ROLE OF THE MITOCHONDRIAL ADENYLATE KINASE AND BIVALENT CATIONS IN THE REGULATION OF ADENINE NUCLEOTIDE METABOLISM

By

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DECLARATION

This Thesis is my original work and has not been presented for a degree in any other University.
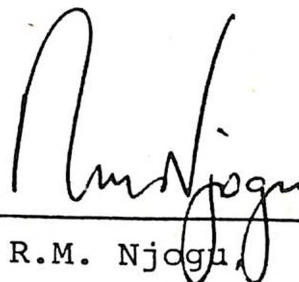
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LIST OF CONTENTS

CHAPTER II

LIST OF FIGURES

PAGE

LIST OF TABLES

ABBREVIATIONS

ADP	Adenosine 5' - diphosphate
AMP	Adenosine 5' - monophosphate
ATP	Adenosine 5' - Triphosphate
ATPase	Adenosine triphosphatase
AMP-PNP	Adenylyl imidophosphate
ANS	1-anilino-8-napthalenesulfonic acid
AP^{5}A	P^{1}P^5- di (adenosine - 5') pentaphosphate
CAT	Carboxyatractylate
CCCP	Carbonyl cyanide m-chlorophenyl hydrazone
CMP	Cytosine 5' - monophosphate
CTP	Cytosine 5' - triphosphate
DCCD	N N' - dicyclohexyl carbodiimide
DNA	Deoxyribose nucleic acid
EDTA	Ethylene diamine tetra acetate
GMP	Guanosine 5' - monophosphate
GTP	Guanosine 5' - triphosphate
ITP	Inosine 5' - triphosphate
NAD$^+$	Oxidized nicotinamide - adenine dinucleotide
NADH	Reduced nicotinamide - adenine dinucleotide
NADP$^+$	Oxidized nicotinamide - adenine dinucleotide phosphate
NEM	N-ethylene malemaide
Pi	Inorganic phosphate
PPi	Inorganic pyrophosphate

RNA   Ribose nucleic acid

TEA   Triethanolamine

Tris   Tri (hydroxymethyl) amino ethane

UTP   Uridine 5' - triphosphate.

SUMMARY OF THE THESIS

Introduction

The purpose of this study is to characterize further, the manner in which the mitochondrial enzyme, adenylate kinase and the mitochondrial bivalent cation transport interact functionally with the oxidative phosphorylation machinery of the cell in the regulation of adenine nucleotide metabolism.

The work has been carried out in two parts.

PART ONE

Various parameters of the mitochondrial adenylate kinase have been investigated, with an aim of understanding further how its interaction with intra - and extramitochondrial adenine nucleotides together with the process of oxidative phosphorylation may participate in regulating the metabolic interplay between the cytosol and the mitochondria, with special emphasis on adenine nucleotide metabolism. Most of the work has been carried out with intact rat liver mitochondria, which allowed a study of the functional relationship between the adenylate kinase (located in the mitochondrial outer compartment) and the oxidative phosphorylation machinery of the mitochondria on one hand, and that between adenylate kinase and the

extramitochondrial environment.

The results show that both the direction and velocity of the adenylate kinase reaction are influenced by the relative concentrations of the adenine nucleotides in its environment. This in turn has a strong influence on the rate of oxidative phosphorylation.

With a high concentration of ATP and AMP in the extramitochondrial environment the forward reaction of the adenylate kinase is strongly stimulated. On the other hand, with a high concentration of ADP, the reverse reaction is strongly stimulated.

In the presence of a respiratory substrate and inorganic phosphate, AMP and ADP stimulate both the adenylate kinase reaction and oxidative phosphorylation while ATP has no effect on either of the reactions.

With AMP, oxidative phosphorylation is stimulated, with a respiratory control ratio similar to that for ADP. This effect is due to the presence of small amounts of ATP in the mitochondrial outer compartment which react with AMP forming ADP under the catalysis of the adenylate kinase, with the ADP so formed initiating oxidative phosphorylation. A comparison of the amount of ATP formed, with AMP as the substrate

for oxidative phosphorylation to that formed with ADP

as the substrate showed that much more ATP is formed

with ADP as the substrate. This is due to the fact

that most of the ATP formed with AMP as the substrate

is used to phosphorylate more AMP to ADP via the

adenylate kinase, as shown in scheme 1 (a)

Scheme 1 (a)

Adenylate   Oxidative
kinase   phosphorylation

AMP ⟶ 2ADP ⟶ 2ATP

        2Pi

ATP
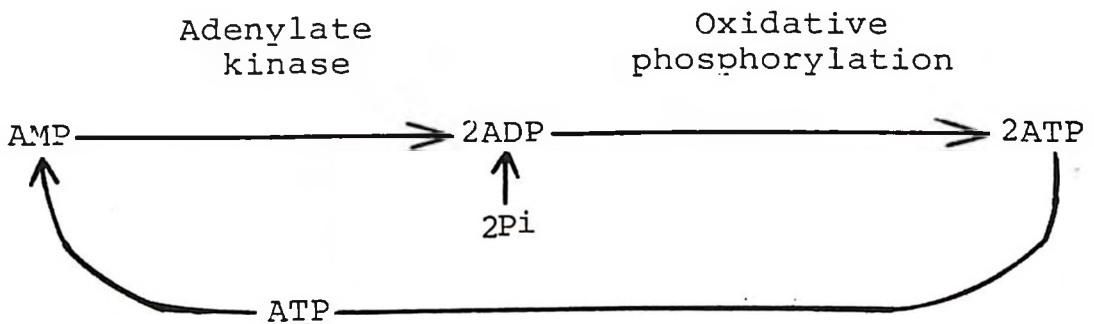
With ADP, ATP is formed by both the adenylate

kinase reaction and oxidative phosphorylation, the

relative amounts formed by either of the reactions

depending on the concentration of ADP. At low concen-

trations of ADP much more ATP is formed by oxidative

phosphorylation than by the adenylate kinase reaction,

while the reverse happens with high concentrations of

ADP. This was shown to be due to the fact that

oxidative phosphorylation has a lower K_m value for ADP,

than the adenylate, while the adenylate kinase reaction

has a much higher V_{max}.   Scheme 1 (b)

Adenylate kinase

ADP $\longrightarrow$ ATP + AMP

ADP

Pi

Oxidative
phosphorylation

ATP
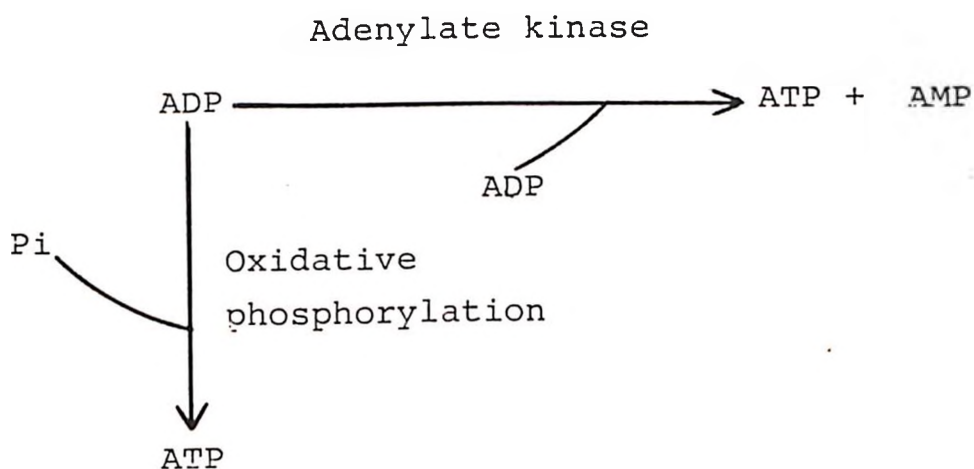
PART TWO

The effect of various bivalent cations on ATP synthesis by intact rat brain mitochondria, and ATP hydrolysis by submitochondrial particles was tested.

The results showed that rat brain mitochondria were capable of supporting a respiratory dependent uptake of Ca^{2+}, Mn^{2+} and Ba^{2+}.   Uptake of the cations took precedence over oxidative phosphorylation with no ATP being synthetized during the uptake process. Accumulation of large amounts of either Ca^{2+} or Ba^{2+} led to a complete inhibition of ATP synthesis, while accumulation of Mn^{2+} had no effect on oxidative

phosphorylation and could even reverse the inhibitory effects of Ca^{2+} and Ba^{2+}.

Experiments carried out with beef heart Mg ATP submitochondrial particles showed that bivalent cations had a strong influence on the ATPase activity of the particles.

In the presence of ADP and either Mn^{2+} or Mg^{2+} the ATPase activity of the particles was strongly stimulated. This effect was more pronounced in the presence than in the absence of a respiratory substrate. A similar effect was also observed with the purified H^{+}- ATPase, but this was independent of the presence or absence of a respiratory substrate. ADP and Ca^{2+} had no effect on the ATPase activity of either the sub-mitochondrial particles or the purified ATPase. The stimulation observed with ADP and either Mn^{2+} or Mg^{2+} is most probably due to the ADP bivalent cations binding to a regulatory site on the inhibitor free ATPase molecules, altering its conformation to a more reactive state. That the H^{+}- ATPase inhibitor may be involved in this stimulation is shown by the fact that, the stimulation is more pronounced in the presence of a respiratory substrate, which is known to cause a release of the ATPase - inhibitor from its inhibitory site (Gomez - Puyou, et al., 1979)

Conclusions

The results described above show that both the mitochondrial enzyme, adenylate kinase and bivalent cations play a significant role in the regulation of adenine nucleotide metabolism.

Adenylate kinase acts by monitoring the adenylate charge of the cell (Atkinson, 1968) and responds by altering both the direction and velocity of its reaction, an effect that in turn has a strong influence on oxidative phosphorylation.

Bivalent cations regulate the metabolism of adenine nucleotide metabolism by (i) Competing with oxidative phosphorylation for the electrochemical gradient during the uptake process ; (ii) By competing with intramitochondrial Mg^{2+} for binding to intramitochondrial adenine nucleotides to form bivalent cation-nucleotide complexes, whose effects will depend on the suitability of the complex to act as a substrate for the H^{+}-ATPase and to act as a modulator for the enzyme.

C H A P T E R   I

INTRODUCTION

(i)   BACKGROUND

The most outstanding feature of the mitochondrion
is its intrinsic ability to perform integrated
enzymatic activities. The most important of these is
the oxidation of respiratory substrates, and transport
electrons, to molecular oxygen, coupled to oxidative
phosphorylation with concomitant synthesis of ATP for
use by the rest of the cell. Other activities include,
β-oxidation of fatty acids, part of the urea cycle,
protein biosynthesis, nucleic acid synthesis, and the
transport of cations and anions. The mitochondrion
contains all the necessary enzymes, coenzymes and
cofactors for these activities.

Morphologically, the mitochondrion consists of
two compartments, delineated by two membrane systems
(Palade 1956). The outer membrane and the intermembrane
space forms the outer compartment, while the inner
membrane and the matrix form the inner compartment.
The activities that take place in the two compartments
also vary, a situation that is reflected in the
intramitochondrial localization of the various enzymes.

The enzymes located in the outer membrane represent a rather heterogeneous group, from a functional point of view. These include monoamine oxidase (Cotzias and Dole 1951), Kynurenine hydroxylase (Okamoto, et al., 1967), NAD^+ - specific xylitol dehydrogenase (Arsenis et al., 1968), a rotenone insensetive NADH - cytochrome c reductase (de Duve et al., 1955, Ernster, 1956), an ATP-dependent fatty acyl-CoA synthetase which is specific for long chain fatty acids (Norum et al., 1966) and a hexokinase binding protein (Rose and Warms, 1967, Linden, et al., 1982). The outer membrane is freely permeable to several kinds of low molecular weight substances upto an approximate molecular weight of 10,000 (Werkheiser and Brierley, 1957, O'Brien and Brierley 1965). This non-specific permeability is due to the presence of aqueous pores with a diameter of 20 - 30 $\overset{o}{A}$ in the outer membrane (Parsons et al., 1966). Zalman, et al., (1980) have reported the isolation of a partially purified 30,000 molecular weight component from the outer membrane of mung bean mitochondria which produced non-specific diffusion channels across a phospholipid bilayer with an exclusion limit of 10,000. A similar protein has also been isolated from the outer membrane of rat liver mitochondria by Linden et. al., (1982) who showed it to be the same protein as the hexokinase binding protein.

The intermembrane space contains the phospho-
transferases, adenylate kinase, nucleoside monophospho-
kinase, nucleoside diphosphokinase (Brdiczka et al.,
1968, Shnaitman and Greenawalt 1968) and xylitol
dehydrogenase (Arsenis et al., 1968).

The inner membrane is the site of the respira-
tory chain enzymes (Levy et al., 1967, Parson et al.,
1966) and the enzyme system responsible for oxidative
phosphorylation (Racker, et al., 1965). The nicoti-
namide nucleotide transhydrogenase (Danielson and
Ernster, 1963) and β-hydroxybutyrate dehydrogenase
(Norum, et al., 1966) are firmly associated to the
inner membrane. The inner membrane has a strict
orientation of its constituent enzymes as illustrated
by the fact that succinate (Harris et al., 1967) and
NADH (Lehninger, 1951) interacts with the respiratory
chain from the inside (matrix) surface of the inner
membrane, whereas cytochrome c appears to be accessible
preferentially from the outside surface (Lenaz and
McLennan, 1966). The inner membrane is only freely
permeable to uncharged low molecular weight substances
of upto 150, such as ammonia, glycerol and carbon
dioxide (Klingengberg 1963; Chappell and Hearhoff
1967, Chappell 1968). However the membrane contains
specific translocators for various charged molecules
of physiological importance. These include the

translocators for phosphate (arsenate), malate, succinate, α-ketoglutarate, citrate, isocitrate, cisaconitase, glutamate, aspartate and the atracylate sensitive ATP-ADP carrier (Chappell, 1968) as well as that for carnitine esters of fatty acids (Norum et al., 1966). The inner membrane also contains the binding sites for various divalent cations, involved in their energy linked translocation across the inner mitochondrial membrane (Brierley et al., 1964), as well as those for different ionophoric antibiotics such as valinomycin and gramicidin that facilitate the transport of univalent cations (Lehninger et al., 1967).

The matrix contains the enzymes responsible for the citric acid cycle (except succinate dehydrogenase) and related processes such as substrate level phosphorylation (Brdiczka, et al., 1968, Parsons et al., 1967) pyruvate and phosphoenol pyruvate carboxylase reactions (. Pfaff, et al., 1968), glutamate transamination and citrulline synthesis (Shnaitman and Greenawalt, 1968), the GTP and ATP dependent fatty acyl CoA synthetase reactions, which are specific for medium and short chain fatty acids (Aas and Bremer, 1968) and fatty acid oxidation (Beatie, 1968, Brdiczka et al., 1968). The mitochondrial DNA (Naas and Naas 1963) the mitochondrial RNA, and the protein synthetising

systems are located in the matrix (Beatie, et al., 1967).

Despite the apparently wide variety of events that take place in the mitochondrion, most of its activities are ultimately aimed at the provision of energy in the form of ATP to the cell.

(ii) METABOLISM OF ADENINE NUCLEOTIDES

The adenine nucleotides play a major role in cellular energy conservation,with ATP being the major energy source for most biological processes. A relatively large amount of energy is stored in each of the high energy phosphate bonds of ATP, and can be released on hydrolysis of the bonds (see section ii, b).

(ii) a. Synthesis of ATP

The major bulk of ATP is synthesised by the mitochondrial H^+ - ATPase (E.C.3.6.1.3.),also referred to as the ATP synthetase, which is located in the inner mitochondrial membrane (Racker, et al., 1965) The enzyme catalyzes the reaction :-

$$ATP + H_2O \rightleftharpoons ADP + Pi$$
$$\Delta G^O = -7.3 \text{ Kcal/mol.}$$
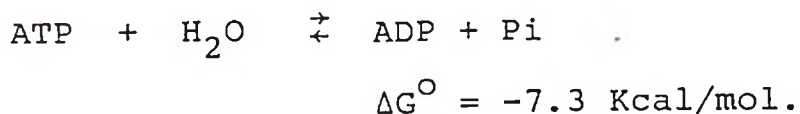
The forward reaction occurs with loss of energy and is spontaneous. It is this energy that is available for driving the various energy dependent reactions in the cell. However, the reverse reaction i.e. ATP synthesis, which actually predominates under intramitochondrial conditions requires an input of energy as described below.

The H^+-ATPases are found throughout the phylogenetic scale:- in mitochondria of eukoryotic cells and in both aerobic and anaerobic bacteria. The structure of the mitochondrial H^+- ATPase (Figure 1) is very similar to that of the chloroplast and bacterial H^+- ATPases (Futai and Kanazawa, 1980) but distinct from other ATP hydrolysing ion pumps such as the $Na^+ - K^+$ ATPase of the eukaryotic plasma membrane and the Ca^{2+}- ATPase of the sarcoplasmic reticulum as well as the actomyosin ATPase.

The H^+- ATPase consist of 3 morphologically distinguishable parts. An extrinsic hydrophilic part called the F_1, which forms projections on the inner mitochondrial membrane and which contains the centre for catalytic activity (Pullman, et al., 1960) and an

FIGURE 1

A model for the organisation of the subunits in the mitochondrial H^+- ATPase, depicting the α_3 β_3 γ δ and ε structure. The dotted lines through F_O indicate the proton channel.

FIG. 1
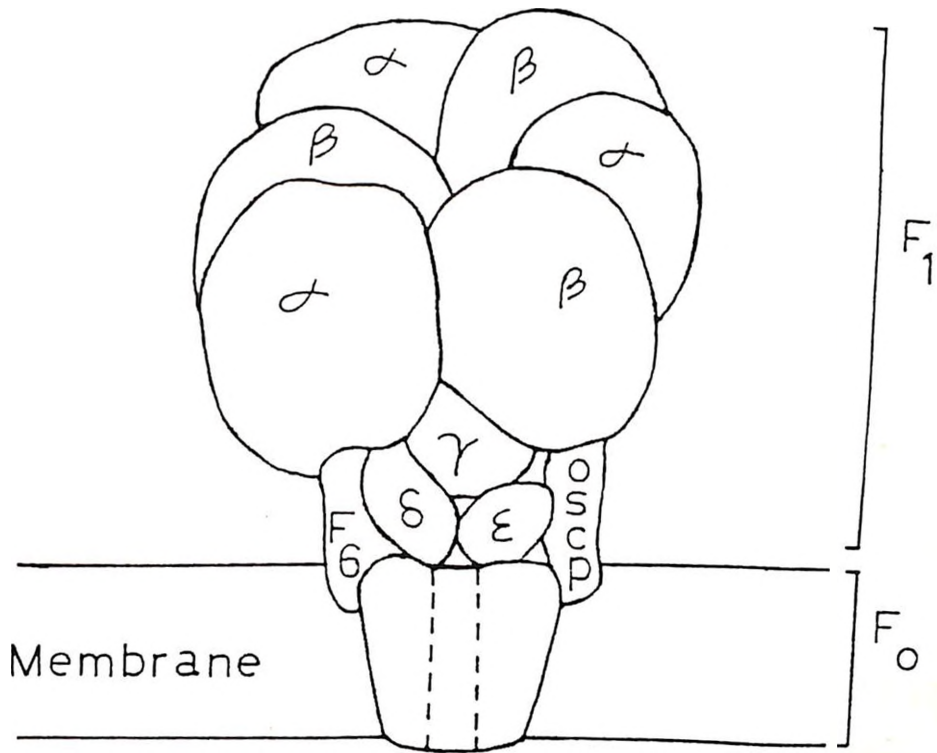
extrinsic hydrophobic part called F_O embedded in the membrane and containing the proton translocating sector of the enzyme (Mitchell, 1976; Kagawa, 1978). The two parts, F_1 and F_O are connected by a third part referred to as the stalk.

The F_1 part of the mitochondrial H^+ - ATPase contains 5 major types of polypeptides in sodium dodecyl sulfate polyacrylamide gel electrophoresis systems. The are most commonly designated as α, β, γ, δ and ϵ in order of decreasing molecular weight. The stoichiometry of the F_1 subunits is still a controversy, with two schools of thought, one favouring a completely dimeric structure, α_2, β_2, γ_2, δ_2 and ϵ_2 and another that favour the α_3, β_3, γ, δ and ϵ. Most of the evidence (Semer and Brooks, 1971, Caterall et al., 1973; Pedersen, 1975) centres on the latter structure. For ATP hydrolysis, the β-subunit has been found to contain the catalytic centre (Penefsky, 1979; Nelson, 1981; Fillingame, 1981). The β-subunit alone is not an active ATPase as evidenced by reconstitution experiments with bacterial F_1 (Yoshinda et al., 1977) and immunochemical studies on chloroplast F_1 (Nelson et al., 1973) which show that the interaction between the α and the β- subunits and perhaps the γ -subunits is necessary for catalytic activity. Nucleotide binding has been shown to be restricted to the α and

the β-subunits of the enzyme (Lunardi et al., 1977, Vagenvood et al., 1977). The γ-subunit (Nelson and Karny 1976; Younis et al., 1976) and the δ-subunit (Dunn and Heppel, 1980) are required for binding the enzymatically active parts i.e. α and β subunits to the F_O.

The F_O consists of a DCCD binding protein, a peptide called the oligomycin sensitivity conferring protein, an uncoupler binding protein, an -SH containing peptide called factor B and a small protein called F_6. There also may be a 28,000 dalton protein (Alfonzo and Racker, 1979). The F_O has the characteristic property of inducing an increase in the proton conductivity when incorporated into liposomes and this can be prevented by binding of oligomycin, DCCD or F_1 (Glaser et al., 1977; 1980; Okamoto et al., 1977; Schipakin et al., 1976). The mechanism by which F_O directs protons to the F_1 part of the enzyme is still unclear and may involve diffusion through an aqueous channel, "H^+- hopping", proton tunneling or simple H^+-binding followed by a conformational change (Pedersen et al., 1981).

The F_1 part of the molecule has rather specific binding sites for ADP and inorganic phosphate and is capable of utilizing an electrochemical gradient, formed by the unidirectional flow of electrons along

the inner membrane as the driving force for
ATP synthesis using ADP and inorganic phosphate. The
mechanism by which this process occurs is still
unclear but several theories have been put forward.
According to the Mitchell's chemiosmotic theory
(Mitchell, 1979) proton conducted through F_o interact
directly with the active site of F_1 where Pi is
activated to "Pi^+" and simultaneously attacked by
ADP to form ATP. Alternatively, Boyer (1974) and
Slater (1974) have proposed that proton conduction
through F_o indirectly alters the conformation of F_1
so as to release bound ATP, the synthesis of the latter
from bound ADP and Pi taking place at the expense of
the energy stored in the "energised" conformation
state of the enzyme. Later Boyer and his associates
(Kayalar et al., 1977) extended this hypothesis to
involve catalytic cooperativity between identical
subunits of F_1 whereby binding of ADP and Pi to one
subunit is accompanied by release of ATP from another
subunit.

(ii) b. Utilization of ATP

Most of the cellular energy requirement is
derived from the hydrolysis of ATP. About 7300 calories
per mole is stored in each of the two pyrophosphate
bonds of ATP (Scheme 2), and can be released by

SCHEME II

Structure of adenosine triphosphate (ATP)

A - refers to the point of cleavage leading to formation of adenosine diphosphate (ADP)

B - refers to the point of cleavage leading to the formation of adenosine monophosphate (AMP).

Scheme II
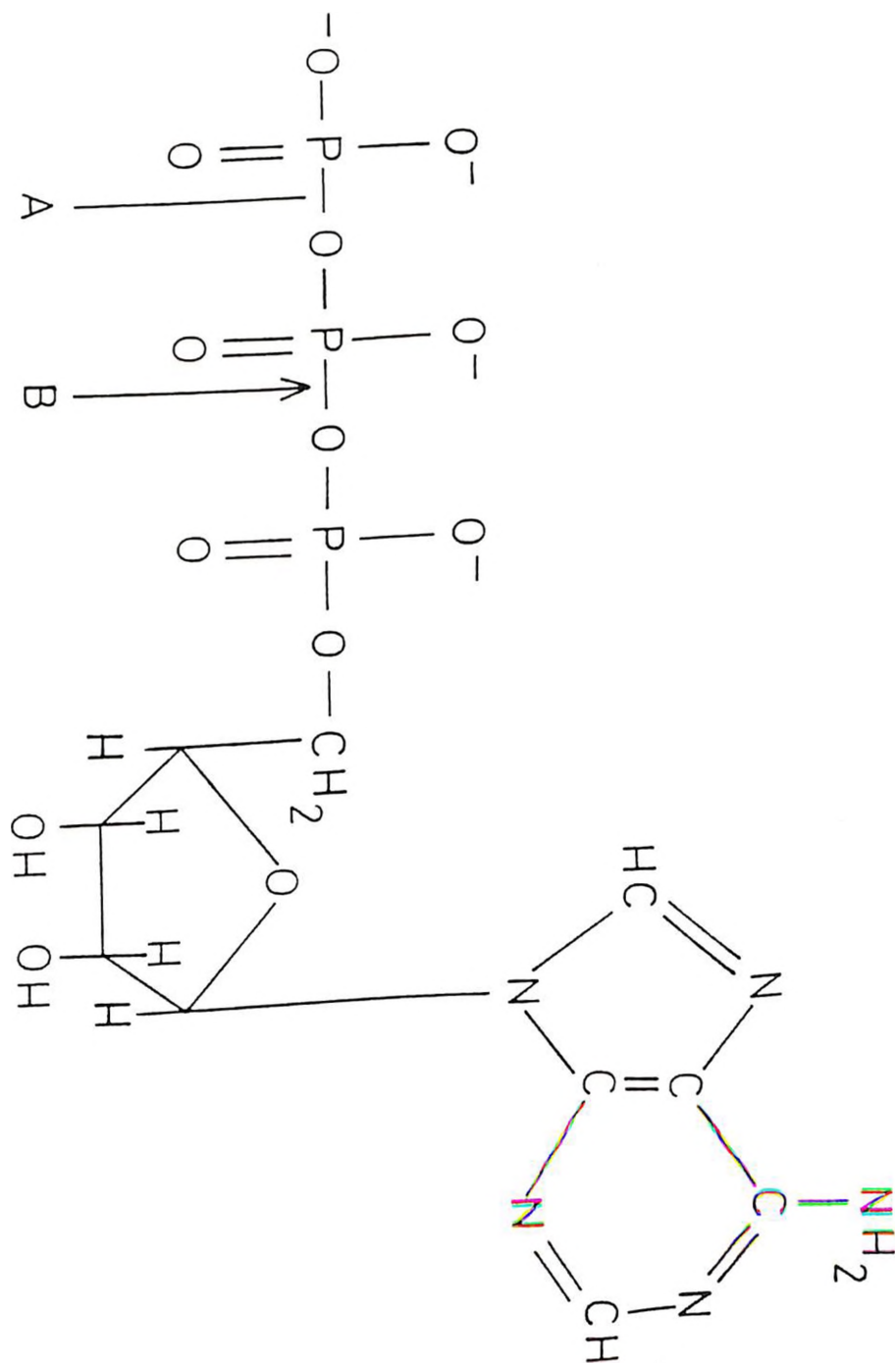
splitting of these bonds. After ATP is formed by mitochondrial H^+- ATPase (ATP-synthetase) it may either be used in the mitochondria or translocated to the cytosol through the ATP-ADP exchange carrier. In the mitochondria ATP may be hydrolysed by the H^+- ATPase with subsequent generation of an electrochemical gradient which can be used to drive processes like metabolite transport, NADH - $NADP^+$ transhydrogenase, reverse electron flow or cation transport (Pedersen et al., 1981). It may also be used to drive other mitochondrial processes like fatty acyl CoA synthesis. However, most of the ATP is translocated to the cytosol where most of the energy requiring processes occur.

Some of the energy requiring processes break down ATP to ADP and Pi (see Scheme II)

Eq. 1

$$ATP + H_2O \longrightarrow ADP + Pi$$

$$\Delta G^O = -7.3 \text{ Kcal/mol}$$
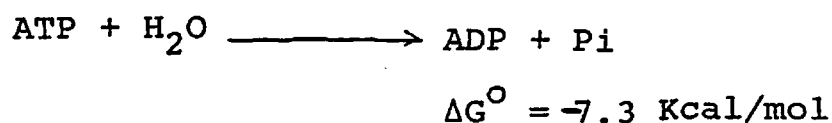
Other energy requiring processes break down ATP to AMP and inorganic pyrophosphate (PPi)

Eq. 2

$$ATP \longrightarrow AMP + PPi$$

$$\Delta G^O = -7.7 \text{ Kcal/mol}.$$
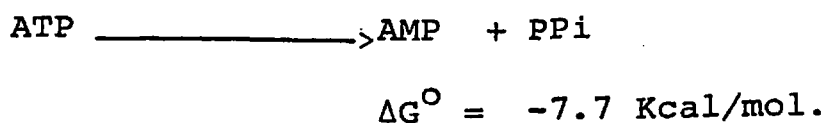
The PPi is subsequently hydrolyzed by pyrophos-
phatase to yield two molecules of inorganic phosphate

Eq. 3

$$PPi + H_2O \longrightarrow 2Pi$$

$$\Delta G^O = -6.9 \ Kcal/mol.$$

The overal reaction

Eq. 4

$$ATP + 2 H_2O \longrightarrow AMP + 2Pi$$

$$\Delta G^O = -14.6 \ Kcal/mol.$$
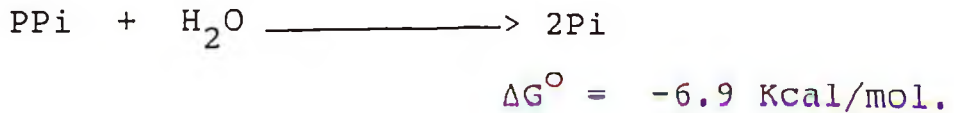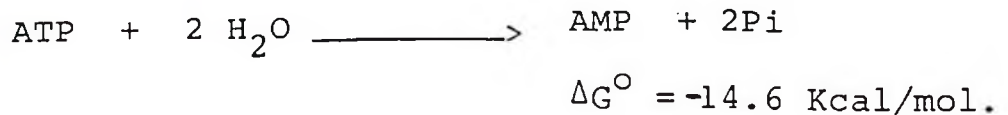
Most of the ADP arising from the hydrolysis of
ATP goes back to the mitochondria where it is
phosphorylated to ATP, while some of it is phosphory-
lated in the cytosol by the substrate level reactions
of glycolysis or by the adenylate kinase, which
catalyzes the reaction between 2 molecules of ADP to
form one molecule of ATP and one of AMP. AMP formed
either by the hydrolysis of ATP or by the adenylate
kinase reaction cannot permeate the inner mitochond-
rial membrane, and has to be converted to ADP by the
adenylate kinase reaction, at the expense of
mitochondrial ATP. This flux through the adenine
nucleotide system is rapid and very variable (Atkinson
and Champman, 1979). Thus the relative concentrations
of ATP, ADP and AMP may be expected to fluctuate widely.
Actually the concentration ratios of the adenine

nucleotides are remarkably constant. Several
regulatory factors are responsible for the maintenance
of this near constancy of the adenine nucleotide
concentration ratios as described below.

(iii) <u>REGULATION OF ADENINE NUCLEOTIDE METABOLISM</u>

Most of the cellular ATP is synthesised in the
mitochondria, while the major part of it is utilized in
the cytosol. Like other physiological metabolites,
the metabolism of adenine nucleotides has to be finely
regulated to ensure efficient conservation of cellular
energy. Unlike most other metabolites, the products
of ATP hydrolysis, ADP, AMP and Pi, are the precursors
of ATP. This then requires that the metabolism of
adenine nucleotides be regulated at various levels
including the level of ATP synthesis, transport of
ATP and ADP between the mitochondria and the cytosol
and the recycling of ADP, AMP, the precursors of ATP.
This multiplicity of levels of regulation calls for
a multiplicity of factors involved in the regulation
of adenine nucleotide metabolism. The factors
believed to be involved in the regulation of adenine
nucleotide metabolism include:-

a) The enzyme adenylate kinase
b) The ATP-ADP translocator
c) The ATPase inhibitor peptide

d) Mitochondrial bivalent cation transport

(iii)a <u>The Enzyme Adenylate Kinase E.C. 2.7.</u>
<u>4.3.</u>

As mentioned in the preceding section, ADP and AMP are the precursors of ATP. ADP is directly phosphorylated either by substrate level phosphorylation, or by the ATPase to ATP. AMP can neither be directly phosphorylated to ATP, nor can it permeate the mitochondrial membrane and has to be converted to ADP first. A small amount of AMP is formed in the mitochondrial matrix by such processes as short chain fatty acyl CoA synthesis, and this AMP is phosphorylated to ADP by the mitochondrial GTP - AMP phosphotransferase (Chiga et al., 1961). Most AMP is however formed in the cytosol. This AMP has to be converted to ADP before it can permeate the inner mitochondrial membrane, for phosphorylation to ATP. This is done by the enzyme adenylate kinase at the expense of mitochondrial ATP.

Adenylate kinase commonly referred to as myokinase and designated by the enzyme commission as ATP - AMP phosphotransferase catalyzes the reaction

<u>Eq. 5</u> $ATP + AMP \rightleftharpoons 2ADP$
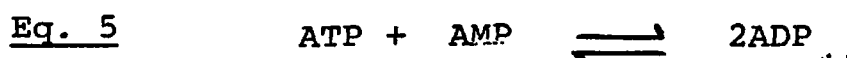
There are two major types of isoenzymes of adenylate kinase:- a "muscle type" of the enzyme, which is mainly found in skeletal muscle, erythrocytes and brain, and a "liver type" which is mainly found in liver, kidney, spleen and heart (Khoo and Russel, 1972). The two isoenzymes have different subcellular localization. The muscle type is located in the cytosol, while the liver type is located in the mitochondrial outer compartment. The isoenzymes are believed to serve different physiological functions, with the muscle type, serving mainly to regenerate ATP from ADP, thus making ADP available as an energy source for, e.g. muscle contraction, while the liver type which has a relatively high affinity for AMP, serves mainly to regenerate ADP from AMP making the latter available for oxidative phosphorylation (Noda, 1973). The two isoenzymes can be identified on the basis of sulfhydryl reactivity and antibody inhibition studies (Khoo and Russel, 1972, Tamura et al., 1980).

Occurrence

Adenylate kinase is an ubiquitous enzyme (Noda 1973) found in many tissues of the mammalian body, plants and other lower forms of life. The enzyme has been purified from a variety of mammalian tissues

including rabbit muscle (Noda and Kuby, 1975), porcine liver mitochondria (Chiga and Platt, 1960), carp muscle (Itakura et al., 1978) bovine liver mitochondria (Noda et al., 1975; Markland and Wadkins, 1966), rat liver mitochondria (Criss et al., 1975, Sapico et al., 1972), rat hepatomas (Criss et al., 1974), rat brain (Pandhan and Criss, 1976), human erythrocytes (Thurme et al., 1972) and from bakers yeast (Chiu et al., 1967; Su and Russel, 1967).

The enzyme has also been shown to be present in lemon leaves (Noort and Wallace, 1967) in wheat (Bomsel and Pladet, 1967) and in E.coli (Peterson et al., 1964).

Nucleotide Specificity

The nucleotide specificity varies depending on the source of the enzyme. However the adenine nucleotides have the lowest K_m values and are the natural substrates. Base substitutions as well as substitition of hydrogen for hydroxyl in the 2'- ribose lead in general to decreased activity. Several adenylate kinases are able to utilize ATP, 2' - dATP, CTP, GTP, UTP and ITP as the phosphate donor, while others only utilize ATP or dATP (with a higher K_m) (Noda 1973)

The enzyme seems to be more specific for AMP.
Rat liver enzymes are only capable of utilizing
5' - AMP as the phosphate acceptor (Criss et al., 1970;
Sapico et al., 1972). Infact, only with the yeast
enzyme has d-AMP been reported to be active (Chiu et al.,
1967; Su and Russel, 1968).

Since the early investigations on the enzyme,
adenylate kinase has been believed to contain two
substrate binding sites (Noda 1962) one for the
magnesium-nucleotide complex, and one for the
uncomplexed nucleotide. This suggestion has been
verified further by Rhoads and Lowenstain, (1968),
using isotope exchange rates at equilibrium and more
recently by Hamada et al., (1979) using fluorescent-
quenching technique and UV-spectral methods for the
study of substrate binding. It is nowadays believed
that the two substrate sites are distinct and specific,
one for the uncomplexed AMP or ADP and the other for
MgATP or MgADP. Thus, the forward reaction may be
presented as one between the magnesium complex of
ATP and uncomplexed AMP, and the reverse reaction
as that between the magnesium complex of ADP and
uncomplexed ADP (Hamada et al., 1979)

$$MgATP + AMP \rightleftharpoons MgADP + ADP.$$
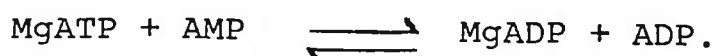
The K_m ATP and K_m ADP values for both rat liver and rat muscle enzymes has been reported to be the same (Tamura et al., 1980), while the K_m AMP value for the liver enzyme is about one-fifth that of the muscle enzyme. Several authors have reported inhibition of the enzyme by high concentration of AMP (Tamura et al., 1980; Font and Gautheron, 1980; Rhoads and Lowenstain 1968). This inhibition has been thought to be due to the fact that, at high concentration of AMP, a substantial amount of it will complex with Mg^{2+}, which then competes with MgATP for the ATP binding site (Font and Gautheron 1980).

Metal Requirements

Adenylate kinase catalyzes the transfer of a phosphate group between a bivalent cation - ATP complex and AMP in the forward reaction and between a bivalent cation - ADP complex and ADP in the reverse reaction.

bivalent cation - ATP + AMP $\rightleftarrows$ bivalent cation-ADP+ADP
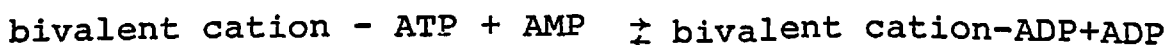
The enzyme can utilize various bivalent cations, but the order of reactivity varies from one enzyme source to another. For the rat muscle enzyme Mg>, Mn^{2+}> Ba^{2+} (O'Sulliv n and Noda, 1968), yeast enzyme Mg^{2+}> Ca^{2+}> Mn^{2+}> Ba^{2+} (Su and Russel, 1967) and

bovine liver enzyme $Mg^{2+} > Mn^{2+} > Ca^{2+} > Co^{2+}$

(Markland and Wadkins, 1966).

Despite the individual differences it is seen that Mg^{2+} has the highest activity followed by Ca^{2+}, Mn^{2+}, Ba^{2+} and Co^{2+}. Sometimes Ba^{2+} and Co^{2+} only shows a fraction of the activity given by the most reactive metal. The metal ion is bound to the di- or triphosphate to give a metal nucleotide complex.

Kinetic and NMR studies have established that there are two substrate sites per enzyme active site, a site for binding the nucleotide monophosphate and another for binding a metal nucleotide di- or triphosphate (Noda, 1973).

Physical Properties

Most adenylate kinases have molecular weights of between 21,000 and 23,000. This is true for human, pig and rabbit muscle enzymes, as well as rat liver mitochondrial enzymes (Noda 1973). At high protein concentrations, the rat liver enzyme is known to exist as a dimer or trimer with molecular weights of 46,000 and 68,000 respectively (Criss et al., 1970). At low protein concentrations it occurs as a monomer, with a molecular weight of 23,000, with no alteration of activity. The yeast enzyme has the highest

molecular weights of adenylate kinases prepared so far

(Su and Russel, 1967). Beef heart mitochondrial enzyme

has a molecular weight of 31,000 while the correspond-

ing cytosolic enzyme has a molecular weight of

21,000 (Tomasseli and Noda, 1980).

The isoelectric point (pI) value of the rabbit

muscle is at pH 6.1, rat muscle enzyme, 7.0 and rat

liver enzyme at 8.1.

The rat muscle enzyme (Noda, 1958) and the rat

liver enzymes have a broad pH optimum of 6.0 - 9.0.

Beef heart mitochondrial enzyme has optimum activity

at pH 5.8 in the forward and pH 8.0 in the reverse

reaction.

Metabolic Properties

The "liver type" of the enzyme is very sensitive

to alterations in the metabolic conditions while

the "muscle type" of the enzyme is less sensitive

(Criss, 1970). Adelman et al., (1968) found that the

activity of the rat liver enzyme is influenced

by diets as well as by hormones. On fasting for 48

hours, the level of adenylate kinase activity increased

from 135 - 375 units per gram of liver tissue, and on

refeeding a high glucose diet the enzymatic activity

in 16 hours was 40 units per gram of liver tissue.
With fed alloxan diabetic rats, the adenylate kinase
activity was as high as in the starving level and
this was reduced to normal levels by the administration
of insulin.   Criss (1970) showed that it is mainly the
mitochondrial enzyme that was responsive to diets and
hormones.   In adrenalectomized rats (Adelman et al.,
1968), liver adenylate kinase activity increased but not
as much as in fasting rats.   Criss et al., (1970) and
Criss (1970) showed that the mitochondrial adenylate
kinase activity decreased with increased differentia-
tion of tumours, and was found to be high in tissues
which had high respiratory rates, and low in tissues
which had low respiratory rates (Criss 1971).   The
activity of the isoenzyme was also found to be low
in hypothyroid rats (Muchiri and Nelson, 1982).

(iii)b.   The ATP-ADP Translocator

In the aerobic eukaryotic cell, most of the
ATP consumed in the cytosol is synthesised in the
mitochondrial inner compartment, which is separated
from the cytosol by both the outer and inner
mitochondrial membranes.   The outer membrane is
freely permeable to adenine nucleotides via the pore
protein (Parsons et al., 1966; Zalman, et al., 1980),
whereas the inner membrane is impermeable to adenine

nucleotides. During oxidative phosphorylation ATP and ADP' have to shuttle between the two compartments. This requires that there be a transport system capable of transporting adenine nucleotides across the inner mitochondrial membrane.

Upto 1965, transphosphorylation reactions i.e. a direct coupling between intramitochondrial ATP and extramitochondrial ADP, whereby a phosphate group is transferred from intramitochondrial ATP to the extramitochondrial ADP, or a direct access of ADP to the ATPase were predicted (Brierly and Green, 1965). Later on, with improved methods of measuring adenine nucleotide transport in mitochondria (Klingengberg, 1970, 1976) a specific adenine nucleotide carrier was discovered. This discovery was later reinforced by the finding, that cellular adenine nucleotides exist in two distinct pools; an intramitochondrial pool, that is in direct contact with the system that phosphorylates ADP, and a cytosolic pool (Klingengberg 1979).

Properties of the ATP/ADP Carrier

The ATP-ADP transport is the most active transport system in many eukaryotic cells, and forms the most vital link in the compartmentation of

metabolism (Klingengberg 1979). It is also the most abundant protein in mitochondria, and the most abundant membrane protein in many eukaryotic cells.

The ATP-ADP carrier operates a 1:1 exchange between cytosolic and mitochondrial nucleotides. The transport system has a very high specificity for ATP and ADP and does not tolerate any structural aberrations in the base, ribose and phosphate sections of the molecule (Pfaff and Klingengberg 1968). Neither AMP, nor other nucleotides can be transported by the carrier. AMP formed in the cytosol has to be converted to ADP by the enzyme adenylate kinase (Noda, 1973) while AMP formed in the mitochondrial matrix has to be converted to ADP by the enzyme GTP-AMP phosphotransferase (Chiga et al., 1961) Unlike the ATP-ADP carrier, the H^+- ATPase is less specific, accepting other nucleotides as well. The apparent high specificity of the mitochondrial oxidative phosphorylation is an expression of the ATP-ADP carrier, rather than that of the phosphorylation mechanism per se.

The carrier operates without Mg^{2+} or any other bivalent cations, which is in contrast to most adenine nucleotide utilizing proteins. Infact, it has been demonstrated that the presence of Mg^{2+} retards the transport activity of the carrier (Pfaff et al., 1969). This is due to the formation of the complexes,

ADP-Mg^{2+} or ATP-Mg^{2+}, which effectively lowers the concentrations of free ADP and ATP, which are the actual substrates of the carrier.

The transport system is highly dependent on temperature, a low temperature inhibiting the exchange (Heldt et al., 1965).

The ATP-ADP carrier is influenced by the mitochondrial membrane potential with respect to the preference of ADP or ATP (Klingengberg and Lubbers, 1966). The carrier is however fully active in the absence of membrane energisation, and in accordance with the second law of the thermodynamics, symetrical in both directions with respect to the specificity for ADP or ATP. However such a transport may not serve oxidative phosphorylation efficiently since ADP and ATP will be exchanged at equal rates in both directions. This means that the four possible modes of exchange, ATP-ATP, ADP-ADP, ATP-ADP and ADP-ATP will occur at equal rates. In the energized membrane, which exists in phosphorylating mitochondria, the exchange is regulated in such a manner that it prefers largely the ADP uptake against ATP release. Under these conditions the ADP uptake versus ATP release is more than 20-fold preferred. Thus the exchange is directed towards the requirements for oxidative

phosphorylation. Such an "asymmetric" mode of transport can only be accomplished by investiment of energy. The driving force for this regulation is membrane potential (Pfaff and Klingengberg, 1968; Klingengberg, 1970). The transport can be considered to be electrical in such a manner that in the exchange of ADP^{3-} against ATP^{4-}, one negative charge difference is transported through the membrane. With the membrane potential outside being positive, the ADP against ATP exchange would be driven electrophoretically to the outside. This mode of exchange generates a higher ATP/ADP ratio outside the mitochondria than inside it. With a membrane potential of about 180 mV. a 100-fold difference in the ATP/ADP ratio may result (Klingengberg, 1975). This accounts for 2 - 4 Kilo-calorie difference in the free energy of ATP between the cytosol and the mitochondria, which is in accordance with the cellular requirements, i.e. a low ATP/ADP ratio in the mitochondria which favours ATP synthesis, and a high ATP/ADP ratio in the cytosol which favours the utilization of ATP.

Inhibitors of ATP-ADP Transport

There exists two groups of inhibitors for the ATP-ADP carrier. These are (a) the atractyloside group including atractylate, epiatractylate and

carboxyatractylate and (b) the bongkrekate group,
which includes bongkrekate and isobongkrekate (Heldt,
1969; Klingengberg et al., 1972). The two groups of
inhibitors interact with the carrier in a different
manner (Klingengberg, 1971). Atractyloside is
impermeable to cell membranes, and binds to the
cytosolic surface of the carrier, whereby it prevents
binding of adenine nucleotides to the carrier.
Bongkrekate is lipophilic and thus permeable to cell
membranes. It binds to the mitochondrial matrix
surface of the carrier, whereby it prevents dissocia-
tion of adenine nucleotides from the carrier.

(iii)c. The H^+-ATPase Inhibitor Peptide

The H^+- ATPase is capable of utilizing the
electrochemical gradient generated by the respiratory
chain as the driving force for ATP synthesis. The
same enzyme is capable of hydrolysing ATP to ADP and
inorganic phosphate. Nothing would be accomplished
if both ATP synthesis and ATP hydrolysis occurred at
equal rates, or if the newly synthesized ATP is then
hydrolysed by the ATPase. It is well documented that,
in addition to acting as the driving force for ATP
synthesis, energization of the inner mitochondrial
membrane also stimulates the ATPase (Gomez-Puyuo et al.,
1979, 1980; Harns et al., 1979). This kind of a
situation seems paradoxical, in that the conditions

that favour ATP synthesis also favour ATP hydrolysis.
The solution to this paradox lies in the fact that,
all biological systems have evolved what appear to be
natural peptide regulators of H^+-ATPases, commonly
referred to as H^+-ATPase inhibitors. The ATPase
inhibitor was first purified from bovine heart mito-
chondria by Pullman and Monroy (1963). Similar prot-
eins have subsequently been found in rat liver mito-
chondria (Chain and Barbour, 1976; Citron and Pedersen,
1979), yeast mitochondria (Satre, et al., 1975), from
chloroplasts (Nelson, et al., 1972) and from several
bacteria species including E. coli (Smith et al., 1975).
The reported molecular weights are in the range of
7,000 - 12,000.

Purification of the ATPase inhibitor

This involves rather harsh "denaturating"
conditions, which raise the question about the extent
to which the secondary and tertiary structural organi-
sation is required for their mode of action. To purify
the bovine heart inhibitor, Pullman and Monroy (1963)
subjected mitochondria to alkaline treatment. After
centrifugation and neutralization, further purification
of the supernatant was achieved in a sequence of steps
involving trichloroacetic acid precipitation, ammonium
sulfate precipitation and DEAE cellulose chromatography.
Most other workers have patented purification schemes

of the inhibitor peptide after the original Pullman-Monroy procedure.

Localization of the H^+- ATPase Inhibitor

In animal systems the precise location of the ATPase inhibitor is not very clear although there is a tendency of describing it as attached to the F_1. However, a dual interaction between the F_1 and F_0 is possible as evidenced by the fact that solubilization of the F_1-ATPase by diphosphoglycerol in MgATP particles is preceded by a stimulation of ATPase activity (Bruni and Bigon, 1974). Secondly acidic phospholipids (in contrast to phosphatidyl choline) prevent inhibition of both soluble and membrane bound F_1-ATPase by the purified ATPase inhibitor (Dabbeni-Sala, et al., 1974). Since phospholipids are components of the F_0, it is possible that specified phospholipids compete with phospholipids of F_0 important for binding F_1-APase inhibitor to the F_0.

Requirments for optimal inhibitory response

A peculiar feature of the F_1-ATPase inhibitors isolated to date is their inability to inhibit rapidly the ATPase activity of H^+-ATPases, and

require preincubation with F_1 prior to assay to elicit maximal inhibitory response. For the rat liver inhibitor about 10 minutes incubation is required for maximal inhibitory response (Pedersen, et al., 1981).

Another feature of the H^+-ATPase inhibitor is that, their interaction with the H^+-ATPase require the presence of a hydrolysable nucleoside triphosphate and a divalent cation in the incubation medium to affect maximal inhibition of the ATPase activity. A number of nucleoside triphosphates and divalent cations support inhibitor-F_1, interactions. ITP, UTP, GTP, CTP and several hydrolysable ATP analogs (Gomez - Ferandez and Harris, 1978) support the interactions in the presence of divalent cations Mn^{2+}, Ca^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+}, Co^{2+} and Zn^{2+}. The ATP analog AMP-PNP which is not hydrolysable does not promote H^{2+}-ATPase-inhibitor interaction. In the study by Gomez-Ferandez and Harris (1978) they suggest that hydrolysis of a nucleoside triphosphate may be required to promote interaction between the F_1- ATPase and the inhibitor. Infact they calculated that 200 moles of ATP are hydrolysed per mole of F_1 inhibited. These workers suggest that NTP-hydrolysis may result in a transient conformation state of the F_1-ATPase that interacts with the inhibitor. Van de

Stadt et al., (1973) have compiled some data indicating that interaction of the H^+- ATPase with its inhibitor is dependent on the ATP/ADP ratio with a low ratio favouring interaction.

Another feature of the F_1- ATPase inhibitor (with the exception of E.coli) is that maximum interaction is promoted at a pH of 6.9 or lower, although, significant inhibition does occur at physiological pH (Pullman and Monroy 1963, Citron, 1978).

Effect of the H^+-ATPase inhibitor on the kinetics of ATP hydrolysis, and its possible localization in relation to F_1 active site

The ATPase inhibitor of bovine heart is non-competitive with respect to ATP (Van de Stadt et al., 1973; Ernster et al., 1977. Ernster et al., (1977) and Junti et al., (1971) stated that neither the K_m (ATP) nor the K_i (ADP) is changed upon binding of the inhibitor to submitochondrial particles of bovine heart or to bovine heart F_1. Gomez Puyou et al., (1977) also stated that the peptide inhibitor of bovine heart is uncompetitive with ATP. It seems clear from these kinetic studies and from other studies that the ATPase does not interact directly with the

hydrolytic site of F_1. It is not clear whether it binds to the free enzyme, to a transient intermediate involved directly in the hydrolytic pathway or to a transient intermediate involved as an offshoot of hydrolytic pathway. Galante, et al., (1980) have found that when bovine heart ATPase is inhibited by the bovine heart inhibitor, it can still react with covalent labelling agents that presumably react with ATP hydrolytic site(s) thus reinforcing the conclusion that the inhibitor interacts at a site(s) different from the ATP hydrolytic site(s). These studies together with those of Kozlov and Skulachev (1977), showed that the inhibitor interacts with a tyrosine residue of the β-subunit which is not part of the hydrolytic site.

Effect of the H^+-ATPase inhibitor on ATP dependent activities

The F_1-ATPase inhibitor, inhibits all ATP dependent activities catalysed by the inner membrane vesicles of rat liver (Citron, 1978; Citron and Pedersen, 1979). The ATP dependent activities inhibited include the ATPase, ATP dependent trans-hydrogenase,

$$(NADH + H^+ + NADP^+ \rightleftharpoons NAD^+ + NADPH + H^+)$$
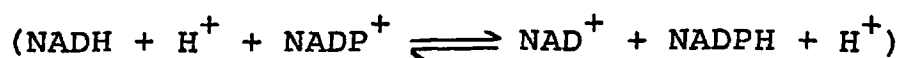
ATP dependent reverse electron flow (Succinate $\longrightarrow$ NAD$^+$) and ATP-Pi exchange. Respiration driven transhydrogenase and reverse electron flow were not affected by the rat liver inhibitor. In the bovine heart Pullman and Monroy (1863) showed that the inhibitor inhibited the ATPase, ATP-dependent ANS fluoresence, and ATP dependent reverse electron flow in EDTA-submitochondrial particles. However, Ernster and coworkers (1973) indicated that MgATP submitochondrial particles, which are supposed to contain a high content of the inhibitor due to their low ATPase activity catalyse maximal ATP-dependent transhydrogenase and reverse electron flow.

Role of the H$^+$-ATPase inhibitor in ATP synthesis

Until recently the ATPase inhibitor was thought to be a unidirectional inhibitor of ATP dependent functions catalysed by the H$^+$-ATPases, but not an inhibitor of ATP synthesis. This is based on the findings of Pullman and Monroy (1963), Ernster and coworkers (Asami, et al., 1970, Ernster et al., 1973), both of who found that the ATP dependent inhibitor failed to inhibit ATP synthesis. More recent work from both Ernster's laboratory (Gomez-Puyuo et al., 1979) and the laboratory of Harris. (Harris and Crofts 1978; Harris et al., 1979) indicated that the

unidirectional view may need some slight
modification. These workers believe that the inhibitor
has no effect on the steady phase of ATP synthesis,
but it inhibits the initial phase of the synthesis,
when the assay is initiated with a respiratory
substrate in mitochondria (or light in chloroplasts).
Their view is that the inhibitor is tightly bound to
the ATPase, and energisation with a respiratory substrate
in mitochondria or light in chloroplasts is envisioned
to displace the inhibitor from its site of action.
The evidence for this, is that, after energisation by
substrate, ATP synthesis occurs with a lag phase
before a steady state is achieved. Another evidence
is that EDTA-Sephadex particles of bovine heart
mitochondria which presumably have little or no
inhibitor present no lag phase in ATP synthesis, but
present such a lag when supplemented with the purified
inhibitor.

It is thus clear from the preceding that, the
binding ATPase inhibitor peptide to the ATPase inhibits
all ATPase dependent reactions including both ATP
synthesis and ATP hydrolysis. Release of the inhibitor
from the ATPase brought about by energisation of the
inner mitochondrial membrane activates the ATPase, and
the direction of its reaction is then dependent on
other factors in its immediate environment. The presence
of low ATP/ADP ratio and inorganic phosphate drives the

reaction towards ATP synthesis. Conversely the presence of a high ATP/ADP ratio may be expected to drive the reaction in the direction of ATP hydrolysis. However, as indicated by Gomez-Ferandez and Harris (1978), the presence of a hydrolysable nucleoside triphosphate and a bivalent cation causes binding of the ATPase inhibitor to the ATPase, thus inhibiting it. This means that although the inhibitor inhibits both ATP synthesis and ATP hydrolysis, it is released from the ATPase under conditions favouring ATP synthesis, and gets bound under conditions favouring ATP hydrolysis. It is thus apparent that the main physiological role of the ATPase inhibitor is to prevent hydrolysis of the newly synthetized ATP after a burst of phosphorylating respiration.

(d) Mitochondrial Bivalent Cation Transport

Most of the enzymes involved in the metabolism of adenine nucleotides require bivalent cations for their activity. This includes the H^+-ATPase, and the many other enzymes both in the mitochondria and in the cytosol that are involved in the utilization of adenine nucleotides. One exception to this rule is the ATP-ADP translocator whose activity is, infact, lowered by the presence of bivalent cations (Pfaff et al., 1969). Most of these enzymes can utilize several bivalent cations, but generally Mg^{2+} is the most preferred cation.

This means that concentrations of various bivalent cations have a strong influence on the rates of various adenine nucleotide metabolizing enzymes. In addition bivalent cations serve to regulate many other enzymatic reactions in the cell. For this reason the cell has evolved transport system for bivalent cations which regulate the concentration of bivalent cations in the cells. These include, the Ca^{2+}- Mg^{2+} ATPase (the Ca^{2+} pump) of the cell membrane (Schatzmann and Burgen, 1978) and the Na^+- Ca^{2+} exchange mechanism also of the cell membrane (Baker, 1976;). The Ca^{2+}-Mg^{2+}-ATPase is dependent on ATP as its source of energy, while the Na^+-Ca^{2+} exchange mechanism is dependent on the assymetric distribution of Na^+ across the plasma membrane with a high Na^+ concentration outside and a low Na^+ concentration inside. This Na^+ gradient is also maintained at the expense of ATP, by the action of a specific Na^+- pump or the Na^+- K^+ ATPase. This Na^+ gradient represents an electro-chemical gradient which can serve to drive a variety of other transport reactions. For example, the Na^+ gradient is the immediate energy source for the concentrative uptake of amino acids, glucose, phosphate and other substances by cells (Rasmussen, 1981). It is also the driving force, via Na^+- Ca^{2+} exchange, for the energy-dependent efflux of calcium

from the cell. Both the $Ca^{2+}-Mg^{2+}$ ATPase and the Na^+-Ca^{2+} exchange mechanism, serve to pump out Ca^{2+} from the cytosol to the extracellular fluid and are responsible for the maintenance of the approximately 10,000-fold difference in the concentration of Ca^{2+} between the cytosol and the extracellular fluid.

Another well characterized bivalent cation transport system is the calcium pump of the endoplasmic reticulum or sarcoplasmic reticulum. The endoplasmic or sarcoplasmic reticulum is well developed in fast skeletal muscle, somewhat less developed in cardiac muscle, and even less developed but obviously present in smooth muscle and in a variety of other cells including those of the liver, kidney, brain, salivary glands and platelets, (Rasmussen, 1981). The pump operates to catalyze the influx of Ca^{2+} into the endoplasmic or the sarcoplasmic reticulum. The calcium pump of sarcoplasmic reticulum of skeletal muscle and cardiac muscle is involved in the stimulus - response coupling responsible for muscle contraction. It is not clear what purpose the Ca^{2+} pumped into the endoplasmic reticulum of other cells serve.

Both the plasma membrane and the membrane of the endoplasmic reticulum are partially permeable to

Ca^{2+} ions. This means that Ca^{2+} is continuously leaking into the cytosol from the extracellular fluid and the interior of the endoplasmic and reticulum. The various Ca^{2+} pumps are therefore involved in the maintenance of required level of Ca^{2+} in the cytosol.

Of more significance in relation to the regulation of adenine nucleotide metabolism is the respiration dependent uptake of bivalent cations by mitochondria. In 'addition to regulating the levels of various bivalent cations in the cytosol, the process has of late received attention as a possible mechanism for the regulation of ATP synthesis and ATP hydrolysis by the ATPase. This has come about as a result of observations made in several laboratories relating to the effect of bivalent cation transport by mitochondria on oxidative phosphorylation and ATP hydrolysis. Horstman and Racker (1970) showed that the binding of the ATPase inhibitor of bovine heart to bovine heart F_1-ATPase is supported equally well when the preincubation is carried out with ATP-Ca^{2+} as with ATP-Mg^{2+}. Similar findings were reported by Gomez-Puyuo et al., (1980) who also showed that the ATPase activity of rat heart mitochondria fell after the mitochondria had accumulated Ca^{2+}. These authors also observed a 60%

decrease in the rate of ATP synthesis after uptake of Ca^{2+}. It is therefore reasonable to suggest from these findings that, Ca^{2+} accumulated in the mitochondrial matrix may bind to ATP and then to the ATPase inhibitor, which in turn binds to the ATPase inhibiting it.

Yamada et al., (1980) showed that free Ca^{2+} concentrations of upto 50 μM stimulates the ATPase activity of rat skeletal muscle submitochondrial particles. These authors were able to effect the release of a highly purified F_1-ATPase inhibitor by incubating the submitochondrial particles at 0° with Ca^{2+} concentrations ranging from 0.085 - 0.45 nMol. of Ca^{2+} per microgram of particle protein.

(iv)a. <u>Characteristics of the mitochondrial bivalent cation transport</u>

<u>Historical background</u>

Mitochondrial uptake of cations has been a subject of investigations since the early 1950's. The history of the problem began in 1952 - 54, with the first observation that isolated mitochondria can retain certain cations such as K^+ or Mg^{2+} as long as respiration is occurring (MacFarlane et al.,

1953; Spector, 1953). However, only slight accumulation of K^+ or Mg^{2+} from the medium was observed. When respiration was blocked, K^+ and Mg^{2+} leaked into the medium suggesting that retention of these cations involved a respiration dependent accumulation process.

Sieskevitz and Potter (1953) reported an approximately 3-fold stimulation of respiration on addition of 500 μM Ca^{2+} to rat liver mitochondria. This observation was later confirmed by Lindberg and Ernster (1954). Lardy et al., (1958) reported that mitochondria could utilize energy from the three energy conservation sites of the respiratory chain for the accumulation of Ca^{2+} and that the reaction was insensitive to oligomycin. Vasington and Murphy (1962), observed that isolated mitochondria can bring about net accumulation of large amounts of Ca^{2+} in a respiration linked process. Drahota et al., (1965) reported that mitochondria could utilize energy derived from ATP hydrolysis, to drive Ca^{2+} uptake, a reaction that was sensitive to oligomycin. Further investigations (Lehninger et al., 1967; Carafoli and Crompton, 1976) showed that in addition to Ca^{2+}, mitochondria could catalyse an energy dependent uptake of other bivalent cations including Mn^{2+}, Sr^{2+} and Ba^{2+} but not Mg^{2+}, utilizing energy from the three

coupling sites of the respiratory chain and/or from ATP hydrolysis.

Lehninger et al., (1963) observed that phosphate accompanied the respiratory linked accumulation of Ca^{2+}. This uptake of Ca^{2+} and phosphate occurred in a stoichiometric manner with 1.7 - 2.0 Ca^{2+} ions being accumulated with 1.0 phosphate ion, per pair of electrons transversing each of the three energy conserving sites of the respiratory chain. Rossi and Lehninger (1964) and Chance, (1965) observed that Ca^{2+} stimulated respiration in a cyclic fashion, in such a manner that 2 calcium ions caused an extra uptake of oxygen equal to that caused by one molecule of ADP.

Saris (1963) and Lehninger et al., (1967) observed that accumulation of Ca^{2+} during electron transport is accompanied by H^+-ejection. In the absence of phosphate or other "permeant" anions at least one proton is ejected per pair of calcium ions accumulated. Accumulation of Ca^{2+} in the presence of phosphate led to the formation of electron dense granules in the mitochondrial matrix (Chappell and Crᵒfts, 1965) , a phenomenon that was not observed in its absence. The formation of these electron dense granules was also found to be promoted by the presence of ATP or ADP (Rossi and Lehninger, 1964; Carafoli et al., 1965).

Specificity and Affinity for Mitochondrial

cation uptake

Mitochondria isolated from rat liver are able to support an energy dependent uptake of Ca^{2+}, Sr^{2+}, Mn^{2+} and Ba^{2+} but not Mg^{2+} (Lehninger et al., 1967; Mela and Chance, 1968). Ca^{2+} and Sr^{2+} are taken up very rapidly and at almost equal rates, while Mn^{2+} and Ba^{2+} are only slowly taken up.

Monovalent cations including K^+ and Na^+ are not normally accumulated by isolated mitochondria except in the presence of the ionophorous antibiotics such as valinomycin (Moore and Pressman, 1964) and gramicidin (Chappell and Crofts, 1965). ·

The high affinity for the respiratory linked accumulation of Ca^{2+} and its presence in large amounts in various tissue fluids (compared with other cations such as Sr^{2+}, Ba^{2+} or Mn^{2+} suggest a regulatory action for the cation in mitochondrial function. This suggestion is strongly supported by the fact that mitochondria have an exceedingly high affinity for Ca^{2+} which greatly exceeds that for ADP (Rossi and Lehninger 1964; Vercesi et al., 1978). Infact, when an equimolar mixture of Ca^{2+} and ADP is presented to intact liver mitochondria, at very low physiological

concentrations, Ca^{2+} is accumulated in prevalence to
ADP. No ATP is formed until all the added Ca^{2+} has
been accumulated.

Tissue Specificity

Mitochondria isolated from rat liver, kidney,
brain and heart were early shown to support a
respiration dependent accumulation of Ca^{2+} (Rossi
and Lehninger, 1963). In a more recent survey
(Lehninger, 1970) mitochondria from all mammalian
tissues examined including the adrenal cortex, testis,
spleen and skeletal muscles of such animals as mouse,
guinea pig and rabbit showed the capacity for an
energy dependent accumulation of Ca^{2+}. Ca^{2+}
accumulation also occurs in mitochondria of at least
some higher plants (Bonner and Pressman, 1965), but
not mitochondria isolated from the yeasts
Saccharomyces cerevisae and Turula utilis (Kenefic
and Hanson, 1966). However mitochondria from
Neurospora crassa (Carafoli et al., 1970) show some
Ca^{2+} uptake activity.

Mechanism of Mitochondrial Bivalent Cation
uptake.

A lot of work has been carried out with the aim

of determining the mechanism by which bivalent cations
are transported by isolated mitochondria (Mitchell,
1969; Carafoli and Crompton 1978; Mitchell 1976;
Nicholls 1978). Most of these investigators have
more or less restricted themselves to a study of the
transport of Ca^{2+}. Very little work has been done
regarding the transport of other cations, except for
Mn^{2+}, the transport of which has received some
attention (Ernster and Nordenbrand, 1967, Vinogradov
and Scarpa 1973, Hughes and Exton 1983).

The establishment of the chemiosmotic theory by
Mitchell (1961) provided a simple explanation, for the
high capacity of mitochondria to accumulate large
quantities of Ca^{2+}. The mitochondrial electron
transport generates a proton electrochemical gradient
($\Delta\mu_H+$) on the inner mitochondrial membrane which is
positive on the outside and negative on the inside.
The $\Delta\mu_H+$ has two components i.e the membrane potential
($\Delta\psi$) which is contributed by the unequal distribution
of ions across the mitochondrial inner membrane, and
the pH gradient (ΔpH) which is acidic on the outside
and alkaline on the inside. The two components can
be combined to form the equation.

$$\Delta\mu_H+ = \Delta\psi - 60 \Delta pH$$

$\Delta\mu_H+$ is usually expressed in millivolts, and is about
180.

In normal respiring mitochondria $\Delta\Psi$ contributes about 75% of the energy of $\Delta\mu_H^+$. In addition to respiration, the hydrolysis of ATP by the ATPase can also create a proton electrochemical gradient across the inner mitochondrial membrane (Pedersen et al., 1978).

Uptake of Ca^{2+} is known to be an energy dependent process which can be driven by energy derived either from substrate oxidation by the respiratory chain, or by ATP hydrolysis (Bygrave, 1978; Carafoli and Crompton 1978; Scarpa, 1979; Mitchell 1969). It is nowadays believed that the primary driving force for Ca^{2+} entry is the $\Delta\Psi$ component of the total electrochemical gradient, generated by either substrate oxidation or by ATP hydrolysis. In this view Ca^{2+} uptake is driven electrophoretically (in response to an electrical gradient) into the matrix space via a Ca^{2+} uniport (Nicholls, 1978, Nicholls, 1978.a). Uptake of Ca^{2+} will tend to dissipate the $\Delta\Psi$ and hence lower the $\Delta\mu_H^+$. The respiratory chain responds to the lowered $\Delta\mu_H^+$ by a further net extrusion of proton, thus increasing the ΔpH. For every calcium ion taken up, two protons are extruded (Nicholls, 1982). Since respiration can only achieve the same $\Delta\mu_H^+$

as before, this means that increasing uptake of Ca^{2+} will lower the $\Delta\Psi$ component and increase the ΔpH component of the total $\Delta\mu_H^+$. The lowered $\Delta\Psi$ means that Ca^{2+} uptake under these conditions becomes self limiting, as the driving force steadily decreases until electrochemical equilibrium is attained i.e. ΔG is zero, and hence $\Delta\mu_{Ca}^{2+} = 0$. At this point the ΔpH is very high, and acts as a backward force on the respiratory proton pump, which leads to an inhibition of respiration i.e. state VI of Chance and Schoener (1966). If at this point a "permeant" anion which can donate protons within the matrix is added, the anion is taken up, and dissipates the ΔpH, allowing respiration to resume, and restoring the $\Delta\Psi$, which leads to further Ca^{2+} uptake. Under physiological conditions, the major anion is phosphate, which upon entering the matrix space interacts with Ca^{2+} to form a non-ionic calcium phosphate complex which is osmotically inactive. Formation and/or stabilization of this complex requires the presence of ATP and/or ADP (Rossi and Lehninger, 1964; Carafoli, et al., 1965), and the complex appears to have the following stoichiometry $Ca_{15}P_{10}ATP$ (Rasmussen, 1981). The formation of this non-ionic complex means that, the presence of phosphate and adenine nucleotides in the mitochondrial matrix enables the mitochondria to have a very high capacity for buffering Ca^{2+} accounting for

the extremely large quantities of Ca^{2+} that can be taken up by isolated mitochondria.

Uptake of Adenine Nucleotides during Ca^{2+} accumulation

Accumulation of amorphous granules of calcium phosphate in mitochondria has been found to be accompanied by a significant accumulation of an organic phosphate derivative or derivatives (Carafoli et al., 1965). This is largely contributed by adenosine di- and triphosphate, which accumulate at levels far much in excess of those found in normal mitochondria. The accumulation of the adenine nucleotides occurs proportionally and is parallel to calcium accumulation. This accumulation of adenine nucleotides is thought to be related to the requirement of ATP for supporting Ca^{2+} accumulation and the formation of amorphous granules of calcium phosphate in the presence of inorganic phosphate (Vasington and Murphy, 1962; Rossi and Lehninger, 1964; Rasmussen, 1981).

Carafoli et al., (1965) observed that the adenine nucleotide uptake which accompanied Ca^{2+} accumulation was inhibited by respiratory inhibitors and uncouplers, factors that also inhibited Ca^{2+}

accumulation. The Ca^{2+} effect of stimulating massive accumulation of adenine nucleotides was also observed with Sr^{2+}, but not Ba^{2+}, Mn^{2+} or Mg^{2+}. The nucleotide uptake is very specific for ATP and ADP, in that neither AMP nor other nucleotide di- or triphosphates were taken up during Ca^{2+} accumulation. This effect is also dependent on the presence of inorganic phosphate in that very small amounts of adenine nucleotides are taken up, during the limited uptake of Ca^{2+} which occurs in the absence of inorganic phosphate. The effect of atractyloside on the Ca^{2+} stimulated uptake of adenine nucleotides is dependent on the Ca^{2+} concentration in the reaction medium. Atractyloside inhibited adenine nucleotide uptake when Ca^{2+} concentration was below 300 μM, but had no inhibitory effect when Ca^{2+} concentration was above 3mM.

Accumulated Ca^{2+} has a rather different effect on adenine nucleotide translocation as compared to the effect seen during active Ca^{2+} accumulation. Once Ca^{2+} has been accumulated, the adenine nucleotide translocase activity is inhibited (Gomez-Puyuo et al., 1979.a) with a half maximal inhibition being observed on the accumulation of approximately 40 nMoles Ca^{2+} per mg. of mitochondrial protein. That this inhibitory effect is due to accumulated Ca^{2+}, was

shown by the fact that inhibition of respiration, uncoupling or the presence of ruthenium red all of which inhibit Ca^{2+} uptake, prevented the inhibition of the adenine nucleotide translocase activity.

It has also been shown that the presence of adenine nucleotides in the mitochondria is of prime importance in the mitochondrial Ca^{2+} uptake activity. Asmakis and Sordhal (1981) found that rat liver mitochondrial which had their adenine nucleotide content partially depleted by incubating them with inorganic pyrophosphate, had an impaired ability to retain Ca^{2+}. This was observed as a premature Ca^{2+} efflux associated with swelling and altered energy coupling. Exogenous ATP or ADP added prior to Ca^{2+} efflux restored Ca^{2+} retention in these mitochondria an effect that was inhibited by atractyloside. This effect of Ca^{2+} retention was also observed on addition of large amounts of AMP, but not GTP. The effect of AMP is most probably due to formation of ADP and/or ATP by adenylate kinase.

Effect of Mitochondrial Ca^{2+} accumulation on oxidative phosphorylation

In most of the tissues tested including rat liver (Rossi and Lehninger, 1964) and heart (Vercesi et al.,

1978), uptake of Ca^{2+} takes precedence over oxidative phosphorylation, when both Ca^{2+} and ADP are added together in the presence of a respiratory substrate and inorganic phosphate. ATP synthesis occurs only after all the added Ca^{2+} has been accumulated.

The effect of accumulated Ca^{2+} on the subsequent stimulation of oxidative phosphorylation depends on the tissue source of the mitochondria.

Uptake of small amounts of Ca^{2+} by rat liver mitochondria has no effect on the subsequent stimulation of oxidative phosphorylation (Rossi and Lehninger, 1964). However, uptake of larger amounts of Ca^{2+} is sometimes observed to destroy the mitochondrial respiratory control, causing swelling and structural damage to the mitochondria leading to a loss of accumulated Ca^{2+} (Lehninger, et al., 1967). The reasons behind this effect are so far not clear.

Accumulation of Ca^{2+} in mitochondria isolated from rat brain (Roman et al., 1981, Nowicki, et al., 1982, Hillered et al., 1983), Ehrlich ascites tumour (Thorne and Bygrave, 1974; Villalobo, 1978) and heart (Gomez-puyuo et al., 1980) has been shown to cause an inhibition of the subsequent stimulation of oxidative phosphorylation. It is not clear how

accumulated Ca^{2+} in these mitochondria inhibit oxidative phosphorylation. However, several explanations have been proposed including the inhibition of release of the ATPase inhibitor protein, which becomes associated with the ATPase during active Ca^{2+} uptake (Gomez-Puyuo, et al., 1980), a competition with intramitochondrial Mg^{2+} for binding to ADP (Roman et al., 1981) or an inhibition of the ATP-ADP translocator (Thorne and Bygrave, 1974; Gomez-Puyuo, et al., 1979). However, so far there has been no compromise, and more investigations are still required.

Efflux of Ca^{2+} from mitochondria

Addition of Na^+ to a medium containing mitochondria that have accumulated Ca^{2+}, has been shown to cause release of the accumulated Ca^{2+} (Carafoli et al., 1974; Crompton et al., 1976; Crompton et al., 1977; Crompton et al., 1978). This effect has been observed in mitochondria isolated from heart, brain, adrenal cortex and skeletal muscle i.e. excitable tissues but not in mitochondria isolated from non-excitable tissues such as liver, kidney, lung, uterus and ileum muscle. The rate of the Na^+ dependent Ca^{2+} efflux is a function of Na^+ concentration, and is stimulated by energy dependent respiration. This apparently

indicates the existence of a system capable of catalysing an exchange between Na^+ and Ca^{2+} across the inner mitochondrial membrane. The system is thought to be an electroneutral exchange between $2Na^+$ and one Ca^{2+} (Crompton, et al., 1977)

The rate of Ca^{2+} uptake by mitochondria is also dependent on the external concentration of Na^+. The rate of uptake decreases with an increase in the external Na^+ concentration upto a concentration of 20 mM, after which no net uptake occurs (Crompton et al., 1976). Unlike the Ca^{2+} uptake system, the Ca^{2+} efflux system is not inhibited by ruthenium red, but is inhibited by La^{3+} (Crompton, et al., 1977). This indicates that the two processes may be catalysed by two different membrane carrier systems. This is further emphasized by the fact that Ca^{2+} efflux is complete in the presence of ruthenium red, but not in its absence. This is due to the fact that, in the presence of ruthenium red, re-uptake of the released Ca^{2+} is inhibited, while in its absence efflux is accompanied by a re-uptake.

This means that in the presence of Na^+, Ca^{2+} moves in a cyclic fashion, the rates of which are dependent on the Na^+ concentration. This is most

Inner mitochondrial
membrane

Ca^{2+} influx

Ruthenium red
sensitive

Matrix

Na^{+}-dependent Ca^{2+}
efflux

Ruthenium red
insensitive
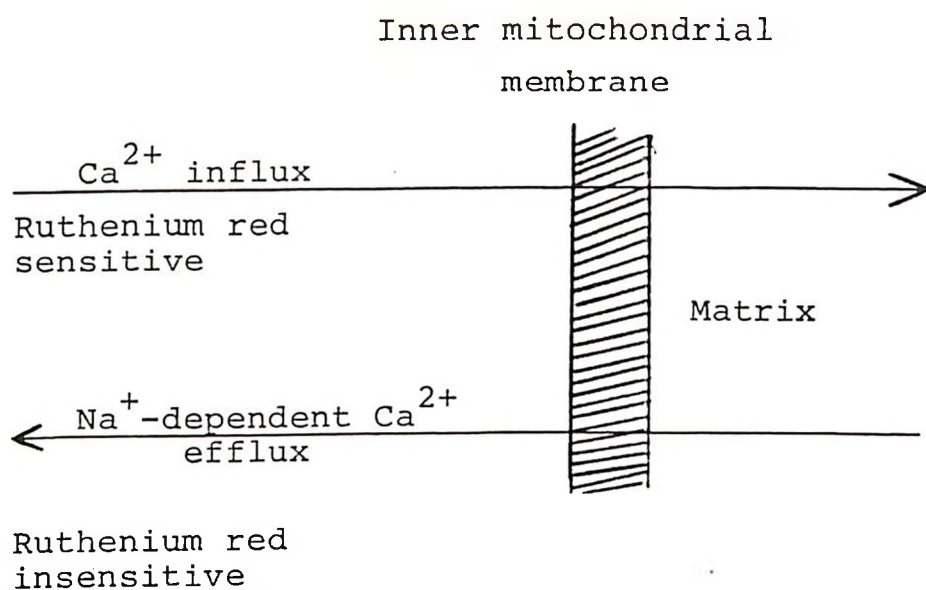
evident when Ca^{2+} movements are assayed using the
oxygen electrode. Addition of limited amounts of
Ca^{2+} to respiring mitochondria causes an increase in
the rate of oxygen uptake, which ceases when all the
Ca^{2+} is taken up. Addition of Na^{+} at this point
causes uncoupling of the mitochondrial respiration
due to cyclic Ca^{2+} movements. However this
uncoupling is inhibited by ruthenium red which
inhibits re-uptake of the released Ca^{2+}. The sodium
induced efflux of accumulated Ca^{2+} has been shown to
be an energy dependent process (Crompton et al., 1976)
by the fact that addition of antimycin to mitochondria
that have accumulated Ca^{2+} show a slow efflux
accumulated Ca^{2+}, which is only slightly increased
by the addition of Na^{+}.

(iv) AIM OF THE STUDY

The aim of the current investigation is to characterize further the manner in which the mitochondrial enzyme adenylate kinase and mitochondrial cation uptake may interact functionally with the oxidative phosphorylation machinery of the cell in the regulation of adenine nucleotide metabolism.

The work has been carried out in the form of two distinct but closely related subjects.

(a) Various parameters of the rat liver mitochondrial adenylate kinase has been investigated with an aim of elucidating the manner in which its interaction with extra- and intramitochondrial adenine nucleotides may regulate and harmonize the activity of the ATPase (ATP synthetase). For this subject, rat liver mitochondria were chosen because they are easy to prepare and they contain a very high activity of the mitochondrial (liver type) enzyme as compared to most other tissues.

(b) The effect of rat brain mitochondrial cation transport and cation accumulation has been investigated with an aim of elucidating further the manner in which various bivalent cations and their adenine nucleotide

complexes may regulate oxidative phosphorylation. For these experiments rat brain mitochondria and coupled beef heart MgATP submitochondrial particles were used.

It is hoped that this study will provide a further insight into the role of the mitochondrial adenylate kinase and bivalent cation transport in the regulation of adenine nucleotide metabolism.

C H A P T E R   I I

MATERIALS AND METHODS

Preparation of rat liver mitochondria

Rat liver mitochondria were prepared from male Sprague-Dawley rats by a modification of the method described by Hogeboom (1962).

The rats were decapitated using a guillotine, and were allowed to bleed completely. The livers were then removed and washed several times in ice cooled 0.25 M. sucrose. They were minced using a tissue mincer which also separated the soft tissues of the liver from connective tissue. The minced soft tissues were washed several times in ice cooled 0.25 M. sucrose to remove the blood. The mince from one whole liver was suspended in 80 ml. of ice cooled 0.25 M. sucrose. This was then divided into two lots of 40 ml. each and homogenized using a chilled Potter Elvehjem homogeniser with a polytetra-gluoroethylene (Teflon) pestle. The liver homogenate was then centrifuged for 5 minutes at 600 x g using an internally refrigerated MSE High Speed 18 centrifuge, to remove nuclei, cell debris and red blood cells. The supernatant was decanted and re-centrifuged for 25 minutes at 4000x g. The mitochondrial pellet was

resuspended in 0.25 M. sucrose, and washed twice by centrifugation at 4000 x g. for 15 minutes. The residual mitochondrial pellet was resuspended in cold 0.25 M. sucrose to give the required protein concentration. All the procedures described above were carried out at 0 - 4°C.

The quality of the mitochondria was always tested by measuring the respiratory control ratio using a clark type oxygen electrode. The respiratory control ratio was always between 5 and 6.

Fractionation of the liver mitochondrial intermembrane space components

This was carried out as described by Bogucka and Wojtczak (1976). About 100 mg. of mitochondrial protein was suspended in 10 ml. of 10 mM. potassium phosphate buffer, pH 7.4 for 10 minutes at 0°C. This treatment results in swelling, followed by rupture of the outer membrane and leakage of the intermembrane space components. This suspension was then centrifuged for 15 minutes at 20,000 x g. to sediment the mitoplasts. The supernatant was decanted and re-centrifuged for 60 minutes at 105,000 x g. to sediment the outer mitochondrial membranes. The resulting supernatant which contained mainly the

soluble proteins and other low molecular weight
components of the intermembrane space was decanted.

Preparation of rat brain mitochondria

Rat brain mitochondria were prepared from male
Sprague-Dawley rats as described by Roman et al.,
(1981).

The brains were removed following decapitation
and cooled in ice cold isolation medium which contained
0.3 M. Mannitol, 10mM. Tris - HCl pH, 7.4 and 0.3 mM.
Tris - EDTA. The brains were washed twice in cold
isolation medium, and then cut into tiny pieces using
a pair of scissors. The tiny pieces were then
homogenized in a chilled Potter Elvehjem homogeniser
with a teflon pestle. The homogenate was centrifuge
for 10 minutes at 600 x g. and the supernatant was
decanted. The supernatant was re-centrifuged for
20 minutes at 5000 x g. The resulting mitochondria-
pellet was resuspended in the homogenising medium
and applied to a continuous ficoll gradient (3% and
10%). This was centrifuged for 20 minutes at
43,000 x g. The resulting pellet consisting of
purified mitochondria was collected after sedimenta-
tion through the 10% layer.

Preparation of heavy beef heart mitochondria

Heavy beef heart mitochondria were prepared as described by Low and Vallin (1963).

Ice cooled beef hearts straight from slaughter were minced and mixed with 0.25 M. sucrose, 5 mM. Tris-HCl, 2 mM. Tris-EDTA pH 7.5. The mince was shaken for about 10 minutes in a plastic anchor stirrer. The pH of the mince was controlled with a glass electrode and readjusted to pH 7.5 with 5 M. ethanolamine buffer. The mince was then squeezed through a cheese cloth and completely freed of the washing solution. The mince was then mixed with 0.25M. sucrose, 10mM. Tris-HCl, buffer, pH 7.5, 1mM. Tris-succinate and 2mM. EDTA, - Tris, pH 7.5 (solution A). The mince was then treated with a brown blender set at medium speed for 45 seconds. The temperature was controlled at 0 - 4°C and pH at 7.5 by adding Triethanolamine buffer. The blended mince was then centrifuged in 1 litre bottles for 20 minutes at 350 x g. The supernatant is decanted without disturbing the upper layer of the precipitate. The supernatant was filtered through a triple layer of cheese cloth. The filterate was centrifuged for 15 minutes at 36,400 x g. The resulting pellet was composed of three layers (a) a loosely packed, "light brown layer

(light mitochondria)   (b) a tightly packed, dark
brown layer (heavy mitochondria) and   (c) a
brown-red button at the bottom of the tube (red cells
and cell debris).   The upper layer was removed by
adding some solution A and shaking gently. The tightly
packed dark brown layer was dislodged by first
adding solution A, and vigorously shaking the centrifu-
ge tubes, and then removing it with the aid of a
stirring rod, avoiding the brown-black-red button.

The heavy mitochondrial were then mixed with a
5 times volume of solution A, and resuspended with
the use of a glass-teflon homogeniser by two full
passes. The pH was readjusted to 7.5 using 5 M.
triethanolamine buffer. The suspension was centrifu-
ged twice at 15,000 x g for 15 minutes. The pellet
was then resuspended in 0.25 M. sucrose, 10mM. Tris-
HCl buffer, pH 7.5 to a final concentration of
60 - 70 mg. protein per ml. The mitochondria were
stored at -20°C until use.

Preparation of beef heart MgATP submitochondrial particles

MgATP submitochondrial particles were prepared
as described by Lee and Ernster (1967). Heavy beef
heart mitochondria were diluted to a protein
concentration of 20 - 30 mg. per ml.   8ml. of these

mitochondria were diluted to 25 ml with 0.25 M. sucrose and the pH was adjusted to 7.5 using 5 M. Triethanolamine buffer. MgCl and ATP to a final concentration of 15 mM. and 1 mM. respectively were then added to the suspension. The suspension was then sonicated using a Branson sonifier set at 4 for 35 seconds. This suspension was then diluted to 40 ml. with 0.25 M. sucrose and centrifuged at 9000 x g. for 10 minutes. The supernatant was decanted and recentrifuged for 45 minutes at 105,000 x g. The resulting pellet consisting of MgATP submitochondrial particles was resuspended in 0.25 M. sucrose and centrifuged twice for 45 minutes.

The pellet was resuspended in 0.25 M. sucrose to give the required protein concentration.

Preparation of beef heart state III submitochondrial particles

State III submitochondrial particles were prepared as described by Van de Stadt et al., (1973). Heavy beef heart mitochondria were diluted to a protein concentration of 20 - 30 mg/ml. 8ml. of these mitochondria were diluted to 25 ml. with 0.25M. sucrose and the pH was adjusted to 7.5 with 5M. Triethanolamine buffer. The following additions were

then made to the suspension:- 15mM MgCl, 5mM
succinate, 3mM, inorganic phosphate and 3mM. ADP
(final concentrations). This was then followed by a
35 seconds sonication of the suspension, using a
Branson sonifier set at 4, after which the suspension
was incubated for 10 minutes. The suspension was then
diluted to 40 ml. with 0.25 M. Sucrose, and centri-
fuged at 9000 x g for 10 minutes. The supernatant was
decanted and recentrifuged for 45 minutes at
105,000 x g. The resulting pellet consisting of
state III submitochondrial particles was resuspended
in 0.25 M. sucrose and centrifuged twice per 45 minutes.

The pellet was resuspended in 0.25 M. sucrose to
give the required protein concentration.

Preparation of beef heart mitochondrial purified
F_1-ATPase

Purified F_1 ATPase was prepared according to
Penefsky (1979). 75ml of heavy beef heart mitochond-
ria at a protein concentration of 30mg/ml, in a plas-
tic tube, were placed in an ice water bath, and
sonicated for 4 minutes, using a Branson sonfier set
at 4. The suspension was then centrifuged for 15
minutes at 17,000 rpm, using a spinco preparative

centrifuge at 5°C. with a number 30 rotor. The
supernatant which contained submitochondrial particles
was recentrifuged at 30,000 rpm. for 90 minutes. The
pellet was suspended in 0.25 M. sucrose containing
10 mM. Tris-HCl buffer, pH 7.5. This suspension could
be stored for upto 8 weeks at -70°C.

The above suspension of submitochondrial particles
was diluted with 0.25 M. sucrose, containing 10 mM.
Tris-HCl buffer, pH 7.5 to a protein concentration of
20 mg/ml. 75 ml of the suspension was put in a 100 ml
beaker and sonicated for 4 minutes with the sonifer
set at 4. This was followed by centrifugation of the
suspension at 30,000 rpm. for 90 minutes. The pellet
was suspended to a protein concentration of 30 mg/ml.
with 0.1 M sucrose, 4 mM. ATP, 2 mM EDTA, and the
pH was adjusted to 9.2 with ammonium hydroxide. The
suspension was then placed in a 1 litre graduated
cylinder which was covered with paraffin wax and
stored overnight at room temperature. On the
following morning, the pH of the suspension was
adjusted to 9.2. The suspension was then transfer-
red to a stainless steel beaker, and immersed in a
water bath at 35°C., followed by sonication of
7 minutes with the sonifer set at 7. The suspension
was then centrifuged at 25°C., for 90 minutes at
30,000 rpm.

The pH of the supernatant was adjusted to 5.4 with 3N acetic acid. It was then centrifuged for 5 minutes at 15,000 rpm., using a sorvall centrifuge with a 55 - 34 rotor. The pH of the supernatant was adjusted to 8.0 with undilited Tris.

The supernatant was passed through a DEAE-Sephadex-A50 column which had been washed previously with 100 ml. TEA buffer containing 20 mM. Tris-HCl, pH 8.0, 4 mM. ATP and 2 mM. EDTA . The supernatant was allowed to pass for 80 minutes. The enzyme was retained in the column. The column was then washed with 100 ml. TEA buffer, followed by another washing with 400 ml. of the TEA buffer containing 0.10 M. Na_2SO_4. F_1-ATPase is then eluted with a buffer containing TEA and 0.15 Na_2SO_4. The effluent is collected at a flow rate of 3 - 4 ml/min and the samples containing most of the F_1 were mixed. Solid $(NH_4)_2SO_4$ was added to the F_1 containing effluent to a final concentration of 2.8 M. This was left overnight at 5°C. The precipitate was collected the following morning and dissolved at room temperature in a minimal volume 0.25 M. sucrose containing 50 mM. Tris-HCl buffer, pH 8.0, 2 mM EDTA and 4 mM. ATP. To this is added an equal volume of saturated $(NH_4)_2SO_4$ solution pH 8.0. The $(NH_4)_2SO_4$ suspension of the enzyme was stored at 5°C.

Measurement of Mitochondrial respiration

Mitochondrial respiration was measured by following the rate of oxygen uptake polarographically using a Clark type oxygen electrode (Yellow-Springs, OHIO) and recorded on a Sargent - Welch recorder. The reaction mixture at 30°C contained (unless otherwise stated) 50 mM. Tris-HCl buffer, pH 7.5, 0.25 M. sucrose, 100 mM. KCl, 3 mM. Tris-phosphate, pH 7.5 and 2 - 3 mg. of mitochondrial protein, in a 2 ml. total volume. Other additions are as indicated in individual experiments. Stimulation of oxygen uptake was initiated by the addition of ADP (or AMP in some cases). The solubility of oxygen in the media was determined polarographically by measuring NADH oxidation using beef heart submitochondrial particles. The solubility of the oxygen was equal to 400± ug. atoms oxygen per ml. of the media at 30°C.

Determination of the kinetic constants of the adenylate kinase reaction

The kinetic constants of the adenylate kinase reaction, K_m and maximum velocities were determined by measuring the initial reaction velocities versus changing initial substrate concentrations in the

absence of reaction products. Initial reaction velocities were determined by assaying the rate of formation of either ADP (forward reaction) or ATP (reverse reaction).

In the forward reaction the rate of ADP formation was determined in a reaction mixture containing 50 mM. Tris-HCl buffer, pH 7.5, 0.25 M. sucrose, 133 mM. KCl, 3 mM.$MgCl_2$, 1.1 mM. phosphoenol pyruvate, 5 units lactate dehydrogenase, 0.1 mM. NADH, 5ug. oligomycin to inhibit the ATPase activity, 5ug. rotenone to inhibit NADH oxidase activity and 0.4 mg. of rat liver mitochondrial protein at 30^OC. in a 3 ml. final volume. The concentration of ATP and AMP were varied to observe the effect of initial substrate concentration on the initial reaction velocity. The reaction was started by the addition of AMP, after absorption at 340 nM. had stabilized. Decrease in absorbance was recorded continuously spectrophotometrically. A unit of enzyme activity was taken as the number of μmoles NADH oxidized per minute per mg. of mitochondrial protein.

The reverse reaction was assayed by measuring the rate of ATP formation. The reaction mixture at 30° contained 50 mM. Tris-HCl buffer, pH 7.5, 0.25 M. sucrose, 133 mM. KCl, 3.0 mM. $MgCl_2$, 0.4 mM. $NADP^+$,

1.0 mM. glucose, 5 units glucose-6-phosphate
dehydrogenase, 5 units hexokinase, 5 ug. oligomycin
and 0.4. mg. of mitochondrial protein, at 30^OC in a
3 ml. final volume. The reaction was started by the
addition of varying concentrations of ADP to observe
the effect of initial ADP concentration on the initial
reaction velocity. Increase in absorbance was
recorded continuously spectrophotometrically at
340 nM. A unit of enzyme activity was taken as twice
the number of μMoles of $NADP^+$ reduced per minute
per mg. of mitochondrial protein.

In both the forward and the reverse reactions
the absorption coefficient of NADH and $NADP^+$ was
taken as 6.22 mM^{-1} cm^{-1}.

Determination of the equilibrium constant of
the adenylate kinase reaction

This was determined in a reaction mixture
containing 50 mM. Tris-HCl buffer, pH 7.5, 0.25 M
sucrose, 3.0 mM. $MgCl_2$, 133 mM. KCl, 0.3 mM ATP,
0.3 mM. AMP, 0.3 mM. ADP, 5 ug. oligomycin and
0.4 mg. of mitochondrial protein in a final volume
of 3 ml. The reaction mixture was incubated for
one hour, and the reaction was stopped by addition
of perchloric acid to a final concentration of 5%.

This was then followed by neutralization using 5 M. KOH, after which the adenine nucleotide composition of the mixture was determined as described below.

Effect of Mg^{2+} concentration on the equilibrium position of the adenylate kinase reaction

The reaction mixture was similar to that described for the determination of the equilibrium constant for the adenylate kinase reaction(see above). However the Mg^{2+} concentration was varied from one experiment to another to observe the effect of Mg^{2+} concentration on the equilibrium constant of the reaction.

Determination of the effect of H^{+} concentration on the adenylate kinase reaction

The reaction mixture was similar to that described for the determination of the kinetic constants for the adenylate kinase reaction. However, the concentrations of ATP and AMP were kept constant at 0.7 and 0.3 mM respectively for the forward reaction, and the concentration of ADP was kept constant at 1.0 mM for the reverse reaction. The pH of the buffer was varied between 5.5 and 9.0.

Measurement of ATPase activity

ATPase activity was measured as described by
Pullman et al., (1960). The reaction mixture at
30°C. contained 25 mM. Tris-acetate buffer, pH 7.5,
30 mM. potassium acetate, 3.0 mM. magnessium acetate,
1 mM. phosphoenol pyruvate, 0.2 mM. NADH, 5 ug.
rotenone, 5 μM. CCCP, 5 units pyruvate kinase, 5
units lactate dehydrogenase and either 50 - 100 ug.
of MgATP particles or the purified ATPase in a 3 ml.
final volume. The reaction was started by the
addition of 3.0 mM. ATP and NADH oxidation was recorded
spectrophotometrically at 340 nM. A unit of ATPase
activity was taken as the number of μMoles of NADH
oxidized per minute per mg. of particle protein.

Alternatively ATPase activity was measured
directly by measuring the amount of inorganic
phosphate formed, as described by Lindberg and
Ernster (1956). After the ATPase reaction was
complete, the sample was first treated with trichlo-
roacetic acid to precipitate protein as follows:-
1 ml. of the test sample was mixed with 0.6 ml. of
ice cold 15% trichloroacetic acid, and left to stand
for 10 minutes, in an ice bath, followed by
neutralization, by dropwise addition of 5m.KOH. The
mixture was then centrifuged in the cold room (4-5°C)

for 10 minutes using a bench centrifuge at maximum
speed to sediment the denatured proteins. The
supernatant was carefully decanted and used for
inorganic phosphate assay. Phosphate assay was carried
out in a reaction mixture containing 1.1 mM. sodium
acetate buffer, pH 7.2, 1.4% formaldehyde, 0.6%
ammonium molybdate and 0.4 mM. stannous chloride.

The reaction was started by the addition of the
inorganic phosphate containing sample to a 3 ml. final
volume. The mixture was incubated for 15 minutes at
room temperature and the optical density was read
spectrophotometrically at 735 nM. The amount of
inorganic phosphate was calculated using standard
inorganic phosphate solutions.

Measurement of monoamine oxidase activity

Monoamine oxidase activity was measured by the
method of White-Tabor and Rosenthal (1955). The
reaction mixture contained 0.2 mM. phosphate buffer,
pH. 7.2, 10 µMoles benzylamine, 100 µl. of 1%
deoxycholate, 1.8 ml. H_2O and 100 µl. of the test
sample in a 3.1 ml. final volume. The reaction was
followed at 250 nM. using a Ziess PM QII spectro-
photometer for 10 minutes. A reagent blank minus

benzylamine was also run. The activity was expressed as ΔE_{250} per minute per mg. protein.

Measurement of malate dehydrogenase activity

Malate dehydrogenase activity was assayed as described by Bergmeyer and Bernt (1965). The reaction mixture contained 91 mM. phosphate buffer and 38 mM. aspartate, 1.2 mM. 2-oxoglutarate, 10 mM. malate, 0.2 mM. NADH and 3.3 units GOT suspension. After incubation for 5 minutes, the sample was added. Change in optical density is read at 340 nM.

Measurement of cytochrome oxidase activity

Cytochrome oxidase activity was assayed polarographically as described by Cameino and King (1960). The reaction mixture contained 30 mM. ascorbate, 1 mM. EDTA, 30 μM cytochrome c, and 50 mM. phosphate buffer, using an oxygen electrode. Activity of cytochrome oxidase was calculated as micromoles of oxygen consumed per minute per mg. protein at $25^{\circ}C$.

Measurement of cytochrome c content

Cytochrome c content was measured in accordance with the method of Paul (1955). The reaction mixture

contained 100 mM. phosphate buffer, pH 7 and the sample is reduced with sodium dithionite. Spectra of reduced cytochromes were made between 400 and 600 nM. The cytochrome c content was calculated as the absorption difference at 540 and 550 nM, using the formula.

$$\frac{A}{E \times L} :-$$

where :— A = Absorption difference at 540 and 550 nM.

E = Absorption coefficient of cytochrome c which is $19.1 \text{ mM}^{-1}\text{cm}^{-1}$.

L = Volume in the cuvette.

Measurement of mitochondrial oxidative phosphorylation

Mitochondrial oxidative phosphorylation was measured by measuring the rate of ATP formation using a coupled enzyme assay. The reaction mixture at $30^{O}C$ contained 50 mM. Tris-HCl buffer, pH 7.5, 0.25 M. sucrose, 3.0 mM. Tris-succinate, pH 7.5, 3.0 mM. Tris-phosphate, pH 7.5, 3 ug. rotenone, 3.0 mM. $MgCl_2$, 0.4 mM. $NADP^{+}$, 5 units of hexokinase, 1.0 mM. glucose, 5 units glucose-6-phosphate dehydrogenase, 0.5 mg. mitochondrial protein and 500 μM $P^{1}P^{5}$- Di- (Adenosine-

5)- pentaphosphate ($A_{p5}A$) which has been reported
to be a potent inhibitor of adenylate kinase
competitive with both ATP and AMP (Lienhard and
Secemski, 1973), ADP (Feldhanc et al., 1975, Melnic
et al., 1979) and does not affect oxidative phosphory-
lation (Feldhanc et al., 1975 Melnic et al., 1979),
in a 3 ml. final volume. The reaction was started by
the addition of ADP (or AMP in some cases) after
absorption at 340 nM. had stabilized. Increase in
absorbance was continuously recorded spectrophoto-
metrically.

Comparison of the rates of ATP formation by the adenylate kinase reaction and by oxidative phosphorylation

The reaction mixture was similar to that
described for measurement of oxidative phosphorylation
except that $A_{p5}A$ was omitted. The reaction was
started by the addition of varying concentrations of
ADP, and the rate of ATP formation was followed
spectophotometrically by following the rate of $NADP^+$
reduction at 340 nM. Under these conditions the rate
of ATP formation was taken as the combined effects of
adenylate kinase and oxidative phosphorylation.
Inclusion of carboxyatractylate, an inhibitor of
adenine nucleotide transport, allowed separate
measurements of ATP production by adenylate kinase.

The difference in ATP production in the presence and in the absence of carboxyatractylate was taken as the contribution by oxidative phosphorylation.

Preincubation of MgATP submitochondrial particles

MgATP particles were preincubated at $30^{\circ}C$ in a 3 ml. total volume, in a reaction mixture containing (unless otherwise indicated) 25 mM. Tris-acetate buffer, pH 7.5, 125 mM. Sucrose, 5 mM. Tris-succinate, 5 ug. rotenone and 50 - 100 µg. of the MgATP particle protein. Other additions are as indicated in individual experiments. 5µM. CCCP was then added followed by measurement of ATPase activity.

Determination of mitochondrial Mg^{2+}

The Mg^{2+} content of mitochondria was determined after precipitation of mitochondrial protein with perchloric acid as follows:- 50 - 100 µg of mitochondrial protein was suspended in 5 ml. of de-ionized water at room temperature. The suspension was then mixed with 2.5 ml. of 20% perchloric acid dissolved in de-ionized water. The mixture was incubated at room temperature for 5 minutes, and this was followed by a 10 minute centrifugation using a bench centrifuge at maximum speed, to sediment the

denatured protein. The supernatant was carefully decanted without disturbing the mitochondrial protein pellet, and this was used for Mg^{2+} assay.

Assay of the Mg^{2+} content was carried out using a Beckman atomic absorption spectrophotometer. Absorption measurements were carried out in accordance with the instrument manual instructions, using stand-ard $MgCl_2$ solutions dissolved in de-ionized water.

Determination of the adenine nucleotides

The adenine nucleotides were assayed after precipitation of mitochondrial protein with perchloric acid, followed by neutralization with 5 M. potassium hydroxide as follows:-

500 µl. of the adenine nucleotide containing reaction mixtures was diluted to 1 ml. in ice cold distilled water. The samples were then mixed with 0.5 ml. of ice cold 15% perchloric acid, and the mixture was incubated for 3 minutes in an ice bath. After the incubation the samples were then neutralized by dropwise addition of 5 M. potassium hydroxide to a pH of between 6 and 7 (indicator paper). After this 0.5 ml. of 1M. Tris-HCl buffer, pH 7.5 was added. The samples were then centrifuged for 10 minutes, using a bench centrifuge in the cold room to

sediment the potassium perchlorate and the denatured proteins. The supernatant was carefully decanted and used for adenine nucleotide assay.

Assay of the individual adenine nucleotides, i.e. ATP, ADP and AMP was carried out using a coupled enzyme as described by Williamson and Corkey (1969). For ADP and AMP determination the reaction mixture at room temperature contained 100 mM. Tris-HCl buffer, pH 7.5, 133 mM KCl, 3 mM. $MgCl_2$, 0.2 mM. NADH, 1 mM. phosphoenol pyruvate and 5 units of lactate dehydrogenase and between 200 and 500 μl. of the adenine nucleotide containing sample in a 3 ml. total volume. The reaction was started by the addition of 5 units pyruvate kinase. Decrease in absorption was followed spectrophotometrically at 340 nM. using a Unicam SP 1800 Ultraviolet spectrophotometer attached to a Unicam AR 25 linear recorder. This decrease in absorption is due to ADP. After the absorption decrease had stopped 5 units of myokinase was added. This gave a further decrease in absorption which was due to AMP.

ATP was determined in a reaction mixture containing 100 mM. Tris-HCl buffer, pH 7.5, 133 mM KCl, 3.0 mM. $MgCl_2$, 1.0 mM. glucose, 0.5 mM. $NADP^+$, 5 units glucose-6-phosphate dehydrogenase and

200-500 µl. of the sample in a 3 ml. final volume at room temperature. The reaction was started by the addition of 5 units hexokinase, and increase in absorption was followed spectrophotometrically at 340 nM.

The amounts of ADP, AMP and ATP in the samples were calculated using 6.22 as the absorption coefficient of both NADH and $NADP^+$.

Determination of nicotinamide adenine nucleotide content

Nicotinamide adenine nucleotide content was determined after precipitation of the mitochondrial protein with perchloric acid followed by neutralization with 5M. KOH as described for the determination of adenine nucleotides.

NAD^+ and $NADP^+$ were determined using an Aminco-Bowman spectro-flourimeter at an excitation wavelength of 360 nM. and an emission wavelength of 450 nM. The incubation medium contained 25 mM. Tris-HCl buffer, pH 8.4, semicarbazide 0.025% (w/v) ethanol 5% (v/v), 5 mM. glucose-6-phosphate and 500 µg. of the neutral extract in a final volume of 1 ml. NAD^+ reduction was initiated by the addition of 5 units

alcohol dehydrogenase. After reduction of all the NAD$^+$, 5 units glucose-6-phosphate dehydrogenase was added, to reduce the NADP$^+$.

For all the determinations, fluorescent units were calibrated with standard solutions of NADH assayed spectrophotometrically.

Measurement of mitochondrial bivalent cation transport

Mitochondrial bivalent cation uptake was measured by following the rate of stimulation of oxygen uptake using Clark - type oxygen electrode attached to a recorder. The reaction mixture at 30°C. contained (unless otherwise indicated) 50 mM. Tris-HCl buffer, pH 7.5, 0.25 M. sucrose, 100 mM. KCl, 5 mM. malate, 5 mM. glutamate and 2 - 3 mg. of mitochondrial protein in 3 ml. final volume. Stimulation of oxygen uptake was initiated by addition of the cations.

Alternatively bivalent cation uptake was measured spectrophotometrically using an Aminco DW-2 dual wavelength spectrophotometer, attached to a recorder in a reaction mixture containing 50 mM. Tris-HCl buffer, pH 7.5, 0.25 M. sucrose, 100 mM.

KCl, 30 mM. Tris-phosphate, pH 7.5, 5 mM malate, 5 mM glutamate 100 μM Arrenazo III as an indicator of extramitochondrial cations, (Harris, 1979) and the indicated amounts of the cations in a 3 ml. final volume. The reaction was started by the addition of 1 mg. of mitochondrial protein, and change in optical density was recorded continuously at 665 and 685 nM.

Protein determination

For intact mitochondria and membrane proteins, protein determination was carried out by the Biuret method (Jacobs et al., 1956) in the presence of 3% deoxycholate. For soluble proteins like those of the mitochondrial intermembrane space, protein determination was carried out using the method of Lowry, et al., (1951). In both cases bovine serum albumin was used as the standard.

C H A P T E R   III

CHARACTERIZATION OF THE RAT LIVER MITOCHONDRIAL INTER MEMBRANE SPACE WITH SPECIAL REFERENCE TO THE ENZYME ADENYLATE KINASE

Table I shows the activities of various marker enzymes recovered from isolated intermembrane space contents as a percentage of the total mitochondrial activity. It is evident from the data that the mitochondrial intermembrane space contains the bulk of the mitochondrial adenylate kinase, and is virtually free from marker enzymes of the outer membrane (monoamine oxidase) inner membrane (cytochrome $\underline{c}$ oxidase) and matrix (malate dehydrogenase). In addition it was found that the activity of adenylate kinase in intact mitochondria was unaffected by the presence of 0.1 mM. carboxy-atractylate, which completely inhibited respiration stimulation induced by ADP, providing further evidence for its localization in the intermembrane space. The activity of the enzyme in intact mitochondria was unaffected by trypsin, while the enzyme solubilized after disruption of the outer membrane was highly trypsin sensitive, thus ruling out localization of the enzyme on the outer surface of the outer mitochondrial membrane. It thus seems apparent from

TABLE I

Marker enzyme activities recovered from isolated
intermembrane space fraction of rat liver
mitochondria

The intermembrane space of rat liver mitochondria
was isolated according to Bogucka and Wojtczak (1976)
and the enzyme activities were determined as described
in materials and methods.

The maximum velocity of the forward reaction of
the adenylate kinase was 1100 nMoles ADP formed per
minute per mg. of mitochondrial protein.

Marker enzyme	Percent activity recovered in the intermembrane space
Adenylate kinase	94
Mono amine oxidase	0
Cytochrome oxidase	0
Malate dehydrogenase	6

these results that adenylate kinase is a soluble enzyme in the mitochondrial intermembrane space. The possibilities that it may be loosely attached to the inner surface of the outer membrane, or the outer surface of the inner membrane are unlikely in that very little activity was recovered in the outer membrane or the mitoplast fractions. The little activity present in these fractions could have been due to non-specific binding.

Table II shows that the intermembrane space contained 8% of the total mitochondrial protein. In addition it contained 35% of the mitochondrial cytochrome c which is in agreement with an earlier report by Pfaff and Schwalbach (1967) who found that 35 - 40% of the mitochondrial cytochrome c was solubilized upon disruption of the outer membrane. This probably represents cytochrome c that is loosely bound to the outer surface of the inner mitochondrial membrane. The intermembrane space was virtually free of nicotinamide nucleotides, but contained about 10% of the total mitochondrial adenine nucleotides (NB. total mitochondrial adenine nucleotide content was 12 nMoles per mg. of mitochondrial protein). In addition it contained 47% of the mitochondrial Mg^{2+}, which is in agreement with data reported by Bogucka and Wojtczak (1981) who reported that about half of the mitochondrial Mg^{2+} is present in the intermembrane space fraction. Both the adenine

TABLE II

Recovery of certain mitochondrial components

from isolated intermembrane space of rat liver

mitochondria

The intermembrane space fraction of rat liver

mitochondria was isolated according to Bogucka and

Wojtczak (1976) and the various components were

assayed as described in materials and methods.

Mitochondrial component	Percent recovered in the intermembrane space fraction
Adenine nucleotides	10
Nicotinamide nucleotides	0
Mg^{2+}	47
Cytochrome c	35
Total protein	8

nucleotides and the Mg^{2+} found in the intermembrane space are present in some bound form as they could not be washed even after prolonged incubation of the mitochondria with 0.25 M. sucrose. However, as table III indicates, the presence of substances that can chelate Mg^{2+}, including EDTA, ATP and ADP caused release of about half the mitochondrial Mg^{2+}.

DISCUSSION

These results show that in agreement with earlier reports (Criss, 1970, Brdieczka, 1968) the mitochondrial adenylate kinase is located in the mitochondrial outer compartment, and specifically exists as a soluble enzyme in the intermembrane space. The enzyme is believed to play a major role in adenine nucleotide interconversions between energy generating and energy utilizing reactions, and thereby regulating the adenylate energy charge (Atkinson and Champman 1979). Its location in the mitochondrial intermembrane space is very precise for this function since the enzyme is always in contact with adenine nucleotides shuttling between the mitochondrial matrix and the cytosol, and vice-versa.

TABLE III

Release of mitochondrial Mg^{2+} upon incubation under different conditions

Rat liver mitochondria were incubated in a medium containing 0.25 M. sucrose, 50 mM. Tris-HCl buffer, pH 7.5, and 0.5 mg. mitochondrial protein in a 1 ml. volume for 10 minutes, at 30°C. Other additions are as indicated in the table. After the incubation the samples were centrifuged at 1200 x g for 20 minutes and the Mg^{2+} content of the supernatant was determined by atomic absorption. The Mg^{2+} content of the mitochondria prior to the incubation was approximately 30 nMoles per mg. protein.

Additions	% of total Mg^{2+} released
None	0
2 mM EDTA	51.3
2 mM ADP ± CAT	49.3
2 mM ATP ± CAT	48.0

The intermembrane space also contains bound adenine nucleotides, which as will be discussed in chapter V are capable of initiating the activity of adenylate kinase under varying energy conditions.

The space also contains large quantities of bound Mg^{2+}, which is a necessary cofactor in the activity of adenylate kinase and other adenine nucleotide metabolising enzymes present in this space such as nucleoside monophosphokinase and nucleoside diphosphokinase.

C H A P T E R   IV

PROPERTIES OF RAT LIVER MITOCHONDRIAL ADENYLATE
KINASE

a) Kinetic parameters

The kinetic parameters of the mitochondrial
adenylate kinase were assayed under conditions when
initial concentrations of the substrates were
systematically varied, and some or all the products
were kept at near zero concentration by a trapping
system. The results of the measurements are presented
in figures 2, A-C. It is evident from the plots
that at non-saturating initial concentrations of
adenine nucleotides, the initial reaction velocity
increases with increase in adenine nucleotide
concentrations. However, at high concentrations the
reaction velocity levelled off for both ADP and ATP,
while high concentrations of AMP had an inhibitory
effect. This inhibition was seen at an AMP concent-
rations higher than 0.5 mM. Double reciprocal
plots drawn from the kinetic data showed that the
K_m values for AMP and ATP were 50 and 70 μM
respectively, and the maximum velocity for the
forward reaction was 1100 nMoles ADP formed per
minute per mg. of mitochondrial protein. In
the reverse reaction the K_m value for ADP was 180 μM

FIGURE 2 (A) and (B)

Kinetic parameters of the mitochondrial
adenylate kinase

The effect varying initial concentrations of
AMP, and ATP on the initial reaction velocity of
the mitochondrial adenylate kinase was determined
by following the rate of ADP formation in a reaction
mixture consisting of 50 mM. Tris-HCl buffer pH. 7.5,
0.25 M. sucrose, 133 mM. KCl, 3 mM. $MgCl_2$, 1 mM.
phosphoenolpyruvate, 5 units lactate dehydrogenase,
5 units pyruvate kinase, 5 ug. oligomycin, 0.1 mM.
NADH and 0.4 mg. of rat liver mitochondrial protein
in a 3 ml. final volume. The reactions were started
by the addition of AMP and assayed by following the
rate of NADH oxidation spectrophotometrically at
340 nM. A unit of enzyme activity was taken as the
number of uMoles of NADH oxidized per minute per mg.
of mitochondrial protein. In·Figure 2A the concentra

In Figure 2A the concentration of ATP was fixed
at 0.7 mM while in Figure 2B the concentration of AMP
was fixed at 0.3 mM.

2A

$\dfrac{1}{\text{Enzyme Activity}}$

$-\dfrac{1}{K_m}$

$\dfrac{1}{[ATP]}$ mM.

Enzyme Activity vs. ATP Conc. mM.

2B
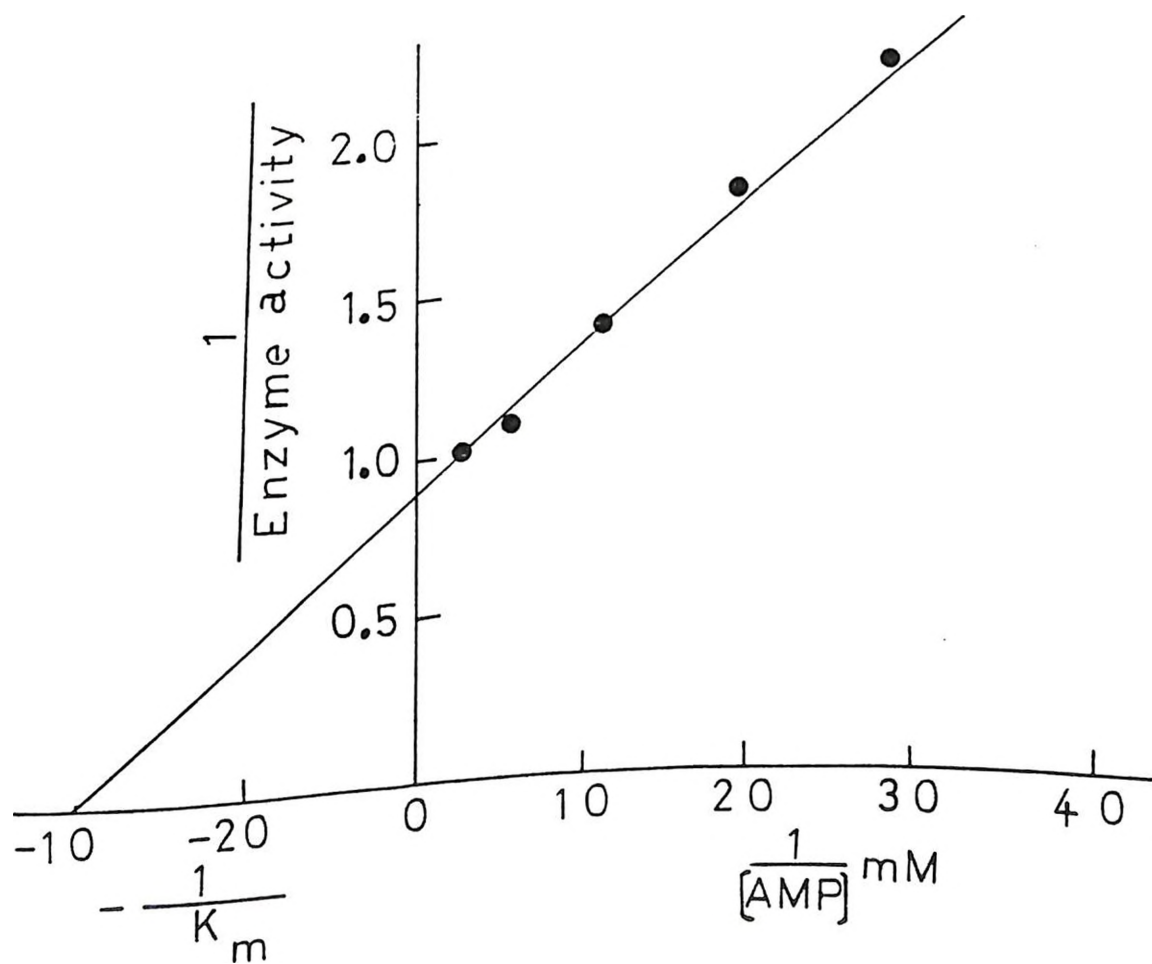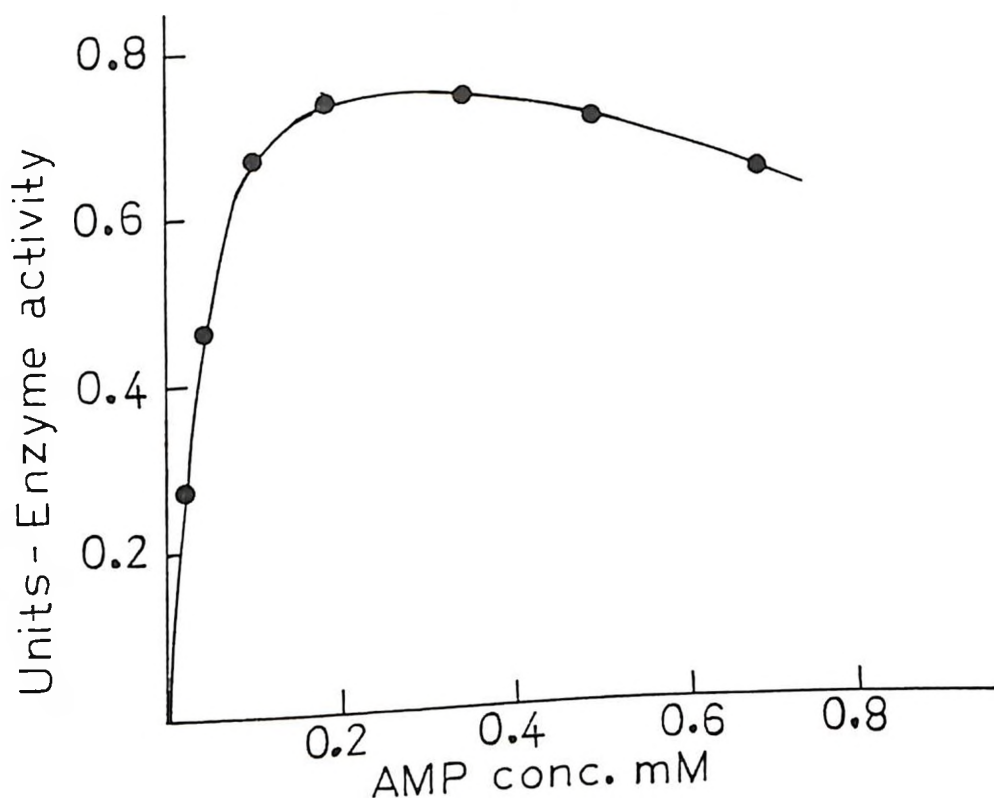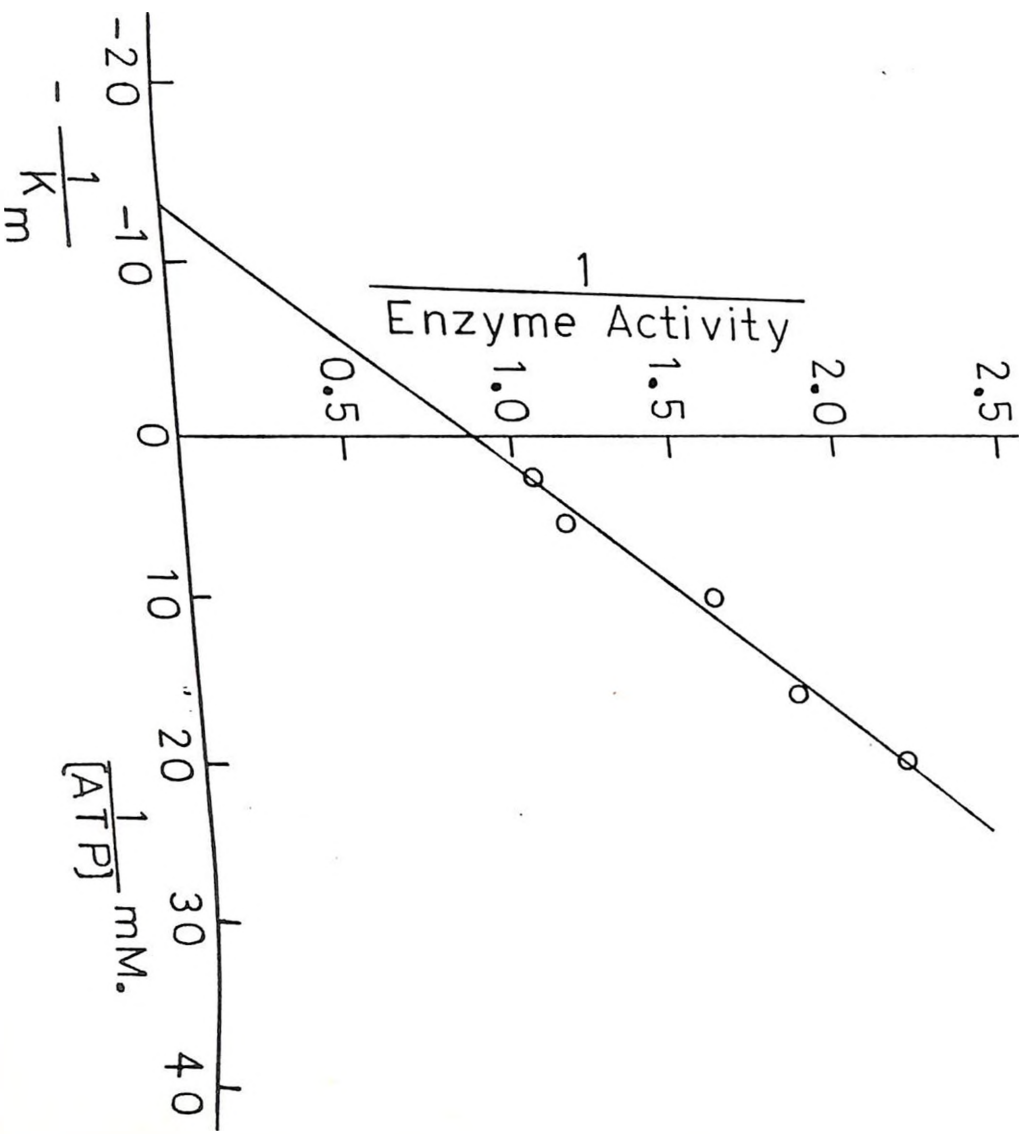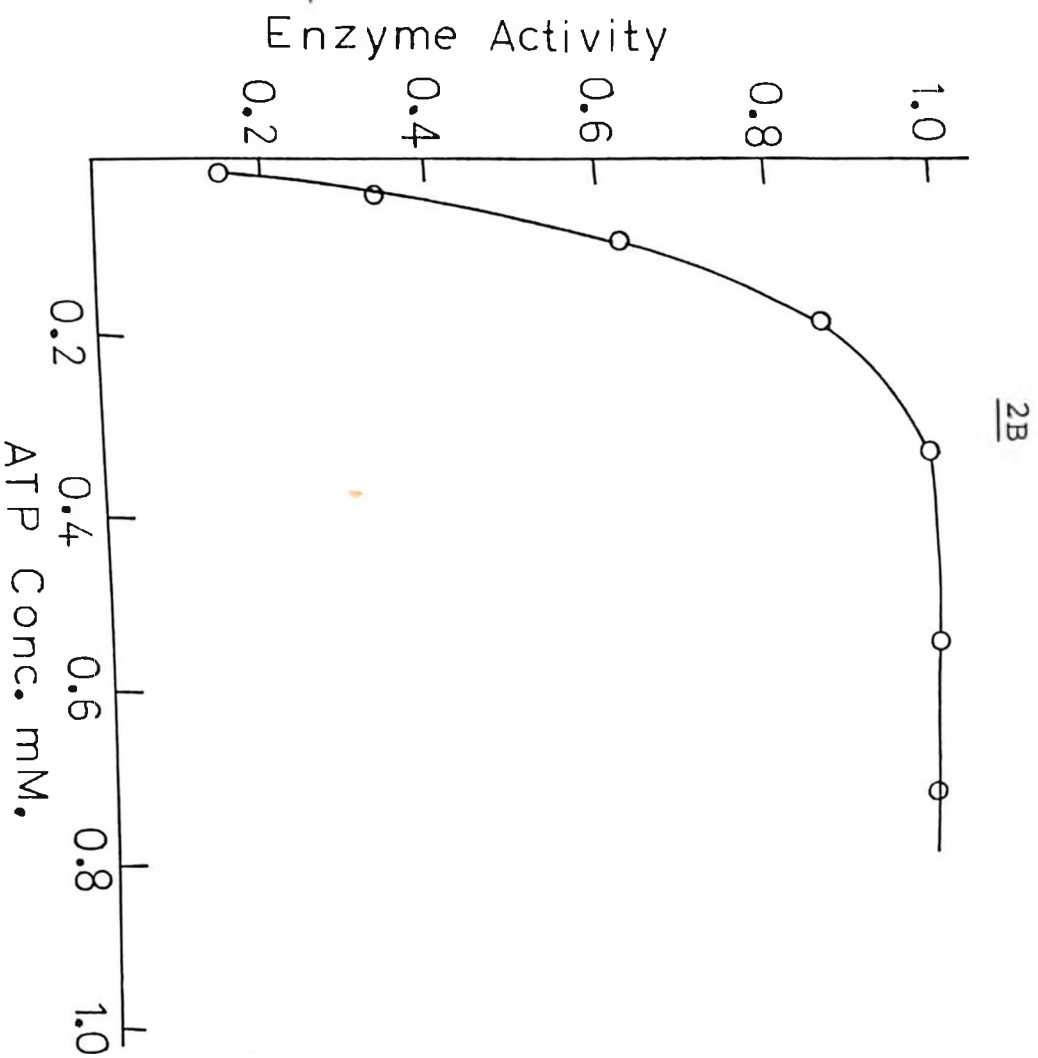
FIGURE 2 (C)

Kinetic parameters of the mitochondrial

adenylate

The effect of varying initial concentrations of

ADP on the initial reaction velocity of the mitocho-

ndrial adenylate kinase was determined by following

the rate of ATP formation in a reaction mxiture

containing 50 mM. Tris-HCl buffer pH 7.5, 3.0 mM.

$MgCl_2$, 0.4 mM. $NADP^+$, 1.0 mM. glucose, 5 units

glucose-6-phosphate dehydrogenase, 5 units hexokinase,

5 ug. oligomycin, and 0.4 mg. of rat liver mitochond-

rial protein at 30°C in a 3 ml. final volume. The

reaction was assayed by following the rate of $NADP^+$

reduction at 340 nM. A unit of enzyme activity was

taken as the number of uMoles of $NADP^+$ reduced per

minute per mg. of mitochondrial protein.

2C
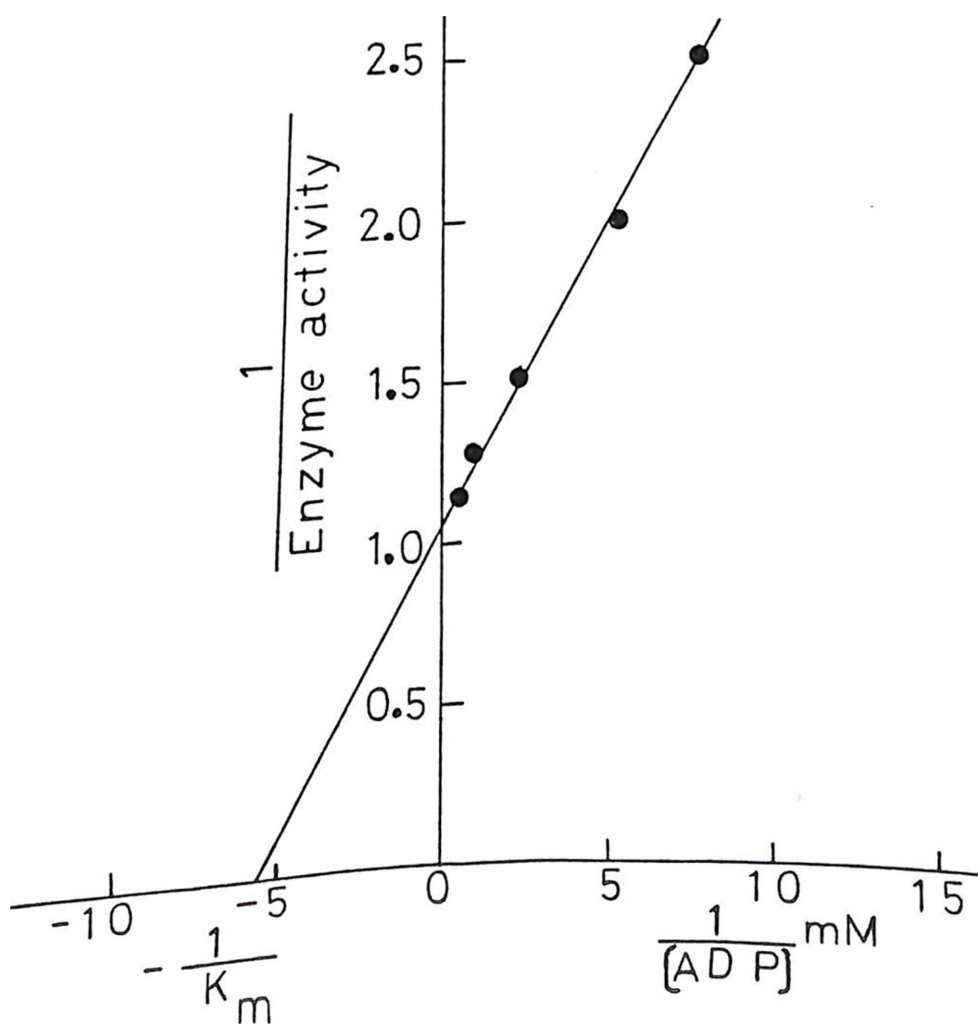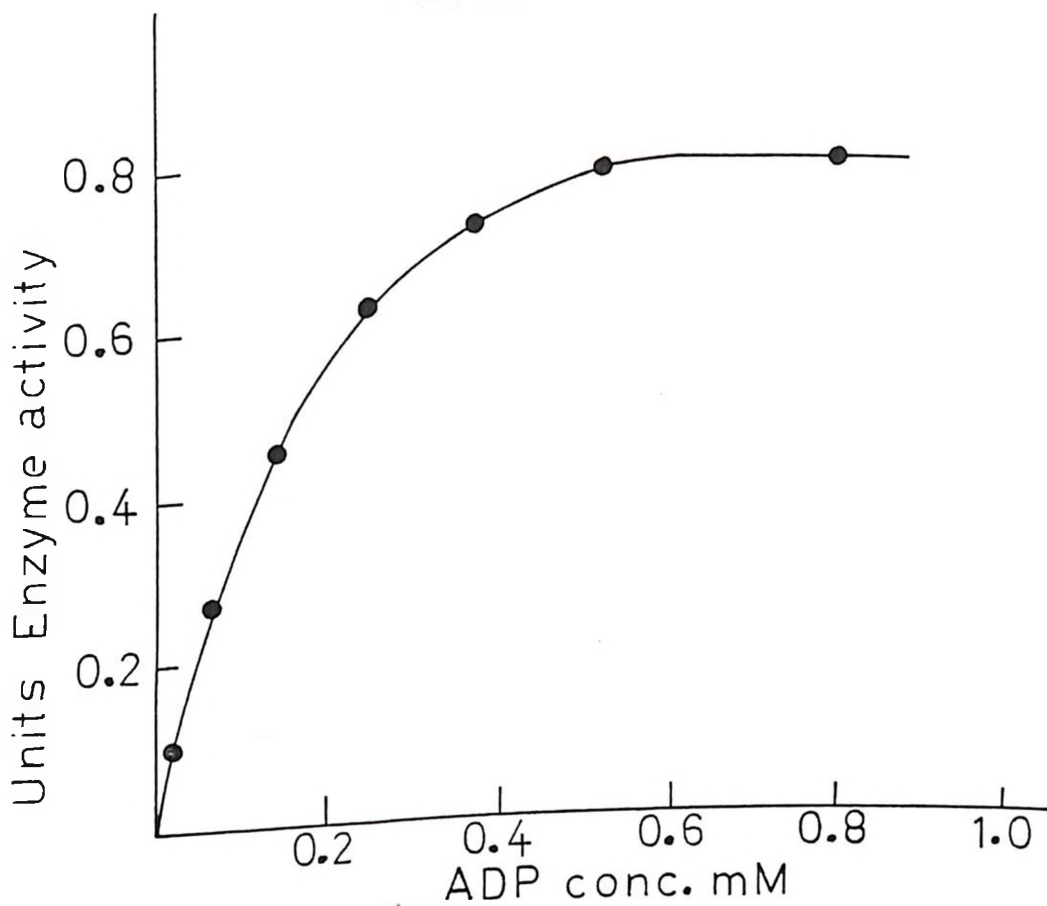
with a maximum velocity of 900 nMoles ADP utilized per minute per mg. of mitochondrial protein.

b) Nucleotide specificity

Table IV shows the effect of various substrates on adenylate kinase activity. As the data indicates, adenylate kinase has a high specificity for the adenine nucleotides. In the presence of ATP the monophosphate site had a high degree of specificity. Other nucleoside monophosphates tested including GMP and CMP had no activity. The triphosphate site was less specific although other nucleoside triphosphates tested including GTP, CTP and UTP had only between 15 - 20% of the ATP activity.

c) Effect of H^+ concentration on the adenylate kinase activity

Figure 3 shows the effect of pH on adenylate kinase activity. It can be seen that, for the forward reaction, the optimum pH is at pH 6.5, while for the reverse it is between 7.0 and 8.0. It is also evident that the forward reaction is more sensitive to alterations of pH than the reverse reaction.

d) Effect of Mg^{2+} concentration on the adenylate kinase reaction

TABLE IV

Effect of various substrates on adenylate kinase

activity of rat liver mitochondria

The reaction mixture at 30°C consisted of 50 mM.
Tris-HCl buffer, pH 7.5, 0.25 M. sucrose, 133 mM. KCl
3.0 mM. $MgCl_2$, 1.1 mM. phosphoenol pyruvate, 5 units
pyruvate kinase, 5 units lactate dehydrogenase, 0.1
mM. NADH, 5 μg oligomycin, 5μg rotenone and 0.4 mg.
mitochondrial protein in a final volume of 3.0 ml.
The reaction was assayed by following the oxidation
of NADH spectrophotometrically at 340 nM.   The
concentration of the nucleoside triphosphates was
0.7 mM while that of the nucleoside monophosphates
was 0.3 mM.

Substrates	Adenylate kinase activity nMoles/min/mg. protein
ATP + AMP	1100
ATP + GMP	0
ATP + CMP	0
AMP + GTP	220
AMP + CTP	197
AMP + UTP	165

FIGURE 3

Effect of H⁺ concentration on the adenylate kinase activity

The effect of pH on the forward —o—o— and the reverse —●—●— reactions of the adenylate kinase were tested by following the rates ADP formation (forward reaction) and ATP formation (reverse reaction) in an assay medium similar to that described for figure 1, except that the concentrations of AMP and ATP were kept constant at 0.3 mM and 0.7 mM, respectively for the forward reaction, and the concentration of ADP was kept constant at 1.0 mM for the reverse reaction. The pH of the buffer was varied between 5.5 and 9.0.
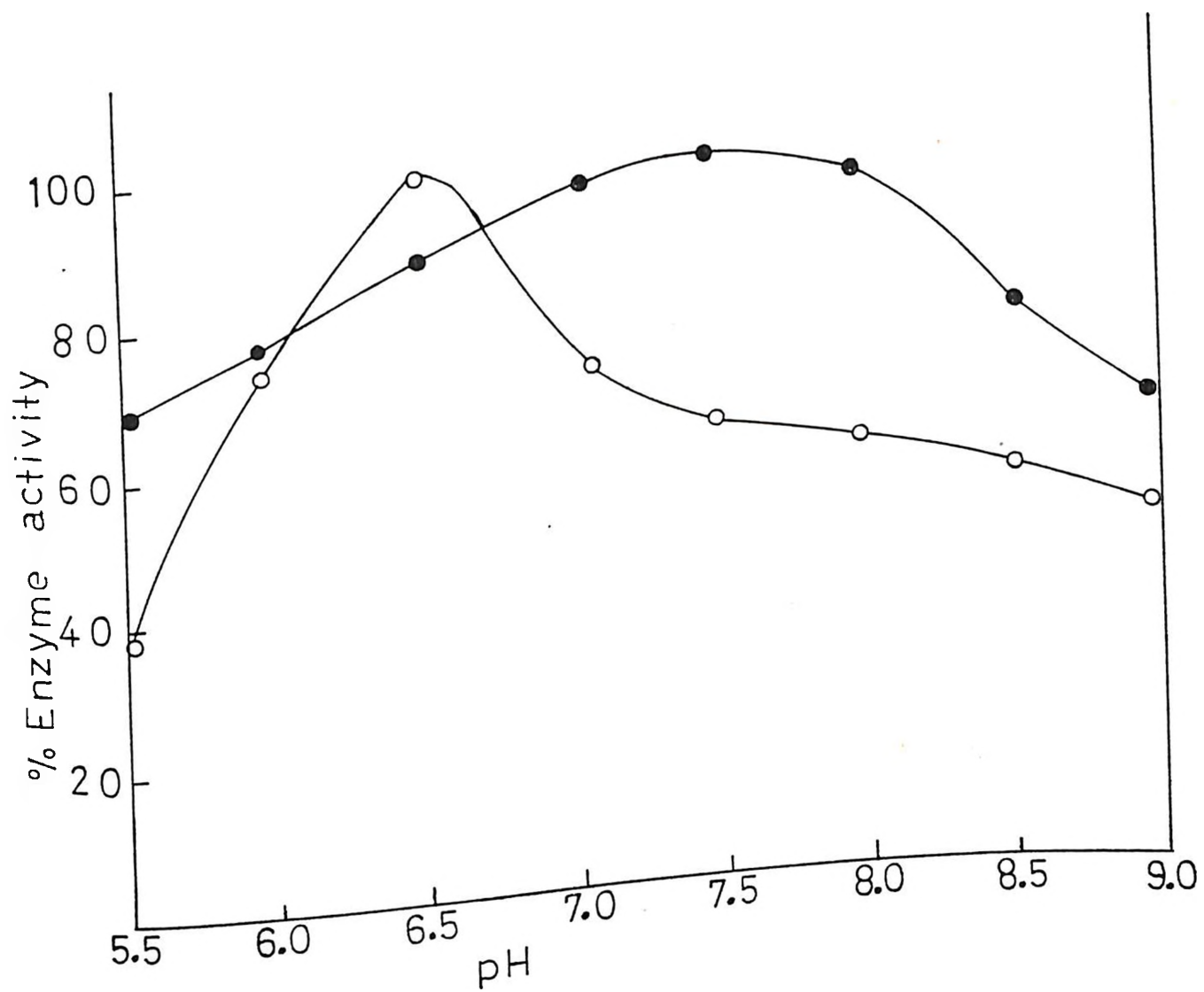
Figure 4 shows the effect of Mg^{2+} on the forward reaction of the adenylate kinase reaction. The reaction velocity increases with increase in the Mg^{2+} concentration upto a Mg^{2+} concentration of 3.0 mM, after which the reaction velocity levels off.

e) Effect of Mg^{2+} concentration on the equilibrium constant of the adenylate kinase reaction

Figure 5 shows the effect of Mg^{2+} concentration on the equilibrium constant of the adenylate kinase reaction. It is evident from the plot that the equilibrium constant increases with increase in Mg^{2+} concentration upto 2.0 mM and then drops at higher concentrations.

DISCUSSION

The results of figures 2 A-C show that the mitochondrial adenylate kinase obeys the Michaelis-Menten Kinetics. The reaction rates increase exponentially with increase in substrate concentration, until the enzyme becomes saturated. For both ATP and ADP, the reaction rate levels off at high nucleotide concentrations. However, with AMP the reaction rate falls at high concentrations. A similar inhibition

FIGURE 4

Effect of Mg^{2+} concentration on the forward
reaction of adenylate kinase

The reaction mixture at 30°C contained 50 mM
Tris-HCl buffer, pH 7.5, 133 mM. KCl, 1 mM phospho-
enol pyruvate, 5 units lactate dehydrogenase, 5
units pyruvate kinase, 5 µg. oligomycin, 3 ug
rotenone, 0.1 mM NADH, 0.3 mM AMP, 0.7 mM ATP,
varying concentrations of MgCl$_2$ and 0.4 mg. rat
liver mitochondrial protein in a 3 ml final volume.
The reaction was started by the addition of AMP, and
enzyme activity was assayed by following the rate of
NADH oxidation spectrophotometrically at 340 nM.
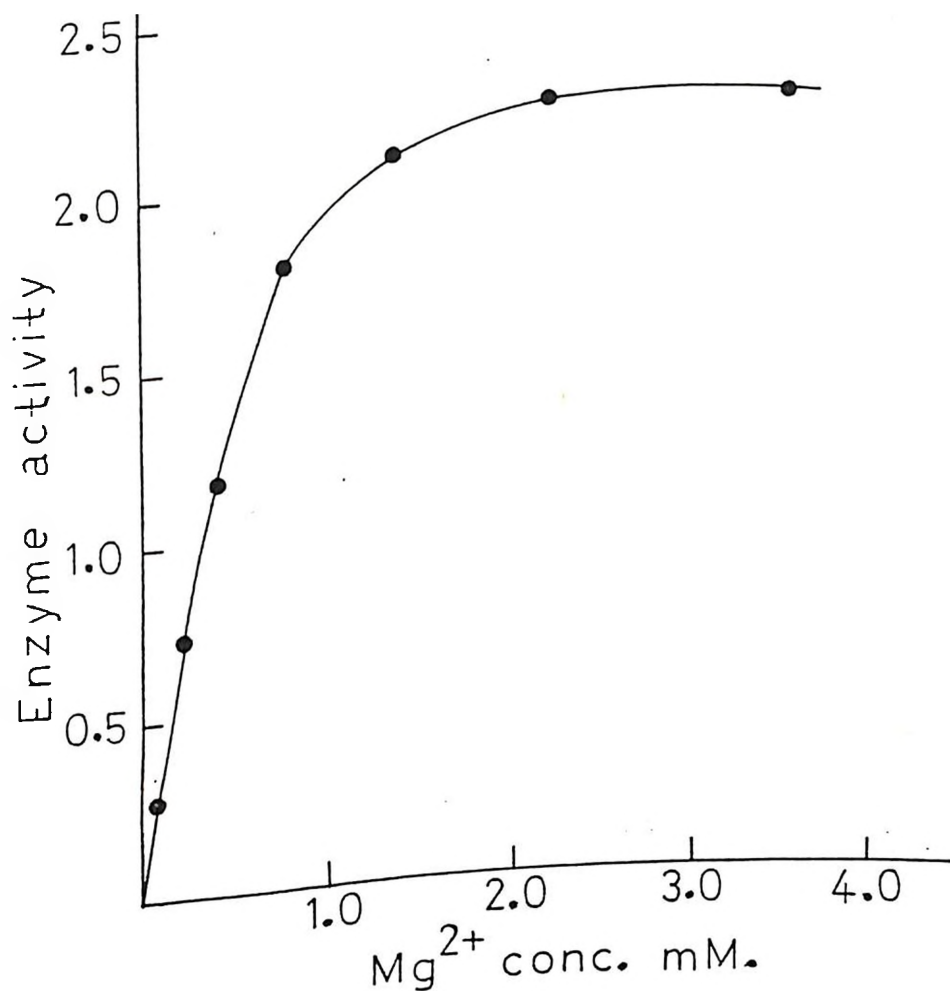
FIGURE 5

Effect of Mg^{2+} concentration on the equilibrium constant of the adenylate kinase reaction

The reaction mixtures at $30^{\circ}C$ contained 50 mM Tris-HCl buffer, pH 7.5, 0.25M. sucrose, 133 mM KCl, 0.3 mM AMP, 0.3 mM ATP, 0.3 mM ADP, 5 μg oligomycin, 0.4 mg. of rat liver mitochondrial protein and varying concentrations of $MgCl_2$. The reaction mixtures were incubated for one hour, and the reaction was stopped by the addition of perchloric acid to a 5% final concentration, followed by neutralization with 5 M. KOH. The concentration of AMP, ATP and ADP in the various samples was then assayed, as described in material and methods.
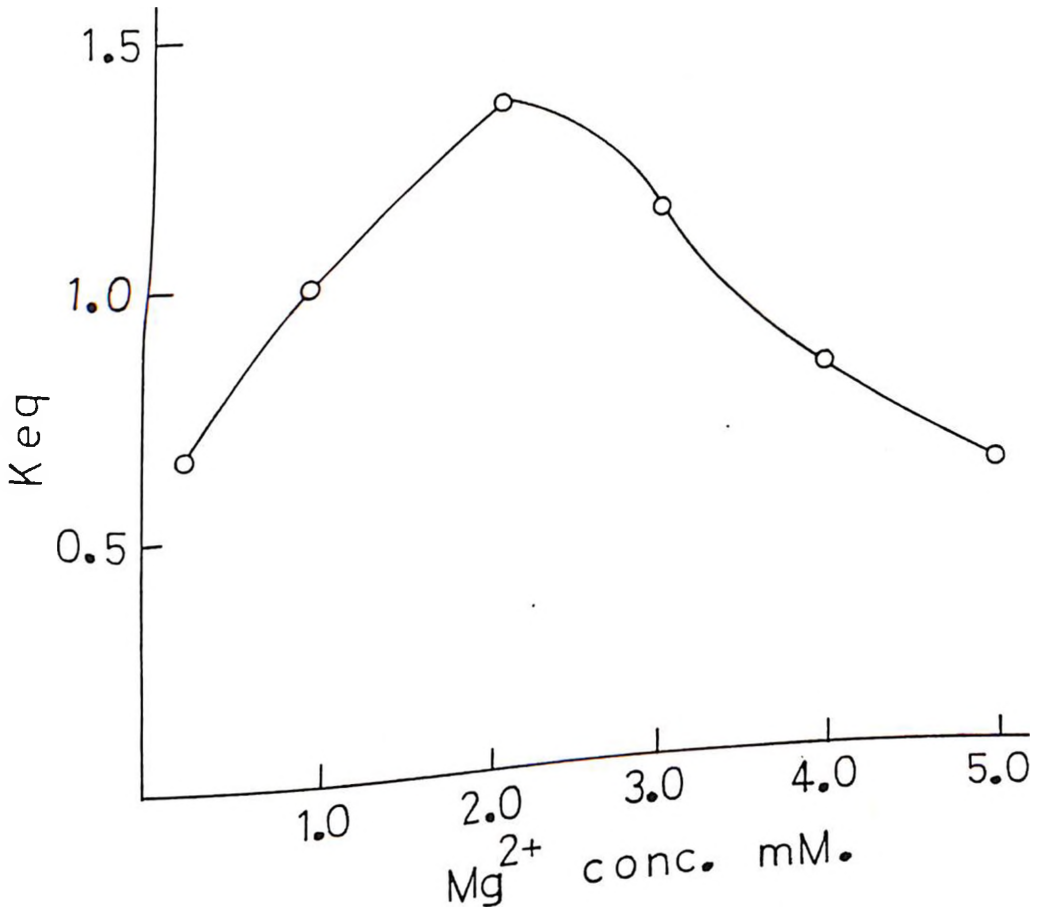
of the adenylate kinase activity by high levels of
AMP was reported earlier with the purified enzyme
(Tamura et al, 1980; Font and Gautheron, 1980). The
reason for this inhibition is most probably related to
the fact that, the enzyme active site has two
subsites (Noda, 1962) one for MgATP or MgADP, and
one for the uncomplexed ADP or AMP. In this view it
may be argued that at high AMP concentrations, and
excess Mg^{2+}, part of the AMP will bind to Mg^{2+} to
form MgAMP which could then compete with MgATP for
the MgATP subsite, thus lowering the availability of
ATP to the enzyme. The enzyme has much lower K_m
values for both ATP and AMP, than for ADP showing
that at least under the experimental conditions the
forward reaction is favoured.

Adenylate kinase was also found to have a very
high specificity for AMP, while it was less specific
for the nucleoside triphosphates. This means that
other nucleoside triphosphates can be used to
regenerate ADP from AMP, when the supply of ATP is
low.

Both the activity and the equilibrium position
of the adenylate kinase were seen to be highly depen-
dent on the Mg^{2+} concentration. At low Mg^{2+}
concentration, the equilibrium constant increased with
increase in the Mg^{2+} concentration, and then declined

at high Mg^{2+} concentration. This effect is most
probably related to the fact that the stability
constant of MgATP. is higher than that for MgADP
(Watts, 1973). At low Mg^{2+} concentrations, most of
the Mg^{2+} will be bound to ATP, thus favouring the
the forward reaction, while at higher Mg^{2+} concentra-
tions a substantial amount of Mg^{2+} will be bound to
ADP, increasing the rate of the reverse reaction.

C H A P T E R   V

UTILIZATION OF INTERMEMBRANE SPACE ADENINE NUCLEOTIDES AND Mg^{2+} BY ADENYLATE KINASE

As mentioned in Chapter III, the mitochondrial intermembrane space contains bound adenine nucleotides and Mg^{2+}. The bound ATP can be used for initiating the activity of adenylate kinase upon addition of external AMP. However, due to the small amounts of ATP present it is necessary to have an ATP regenerating system in order to observe the reaction. The regenerating systems that can be used include:-

Pyruvate kinase and phosphoenol pyruvate

Figure 6(A) is a control experiment showing that addition of AMP and ATP to intact mitochondria induces an immediate activity of adenylate kinase.

In Figure 6(B) AMP was added to intact mitochondria in the absence of added ATP, but in the presence of pyruvate kinase and phosphoenol pyruvate. The trace shows that there was a lag period before activation of a maximum steady state activity of adenylate kinase.

FIGURE 6

Utilization of intramitochondrial adenine nucleotides by adenylate kinase with pyruvate kinase and phosphoenol pyruvate as an ATP regenerating system

In (A) the reaction mixture at 30°C contained 50 mM Tris-HCl buffer, pH 7.5, 133 mM. KCl, 3 mM $MgCl_2$, 1 mM phosphoenol pyruvate, 5 units pyruvate kinase, 5 ug. oligomycin, 5 μg rotenone, 0.1 mM NADH, 0.7 mM ATP and 0.4 mg rat liver mitochondrial protein in a 3 ml final volume. The reaction was started by the addition of 0.3 mM AMP, and was assayed by following the rate of NADH oxidation spectrophotometrically at 340 nM.

In (B) the reaction mixture was similar to that described for (A) except that ATP was omitted. The reaction was started by the addition of 0.3 mM AMP.

In (C) the reaction was similar to that described for (A) except that ATP was omitted, and the mixture was preincubated for 10 minutes before addition of AMP

AMP →

(A)

AMP →

(B)

$A_{340} = 0.1$

1 Min.

AMP →

(C)
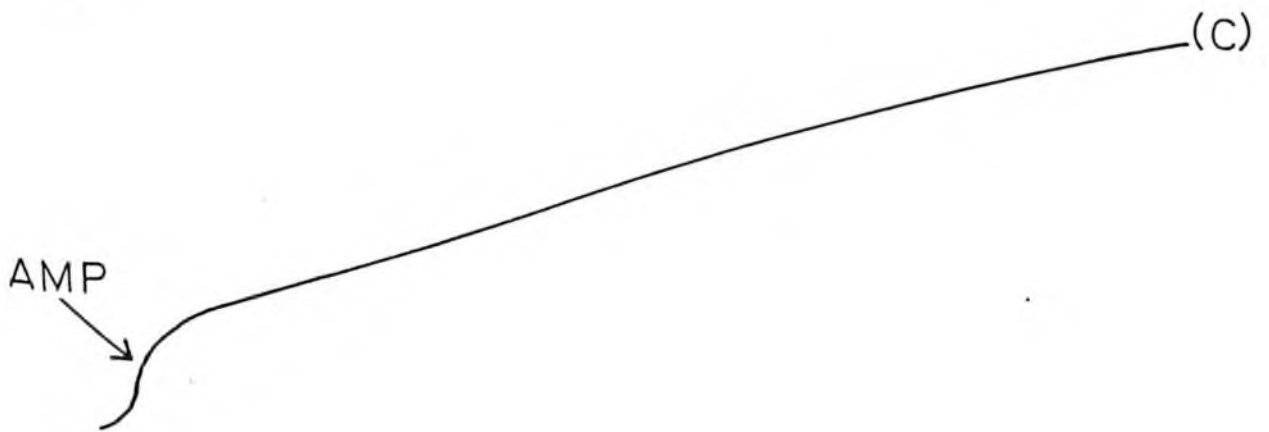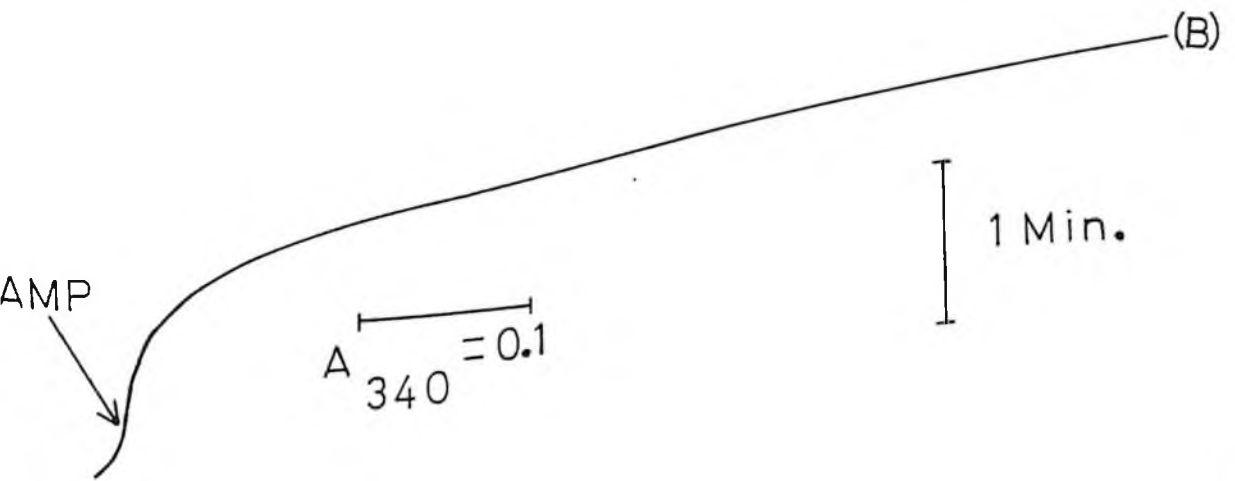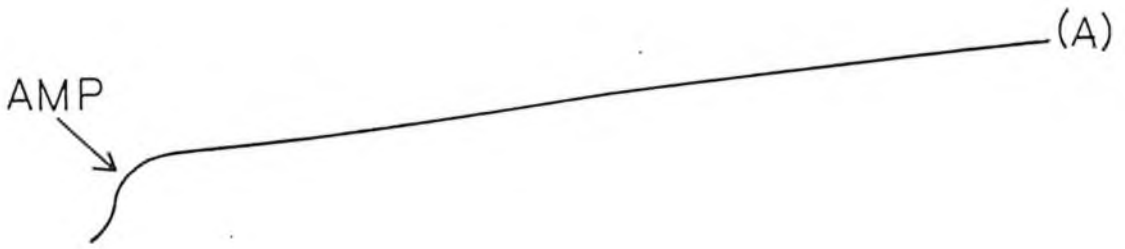
In Figure 6(C) intact mitochondria were
preincubated for 10 minutes in the presence of
pyruvate before addition of AMP. The trace shows
that addition of AMP after the preincubation induced
a maximum steady state activation of the adenylate
kinase after a very short lag period (cf. fig. 6B).

The reactions illustrated in figures 6(A - C)
were insensitive to carboxyatractylate.

Oxidative phosphorylation

Figures 7(A - E) are oxygen electrode recordings
of rat liver mitochondria respiring under various
conditions. Trace A is a control experiment showing
the stimulation of respiration upon addition of
ADP.

Trace B shows that, like ADP, AMP is also able
to stimulate mitochondrial respiration. The respira-
tory control ratio obtained with AMP is similar to
that obtained with ADP whereas the AMP/O ratio is
half the ADP/O ratio.

Trace C shows that the respiratory stimulation
induced by AMP is inhibited by EDTA or P^1P^5-Di-
(adenosine-5') pentaphosphate, a specific inhibitor
of adenylate kinase (Lienhard and Secemski, 1973)

FIGURE 7

Utilization of intramitochondrial adenine
nucleotides by adenylate kinase with oxidative
phosphorylation as an ATP regenerating system

The incubation mixture at 30°C consisted of
50 mM Tris-HCL buffer, pH 7.5, 100 mM KCl, 0.25 M
sucrose, 3 mM Tris-phosphate and 2.5 mg. rat liver
mitochondrial protein in a 2 ml final volume. The
reaction was assayed by following the rate of
oxygen uptake polarographically using a clark type
oxygen electrode. Additions when indicated were
3 mM Tris-succinate (succ) 170mM ADP, 170 mM AMP,
2 mM EDTA and 2 mM $MgCl_2$. In traces D and E, the
mitochondria were washed with 2 mM EDTA before
incubation.

(A) Succ. ADP

(B) Succ. AMP ADP

(C) Succ. AMP EDTA or Ap$_5$A ADP AMP

(D) Succ. AMP ADP

(E) Succ. AMP Mg^{2+}

1 Min

$\Delta O_2 =$ 50 nMole atoms
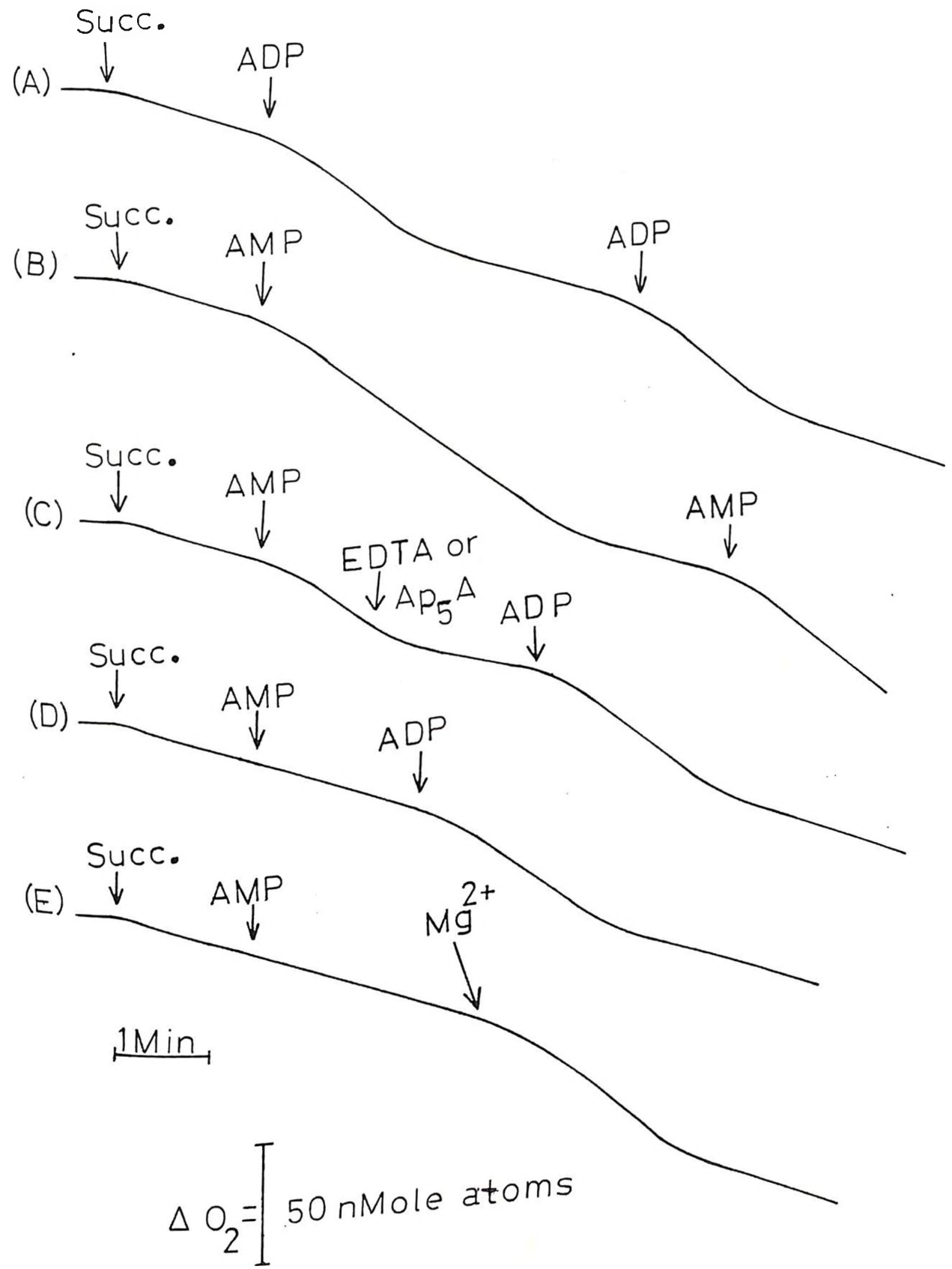
while that of ADP was not inhibited.

Trace D shows that the respiratory stimulation induced by AMP was abolished by prior washing of the mitochondria in the presence of EDTA, while that of ADP was not.

Trace E shows that addition of external Mg^{2+} to EDTA washed mitochondria caused a restoration of the AMP induced respiratory stimulation.

DISCUSSION

These results show that, in agreement with data tabulated in table II, the intermembrane space contains adenine nucleotides which can be used to initiate the adenylate kinase reaction. Due to the small amounts of adenine nucleotides present, the reaction takes some time before a maximum steady state rate is observed during which time the regenerating system makes enough ATP to react with the added AMP. Preincubation of the reaction medium prior to addition of AMP allows conversion of intermembrane ADP to ATP by the pyruvate kinase reaction, thus accelerating the attainment of a maximum steady state rate upon addition of AMP. That the ATP utilized in this reaction comes from the intermembrane space is shown by the fact that the

presence of carboxyatractylate, an inhibitor of the adenine nucleotide carrier has no effect on the reactions.

The results also show that like ADP, AMP is also able to stimulate mitochondrial respiration with a respiratory control ratio similar to that for ADP. This effect of AMP is due to its conversion to ADP by adenylate kinase, using mitochondrial ATP as shown by the fact that its effect was inhibited by the presence of EDTA or P^1P^5-Di-(adenosine-5') pentaphosphate, while that for ADP was not inhibited. The AMP/O ratio was half the ADP/O ratio which is consistent with the fact that one molecule of AMP reacts with one molecule of ATP to form two molecules of ADP. The inhibition of the AMP induced respiratory stimulation is in agreement with data tabulated in Table II which showed that the mitochondrial inter-membrane space contains large amounts of Mg^{2+} which can be used by the adenylate kinase. Prior washing of the mitochondria in the presence of EDTA selectively removes intermembrane space Mg^{2+} as shown by the fact that, the treatment inhibited the AMP induced respiratory stimulation, but had no effect on the ADP induced respiratory stimulation. The transloca-tion of ADP across the mitochondrial inner membrane does not require Mg^{2+} (Klingengberg, 1977) but ATP

synthesis does, indicating that the EDTA treatment did not affect the mitochondrial matrix Mg^{2+}.

These findings thus show that, the mitochondrial adenylate kinase can utilize both bound adenine nucleotides and Mg^{2+} in the intermembrane space for its activity.

<u>C H A P T E R   V I</u>

<u>FUNCTIONAL INTERACTION BETWEEN THE MITOCHONDRIAL</u>

<u>ADENYLATE KINASE AND OXIDATIVE PHOSPHORYLATION</u>

As described in the preceding chapter, like ADP, AMP is also able to stimulate mitochondrial respiration. However, the effect of AMP is dependent on the activity of adenylate kinase which furnishes ADP for the mitochondrial oxidative phosphorylation. The present chapter illustrates how adenylate kinase interacts with oxidative phosphorylation for the efficiency of ATP supply to the cell.

<u>Kinetics of mitochondrial oxidative phosphorylation</u>

Figure 8 shows the effect of varying initial concentrations of ADP and AMP on the initial rates of oxidative phosphorylation, measured as the amount glucose-6-phosphate formed in the presence of hexokinase and glucose-6-phosphate dehydrogenase. The figure shows that the amount of glucose-6-phosphate formed per minute increased with increase in the concentrations of both ADP and AMP, and then levelled off at high concentrations. With succinate the K_m value for ADP was 28 µM, a value that agrees well with earlier data (Chance, 1959) and the

FIGURE 8

Comparison of oxidative phosphorylation with ADP and AMP as the substrates

The reaction mixture, at 30°C contained 50 mM Tris-HCl buffer, pH 7.5, 0.25 M. sucrose, 133 mM KCl, 3 mM $MgCl_2$, 3 mM succinate, 3 mM phosphate, 3 µg rotenone, 0.4 mM $NADP^+$, 1 mM glucose, 5 units hexokinase, 5 units glucose-6-phosphate dehydrogenase and 0.5 mg. rat liver mitochondrial protein, in a 3 ml final volume. The reaction was started by the addition of varying concentrations of ADP ─o─o─ or varying concentrations of AMP ─●─●─, and the rate of glucose-6-phosphate formation was assayed by following the rate of $NADP^+$ reduction spectrophotometrically at 340 nM.
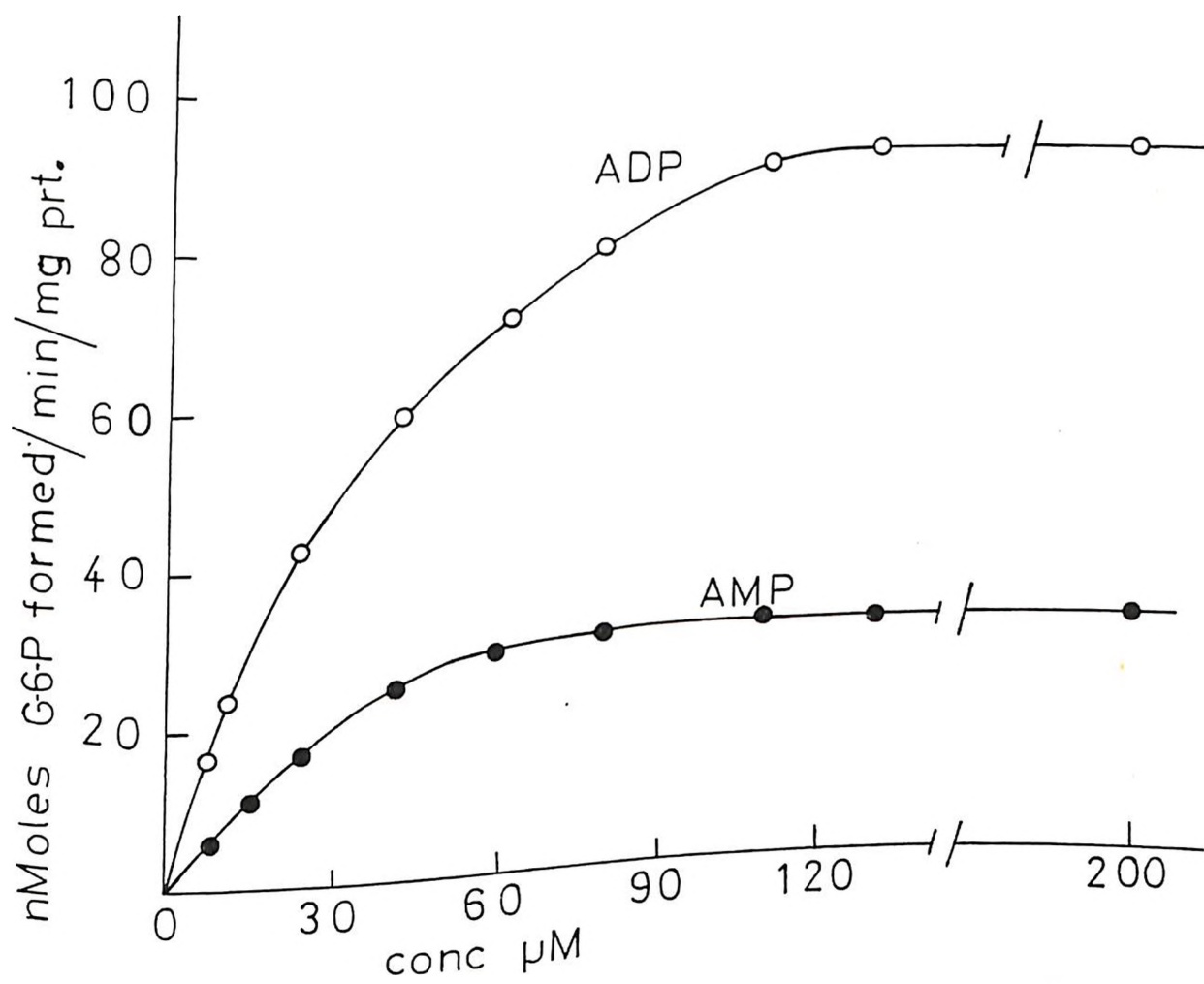
maximum velocity was 91 nMoles ATP formed per minute per mg. of mitochondrial protein. For AMP, the K_m value was apparently 19 μM with a maximum velocity of 25 nMoles ATP formed per minute per mg. of mitochondrial protein.

ATP formation by oxidative phosphorylation and by adenylate kinase

Table V summarises the kinetic parameters of the adenylate kinase together with those of oxidative phosphorylation. It is evident from the table that oxidative phosphorylation has a considerably higher affinity for ADP than adenylate kinase. On the other hand the maximum velocity for the adenylate kinase in either direction is about 10 times higher than that for oxidative phosphoryla- tion. It would thus appear that, in the intact cell, more ATP will be formed by oxidative phosphorylation when ADP concentrations are low, i.e. a high adeny- late charge, while more ATP will be formed by adenylate kinase at high ADP concentrations, i.e. a low adenylate charge. That this is so, is shown in the results of Figure 9 in which the rates of ATP formation by oxidative phosphorylation and by adenylate kinase are compared at varying concentrations of ADP. The data shows that, at low ADP concentra- tions most of the ATP formed comes from oxidative

TABLE V

Kinetic parameters of adenylate kinase and
oxidative phosphorylation in rat liver
mitochondria

Reaction	Substrate	K_m (uM)	V_{max} (nMoles/min/ mg. protein)
Adenylate kinase	AMP	50	1100
	ATP	75	
	ADP	180	900
Oxidative phosphorylation	ADP	28	91
	AMP	19	25

FIGURE 9

Relative contributions of adenylate kinase and oxidative phosphorylation to ATP formation by rat liver mitochondria at varying concentrations of ADP.

The reaction mixture at 30°C contained 50 mM Tris-HCl buffer, pH 7.5, 0.25 M sucrose, 133 mM KCl, 3.0 mM MgCl, 3 mM succinate, 3 mM phosphate, 3 μM rotenone, 0.4 mM NAD^+, 1.0 mM glucose, 5 units hexokinase, 5 units glucose-6-phosphate dehydrogenase and 0.5 mg of mitochondrial protein. The relative contributions of adenylate kinase —o—o— and oxidative phosphorylation —•—•— were determined by including 5 μg carboxyatracylate and 0.5 mM diadenosine pentaphosphate respectively. The rate of ATP formation was assayed by following the rate of $NADP^+$ reduction spectrophotometrically at 340 nM. Total ATP formed —o—o— is expressed in nMoles/ min/mg. protein.
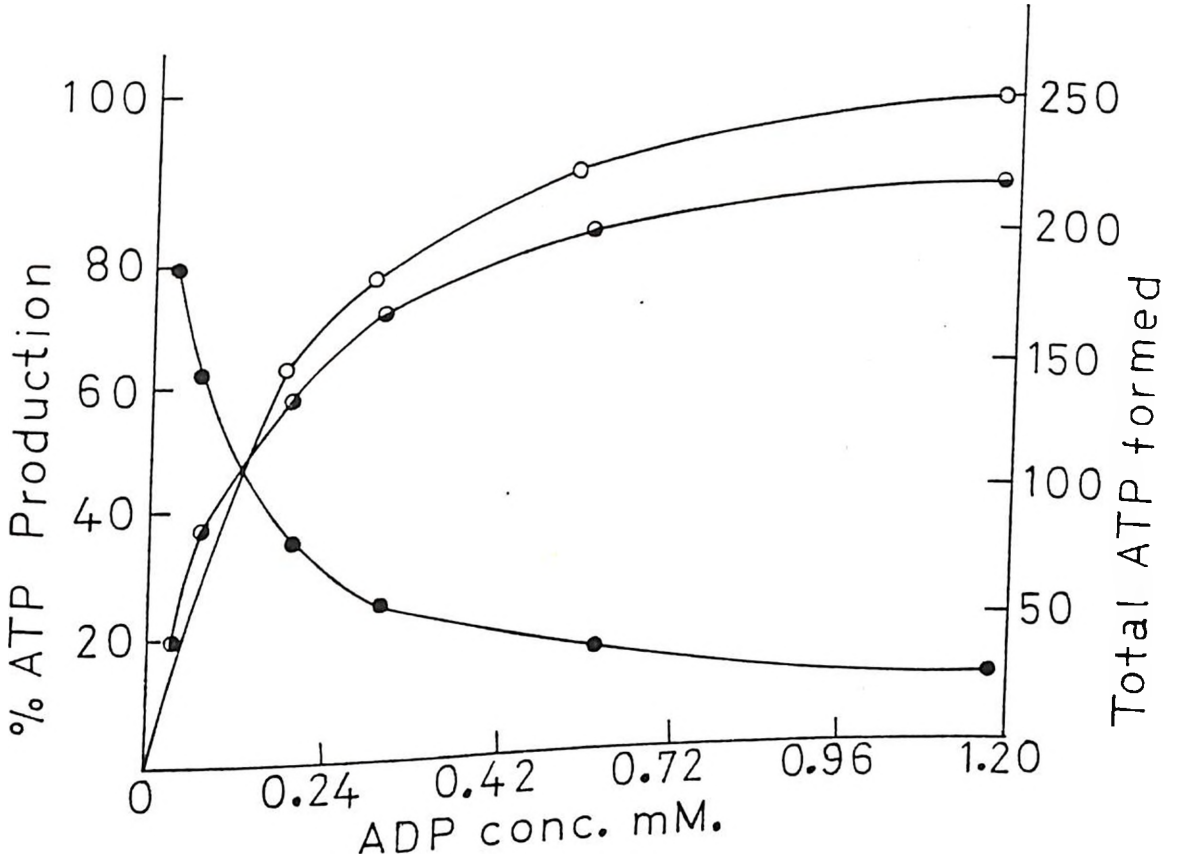
phosphorylation, while at high ADP concentrations most of the ATP formed comes from the adenylate kinase.

DISCUSSION

The results of Figure 8 show that both ADP and AMP are able to stimulate oxidative phosphorylation with concomittant ATP synthesis. However, the rate of ATP formation from AMP is apparently much lower than the rate of ATP formation from ADP. This is in contrast with the results of Figure 7 where it was observed that both ADP and AMP give similar respiratory control ratios. The reason for this discrepancy is that part of the ATP formed with AMP as the substrate is used by the adenylate kinase to phosphorylate the latter to ADP, which is then utilized by oxidative phosphorylation. As such only part of the ATP formed under these conditions is available for the hexokinase reaction.

The results of Figure 9 show that the rates of ATP formation by oxidative phosphorylation and by adenylate kinase depend on the concentration of ADP. At low ADP concentrations more ATP is formed by oxidative phosphorylation while at high ADP concentrations more ATP is formed by adenylate kinase.

This is consistent with data on Table V which show
that oxidative phosphorylation has a considerably
lower K_m value for ADP, and a much lower maximum
velocity than adenylate kinase. However, it should
be mentioned that adenylate kinase does not result
in any net phosphorylation of the adenylate system,
since for every molecule of ATP formed, a molecule
of ADP is dephosphorylated to AMP. This AMP must be
rephosphorylated back to ADP before it can
continue in the adenine nucleotide cycle. Thus
adenylate kinase is only used for a transient
synthesis of ATP, under conditions whereby the rate
of ATP utilization in the cytosol is higher than the
rate of its formation by oxidative phosphorylation.

C H A P T E R   VII

CATION TRANSPORT BY RAT BRAIN MITOCHONDRIA

Transport of calcium

Figure 10(A) is a control experiment showing
the stimulation of rat brain mitochondrial respiration
induced by the addition of ADP. ADP induced a 4-5
fold increase in the rate of oxygen uptake and this
was reversed on depletion of ADP. A further stimula-
tion of respiration could be induced by the addition of
more ADP or addition of the uncoupler CCCP.

In Figure 10(B) stimulation of mitochondrial
respiration was induced by the addition of Ca^{2+}.
This addition caused a 4-fold increase in the rate
of oxygen uptake and was reversed after all the added
Ca^{2+} was taken up. A further stimulation of
respiration could be induced by addition of more Ca^{2+}
or addition of the uncoupler CCCP. With malate and
glutamate as the respiratory substrates the Ca^{2+}/O
ratio (nMoles of Ca^{2+} taken up per nMole of oxygen
consumed) was 5.2, while with succinate or ascorbate
+ TMPD as the substrates the observed Ca^{2+}/O ratios
were 3.8 and 1.85 respectively. These parameters
are similar to those that have been described for

FIGURE 10

Respiratory stimulation by ADP and Ca^{2+} in rat brain mitochondria

The incubation mixture at 30°C consisted of 50 mM Tris-HCl buffer, pH 7.5, 0.3 M mannitol, 100 mM KCl, 3 mM Tris-phosphate and 2 mg. mitochondrial protein in a 1.7 ml. final volume. The reaction was assayed by following the rate of oxygen uptake polarographically using a clark-type oxygen electrode. Additions when indicated are 5 mM malate (MAL), 5 mM glutamate (GLU) 180 nMoles ADP, 5 µM CCCP and 500 nMoles Ca^{2+}.
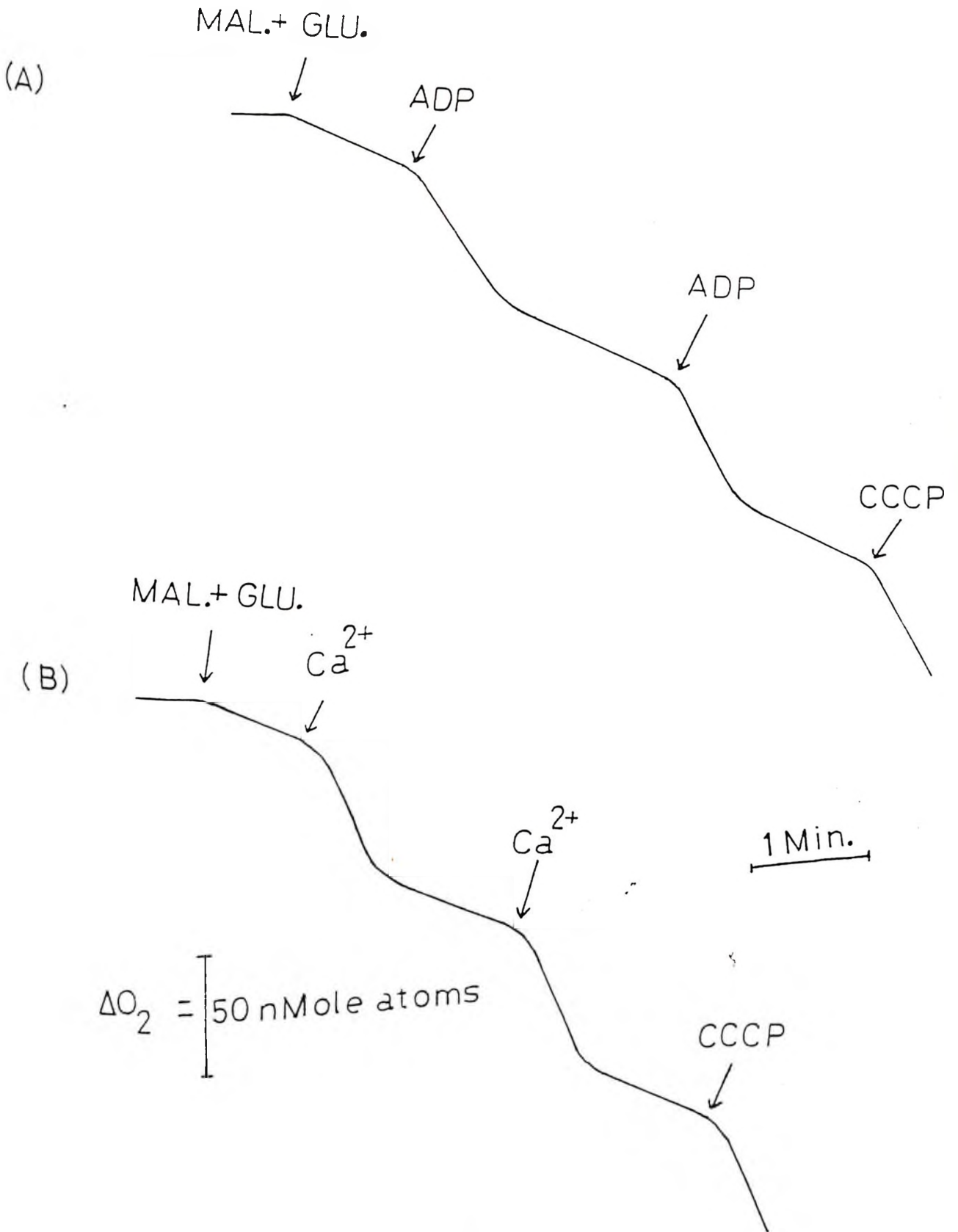
mitochondria isolated from rat liver (Rossi and Lehninger, 1964) and from rat heart (Vercesi et al., 1978).

In Figure 11(A) uptake of Ca^{2+} by rat brain mitochondria was followed spectrophotometrically using an Aminco DW-2 dual wavelength spectrophotometer set at 685 - 665 nM. The reaction mixture contained 100 µM arsenazo III as an indicator of extramitochondrial Ca^{2+}. Addition of Ca^{2+} to the medium caused an increase in absorbance which was reversed upon addition of mitochondria. That this decrease in absorption on addition of mitochondria was due to uptake of Ca^{2+} was shown by the fact that it was prevented by prior addition of ruthenium red an inhibitor of the Ca^{2+} carrier (Moore, 1971) or by prior addition of the respiratory inhibitor rotenone.

In Figure 11(B) the uncoupler CCCP or the respiratory inhibitor rotenone were added after uptake of Ca^{2+} by the mitochondria. The figure shows that these additions caused a rapid efflux of accumulated Ca^{2+}. A similar effect was observed when the reaction mixture was incubated for 7 minutes after uptake of the Ca^{2+}. This effect was most probably due to depletion of dissolved oxygen.

FIGURE 11

Ca^{2+} uptake by rat brain mitochondria

The incubation mixture at 30°C consisted of 50 mM Tris-HCl buffer, pH 7.5, 100 mM KCl, 0.3 M. mannitol, 3 mM Tris-phosphate and 100 µM arsenazo III in a 3 ml. final volume. The reaction was assayed by following the changes in absorption using an Aminco DW-2 dual wavelength spectrophotometer set at 685 and 665 nM. Additions when indicated are :- 30 nMoles $CaCl_2$, 1 mg mitochondria, 1 µM ruthenium red (RR), 3 µg rotenone and 5 µM CCCP.
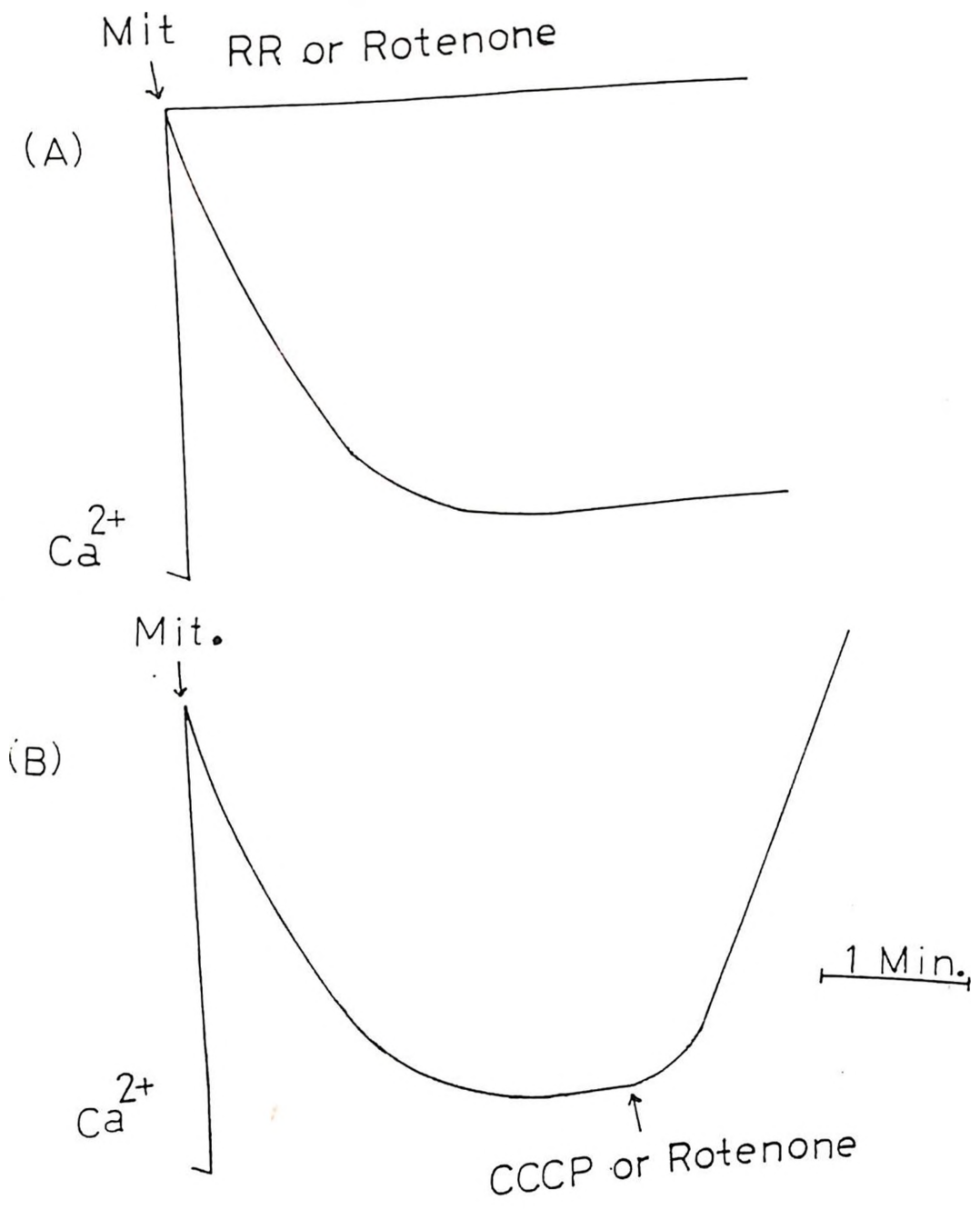
$$A_{685-665} = \left[0.02 \right.$$

Figure 12(A) shows the respiratory stimulation induced by the addition of 4 cycles of 250 nMoles of Ca^{2+} per mg. of mitochondrial protein each. The figure shows that the first 3 cycles resulted in a full stimulation of respiration, indicating that all the added Ca^{2+} was taken up. However, the 4th cycle resulted in only a slight stimulation followed by a reversal to state IV respiration. A further stimulation of respiration could be induced by addition of the uncoupler CCCP. This shows that these mitochondria are only capable of accumulating about 800 nMoles of Ca^{2+} per mg. of mitochondrial protein.

In Figure 12(B) respiratory stimulation was induced by the addition of 250 nMoles Ca^{2+} per mg. of mitochondrial protein in the absence of added phosphate. The figure shows that this addition of Ca^{2+} resulted in only a small stimulation of respiration which was followed by a reversal to state IV respiration. This was followed by addition of inorganic phosphate which resulted in a further stimulation of respiration apparently due to Ca^{2+} uptake since it shortly reverted to state IV respiration. Addition of more Ca^{2+} at this point resulted in a further stimulation of respiration. A similar effect, but to a lesser extent was seen when ATP

FIGURE 12

Effect of inorganic phosphate on Ca^{2+} uptake by rat brain mitochondria

The incubation mixture at 30°C consisted of 50 mM Tris-HCl buffer pH 7.5, 100 mM KCl, 0.3 M. mannitol and 2 mg. mitochondrial protein in a 1.7 ml final volume. The reaction was assayed by following the rate of oxygen uptake polarographically using a clark-type oxygen electrode. Additions when indicated are :- 3 mM Tris-phosphate-(Pi) 5 mM malate (MAL) 5 mM glutamate (GLU) 500 nMoles $CaCl_2$, 5 µM CCCP, and 80 nMoles N-ethylene malemaide (NEM).

(A) MAL.+ GLU.

Ca^{2+}

Ca^{2+}

Ca^{2+}

1 Min.

$\Delta O_2 = $ 50 nMole atoms

Ca^{2+}

(B) MAL.+ GLU.

Ca^{2+}

Pi

Ca^{2+}

CCCP

(C) MAL.+GLU.

NEM

Ca^{2+}
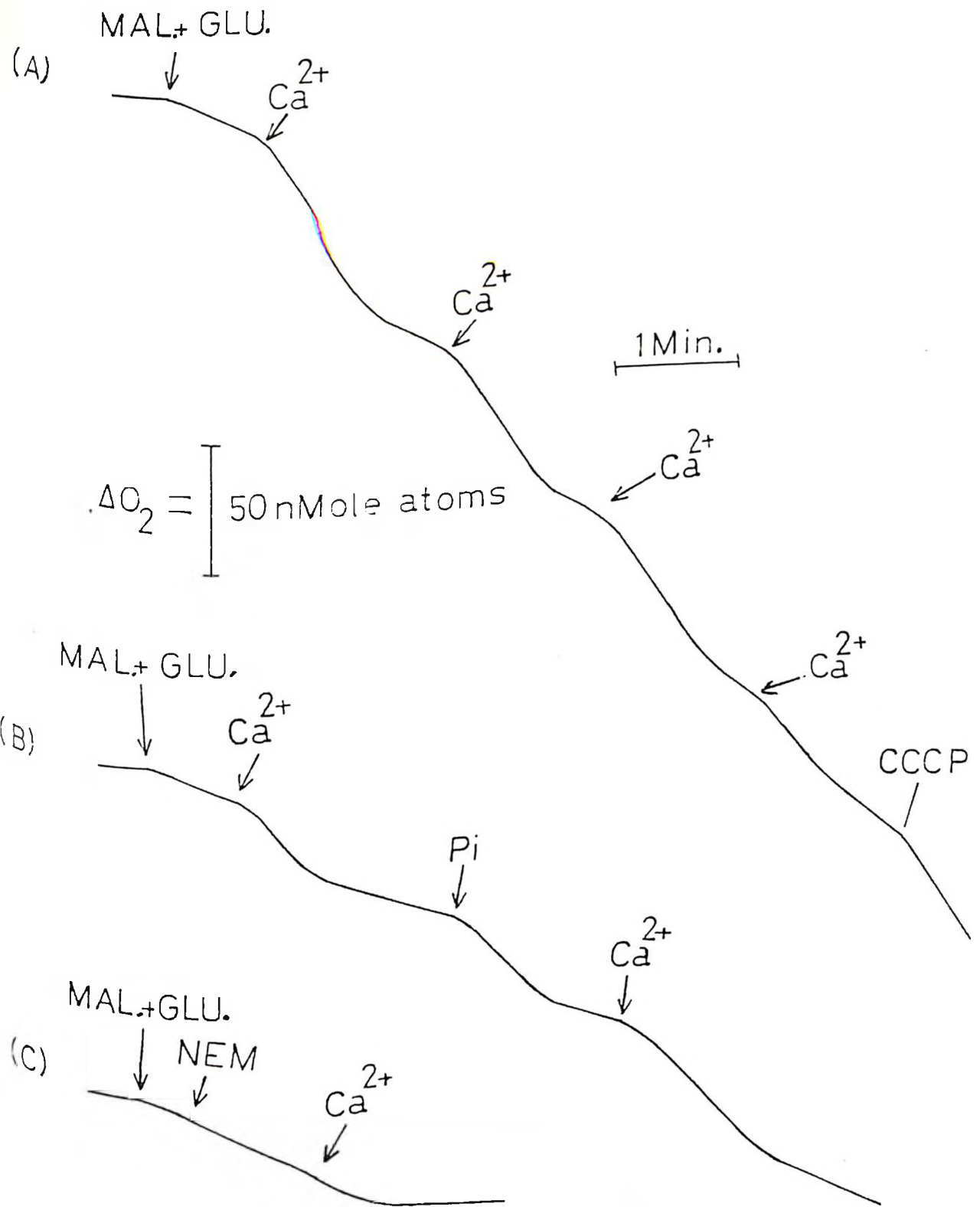
was added instead of inorganic phosphate, most probably due to hydrolysis of the ATP to ADP and inorganic phosphate by the ATPase.

In Figure 12(C) N-ethylene-malemaide an inhibitor of the phosphate carrier (Coty and Pedersen 1974) was added prior to addition of Ca^{2+}. Addition of Ca^{2+} resulted in only a very slight stimulation of respiration followed by a complete inhibition of respiration.

Figure 13(A) shows the respiratory stimulation induced by the addition of 4 cycles of 250 nMoles Ca^{2+} per mg. of mitochondrial protein each. The figure shows that the first 3 cycles caused a full stimulation of respiration, while the 4th cycle only caused a very slight stimulation. Addition of ATP at this point caused a further stimulation of respiration which then reversed to state IV. That this further stimulation of respiration was due to Ca^{2+} uptake was shown by the fact that addition of ruthenium red prior to addition of ATP prevented the respiratory stimulation (Figure 13 B). This effect was also dependent on the entry of ATP into the mitochondrial matrix as was shown by the fact that addition of carboxyatractylate before addition of ATP also prevented the respiratory stimualtion (Figure 13C). In Figure 13(D) ATP was added before

FIGURE 13

Effect of ATP on Ca^{2+} uptake by rat brain
mitochondria

The incubation mixture at 30°C consisted of 50 mM
Tris-HCl buffer, pH 7.5, 0.3 M mannitol, 100 mM KCl,
3 mM Tris-phosphate and 2 mg mitochondrial protein in
a 1.7 ml final volume. The reaction was assayed by
following the rate of oxygen uptake polarographically
using a clark-type oxygen electrode. Additions when
indicated are 500 nMoles Ca^{2+}, 1 mM ATP, 5 mM malate
(MAL) 5 mM glutamate (GLU) 1 µM ruthenium red (RR)
and 3 µM carboxyatractylate (CAT).

MAL.+ GLU.

(A)

Ca^{2+}

Ca^{2+}

Ca^{2+}

MAL+ GLU

(B)

Ca^{2+}

Ca^{2+}

Ca^{2+}

Ca^{2+}

ATP

Ca^{2+}

1 Min.

Ca^{2+}

$\Delta O_2 = \left|50\,nMole\ atoms\right.$

RR

ATP

(C) MAL + GLU, Ca²⁺ traces

(D) MAL + GLU, ATP, Ca²⁺, CAT, ATP traces

1 Min

$\Delta O_2 = $ 50 nMole atoms
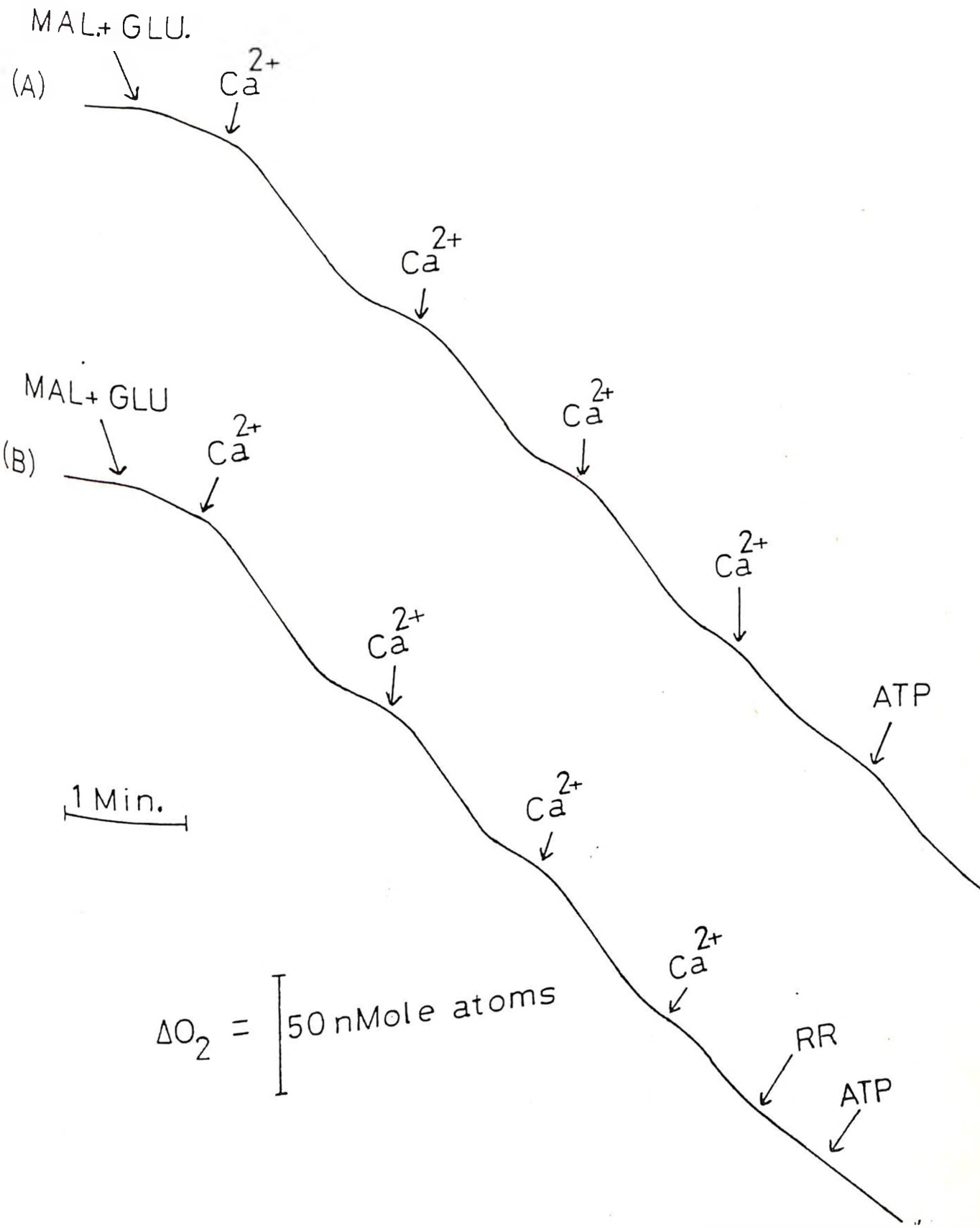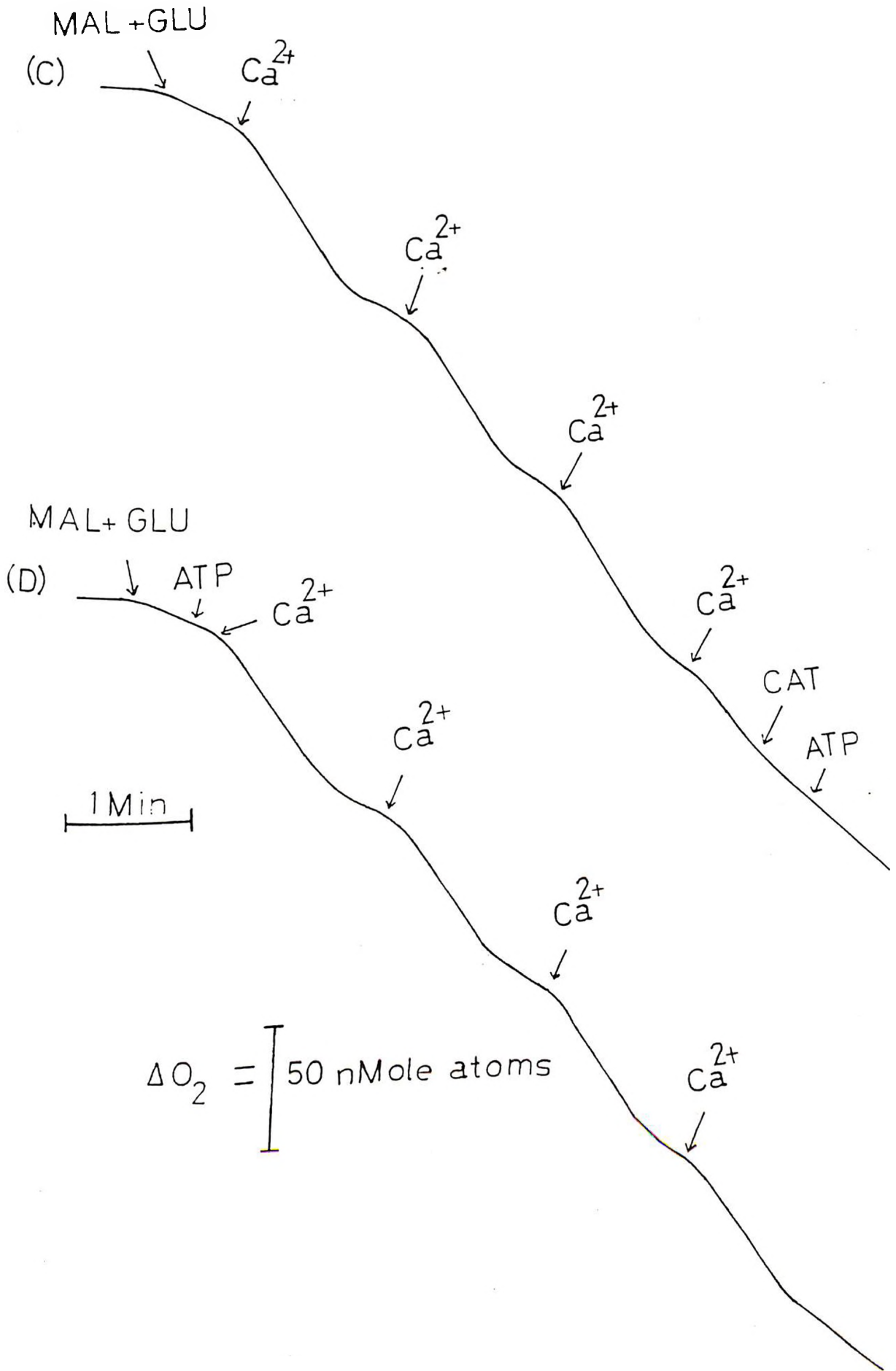
addition of Ca^{2+}. The trace shows that under these conditions all the 4 cycles of Ca^{2+} addition resulted in a full stimulation of respiration.

Uptake of other bivalent cations by rat brain mitochondria

In Figure 14(A) stimulation of respiration was induced by the addition of 150 nMoles of Ba^{2+} per mg. of mitochondrial protein. This addition of Ba^{2+} induced a 2.4 fold stimulation of respiration which proceeded until all the added Ba^{2+} was taken up. A further stimulation of respiration could be induced by addition of more Ba^{2+} or the uncoupler CCCP. With malate and glutamate as the respiratory substrates, the Ba^{2+}/O ratio (nMoles of Ba^{2+} taken up per nMole of oxygen consumed was 7.65.

In Figure 14(B) stimulation of respiration was induced by the addition of 150 nMoles Mn^{2+}. This addition of Mn^{2+} caused a 3.2 fold stimulation of respiration which reverted to state IV respiration after all the added Mn^{2+} was taken up. A further stimulation of respiration could be induced by the addition of more Mn^{2+} or the uncoupler CCCP. With malate and glutamate as the respiratory substrates the Mn^{2+}/O ratio was 2.55.

FIGURE 14

Respiratory stimulation by Ba^{2+} and Mn^{2+} in rat brain mitochondria

The incubation mixture consisted of 50 mM Tris-HCl buffer, pH 7.5, 100 mM KCl, 0.3 M. mannitol, 3 mM Tris-phosphate and 2 mg. mitochondrial protein in a 1.7 ml. final volume. The reaction was assayed by following the rate of oxygen uptake polarographically using a clark-type oxygen electrode. Additions when indicated are 5 mM malate (MAL) 5 mM glutamate (GLU) 300 nMoles $BaCl_2$, and 300 nMoles $MnCl_2$.
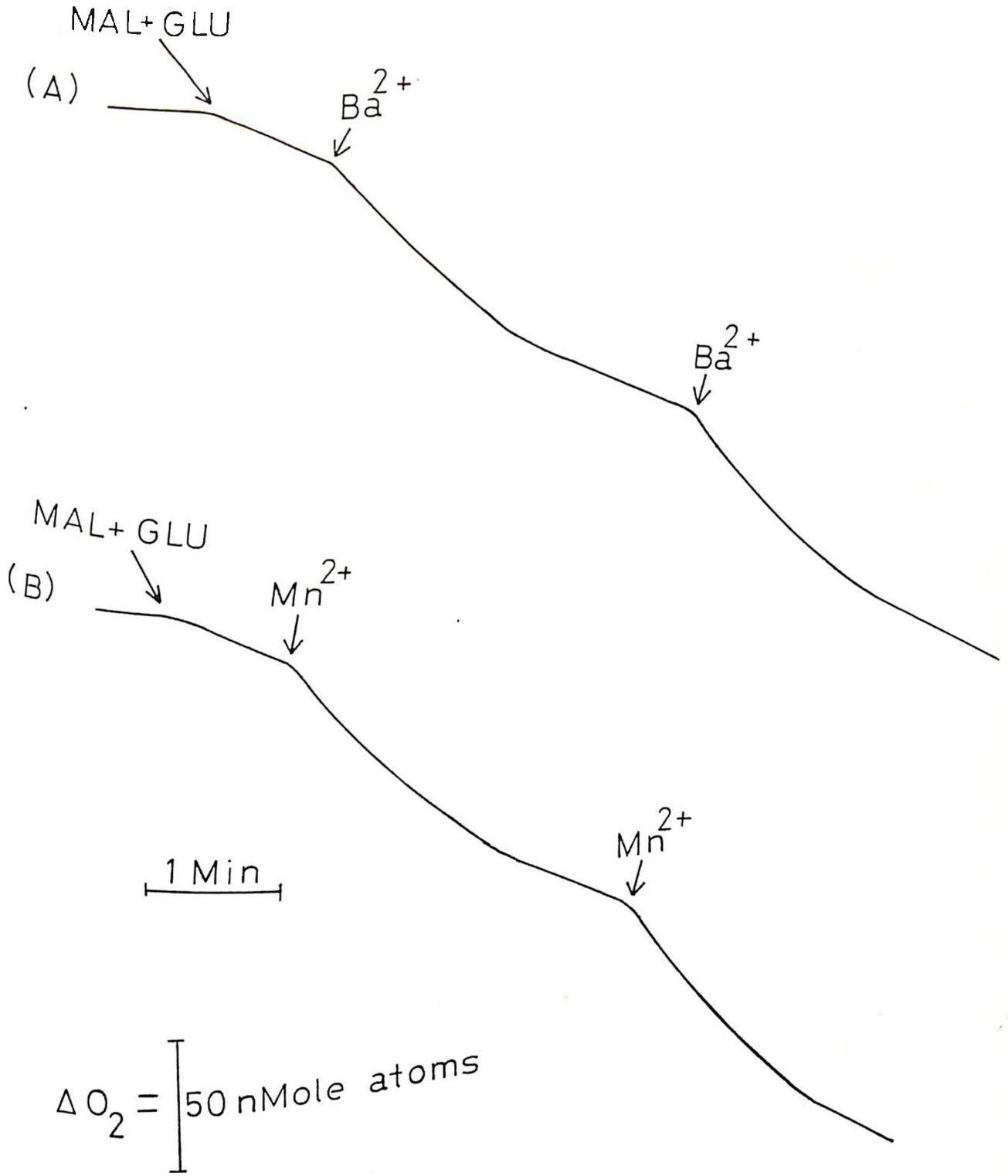
In Figure 15(A) stimulation of respiration was induced by the addition of 150 nMoles Ca^{2+} and 150 nMoles of Ba^{2+} per mg. of mitochondrial protein. Both cations were taken up as evidenced by the fact that the extent of respiratory stimulation was much more than that induced when the cations were added individually.

In Figure 15(B) respiratory stimulation was induced by the addition of 150 nMoles of Ca^{2+} and 150 nMoles of Mn^{2+} per mg. of mitochondrial protein. In agreement with earlier reports (Ernster and Nordenbrand, 1967; Mela and Chance, 1968) both cations were taken up together with Ca^{2+} accelerating the uptake of Mn^{2+}, and Mn^{2+} retarding the uptake of Ca^{2+}.

Na^+ induced efflux of accumulated Ca^{2+}

In Figure 16 Ca^{2+} uptake by rat brain mitochondria was followed spectrophotometrically using an Aminco DW-2 dual wavelength spectrophoto- meter. After uptake of all the Ca^{2+} 1µM ruthenium red and NaCl to a final concentration of 20 mM were added. This resulted in an efflux of all the accumulated Ca^{2+}.

Figure 17 shows that addition of NaCl to a final concentration of 30 mM to respiring brain

FIGURE 15

Respiratory stimulation by the combined uptake of
cations in rat brain mitochondria

The reaction mixture at 30°C consisted of 50 mM
Tris-HCl buffer, pH 7.5, 0.3 M mannitol, 100 mM KCl,
3 mM Tris-phosphate and 2 mg mitochondrial protein in
a 1.7 ml. final volume. The reaction was assayed by
following the rate of oxygen uptake polarographically
using a clark-type oxygen electrode. Additions when
indicated are 5 mM malate (MAL) 5 mM glutamate(GLU)
300 nMoles Ca^{2+}, 300 nMoles Ba^{2+} and 300 nMoles Mn^{2+}.

(A) MAL+ GLU $Ca^{2+} + Ba^{2+}$

(B) MAL+ GLU $Ca^{2+} + Mn^{2+}$

1 Min.

ΔO_2 50nMole atoms
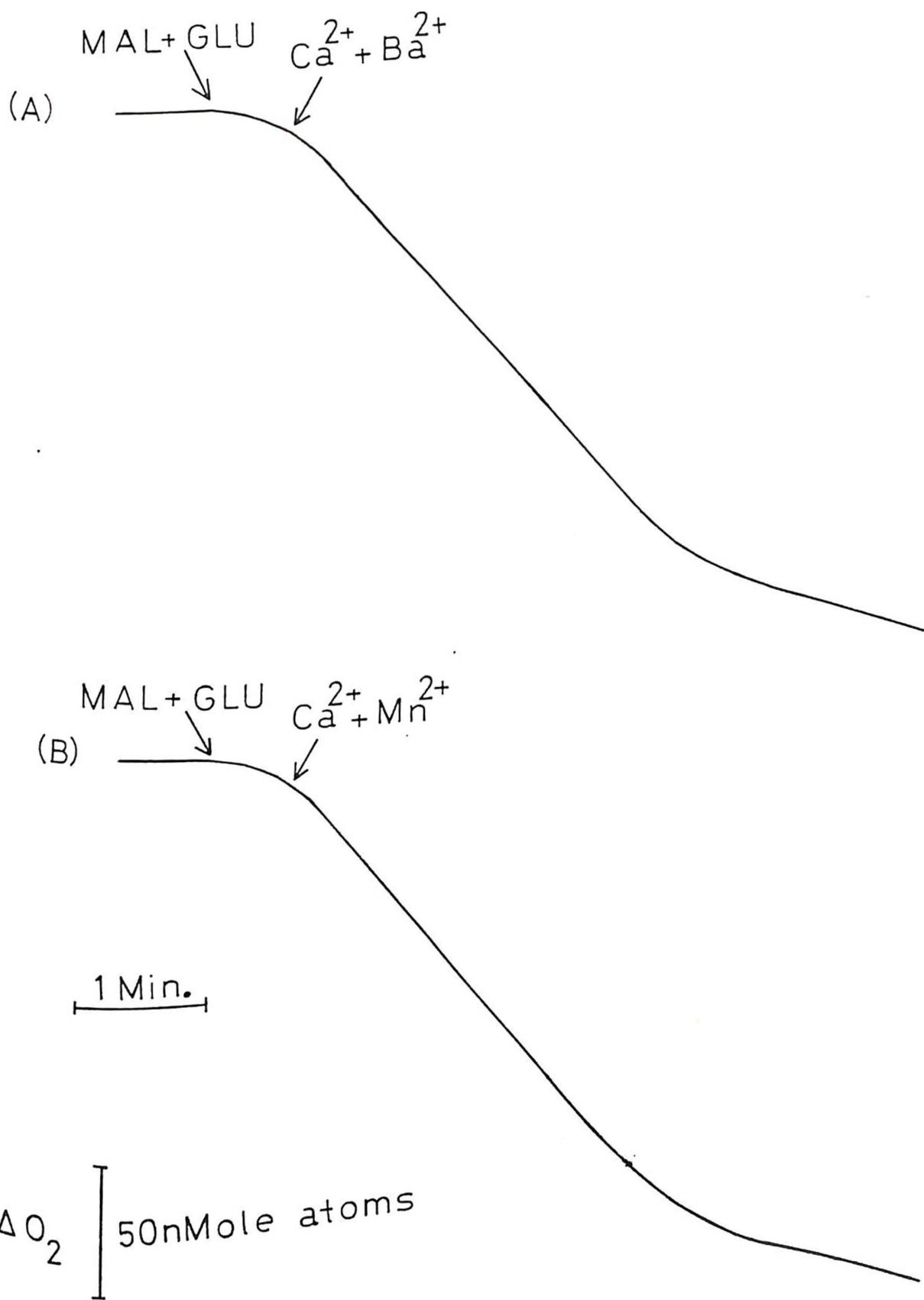
FIGURE 16

Effect of Na^+ on Ca^{2+} accumulation by rat brain mitochondria

The incubation medium at 30°C consisted of 50 mM Tris-HCl buffer, pH 7.5, 0.3 M mannitol 100 mM KCl, 3 mM Tris-phosphate, 5 mM malate, 5 mM glutamate and 100 µM arsenazo III. The reaction was assayed by following the changes in absorption using an Aminco DW-2 dual wavelength spectrophotometer set at 685 and 665 nM. Additions when indicated are 30 nMoles $CaCl_2$, 1 mg. mitochondrial protein, 1 µM ruthenium red (RR) and NaCl to a final concentration of 20 mM.
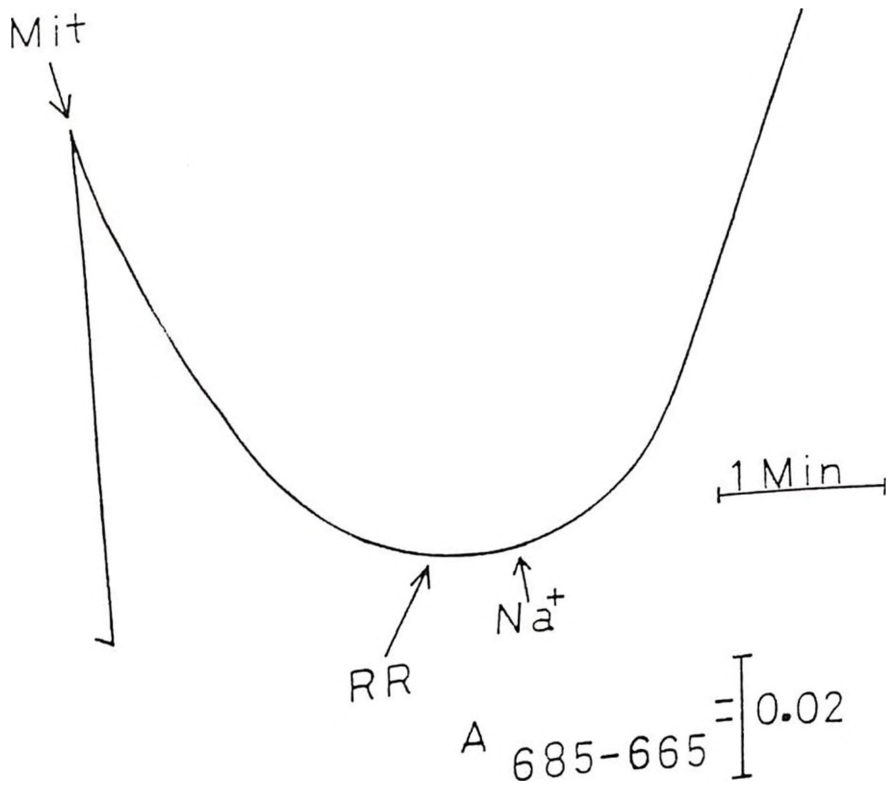
FIGURE 17

Effect of Na$^+$ on rat brain mitochondrial respiration

The incubation mixture at 30° consisted of 50 mM Tris-HCl buffer, pH 7.5, 100 mM KCl, 0.3 M mannitol, 3 mM Tris-phosphate and 2 mg mitochondrial protein in a 1.7 ml final volume. Additions when indicated are 5 mM malate (MAL) 5 mM glutamate (GLU) 180 nMoles ADP, and NaCl to a final concentration Of 20 mM.

MAL-GLU

Na$^+$

1 Min

ADP

$\Delta O_2 =$ 50 nMole atoms
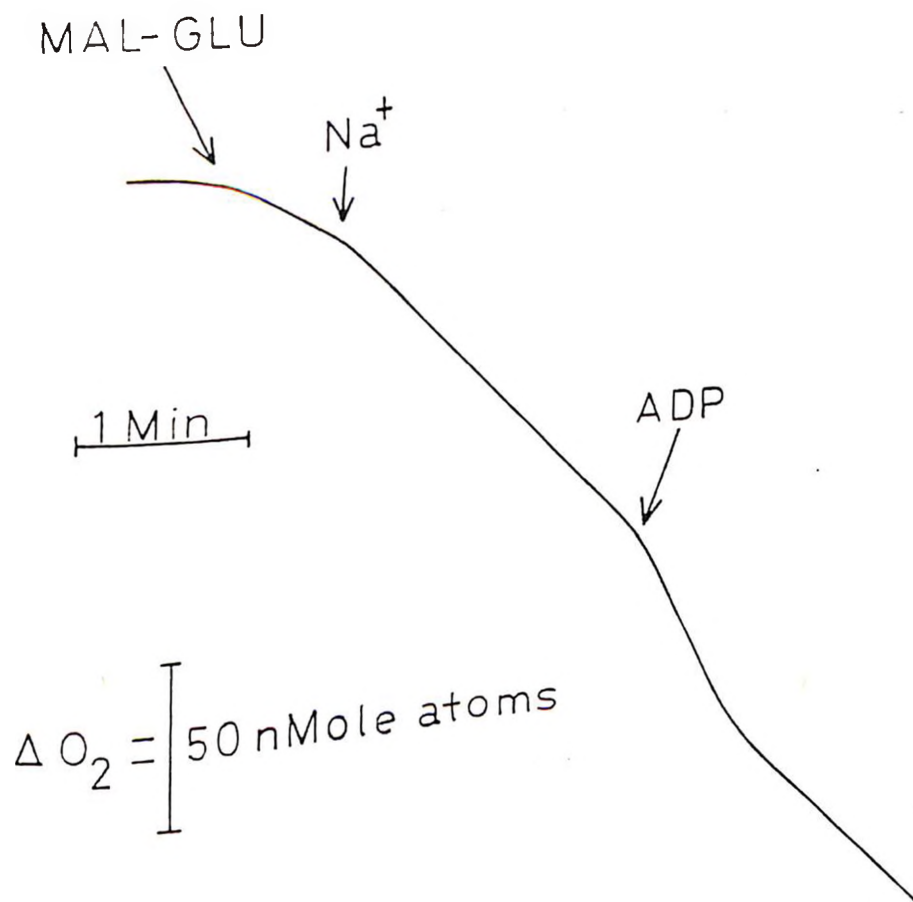
mitochondria led to a 2-fold stimulation of respiration. Table VI shows that the extent of respiratory stimulation induced by Na^+ was dependent on the concentration of Na^+ in the reaction medium.

Figure 18 shows that addition of Na^+ to a final concentration of 20 mM to respiring mitochondria caused an increase in the acidity of the medium due to an efflux of protons. This indicates that these mitochondria are capable of supporting an uptake of Na^{2+} which is coupled to an efflux of proton.

DISCUSSION

The results of Figures 10 - 15 show that rat brain mitochondria are capable of supporting an energy dependent uptake of the bivalent cations Ca^{2+}, Mn^{2+} and Ba^{2+}. The transport of bivalent cations is an electrophoretic process which takes place in response to the proton electrochemical gradient ($\Delta\mu_H+$) that exists across the inner mitochondrial membrane generated by electron transport. The electrical component ($\Delta\Psi$) of the total proton electrochemical gradient is believed to be the primary driving force for bivalent cation transport. Uptake of bivalent cations tends to dissipate the

FIGURE 18

Effect of Na$^+$ on mitochondrial respiration

The incubation mixture at 30°C consisted of
5 mM Tris-HCl buffer, pH 7.5, 0.3 M mannitol, 100
mM KCl and 2 mg. mitochondrial protein. The
reaction was assayed by following the rate of
acidification of the medium using a sensitive pH
electrode. The reaction was initiated by the
addition of NaCl to a final concentration of 30 mM.

Na^+

$\Delta pH =$ 40 nMoles H^+

1 Min
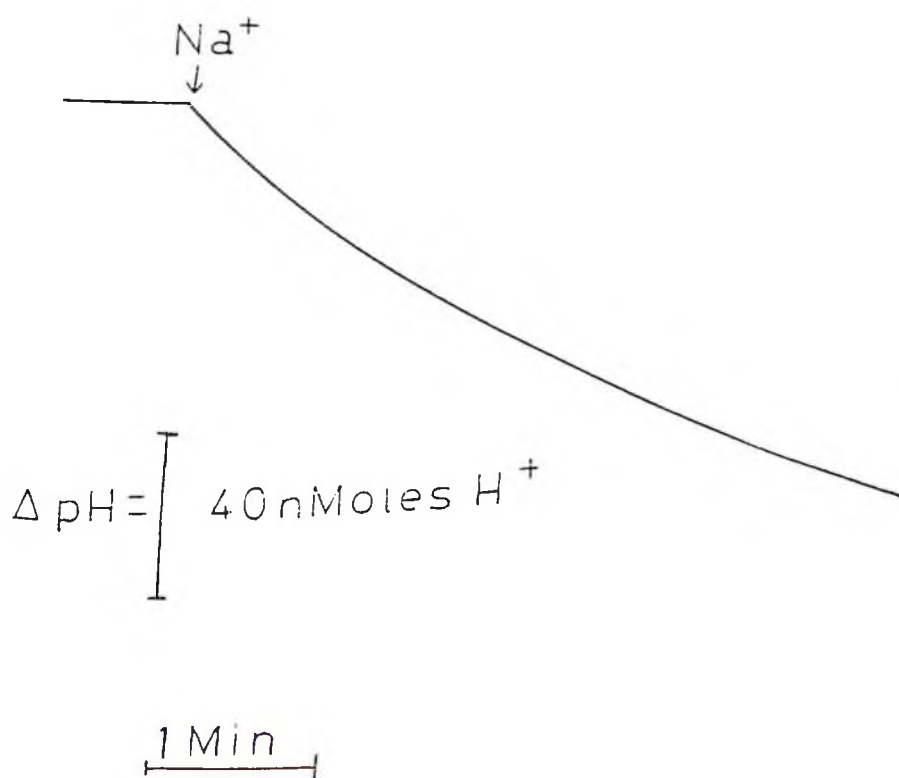
($\Delta\Psi$) hence lowering the $\Delta\mu_H^+$. The respiratory chain responds to the lowered $\Delta\mu_H^+$ by a net extrusion of protons. This effect is what was observed as an increase in the rate of mitochondrial oxygen consumption during the cation uptake process. That the uptake of bivalent cations is dependent on an active electron transport chain was shown by the fact that the presence of a respiratory inhibitor inhibited the uptake process (Figure 11 A). Maintenance of the accumulated cations inside the mitochondria is also an energy dependent process as shown by the fact that addition of the uncoupler CCCP, the respiratory inhibitor rotenone or depletion of oxygen led to an efflux of accumulated Ca^{2+} (figure 11 B).

The results also show that there is very limited uptake of Ca^{2+} by brain mitochondria in the absence of added phosphate. Inorganic phosphate is believed to complex with Ca^{2+} in the mitochondrial matrix, forming a non-ionic calcium phosphate complex (Rasmussen, 1981) which offsets the decrease in $\Delta\Psi$ caused by ionic Ca^{2+} in the matrix, thus accounting for the very large capacity for Ca^{2+} uptake in the presence of inorganic phosphate. In the presence of N-ethylene malemaide which inhibits the phosphate/ H^+ symporter (Vercesi et al., 1978) there is a rapid build up of the ΔpH component of the total $\Delta\mu_H^+$,

as Ca^{2+} is taken up, which leads to a complete inhibition of respiration i.e. state VI of Chance and Shnoener (1966). In addition the results of Figure 13 show that addition of external ATP may raise the capacity of brain Ca^{2+} accumulation. Asikamis and Sardahl (1981) had shown that the capacity of heart mitochondrial Ca^{2+} accumulation is dependent on the intramitochondrial adenine nucleotide content. In their experiments that had shown that prior incubation of mitochondria with inorganic pyrophosphate which lowered the adenine nucleotide content of the mitochondria lowered the capacity of mitochondria to accumulate Ca^{2+}, a situation that was reversed by addition of external ATP or ADP. These findings are consistent with the report by Rasmussen (1981) who showed that the calcium phosphate complex in mitochondria requires ATP and/or ADP for stabilization.

The results of Figure 16 show that addition of Na^+ to mitochondria that have accumulated Ca^{2+} resulted in efflux of all the accumulated Ca^{2+}. This is in agreement with earlier reports (Carafoli et al, 1974, Crompton et al., 1976, 1978) who showed that Na^+ is able to cause an efflux of accumulated Ca^{2+} in mitochondria isolated from excitable tissues such as heart, brain, adrenal cortex and skeletal muscle. This the authors claimed is due to the

presence of Na^+-Ca^{2+} exchange carrier present in such tissues. Figure 17 showed that Na^+ are able to induce a stimulation of mitochondrial respiration. A similar Na^+ induced stimulation of mitochondrial respiration has also been described (Cromptom et al., 1976) in heart mitochondria. However, these authors only observed the effect following accumulation of Ca^{2+}, whereas the stimulation observed in the current experiments was independent of prior accumulation of Ca^{2+}. This thus means that brain mitochondria have some capacity for Na^+ accumulation which as shown in Figure 18 is accompanied by an efflux of protons. As indicated earlier, the $\Delta\Psi$ component of the total $\Delta\mu_H^+$ is believed to be the primary driving force for bivalent cation uptake. Uptake of Na^+ by mitochondria coupled to an efflux of protons may be expected to raise the ΔpH component of the $\Delta\mu_H^+$. Since the $\Delta\mu_H^+$ has to remain constant an increase in the ΔpH component will be accompanied by a decrease in the $\Delta\Psi$ component. This fall in the $\Delta\Psi$ will lower the energy available for both Ca^{2+} uptake and its retention, resulting in an efflux of the accumulated Ca^{2+}. This effect is somehow similar to that which was observed upon de-energisation of the mitochondrial membrane by addition of an uncoupler or a respiratory inhibitor.

C H A P T E R   VIII

INTERACTION OF BIVALENT CATIONS WITH THE
MITOCHONDRIAL ATP SYNTHETASE (ATPase)

Effect of bivalent cation accumulation on
oxidative phosphorylation

In Figure 19(A) respiratory stimulation was
induced by the addition of 180 nMoles ADP. After
exhaustion of all the added ADP, and a resumption of
state IV respiration 250 nMoles $CaCl_2$ per mg. of
mitochondrial protein was added. This addition of
Ca^{2+} caused a further stimulation of respiration which
reversed to state IV after uptake of all the Ca^{2+}.
This was followed by the addition of 180nMoles ADP.
The figure shows that this addition of ADP failed
to stimulate respiration. However, a further
stimulation of respiration could be induced by the
addition of more Ca^{2+} or addition of the uncoupler
CCCP. That the inhibition of the ADP response is
due to intramitochondrial rather than extramitochon-
drial Ca^{2+} is shown in Figure 19(B). Here ruthenium
red was added after depletion of the ADP and prior to
addition of Ca^{2+}. Addition of Ca^{2+} at this point
failed to stimulate respiration showing that the
presence of ruthenium red inhibited its uptake. This

FIGURE 19

Effect of Ca^{2+} accumulation on brain mitochon-
drial oxidative phosphorylation

The incubation mixture at 30°C consisted of 50
mM Tris-HCl buffer, pH 7.5, 0.3 M mannitol, 100 mM
KCl, 3 mM Tris-phosphate and 2 mg. mitochondrial
protein in a 1.7 final volume. The reaction was
assayed by following the stimulation of oxygen
uptake polarographically using a clark-type oxygen
electrode. Additions when indicated are :- 5 mM
malate (MAL), 5 mM glutamate (GLU), 500 nMoles Ca^{2+},
180 nMoles ADP, 1 µM ruthenium red (RR) and 5· µM
CCCP.

MAL-GLU
ADP
(A)
Ca^{2+}
ADP
Ca^{2+}
CCCP

MAL-GLU
ADP
(B)
RR
Ca^{2+}
ADP

$\Delta O_2 = \bigg|$ 50 nMole atoms

1 Min
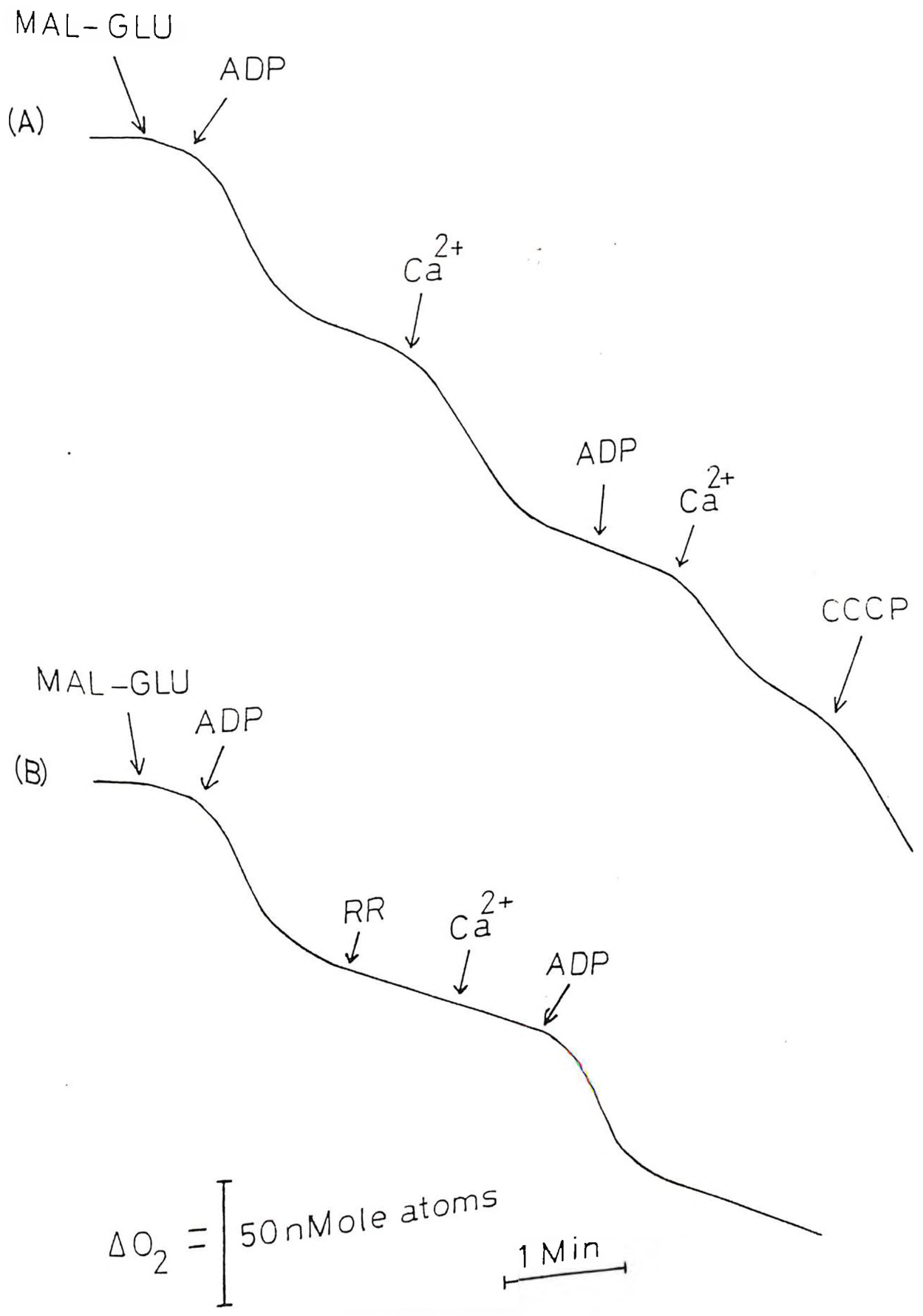
was then followed by the addition of 180 nMoles ADP which resulted in a full unihibited respiratory stimulation.

In Figure 20(A) stimulation of respiration was induced by the addition of 1.5 μMoles ADP. This amount of ADP was capable of stimulating respiration continuously until all the dissolved oxygen was depleted. This was then followed by the addition of 250 nMoles Ca^{2+} per mg. of mitochondrial protein. The figure shows that this addition of Ca^{2+} under these conditions also caused an inhibition of the ADP induced respiratory stimulation. In Figure 20(B) ruthenium red was added after respiratory stimulation was induced by the addition of 1.5 μMoles ADP. This was then followed by the addition of 250 nMoles Ca^{2+} per mg. of mitochondrial protein. The figure shows that unlike the effect seen in Figure 20(A) this addition of Ca^{2+} did not result in any inhibition of the ADP induced respiratory stimulation.

Figures 21 (A - D) are spectrophotometric recordings showing the effect of Ca^{2+} on mitochondrial ATP synthesis, measured as the amount of glucose -6-phosphate formed.

FIGURE 20

Effect of Ca^{2+} uptake on active brain
mitochondrial oxidative phosphorylation

The reaction mixture at 30°C consisted of 50 mM Tris-HCl buffer, pH 7.5, 0.3 M mannitol, 3 mM Tris-phosphate and 2 mg mitochondrial protein in a 1.7 ml. final volume. The reaction was assayed by following the stimulation of oxygen uptake polarographically using a clark-type oxygen electrode. Additions when indicated are :- 5 mM malate (MAL), 5 mM glutamate (GLU), 1.5 µMoles ADP, 500 nMoles Ca^{2+} and 1 µM ruthenium red (RR).
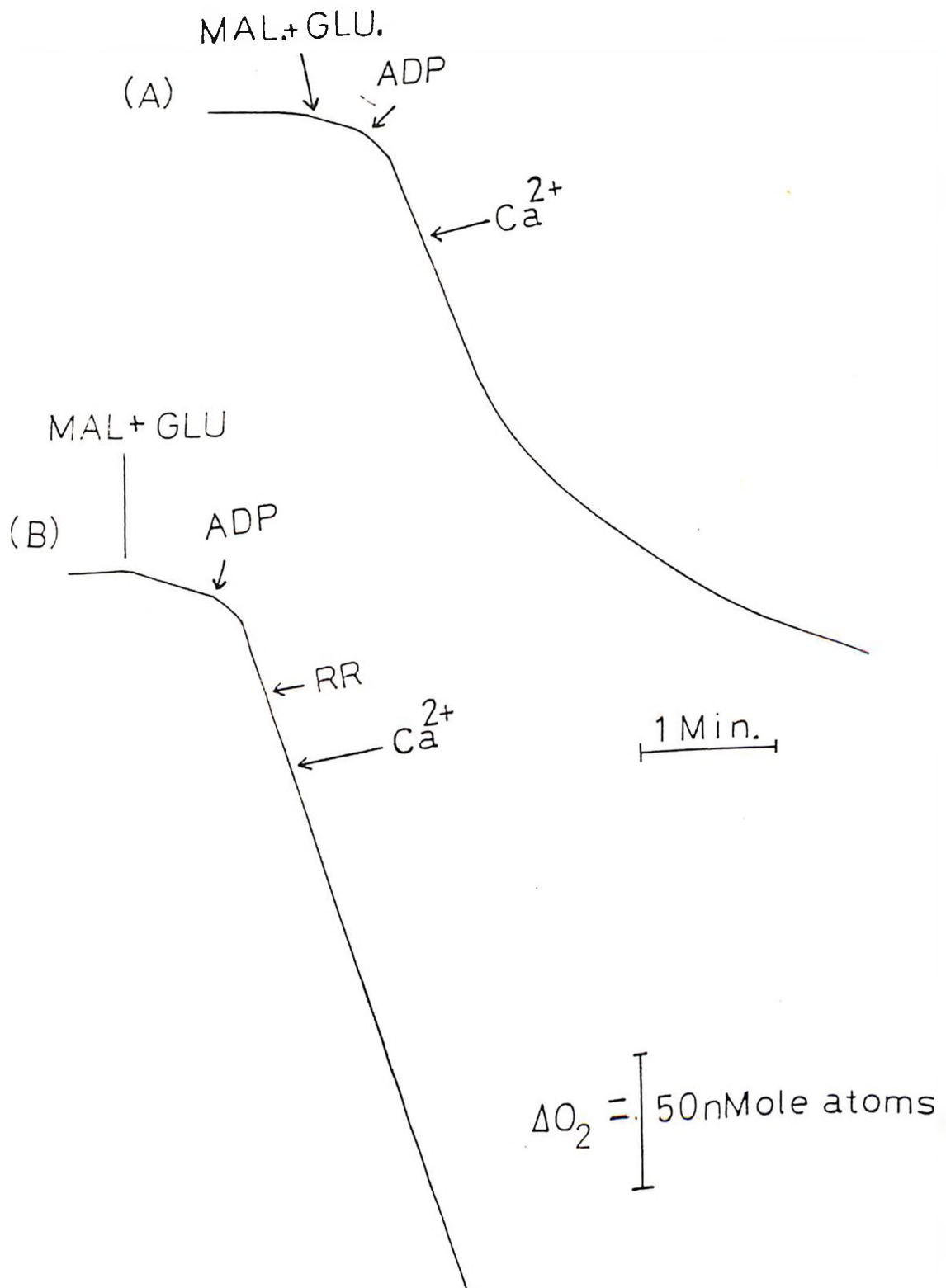
Figure 21(A) is a control experiment showing that addition of ADP to the medium caused an immediate stimulation of ATP synthesis.

In Figure 21(B) 65 nMoles Ca^{2+} was added, followed by the addition of ADP. The plot shows that this addition of ADP failed to stimulate ATP synthesis.

In Figure 21(C) stimulation of ATP synthesis was induced by the addition of ADP, followed by the addition of Ca^{2+}. The plot shows that even under these conditions Ca^{2+} caused an inhibition of ATP synthesis.

In Figure 21 (D) stimulation of ATP synthesis was induced by the addition of ADP, followed by the addition of ruthenium red and Ca^{2+}. The plot shows that under these conditions Ca^{2+} failed to inhibit ATP synthesis, indicating that it is the intramito-chondrial Ca^{2+}, and not the extramitochondrial Ca^{2+} that is responsible for inhibition of ATP synthesis.

The extent of inhibition of the ADP response was dependent on the amount of Ca^{2+} accumulated by the mitochondria. This is shown in Figure 22 by the saturation and Linear-Weaver Burk plots. Complete inhibition of the ADP response was seen upon uptake of 250 nMoles per mg. of mitochondrial protein, and
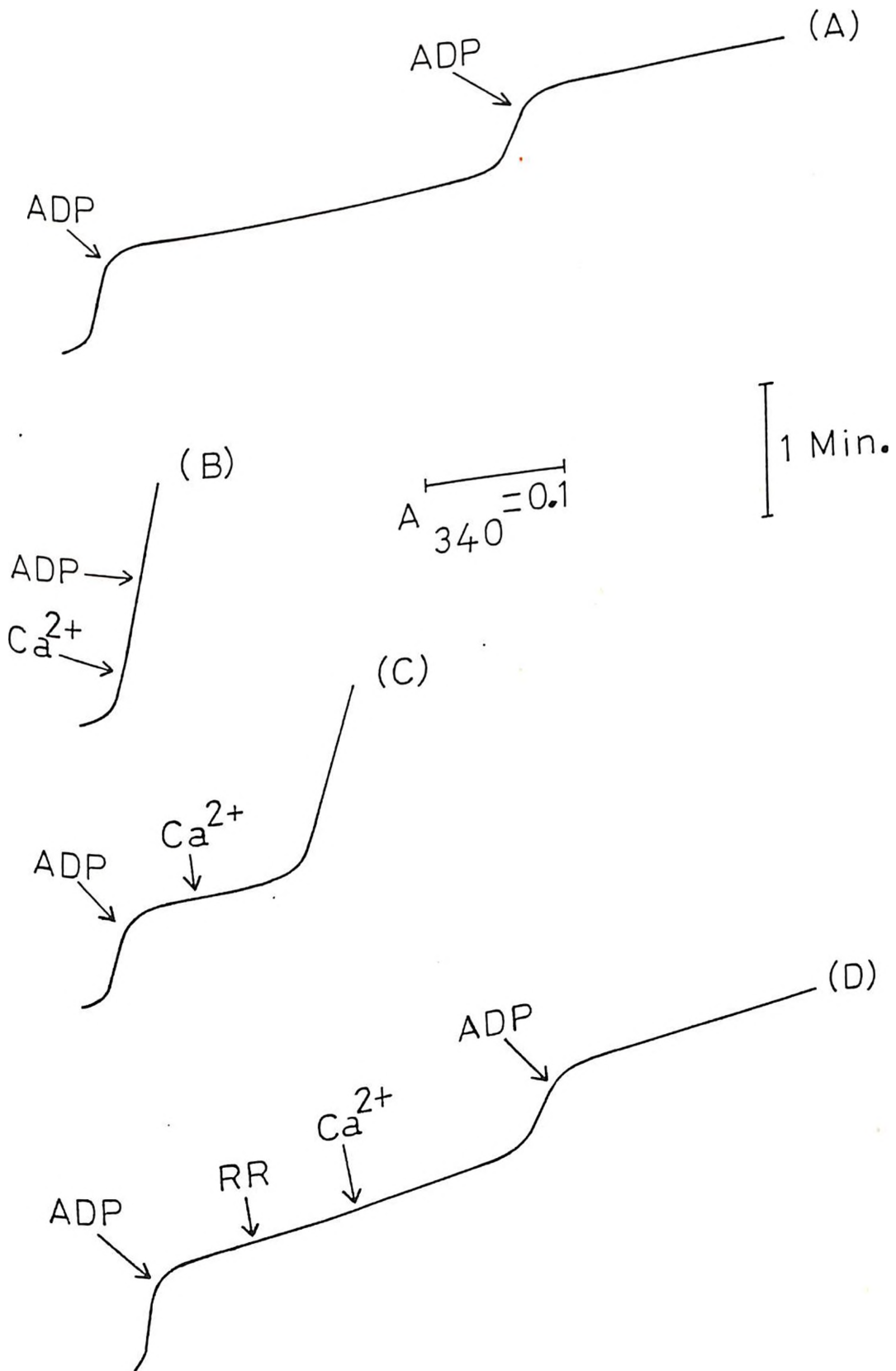
FIGURE 21

Effect of Ca^{2+} uptake on brain mitochondrial
oxidative phosphorylation

The reaction mixture at 30°C contained 50 mM
Tris-HCl buffer, pH 7.5, 0.3 M mannitol, 100 mM KCl,
3 mM Tris-phosphate, 5 mM malate, 5 mM glutamate,
3.0 mM $MgCl_2$, 0.4 mM $NADP^+$, 1.0 mM glucose, 5 units
hexokinase, 5 units glucose-6-phosphate dehydrogenase
500 µM diadenosine pentaphosphate and and 0.5 mg.
mitochondrial protein. The reaction was assayed
by following the reduction of $NADP^+$ spectrophoto-
metrically at 340 nM. Additions when indicated
are 150 nMoles ADP, 65 nMoles Ca^{2+} and 1 µM
ruthenium red (RR).

FIGURE   22

Saturation and Linear-Weaver Burk plots, showing
the extent of inhibition of oxidative phosphory-
lation upon uptake of varying amounts of Ca^{2+}

The extent of inhibition of oxidative
phosphorylation was determined by assaying the extent
of inhibition of the ADP induced respiratory stimula-
tion upon uptake of varying amounts of Ca^{2+} as
described for figure 20(A).

(A)
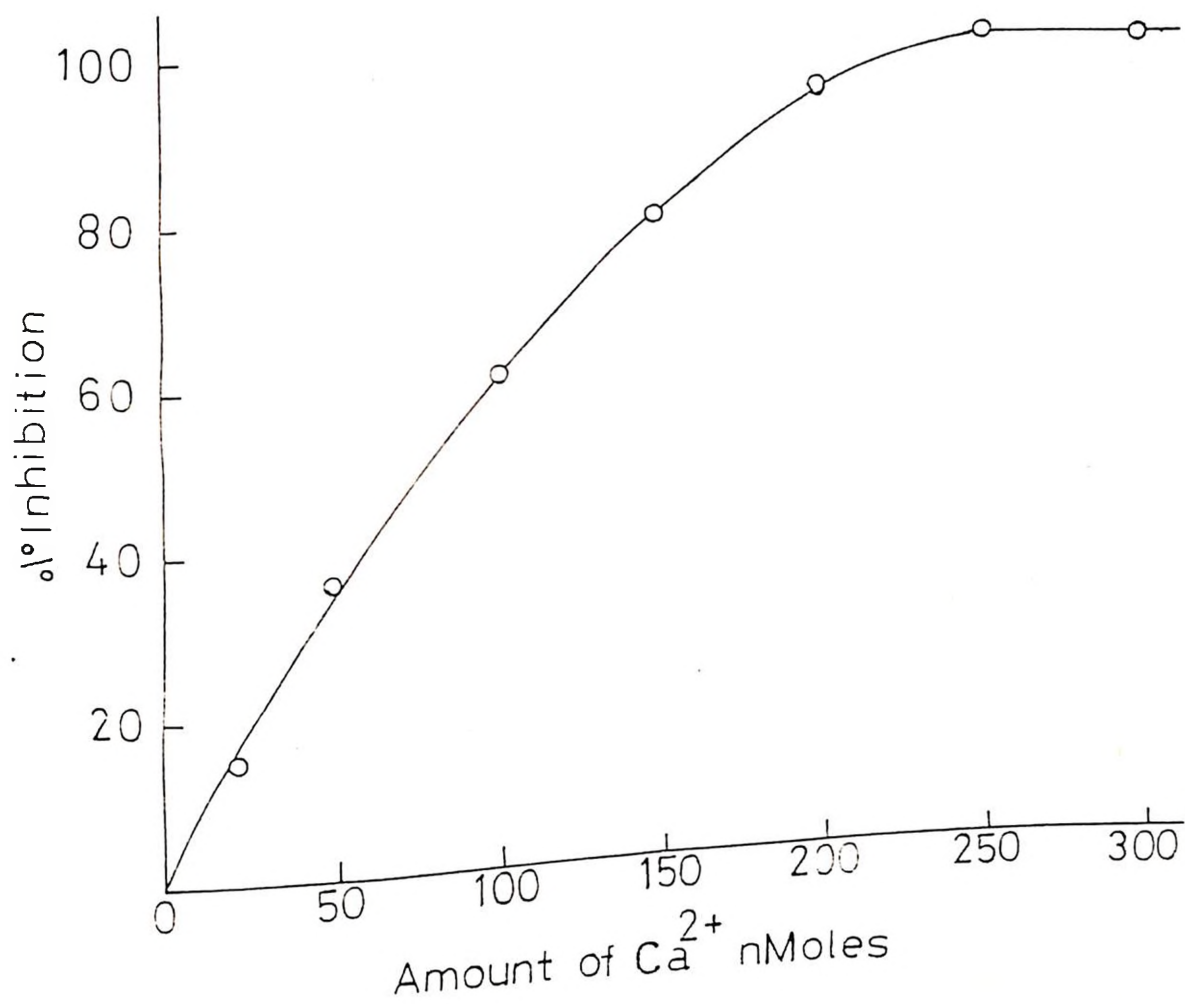
(B)
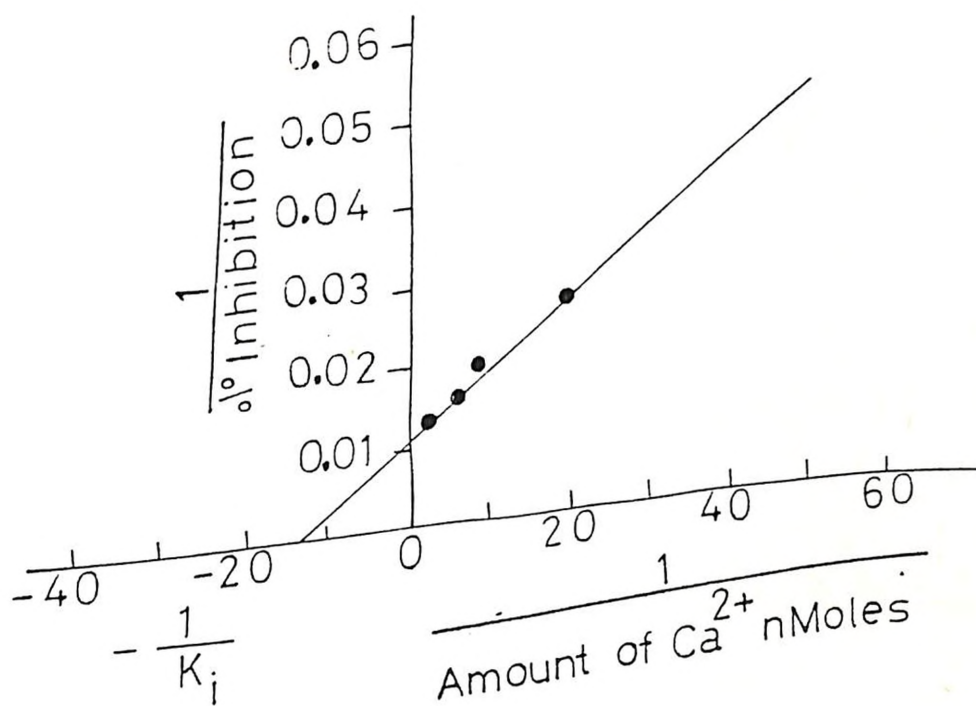
a half maximal inhibition was seen upon uptake of
75 nMoles Ca^{2+}.

In Figure 23 respiratory stimulation was
induced by the addition of 250 nMoles Ca^{2+} per mg. of
mitochondrial protein. After uptake of the Ca^{2+} and
a resumption of state IV respiration 180 nMoles ADP
was added. The trace shows that this addition of
ADP failed to stimulate respiration. This was
followed by the addition of 1 μM. ruthenium red and
NaCl to a final concentration of 20 mM. This
caused a gradual resumption of respiratory stimulation
which eventually was similar to that caused by ADP.
This is apparently due to efflux of accumulated Ca^{2+}
caused by the presence of Na^+, which also shows that
Ca^{2+} accumulation does not result in a permanent
damage to mitochondria, and its inhibitory effect on
oxidative phosphorylation can be reversed when it is
released from the mitochondria.

In Figure 24(A) stimulation of respiration was
induced by the addition of 180 nMoles ADP. After
depletion of the ADP and a resumption of state IV
respiration, 150 nMoles Ba^{2+} per mg. of mitochondrial
protein was added. This caused a stimulation
of respiration which continued until all the added
Ba^{2+} was taken up. This was followed by a further
addition of 180 nMoles ADP. This addition of ADP

FIGURE 23

Effect of Na$^+$ on the Ca^{2+} induced inhibition
of oxidative phosphorylation in brain
mitochondria

The reaction mixture at 30°C consisted of 30 mM
Tris-HCl buffer, pH 7.5, 0.3 M mannitol, 3 mM Tris-
phosphate, 100 mM KCl and 2 mg. mitochondrial protein
in a 1.7 ml final volume. The reaction was assayed
by following the stimulation of oxygen uptake
polarographically using a clark-type oxygen electrode.
Additions when indicated are :- 5 mM malate (MAL),
5 mM glutamate (GLU) 500 nMoles Ca^{2+}, 180 nMoles ADP,
1 μM ruthenium red (RR) and NaCl to a final concentration of
20 mM.

MAL + GLU

Ca^{2+}

ADP

RR

Na$^+$

1 Min

$\Delta O_2 = $ 50 nMole atoms
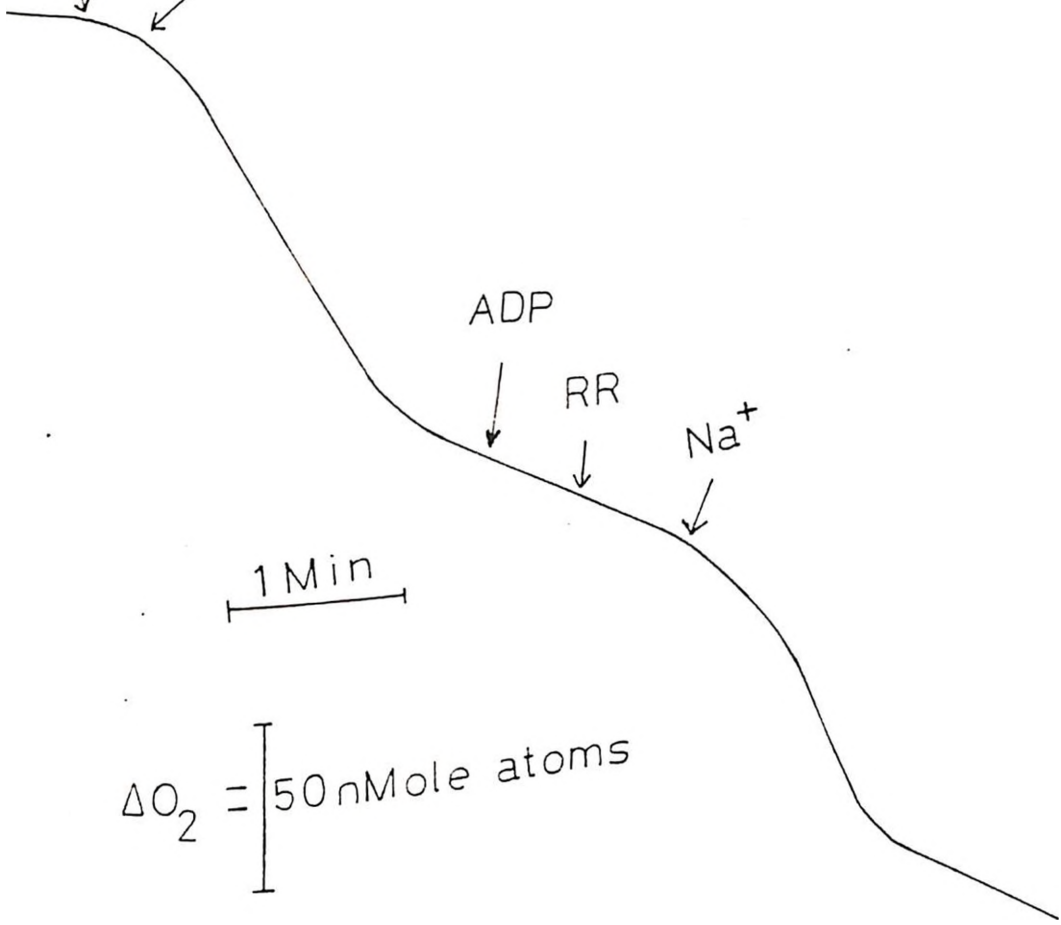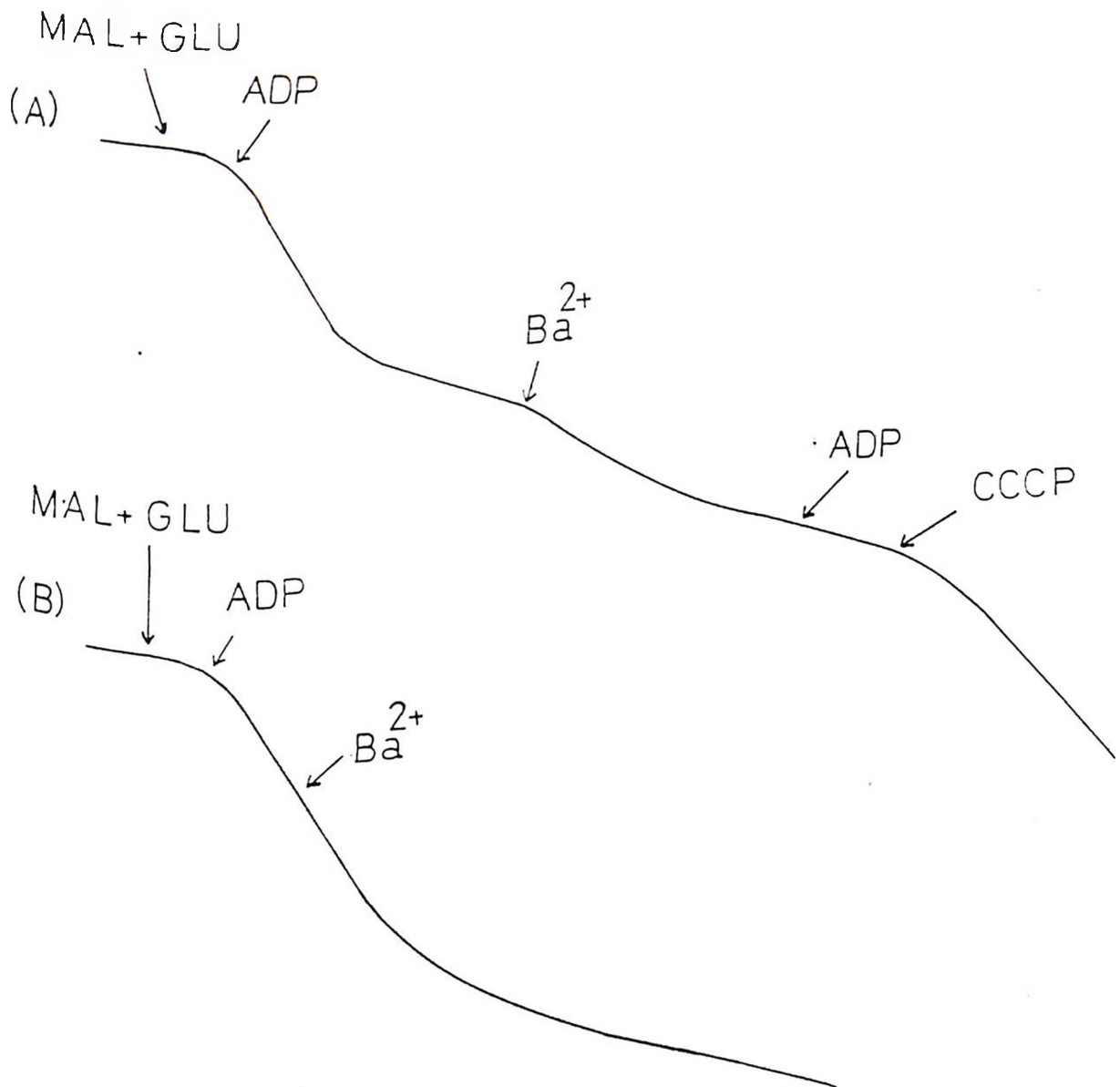
FIGURE 24

Effect of Ba^{2+} accumulation on brain mitochondrial oxidative phosphorylation

For figures A and B the incubation mixture consisted of 50 mM Tris-HCl buffer, pH 7.5, 0.3 M mannitol, 100 mM KCl, 3 mM Tris-phosphate and 2 mg. mitochondrial protein in a 1.7 ml. final volume. The reaction was assayed by following the stimulation of oxygen uptake polarographically using a clark-type oxygen electrode. Additions when indicated are:- 5 mM malate (MAL) 5 mM glutamate (GLU), 300 nMoles Ba^{2+}, 5 µM CCCP, 180 nMoles ADP in figure A and 1.5 µMoles ADP in figure B.

Figures C and D are saturation and Linearweaver Burk plots showing the extent of inhibition of oxidative phosphorylation upon uptake of varying amounts of Ba^{2+}. This was assayed by determining the extent of inhibition of the ADP induced respiratory stimulation upon uptake of varying amounts of Ba^{2+} as described for figure A.

(D)

Amount of Ba^{2+} nMoles

$$\frac{1}{\% \text{ Inhibition}}$$

0.06
0.05
0.04
0.03
0.02
0.01

-40 -30 -20 -10 0 10 20 30 40 50 60

$-\dfrac{1}{K_i}$

$$\frac{1}{\text{Amount of Ba}^{2+} \text{ nMoles}}$$

(C) 100

% Inhibition
80
60
40
20
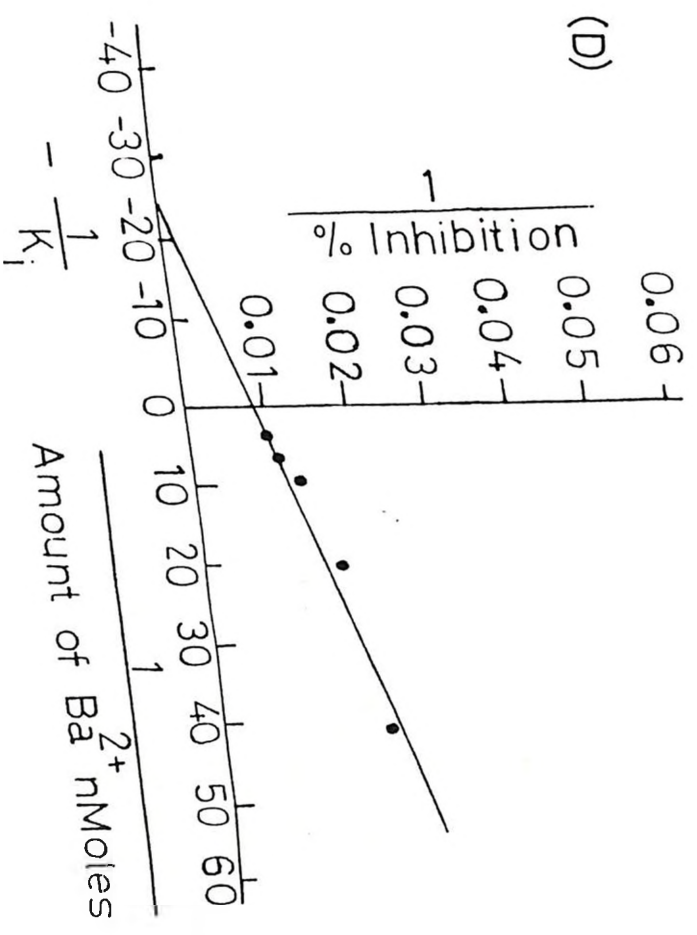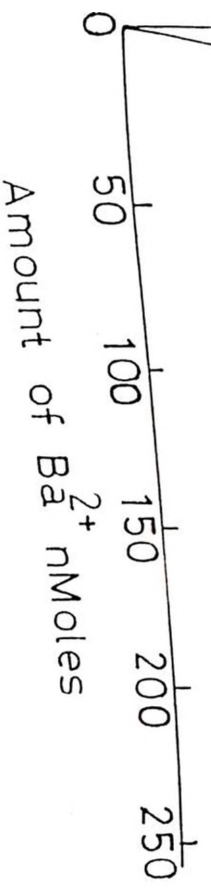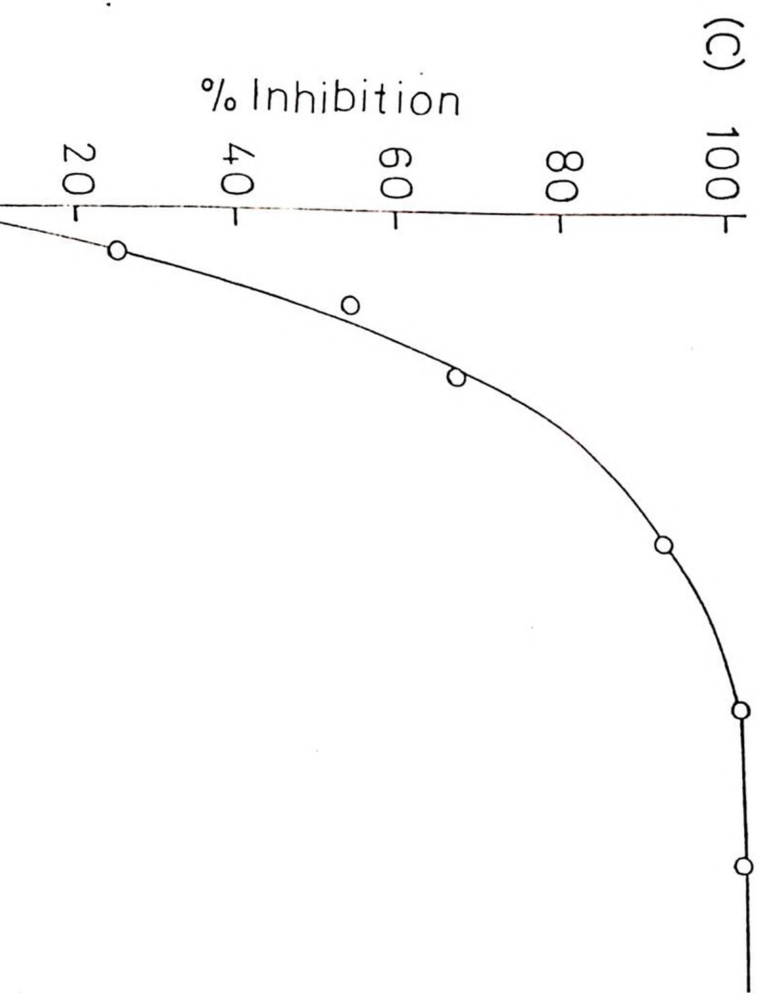
failed to stimulate respiration. A further stimulation of respiration could be re-induced by the addition of more Ba^{2+} or addition of the uncoupler CCCP. In figure 24(B) stimulation of respiration was induced by addition of 1.5 µMoles ADP. This was then followed by the addition of 150 nMoles Ba^{2+} per mg. of mitochondrial protein. The figure shows that this addition of Ba^{2+} also caused an inhibition of the ADP induced respiration.

Figure 24(C) and (D) shows that the Ba^{2+} induced inhibition of the ADP response was dependent on the amount of Ba^{2+} taken up by the mitochondria. Uptake of 150 nMoles Ba^{2+} per mg. of mitochondrial protein resulted in a complete inhibition of the ADP response while a half maximum inhibition was seen on uptake of 50 nMoles Ba^{2+}. .

In Figure 25 respiratory stimulation was induced by the addition of 150 nMoles Ba^{2+} and 150 nMoles Ca^{2+} per mg. of mitochondrial protein. Both cations were taken up as evidenced by the extent of the respiratory stimulation. Addition of ADP after uptake of the cations failed to stimulate respiration.

In Figure 26(A) respiratory stimulation was induced by the addition of 250 nMoles Mn^{2+} per mg.

FIGURE 25

Effect of combined uptake of Ca^{2+} and Ba^{2+} on mitochondrial oxidative phosphorylation

The incubation mixture consisted of 50 mM Tris-HCl, pH 7.5., 0.3 M mannitol 100 mM KCl, 3 mM Tris-phosphate and 2 mg. of mitochondrial protein in a 1.7 ml final volume. Additions when indicated are:- 5 mM malate (MAL) 5mM glutamate (GLU), 300 n Moles Ca^{2+}, 300 nMoles Ba^{2+} and 180 nMoles ADP.
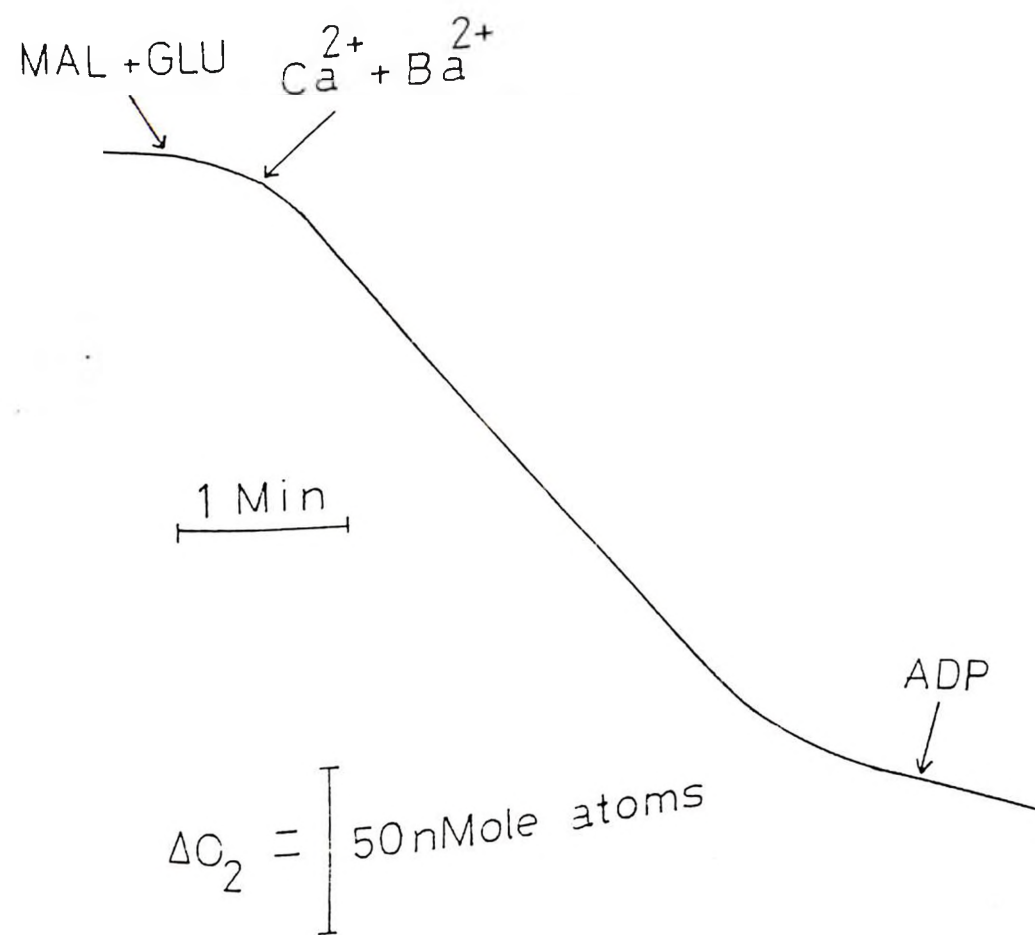
FIGURE 26

Effect of Mn^{2+} uptake, and a combined uptake of Ca^{2+} and Mn^{2+}, Ba^{2+} and Mn^{2+} on brain mitochondrial oxidative phosphorylation

The incubation mixture at 30°C consisted of 50 mM Tris-HCL buffer, pH 7.5, 0.3 M. mannitol, 100 mM KCl, 3 mM Tris-phosphate and 2 mg. of brain mitochondrial protein in a 1.7 ml final volume. Additions where indicated are 5 mM malate (MAL), 5 mM glutamate (GLU) 500 nMoles Mn^{2+} for Figure A, 300 nMoles Mn^{2+} and 300 nMoles Ca^{2+} for Figure B, 300 nMoles Mn^{2+} and 300 nMoles Ba^{2+} for Figure C, and 180 nMoles ADP.

(A)

MAL + GLU

Mn^{2+}

1 Min

ADP

$\Delta O_2 = \Big|$ 50 nMole atoms

(B)

MAL.+ GLU. Ca^{2+} + Mn^{2+}

1 Min

ADP

ΔO_2 = | 50 nMole atoms

(C)
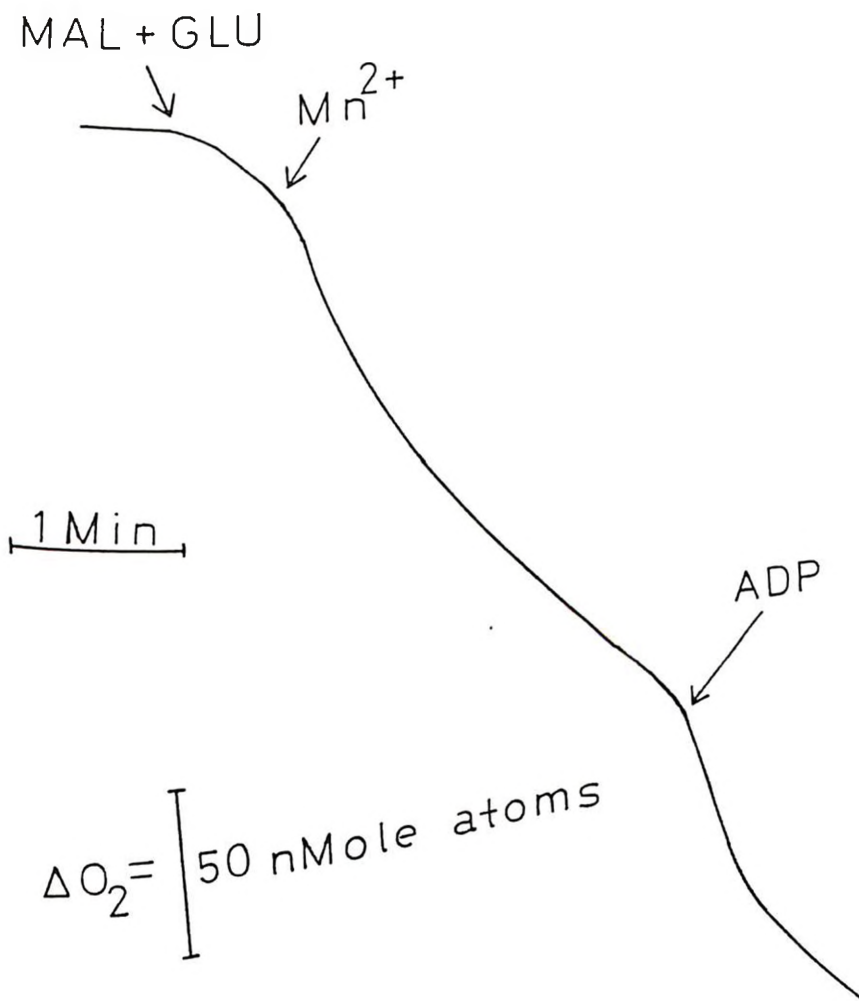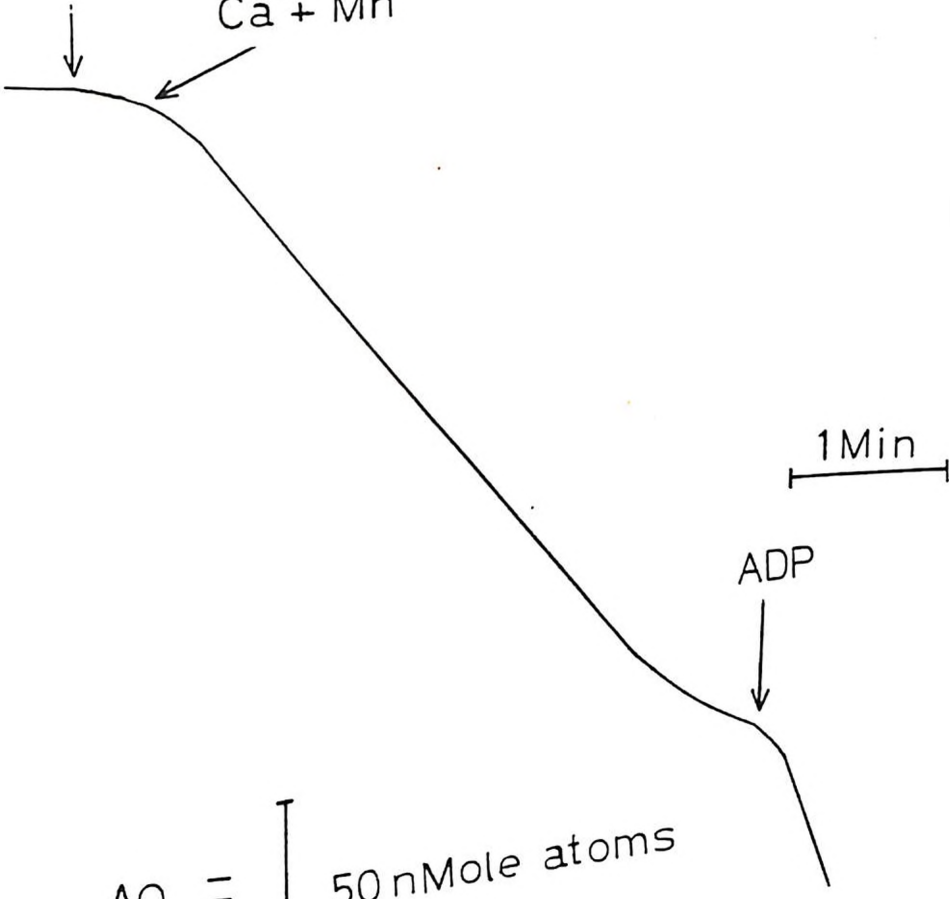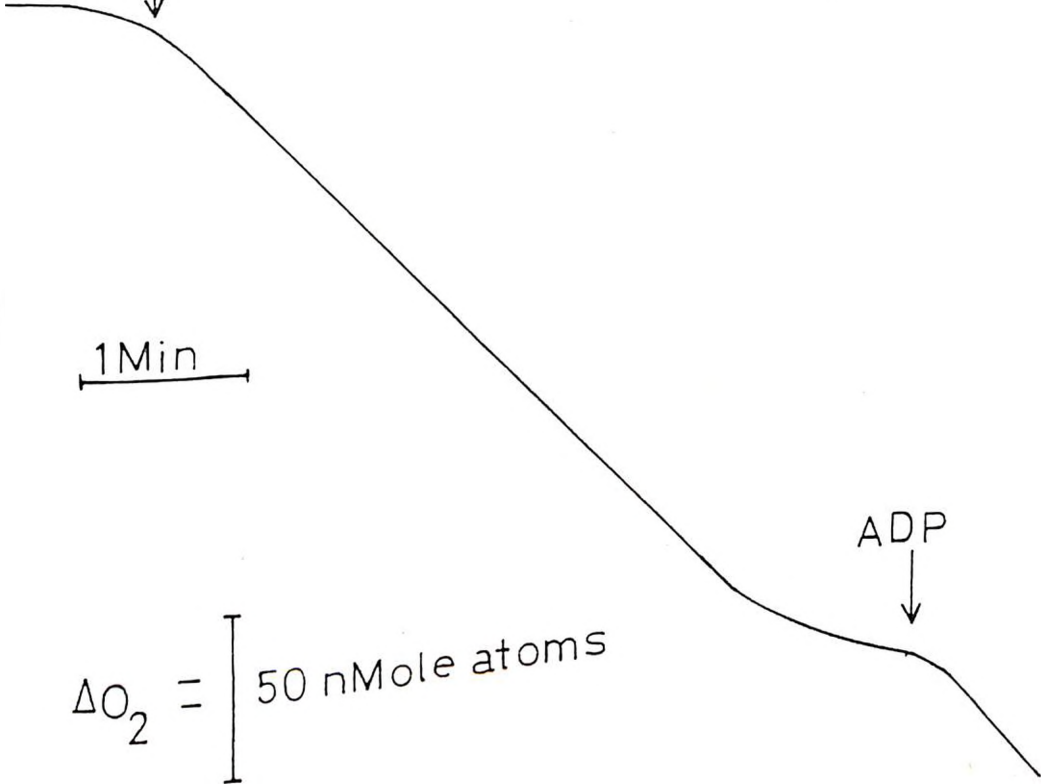
of mitochondrial protein. After uptake of all the Mn^{2+} and a resumption of state IV respiration 180 nMoles ADP was added. The figure shows that unlike the effects of Ba^{2+} and Ca^{2+} accumulation of Mn^{2+} did not have any effect on the ADP induced respiratory stimulation.

In Figure 26(B) respiratory stimulation was induced by the addition of 150 nMoles Ca^{2+} and 150 nMoles Mn^{2+} per mg. of mitochondrial protein. The figure shows that both cations were taken up after their uptake 180 nMoles ADP was added. This resulted in a full and an uninhibited respiratory stimulation which is unlike the effect observed when Ca^{2+} was taken up alone. This shows that the presence of Mn^{2+} reverses the inhibitory effect of Ca^{2+} on oxidative phosphorylation. A similar effect was observed when Mn^{2+} and Ba^{2+} were taken up together (Figure 26 C).

Effect of bivalent cations on beef heart MgATP submitochondrial ATPase activity

Table VI shows that preincubation of MgATP submitochondrial particles with a respiratory substrate such as succinate or NADH caused a twofold increase in the ATPase activity. This activation of

TABLE VI

Effect of preincubating MgATP submitochondrial particles under various conditions on the ATPase activity

50 μg MgATP particles were preincubated at 30°C for 3 minutes in a medium containing 25 mM Tris- acetate buffer, pH 7.5,   125 mM sucrose, 30 mM K^+-acetate in a 3 ml. final volume. Other additions where shown are 3 ug antimycin, 5 μM CCCP, 1 mM NADH and 1 mM Ca^{2+}, Mg^{2+} or Mn^{2+}. After the preincubation 5 μM CCCP was added to the samples and the ATPase activity tested by following the rate of ATP hydrolysis with a pyruvate kinase – lactate dehydrogenase system and, measuring the rate of NADH oxidation spectrophotometrically at 340 nM. A unit of ATPase activity was expressed as the number of nMoles of ATP hydrolysed per minute per mg of particle protein.

Preincubation conditions (additions)	ATPase activity
No addition	200
Rotenone	200
Rotenone + Succinate	430
Rotenone + Succinate + Antimycin	240
Rotenone + Succinate + CCCP	200
NADH	440
NADH + Rotenone	200
Rotenone + Succinate + Ca^{2+} or Mg^{2+} or Mn^{2+}	430

the ATPase activity was inhibited when the respiratory
inhibitors antimycin or rotenone or the uncoupler
CCCP were added to the medium prior to the preincuba-
tion, but not when they were added after the preincuba-
tion. This thus shows that the activation of the
ATPase is as a result of an establishment of an
electrochemical gradient across the submitochondrial
membranes. The ATPase remained activated for upto
10 minutes after addition of a respiratory inhibitor.
These observations are in agreement with those of
Van de Stadt et al., (1973) and those of Gomez-Puyuo,
et al., (1979) who also reported an activation of the
ATPase activity of submitochondrial particles upon
establishment of an electrochemical gradient. These
authors claimed that the observed activation of the
ATPase was most probably due to a release of the
endogenous ATPase inhibitor protein from its
inhibitory site.

That the inhibitor protein is really involved
in this activation is shown in table VII. Here
particles in which most of the inhibitor had been
removed (state III particles) according to Van de
Stadt, et al., (1973) were used. These particles were
characterized by a high succinate insensitive ATPase
activity, which could be lowered by incubating them
with the purified inhibitor in the presence of ATP
and Mg^{2+}. The ATPase of the inhibitor supplemented

TABLE VII

Activation of inhibitor supplemented state III submitochondrial particles, by preincubation, with succinate

Inhibitor depleted submitochondrial particles were prepared according to Van de Stadt et al., (1973) as described in materials and methods. 50 µg of the inhibitor depleted were preincubated at 30°C for 3 minutes in a medium containing 25 mM Tris-acetate buffer, pH 6.5, 125 mM sucrose, 3 µg rotenone, in a 3 ml final volume. Where indicated the following additions were made:- 3 mM ATP, 3 mM $MgCl_2$, 5 mM succinate and 5 µg of the purified ATPase inhibitor. After the preincubation 5 µM CCCP was added and the ATPase activity was assayed as described for Table VI. ATPase activity was expressed as the number of µMoles of ATP hydrolysed per minute per mg. of submitochodnrial protein.

Additions	ATPase activity
None	3.53
Succinate	3.54
Inhibitor protein + ATP + Mg^{2+}	0.70
Inhibitor protein + succinate+ATP+Mg^{2+}	2.56

FIGURE 27

Effect of inorganic phosphate on beef heart
MgATP submitochondrial particle ATPase activity

This effect was tested after preincubating 50
ug MgATP submitochondrial particles in a medium
consisting of 25 mM Tris-acetate buffer, pH 7.5,
125 mM sucrose, 30 mM K^+-acetate, 3 ug rotenone and
varying concentrations of Tris-phosphate for 3 min-
utes at 30°C in a 3 ml volume in the absence of
succinate —•—•— and in the presence of 5 mM
succinate —o—o—. After the preincubation 5μM
CCCP was added and the ATPase activity was assayed
enzymatically by following the rate of ATP hydrolysis
with a pyruvate kinase, lactate dehydrogenase system
and measuring the rate of NADH oxidation spectrophoto-
metrically at 340 nM. ATPase activity was expressed
as μMoles of ATP hydrolysed per minute per mg. of
submitochondrial protein.

1.2

1.0
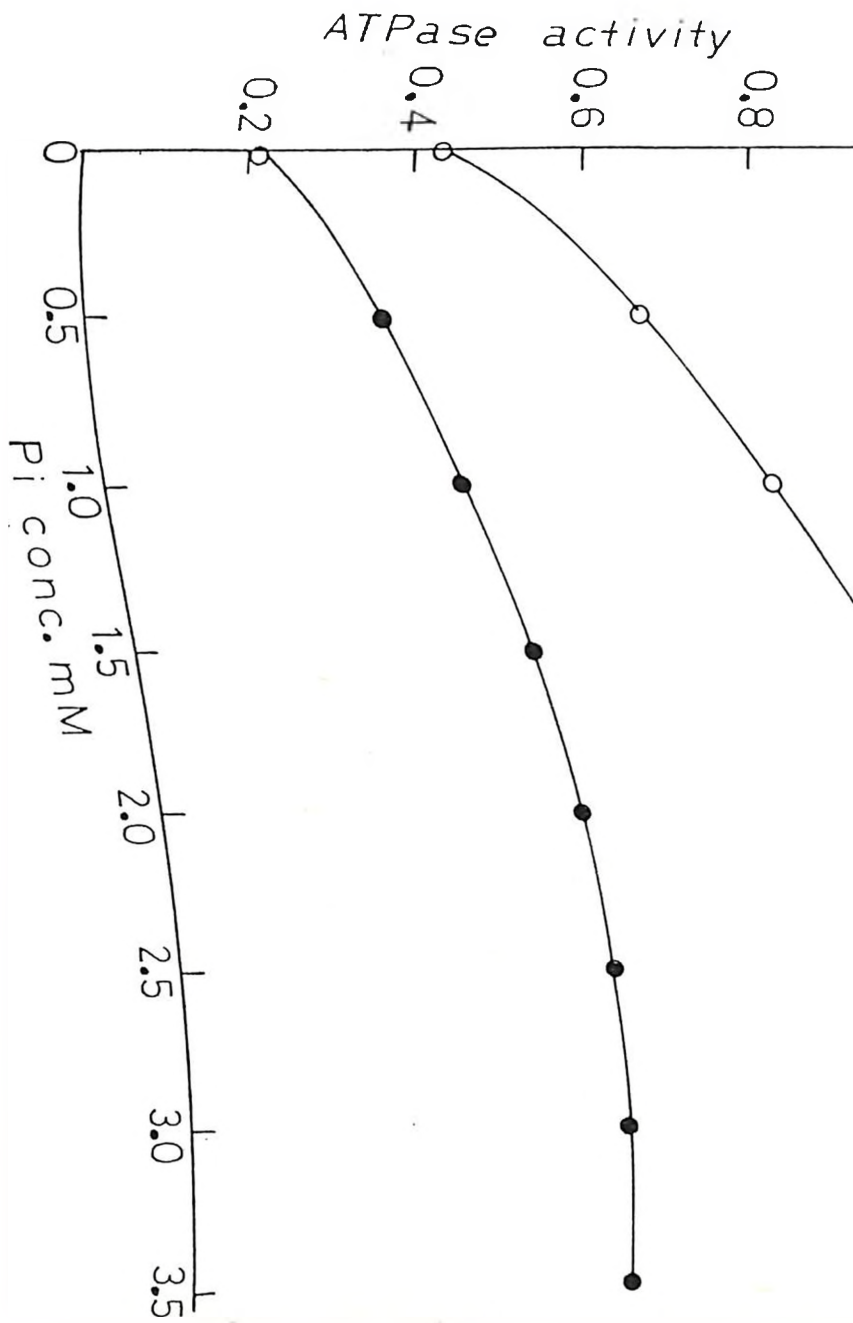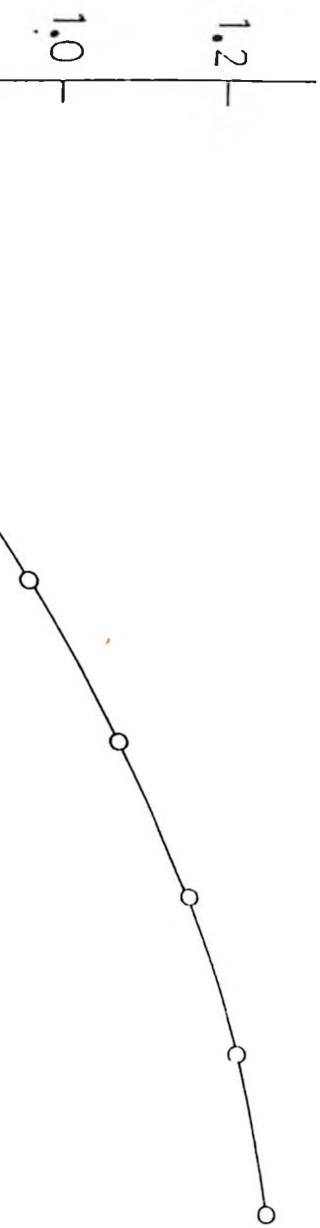
TABLE VIII

Activation of MgATP submitochondrial particle
ATPase activity by inorganic phosphate and
ADP-bivalent cation complexes

50 μg of MgATP submitochondrial particles were
preincubated for 3 minutes in a medium consisting of
25 mM Tris-acetate buffer, pH 7.5, 125 mM sucrose,
30 mM K^+-acetate, 3 μg rotenone, at 30°C in a 3 ml
final volume. Other additions where indicated are
5 mM Tris-succinate, 33 μM ADP, 3 mM Tris-phosphate
(Pi), 1 mM Ca^{2+}, 1 mM Mg^{2+} and 1 mM Mn^{2+}. After
the preincubation 5 μM CCCP was added and the ATPase
activity was assayed as described for Table VI,
ATPase activity was expressed as the number of nMoles
of ATP hydrolysed per minute per mg. of submitochond-
rial protein.

Additions	ATPase activity
No additions	200
Succinate	430
Succinate + Pi	810
Succinate + Pi + ADP	810
Succinate + Pi + ADP + Ca^{2+}	880
Succinate + Pi + ADP + Mg^{2+}	1220
Succinate + Pi + ADP + Mn^{2+}	1800

like the effect of inorganic phosphate alone was
more pronounced in the presence than in the absence
of succinate.

Figure 28 shows the effect of varying concentra-
tions of Mn^{2+}, Mg^{2+} and Ca^{2+} on the phosphate and
ADP induced activation of the ATPase activity. Mn^{2+}
showed the highest stimulatory effect at all the
concentrations tested, followed by Mg^{2+}. Ca^{2+} had
only a very small effect.

Figure 29(A) shows that preincubation of the
purified F_1-ATPase in the presence of inorganic
phosphate also caused an increase in ATPase activity.
This effect strengthens the above argument that
inorganic phosphate may bind to the inhibitor free
ATPase molecules, altering their conformation to a
more reactive state.

Figure 29(B) shows that incubation of the
purified F_1-ATPase with inorganic phosphate and
ADP in the presence of Mn^{2+} or Mg^{2+} caused a further
increase in the ATPase activity, with Mn^{2+} being
the more potent cation. Under these conditions
the presence of Ca^{2+} had no effect.

Figure 30 shows that for the activation of
submitochondrial ATPase to be observed under the

FIGURE 28

Effect of bivalent cations on beef heart MgATP

submitochondrial particle ATPase activity

This effect was tested after preincubating 50

ug MgATP particles in a medium consisting of 25 mM

Tris-acetate buffer, pH 7.5, 125 mM sucrose, 30 mM

K^+-acetate, 3 ug rotenone 1 mM inorganic phosphate,

5 mM succinate, 33 µM ADP, for 3 minutes at 30°C in

a 3 ml final volume. Other additions were:-

none ——•——•—— varying concentrations of $CaCl_2$

——o——o——, varying concentrations of $MgCl_2$ ——ö——ə——

and varying concentrations of $MnCl_2$ ——o——•—— .

After the preincubation 5 µM CCCP was added and the

ATPase activity was assayed as described for figure

27.
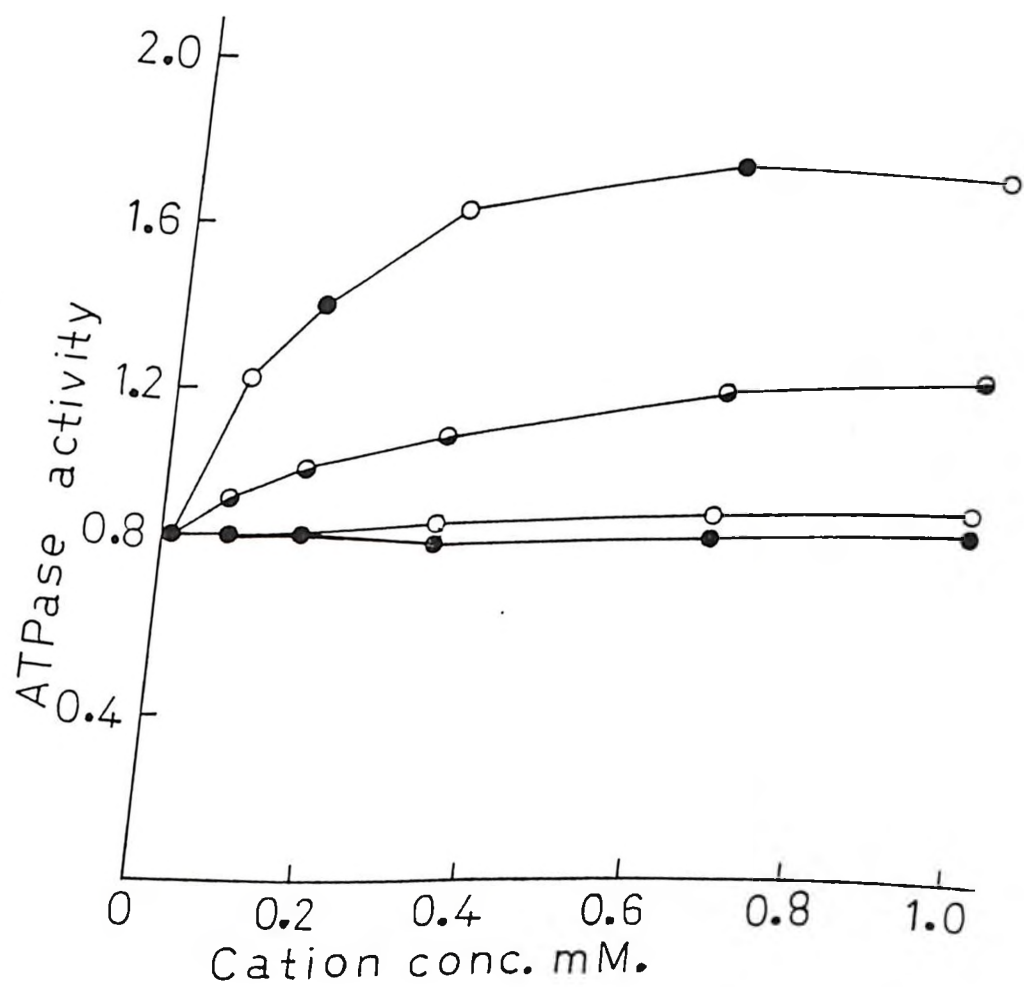
FIGURE 29

Effect of inorganic phosphate and bivalent cations on the ATPase activity of the purified enzyme

This effect was tested after preincubating 50 µg of the purified F_1-ATPase for 3 minutes in a medium containing 25 mM Tris-acetate buffer, 125 mM sucrose, 30 mM K^+-acetate, varying concentrations of Tris-phosphate for figure (A), and 1 mM Tris-phosphate in the presence of 33 µM ADP and varying concentrations of Ca^{2+} —o—o—, varying concentrations of Mg^{2+}, —•—•—, and varying concentrations of Mn^{2+} —o—o— for figure B. The temperature was 30°C and the volume was 3 ml. After the preincubation, the ATPase activity was tested as described for figure 27. ATPase activity is expressed as the µMoles ATP hydrolysed per minute per mg. protein.

ATPase activity vs Cation conc. mM

(A)

ATPase activity

(B)

Pi conc. mM
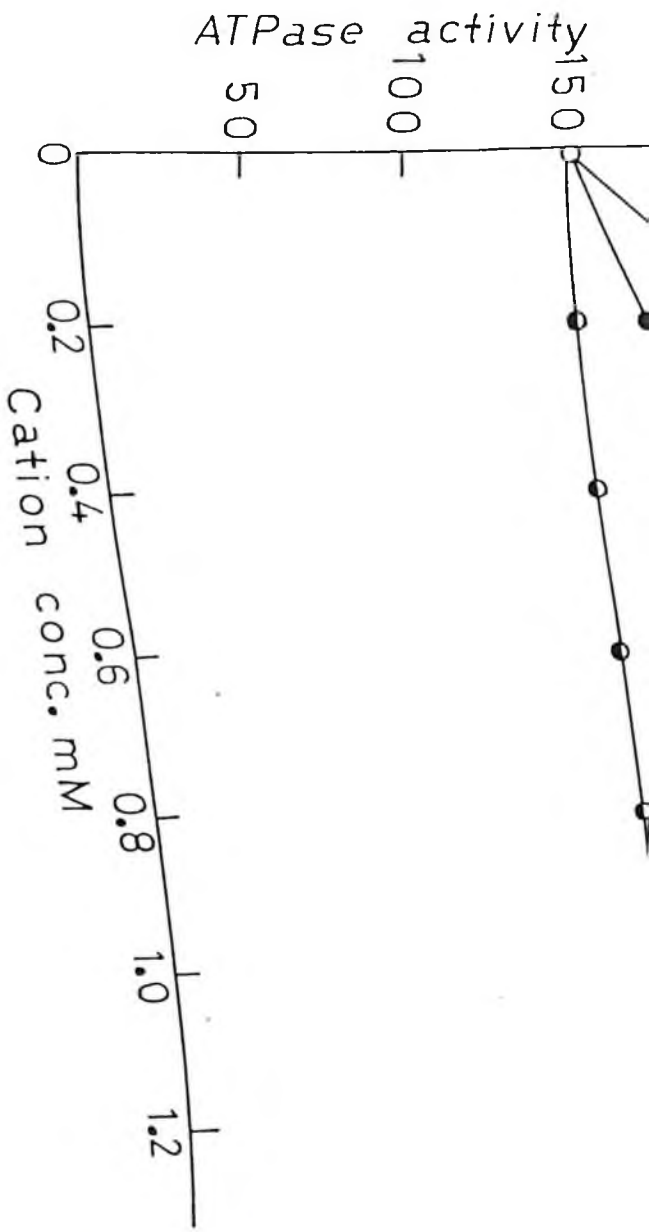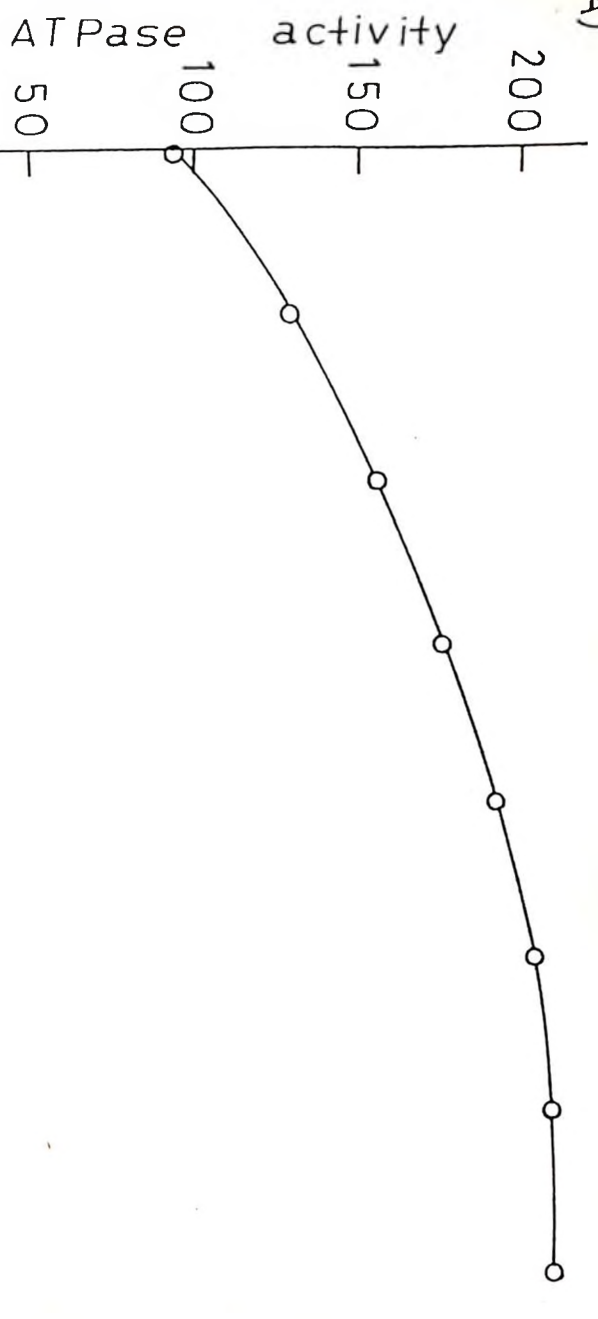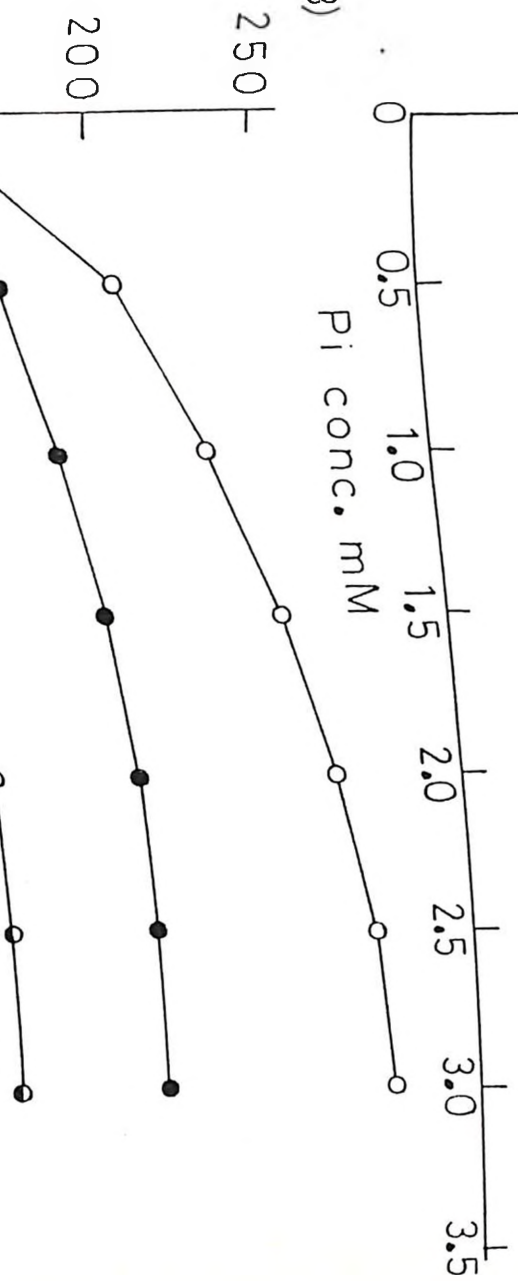
FIGURE 30

Effect of preincubating MgATP submitochondrial

particles at varying lengths of time on the

ATPase activity

300 μg of MgATP particles were preincubated at

30°C · in a medium consisting of 25 mM acetate buffer,

pH 7.5, 125 mM sucrose in the presence of :- 1 mM Tris-

phosphate + 0.4 mM $MnCl_2$ + 33 μM ADP ——△——△——, 1 mM

Tris-phosphate ——●——●—— , 1 mM Tris-phosphate + 33 μM

ADP ——⊖——⊖——, and no addition ——○——○——. At the

indicated times samples containing 50 μg particles were

taken and 5 μM CCCP added after which the ATPase activity

was assayed as described for figure 27.
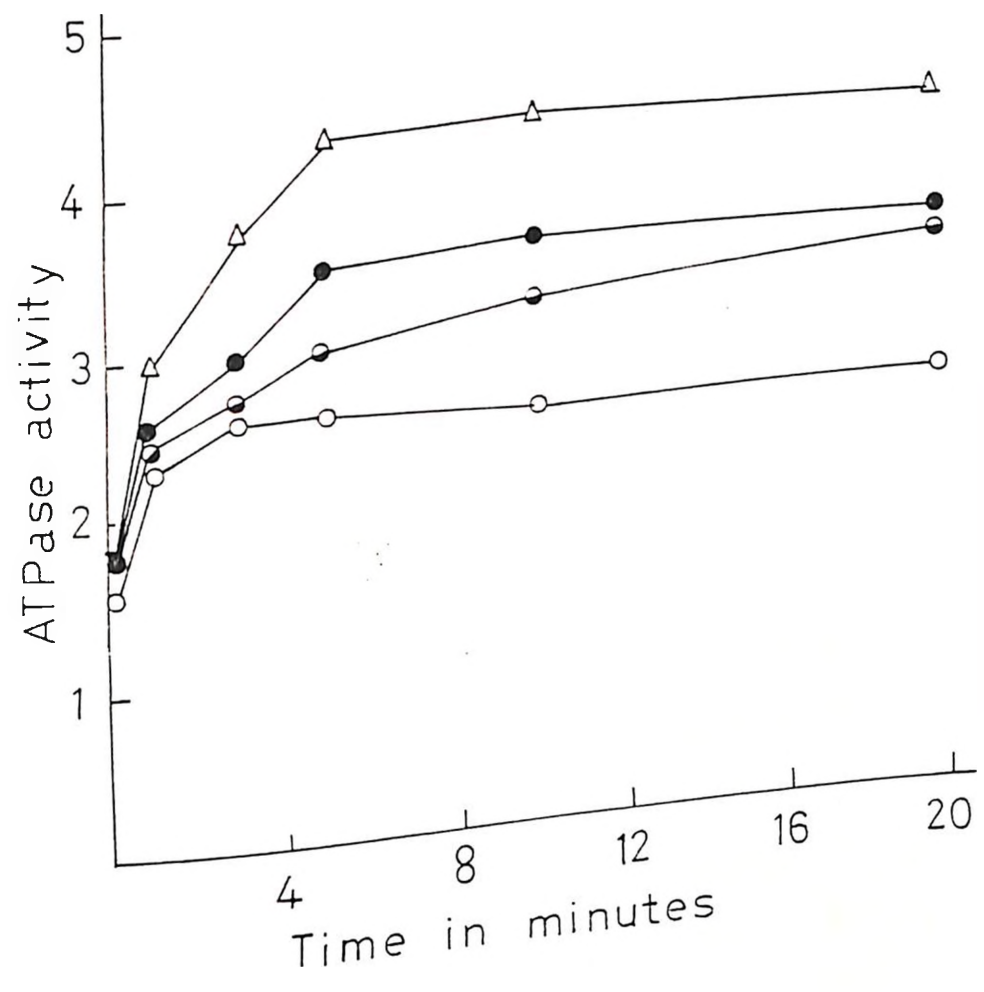
conditions described above, a certain preincubation period is required, with maximum activation occurring only after a preincubation period of 5 minutes.

Figures 31 and 32 shows that unlike the effect seen when submitochondrial particles were first preincubated with a bivalent cation and ADP, Mg^{2+} is a more potent cation for both ATP synthesis and ATP hydrolysis, when these were tested without preincubation of the particles. Ca^{2+} was not a good cofactor for either ATP synthesis or ATP hydrolysis.

Table IX shows that preincubation of MgATP submitochondrial particles with ATP in the presence of the bivalent cations Mn^{2+}, Mg^{2+} and Ca^{2+} led to an inactivation of the succinate and phosphate induced activation of the ATPase with Mn^{2+} being the more superior cation. This observation is in agreement with that of Gomez-Ferandez and Harris, (1978) who also showed that the presence of Mg and ATP led to an inactivation of submitochondrial ATPase activity. This inhibition of the ATPase activity is most probably due to a binding of the ATPase inhibitor protein on its inhibitory site. This is shown by the data, plotted in table X. The table shows that preincubation of inhibitor depleted

FIGURE 31

Effect of bivalent cations on the ATP
Synthesis of MgATP submitochondrial particles

This was tested during a 3 minute incubation in a medium containing 100 ug particle protein, 50 mM Tris-HCl buffer pH 7.5, 125 mM sucrose, 30 mM K^+-acetate, 5 mM succinate, 3 ug rotenone, 5 mM Tris-phosphate, 1 mM ADP, 500 µM diadenosine pentaphosphate and varying concentrations of Ca^{2+} —o—o—, Mn^{2+} —•—•— and Mg^{2+} —o—o— . After the incubation, the reaction was stopped with 5% perchloric acid followed by neutralization with KOH, and the amount of ATP formed was assayed using a hexokinase, glucose-6-phosphate dehydrogenase system by following the reduction of $NADP^+$ spectrophotometrically at 340 nM.
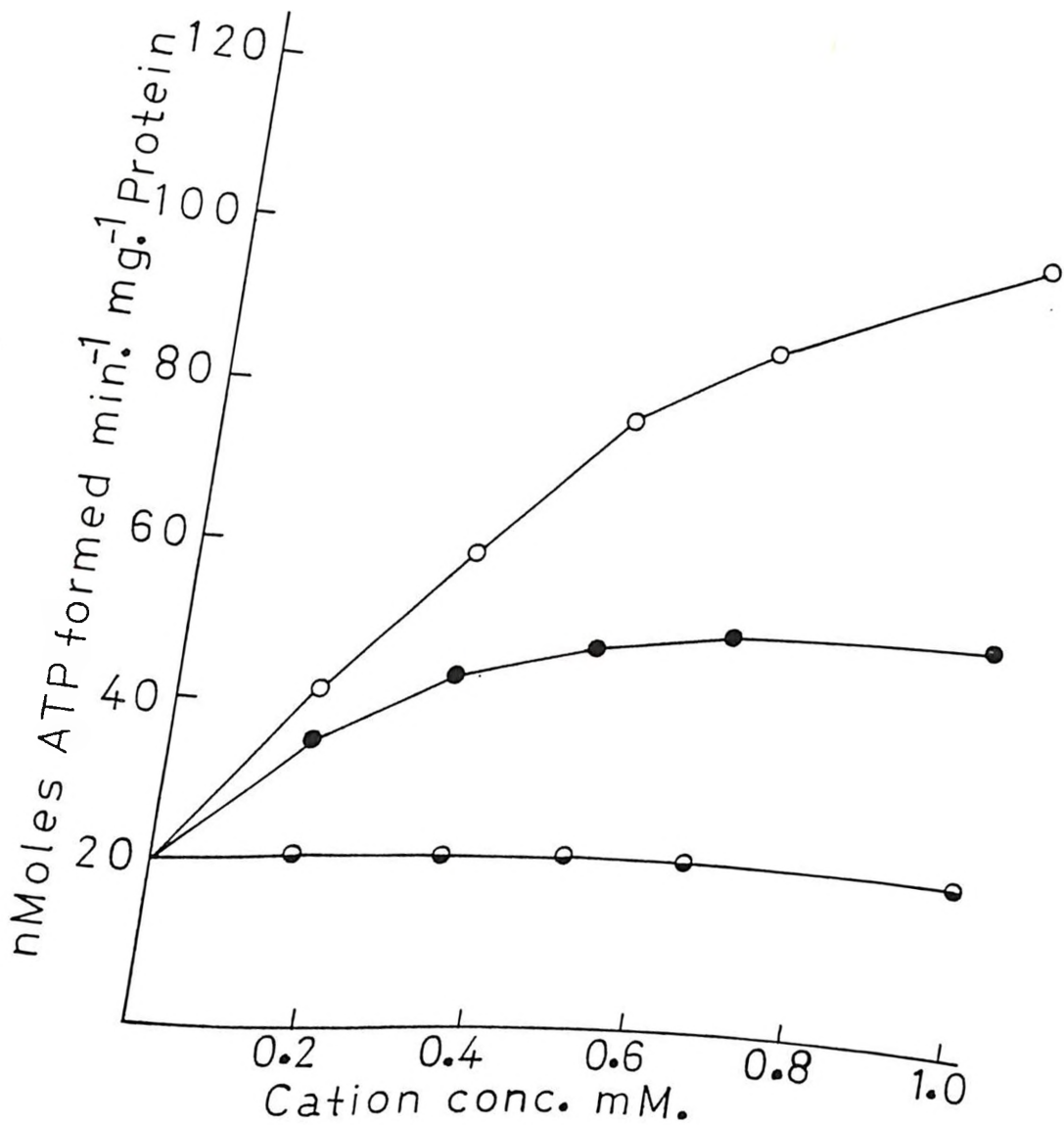
FIGURE 32

Effect of bivalent cations on ATP hydrolysis by MgATP particles

36 μg of MgATP submitochondrial particles were incubated for 3 minutes in a medium consisting of 25 mM Tris-acetate buffer pH 7.5, 125 mM sucrose, 30 mM K^+-acetate, 3 mM ATP, 5 μM CCCP in the presence of varying concentrations of Ca^{2+} —▲—▲—, Mn^{2+} + Ca^{2+} —△—△—, Mg^{2+} + Ca^{2+} —o—o—, Mn^{2+} —●—●— and Mg^{2+} —●—●—. After the incubation the reaction was stopped with 9.4% trichloacetic acid followed by neutralization with KOH. The extent of ATP hydrolysis was measured by assaying the amount of inorganic phosphate formed using a formaldehyde, ammonium molybdate system as described in materials and methods.
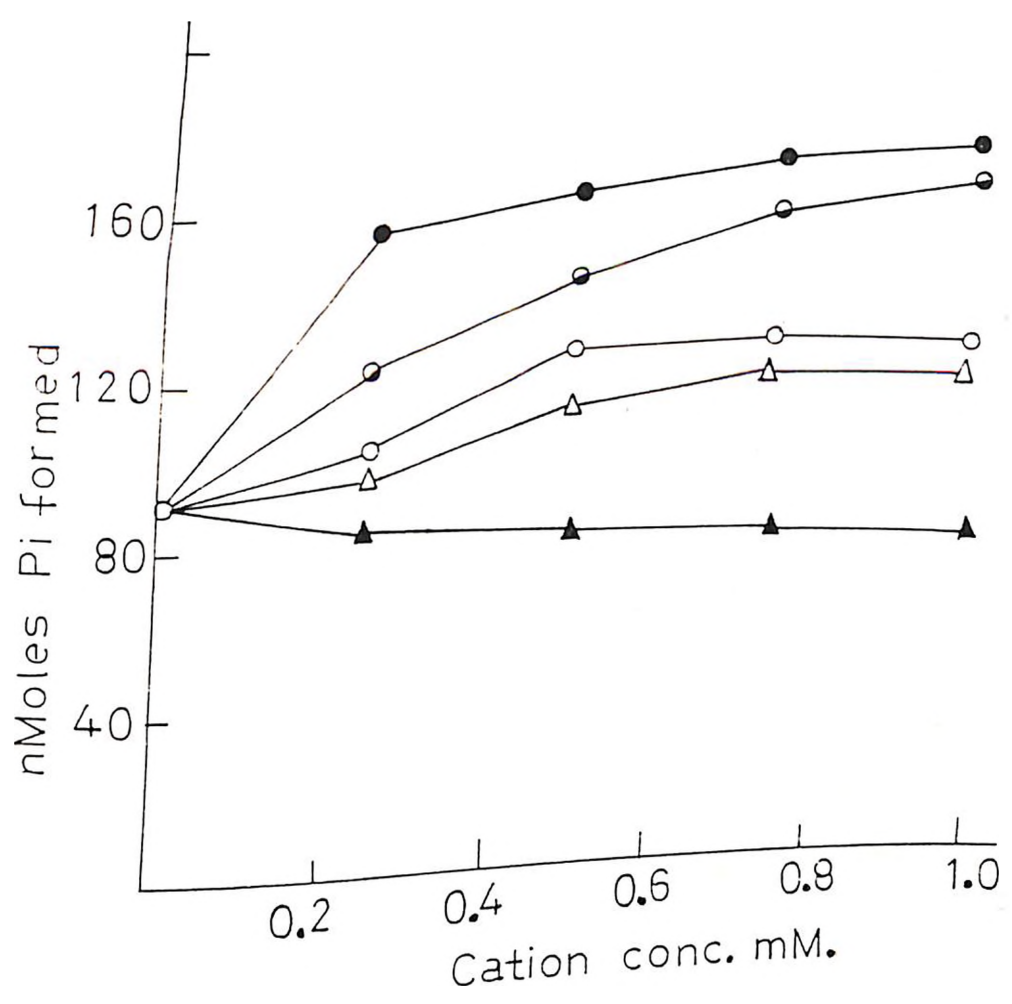
TABLE IX

Inactivation of the succinate and phosphate induced activation of MgATP submitochondrial particle ATPase by ATP-bivalent cation complexes

50 µg of MgATP particles were preincubated in a medium consisting of 25 mM Tris-acetate buffer, pH 7.5, 125 mM sucrose, 30 mM K^+-acetate at 30°C for 3 minutes. Additions where indicated are 5 mM succinate (+ 3 µg rotenone), 1 mM Tris-phosphate, 3 mM ATP, 1 mM Ca^{2+}, 1 mM Mg^{2+} and 1 mM Mn^{2+}. After the preincubation 5 µM CCCP was added and the ATPase activity was assayed as described for table VI. ATPase activity was expressed as the number of nMoles of ATP hydrolysed per minute per mg. of the submitochondrial protein.

Additions	ATPase activity
No addition	200
Succinate	430
Succinate + Pi	810
Succinate + Pi + ATP	810
Succinate + Pi + ATP + Ca^{2+}	720
Succinate + Pi + ATP + Mg^{2+}	560
Succinate + Pi + ATP + Mn^{2+}	200

submitochondrial particles (state III particles),
with the purified inhibitor in the presence of ATP
and bivalent cations led to an inactivation of the
ATPase activity with Mn^{2+} showing the highest
inhibitory effect followed by Mg^{2+} and Ca^{2+}.

Figure 33 shows the effect of varying concentra-
tions of the cations Mn^{2+}, Mg^{2+} and Ca^{2+} on the ATP
induced inactivation of the ATPase activity. Mn^{2+}
showed the highest inhibitory effect at all the
concentrations tested followed by Mg^{2+} and Ca^{2+}.

DISCUSSION

The results of figures 19 - 26 show that
accumulation of large amounts of Ca^{2+} and Ba^{2+} by
rat brain mitochondria led to a complete inhibition
of ADP induced respiratory stimulation. The uptake
of these cations took precedence over oxidative
phosphorylation in the utilization of respiratory
energy as shown by the fact that they caused a
inhibition of oxidative phosphorylation when they
were added, after addition of large quantities of
ADP. Accumulation of Mn^{2+} did not result in any inhibition
of the subsequent ADP induced respiratory stimulation,

FIGURE 33

Effect of preincubating MgATP particles in the

presence of bivalent cations and ATP on the

ATPase activity.

50 ug of MgATP particles were preincubated for

3 minutes in a medium containing 25 mM Tris-acetate

buffer, pH 7.5, 125 mM sucrose, 30 mM K^+-acetate,

1 mM Tris-phosphate, 5 mM succinate, 3 mM ATP and

——•——•—— no further addition, ——•——•—— varying

concentrations of Ca^{2+}, ——o——o—— varying concentrations

of Mg^{2+} ——o——•—— varying concentration of Mn^{2+}. After

the preincubation 5 µM CCCP was added and the ATPase

activity of the particles was tested as described for

figure 27.
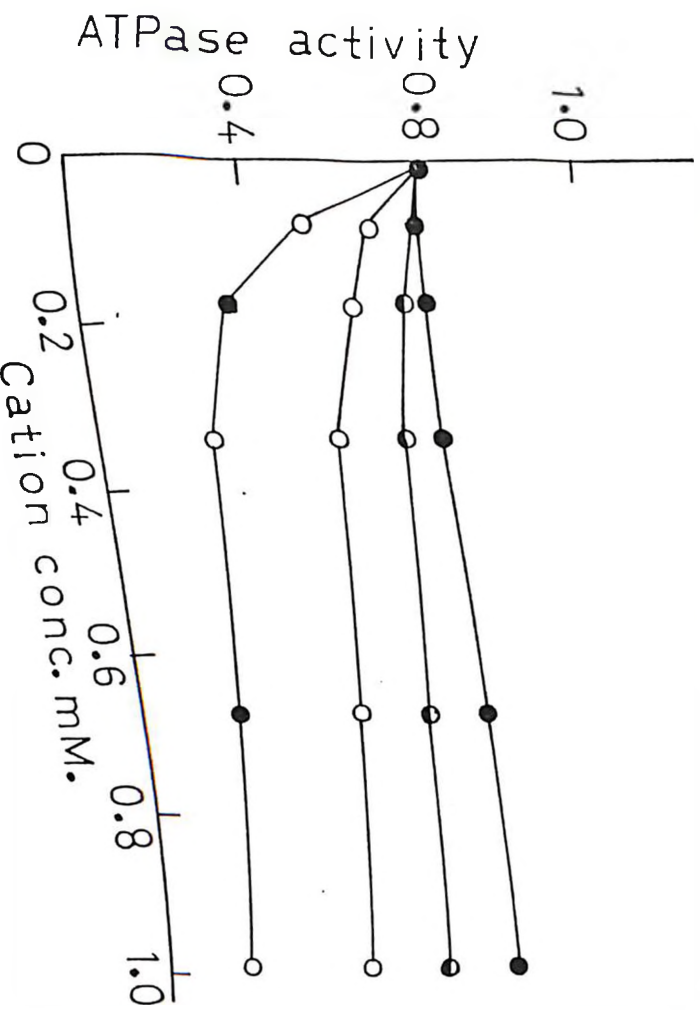
TABLE X

Deactivation of submitochondrial ATPase activity by addition of the purified ATPase inhibitor to inhibitor depleted particles in the presence of ATP and bivalent cations

Inhibitor depleted submitochondrial particles were prepared according to Van de Stadt et al., (1973) as described in materials and methods. 50 µg. of the inhibitor depleted particles were preincubated at 30°C for 3 minutes in a medium containing 25 mM Tris-acetate buffer, pH 6.5, 125 mM sucrose, 3 µg rotenone, in a 3 ml final volume. Where indicated the following additions were made:- 3 mM ATP, 5 µg of the purified ATPase inhibitor, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM $MnCl_2$. After the preincubation 5 µM CCCP was added and the ATPase activity assayed as described for Table VI. ATPase activity was expressed as the number of µMoles of ATP hydrolysed per minute per mg. of submitochondrial protein.

Additions	ATPase activity
None	3.53
ATP	3.49
Inhibitor	3.42
Inhibitor + ATP	3.34
Inhibitor + ATP + Ca^{2+}	2.56
Inhibitor + ATP + Mg^{2+}	0.70
Inhibitor + ATP + Mn^{2+}	0.21

and infact the presence of accumulated Mn^{2+} reversed
the inhibitory effects of Ca^{2+} and Ba^{2+}. That the
inhibitory effect of Ca^{2+} is due to intramitochondrial
and not extramitochondrial Ca^{2+} is shown by the fact
that the presence of ruthenium red, prevented the
inhibitory effect of Ca^{2+}. Furthermore accumulated
Ca^{2+} did not cause any permanent damage to the mitocho-
ndria, since when it was released by addition of Na^+,
mitochondrial oxidative phosphorylation was restored.
A similar inhibitory effect of accumulated Ca^{2+} on
oxidative phosphorylation has been reported earlier in
mitochondria isolated from brain (Roman et al, 1981,
Nowicki et al., 1982) Ehlrich ascites tumours (Thorne
and Bygrave 1974, Villalobo, 1978) but not those from
liver (Jacobus et al., 1975, Vercesi, et al., 1978)
which had their ADP response restored after Ca^{2+} uptake
was complete. The precise mechanism by which
accumulated Ca^{2+} causes an inhibition of oxidative
phosphorylation is not clear. However, several
mechanisms have been proposed, including an inhibition
of the ATP-ADP translocator (Thorne and Bygrave, 1974;
Gomez-Puyou, et al., 1979) a competition with
intramitochondrial Mg^{2+} for binding to ADP, (Roman
et al., 1981) or an inhibition of the release of the
ATPase inhibitor protein, which becomes associated
with the ATPase during active Ca^{2+} uptake. However,
none of these mechanisms have been thoroughly investi-
gated.

To test the possible mechanism by which Ca^{2+} accumulation could inhibit oxidative phosphorylation, in a manner that could be reversed by Mn^{2+} MgATP submitochondrial particles from beef heart mitochondria were used. These were chosen because they allowed a direct accessibility of the cations to the ATPase. The choice of beef heart mitochondria as the source of the particles was because it was found difficulty to make coupled submitochondrial particles from brain, and that beef heart mitochondria could be made in large quantities. Furthermore heart mitochondria have been shown to behave in a similar manner to brain mitochondria with regard to the effects of Ca^{2+} accumulation on oxidative phosphorylation (Gomez-Puyou et al., 1979, Roman et al, 1981).

In some of the experiments the effects of Ca^{2+} and Mn^{2+} on the submitochondrial ATPase was compared to that of Mg^{2+} which is the physiological cation in the activity of the ATPase.

The results of these experiments show that the ATPase activity of the submitochondrial particles could be raised twofold by preincubating the particles in the presence of a respiratory substrate such as succinate or NADH. This effect is apparently due to a conformational change of the ATPase as shown by the fact that addition of an uncoupler or a respiratory

inhibitor after the preincubation did not reverse the activation of the ATPase. These observations are in agreement with those of Van de Stadt et al., (1973) and those of Gomez-Puyuo, (1979) who also reported an activation of submitochondrial ATPase upon establishment of an electrochemical gradient. These authors claimed that the observed activation of the ATPase was most probably due to a release of the ATPase inhibitor protein from its inhibitory site on the ATPase. The results of the current work also point to the same conclusion, since the ATPase activity of inhibitor depleted submitochondrial part- icles (state III particles) was not affected by the presence of a respiratory substrate, while, the ATPase activity of inhibitor supplemented state III particles was raised by preincubation with a respiratory substrate. The activity of both the submitochondrial ATPase and that of the purified F_1-ATPase was further raised by the presence of inorganic phosphate. This effect is apparently due to a binding of inorganic phosphate on the inhibitor free ATPase, and causing a conformational change, which allows the ATPase to bind ATP at a much faster rate. In connection with this observation, Penefsky and Grubmeyer (1984) showed that the soluble F_1-ATPase was activated by incubating it in the presence of inorganic phosphate, an effect that was due to the ATPase

binding ATP at a much faster rate on the catalytic site. In addition to inorganic phosphate, the presence of ADP and the bivalent cations Mn^{2+} and Mg^{2+} resulted in a further activation of the ATPase, with the presence of Mn^{2+} resulting in a stronger activation. However, when the effect of these cations on ATP synthesis and ATP hydrolysis by the submitochondrial particles were tested without preincubation of the particles, Mg^{2+} was found to be a more potent cofactor than Mn^{2+}. Ca^{2+} did not support either ATP synthesis or ATP hydrolysis.

These observations thus suggest that under intramitochondrial conditions, the establishment of an electrochemical gradient causes a release of the ATPase inhibitor from its inhibitory site of the ATPase. Inorganic phosphate and an ADP-bivalent cation complex binds on a regulatory site of the inhibitor free ATPase molecules altering its conformation in such a way that it gets activated.

On the other hand when submitochondrial particles were preincubated in the presence of ATP and the bivalent cations Mn^{2+}, Mg^{2+} and Ca^{2+} the ATPase inactivated. This is shown in table X to be due to the fact that the presence of ATP and a bivalent cation causes the binding of the ATPase inhibitor

onto the ATPase thus inactivating it. As such it
may be argued that, the presence of ATP and a
bivalent cation results in a conformational change
on the ATPase which allows the inhibitor protein to
bind easily on the ATPase.

C H A P T E R   IX

GENERAL DISCUSSIONS AND CONCLUSIONS

The regulation of adenine nucleotide metabolism is a very important aspect of the overall cellular metabolism, since most of the energy dependent reactions in the cell utilize ATP as their energy source.

ATP is converted to either ADP or AMP with the release of chemical energy (see Equations 1 - 4 on pages 11 and 12). It is this energy that is used to drive energy requiring processes.

ADP and AMP cannot be used as energy sources as such. The ADP may enter the mitochondrial matrix where it is phosphorylated to ATP by the ATP synthetase (ATPase). Alternatively, two molecules of ADP may react under the catalysis of adenylate kinase to form one molecule of ATP and one of AMP. AMP cannot penetrate the inner mitochondrial and has to be converted to ADP at the expense of ATP. For these reasons ADP may be considered as containing half the amount of energy available from ATP, and AMP has zero energy relative to ATP.

With these considerations, Atkinson (1968) derived an equation illustrating the energy content of a system,

in relation to the concentrations of the various
adenine nucleotides. This he referred to as the
adenylate energy charge and is expressed as:-

$$\text{Adenylate energy charge} = \frac{(ATP + \frac{1}{2}ADP)}{(ATP + ADP + AMP)}$$

This means that the amount of energy available in
a system is determined not simply by the absolute
concentration of ATP, but by the relative concentrations
of ATP, ADP and AMP. If all the adenine nucleotides in
the system existed in the form of ATP, the system would
have an energy charge of 1 and would be analogous to a
fully charged storage battery. The system would have
an energy charge of 0.5 and 0 if the adenine nucleotides
existed in the form of ADP and AMP respectively.

To ensure efficiency in cellular function, a
given cell requires a particular level of energy charge
which has to be maintained relatively constant. This
constancy is ensured by the continuous synthesis of
ATP by oxidative phosphorylation and to a lesser extent
by substrate level phosphorylation, hydrolysis of ATP
by the various energy requiring processes, and the
interconversion of the various adenine nucleotides.
It is therefore, very important that the metabolism
of adenine nucleotides is carefully regulated both
at the level of ATP synthesis, ATP hydrolysis and the

interconversion of the various adenine nucleotides

if efficiency of cell function is to be maintained.

For the purpose of this thesis, two factors that

are very important in the regulation of adenine

nucleotide metabolism have been considered.

(i) The mitochondrial adenylate kinase

This enzyme catalyzes the reversible reaction

$$ATP + AMP \rightleftharpoons 2\ ADP$$

and as such serves an important function in the

interconversion of the adenine nucleotides.

The enzyme is located in the mitochondrial

intermembrane space, a very strategic position in

that it is able to communicate with the extramitochon-

drial environment via the pore protein (Linden, et al.,

1982) and the intramitochondrial environment via the

ATP-ADP translocator (Klingengberg, 1970). Due to

this location the enzyme is able to monitor the level

of the various adenine nucleotides in the cell, and

responds by altering both the velocity and direction

of its activity in relation to the adenylate charge

equirements of the cell.

The enzyme is able to interact with both the

extramitochondrial and intramitochondrial adenine

nucleotides as illustrated in Figure 34.

ADP arising from ATP hydrolysis in the cytosol may either penetrate the mitochondrial matrix where it is phosphorylated to ADP by the ATP synthetase. Alternatively, 2 molecules of ADP may react under the catalysis of adenylate kinase to form one molecule of ATP and one molecule of AMP. As shown in Figure 9, either of these two reactions may predominate depending on the concentration of ADP. At low ADP concentrations, most of the ADP penetrates the inner mitochondrial membrane, to the matrix where it is phosphorylated to ATP. However, at high ADP concentrations, the ATP-ADP translocator becomes saturated, and most of the ADP reacts under the catalysis of adenylate kinase forming ATP and AMP.

AMP arising from ATP hydrolysis in the cytosol cannot penetrate the inner mitochondrial membrane. It reacts with intermembrane ATP forming ADP which then enters the mitochondrial matrix where it is phosphory-lated by ATP. This is illustrated in Figure 6 which show that externally added AMP is able to initiate the adenylate kinase activity in the presence of an ATP regenerating system, and in Figures 7 and 8 which show that like ADP, AMP is able to stimulate mitochon-drial ATP synthesis. ATP synthesis induced by AMP is dependent on an active adenylate kinase as shown by the

FIGURE 34

Schematic view of the functional relationship of adenylate kinase to extra- and intramitochondrial adenine nucleotides and oxidative phosphorylation in rat liver mitochondria.

OM	-	Outer membrane
IM	-	Inner membrane
IMS	-	Intermembrane space
AK	-	Adenylate kinase
A	-	ATP synthetase
B	-	ADP - ATP translocator
C	-	Phosphate carrier.
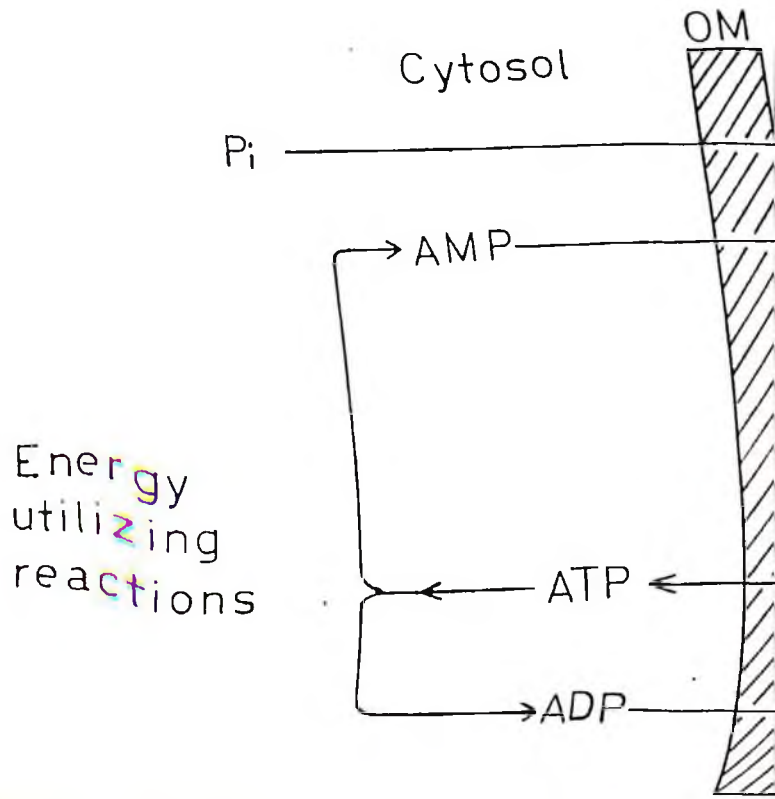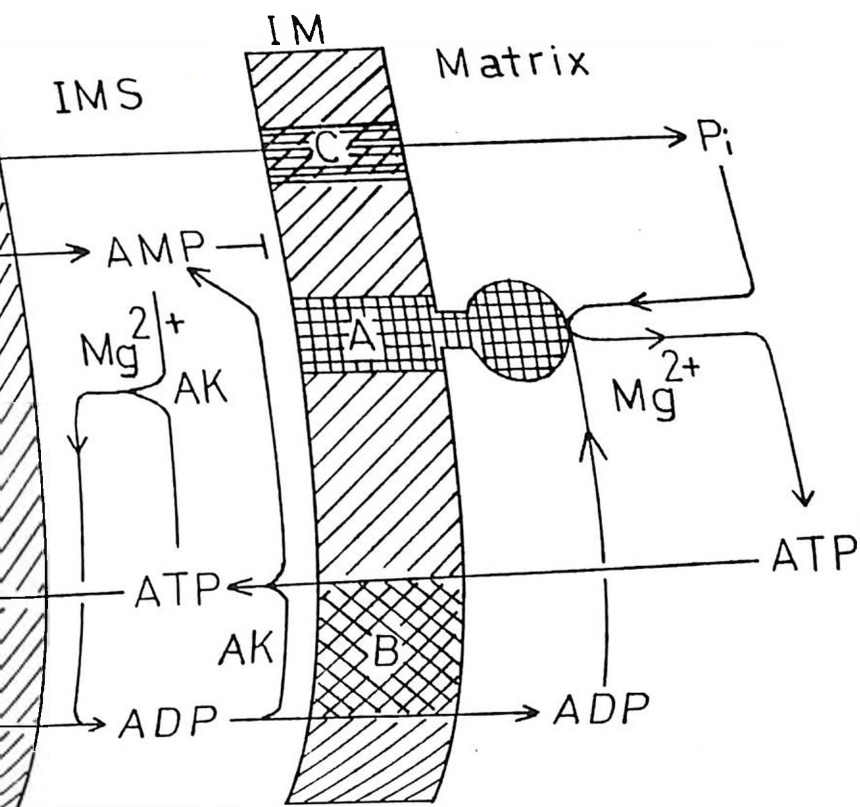
fact that, respiratory stimulation by the latter was
inhibited by the presence of EDTA, and di adenosine
pentaphosphate, a specific inhibitor of the adenylate
kinase. ADP formed from the AMP and ATP goes back to
the mitochondrial matrix where it is phosphorylated
to ATP by the ATP synthetase. The ATP then comes out
of the matrix to the intermembrane space where part
of it reacts with more ATP to form ADP and part of it
moves to the extramitochondrial space. These factors
account for the observations in Figure 7 that the
respiratory control ratio induced by AMP, is similar
to that for ADP, and that the AMP/$_O$ ratio is half the
ADP/$_O$ ratio since two molecules of ADP are formed for
every molecule of AMP that reacts with ATP. They also
account for the observation in Figure 8 that more ATP
is formed when synthesis of the latter is induced by
ADP than when induced by AMP since when it is induced
by AMP, part of the ATP formed reacts with more AMP,
and only part of it is available to the hexokinase
reaction in the extramitochondrial space. These
activities of adenylate kinase are aided by the
presence of catalytic amounts of bound adenine nucle-
otides in the intermembrane space, e.g. the initiation
of the AMP + ATP reaction is aided by the presence of
catalytic amounts of ATP in space, and by the presence
of large amounts of bound Mg^{2+} in the space which are
an essential factor in the activity of enzyme.

2. Bivalent cation uptake

Mitochondria from a wide variety of tissues are known to support an energy dependent uptake of various bivalent cations including Ca^{2+}, Sr^{2+}, Mn^{2+} and Ba^{2+} but not Mg^{2+} (Rossi and Lehninger, 1963; Lehninger, et al., 1967, Carafoli and Crompton, 1967). Uptake of the bivalent cations is an elctrophoretic process, which utilizes the electrochemical gradient generated by electron transport. The results of this work show that rat brain mitochondria are capable of supporting, an uptake of Ca^{2+}, Mn^{2+} and Ba^{2+}. Uptake of these cations was dependent on the presence of an electrochemical gradient generated by electron transport as shown by the fact that, the process was inhibited by the presence of a respiratory inhibitor, or by the presence of an uncoupler. Retention of the accumulated cations was also dependent on the presence of an energized membrane since additon of a respiratory inhibitor, uncoupler or depletion of oxygen caused a rapid efflux of accumulated cations. In addition the accumulation of large quantities of Ca^{2+} was dependent on the presence of inorganic phosphate. The presence of ATP further raised the capacity of these mitochondria to accumulate Ca^{2+}.

Accumulation of large amounts of both Ca^{2+} and Ba^{2+} caused a complete inhibition of oxidative phosphorylation. This inhibition occurred even when these cations were added during active ATP synthesis. It has been reported earlier that respiratory linked uptake of bivalent cations is able to compete with other respiratory linked mitochondrial processes such as the energy linked transhydrogenase (Lee and Ernster, 1968) and with ATP synthesis (Jacobus et al., 1975; Vercesi, et al., 1978). Accumulated Ca^{2+} has also been shown to cause a complete inhibition of oxidative phosphorylation in mitochondria isolated from brain (Roman et al., 1981) and from Ehlrich ascites tumours (Thorne and Bygrave, 1974), and the inhibition is relieved upon release of the accumulated Ca^{2+}.

Unlike the effects of Ca^{2+} and Ba^{2+}, accumulation of Mn^{2+} did not result in any inhibition of oxidative phosphorylation, and could even reverse the inhibitory effects of Ca^{2+} and Ba^{2+}. Accumulation of these cations did not cause any permanent damage to mitochondria since release of accumulated Ca^{2+} induced by Na^+ restored oxidative phosphorylation.

So far the mechanism by which accumulated Ca^{2+} or Ba^{2+} causes and inhibition of oxidative phosphory-lation is not clear.

To test the possible mechanism by which Ca^{2+} could inhibit oxidative phosphorylation in a manner that could be reversed by Mn^{2+}, experiments were carried out with beef heart MgATP submitochondrial particles. Use of submitochondrial particles enabled direct accessibility of the cations to the ATPase.

The results of these experiments showed that the ATPase activity of the submitochondrial particles could be raised two-fold, by incubating them with a respiratory substrate, an effect that was shown to be due to a release of the ATPase inhibitor protein from its inhibitory site on the ATPase. Similar observations have also been reported before (Van de Stadt et al., 1973; Gomez-Puyou, et al., 1979). It is thus possible that active bivalent cation transport utilizes all the energy derived from respiration, and none is available to cause release of the ATPase inhibitor, thus accounting for the inhibition of ATP synthesis observed during bivalent cation uptake.

It was also observed that inclusion of inorganic phosphate and ADP in the preincubation medium led to a further activation of the ATPase activity. However, the effect of ADP was only apparent in the presence of bivalent cations with Mn^{2+} being the more potent cation followed by Mg^{2+}, while Ca^{2+} was without effect. However, when both ATP synthesis and ATP hydrolysis of

the particles was tested without preincubation, Mg^{2+} was found to be the more potent cation, followed by Mn^{2+}, while Ca^{2+} did not act as a cofactor for either of the two processess. These observations thus mean that during the preincubation inorganic phosphate and the ADP-bivalent cation complex bind onto the ATPase, in such a way that it is activated. This is most probably due to a binding of the complexes onto a regulatory site on the ATPase, leading to a conformational change which allows the ATPase to accept ATP at a much faster rate (Figure 35). It is thus possible that when bivalent cations are taken up by mitochondria, they compete with intramitochondrial Mg^{2+} for binding to adenine nucleotides, the resulting effect being dependent on the accumulated cation. Since Mn^{2+} has an almost similar effect with Mg^{2+} on the ATP-synthetase its accumulation may not be expected to affect oxidative phosphorylation. On the other hand accumulated Ca^{2+} may be expected to cause an inhibition of oxidative phosphorylation since the Ca^{2+}-ADP complex formed neither stimulates nor does it act as a substrate for the ATP-synthetase. The results have shown that the amount of Ca^{2+} required to cause a complete inhibition of ATP synthesis is more than 8 times the mitochondrial content of Mg^{2+}. It is thus possible that when such large amounts of Ca^{2+} are accumulated, most of the ADP in the

FIGURE 35

Transation states of the mitochondrial H^+-ATPase as regulated by respiration, inorganic phosphate, ADP, ATP and bivalent cations.

$E°$ = Inactive ATPase

E = Semiactivated ATPase

$E+$ = Activated ATPase

$$E \overset{\circ}{I} \xrightarrow{\text{Respiration}} E + I$$

$$E + I \xrightarrow{\text{ATP+Mn}^{2+} \text{ or Mg}^{2+} \text{ or Ca}^{2+}} \overset{\circ}{E} \underset{I}{\overset{\text{ATP-Mn}^{2+}}{\diagup}}$$

$$E + I \xrightarrow{\text{ADP+Ca}^{2+}\text{+Pi}} \overset{\circ}{E} \underset{Pi}{\overset{\text{ADP-Ca}^{2+}}{\diagup}}$$

$$E + I \xrightarrow{\text{Pi+ADP+ Mg}^{2+} \text{ or Mn}^{2+}} \overset{+}{E} \underset{Pi}{\overset{\text{ADP-Mn}^{2+}}{\diagup}}$$
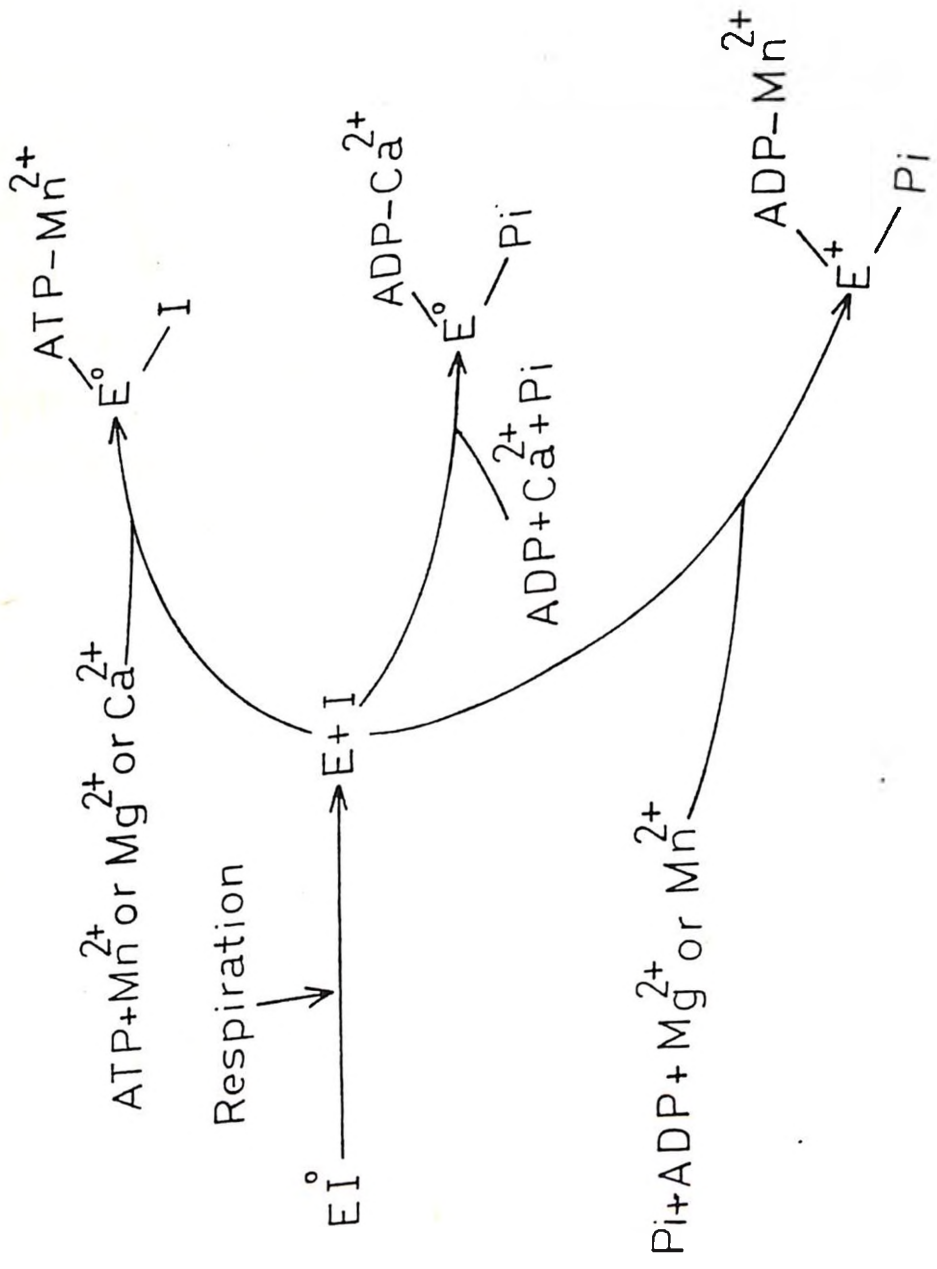
mitochondria is bound to Ca^{2+}, and only small amounts are bound to Mg^{2+}, with the result that the effect of Mg^{2+}-ADP is diminished by that of Ca^{2+}-ADP. When both Ca^{2+} and Mn^{2+} are taken up together, more of the ADP gets bound to Mn^{2+} due to the higher stability constant of Mn^{2+}-ADP as compared to that of Ca^{2+}-ADP (Watts, 1973) and as such complete inhibition of oxidative phosphorylation will not occur. It is possible that accumulated Ba^{2+} acts in the same way as Ca^{2+}.

When submitochondrial particles were preincubated with ATP in the presence of bivalent cations, the ATPase activity was lowered, with Mn^{2+} being the more potent cation followed by Mg^{2+} and then Ca^{2+}. This effect was shown to be due to the presence of ATP and bivalent cations causing a binding of the ATPase inhibitor protein onto the ATPase. This might thus mean that the presence of an ATP-bivalent cation alters the conformation of the ATPase in such a way that it binds the inhibitor more readily, thus lowering its activity (Figure 35).

CONCLUSIONS

The results of the present work show clearly that both the mitochondrial enzyme, adenylate kinase and the

mitochondrial bivalent cations play a significant role in the regulation of adenine nucleotide metabolism.

Adenylate kinase plays its role by monitoring the cellular adenylate charge, and altering both the direction and velocity of its reaction in accordance with the energy charge requirements of the cell. In a case of excess AMP as may result during excessive activation of fatty acids, or amino acids, the enzyme catalyzes the phosphorylation of the AMP to ADP at the expense of ATP derived from oxidative phosphory-lation. The ADP so formed is then phosphorylated to ATP by the ATP synthetase.

In the presence of excess ADP as may result during excessive work or during ischaemia, adenylate kinase catalyzes the reaction between 2 molecules of ADP to form a molecule of ATP and a molecule of AMP. This way it makes the β-phosphate of ADP available as an energy source.

Mitochondrial bivalent cations regulate the metabolism of adenine nucleotides in two ways:-
(i) Active bivalent cation uptake competes with oxidative phosphorylation for the electrochemical gradient, since both process utilize the latter as an energy source. In fact, bivalent cation transport takes precedence over oxidative phosphorylation in

the utilizing of the electrochemical gradient, with no
ATP being formed during active cation uptake.

(ii)   Accumulated bivalent cations compete with
intramitochondrial Mg^{2+} for binding to adenine nucleo-
tides, the resulting effect being dependent on the
accumulated cation.

Accumulated Mn^{2+} has the same effect as intramito-
chondrial Mg^{2+}.   This was shown by the fact that both
the Mn^{2+} and Mg^{2+}- ATP complexes could support ATP
hydrolysis by MgATP submitochondrial ATPase, while
both Mn^{2+} and Mg^{2+} - ADP complexes could support ATP
synthesis.   In addition preincubation of MgATP
submitochondrial particles in the presence of an
electrochemical gradient, inorganic phosphate and Mg^{2+}
or Mn^{2+}-ADP complexes resulted in the activation of
the ATPase activity.   This effect is conversant with
the requirements for oxidative phosphorylation, since
it is under these conditions that the ATPase (ATP
synthetase) should be activated to synthesise ATP.
On the other hand, preincubation of MgATP submitochon-
drial particles with either Mn^{2+} or Mg^{2+}-ATP complexes
resulted in the inactivation of the submitochondrial
ATPase.   This effect is also conversant with the
cellular requirements, since inactivation of the
ATPase in the presence of ATP, prevents hydrolysis
of the latter.

These effects thus explain the observation
that accumulated Mn^{2+} had no effect on the mitochon-
drial respiratory stimulation induced by ADP.

The effect of Ca^{2+} on the MgATP submitochondrial
ATPase activity was different from that of Mn^{2+} or
Mg^{2+}. Ca^{2+}-ADP did not act as a substrate for ATP
synthesis, while Ca^{2+}-ATP did not also act as a
substrate for ATP hydrolysis. Preincubation of
submitochondrial particles in the presence of Ca^{2+}
and ADP did not stimulate the submitochondrial
ATPase activity. As such the Ca^{2+}-ADP complex is
neither a substrate for the ATPase, nor does it
modulate the activity of the ATPase. Thus accumulated
Ca^{2+} competes with intramitochondrial Mg^{2+} for
binding to ADP, its effect being the reverse of that
of Mg^{2+}. This explains the observation that accumu-
lated Ca^{2+} led to an inhibition of the ADP induced
respiratory stimulation.

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