tris ascorbate in (1) and 0.35 mM TMPD plus 1.4 mM Tris ascorbate in (2) at 4 min. intervals.

			Glucose-6-phosphate (umoles/mg N)	
	Succinate		+ cytochrome c	- cytochrome c
	TMPD (mM)	Tris Ascorbate (mM)	26.4	25.3
(1)	0.36	1.4	42.6	39.7
(2)	0.36	1.4	43.6	42.0
	0.36	14	43.8	41.1
	3.6	14	41.7	41.2
	0.36	28	44.7	41.6
	7.2	28	41.1	39.2

Fig. VIII.1. The effect of TMPD and ascorbate concentrations on Mg^{++} and Pi uptake by beetroot mitochondria, incubated in a medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 3 mM Tris-phosphate buffer pH 7.2, 16 mM $MgCl_2$, 4 uM cytochrome c and 120 ug mitochondrial N/ml.

o -- o no addition

Δ -- Δ with 0.36 TMPD and 1.4 mM Tris ascorbate. This concentration of ascorbate was maintained by additions of 1.4 mM Tris ascorbate at 3 min. intervals.

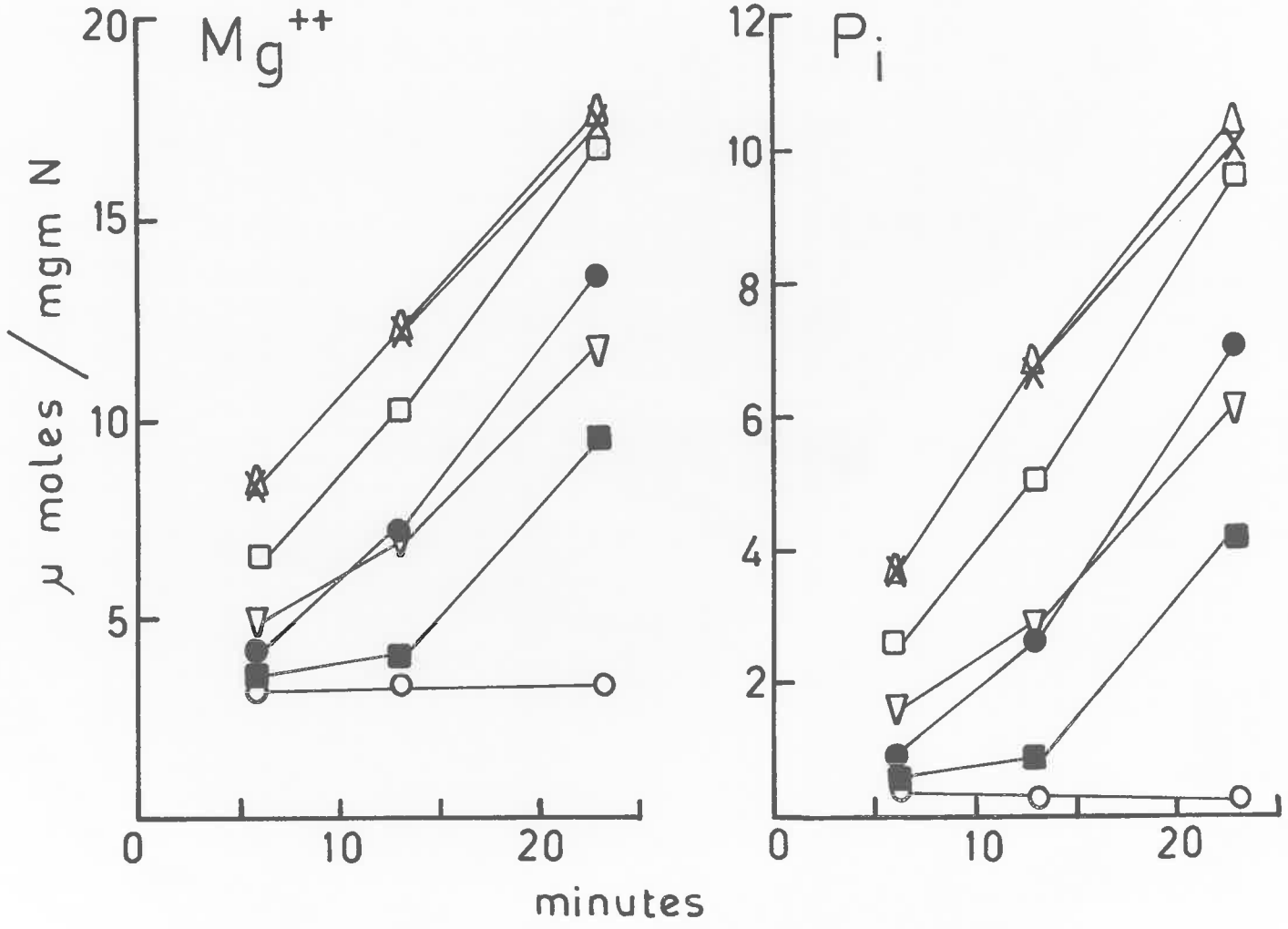
x -- x 0.36 mM TMPD and 1.4 mM Tris ascorbate. This concentration of ascorbate was maintained by additions of 0.36 mM TMPD plus 1.4 mM Tris ascorbate at 3 min intervals.

□ -- □ with 0.36 mM TMPD and 14.3 mM Tris ascorbate.

▽ -- ▽ with 0.36 mM TMPD and 28.6 mM Tris ascorbate.

● -- ● with 3.6 mM TMPD and 14.3 mM Tris ascorbate.

■ -- ■ with 7.2 mM TMPD and 28.6 Tris ascorbate.


Mg++
Pi
μ moles / mgm N
minutes
0
10
20
5
10
15
2
4
6
8
12

b. Glucose-6-phosphate formation:- Table VIII 7 shows that increasing ascorbate and TMPD concentrations had no effect on glucose-6-phosphate formation (with 13mM $MgCl_2$). The increase in P/O ratios (Table VIII 6) on reduction of ascorbate and TMPD concentrations was probably a result of lower ascorbate oxidation rates although the different mitochondrial preparations could have contributed to the change in ratios. Increase in ascorbate oxidation as TMPD concentration increased (Table VIII 5) must have resulted from an increase in uncoupled or non-phosphorylative oxidation not linked to ATP formation.

c. Ion uptake:- Fig VIII 1 shows the effect of ascorbate and TMPD concentrations on magnesium and phosphate uptake by beetroot mitochondria. A rapid linear rate of uptake was recorded for 20 minutes when low ascorbate and TMPD concentrations (1.4mM and 0.36mM) were initially added. Further additions of either 1.4mM ascorbate, or 1.4mM ascorbate plus 0.36mM TMPD were made at 3-4 minutes intervals to maintain a continuous supply of ascorbate. 1.4mM ascorbate was oxidized in 5-6 minutes by this concentration of mitochondria. Increasing the initial ascorbate concentration from 1.9mM to 14mM inhibited ion uptake (Fig VIII 1) when the TMPD concentration remained at 0.36mM. This inhibition decreased with time. Only a small proportion of the inhibition could have been due to Tris added with the 14mM ascorbate because 10mM Tris-HCl inhibited substrate-dependent ion uptake by less than 10% (Chapter 7.) Ion uptake was inhibited further by 28.6mM Tris ascorbate and the inhibition again decreased with time (Fig VIII 1). Ascorbate concentrations did not

affect oxidation rate (Table VIII 5) or glucose-6-phosphate formation (Table VIII 7). The recovery in ion uptake with time would be produced if the reduced form of ascorbate inhibited ion uptake but the oxidized form did not. Approximately 70% of 14mM Tris ascorbate and 35% of 28.6mM ascorbate would have been oxidized within 23 minutes.

Increasing TMPD concentration from 0.36mM to 3.6mM with 14mM Tris ascorbate inhibited ion uptake (Fig VIII 1). The recovery of ion uptake associated with this ascorbate concentration was observed. Further TMPD concentration increased (7.2mM with 28.6mM ascorbate) almost completely inhibited ion uptake (Fig VIII 1) and again ion uptake recovered with time.

Thus high concentrations of both TMPD and ascorbate inhibited ion uptake and the inhibitions were approximately additive. The combined effects of inhibited ion uptake and stimulated oxidation at high TMPD concentrations produced the low Mg^{++}/O ratios recorded in Table VIII 4. 36mM and 7.2mM TMPD inhibited ion uptake by approx. the same amount above any inhibition by ascorbate (Fig VIII 1). This could be related to the TMPD concentration effect on ascorbate oxidation when increase from 0.36mM to 3·6 mM stimulated oxidation by 150% but 7.2mM TMPD had little further effect on oxidation (Table VIII 4). Contaminating substances could account for some or all of these effects of ascorbate and TMPD.

6. Cytochrome c Effect.

a. Oxidation:- Cytochrome c (3-5uM) stimulated succinate oxidation occasionally but had no effect on APD/O or R.C. ratios (Chapter 3.)

Table VIII. 8. Effect of Cytochrome c and Bovine Serum Albumin on Ascorbate - Supported Ion Uptake.

Beetroot mitochondria were incubated at 25°C in a medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 3 mM Tris-phosphate buffer pH 7.2, 15 mM $MgCl_2$, 0.36 mM TMPD, 1.3 mM Tris ascorbate and 77 ug mitochondrial N/ml. Additions of 1.3 mM ascorbate were made at 4 min. intervals. The results have been corrected for the uptake of ions in the absence of added substrate.

6

	Mg^{++} (umoles/mg N)		Pi (umoles/mg N)	
	10 min.	15 min.	10 min.	15 min.
Control	20.9	29.3	15.2	20.9
+ Cytochrome c (4 uM)	28.0	37.9	19.4	26.6
+ BSA (0.01%)	29.1	39.1	20.1	27.8
+ Cytochrome c (4 uM) + BSA (0.01%)	31.4	41.8	22.7	29.6

Fig. VIII. 2. The effect of cytochrome c and of TMPD and ascorbate concentrations on Mg^{++} and Pi uptake by beetroot mitochondria incubated in a medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 3 mM Tris-phosphate buffer pH 7.2, 16 mM $MgCl_2$ and 72 ug mitochondrial N/ml.

x -- x no addition

● -- ● with 14 mM Tris succinate

o -- o with 14 mM Tris succinate plus 5 uM cytotochrome c.

▼ -- ▼ with 0.36 mM TMPD and 1.4 mM Tris ascorbate. Additions of 1.4 mM Tris ascorbate were made at 4 min. intervals.

▽ -- ▽ with 0.36 mM TMPD and 1.4 mM Tris ascorbate plus 5 uM cytochrome c. Additions of 1.4 mM Tris ascorbate were made at 4 min intervals.

■ -- ■ with 0.36 mM TMPD and 14 mM Tris ascorbate.

□ -- □ with 0.36 mM TMPD and 14 mM Tris ascorbate. plus 5 uM cytochrome c.

▲ -- ▲ with 3.6 mM TMPD and 14 mM Tris ascorbate.

△ -- △ with 3.6 mM TMPD and 14 mM Tris ascorbate plus 5 uM cytochrome c.

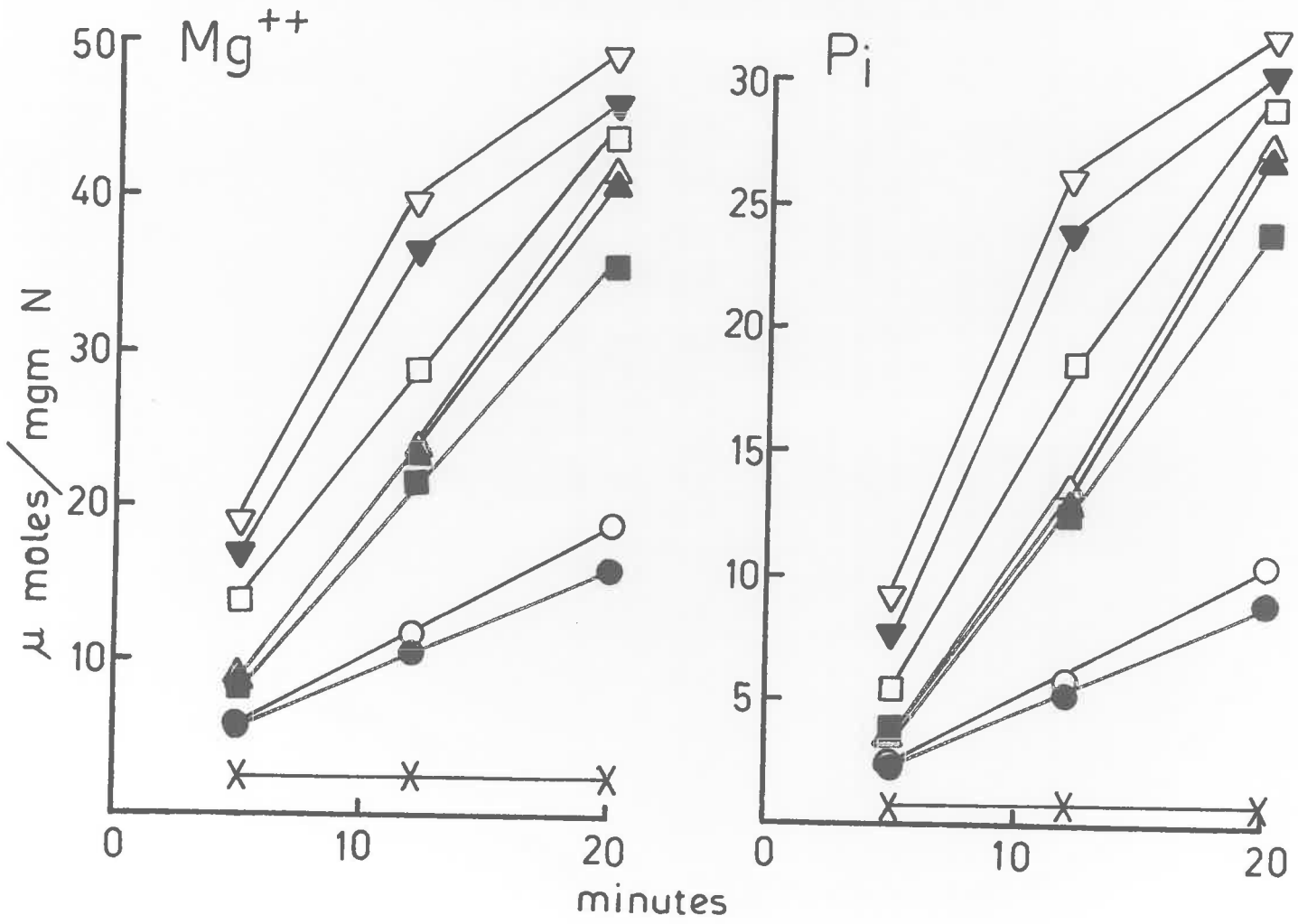

Mg++
Pi
μ moles / mgm N
50
40
30
20
10
30
25
20
15
10
5
0
10
20
minutes

Table VIII. 9. Effect of Antimycin A on Ascorbate Oxidation.

Beetroot mitochondria were incubated at 25°C in a medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 5 mM $MgCl_2$, 3 mM Tris-phosphate buffer pH 7.2, 4 uM cytochrome c, 0.36 mM TMPD, 1.4 mM Tris ascorbate, 11 ug mitochondrial N/ml in Expts. 1 and 2 and 27 ug mitochondrial N/ml in Expts. 3 and 4.

Treatment	Oxidation rate (mumoles O_2/min)			
	Expt. 1	Expt. 2	Expt. 3	Expt. 4
Control	105	400	197	260
+ 1 ug/ml Antimycin A	120		270	282
+ 2 ug/ml Antimycin A	165	530	300	
+ 5 ug/ml Antimycin A		618		

Table VIII. 10. Effect of Antimycin A on Glucose-6-Phosphate Formation.

Beetroot mitochondria were incubated at 25°C in a medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 13 mM Tris-phosphate buffer pH 7.2, 3 uM cytochrome c, 12 mM $MgCl_2$, 16 mM glucose, 0.5 mM ADP, excess hexokinase, 87 ug mitochondrial N/ml and where indicated 11 mM Tris succinate, 0.3 mM TMPD and 1.1 mM Tris ascorbate. Additions of 1.1 mM Tris ascorbate were made at 4 minute intervals. The results have been corrected for the formation of glucose-6-phosphate in the absence of substrate.

Substrate	Antimycin A (ug/ml)	Glucose-6-phosphate (umoles/mg N)	
		11 min.	23 min.
Succinate	-	14.6	29.0
Succinate	0.8	0.7	0.5
Ascorbate	-	18.9	37.4
Ascorbate	0.8	20.0	39.1
Ascorbate	1.6	20.9	40.9
Ascorbate	3.1	19.4	38.4
Ascorbate	4.7	19.8	37.6

Ascorbate oxidation was always stimulated 10-30% by cytochrome c (Table III 3) independent of the initial rate of TMPD-induced ascorbate oxidation. The response to cytochrome c varied from one mitochondria preparation to another. BSA (0.1%) had no effect on ascorbate oxidation.

b. Glucose-6-phosphate formation:- Table VIII 7 shows that 4uM cytochrome c did not affect glucose-6- phosphate formation with succinate or ascorbate.

c. Ion Uptake:- Table VIII 8 shows thatascorbate-supported ion uptake was stimulated by cytochrome c (4uM) and by B.S.A. (0.01%) and cytochrome c plus B.S.A. further stimulated ion uptake but not additively. The effects of cytochrome c and B.S.A. on succinate-supported ion uptake were discussed in Chapter 4.

Fig. VIII 2 also shows that cytochrome c stimulated ascorbate-supported ion uptake. When ascorbate was 1.4mM and TMPD o.36mM cytochrome c stimulated ion uptake by approx. 10% but with higher ascorbate and TMPD concentrations cytochrome c stimulated ion uptake significantly (Fig VIII 2).

7. Antimycin A Effect.

a. Oxidation:- Table VIII 9 shows that antimycin A stimulated ascorbate oxidation by 20-40% at a concentration which completely inhibited succinate oxidation (lug/ml). Higher antimycin A concentrations further stimulated ascorbate oxidation (Table VIII 9). Antimycin A did not affect ascorbate ADP/O ratios or respiratory control ratios (Table III 3). HOQNO stimulated ascorbate oxidation slightly (10%).

b. Glucose-6-phosphate Formation:- Table VIII 10 shows that 0.8ug/ml

Fig. VIII. 3. The effect of antimycin A on Mg^{++} and Pi uptake by beetroot mitochondria incubated in a medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.7, 3 mM Tris-phosphate buffer pH 7.2, 13 mM $MgCl_2$, 4 uM cytochrome c and 112 ug mitochondrial N/ml. Ascorbate concentration was maintained by additons of 1.4 mM Tris ascorbate at 3 min. intervals.

x -- x no addition.

o -- o with 14 mM Tris-succinate plus 1 ug/ml antimycin A.

▼ -- ▼ with 14 mM Tris succinate.

■ -- ■ with 0.36 mM TMPD and 1.4 mM Tris ascorbate.

△ -- △ with 0.36 mM TMPD and 1.4 mM Tris ascorbate. plus 1 ug/ml antimycin A.

● -- ● with 0.36 mM TMPD and 1.4 mM Tris ascorbate. plus 2 ug/ml antimycin A.

□ -- □ with 0.36 mM TMPD and 1.4 mM Tris ascorbate plus 3 ug/ml antimycin A.

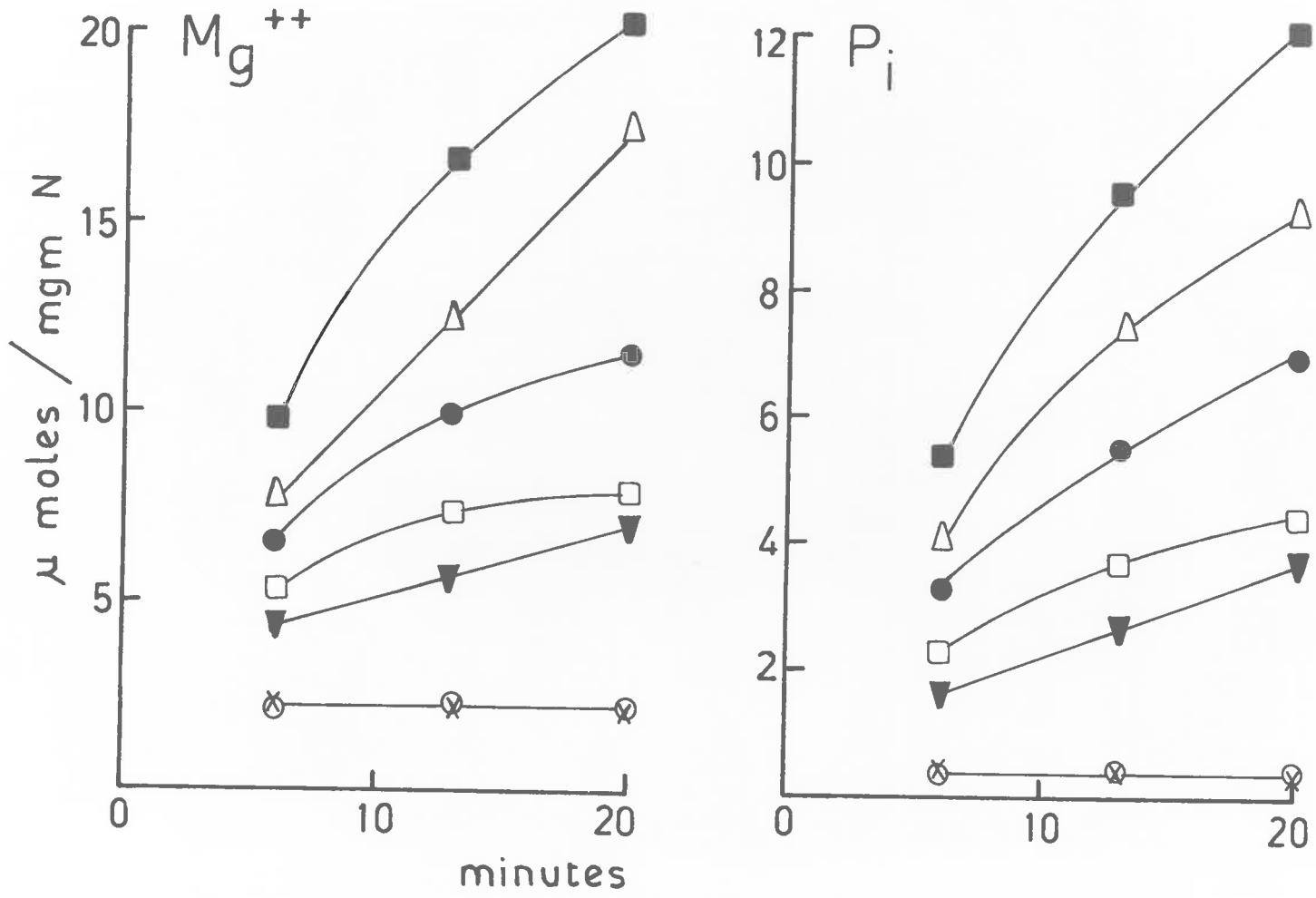

Mg ++
μ moles / mgm N
20
15
10
5
0
10
20
minutes
Pi
12
10
8
6
4
2
0
10
20

Table VIII. 11. Effect of Antimycin A and Cytochrome c on Mg^{++} and Pi Uptake.

Beetroot mitochondria were incubated at 25°C for 19 minutes in a medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 3 mM Tris-phosphate buffer pH 7.2, 12 mM $MgCl_2$, 63 ug mitochondrial N/ml and where indicated 11 mM Tris succinate, 0.3 mM TMPD plus 1.1 mM Tris ascorbate and 3 uM cytochrome c. Addition of 1.1 mM Tris ascorbate were made at 4 min. intervals. The results were corrected for ion uptake in the absence of substrate.

		Mg^{++} (umoles/mg N				Pi (umoles/mg N)			
Substrate	Antimycin A (ug/ml)	+ cyto c	% Inhibition	- cyto c	% Inhibition	+ cyto c	% Inhibition	- cyto c	% Inhibition
Succinate	-	6.5	-	5.9		4.7		4.2	
Succinate	0.8	0	100	-		0.2	96	-	
Ascorbate	-	22.2	-	14.5	-	18.1	-	12.4	-
Ascorbate	0.8	17.4	22	11.6	20	13.9	23	9.4	24
Ascorbate	1.6	12.8	42	7.4	49	9.8	46	6.2	50
Ascorbate	3.1	5.2	77	3.5	75	3.7	80	2.5	80

antimycin A completely inhibited glucose-6-phosphate formation with succinate but had no effect on formation with ascorbate. Increasing the antimycin A concentration to 7.2ug/ml did not affect glucose-6-phosphate formation with ascorbate. Cytochrome c did not affect ascorbate-supported glucose-6-phosphate formation in the presence or absence of antimycin A (Table VIII 10.) HOQNO had no effect on ascorbate-supported glucose-6-phosphate formation at a concentration (2uM) which completely inhibited formation with succinate. These results were expected from the postulated action sites of these inhibitors (between cytochromes b and c) (Chapter 1) and from the site of electrons entering the oxido-reduction chain from ascorbate via TMPD (cytochrome c or c_1)(Tyler et al 1966, Howland 1963b, Packer et al 1963).

c. Ion Uptake:- Fig. VIII 3 shows the effect of antimycin A concentration on ascorbate-supported magnesium and phosphate uptake. Ion uptake supported by succinate was completely inhibited by lug/ml antimycin A. and ascorbate-supported ion uptake was progressively inhibited as antimycin A concentration increased. Table VIII 11 shows that cytochrome c stimulated ascorbate-supported ion uptake but did not affect the % inhibitions of ion uptake by different concentrations of antimycin A.

C. DISCUSSION.

1. Substrate Efficiency.

The state 4 oxidation rates of malate plus glutamate, succinate,

and ascorbate plus TMPD were very different, increasing from an extremely low rate with malate to a very fast rate with ascorbate. State 3 oxidation rates also increased from malate to ascorbate. The state 4 oxidation rate is produced when ADP-phosphorylation has ceased and one of the phosphorylation sites in the electron transfer chain is controlling the oxidation rate. The R.C. ratios give a measure of phosphorylation control of oxidation and they decreased from malate (5) to succinate (2-3) to ascorbate (1). Thus the first phosphorylation site associated with malate oxidation was most tightly coupled to oxidation, the second site (succinate) less so and the last site associated with cytochrome c (ascorbate) oxidation was very poorly coupled (Chapter 3).

The effect of oligomycin on the oxidation rates of malate, succinate and ascorbate indicated that differences in state 4 rates were not produced by different ATPase activity. The ATPase activity was approximately the same for each substrate, contributing to more than half of the very slow state 4 malate oxidation rate, but to only 30% of the succinate state 4 rate and to a smaller proportion of the ascorbate rate.

State 3 oxidation rates are limited by the rate of electron movement along the respiratory chain (Chance and Williams 1955) and reduced electron flow in the region of malate would produce a slower oxidation rate. Electron flow should not be limited by chain components during state 4 oxidation (ADP limiting, (Chance and Williams 1955) but would depend on spontaneous high-energy intermediate breakdown by the classical

hypothesis of oxidative phosphorylation. The different state 4 rates of malate, succinate and ascorbate oxidation would be produced by different breakdown rates of the intermediates produced at the 3 phosphorylation sites. The rapid ascorbate oxidation compared with malate would thus be due to faster breakdown of the site 3 intermediate (uncoupled oxidation) than the site 1 intermediate, or to oxidation through a non-phosphorylating pathway. However, ascorbate used the site 3 high-energy intermediates to form ATP (when ADP was added) as efficiently as malate, and the ascorbate state 3 - state 4 transition must involve a change from non-phosphorylating or uncoupled oxidation to coupled oxidation with complete use of intermediates for ATP formation.

The problem of such a change-over during state 4 - state 3 transition arises from the direct chemical link between oxidation and phosphorylation in the classical scheme for oxidative phosphorylation. The chemiosmotic hypothesis postulates no chemical intermediate between the oxido-reduction system and the ATP formation system (Mitchell 1966, Chapter 1.) State 3 oxidation rates would be limited by electron movement along the oxido-reduction chain but state 4 rates would depend on the amount of PMF required to inhibit electron flow and H^+ and e^- separating on and thus control respiration. The PMF required to prevent protein and electron separation in the NAD-non-heme iron couple could be less than that required in the CoQ-cytochrome c,a,a_3 couple (Fig I 6A). The redox potential of NADH is more negative than that of cytochrome c and state 3 - state 4 transitions with β-hydroxybutyrate produced from 50%->100% reduction of NADH but from only 5->15% reduction of cytochrome c

(Chance and Williams 1955). PMF built up in the absence of phosphate acceptor could be partially relieved by H^+ leakage (or cation uptake) back across the membrane. This leakage would be greater under the influence of the high PMF maintained during ascorbate acceptorless oxidation than under the influence of malate state 4 PMF. The rapid ascorbate state 4 oxidation would thus result from high PMF build up and more H^+ leakage. The PMF built up during acceptorless succinate oxidation would be intermediate between malate and ascorbate from the intermediate redox potential. Addition of ADP would produce very fast and complete collapse of all PMF through the reversible ATPase system. Thus the state 3 oxidation rates and phosphorylation efficiency would be independent of the state 4 PMF build up and any H^+ leakage under the influence of this PMF i.e. of the state 4 oxidation rate. The use of ascorbate PMF would be as efficient as use of malate PMF for ATP-formation which would provide less resistance to PMF collapse than H^+ leakage.

More glucose-6-phosphate was formed with malate than with succinate or ascorbate over the same incubation period. The P/O and ADP/O ratios approached the theoretical values of 3,2 and 1 for malate, succinate and ascorbate. Thus, although the absolute phosphorylation efficiency per atom of oxygen consumed decreased from malate (3) to ascorbate (1), one phosphorylation site was as efficient as any other in producing the required amount of ATP. The use of the high-energy intermediates produced during ascorbate oxidation for ATP formation was

as efficient as the use of intermediates produced during malate oxidation (classical hypothesis of oxidative phosphorylation) or the collapse of PMF produced by ascorbate oxidation by operation of the reversed ATPase system was as efficient as collapse of PMF produced by malate oxidation (of the chemiosmotic hypothesis).

Ion uptake by beetroot mitochondria was high with ascorbate lower with succinate and extremely low with malate plus glutamate. The Mg^{++}/O ratios calculated indirectly were 0.4 (ascorbate) o.5 (succinate) and 0.1 (malate) while the Mg^{++}/P ratios derived from Mg^{++}/O and P/O ratios were 0.8, 0.6 and 0.1 respectively. Oligomycin increased malate-supported ion uptake and increased the Mg^{++}/O ratio to 0.3 - 0.4 but had little effect on succinate-and ascorbate-supported ion uptake and Mg^{++}/O ratios. Thus, although more ATP was formed during malate oxidation than during ascorbate oxidation, and P/O ratios were higher with malate than with succinate or ascorbate, magnesium and phosphate uptake was much lower and less efficient with malate than with succinate and ascorbate. Malate oxidation by beetroot mitochondria was not efficiently coupled to ion uptake (although it was efficiently coupled to ATP formation), whereas succinate and in particular ascorbate oxidation supported massive magnesium and phosphate uptake. Ion uptake was directly related to state 4 (and state 3) rates of substrate oxidation and indirectly related to respiratory control and P/O ratios.

The chemical hypothesis of oxidative phosphorylation (Chapter 1) energises ion uptake by a high-energy intermediate (Chance 1965,

Rasmussen et al 1965). The results obtained with beetroot mitochondria suggest that ion uptake was not correlated with the amount of high-energy intermediates formed. Malate produced high-energy intermediates which were used efficiently for ATP formation but in beetroot mitochondria these were not used efficiently or could not be used for ion uptake. It has been proposed that the 3 high-energy intermediates produced during malate oxidation (one at each phosphorylation site) are different (Slater 1966). Intermediates with different abilities to move ions (by structure or position) could account for the small ion uptake and the low Mg^{++}/O ratio with malate compared with ascorbate uptake and ion/O efficiency in beetroot mitochondria.

The chemiosmotic hypothesis produces PMF by the oxidation-reduction chain which can be used for ATP production or ion uptake. The PMF produced in the absence of phosphate accepter by malate oxidation could be too small to promote ion uptake. Succinate and ascorbate, by maintaining higher PMF during acceptorless oxidation (and producing higher state 4 rates) could support massive ion uptake more efficiently. Phosphorylation through the reversed ATPase system would not be affected by ion uptake if the former process provided less resistance to PMF collapse. Ion uptake would provide less resistance to PMF collapse than H^+ leakage across the membrane.

Investigations with animal mitochondria have indicated that malate and related substrates can support ion uptake as efficiently as other substrates connected with the second and third phosphorylation sites

(Chance 1963, 1965, Chappell et al 1963, Rossi and Lehninger 1964). Mn^{++} or Ca^{++}/O ratios of 6 with substrates related to malate, 4 with succinate and 2 with ascorbate have been recorded in animal mitochondria and cation/ADP ratios of 1.8 - 2.0 have been calculated (Chance 1965, Rossi and Lehninger 1964). These ratios are the theoretical values expected by both the classical mechanism (Chance 1965) and the chemiosmotic mechanism for ion uptake (Mitchell 1966) for tightly coupled or nonpermeable mitochondria (Chapter 1). The higher permeability of plant mitochondrial membranes to ions could account for lower ion uptake/oxygen ratios recorded for all substrates in beetroot mitochondria while ADP/O ratios were as high as in animal mitochondria.

2. Ascorbate, TMPD, Antimycin A and Cytochrome c Effects.

Ion uptake was inhibited when ascorbate concentration was increased above 1.4mM but oxidation and glucose-6-phosphate formation were not affected. The inhibition of ion uptake decreased with time and did not occur when ascorbate was added in low concentrations at short intervals throughout the experiment. Reduced ascorbate appeared to inhibit ion uptake whereas the oxidized form had little effect. Impurities added with the ascorbate could also have affected ion uptake.

Ascorbate oxidation was stimulated as TMPD concentration was increased above 0.4mM, glucose-6-phosphate formation and ADP/O ratios were not affected and ion uptake was inhibited. These effects of TMPD cannot be easily accounted for by the classical mechanism in which a non-phosphorylated high-energy intermediate energises both ATP formation

and ion uptake and in which oxidation stimulation is produced by an increase in coupled or uncoupled oxidation or by operation of a non-phosphorylating oxidation pathway. If TMPD promoted coupled oxidation by acting as an electron carrier between ascorbate and cytochrome c then ATP formation and ion uptake would be stimulated and ADP/O ratios would remain constant. If uncoupled or non-phosphorylating oxidation was stimulated by TMPD then ADP/O ratios should decrease and ATP formation and ion uptake would remain constant or decrease. TMPD could affect the stability or structure of the high-energy intermediate and prevent this intermediate supporting ion uptake but not affect support of ATP formation.

The chemiosmotic mechanism suggests that ATP formation provides a pathway with less resistance to collapse of substrate-induced PMF than ion uptake. ATP formation was the favoured process in competition with ion uptake. TMPD would stimulate oxidation by promoting electron transfer between ascorbate and cytochrome c but could also affect membrane permeability to H^+. TMPD could thus produce a pathway for PMF collapse with more resistance than the ATP-formation pathway but with less resistance than ion uptake. This would account for TMPD stimulating oxidation, not affecting ATP formation and ADP/O ratios, and inhibiting ion uptake. State 4 ascorbate oxidation would be increased by TMPD according to this scheme and R.C. ratios should decrease. R.C. ratios (1.1 - 1.3) were not affected by TMPD from 70 - 400uM but controlled respiration was not recorded when TMPD was higher than 4mM.

Antimycin A stimulated ascorbate oxidation by 20 - 40% and inhibited ion uptake but had no effect on glucose-6-phosphate formation or ADP/O ratios. Antimycin A does not act as an electron carrier (unlike TMPD) and ascorbate oxidation was only slightly stimulated by antimycin A. If antimycin A increased the permeability of the mitochondrial membranes to H^+ in the same way as TMPD, then oxidation would be stimulated slightly, ion uptake would be inhibited, but ATP formation and ADP/O ratios would not be affected. It is difficult to account for the antimycin A effects in beetroot mitochondria by the classical scheme for oxidative phosphorylation and ion uptake although antimycin A could also reduce the ability of the high-energy intermediate to support ion-uptake but not the ability to support ATP formation.

The small stimulation in ascorbate-supported ion uptake by horse heart cytochrome c can be related to a stabilising protein effect (Chapter 4), or to the stimulation of ascorbate oxidation by cytochrome c. This latter effect was always recorded (succinate oxidation was not always increased by cytochrome c, Chapter 4) and could have been due to the low cytochrome c level of washed beetroot mitochondria limiting ascorbate oxidation. Glucose-6-phosphate formation and ADP/O ratios with ascorbate were not affected by cytochrome c. The marked stimulation of ion uptake by cytochrome c at high TMPD and ascorbate concentrations (Fig. VIII.2) could have been due to decrease in the concentration of the inhibitory reduced form of ascorbate by reduction of excess cytochrome c. Cytochrome c could also stabilise membrane structure and prevent or reduce any TMPD effects on membrane permeability (or high-energy intermediate

stability) and the associated decrease in ion uptake.

D. CONCLUSIONS.

Beetroot mitochondria oxidized malate, succinate and ascorbate and coupled oxidation to phosphorylation of ADP. The ADP/O and P/O ratios approached the theoretical values of 3, 2 and 1 for these substrates indicating that each of the 3 respiratory chain phosphorylation sites used the high-energy intermediates (classical) or PMF (chemiosmotic) produced efficiently to form ATP. Ascorbate supported massive magnesium and phosphate uptake whereas malate supported very little ion uptake. Mg^{++}/O ratios did not increase like the ADP/O ratios from ascorbate to malate indicating that malate did not use the energy of oxidation efficiently for ion uptake. Substrate support of ion uptake was directly related to the state 4 (and state 3) rates of oxidation and indirectly related to the respiratory control and ADP/O ratios. These results were discussed in relation to the classical mechanisms (different abilities of the 3 intermediates to move ions) and the chemiosmotic mechanisms (different PMF build up to produce respiratory control) for oxidative phosphorylation and ion uptake.

The Mg^{++}/O ratios in beetroot mitochondria were 0.5, 0.4 and 0.1 for ascorbate, succinate and malate whereas the ion/O ratios in animal mitochondria were 2.0, 4.0 and 6.0 for related substrates. The ratios in animal mitochondria agree with the stoichiometry of ion uptake mechanisms based on both hypotheses for oxidative phosphorylation. The low Mg^{++}/O ratios for beetroot mitochondria (and the low Mg^{++}/ADP ratios)

would be produced if the mitochondria membranes were more permeable to ions than those of animal mitochondria.

The effects of TMPD, antimycin A and cytochrome c on ascorbate oxidation, ATP formation and ion uptake were discussed in relation to both hypotheses for oxidative phosphorylation. TMPD and antimycin A could react with high-energy intermediates preventing support of ion uptake but not ATP formation (classical), or increase membrane permeability to H^+ providing a pathway with more resistance to PMF collapse than ATP formation but with less resistance than ion uptake (chemiosmotic).

CHAPTER NINE

BASE = ACID TREATMENT.

A. INTRODUCTION.

The chemiosmotic hypothesis proposed by Mitchell (1966) couples the hydro-dehydration or ATPase system of mitochondria and chloroplasts to the oxido-reduction or respiratory chain system by means of proton current and membrane potential. No chemical link exists between these two systems as postulated for the classical hypothesis of oxidative phosphorylation. If a proton-motive force corresponding to 200 to 250mV or -3 to -4 units (for the ATPase II system, Chapter 1) were artificially imposed across the coupling membrane for a short time then there should theoretically be some synthesis of ATP. This artificial PMF could be supplied by equilibrating a suspension of mitochondria in a high pH and then lowering the pH in the presence of ADP and phosphate or vice versa for a chloroplast suspension.

Hind and Jagendorf (1965) reported that when the pH of the first energy-or light-accepting stage of photosynthetic phosphorylation was 4.6 and the pH of the second dark stage (containing ADP and phosphate) was 8.0, controls which had not been illuminated in the first stage synthesized a considerable amount of ATP in the second stage. Jagendorf and Uribe (1966) found that the amount of ATP synthesized by the chloroplasts was more dependent on the range over which the pH was changed than upon the absolute initial and final pH values implying that the pH differential was of major importance. Anionic buffers increased the amount of ATP synthesized.

The chemical hypothesis of oxidative phosphorylation could postulate pH-dependent breakdown of an H_2-containing high-energy intermediate (Chance 1965, Chance and Mela, 1966). However, pH differential was more important than absolute pH values and Jagendorf and Uribe (1966) calculated that the amount of ATP synthesized was equivalent to 100ATP molecules per cytochrome molecule making the synthesis of a pH-dependent high-energy intermediate unlikely.

Movement of protons through the mitochondrial ATPase II system in response to a pH differential would synthesize ATP but would also build up an opposing membrane potential if there was no compensating flow of ions through the coupling membrane. This membrane is presumed to be relatively impermeable to ion movement (Mitchell 1966, Chappell and Crofts 1966). Mitchell (1966) summarised the requirements for ATP-production as follows:-

(1) the driving force of ATP synthesis is the P.M.F. and not the pH differential.

(2) A P.M.F. of some 210mV (equivalent to - 3.5pH units with no membrane potential) should be required to drive ATP synthesis via ATPase II (see Fig.I 5) in the presence of 10mM phosphate.

(3) the amount of ATP synthesized via ATPase II should be given by the total number of protons passing across the coupling membrane at this P.M.F. multiplied by the proportion of the total proton flux which passes specifically through the ATPase system.

(4) The synthesis of ATP by a pH differential may be stimulated by specific reagents such as gramicidin or valinomycin that can collapse the

Table IX. 1. ATP Formation by Base-Acid Treatment of Beetroot Mitochondria.

	mu moles ATP/mg N		
(a) pH Change	1) 10 - 6.5	2) 9.5 - 7.1	3) 10.0 - 7.1
Control	416	290	417
+ Oligomycin 2 ug/ml	267	-	380
+ Ethanol	-	310	409
+ DNP (1×10^{-4} M)	244	-	344
pH Change 7.2 - 7.0	272	189	-

(b) pH Change	4) 9.8 - 6.3	5) 9.5 - 6.5
Control	1150	1050
+ Oligomycin 2 ug/ml	875	913
+ DNP (1×10^{-4} M)	900	870
pH 7.2	-	787
pH 9.5	-	890

Mitochondria were shaken with 0.2 M Tris for 1 min. (a), or 2 min. with 2 ug/ml Antimycin A (b) and then transferred to a medium containing 10 mM Tris-phosphate buffer (pH 7.2), 3 mM $MgCl_2$, 15 mM glucose, 0.01% BSA, excess hexokinase, 0.3 mM ADP and 2.5 mM AMP. 0.1 N HCl was added immediately (a) or after 15 secs. (b), and after 1 min. shaking the reaction was stopped by boiling (a) or by 2.5% $HClO_4$ (b). Oligomycin and DNP were added to the alkaline phase (b) or to the reaction medium (a). Mitochondria were shaken where indicated (pH 7.2) with 0.2 M Tris-HCL buffer (pH 7.2) instead of Tris, and Tris-HCL was added in place of HCl. Where indicated (pH 9.5) mitochondria were shaken with 0.2 M Tris and transferred to the medium but HCl was not added. The concentrations of mitochondria in the acid phase were (1) 520; (2) 595; (3) 390; (4) 950; (5) 965 ug N/ml.

membrane potential without collapsing the pH differential. (Chapter 1). Uncoupling agents e.g. DNP, by collapsing pH differential (but not membrane potential) would prevent ATP synthesis by this system (Chapter 1). This was demonstrated by Jagendorf and Uribe (1966) with the fragmented spinach chloroplast system.

Reid et al (1966) reported synthesis of ATP by base-acid treatment of rat liver mitochondria. ATP formation was measured by P_{32} uptake from a medium. The mitochondria were pre-incubated in rotenone and antimycin A (to inhibit endogenous substrate oxidation), EDTA (to inhibit adenylate kinase, Chappell 1964) and valinomycin (with KCl in the alkaline phase to collapse membrane potential). The alkaline phase pH (KOH) was 8.8 - 9.0 and addition of HCl produced a drop to pH 4.2 - 4.3 and then further fall to zero pH. 500-600 mu moles ATP/g protein were produced by the 4.6pH span when the acid phase was of 1-2 sec. duration and extended acid incubation (5-10 sec.) or a 5.6 pH span reduced ATP formation to zero. Oligomycin or uncoupling agents added during the pre-incubation phase reduced ATP production to 23% at 1 sec. or zero at 2 sec. in the acid phase. The transient ATP synthesis was attributed in part to vigorous ATPase activity.

B. RESULTS.

1. ATP Formation during Base-Acid Treatment.

Table IX 1 (a) shows that ATP was formed on base-acid treatment of beetroot mitochondria. Oligomycin, DNP and constant pH treatment at either 7.2 or 9.5 reduced the amount of ATP formed by 10% - 40% in

Table IX. 2. The effect of AMP on ATP formation by Adenylate Kinase.

AMP/ADP	△ OD/Min.	% Inhibition
0	0.165	0
1.8	0.074	36
3.6	0.046	60
7.3	0.024	80
9.1	0.023	81

Adenylate Kinase was assayed in 3.0 ml of medium containing 0.2 M sucrose, 8 mM Tris-HCl buffer (pH 7.2), 4 mM $MgCl_2$, 3 mM Tris-phosphate buffer (pH 7.2), excess hexokinase, 17 mM glucose, 500 ug NADP, 0.01% BSA, 0.3 mM ADP, glucose-6-phosphate dehydrogenase and 74 ug mitochondrial N.

Table IX. 3. Mg^{++} and Pi Uptake During Base-Acid Treatment of Beetroot Mitochondria.

	mu moles/mg N							
	Mg^{++}	Pi	Mg^{++}	Pi	Mg^{++}	Pi	Mg^{++}	Pi
pH Change	[1] 9.5 - 7.0		[2] 9.5 - 7.0		[3] 9.5 - 5.5		[4] 9.5 - 3.7	
Control	547	256	840	220	289	187	377	233
+ Oligomycin	550	250	855	218	302	201		
+ DNP	512	224	779	190	231	142	311	185
pH 7.2	513	224	730	167	(515)	142		

Mitochondria were shaken for 1 min. with 0.2 M Tris and 2 ug/ml Antimycin A and then transferred to a medium containing 3 mM Tris-phosphate buffer (pH 7.2), 10 mM $MgCl_2$, 0.01% BSA and where indicated 2 ug/ml oligomycin and 1 x 10^{-4} M DNP. 0.1 N HCL was added immediately and after 1½ min. shaking the mitochondria were collected by filtering onto 1.2 u Millipore filters. 0.2 M Tris-HCl buffer (pH 7.2) was used in place of 0.2 M Tris where indicated (pH 7.2). The mitochondrial concentrations in the acid phase were (1) 400; (2) 244; (3) 300; (4) 280 ug N/ml.

different experiments. The large amount of ATP formed during the constant pH treatments was probably due to adenylate Kinase. AMP was included in the acid phase reaction medium in the ratio 8 AMP/1 ADP which inhibited adenylate kinase by 80% (Table IX 2). Antimycin A (2ug/ml) was included in Table IX 1(b) to inhibit oxidation of any endogenous substrate.

2. Ion Uptake during Base-Acid Treatment.

Table IX 3 shows that some magnesium and phosphate was taken up during base-acid treatment of beetroot mitochondria. DNP and treatment throughout at pH 7.2 reduced magnesium and phosphate contents but oligomycin had no effect. The difference between pH change-induced magnesium level and DNP-treated level was small (10 - 20%) and was smaller than substrate-induced magnesium uptake from a medium containing phosphate but no magnesium (Table IV 3). The high concentration (0.2M) of Tris transferred from the alkaline to the acid phase would have inhibited magnesium binding (Table VII 1) to the very low levels in Table IX 3. The 1½ minute incubation in the acid phase should have allowed equilibration of bound magnesium. When the acid phase was at a pH lower than 5.5 - 6.0 bound magnesium was reduced (Table IX 3(3)) probably as a result of replacement of magnesium by H^+ at cation binding sites. The pH differential-induced phosphate uptake was approximately the same as the substrate-induced phosphate uptake from a medium containing magnesium but no added phosphate (Table IV 4). The ratios of pH change-induced magnesium/phosphate uptake were between 1 and 2.

Table IX. 4. Ion Uptake by a Range of pH Differentials.

	mumoles/mg N					
	Mg^{++}	Pi	Mg^{++}	Pi	Mg^{++}	Pi
pH Change	9.5 - 7.3		9.5 - 6.0		9.5 - 5.5	
Control	491	145	640	300	204	142
+ DNP	424	100	429	134	203	139

Mitochondria were shaken for 1 min. with 0.2 M Tris and then transferred to a medium containing 3 mM Tris-phosphate buffer (pH 7.2), 10 mM $MgCl_2$, 0.01% BSA and where indicated 1×10^{-4} M DNP. 0.1 N HCl was added immediately and after 1½ min. shaking the mitochondria were collected by filtering. 420 ug mitochondrial N/ml was present in the acid phase.

Table IX 4 shows the effect of pH differential on ion uptake by one preparation of beetroot mitochondria. A pH change of 3.5 supported more ion uptake than pH changes of 2.2 or 4.0. Magnesium content was again reduced when the pH of the acid phase was 5.5. The mitochondria precipitated when the pH of the acid phase was reduced below 4 - 5 although the pH at which flocculation occurred varied greatly from one mitochondrial preparation to another.

C. DISCUSSION.

ATP formation by pH change across beetroot mitochondrial membranes was 20 - 70 times ~~less~~ more than ATP formed by rat liver mitochondria (Reid et al 1966). A pH change of 3.5 in the absence of any membrane potential should be required to synthesize ATP according to the chemiosmotic hypothesis (Mitchell 1966). ATP formation was induced by a pH differential as low as 2.5 in beetroot mitochondria but membrane potential could have supported this pH differential. Reid et al (1966) included valinomycin with KCl in the alkaline phase to breakdown opposing membrane potential set up by H^+ movement. The more permeable beetroot mitochondria would not require this artificial means of inducing ion movement across the membrane. The ATP formation supported by pH differential in beetroot mitochondria could possibly be increased by using KOH in place of Tris in the alkaline phase and preventing binding or structural changes by this molecule.

Significant amounts of ATP were formed in beetroot mitochondria on base-acid treatment and these were reduced by oligomycin, DNP and incubation at a constant pH of 7.5 or 9.5. However, the changes above the

adenylate kinase-induced ATP formation were very small and the results could possibly be accounted for by changes in adenylate kinase activity under the different conditions. If the results are due to base-acid treatment then they support those of Reid et al (1966) and Jagendorf and Uribe (1966) in suggesting that oxidative and photosynthetic phosphorylation are associated with pH differential and membrane potential set up across the whole or a part of the mitochondrial or chloroplast-membrane. These results support the chemiosmotic hypothesis of oxidative and photosynthetic phosphorylation although the oxido-reduction pathway and ATP-forming system need not be set up and act precisely as proposed by Mitchell (1966). The classical hypothesis of oxidative phosphorylation postulates the existence of an H - containing high-energy intermediate involved in ion uptake (Chance 1965, Chance and Mela 1966) and ATP formation. ATP formation induced by pH differential could probably be accounted for by reactions of this (or a related) compound in spite of the discrepancies between molecules of ATP formed and number of cytochrome molecules (Jagendorf and Uribe (1966).

Magnesium and phosphate uptake by beetroot mitochondria was supported by pH differentials of 2.5 - 5.3. DNP or a constant pH (7.2) reduced the ion levels but oligomycin had no effect. These results support the chemiosmotic scheme for ion uptake which can be supported by PMF (pH differential and membrane potential) produced by substrate oxidation or ATP breakdown. ATP breakdown did not, however, support ion uptake by beetroot mitochondria (Chapter 4). Ion uptake by beetroot

mitochondria supported by base-acid treatment could possibly be increased if the alkaline phase were produced by KOH rather than Tris. Tris greatly reduced magnesium binding and probably reduced pH change-supported magnesium uptake as a result of this.

D. CONCLUSIONS.

Base-acid treatment of beetroot mitochondria supported ATP formation and ion uptake. The action of oligomycin and DNP on ATP formation and ion uptake induced by pH differential supported the chemiosmotic scheme for oxidative phosphorylation and ion uptake.

CHAPTER TEN

ION UPTAKE BY BEETROOT MITOCHONDRIA

Magnesium and phosphate were taken up by beetroot mitochondria by a process dependent on the oxidation of substrate. Substrate-dependent ion uptake was inhibited by inhibitors of electron transfer chain, e.g. cyanide, antimycin A, HOQNO and by uncouplers of oxidative phosphorylation, e.g. DNP, CCP. However, oligomycin, which is a powerful inhibitor of oxidative phosphorylation, did not affect ion uptake. These results were similar to those obtained with animal and other plant tissue mitochondria and indicated that movement of electrons along the respiratory chain was necessary for ion uptake and that this process was closely associated with oxidative phosphorylation but ATP as such was not required. A mechanism to account for these results based on the classical hypothesis of oxidative phosphorylation uses the high-energy intermediates formed during this process for ion uptake. The chemiosmotic hypothesis uses the PMF set up by the oxido-reduction chain for promoting ion uptake. These mechanisms would account for the action of inhibitors, uncouplers and oligomycin on ion uptake.

Conditions allowing both ion uptake and continuous ATP formation produced only ATP formation and no ion uptake, suggesting a competition between these two processes, favouring ATP formation. Oligomycin inhibited ATP formation and allowed ion uptake to proceed. The common intermediates of the classical mechanism would account for competition, and breakdown of these more rapidly by ATP formation than ion uptake

would produce favoured oxidative phosphorylation. Oligomycin by blocking ATP formation would promote ion uptake energised by these intermediates. The PMF set up across the coupling membrane in the chemiosmotic hypothesis would support ATP formation and ion uptake, but if resistance to PMF collapse via the reversible ATPase system was less than via ion uptake, oxidative phosphorylation would be favoured. Oligomycin would block the ATPase system and allow support of ion uptake by the PMF.

ATP did not support magnesium and phosphate uptake by beetroot mitochondria in contrast to results with animal and corn mitochondria. ATP could be expected to support ion uptake by either production of high-energy intermediates or PMF. The ATPase activity of beetroot mitochondria was very low under the conditions for ion uptake and did not produce sufficient "energy" for support of ion uptake in competition with the favoured process of reformation of ATP. Low concentrations of ATP did, however, stimulate substrate-dependent ion uptake and digomycin did not affect this stimulation. BSA and cytochrome c also stimulated ion uptake and these effects were attributed to stabilisation of the membrane structure or of a precipitated cation-phosphate complex. High concentrations of ATP inhibited substrate-dependent magnesium and phosphate uptake and oligomycin did not relieve inhibition. This was related to the chelating properties of ATP and to the effects of cations (Na or Tris) added with the ATP. EDTA also severely inhibited ion uptake.

Calcium and phosphate uptake by beetroot mitochondria occurred under the same conditions as magnesium and phosphate uptake. Calcium competed with magnesium for binding sites and for substrate-dependent uptake with phosphate. Calcium was the favoured cation in both processes and 4-5 times as much calcium was bound and taken up as magnesium. The apparently selective substrate-dependent process could be produced by membrane cation binding capacity, cation permeability and solubility of any divalent cation-phosphate complex, formed within the mitochondria. The ratio of divalent cation/phosphate taken up approached 1.5 and could indicate formation of $M_3(PO_4)_2$ but electron micrographs did not show any deposits within beetroot mitochondria.

ATP was not required for calcium and phosphate uptake by beetroot mitochondria. Massive calcium and phosphate uptake by animal mitochondria required ATP and it was suggested that bound adenine nucleotide stabilised membrane structure or the calcium phosphate complex. Oxidative phosphorylation was uncoupled in animal mitochondria by 2-4 mM calcium whereas high concentrations of calcium and magnesium had little effect on oxidative phosphorylation in beetroot mitochondria. Magnesium and calcium stimulated substrate oxidation at low concentrations and inhibited at high concentrations while ADP/O and P/O ratios were highest in the absence of added magnesium or calcium. At the calcium and magnesium concentrations used for ion uptake studies ADP/O ratios were reduced by less than 20% in beetroot mitochondria.

Substrate oxidation stimulated by low concentrations of calcium and magnesium did not return to an inhibited oxidation rate in the

presence or absence of phosphate in contrast to results reported for animal and corn mitochondria. This result and the low Mg^{++}/O ratios calculated for beetroot mitochondria could be due to greater permeability of beetroot mitochondrial membranes to ions or to delay in precipitation of cation-phosphate complexes within the mitochondria.

After incubation with magnesium but without phosphate Mg^{++}/P_i ratios were approx. 5-10 indicating that magnesium was taken up without phosphate. Investigations with animal mitochondria have suggested that cation uptake is the primary substrate-dependent act in divalent cation and phosphate uptake. Monovalent cations inhibited magnesium and phosphate uptake by beetroot mitochondria more than monovalent anions suggesting that the cations competed for a common substrate-dependent uptake process. Apparent selectivity of the uptake process for particular ions would depend on cation binding, permeability and on formation of insoluble cation-anion complexes within the mitochondria.

Monovalent cations were taken up in a substrate-dependent manner by beetroot mitochondria and uptake was inhibited by DNP but not affected by oligomycin or KCN, indicating that substrate oxidation was not required. Substrate anion uptake and accumulation has been reported in animal mitochondria and monovalent cation uptake could be associated with uptake and retention of succinate in beetroot mitochondria.

Ascorbate supported more magnesium and phosphate uptake in beetroot mitochondria than succinate, and malate supported very little ion uptake. This capacity for ion uptake was proportional to the state 4 (and state 3) rates of oxidation and indirectly proportional to the

ADP/O ratios and respiratory control ratios. Mg^{++}/O ratios for these substrates were 0.4, 0.5 and 0.1 in contrast to those of 2.0, 4.0 and 6.0 calculated for animal mitochondria. State 4 oxidation was dependent on the control of phosphorylation over oxidation. Site 1 (malate) phosphorylation was thus most tightly coupled to oxidation while site 3 was very loosely coupled. Each site, however, phosphorylated ADP as efficiently as any other and the theoretical ADP/O ratios of 3, 2 and 1 were obtained for malate, succinate and ascorbate respectively. The classical hypothesis of oxidative phosphorylation can account for the different state 4 oxidation rates by different breakdown rates of the high-energy intermediates at each site. This uncoupled oxidation was high for ascorbate but on ADP addition switched completely to coupled oxidation. The chemiosmotic hypothesis can account for the state 4 rates by different PMF's built up before respiration was controlled and leakage of H^+ under the influence of these PMF's. The ATP formation pathway on addition of ADP would provide most rapid collapse of PMF. The ion uptake supported by different substrates in beetroot mitochondria can be accounted for by similar mechanisms. The intermediates would have different ion moving capacities (classical) or ions would only be taken up under the influence of the high PMF built up with succinate and ascorbate (chemiosmotic).

The effects of antimycin A and TMPD on ascorbate-supported ion uptake were accounted for by effects on the reaction of intermediates with ions but not with ADP by the classical hypothesis. The chemosmotic mechanism could postulate that these substances increase membrane

permeability to H^+. Thus the resistance to PMF collapse would decrease from H^+ leakage to ion uptake to TMPD-pathway to ATP formation and could account for the stimulation in oxidation, inhibition of ion uptake and lack of effect on ATP formation by TMPD and antimycin A.

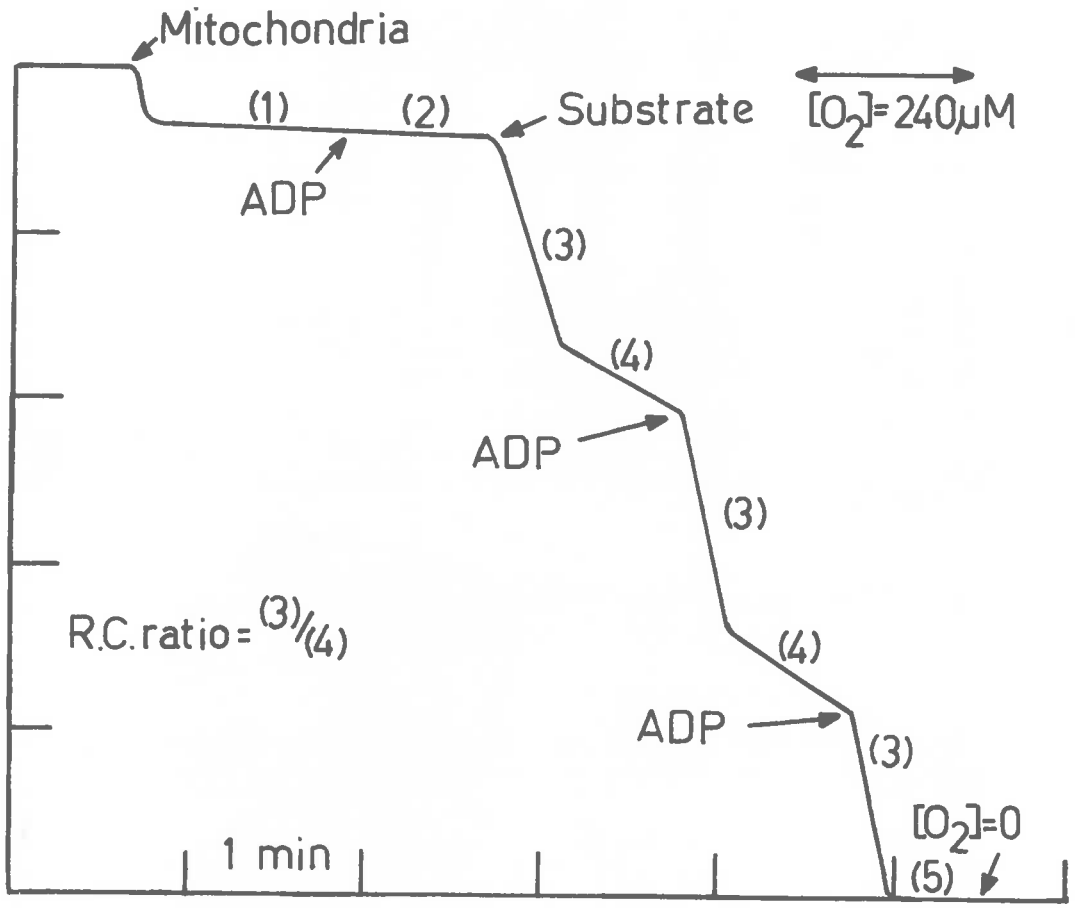
ATP was formed and magnesium and phosphate taken up by beetroot mitochondria subjected to a pH differential by base-acid treatment. These results indicate the importance of pH differential in oxidative phosphorylation and ion uptake mechanisms. They do not conclusively show that H^+ is produced before ATP formation and ion uptake rather than after these processes.

APPENDIX

Table A.1. States of Respiratory Pigments in Mitochondria (from Chance and Williams 1955 p413).

	State 1	State 2	State 3	State 4	State 5
Characteristics	Aerobic	Aerobic	Aerobic	Aerobic	Anaerobic
ADP Level	Low	High	High	Low	High
Substrate Level	Low endogenous	Approaching 0	High	High	High
Respiration Rate	Slow	Slow	Fast	Slow	0
Rate-limiting component	Phosphate Acceptor	Substrate	Respiratory chain	Phosphate acceptor	Oxygen

Fig. A. 1. Hypothetical polarographic tracing of mitochondria oxidizing substrate in a standard medium containing phosphate. Oxidation states (1 - 5) are shown according to the definitions of Chance and Williams (1955) (Table A.1.). Respiratory control (RC) ratios = state 3 rate / state 4 rate.


Mitochondria
(1)
(2)
Substrate
[O2]=240µM
ADP
(3)
(4)
ADP
(3)
R.C.ratio = (3)/(4)
(4)
ADP
(3)
[O2]=0
1 min
(5)

Reprinted from the PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES
Vol. 52, No. 4, pp. 996–1004. October, 1964.

ION UPTAKE BY PLANT MITOCHONDRIA

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Communicated June 30, 1964

Various reports have indicated that plant mitochondria are the sites of ion accumulation.[1, 2] The ability of isolated plant mitochondria to accumulate ions against a concentration gradient has been demonstrated.[2] However, recent reports have shown that divalent metal ions can be accumulated by a respiration-dependent process in mitochondria isolated from both animal[3–6] and plant tissues.[7] This accumulation of divalent metal ions is accompanied by phosphate uptake.[6, 11]

This paper has two objects: (*a*) to present evidence for the accumulation of ions, particularly Mg^{++} and P_i, by mitochondria isolated from red beet (*Beta vulgaris* L.), and (*b*) to discuss hypotheses of the mechanism of ion accumulation in mitochondria.

Methods.—The mitochondria were isolated as previously described.[8] The experimental conditions are specified in the legends to the figures and tables. Constant temperature (25°C) and shaking were maintained by a conventional Warburg apparatus. Aliquots (1.0 ml) were layered over cold 1.0 *M* sucrose and centrifuged at 25,000 × *g* for 5–7 min. The supernatant was removed by suction, and the tubes were carefully rinsed with 1.0 *M* sucrose. The mitochondrial pellet was analyzed for the relevant ions.[9] Oxygen uptake was studied with a Clark oxygen electrode connected to a 1-mv recorder (Varian Associates).

Results.—The oxidative capacity of the isolated mitochondria is shown in Figure 1. Respiratory control was evident and the calculated ADP/O ratios were 1.7 and

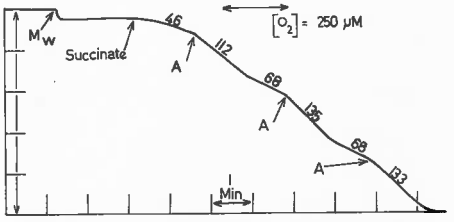

FIG. 1.—Polarographic tracing of oxygen uptake by beet mitochondria—the medium contained 250 mM sucrose, 10 mM Tris-HCl buffer pH 7.2, 10 mM P_i buffer (pH 7.2), 6 mM $MgCl_2$ and 250 μg mitochondrial nitrogen. ADP (0.16 mM) was added at *A*.

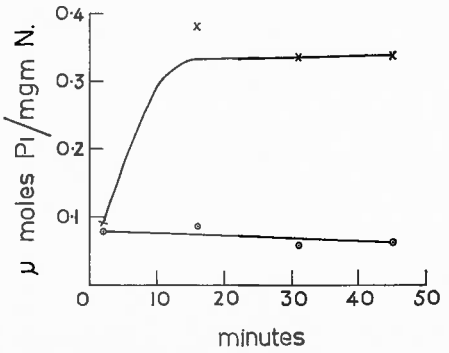

FIG. 2.—P_i accumulation by beet mitochondria incubated in a medium containing 250 mM sucrose, 12 mM Tris-HCl buffer pH 7.2, 3.3 mM P_i (K_2HPO_4/KH_2PO_4 buffer pH 7.2), 5 μM cytochrome *c*, 16 mM $MgCl_2$ with 6 mM sodium succinate (x—x), and without succinate (⊙—⊙).

1.6; the respiratory control ratios were 1.7 and 2.0. Figure 2 shows that the accumulation of P_i was completely dependent on substrate (succinate).

Table 1 shows that the uncouplers 2,4-dinitrophenol (DNP) and carbonyl cyanide *m*-chlorophenyl hydrazone (CCP), as well as the inhibitors of electron transport, antimycin A, 2-heptyl-4-hydroxyquinoline N-oxide (HOQNO), inhibited the accumulation of both P_i and Mg^{++}. Cyanide (10^{-4} *M*) has also been shown to inhibit ion uptake. The percentage inhibition of Mg^{++} uptake was not as great as for P_i uptake. It should be noticed that (*a*) there was a substantial uptake of Mg^{++} in the absence of substrate, and that (*b*) the inhibitors and uncouplers were more effective in preventing Mg^{++} uptake than was the lack of oxidizable substrate. It can be concluded that uptake of these ions was dependent upon the oxidation and coupled phosphorylations of the electron transport chain. The respiration-independent uptake can be considered as a diffusion equilibrium, and thus in all of our studies a correction for the uptake in the absence of substrate has been applied.

Oligomycin, a potent inhibitor of oxidative phosphorylation,[10] stimulated the accumulation of both P_i and Mg^{++} (Table 2). Independent observations in this laboratory have shown that oligomycin will inhibit the state 3 oxidation[11] of isolated beet mitochondria and that the inhibited oxidation can be recovered by uncoupling agents. Since oligomycin prevents ATP formation, it appears that ATP is not directly involved in the uptake of these ions.

TABLE 1

EFFECT OF UNCOUPLERS AND INHIBITORS ON Mg^{++} AND P_i UPTAKE

	Mg^{++}		P_i	
	mμmoles Mg^{++}/mg *N*	% Inhibition	mμmoles P_i/mg *N*	% Inhibition
Control	3,280	—	330	—
No substrate	2,260	31	86	74
Plus antimycin A (1 μg/ml)	1,800	45	92	72
Plus HOQNO (21.5 μM)	2,010	39	124	62
Plus CCP (1.3 μM)	2,010	39	67	80
Plus DNP (1×10^{-4} *M*)	1,910	42	117	65

Mitochondria incubated in medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 10 mM P_i (K_2HPO_4/KH_2PO_4 bufler pH 7.2), 4 μM cytochrome *c*, 15 mM $MgCl_2$, and 11 mM Na succinate for 18 min.

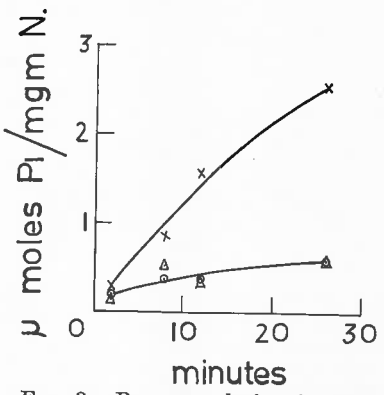

FIG. 3.—P_i accumulation by beet mitochondria incubated in a medium containing 250 mM sucrose, 12 mM Tris-HCl buffer pH 7.2, 3.3 mM P_i (K_2HPO_4/KH_2PO_4 buffer pH 7.2), 5 μM cytochrome *c*, 16 mM $MgCl_2$, x—x control; ⊙—⊙ minus $MgCl_2$, △—△ plus 1 mM ADP, 20 mM glucose, and excess hexokinase.

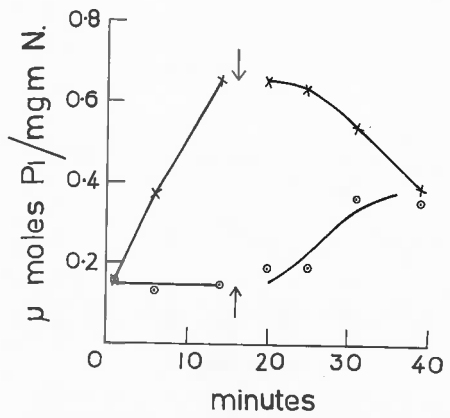

FIG. 4.—The inhibitory effect of ATP formation on P_i uptake. Mitochondria incubated in a medium containing 250 mM sucrose, 12 mM Tris-HCl buffer pH 7.2, 3.3 mM P_i (K_2HPO_4/KH_2PO_4 buffer pH 7.2), 5 μM cytochrome *c*, 16 mM $MgCl_2$, x—x plus 20 mM glucose and excess hexokinase, 1 mM ADP was added at the arrow. ⊙—⊙ plus 1 mM ADP, 20 mM glucose, and excess hexokinase. Oligomycin (2 μg/ml) was added at the arrow.

Under conditions which allowed the continuous formation of ATP (glucose, hexokinase, and ADP present), the accumulation of P_i was completely suppressed (Fig. 3). Similar results have been obtained for the uptake of Mg^{++}. These results are in agreement with the suggestion[3] that P_i and Mg^{++} accumulation compete with ATP formation for the energy-rich intermediates of oxidative phosphorylation. The competition favors ATP formation.

The presence of glucose and hexokinase did not interfere with the accumulation of P_i (Fig. 4). However, when ADP was added to this reaction mixture, P_i accumulation ceased, and the P_i content of the mitochondria began to decrease. A decrease of P_i content has also been observed on applying anaerobic conditions. This loss of P_i differs from the result of Brierley,[12] and suggests that the accumulated P_i can be mobilized. However, the result observed in such an experiment is probably strongly dependent on the morphological intactness of the mitochondria. Figure 4 also shows that, when the formation of ATP prevented P_i accumulation, the addition of oligomycin permitted P_i uptake to proceed.

TABLE 2

THE OXIDATIVE EFFICIENCY OF Mg^{++} AND P_i UPTAKE

	Expt. no.	"O" (mμg atoms/mg *N*)	Mg^{++} (mμmoles/mg *N*)	Mg^{++}/O	P_i (mμmoles/mg *N*)	P_i/O	ADP/O
Control (Na succinate)	1	324	40	0.12	51	0.16	—
	2	286	105	0.37	64	0.22	—
Plus ADP (0.5 mM)	1	316	0	0	23	0.07	1.34
	2	324	0	0	32	0.10	1.25
Plus oligomycin (1 μg/ml)	1	306	130	0.43	158	0.51	—
	2	296	165	0.56	167	0.57	—

Mitochondria incubated in a medium containing 250 mM sucrose, 10 mM Tris-HCl buffer pH 7.2, 10 mM P_i K_2HPO_4/KH_2PO_4 buffer pH 7.2), 5 μM cytochrome *c*, and 8 mM sodium succinate. The results have been corrected or the uptake of ions in the absence of substrate.

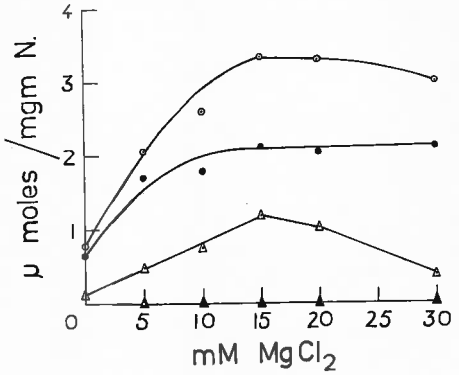

FIG. 5.—The effect of $MgCl_2$ concentration on P_i and Mg^{++} accumulation by beet mitochondria. Incubated for 8 min in a medium containing 250 mM sucrose, 9 mM Tris-HCl buffer pH 7.2, 3.2 mM P_i (K_2HPO_4/KH_2PO_4 buffer pH 7.2), and 4 μM cytochrome *c*. With 11 mM Tris-succinate; ⊙—⊙ Mg^{++} uptake △—△ P_i uptake. Without substrate; ●—● Mg^{++} uptake ▲—▲ P_i uptake.

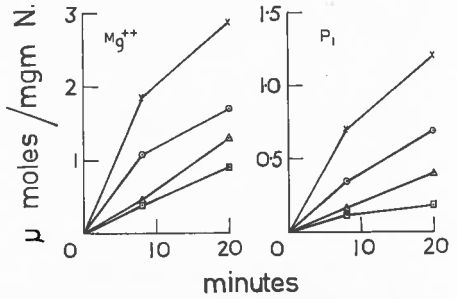

FIG. 6.—The inhibitory effect of sodium and high P_i (K_2HPO_4/KH_2PO_4 buffer) concentration on P_i and Mg^{++} accumulation by beet mitochondria. The medium contained 250 mM sucrose, 9 mM Tris-HCl. x—x with 11 mM Tris-succinate and 3.2 mM P_i; ⊙—⊙ with 11 mM Tris-succinate and 10 mM P_i; △—△ with 11 mM sodium succinate and 3.2 mM P_i; ⊡—⊡ with 11 mM sodium succinate and 10 mM P_i. The results have been corrected for the uptake of ions in the absence of substrate.

The competition between ion uptake and oxidative phosphorylation suggests that common intermediates are involved, but not necessarily the phosphorylated intermediate of oxidative phosphorylation.[3] The results of P_i uptake : $^1/_2O_2$, and Mg^{++} uptake : $^1/_2O_2$ ratios are shown in Table 2.

These ratios, although lower than the ADP/O ratios obtained in the same experiment, are suggestive of an equality between the two processes, i.e., ion accumulation and oxidative phosphorylation. As will be discussed later (Fig. 6), these ratios were not obtained under optimal conditions. Optimal conditions would tend to make the ratios approximate to that reported by Chappell *et al.*,[6] although no respiratory stimulation of the type reported by Chappell *et al.* has been observed. Thus it would appear the ion/O ratios are greater than the expected P/O ratios.

Figure 3 shows that P_i accumulation was dependent on added Mg^{++}. The small amount of P_i accumulated in the absence of added Mg^{++} could have been due to the Mg^{++} present in the mitochondrial suspension. The effect of Mg^{++} concentration on both Mg^{++} and P_i uptake is shown in Figure 5. In this experiment the concentration of P_i was 3.2 mM, and Tris-succinate was used as substrate. The optimum Mg^{++} concentration for ion uptake was 15 mM but P_i uptake appeared to suffer more from a higher Mg^{++} concentration than did Mg^{++} uptake. The effect of other salts in the medium was also investigated.

Figure 6 shows that with a Mg^{++} concentration of 15 mM, 3.2 mM P_i was more favorable than 10 mM P_i for the uptake of both P_i and Mg^{++}. It is also shown in Figure 6 that Tris-succinate was more favorable to Mg^{++} and P_i uptake than sodium succinate. These results are identical with those of Brierley *et al.*[4] The beneficial effect of Tris and of the lower P_i concentration is additive. Thus, it can be concluded that both sodium and high phosphate concentrations interfere with the accumulation of Mg^{++} and P_i. Inhibitions by other salts have been reported.[4, 5]

Discussion.—It has been established that isolated plant mitochondria can accu-

mulate Mg^{++} and P_i ions by a respiration-dependent process. It should be re-emphasized[6] that current evidence provides no proof of active uptake, but only of respiration-dependent uptake. Nevertheless, the mechanism of this uptake may prove to be of fundamental physiological importance.

The results presented here and elsewhere[3-6, 12] show that P_i and Mg^{++} (or some other divalent metal cation) can be accumulated by isolated mitochondria, in a manner dependent on oxidizable substrate and sensitive to respiratory-chain inhibitors or uncouplers of oxidative phosphorylation. Furthermore, ion uptake, which is insensitive to oligomycin, competes unsuccessfully with ATP formation. Thus an intermediate of oxidative phosphorylation is essential in ion uptake, either directly for P_i uptake[3] or indirectly for energy.

Brierley *et al.*[3] have suggested that $Mg_3(PO_4)_2$ precipitates within the mitochondria. The evidence is: (*a*) the Mg^{++}:P_i ratio found within the mitochondria approximates 1.5; (*b*) alkalinity which would precipitate magnesium phosphate increases within the mitochondria; and (*c*) electron micrographs[15] show deposits of what are presumably metal phosphates.

The ratios of accumulated Mg^{++}:accumulated P_i observed in this work approximated to 1.5 and are in accord with the ideas of $Mg_3(PO_4)_2$ precipitation within the mitochondria. However, the precipitation allows the detection of the accumulated ions, and the ratio of 1.5 reflects only the composition of the precipitated compound and in no way reflects the rate of uptake of each ion species. Thus, an ion which is being transported into the mitochondrion but not precipitated may have a rate of efflux such that a build-up of concentration would not be detected.

Unless there is a specific divalent-metal-ion and P_i mechanism, the other ions present must be considered, and thus the results of Figure 6 might be understood. The presence of Tris-succinate instead of sodium succinate might stimulate the uptake of Mg^{++} by markedly reducing the Na^+ concentration which thus offers less competition to the Mg^{++}; this in turn is reflected in P_i uptake. Similarly increasing the P_i concentration from 3.2 mM to 10 mM, which increases the K^+ concentration, might reduce Mg^{++} uptake by competition. Thus, the optimal Mg^{++} concentration would be that which overcame the effects of other cations without having too deleterious an effect on oxidative phosphorylation and the integrity of the mitochondria.[16] Similar deductions cannot be made about H^+, as it appears[4, 6] that there is an exclusion of H^+ from the mitochondria.

The mechanism of ion uptake: In an active transport process it is sufficient to transfer only one of an ion pair.[13, 14] If the cation is transferred by a coupled-respiration process the anion will follow passively. Any anions could participate in this passive movement, but if precipitation of one occurs (as with P_i inside the mitochondria), a concentration gradient favoring that anion would be established. The precipitation of the cation (whose movement is not passive) would not favor its accumulation as the competition with other cations is on the outside.

It is therefore necessary to consider the ion movements due to (1) the respiration-dependent transport, and (2) the effects of precipitation.

Chappell *et al.*[6] have shown that Mn^{++} can be accumulated by rat liver mitochondria in the absence of P_i. This result alone suggests that cation uptake precedes the P_i uptake in the respiration-dependent process, but, further, Chappell

et al.[6] claim that respiration-independent P_i uptake can be induced after Mn^{++} uptake. P_i uptake is therefore secondary to cation uptake and may be of limited value in determining the primary mechanism of respiration-dependent transport. Other evidence supporting cation uptake as the primary act of mitochondrial ion transport is (1) the marked competitive effect of other cations on Mg^{++} uptake as reported here (Fig. 6) and elsewhere,[4] and (2) the secretion of H^+ ions.

Chappell *et al.*[6] and Brierley *et al.*[3] explained the H^+ release in terms of P_i precipitation but did not consider the H^+ release in the absence of P_i.[6] Pressman[17] has shown that the addition of valinomycin to mitochondria (in the absence of P_i) initiated a rapid hydrogen ion release which was substrate-dependent and sensitive to inhibitors and uncouplers. More recently, Moore and Pressman[18] have shown that H^+ release is equivalent to K^+ uptake (when K^+ is the predominant cation in the medium), and under conditions (absence of P_i) where there is no increment of oxygen uptake. They have also shown that addition of P_i is necessary to increase the rate of oxygen uptake. A similar effect was observed by Chance[19] who showed a stimulation of oxygen uptake by Ca^{++} only in the presence of P_i.

The uptake of cations in the absence of phosphate is therefore by exchange for the H^+ released. The necessity of substrate for H^+ release, and the necessity of P_i for O_2 uptake raises the possibility that the substrate is the source of H^+. The release of H^+ could then be the consequence of charge separation in the electron transport system, the H^+ moving out of the mitochondrion leaving a corresponding negative center available to balance the entering cation. Such charge separation would be expected to reach an upper limit which might be the explanation of the transience of the effect observed by Moore and Pressman.[18]

Ion uptake in the absence of phosphate would be a consequence of this charge separation. However, since the electron transport system of mitochondria seems to operate as a one-electron transfer system, the movement of a divalent cation would be accompanied by the movement of a monovalent anion, e.g., Cl^-. The H^+ released would accumulate outside the mitochondria resulting in a decrease of pH. The ratio H^+/cation would be unity for both mono- and divalent cations. However, the mitochondrial resistance to separated charges would not be infinite so that some negative charges would be released. Under these conditions oxygen would be reduced to H_2O and some H^+ ions would no longer contribute to the decrease in pH. Also, the pools of separated charges would be maintained by a steady-state flow of "reducing power" and would not be an equilibrium condition, so that some oxygen uptake would occur, making the system sensitive to inhibitors and uncouplers but not to oligomycin.

Ion uptake by mitochondria, in the presence of phosphate, would occur by the

TABLE 3

INITIAL PHASE OF ION UPTAKE BY MITOCHONDRIA

System	Components	Released	X	Entered	Ion balance	H^+/Ion
A	K Cl	12 H^+	—	12 K^+	12 K^+ 12 e^-	1.0
B	M Cl	12 H^+	—	12 M^{++} 12 Cl^-	12 M^{++} 12 e^- 12 Cl^-	1.0
C	M P	12 H^+	—	12 M^{++} 4 P^- 4 $P^=$	12 M^{++} 12 e^- 4 P^- 4 $P^=$	1.0

X, Mitochondrial membrane. M, Mg. P, Phosphate radical. e^-, Negative center.

TABLE 4

UPTAKE OF IONS BY MITOCHONDRIA IN THE PRESENCE OF PHOSPHATE

Step	Released	X	Entered	Ion balance	Reaction	Ion balance	H^+	M^{++}	H^+/M^{++}
1a	$12\ H^+$	—	$12M^{++}, 4P^-, 4P^{=}$	$12M^{++}, 4P^{=}, 4P^-, 12\ e^-$	$4M_3P_2 + 12H^+$	$12H^+, 12e^-$	12	12	1.0
1b	$12\ e^-$	—	$8P^{=}, 8P^-, 6M^{++}$	$6M^{++}, 12H^+, 8P^{=}, 8P^-$	$2M_3P_2 + 6H^+$	$18H^+, 6P^{=}, 6P^-$	0	18	0
2	$18H^+$	—	$9M^{++}$	$9M^{++}, 6P^{=}, 6P^-$	$3M_3P_2 + 9H^+$	$9H^+, 3P^{=}, 3P^-$	18	27	0.67
3	$9H^+$	—	$\frac{9}{2}M^{++}$	$\frac{9}{2}M^{++}, 3P^{=}, 3P^-$	$\frac{3}{2}M_3P_2 + 9\frac{H^+}{2}$	$\frac{9H^+}{2}, \frac{3P^{=}}{2}, \frac{3P^-}{2}$	27	31.5	0.86
4	$\frac{9H^+}{2}$	—	$\frac{9M^{++}}{4}$	$\frac{9M^{++}}{4}, \frac{3P^{=}}{2}, \frac{3P^-}{2}$	$\frac{3M_3P_2}{4} + \frac{9H^+}{4}$	$\frac{9H^+}{4}, \frac{3P^{=}}{4}, \frac{3P^-}{4}$	31.5	33.75	0.93
5	$\frac{9H^+}{4}$	—	$\frac{9M^{++}}{8}$	$\frac{9M^{++}}{8}, \frac{3P^{=}}{4}, \frac{3P^-}{4}$	$\frac{3M_3P_2}{8} + \frac{9H^+}{8}$	$\frac{9H^+}{8}, \frac{3P^{=}}{8}, \frac{3P^-}{8}$	33.75	34.88	0.97

X, Mitochondrial membrane. M, Mg. P, Phosphate radical. e^-, Negative center.

same mechanism with the following complications: (1) oxygen is consumed more rapidly; (2) with a divalent cation, precipitation occurs; (3) the H^+/Mg^{++} ratio is decreased. The charge separation takes place and substrate-derived H^+ is released, allowing a monovalent cation (or a divalent cation plus a monovalent anion) to enter and balance the corresponding negative center. However, the presence of phosphate allows the negative center to move out and thus allows oxygen reduction[18, 19] resulting in H_2O formation. In such circumstances no H^+ ions derived from substrate would accumulate outside the mitochondria and an extra anion would enter to balance the cation already taken up. If the cation entering is divalent, further H^+ adjustments occur with the precipitation of metal phosphate.[3, 6] Phosphate-derived H^+, accumulating externally, need not be secreted via the electron transport system so that 2 H^+ would exchange for one divalent cation.

This sequence of events in ion transport can now be considered in detail. The uptake of monovalent ions is shown in system A of Table 3. The release of 12 H^+ (taken as an arbitrary figure for all of these considerations) allows the entry of 12 K^+ and yields an H^+/K^+ ratio of 1, with no concomitant oxygen uptake. However, other cations present in the medium may compete with the K^+ so that the observed H^+/K^+ ratio may be higher.[6] Counteracting this effect is the finite limit of charge separation, the leakage of negative centers, and the requirement of energy to maintain the charge separation as discussed already. The reduction of oxygen will recombine the H^+ which otherwise would contribute to a pH change. The electrostatic balance outside the mitochondrion is not shown in Table 3, but the secreted 12 H^+ will be balanced by the 12 Cl^- left by the 12 K^+.

The uptake of divalent cations in the absence of phosphate is shown in system B of Table 3. Here an anion must enter with

Mg^{++} to maintain electrostatic neutrality on exchange for an H^+. The discussion for system A applies to system B.

The initial phases of uptake of divalent cations in the presence of phosphate are shown in system C (Table 3). The existence of two species of phosphate ion complicates the exchange ratios. The further phases of uptake under these conditions are shown in Table 4. In Table 4 the sequential steps are shown as 1–5, and the reactions of each step as occurring in the mitochondria are shown as from left to right. It should be realized that all the reactions shown occur simultaneously and not separately as suggested by Table 4. The precipitation of $Mg_3(PO_4)_2$ yields H^+ which can continue to exchange and so increase the internal Mg^{++} content. This process can be repeated a limited number of times.

This hypothesis explains most results reported to date. (1) The H^+/ion ratio in the absence of phosphate will be unity irrespective of the cation. (2) Little oxygen uptake occurs in the absence of phosphate. (3) Little cation uptake[6, 18] occurs in the absence of phosphate because the system is creating a "battery." (4) In the presence of phosphate, oxygen uptake can occur[18, 19] as the negative centers are released. (5) The Mg^{++}/O can approximate 6,[6, 19] in the presence of phosphate. (6) The H^+/Mg^{++} ratio in the presence of phosphate is approximately 0.9, as reported by Chappell *et al.*,[6] because none of the substrate-derived H^+ is available for a pH change. The competitive effects of other cations, will not be as marked since precipitation of $Mg_3(PO_4)_2$ leads to a deficiency of Mg^{++} in the mitochondria, and Mg^{++} is unavailable for exchange. A similar argument holds for the effects of competing anions. It has been assumed that any ion can compete with the measured ion but some selectivity[18] is likely.

An important feature of this hypothesis is that it establishes the charge separation, with loss of H^+ to the medium, as the essential act in the ion transport system. The reducing energy of the system is sufficient to expel H^+ ions so that the pH of the external medium decreases,[4, 6, 18] while the pH of the mitochondria increases.[4] However, the electrons of the electron-transport chain cannot reduce oxygen unless phosphate is present; in the presence of phosphate (ADP absent or ADP and oligomycin present), the H^+ and the electrons are combined to form water, and the phosphate is simultaneously transported into the mitochondrion. If ADP is present, ATP is formed and the phosphate is not transported. The initial separation of H^+ and e^- thus becomes the basis, as suggested earlier by Mitchell[14] and by Robertson,[13] of both phosphorylation and active transport; an active transport act is an alternative to an oxidative phosphorylation. It is possible that the active transport of all ions depends initially on (1) the H^+/cation exchange and (2) the phosphate transport. Once a small quantity of a soluble phosphate has been accumulated by this process, exchange of phosphate ions for other anions could occur.

We gratefully acknowledge the support of a C.S.I.R.O. senior studentship (D.M.), and the gifts of CCP (Dr. P. G. Heytler, E. I. du Pont de Nemours & Co.,) and of oligomycin (Prof. Elizabeth McCoy, University of Wisconsin).

[1] Jackson, P. C., S. B. Hendricks, and B. M. Vasta, *Plant Physiol.*, **37**, 8 (1962).

[2] Robertson, R. N., M. J. Wilkins, A. B. Hope, and L. Nesztel, *Australian J. Biol. Sci.*, **8**, 164 (1955).

[3] Brierley, G. P., E. Bachmann, and D. E. Green, these PROCEEDINGS, **48**, 1928 (1962).

[4] Brierley, G., E. Murer, E. Bachmann, and D. E. Green, *J. Biol. Chem.*, **238**, 3482 (1963).

[5] Vasington, F. D., *J. Biol. Chem.*, **238**, 1841 (1963).
[6] Chappell, J. B., M. Cohn, and G. D. Greville, *Energy-Linked Functions of Mitochondria* (Academic Press, 1963), p. 219.
[7] Hodges, T. K., and J. B. Hanson, *Plant Physiol.*, (Suppl.), **38**, xxv (1963).
[8] Wiskich, J. T., R. N. Robertson, and R. Morton, *Australian* (*J. Biol. Sci.*, **13**, 109 (1960).
[9] Marsh, B. B., *Biochim. Biophys. Acta*, **321**, 357 (1959); Vogel, A. I., *Quantitative Inorganic Analysis* (London: Longmans, Green and Co., Ltd., 1961), p. 805.
[10] Lardy, H. A., D. Johnson, and W. C. McMurray, *Arch. Biochem. Biophys.*, **78**, 587 (1958).
[11] Chance, B., and G. R. Williams, *Nature*, **176**, 250 (1955).
[12] Brierley, G. P., in *Energy-Linked Functions of Mitochondria* (Academic Press, 1963), p. 237.
[13] Robertson, R. N., *Biol. Rev. Cambridge Phil. Soc.*, **35**, 231 (1960).
[14] Mitchell, P., *Nature*, **191**, 144 (1961).
[15] Lehninger, A. L., C. S. Rossi, and J. Greenawalt, *Biochem. Biophys. Res. Commun.*, **10**, 444 (1963); Brierley, G. P., and D. B. Slautterback, *Biochim. Biophys. Acta*, **82**, 183 (1963).
[16] Purvis, J. L., and E. C. Slater, *Exptl. Cell Res.*, **16**, 109 (1959).
[17] Pressman, B. C., in *Energy-Linked Functions of Mitochondria* (Academic Press, 1963), p. 181.
[18] Moore, C., and B. C. Pressman, *Biochem. Biophys. Res. Commun.*, **15**, 562 (1964).
[19] Chance, B., in *Energy-Linked Functions of Mitochondria* (Academic Press, 1963), p. 253.

Millard, D. L., Wiskich, J. T., & Robertson, R. N. (1965). Ion uptake and phosphorylation in mitochondria: effect of monovalent ions. *Plant Physiology, 40*(6), 1129-1135.

REFERENCES.

Akazawa, T., and Beevers, H. (1957). -- Biochem. J. 67: 115.

Amoore, J.E. (1960). -- Biochem. J: 76, 48.

Amoore, J.E., and Bartley, W. (1958). -- Biochem. J. 69: 223.

Armstrong, W.D. (1952). -- Phosphorus Metabolism, Vol. 2. (Johns Hopkins, Baltimore, p. 698).

Avron (Abramsky), M., and Biale, J.B. (1957). -- J. Biol. Chem., 225: 699.

Azzi, A., and Azzone, G.F. (1966). -- Biochim. Biophys. Acta. 113: 445.

Azzone, G.F., and Ernster, L. (1960). -- Nature 187: 65.

Azzone, G.F., and Ernster, L. (1961a). -- J. Biol. Chem., 236: 1501.

Azzone, G.F., and Ernster, L. (1961b). -- J. Biol. Chem. 236: 1510.

Azzone, G.F., Olofsson, O.E., Ernster, L., Luft, R., and Szabolcsi, G. (1961). -- Exptl. Cell. Res. 22: 415.

Baker, J.E. (1963). -- Arch. Biochem. Biophys. 103: 148.

Baltscheffsky, H. (1957). -- Biochim. Biophys. Acta 25: 382.

Baltscheffsky, H., and Von Stedingk, L.V. (1966). In 'Currents in Photosynthesis.' (Editors Thomas, J.B., and Goedheer,J.C.- Woudschoten - Zeist - The Netherlands).

Bangham, A.D., and Horne, R.W. (1964). J. Mol. Biol. 8: 660.

Bartley, W. (1961). -- Biochem. J. 80: 46.

Bartley, W., and Amoore, J.E. (1958). -- Biochem. J. 69: 348.

Bartley, W., and Davies, R.E. (1952). -- Biochem. J. 52: XX.

Bartley, W., and Davies, R.E. (1954). -- Biochem. J. 57: 37.

Bartley, W., and Enser, M.B. (1964). -- Biochem. J. 93: 322.

Beaudreau, G.S., and Remmert, L.F. (1955). -- Arch, Biochem. Biophys. 55: 469.

Beevers, H. (1961). -- Respiratory Metabolism in Plants (Row Peterson, Evanston, III., 232 pp.).

Beevers, H., and Walker, D.A. (1956). -- Biochem. J. 62: 114.

Bellamy, D., and Bartley, W. (1960). -- Biochem. J. 76: 78.

Bendall, D.S., and de Duve, C. (1960). -- Biochem. J. 74: 444.

Bendall, D.S., and Hill, R. (1956). -- New Phytologist, 55: 206.

Bhagvat, K., and Hill, R. (1951). -- New Phytologist. 50: 112.

Biale, J.B., Young, R.E., Popper, C.S., and Appleman, W.E. (1957). -- Physiol. Plantarum. 10: 48.

Bjorkman, N., and Thorsell, W. (1962). -- Exptl. Cell Res., 27: 342.

Bonner, W.D. Jr., (1961). -- Proc. Intern. Congr. Biochem., 5th, Moscow, 2: 50.

Bonner, W.D. (1959). -- in "Haemitin Enzymes", Symp. Intern. Union. Biochem., Canberra. 1959: 479. (Pergamon, Oxford, 666 pp.)

Bonner, W.D., and Voss, D.O. (1961). -- Nature 191: 682.

Borst, P., and Slater, E.C. (1961). --- Biochim. Biophys. Acta. 48: 362.

Boyer, P.D. (1963). -- Science. 151: 1147.

Boyer, P.D. (1965). -- Proc. Intern. Symp. on Oxidases and Related Oxidation - Reduction Systems, Amherst, 1964, Vol. 2. (Wiley New York, p. 994.)

Brierley, G.P. (1963). -- In "Energy-Linked Functions of Mitochondria" (Ed. Chance, B., Academic Press, New York, p. 237).

Brierley, G.P., Bachmann, E., and Green, D.E. (1962). -- Proc. Natl. Acad. Sci. U.S. 48: 1928.

Brierley, G.P., and Green, D.E. (1965). -- Proc. Natl. Acad. Sci. U.S. 53: 73

Brierley, G.P. and Murer, E. (1964)a. -- Biochem. Biophy. Res. Comm. 14: 437.

Brierley, G.P., Murer, E. and Bachmann, E. (1964b). -- Arch. Biochem. Biophys. 105: 89.

Brierley, G.P., Murer, E., and Green, D.E. (1963). -- Science 140: 60.

Brierley, G.P., Murer, E., and O'Brien, R.L. (1964)a. -- Biochim. Biophys. Acta 88: 645.

Brierley, G.P., and Slautterback, D.B. (1964). -- Biochim. Biophys. Acta 82: 183.

Briggs, G.E., Hope, A.B., and Robertson, R.N. (1961). -- "Electrolytes and Plant Cells" (Blackwell, Oxford).

Briggs, G.E., and Robertson, R.N. (1957). -- Ann. Rev. Plant Physiol. 8: 11.

Brouwer, R. (1965). -- Ann. Rev. Plant Physiol. 16: 241.

Brummond, D.O., and Burris, R.H. (1953). -- Proc. Natl. Acad. Sci. U.S. 39: 754.

Bruni, A. (1966). -- In "regulation of Metabolic Processes in Mitochondria" (Tager, J.M., Papa, S., Quagliariello, E., and Slater, E.C. Editors) (Elsevier Publishing Co., Amsterdam, pp. 275-292).

Bruni, A., and Azzone, G.F. (1964). -- Biochim. Biophys. Acta 93: 462.

Bruni, A., Contessa, R., and Lucinai, S. (1962). -- Biochim. Biophys. Acta 60: 301.

Bruni, A., Lucina, S., and Bortignon, C. (1965). -- Biochim. Biophys. Acta 97: 434.

Burgos, J., and Redfearn, E.R. (1965). -- Biochim. Biophys. Acta 110: 475.

Carafoli, E. (1965). -- Biochim. Biophys. Acta 97: 99.

Carafoli, E. (1965b). -- Biochim. Biophys. Acta 97: 107.

Carafoli, E., and Lehninger, A.L. (1964). -- Biochim. Biophys. Res. Comm. 16: 66.

Carafoli, E., Rossi, C.S., and Lehninger, A.L. (1964). -- J. Biol. Chem. 239: 3055.

Carafoli, E., Rossi, C.S., and Lehninger, A.L. (1965a). -- J. Biol. Chem. 240: 2254.

Carafoli, E., Weiland, S., and Lehninger, A.L. (1965b). -- Biochim. Biophys. Acta 97: 88.

Chance, B. (1952). -- Nature 169: 215.

Chance, B. (1956). -- In "Proceedings of the Third International Congr. of Biochemistry" Brussels 1955. (C. Liebecq Editor, Academic Press, Inc., N.Y. p. 300.

Chance., B. (1958). -- J. Biol. Chem. 233: 1223.

Chance, B. (1963). -- In "Energy-Linked Functions of Mitochondria" (Chance, B. Editor, Academic Press, Inc., N.Y. p. 258)

Chance, B. (1964). -- Fed. Proc. 23; 265.

Chance, B. (1965). -- J. Biol. Chem. 240; 2729.

Chance, B. and Balteschеffsky, M. (1958). -- Biochem. J. 68: 283.

Chance, B,. and Fugmann, U. (1961). -- Biochem. Biophys. Res. Comm. 4: 317.

Chance, B., and Hackett, D.P. (1959). -- Plant Physiol. 34: 33.

Chance, B., and Hollunger, G. (1957). -- Abstracts of the American Chemical Society Meeting, New York, September 1957 p. 43C.

Chance, B., and Hollunger, G. (1963a). -- J. Biol. Chem. 238: 418.

Chance, B., and Hollunger, G. (1963b). -- J. Biol. Chem. 238: 432.

Chance, B., Hollunger, G., and Hagihara, B. (1962). -- Biochem. Biophys. Res. Comm. 8: 180.

Chance, B., Lee, C.P., Oshino, R., and Van Rossum, G.D.V. (1964). -- Am. J. Physiol. 206: 461.

Chance, B., and Mela, L., (1966). -- Nature. 212: 372.

Chance, B., and Parsons, D.F. (1963) -- Science 142: 1176.

Chance, B., Parsons, D.F., and Williams, G.R. (1964). -- Science 143: 136.

Chance, B., and Williams, G.R. (1955a). -- Nature 176: 250.

Chance, B., and Williams, G.R. (1955). -- J. Biol.Chem. 217: 383.

Chance, B., and Williams, G.R. (1956). -- Adv. Enzymol. 17: 65.

Chance, B., and Yoshioka, A.L. (1965). -- Fed. Proc. 24: 425.

Chappell, J.B. (1961). -- IUB - IUBS Symposium on Regulation of Cell Metabolism, Stockholm. (T.W. Goodwin and O. Lindberg Editors). (Academic Press. N.Y.) Vol. II.p. 70-

Chappell, J.B. (1963). -- J. Biol. Chem. 238: 410.

Chappell, J.B. (1964). -- Biochem. J. 90: 225.

Chappell, J.B. (1966). -- In "Regulation of Metabolic Processes in Mitochondria". (Tager, J.M., Papa, S., Quagliariello,E, and Slater, E.C. Editors). (Elsevier Publishing Co., Amsterdam.) pp. 550 and 559.

Chappell, J.B., Cohn, M., and Greville, G.D. (1963). -- In "Energy - linked Functions of Mitochondria" p. 219. (Ed. Chance, Academic Press, New York.)

Chappell, J.B., and Crofts, A.R. (1965a). -- Biochem. J. 95: 393.

Chappell, J.B., and Crofts, A.R. (1965b). -- Biochem. J. 95: 707.

Chappell, J.B., and Crofts, A.R. (1965c). -- Biochem. J. 95: 378.

Chappell, J.B., and Crofts, A.R. (1966). -- In "Regulation of Metabolic Processes in Mitochondria". (Tager, J.M., Papa, S., Quagliariello, E., and Slater, E.C. Editors). (Elsevier Publishing Co., Amsterdam.) pp. 293-316.

Chappell, J.B., and Greville, G.D. (1959). -- Nature 183: 1737.

Chappell, J.B., and Greville, G.D. (1960). -- Biochim. Biophys. Acta 38: 483.

Chappell, J.B., and Greville, G.D. (1961). -- Nature 190: 502.

Chappell, J.B., and Greville, G.D. (1963a). - Symp. Biochem. Soc (Cambridge). 23: 39.

Chappell, J.B., and Greville, G.D. (1963b). - Fed. Proc. 22: 526.

Chappell, J.B., Greville, G.D., and Bicknell, K.E. (1962) -- Biochem. J. 84: 61P.

Childress, C.C., and Stein, H.J. (1965). -- Plant Physiol. 40: 752.

Christie, G.S., Ahmed, K., McSean, A.E.M., and Judah, J.D. (1961). -- Biochim. Biophys. Acta. 45: 121.

Clark, V.M., Hutchinson, D.W., and Todd, A. (1960). -- Nature 187: 59.

Cleland, K.W. (1952). -- Nature 170: 497.

Cohen, M., Ginoza, W., Dorner, R.W., Hudson, W.R. and Wildman, S.G. (1956). -- Science 124: 1081.

Cohn, M. (1953). -- J. Biol. Chem. 201. 735.

Conn, E.E., and Young, L.C.T. (1957). -- J. Biol Chem. 226: 23.

Cooper, C., and Lehninger, A.L. (1956). -- J. Biol Chem. 219:. 489.

Cooper, C., and Lehninger, A.L. (1957). -- J. Biol. Chem. 224: 547.

Crane, F.L. (1957). -- Plant Physiol. 32: 619.

Crane, F.L. (1961). -- Ann. Rev. Plant Physiol. 12: 13.

Crane, F.L., Glenn, J.L., and Green, D.E. (1956). -- Biochim. Biophys. Acta 22:

Crane, R.K1 and Lipmann, F. (1953). - J. Biol Chem. 201: 235.

Crofts, A.R., and Chappell, J.B. (1965). -- Biochem. J. 95: 387.

Dalgarno, L., and Birt, L.M. (1963). -- Biochem. J. 87: 586.

Danielli, J.F. (1942). -- In "Cytology and Cell Physiology." (Ed. G. Bourne, Clarendon Press, Oxford,) Ch. 3, pp. 68-98.

Das, M.L., and Crane, F.L. (1964). -- Biochemistry 3: 696.

David, D.J. (1958). -- Analyst. 83: 655.

David, D.J. (1959). -- Analyst 84: 536.

Davies, R.E., and Ogston, A.G. (1950). - Biochem. J. 46: 324.

Davis. E.J. (1965). -- Biochim. Biophys. Acta 96: 217.

De Luca, H.F., and Engstrom, G.W. (1961). -- Proc. Natl. Acad. Sci. U.S. 47: 1744.

De Luca, H.F., Engstrom, G.W., and Rasmussen, H. (1962). -- Proc. Natl. Acad. Sci. U.S. 48: 1604.

Drahota, Z., and Lehninger, A.L. (1965). -- Biochem. Biophys. Res. Comm. 19: 351.

Drysdale, G.R., and Cohn, M. (1958). -- J. Biol. Chem. 233: 1574.

Ducet, G., and Rosenberg, A.J. (1962). -- Ann. Rev. Plant Physiol. 13: 171.

Ducet, G., Rosenberg, A.J., Vandewale, G., and Andrejew, A. (1958). Compt. Rend. Acad. Sci. 246: 2657.

Engstrom, G.W., and De Luca, H.F. (1964). -- Biochemistry 3: 379.

Epstein, E. (1956). -- Ann. Rev. Plant Physiol. 7: 1.

Ernster, L. (1963). -- Proc. 5th Intern. Congr. Biochem., Moscow, 1961, Vol. 5. p. 115. (MacMillan, New York).

Ernster, L., and Lee, C.P. (1964). -- Ann. Rev. Biochem. 33: 729.

Ernster, L., Low, H., and Lindberg, O. (1955). -- Exptl. Cell. Res. Suppl. 3: 124.

Estabrook, R.W. (1958). -- J. Biol. Chem. 230: 735.

Estabrook, R.W. (1961). -- Biochem. Biophys. Res. Comm. 4: 89.

Estabrook, R.W. (1962). -- Biochim. Biophys. Acta. 60: 236.

Estabrook, R.W., Gonze, J., and Nissley S.P. (1963). -- Fed. Proc. 22: 1071.

Fernandez-Moran, H. (1962). -- Circulation 26: 1039.

Fernandez-Moran, H., Oda, T., Blair, P.V., and Green, D.E. (1964) -- Science 143: 136.

Forti, G. (1957). -- Physiol. Plantarum 10: 898.

Freebairn, H.T., and Remmert, L.F. (1956). -- Plant Physiol. 31: 259.

Freebairn, H.T., and Remmert, L.F. (1957). -- Plant Physiol. 32: 374.

Fritz, G., and Naylor, A.W. (1956). -- Physiol. Plantarum 9: 247.

Furman, N.H., and Low, G.W. (1935). -- J. Am. Chem. Soc. 57: 1585.

Gamble, J.L. Jr. (1962). -- Am. J. Physiol. 203: 886.

Gamble, J.L. Jr. (1963a) -- Biochim. Biophys. Acta 66: 158.

Gamble, J.L. Jr. (1963b). -- Fed. Proc. 22: 213.

Gamble, J.L. Jr. (1965). -- J. Biol. Chem. 240: 2668.

Goddard, D.R., and Bonner, W.D. (1960). -- In "Plant Physiology" I(A), 209-312 (Steward, F.C. Editor) (Academic Press, New York, 331 pp.).

Goddard, D.R., and Stafford, H.A. (1954). -- Ann. Rev. Plant Physiol. 5: 115.

Goodwin, B.C., and Waygood, E.R. (1954). -- Nature 174: 517.

Grabe, B. (1958). -- Biodim. Biophys. Acta 30: 560.

Green, D.E. (1958). -- The Harvey Lectures, Ser. 52: 117.

Green, D.E. (1959). -- Adv. Enzymol. 21: 73.

Green, E.D. (1959b). -- Discussions, Faraday Soc. No. 27: 206.

Green, D.E., Beyer, R.E., Hansen, M., Smith, A.L., and Webster, G. (1963). -- Fed. Proc. 22: 1460.

Green, D.E., and Crane, F.L. (1958). -- Proc. Intern. Symposium on Enzyme Chemistry, Tokyo and Kyoto, 1957: 275.

Green, D.E., and Fleischer, S. (1962). -- In "Horizons in Biochemistry" (Kasha, M., and Pullman, B. Editors) (Academic Press, N.Y.). p. 381.

Green, D.E., and Fleischer, S. (1963). -- Biochim. Biophys. Acta, 70: 554.

Green, D.E., and Wharton, D.C. (1963). -- Biochem. Z. 338: 335.

Greenawalt, J.W., and Carafoli, E. (1966). -- J. Cell Biol. 29: 37.

Greenawalt, J.W., Rossi, C.S., and Lehninger, A.L. (1964). -- J. Cell Biol. 23: 21.

Greengard, P., Minnaert, K., Slater, E.C., and Betel., I. (1959). -- Biochem. J. 73: 637.

Greville, G.D. (1966). -- In "Regulation of Metabolic Processes in Mitochondria" (Tager, J.M., Papa, S., Quagliariello, E., and Slater, E.C. Editors). (Elsevier Publishing Co., Amsterdam). p. 86.

Greville, G.D., Munn, E.A., and Smith, D.S. (1965). -- Proc. Roy. Soc., (London). Ser. B, 161: 403.

Guillory, R.J. and Slater, E.C. (1965). -- Biochim. Biophys. Acta. 105: 221.

Hackett, D.P. (1957). -- J. Exptl. Bot. 8: 157.

Hackett, D.P. (1958). -- Plant Physiol. 33: 8.

Hackett, D.P. (1959). -- Ann. Rev. Plant Physiol. 10: 113.

Hackett, D.P. (1963). -- In "Control Mechanisms in Respiration." (Wright, B. Editor). (Ronald Press, N.Y.) 357 pp.

Hackett, D.P. (1964). -- In "Modern Methods of Plant Analysis" 7: 647. (Linskens, H.F., Sanwal, B.D., and Tracey, M.V. Editors) (Springer, Berlin). 735 pp.

Hackett, D.P., and Haas, D.W. (1958). -- Plant Physiol. 33: 27.

Hackett, D.P., Rice, B., and Schmid, C. (1960). -- J. Biol. Chem. 235: 2140.

Hanson, J.B. (1965). -- In "Genes to Genus, A Biochemical Basis for Tomorrow's Agriculture". (Greer, F.A., and Army, T.J. Editors). (International Minerals and Chemical Corpn. Illinois) p. 65.

Hanson, J.B., Malhotra, S.S., and Stoner, C.D. (1965). -- Plant Physiol. 40: 1033.

Harmon, J.W., and Fiegelson, M. (1952). -- Exptl. Cell Res. 3: 47.

Harris, E.J., Cockrell, R., and Pressman, B.C. (1966). -- Biochem. J. 99; 200.

Hartree, E.F. (1957). -- Adv. Enzymol. 18: 1.

Hatefi, Y. (1959). -- Biochim. Biophys. Acta. 34: 183.

Hatefi, Y., Haavik, A.G., Fowler, L.R., and Griffiths, D.E. (1962). -- J. Biol. Chem. 237. 2661.

Hatefi, Y., Haavik, A.G., and Jurtshuk, P. (1961). -- Biochim. Biophys. Acta 52: 106.

Hatefi, Y., and Lester, R.L. (1958). -- Biochim. Biophys. Acta. 27: 83.

Heldt, H.W., Jacobs, H., and Klingenberg, M. (1965). -- Biochem. Biophys. Res. Comm. 18: 174.

Hemker, H.C. (1962). -- Biochim. Biophys. Acta 63: 46.

Hemker, H.C., and Hulsmann, W.C. (1961). -- Biochim. Biophys. Acta. 48: 221.

Hill, R., and Hartree, E.F. (1953). -- Ann. Rev. Plant Physiol. 4: 115.

Hind, G., and Jagendorf, A.T. (1965). -- J. Biol. Chem. 240: 3195.

Hodges, T.K., and Hanson, J.B. (1965). -- Plant Physiol. 40: 101.

Honda, S.I., and Robertson, R.N. (1956). -- Aust. J. Biol. Sci. 9: 305.

Honda, S.I., Robertson, R.N., and Gregory, J.M. (1958). -- Aust. J. Biol. Sci. 11: 1.

Horne, R.W., and Whittaker, V.P. (1962). -- Z. Zellforsch. Mikroskop. Anat. 58: 1.

Howland, J. L. (1963a). -- Biochim. Biophys. Acta 73: 665.

Howland, J.L. (1963b). -- Biochim. Biophys. Acta 77: 419.

Huijing, F., and Slater, E.C. (1961). J. Biochem. (Tokyo). 49: 493.

Hulme, A.C., and Jones, J.D. (1963). -- In "Enzyme Chemistry of Phenolic Compounds". (Pridham, J.B. Editor). (Pergamon Press, London). p. 73.

Hulme, A.C., and Jones, J.D. (1964). -- Phytochemistry 3: 173.

Hulsmann, W.C., Elliot, W.B., and Slater, E.C. (1960). -- Biochim. Biophys. Acta 39: 267.

Hunter, F. E. (1955). -- In "Methods in Enzymology" (Colowick, S.P. and Kaplan, N. O. Editors, Academic Press Inc., New York). Vol. II, p. 610.

Hunter, F.E. Jr., Levy, J.F., Fink, J., Schutz, B., Guerra, F., and Hurwitz, A. (1959). -- J. Biol. Chem. 234: 2176.

Jackson, P.C., Hendricks, S.B., and Vasta, B.M. (1962). -- Pl. Physiol. 37: 8.

Jackson, F.S., and Lightbown, J.W. (1958). -- Biochem. J. 69: 63.

Jacobs, E.E. (1960). -- Biochem. Biophys. Res. Comm. 3: 536.

Jagendorf, A.T. (1955). -- Plant Physiol. 30: 138.

Jagendorf, A.T. and Hind, G. (1963). -- In "Photosynthesis mechanisms in Green Plants" p. 599. (Kok, B., and Jagendorf, A.T. Editors) (Washington D.C. Nat. Acad. Sci.).

Jagendorf, A.T., and Neumann, J. (1965). -- J. Biol. Chem. 240: 3210.

Jagendorf, A.T., and Uribe, E. (1966). -- Proc. Natl. Acad. Sci. U.S. 55: 170.

James, W.O. (1953). -- Biol Revs. Cambridge Phil. Soc. 28: 245.

James, W.O. (1957). -- Advances in Enzymol. 18: 281.

James, W.O., and Das, V.S.R. (1957). -- New Phytol. 56: 325.

Jennings, D.H. (1963). -- "The Absorption of Solutes by Plant Cells." (Oliver and Boyd, Edinburgh).

Jones, J.D., and Hulme, A.C. (1961). -- Nature 191: 370.

Jones, J.D., Hulme, A.C., and Wooltorton, L.S.C. (1964). -- Phytochemistry 3: 201.

Kaplan, N.O. (1960). -- Advances in Enzymol. 22: 337.

Keilin, D. (1925). -- Proc. Roy. Soc. (Lond.) Ser. B. 98: 312.

Keilin, D., and Hartree, E.F. (1947). --- Biochem. J. 41: 500.

Keilin, D., and King, T.E. (1960). -- Proc. Roy Soc. (Lond.) Ser. B. 152: 163.

King, T.E. (1962). -- Biochim. Biophys. Acta 59: 492.

King, T.E. (1963). -- Nature 198: 366.

Kemp, A., and Slater, E.C. (1964). -- Biochim. Biophys. Acta 92: 178.

Klingenberg, M. (1966). -- In "Regulation of Metabolic Processes in Mitochondria" (Tager, J.M., Papa, S., Quagliariello, E., and Slater, E.C. Editors) (Elsevier Publishing Co., Amsterdam). pp. 535-540.

Kmetec, E., and Newcomb, E.H. (1955). -- Plant Physiol. 30, Suppl. xxxi.

Kolliker, A. (1888). -- Z. Wiss. Zool. 47: 689.

Kornberg, A. (1950). -- J. Biol. Chem. 182:. 805.

Krebs, H.A., and Bellamy, D. (1960). -- Biochem. J. 75: 523.

Kulka, R.G., and Cooper, C. (1962). -- J. Biol. Chem. 237: 936.

Lardy, H.A. (1959). -- In "Manometric Techniques" (Umbreit, W.W., Brussi, R.H., and Stauffer, J.F. Editors). (Burgess Publ. Co. Minneapolis). p. 295.

Lardy, H.A. (1961). -- In "Biological Structure and Function" Vol 2. (Academic Press, New York) p. 265.

Lardy, H.A., and Conelly, J.L. (1961). -- Proc. Intern Congr. Biochem. 5th Moscow. Preprint No. 206.

Lardy, H.A., Connelly, J.L., and Johnson, D. (1964). -- Biochemistry 3: 1961.

Lardy, H.A., and Elvehjem, C.A. (1945). -- Ann. Rev. Biochem. 14: 1.

Lardy, H. A., Johnson, D., and McMurray, W.C. (1958). -- Arch. Biochem. Biophys. 78: 587.

Lardy, H.A., and McMurray, W.C. (1958). -- Fed Proc. 18: 269.

Laties, G.G. (1953). -- Plant Physiol. 28: 557.

Laties, G.G. (1959). -- Ann. Rev. Plant Physiol. 10: 87.

Lee, C.P. (1963). -- Fed. Proc. 22: 2190.

Lee, C.P., Azzone, G.F., and Ernster, L. (1964). -- Nature 201: 152.

Lee, C.P., and Ernster, L. (1966). -- In "Regulation of Metabolic Processes in Mitochondria". (Tager, J.M., Papa, S., Quagliariello, E., and Slater, E.C. Editors). (Elsevier Publishing Co., Amsterdam). p. 218.

Leech, R.M., and Ellis, R.J. (1961). -- Nature 190: 790.

Lehninger, A.L. (1949). -- J. Biol. Chem. 178: 625.

Lehninger, A.L. (1955). -- In "The Harvey Lectures". 1953-54, Ser. 49. p. 176. (Academic Press, New York.).

Lehninger, A.L. (1956). -- In "Enzymes, Units of Biological Structure and Function". (Gaebler, O.H. Editor). (Academic Press, Inc. New York). p. 217.

Lehninger, A.L. (1959). -- Rev. Mod. Phys. 31: 136.

Lehninger, A.L. (1959b). -- J. Biol. Chem. 234: 2465.

Lehninger, A.L. (1962). -- Physiol. Rev. 42: 467.

Lehninger, A.L. (1964). -- "The Mitochondrion" (Benejamin, New York.) 263 pp.

Lehninger, A.L. and Remmert, L.F. (1959). -- J. Biol Chem. 234: 2459.

Lehninger, A.L., Rossi, C.S., and Greenawalt, J.W. (1963). -- Biochem. Biophys. Res. Comm. 10: 444.

Lehninger, A.L., and Wadkins, C.L. (1962). -- Ann. Rev. Biochem. 31: 47.

Lehninger, A.L., Wadkins, C.L., Cooper, C., Devlin, T.M., and Gamble, J.L. Jr. (1958). -- Science 128: 450.

Lehninger, A.L., Wadkins, C.L., and Remmert, L.F. (1959). -- In Ciba Foundation Symposium, "The Regulation of Cell Metabolism" (G.E.W. Wolstenhome and C.M. O'Connor), Churchill, London) p. 130.

Lieberman, M. (1958). -- Science 127: 189.

Lieberman, M. (1960). -- Plant Physiol. 35: 796.

Lieberman, M. (1961). -- In "Recent Advances in Botany" Symp. Intern. Bot. Congr. 9th, Montreal. 1959. Sec. 11: 1168.

Lieberman, M., and Baker, J.E. (1965). -- Ann. Rev. Plant Physiol. 16: 343.

Lieberman, M., and Biale, J.B. (1955). -- Plant Physiol. 30: 549.

Lieberman, M., and Biale, J.B. (1956a). -- Plant Physiol. 31: 420.

Lieberman, M., and Biale, J.B. (1965b). -- Plant Physiol. 31: 425.

Lightbown, J.W., and Jackson, F.L. (1956). -- Biochem. J. 63: 130.

Lindberg, O., and Ernster, L. (1954). -- Nature 173: 1038.

Low, H., Siekevitz, P., Ernster, L., and Lindberg, O. (1958). -- Biochim. Biophys. Acta. 29: 392.

Low, H., Vallin, I., and Alm, B. (1963). -- In "Energy-Linked Functions of Mitochondria" (Chance, B. Editor). (Academic Press, New York). p. 5.

Lund. E.J. (1928). -- J. Exptl. Zool. 51: 327.

Lundegårdh, H. (1945). -- Arkiv. Bot. 32A (12): 1.

Lundegårdh, H. (1954). -- Biochim. Biophys. Acta 35: 340.

Lundegårdh, H. (1955). -- Ann. Rev. Plant Physiol. 6: 1.

Lundegårdh, H. (1958). -- Biochim. Biophys. Acta 27: 355.

Lundegårdh, H. (1960). -- In "Encyclopedia of Plant Physiology" 12 (1): 311-64. (Ruhland, W. Editor), Springer, Berlin). 1121 pp.

Lyons, J.M., Wheaton, T.A., and Pratt, H.K. (1964). -- Plant Physiol. 39:. 262.

McFarlane M.G., and Spencer, A.G. (1953). -- Biochem. J. 54: 569.

McKenzie, H.A., and Wallace, H.S. (1954). - Aust. J. Chem. 7: 55.

McMurray, W., and Begg, R.W. (1959). -- Arch Biochem. Biophys. 84: 546.

Maley, G.F. (1957). -- J. Biol. Chem. 224: 1029.

Maley, G.F., and Johnson, D. (1957). -- Biochim. Biophys. Acta 26: 522.

Marsh, B.B. (1959). -- Biochim. Biophys. Acta 321: 357.

Martin, E.M., and Morton, R.K. (1965a). -- Biochem. J. 62:. 696.

Martin, E.M., and Morton, R.K. (1956). -- Biochem. J. 64: 687.

Martin, E.M., and Morton, R.K. (1957). -- Biochem. J. 65: 404.

Massey, V., and Veeger, C. (1963). -- Ann. Rev. Biochem. 32: 579.

Mazelis, M., and Vennesland, B. (1957). -- Plant Physiol. 32: 591.

Mertz, D., and Levitt, J. (1961). -- Physiol. Plantarum. 14: 57.

Millard, D.L. (1963). -- "Studies in the Phosphate Metabolism of Beetroot Mitochondria". Honours Thesis, University of Adelaide.

Millerd, A., Bonner, J., Axelrod, B., and Bandurski, R.S. (1951). -- Proc. Natl. Acad. Sci. U.S. 37: 855.

Mitchell, P. (1961a). -- Nature,Lond., 191: 144.

Mitchell, P. (1961b). -- Biochem. J. 81: 24P.

Mitchell, P. (1962a). -- Symp. Biochem. Soc. 22: 142.

Mitchell, P. (1962)b. -- J. Gen. Microbiol. 29: 25.

Mitchell, P. (1963). -- In"Cell Interface Reactions" p. 33. (Brown, H.D. Editor). (Scholar's Library, New York.).

Mitchell, P. (1966). -- In "Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation". Research Report No.66/1 Glynn Research Ltd. Publishers, Bodmin.

Mitchell, P. (1966a). -- In "Regulation of Metabolic Processes in Mitochondria". (Tager, J.M., Papa, S., Quagliariello, E., and Slater, E.C. Editors) (Elsevier Publishing Co., Amsterdam) pp. 65 and 551.

Mitchell P, and Moyle, J. (1965a). -- Nature 208: 147.

Mitchell, P., and Moyle, J. (1965b). -- Nature 208: 1205.

Moore, C., and Pressman, B.C. (1964). -- Biochem. Biophys. Res. Comm. 15: 562.

Moor, H., Ruska, C., and Ruska, H. (1964). -- Z. Zellforsch. Microskop. Anat. 62: 581.

Moret, V., Pinamonti, S., and Fornasari, E. (1961). -- Biochim. Biophys. Acta 54: 381.

Morton, R.K. (1958). -- Revs. Pure Appl. Chem. 8: 161.

Myers, D.K., and Slater, E.C. (1957). -- Biochem. J. 67: 572.

Neubert, C., and Lehninger, A.L. (1962). -- Biochim. Biophys. Acta 62: 556.

Neumann, J., and Jagendorf, A.T. (1964a). -- Arch. Biochem. Biophys. 107: 109.

Ogata, E., and Rasmussen, H. (1966). -- Biochemistry 5: 57.

Packer, L., and Jacobs, E.E. (1962). -- Biochim. Biophys. Acta 57: 371.

Packer, L., Marchant, R.H., and Corriden, E. (1963). -- Biochim. Biophys. Acta 78: 214.

Palade, G.E. (1953). -- J. Histochem. and Cytochem. 1: 188.

Pardee, A.B., and Potter, V.R. (1948). -- J. Biol. Chem. 178: 1085.

Parsons, D.F., Bonner, W.D., and Verboon, J.G. (1965). --- Canad. J. Bot. 43: 647.

Pauly, H., and Packer, L. (1960). -- J. Biochem. Biophys. Cytol. 7: 603.

Pauly, H., Packer, L., and Schan, H.P. (1960). -- J. Biochem. Biophys. Cytol. 7: 589.

Peachey. L.D., (1964). -- J. Cell. Biol. 20: 95.

Peters, R.A. (1949). -- Symposia Soc. Exptl. Biol. 3: 36.

Pierpoint, W.S., (1962). -- Biochem. J. 82: 143.

Pierpoint, W.S., (1959). -- Biochem. J. 71: 518.

Pinchot, G.B. (1953). -- J. Biol. Chem. 205: 65.

Pinchot, G.B. (1957). -- J. Biol. Chem. 229: 1.

Potter, V.R., and Reif, A.E. (1952). -- J. Biol. Chem. 194: 287.

Potter, V.R., Siekevitz, P., and Simonson, H.C. (1953). -- J. Biol. Chem. 205: 893.

Pressman, B.C. (1963a). -- In "Energy-Linked Function of Mitochondria." p. 181. (Chance. B., Editor.) New York Academic Press).

Pressman, B.C. (1963b). -- J. Biol. Chem. 238: 401.

Pressman, B.C. (1965). -- Proc. Natl. Acad. Sci., U.S. 53: 1076.

Pressman, B.C., and Park, J.K. (1963). -- Biochem. Biophys. Res. Comm. 11: 182.

Price, C.A., Fonnesu, A., and Davies, R.E. (1956). -- Biochem. J. 64: 754.

Price, C.A., and Thimann, K.V. (1954). -- Plant Physiol. 29: 113.

Pumphrey, A.M., and Redfearn, E.R. (1963). -- Biochim. Biophys. Acta. 74: 317.

Pumphrey. A.M., and Redfearn, E.R. (1962). -- Biochem. Biophys. Res. Comm. 8: 92.

Purvis, J.L., and Slater, E.C. (1959). -- Exptl. Cell Res. 16: 109.

Racker, E. (1961). Adv. Enzymol. 23: 323.

Racker, E. (1964). -- Biochem. Biophys. Res. Comm. 14: 75.

Racker, E. (1965). -- "Mechanisms in Bioenergetics." (New York. Academic Press).

Racker, E., Chance, B., and Parsons, D.F. (1964). -- Fed. Proc. 23: 431.

Racker, E., and Conover, T.E. (1963). -- Fed. Proc. 22: 1088.

Racker, E., and Monroy, G. (1964). -- Proc. Int. Congr. Biochem. 6th, New York. Abstr. X: 760.

Rasmussen, H., Chance, B., and Ogata, E. (1965). -- Proc. Natl. Acad. Sci. U.S. 53: 1069.

Redfearn, E.R., and King, T.E. (1964). -- Nature 202: 1313.

Redfearn, E.R., and Pumphrey, E.R. (1960). -- Biochem. J. 76: 64.

Reed, L.J. (1957). -- Adv. in Enzymol. 18: 319.

Reid, H.B. Jr., Gentile, A.C., and Klein, R.M. (1964). -- Plant. Physiol. 39: 1020.

Reid, R.A., Moyle, J., and Mitchell, P. (1966). -- Nature 212: 257.

Robertson, J.D. (1963). -- In "Regional Neurochemistry: the Regional Chemistry, Physiology and Pharmacology of the Nervous System". p. 497. (Pergamon, Oxford).

Robertson, R.N. (1951). -- Ann. Rev. Plant Physiol. 2: 1.

Robertson, R.N. (1960). -- Biol. Rev. 35: 231.

Robertson, R.N., and Wilkins, M.J. (1948). -- Aust. J. Sci. Res. B. 1: 17.

Robertson, R.N., Wilkins, M.J., and Hope, A.B. (1955). -- Nature. 175: 640.

Robertson, R.N., Wilkins, M.J., Hope, A.B., and Nesztel, L. (1955). Aust. J. Biol. Sci. 8: 164.

Rossi, C.S., and Lehninger, A.L. (1963).a. -- Biochem. Z. 338: 698.

Rossi, C.S., and Lehninger, A.L. (1963b). -- Biochem. Biophys. Res. Comm. 11: 441.

Rossi, C.S., and Lehninger, A.L. (1964). -- J. Biol. Chem. 239: 3971.

Rottenberg. H., and Solomon, A.K. (1965). -- Biochem. Biophys. Res. Comm. 20: 85.

Sallis, J.D., De Luca, H.F., and Martin, D.L. (1965). -- J. Biol. Chem. 240: 2229.

Sallis, J.D., De Luca, H.F., and Rasmussen, H. (1963a). -- Biochem. Biophys. Res. Comm. 10: 266.

Sallis, J.D., De Luca, H.F., and Rasmussen, H. (1963b). -- J. Biol. Chem. 238. 4098.

Sanadi, D.R. (1965). -- Ann. Rev. Biochem. 34: 21.

Sarris, N.E. (1963). -- Acta Chem. Scand. 17: 882.

Schafer, G. (1964). -- Biochim. Biophys. Acta 93: 279.

Schneider, W.C. (1959). -- Advan. Enzymol. 21: 1.

Siekevitz, P., Low, H., Ernster, L., and Lindberg, O. (1958). -- Biochim. Biophys. Acta 29: 378.

Siekevitz, P., and Potter, V.R. (1955). -- J. Biol. Chem. 215: 221.

Siekevitz, P., and Watson, M.L. (1957). -- Biochim. Biophys. Acta. 25: 274.

Sjostrand, F.S. (1953). -- Nature 171: 30.

Slater, E.C. (1953). -- Nature 172: 975.

Slater, E.C. (1957). -- In "Mitochondria and Other Cytoplasmic Inclusions" 110. (Soc. Expt. Biol. Symp. No. 10). (Cambridge).

Slater, E.C. (1958a). -- Advan. Enzymol. 20: 147.

Slater, E.C. (1958b). -- Rev. Pure Appl. Chem. 8: 221.

Slater, E.C. (1961). -- Proc. Intern. Congr. Biochem. 5th Moscow. 5: 325.

Slater, E.C. (1966). -- In "Regulation of Metabolic Processes in Mitochondria." (Tager, J.M., Papa, S., Quagliariello, E, and Slater, E.C. Editors). (Elsevier Publishing Co., Amsterdam). p. 166.

Slater, E.C., and Cleland, K.W. (1952). Nature 170: 118.

Slater, E.C., and Cleland, K.W. (1953). -- Biochem. J. 53: 557.

Smillie, R.M. (1955). -- Aust. J. Biol. Sci, 8: 186.

Smith, D.S. (1963). -- J. Cell Biol. 19: 115.

Smith, L., and Chance, B. (1958). -- Ann. Rev. Plant Physiol. 9: 449.

Snoswell, A.M. (1962). -- Biochim. Biophys. Acta 60: 143.

Solomon, A.K. (1964). -- Abstract of I.O.P.A.B. Reunion. Internationale de Biophysique. No. B 11-3.

Stahy, J.T., and Crane, F.L. (1964). -- J. Cell Biol. 22: 49.

Steward, F.C., and Sutcliffe, J.E. (1961). -- Plant Physiology, II. p. 253. (Steward, F.C. Editor.). (Academic Press, New York).

Strickland, R.G. (1960). -- Biochem. J. 77: 636.

Stiehler, R.D., and Flexner, L.B. (1938). -- J. Biol. Chem. 126: 603.

Stoekenius, W. (1963). -- J. Cell Biol. 17: 443.

Stoner, C.D., and Hanson, J.B. (1966). -- Plant Physiol. 41: 255.

Tager, J.M. (1954). -- Physiol. Plantarum 7: 625.

Tager, J.M., Veldsema - Currie, R.D., and Slater, E.C. (1966) -- Nature. 212: 376.

Tamaoki, T., Hildebrandt, A.C., Burris, R.H., Riker, A.J., and Hagihara, B. (1960). -- Plant Physiol. 35: 942.

Tapley. D.F. (1965). -- J. Biol. Chem. 222. 325.

Tappel, A.L., and Zalkin, H. (1959). -- Arch. Biochem. Biophys. 80: 326.

Tedeschi, H., (1959). -- J. Biochem. Biophys. Cytol. 6: 241.

Tedeschi, H., and Harris, D. (1955). -- Arch. Biochem. Biophys. 58: 52.

Ter Welle, H.F. (1966). -- In "Regulation of Metabolic Processes. in Mitochondria." (Tager, J.M., Papa, S., Quagliariellio, E., and Slater, E.C. Editors). (Elsevier Publishing Co., Amsterdam.) p. 553.

Ter Welle, H.F., and Slater, E.C. (1964). -- Biochim. Biophys. Acta. 89: 385.

Theorell, H. (1941). -- J. Am. Chem. Soc. 63: 1820.

Thomas, R.S., and Greenawalt, J.W. (1964). -- J. Appl. Physics 35: 3083.

Throneberry, G.D. (1961). -- Plant Physiol. 36: 302.

Tyler, D.D., Estabrook. R.W., and Sanadi., D.R. (1965). -- Biochem. Biophys. Res. Comm. 18: 264.

Tyler, D.D., Estabrook. R.W., and Sanadi. D.R. (1966). -- Arch. Biochem. Biophys. 114: 239.

Ulrich, F. (1959). -- Am. J. Physiol. 197: 997.

Urry, D.W., and Eyring, H. (1963). -- Proc. Natl. Acad. Sci. U.S. 49: 253.

Vasington, F.D. (1963). -- J. Biol. Chem. 238: 1841.

Vasington, F.D., and Greenawalt, J.W. (1964). -- Biochem. Biophys. Res. Comm. 15: 133.

Vasington, F.D., and Murphy, J.V. (1961). -- Fed. Proc. 20: 146.

Vasington, F.D., and Murphy, J.V. (1962). -- J. Biol. Chem. 237: 2670.

Veeger, C. (1965). -- Inaugural Lecture as Professor of Biochemistry At the Agriculture High School, Wageningen. Feb.25. 1965.

Vignais, P.V., Vignais, P.M., and Stanilas, E. (1961). -- Biochim. Biophys. Acta 51: 394.

Vignais, P.V., Vignais, P.M., and Stanilas, E. (1962). -- Biochim. Biophys. Acta 60: 284.

Vilkas, M., and Lederer, E. (1962). -- Experimentia 18: 546.

Vogel, A.I. (1961a). -- "Quantitative Inorganic Analysis", p. 805. (Longmans, London).

Vogel. A.I. (1961b). -- "Quantitative Inorganic Analysis" pp. 434, 436. (Longmans, London).

Vogel, A.I. (1961c). -- "Quantitative Inorganic Analysis" p. 808. (Longmans, London).

Wadkins, C.L. (1964). -- Proc. Intern. Congr. Biochem. 6th., New Abstr. X: 762.

Wadkins, C.L., and Lehninger, A.L. (1959). -- J. Biol. Chem. 234: 681.

Wadkins, C.L., and Lehninger, A.L. (1963). -- Fed. Proc. 22: 1092.

Walker, D.A., and Beevers, H. (1956). -- Biochem. J. 62: 120.

Weinbach, E.C., and Garbus, J. (1964). -- Science 145: 824.

Weinbach, E.C., and Von Brand, T. (1965). -- Biochem. Biophys.Res. Comm. 19: 133.

Whittaker, V.P. (1963). -- In "Methods of Separation of Subcellular Components". (University Press, Cambridge). p. 109.

Whittaker, V.P., and Horne, R.W. (1962). -- Abstr. 5th Intern. Congr. For Electron Microscopy.Vol. II Philadelphia. Academic Press, New York). p. P-1.

Whittaker, V.P. (1966). -- In "Regulation of Metabolic Processes in Mitochondria". (Tager, J.M., Papa, S., Quagliariello, E., and Slater, E.C. Editors). (Elsevier Publishing Co., Amsterdam). pp. 1-27.

Williams, G.R., and Parsons, D.F. (1964). -- Abstr. 6th Intern. Congr. Biochem. New York, p. 670.

Willis, J.B. (1960).- Spectrochim. Acta 16: 259.

Wiskich, J.T. (1966). -- Nature 212: 641.

Wiskich, J.T.,and Bonner, W.D. (1963). -- Plant Physiol. 38: 594.

Wiskich, J.T., Morton, R.K., and Robertson, R.N. (1960) --- Aust. J. Biol. Sci. 13: 109.

Wiskich, J.T., Young, R.E., and Biale, J.B. (1964). -- Plant Physiol. 39: 312.

Wojtczak, L., and Lehninger, A.L. (1961). -- Biochim. Biophys. Acta 51: 442.

Yocum, C.S., and Hackett, D.P. (1957). -- Plant Physiol. 32: 186.

Zelitch, I. (1964). -- Ann. Rev. Plant Physiol. 15: 121.