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CHARACTERIZATION OF THE FACTORS REGULATING THE COUPLING AND RESPIRATORY CONTROL OF ISOLATED CHICK HEART MITOCHONDRIA

presented by

PETER PAUL TOTH

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of the requirements for

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Major professor

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CHARACTERIZATION OF THE FACTORS REGULATING THE COUPLING AND RESPIRATORY CONTROL OF ISOLATED CHICK HEART MITOCHONDRIA

By

Peter Paul Toth

A DISSERTATION

Submitted to
Michigan State University
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for the degree of

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1988

ABSTRACT

CHARACTERIZATION OF THE FACTORS REGULATING THE COUPLING AND RESPIRATORY CONTROL OF ISOLATED CHICK HEART MITOCHONDRIA

By

Peter Paul Toth

This study is concerned with identifying and characterizing the factors regulating the degree to which chick heart mitochondria control rates of respiration in both the presence and absence of ADP. In order to preserve the activity of outer membrane proteins, chick heart mitochondria are isolated with the use of collagenase rather than the non-specific protease Nagarse. Up to 70% of the mitochondria in the starting myocardium can be recovered using this method. This isolation procedure also preserves the activity of a novel soluble uncoupling protein. This protein binds to the chick heart mitochondrial outer membrane with high apparent affinity, has a molecular mass of 6-15 kD, and can be inactivated by heat or protease digestion.

These mitochondria are more highly coupled than any yet reported. Under optimal conditions (0.05 nmole cyt aa_3, 20 mM P_i, 5 mM pyruvate/ 2.5 mM malate) the organelles respire with infinite respiratory control. Factors which decrease rates of state 4 respiration include limited Nagarse digestion of the mitochondria, pre-incubating mitochondria with

substrate prior to the addition of ADP, and EGTA. EGTA inhibits the binding of Ca(II) and Mg(II) released from the mitochondria to two contaminating ATPases: actomyosin and the F_1F_0-ATPase of broken mitochondria.

The ADP:O ratios of chick heart mitochondria are fractional and significantly greater than 3.0 during the oxidation of pyruvate/malate (P/M), β-hydroxybutyrate (βHB), or α-ketoglutarate (αKG). Results with these NAD-linked substrates support a 13, and possibly 14, proton model for oxidative phosphorylation. During the oxidation of glutamate/malate (G/M) and succinate, ADP:O ratios approximate to the classic values of 3.0 and 2.0, respectively.

The P_i metabolism of these mitochondria is complex. Based on rates of swelling monitored by 90° light scatter measurements, the P_i transport protein has a K_m for $P_i \leq 50$ μM. P_i transport thus cannot be rate-limiting for oxidative phosphorylation. P_i is inhibitory to respiration during state 4 when either P/M or αKG are used as substrates. The data suggest that P_i inhibits the dehydrogenases for pyruvate and αKG in the absence of ADP. In contrast, during state 3, P_i stimulates respiration. This stimulation is hyperbolic in the presence of P/M and G/M, sigmoidal in the presence of αKG, and biphasic in the presence of βHB.

To Karen,

Ole Building and Loan Buddy Of Mine,

for showing me that 1 + 1 = 1,000,000

ACKNOWLEDGEMENTS

I thank my mentor Dr. Clarence Suelter, G.A.F.C. (Guardian Angel First Class) for giving me the freedom to pursue the type of education I believed I needed and for the privilege of having been one of his students. Thanks are also due to: Dr. Jack Holland (Commander of the Universe), for all the mud, the blood, the beer, and the marvelous lessons in spectroscopy; Dr. Loran Bieber, for a productive and very enjoyable collaboration on mitochondrial carnitine metabolism; Dr. John E. Sell, for his assistance in operating the computerized spectrometer and for making light scattering measurements all the more interesting to perform; my outstanding undergraduate assistants, Kendall Sumerix, Randall Kuntzmann, and Clarissa Stropp, for their diligence and their contributions to this work; Dr. Shelagh Ferguson-Miller, for her willingness to collaborate on parts of this project and for allowing me to use much equipment in her laboratory; Dr. John Wang, for providing me with the two antisera I was in need of; the other members of my guidance committee, Dr. Estelle McGroarty, Dr. Dale Romsos, and Dr. John Wilson for their advice and support; and Dr.'s Britton Chance (University of Pennsylvania) and Antonio Scarpa (Case Western Reserve University), for allowing me to perform experiments in their laboratories. Finally, I wish to thank the American Heart Association of Michigan for funding much of the the work contained in this dissertation. I shall always value the laughter, the difficulties, and the exhilaration embodied within this consuming endeavor.

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LIST OF ABBREVIATIONS

A1	conventional absorbance
A2	absorbance determined spectrofluorometrically
ADP	adenosine 5'-diphosphate
ADP:O	ratio of nmoles of ADP phosphorylated per ng-atom of oxygen consumed
ATP	adenosine 5'-triphospahte
ATPase	adenosine 5'-triphosphatase
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	bovine serum albumin
CCCP	carbonyl cyanide m-chlorophenylhydrazone
cyt aa_3	cytochrome c oxidase (E.C. 1.3.9.1)
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene-glycol-bis(4-amino-ethyl ether)-N,N,N',N'-tetra-acetic acid
F1	fluorescence intensity from cell position 1
F4	Fluorescence intensity from cell position 4
FAD	flavin adenine dinucleotide
ΔG_P	phosphorylation potential or Gibbs free energy change of ATP synthesis
ΔG_{ox}	redox potential or Gibbs free energy change of respiration
G/M	5 mM glutamate and 2.5 mM malate
GDP	guanosine 5'-diphosphate
GTE	50% (v/v) glycerol, 20 mM Tris, pH 7.4, 1 mM EGTA

HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
β-HB	5 mM β-hydroxybutyrate
IDP	inosine 5'-diphosphate
INT	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride
I.U.	international unit of enzyme activity (conversion of one μmole of substrate to product per minute)
J_o	rate of respiration
α-KG	5 mM α-ketoglutarate
λ_{em}	emission wavelength
λ_{ex}	excitation wavelength
L.S.	light scatter
MalNEt	N-ethylmaleimide
MOPS	3-(N-morpholino)propanesulfonic acid
M_r	molecular weight
MS	225 mM mannitol and 75 mM sucrose
MSB	225 mM mannitol, 75 mM sucrose, and 0.2% BSA
MSBE	225 mM mannitol, 75 mM sucrose, 0.2% BSA, and 1 mM EGTA, pH 7.4
MSP_i	225 mM mannitol, 75 mM sucrose, and 20 mM Tris-P_i, pH 7.4
$\Delta\mu_{H+}$	electrochemical potential difference of protons
n	mechanistic stoichiometry
NAD	nicotinamide adenine dinucleotide (oxidized form)
NADH	nicotinamide adenine dinucleotide (reduced form)
$NaDodSO_4$	sodium dodecyl sulfate
NBT	nitro blue tetrazolium
ΔpH	transmembrane pH difference

P/M	5 mM pyruvate and 2.5 mM malate
P_i	inorganic phosphate
$\Delta\psi$	transmembrane electrical potential
R	photon recovery coefficient
RCR	respiratory control ratio (state 3/state 4)
R_F	relative mobility
TEMED	N,N,N',N'-tetramethylethylenediamine
TBS	tris-buffered saline
TTBS	tris-buffered saline enriched with Tween-20
Tris	tris(hydroxymethyl)aminomethane

INTRODUCTION

The nature of mitochondrial respiratory control and the proton, oxygen, and ATP stoichiometries for oxidative phosphorylation have been subjects of intense experimental investigation for the past thirty five years. An understanding of these processes is fundamental to formulating integrative models of how tissues conserve and effectively distribute the energy of substrate oxidation in the form of ATP. Much of our current insight into bioenergetics has been obtained from studies performed on isolated mitochondria. Yet, it is clear from inspection of the available literature that workers in the field of mitochondrial bioenergetics have been somewhat unsuccessful in identifying and controlling many of the factors which compromise the degree to which isolated mitochondria can couple the complex process of substrate oxidation to the phosphorylation of ADP. Consequently, apart from the use of lipophilic protonophores and various types of inhibitors, few systematic methods for non-toxically modulating the magnitude of mitochondrial respiratory control have appeared. Because most published studies have used poorly coupled mitochondria, there is widespread disgreement over what enzymes participate in the phenomenon of respiratory control and what the precise stoichiometries of oxidative phosphorylation can and should be.

This dissertation is concerned with characterizing previously unrecognized factors responsible for modulating the coupling and respiratory control of isolated chick heart mitochondria. This work is divided into seven chapters. The first chapter is a review of the literature relevant to the work presented herein. It is divided into seven sections and is intended to provide the reader with a brief but comprehensive understanding of how mitochondria synthesize ATP at rates

commensurate with the energy demands of the cell. Chapters 2-7 are presented as separate papers detailing the experimental findings of this investigation. Each paper is comprised of separate Introduction, Experimental Procedures, Results, and Discussion sections. Chapter 2 details the isolation of highly coupled chick heart mitochondria with intact outer membrane enzymes using collagenase. The data presented in Chapter 3 demonstrates that uncoupled respiration can be minimized by controlling a number of factors and identifies the extramitochondrial ATPases responsible for the regeneration of ADP under particular conditions. Values of the ATP:O stoichiometries for oxidative phosphorylation during the oxidation of a variety of substrates are proposed. Chapter 4 is an investigation of the light scattering properties of chick heart mitochondria. It details a theoretical basis for the deviation of mitochondrial suspensions from Beer's law and shows that light scattering measurements at 90° are much more sensitive than those at 0°. In Chapter 5 the light scattering properties of these mitochondria are used to obtain estimates for the various kinetic constants of P_i uptake. The effect of P_i on matrix cation content is also explored. Chapter 6 is concerned with the effect of P_i on the activity of various substrate dehydrogenases during state 3 and state 4 respiration and argues that at least two of the substrate dehydrogenases can be rate-limiting for uncoupled respiration. Finally, Chapter 7 presents data which strongly suggests that chick myocardium contains a low molecular weight uncoupler protein which has an apparent high affinity for mitochondria and can completely uncouple substrate oxidation from the phosphorylation of ADP.

Chapter 1

LITERATURE REVIEW

I. THE CHEMIOSMOTIC THEORY

Elucidating the detailed mechanism of mitochondrial oxidative phosphorylation is of concern to many biologists and has been for years. An early description of this process, the chemical coupling hypothesis (Slater, 1953), assumed that, just as in glycolytic substrate-level phosphorylation, a high energy phosphorylated chemical intermediate is formed during electron transport. This compound could then be hydrolyzed and the free energy released used to drive ATP synthesis. No such compound could, however, be found. The inability to identify chemical intermediates in oxidative phosphorylation stimulated the formulation of a novel approach, the chemiosmotic theory. The chemiosmotic theory was a revolutionary and deeply insightful view of how mitochondrial ATP synthesis is driven (Mitchell, 1961, 1975, 1985). The four postulates of chemiosmosis are as follows (Mitchell, 1979 a and b):

(a) The mitochondrial inner membrane contains proton- or hydroxyl-coupled (these are indistinguishable) solute translocases. The carriers are responsible for transport of solutes from the cytosol into the mitochondrial matrix and for osmotic stabilization.

(b) Electron transport chains are intramembrane enzyme systems comprised of an alternating sequence of electron and proton carriers with a characteristic $H^+/2e^-$ stoichiometry. The respiratory chain and the ATPase translocate protons in the same direction (i.e., out of the matrix).

(c) The ATP synthetase is a reversible, intramembrane ATPase with a characteristic H^+/ATP stoichiometry.

(d) The mitochondrial inner membrane is a proteo-lipid osmotic barrier that has a low permeability to solutes in general and to protons and hydroxyl ions in particular.

According to chemiosmosis, the role of the electron transport chain is

to commit the free energy of scalar substrate oxidation reactions toward the vectorial translocation of protons out of the matrix. These oxidation reactions create an electrical potential ($\Delta\psi$, negative inside) and a pH gradient (alkaline inside) across the mitochondrial inner membrane. The resulting electrochemical potential gradient of protons is defined by the following relationship:

$$\Delta\mu_{H+} = F\Delta\psi + RT \ln[c_H^{in}/c_H^{out}] \qquad (1)$$

where F is the Faraday constant (96,490 coulombs/mole of electrons), R is the gas constant, (8.314 joules/°K mole), T is the absolute temperature, and c_H is the concentration of protons internal or external to the matrix, as indicated. The subject matter of this review will be discussed within the context of chemiosmosis. The current evidence which supports its validity as well as its shortcomings will be discussed, and the various steps of mitochondrial energy metabolism will be traced in order to lay a foundation upon which the experiments presented in this dissertation may be interpreted.

II. CARRIER SYSTEMS OF THE MITOCHONDRIAL INNER MEMBRANE

The mitochondrial inner membrane is essentially impermeable to charged solutes and, as a consequence, it ensures that the cytosol and mitochondrial matrix are maintained as chemically distinct compartments. In order for the two compartments to communicate and exchange metabolites, the mitochondrial inner membrane contains a system of transmembrane proteins which specifically transport a broad variety of molecules in and out of the matrix (reviews: LaNoue and Schoolwerth, 1979; Meijer and Van Dam, 1981). Three types of facilitated transport are known to exist:

uniport, in which a single solute is transported by the carrier; symport, two or more different solutes are simultaneously transported in the same direction by the carrier; antiport, two or more different solutes are simultaneously transported in opposite directions by the carrier. The carriers that are relevant to the research presented herein are briefly surveyed below.

The carriers for P_i (Palmieri et al., 1970; Coty and Pederson, 1974), pyruvate (Papa et al., 1971), and glutamate (Hoek and Njogu, 1976) symport protons with their respective substrates in order to maintain electroneutrality. The pyruvate carrier also appears to be responsible for the transport of β-hydroxybutyrate (Pande and Parvin, 1978). The adenine nucleotide translocase and the dicarboxylate carrier are proteins that facilitate antiport. On the former, extramitochondrial ADP is exchanged 1:1 for intramitochondrial ATP (Pfaff and Klingenberg, 1968; Pfaff et al., 1969; Klingenberg and Rottenberg, 1977; Kramer and Klingenberg, 1982); on the latter, dicarboxylic acids (e.g., α-ketoglutarate, malate, and succinate) are exchanged for one another or for HPO_4^{2-} (Palmieri et al., 1971).

There is an elaborate transport system for Ca(II), some aspects of which are still poorly understood. Uptake proceeds via electrophoretic uniport (Rottenberg and Scarpa, 1974; Lotscher et al., 1980), while efflux may proceed on a $Ca(II)/Na^+$ antiporter (Haworth et al., 1980), a $Ca(II)/H^+$ antiporter (Lehninger et al., 1979; Riley and Pfeiffer, 1986), or through an energy-dependent but as yet uncharacterized mechanism (Puskin et al., 1976). K^+, the major cation and osmotic stabilizing agent of the matrix, is transported by a K^+/H^+ antiporter (Martin et al., 1984) whose activity is believed to be regulated by Mg(II)

(Nakashima et al., 1982).

III. SUBSTRATE DEHYDROGENASES

Once transported into the mitochondrial matrix, substrates are channeled into precisely regulated catabolic pathways designed to meet the fluctuating energy demands of myocardium. Fatty acids (Oram et al., 1973; Lysiak et al., 1986 and 1988) and pyruvate (Williamson, 1974) are both important sources of fuel for myocardium. This review, however, will focus on the oxidation of pyruvate and tricarboxylic acid cycle intermediates because the experiments to be detailed in this dissertation are not concerned with fatty acid metabolism. The dehydrogenases for pyruvate, threo-D_s-isocitrate, and α-ketoglutarate catalyze non-equilibrium reactions and are all highly regulated. Because of their importance in determining the rate at which matrix NADH is produced, the known mechanisms regulating the activity of each enzyme will be discussed in turn.

The pyruvate dehydrogenase complex (PDH) catalyzes the irreversible conversion of pyruvate to acetyl CoA. The PDH complex is comprised of three distinct enzymes: pyruvate decarboxylase (E1), dihydrolipoate acyltransferase (E2), and dihydrolipoyl dehydrogenase (E3) (Reed, 1974). The cofactors for E1, E2, and E3 are thiamin pyrophosphate, lipoic acid, and FAD, respectively. In the terminal step of the reaction, E3 transfers electrons from the reduced flavin to NAD^+. PDH is interconverted between a nonphosphorylated active form (PDH_a) and a phosphorylated inactive form (PDH_b) (Linn et al., 1969 a and b). PDH_a is phosphorylated by PDH kinase, an enzyme that uses ATP as phosphate donor. The kinase is associated with E2 (Yeaman, 1986) and phosphorylates a serine residue in

E1 (Yeaman et al., 1978), thereby inactivating the enzyme. PDH kinase is inhibited by pyruvate (Yeaman, 1986; Budde et al., 1988), ADP (Hucho et al., 1972), and NAD^+ and CoA (Pettit et al., 1975); it is activated by NADH and acetyl CoA (Kerbey et al., 1976; Hansford, 1977; Latipaa et al., 1985), the two products of the reaction catalyzed by PDH. PDH_b is converted back to PDH_a via a PDH phosphate phosphatase. This enzyme is activated by Ca(II) (Denton and McCormack, 1980; McCormack and Denton, 1985 and 1986) and inhibited by NADH (Pettit et al., 1975). In addition to covalent modifications, PDH is also subject to product feedback inhibition. NADH and acetyl CoA prevent the binding of NAD^+ and CoA, respectively, to binding sites on the enzyme (Blass and Lewis, 1973; Kerbey et al., 1976; Hansford, 1976). To summarize, increases in the ratios of NADH/NAD^+, acetyl CoA/CoA, and ATP/ADP result in an increase in the ratio of PDH_b/PDH_a.

NAD-isocitrate dehydrogenase is comprised of identical subunits and its activity is subject to allosteric control. ADP increases the affinity (i.e., decreases the K_m) of the enzyme for threo-D_s-isocitrate in both rat (Goebell and Klingenberg, 1963) and bovine (Chen and Plaut, 1963) heart mitochondria. NAD^+ (Goebell and Klingenberg, 1963; Lowenstein, 1967) and Ca(II) (McCormack and Denton, 1986) also decrease the K_m of the rat heart enzyme for threo-D_s-isocitrate. NADH is a competitive inhibitor of NAD^+ binding (Lowenstein, 1967). Consistent with these findings, it is now well established that as the ratios NADH/NAD^+ and ATP/ADP decrease, the activity of the enzyme increases (Hansford, 1980; Bulos et al., 1984).

α-Ketoglutarate dehydrogenase is an enzyme that is structurally and mechanistically similar to PDH (Yeaman, 1986). Unlike PDH, however, the

activity of this enzyme is not modulated by phosphorylation/dephosphorylation reactions. The K_m of the mammalian enzyme for α-ketoglutarate is decreased by either an increase in matrix Ca(II) concentrations or a decrease in the matrix ATP/ADP ratio (McCormack and Denton, 1979 and 1981; Hansford and Castro, 1981). The mechanism by which Ca(II) activates this enzyme is as yet unknown (McCormack and Denton, 1986). The activity of α-ketoglutarate dehydrogenase is negatively modulated by increases in the ratios of $NADH/NAD^+$ and succinyl CoA/CoA, due presumably to feedback inhibition (Hansford, 1980). It is also not known whether ADP activates the dehydrogenase directly (i.e., by inducing a structural transition) or if it activates indirectly by stimulating nucleoside diphosphokinase. The latter enzyme will relieve feedback inhibition by stimulating the hydrolysis of succinyl CoA via the succinate thiokinase reaction (see Fig 8 in Chapter 6 of this dissertation).

The striking parallels in the regulation of these dehydrogenases allows for coordinated stimulation and inhibition, depending upon the metabolic conditions prevailing within the cell. Thus, increases in the redox poise ($NADH/NAD^+$) or energy state (ATP/ADP) adversely affect the rate at which oxidative reactions proceed within the mitochondrial matrix. The importance of dehydrogenase regulation by Ca(II) is gaining increasing recognition. It is now doubtful that mitochondria simply buffer cytosolic concentrations of Ca(II) with their sophisticated set of carriers for Ca(II) uptake and efflux. Rather, it is believed that Ca(II) acts as a second messenger (McCormack and Denton, 1986), mediating the coupling of hormonal stimulation of cardiac contraction to increases in the rate at which dehydrogenases produce NADH. The enhanced rates of NADH output result in higher rates of ATP production by mito-

chondria. It is therefore appropriate to envision the stimulation of cardiac mitochondrial oxidative metabolism as dependent upon the intramitochondrial concentrations of both ADP and Ca(II) (Hansford, 1985). In view of the structural and mechanistic complexity of these dehydrogenases, it would be of interest to ascertain whether additional forms of chemical regulation exist. Elucidating the full range of possible regulation would allow for a more comprehensive model of how mitochondria can so precisely meet the energy demands of myocardium.

IV. ELECTRON TRANSFER CHAIN

The reduced flavin (linked to succinate dehydrogenase) and pyridine nucleotides resulting from oxidative reactions in the matrix are used to transfer protons and electrons from substrates to the electron transfer chain of the mitochondrial inner membrane. The electrons (e-) enter the chain with a high redox potential. The energy of these electrons is defined by the following relationship:

$$\Delta G_{ox} = -nF\Delta E_h \qquad (2)$$

where ΔG_{ox} is the Gibbs free energy of oxidation, n is the number of e- transferred during an oxidation-reduction reaction, F is the Faraday constant, and ΔE_h is the observed difference in potential of the redox couple. ΔE_h is quantitated with use of the Nernst equation:

$$\Delta E_h = E_o' + \frac{2.303RT}{nF} \log \frac{[\text{electron acceptor}]}{[\text{electron donor}]} \qquad (3)$$

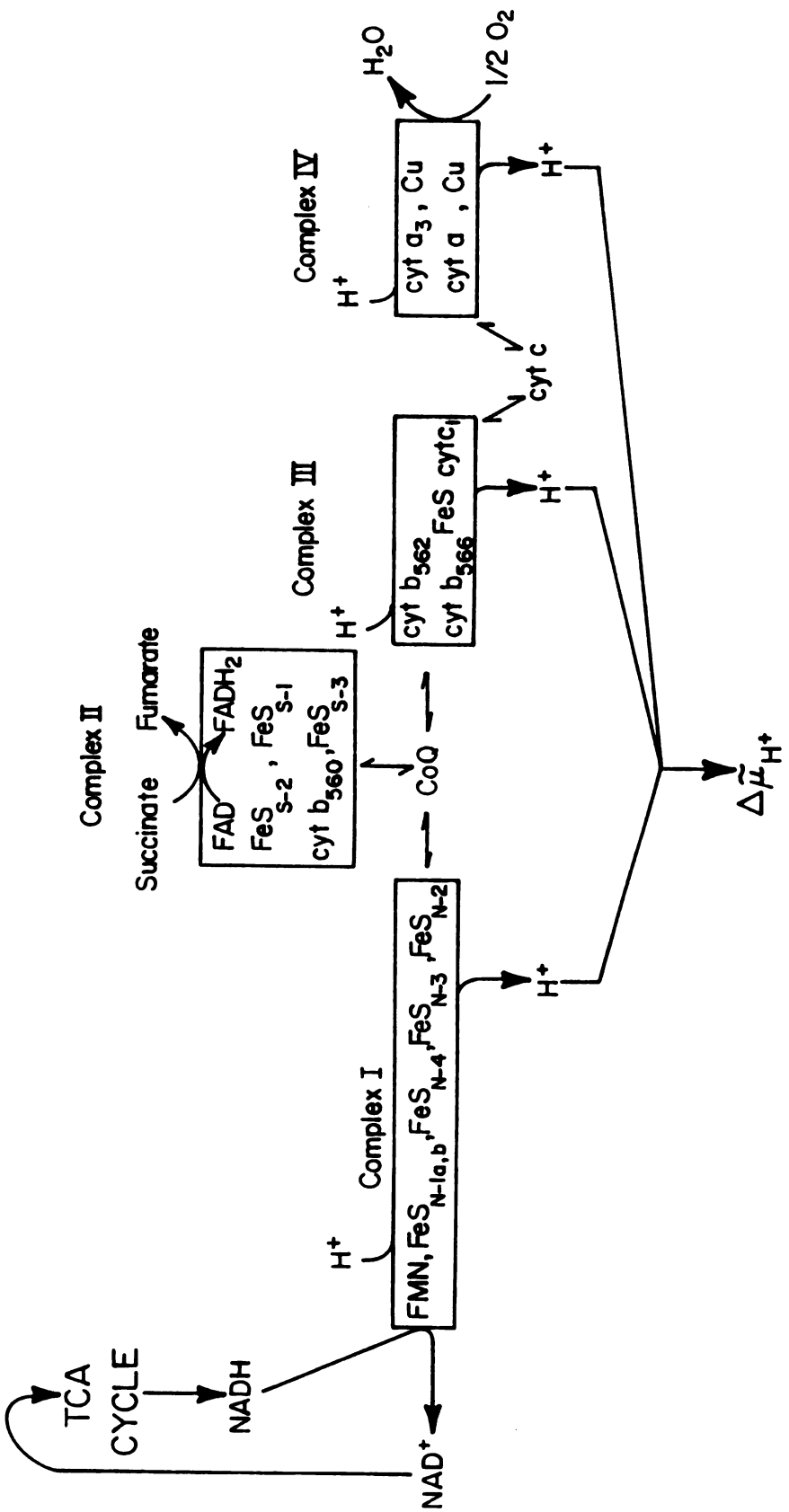
where E_o' is the standard redox (or midpoint) potential. The energy of

the electron is progressively released as it is transferred from one component of the respiratory chain to the next (Dutton et al., 1970). The electron transfer chain is comprised of NADH:ubiquinone oxidoreductase (complex I), coenzyme Q, succinate:ubiquinone oxidoreducatse (complex II), ubiquinol:ferricytochrome c oxidoreductase (complex III), cytchrome c, and ferrocytochrome c: oxygen oxidoreducxtase (cytochrome oxidase or complex IV). A schematic diagram indicating the basic constitution of the respiratory chain is given in Fig 1. Comprehensive reviews detailing the sequence of electron carriers (Chance and Williams, 1956; Ernster and Lee, 1964; Chance, 1967), the nature of transition-metal mediated electron transfer (Williams, 1973), and the molecular constitution and structure of the complexes (Hatefi, 1985) and of cytochrome c (Ferguson-Miller et al., 1979; Margoliash and Bosshard, 1983) have appeared. The reader is referred to these if detail beyond that given here is desired.

The primary biochemical function of heart mitochondria is to produce ATP at steady-state concentrations sufficient to sustain the beat-to-beat energy requirements of myocardium. Electron transport plays a fundamental role in harnessing redox free energy to the synthesis of ATP. Electrons undergo large decreases in redox potential ($\geq$300 mV) during transport through complexes I, III, and IV (Erecinska et al., 1974). The oxidative energy released is coupled to the transport of protons from the matrix to a variety of proposed locations (reviewed below). Thus, the ΔG_{ox} generated from substrate oxidation in the matrix is used to drive the transport of protons against their concentration gradient. Although it is not known how complex I translocates protons, Mitchell (1979) proposes that the flavin moiety of this complex macromolecular assembly ejects

Figure 1. Schematic Depiction of the Mitochondrial Electron Transport Chain

FeS and cyt denote iron-sulfur centers and the cytochromes, respectively. The numerical subscripts on the b cytochromes represent the absorption maxima of their α bands. Complexes I, II, III, and IV and coenzyme Q are located within the mitochondrial inner membrane. Cyt c is a soluble protein found in the inter-membrane space. The FeS cluster in complex III is also known as the Rieske iron-sulfur protein. H^+ on top of each box delineating complexes I, III, and IV are understood to be inside the matrix; H^+ on the bottom of these boxes are understood to be transported out of the matrix to one or more possible locations, as described in the text.


TCA CYCLE
NADH
NAD+
H+
Complex I
FMN, FeS N-1a,b, FeS N-4, FeS N-3, FeS N-2
CoQ
Succinate
Fumarate
Complex II
FAD
FADH2
FeS S-2, FeS S-1
cyt b560, FeS S-3
H+
Complex III
cyt b562
cyt b566
FeS cyt c1
cyt c
H+
Complex IV
cyt a3, Cu
cyt a, Cu
H2O
1/2 O2
H+
H+
H+
Δμ̃H+

protons from the matrix as it is sequentially oxidized and reduced. Proton transport by complex II occurs via either a Q cycle (Mitchell, 1976) or b cycle (Wikstrom et al., 1976), in which CoQ or the b cytochromes are believed to release protons from the inner membrane, respectively. In contrast, both Chance (1972) and Papa (1976) suggest that, by analogy with hemoglobin, the proton translocation catalyzed by complex II is achieved by membrane Bohr effects. Rigorous proof has not yet been promulgated for any of these theories. Cytochrome oxidase, the terminal component of the electron transport chain and the enzyme responsible for transferring e- to oxygen, has been definitively shown to be a redox-driven proton pump (Wikstrom, 1977; reviews: Wikstrom and Krab, 1979; Malmstrom, 1985).

V. ATP SYNTHESIS

Mitochondrial ATP synthesis is catalyzed by the F_1F_0-ATP synthetase. The F_0 component of this enzyme is a transmembrane protein comprised of three subunits known as a, b, and c (Alonzo and Racker, 1979). F_1 is a soluble protein located in the mitochondrial matrix (Racker, 1968) and is made up of 5 subunits (Kagawa, 1978; Baird and Hammes, 1979; Maloney, 1982) with the following stoichiometric ratio (Amzel and Pedersen, 1983): $\alpha_3\beta_3\gamma\delta\varepsilon$. F_1 is connected to F_0 via the oligomycin sensitivity conferring protein (Senior, 1979) and the δ subunit of F_1 (Pedersen and Carafoli, 1987a). The F_0 moiety of the synthetase is a proton channel (Alonzo and Racker, 1979; Seren et al., 1985); F_1 can catalyze both ATP synthesis and ATP hydrolysis (Boyer et al., 1982). It is currently believed that expression of the ATP hydrolytic mode by the enzyme is blocked in vivo by an ATPase inhibitor peptide (Schwerzmann and Pedersen,

1986). The F_1F_0-ATP synthetase can be irreversibly inhibited by treatment with oligomycin A (Bertina et al., 1974), an antibiotic known to bind to a subunit of the F_0 moiety (Senior, 1979).

As to be expected from an enzyme that is structurally this complex, the ATP synthetase is mechanistically quite sophisticated. The energy "stored" in the $\Delta\mu_{H+}$ produced by the electron transport chain is used indirectly to drive ATP synthesis on the ATP synthetase (Hutton and Boyer, 1979). ADP and P_i bind to high affinity catalytic sites on the β subunits or at sites located at α-β interfaces (Pedersen and Carafoli, 1987b). Because the equilibrium constant for ATP synthesis from the bound substrates approximates to unity, the synthesis of ATP proceeds on the enzyme with no external need for free energy (Grubmeyer et al., 1982; Eisenberg and Hill, 1985). Protons flowing down their concentration gradient and back across the mitochondrial inner membrane are believed to protonate functional groups within F_0, particularly glutamate 61 in subunit c (Penefsky, 1985). This induces a conformational change in F_0 which in turn induces a conformational change in F_1. This conformational change in F_1 concomitantly facilitates the dissociation of ATP at one catalytic site and the binding of ADP and P_i with high affinity at another (Boyer, 1975; reviews: Boyer, 1979; Boyer et al., 1977; for a discussion of the thermodynamics of this process, see O'Neal and Boyer, 1984). Thus, in this "alternating site catalysis" model, ATP synthesis occurs by the reversal of ATP hydrolysis with ADP displacing a hydroxyl group from P_i (Boyer, 1984). It is currently believed that the free energy for phosphoanhydride bond formation in ATP results from the capacity of the ATP synthetase to conserve the energy released during the binding of ADP and P_i to the F_1 protein (Jencks, 1975). Protons play

no part in the actual chemistry; rather, H^+ are used to mediate the coupling of conformational changes to changes in the binding of substrates and products on F_1.

In order for oxidative phosphorylation to occur, a sufficient $\Delta\mu_{H+}$ must be avaialable to drive other processes as well. The net internalization of oxidizable substrate and of P_i will dissipate part of the ΔpH component of $\Delta\mu_{H+}$. The exchange of ADP^{3-} in for ATP^{4-} out is electrogenic (Klingenberg and Rottenberg, 1977). Therefore, the magnitude of the transmembrane electrical potential ($\Delta\psi$) decreases during adenine nucleotide translocation. It is clear that $\Delta\mu_{H+}$ is consumed by transport processes and by the need for continuously stimulating conformational changes in or about the F_0 proton channel. The free energy of oxidation is conserved in the sense that ATP, a compound with a relatively large (-7.3 Kcal/mole) negative free energy of hydrolysis, is produced. The continuous synthesis of ATP ensures that the cell's energy requirements can be met. The free energy change associated with the synthesis of ATP from ADP and P_i is defined by the cytosolic phosphorylation potential:

$$\Delta G_P = \Delta G_P^\circ + RT\ln \frac{[ATP]}{[ADP][P_i]} \qquad (4)$$

where ΔG_P° is the free energy of ATP synthesis under standard conditions and all other terms have their usual meaning. The indirect coupling between oxidation and phosphorylation via a proton gradient may be depicted as

$$\Delta G_{ox} \longleftrightarrow \Delta\mu_{H+} \longleftrightarrow \Delta G_P \qquad (5)$$

In equation 5 the arrows are bidirectional because: (a) during the hydrolysis of ATP, the F_1F_0-ATP synthase translocates protons out of the matrix (Nicholls, 1974); and (b) during reverse electron transfer through complex I, NADH is regenerated (Chance, 1961; Chance and Hollunger, 1961).

VI. THE H^+, OXYGEN, AND ATP STOICHIOMETRIES OF OXIDATIVE PHOSPHORYLATION

The stoichiometry of proton ejection by the electron transport chain (H+/O) and the number of protons consumed per molecule of ATP synthesized (H+/ATP) are still not established. The major obstacle to determining these stoichiometries is the fact that in those mitochondria preparations yet studied, substrate oxidation is not completely coupled to the phosphorylation of ADP. Imperfect coupling is a consequence of: (a) secondary proton transport pathways and (b) redox "slips" in the electron transport chain. Mitchell (1961) predicted that the mitochondrial inner membrane has a finite permeability to H+. This prediction was later substantiated (Mitchell and Moyle, 1967; Nicholls, 1974; Stucki, 1976). Because proton transport out of the matrix is an energy dependent process, the non-productive circulation of these particles constitutes a futile cycle. The leak characteristics of the membrane depend upon whether the driving force for proton permeation is an electric field (matrix negative) or a pH gradient (matrix alkaline). As either ΔpH or $\Delta\psi$ are increased, the membrane leak conductance is ohmic (Krishnamoorthy and Hinkle, 1984) and non-ohmic (Zoratti et al., 1986), respectively. In the former case, protons flow down an increasingly steeper concentration gradient back into the matrix; in the latter case, protons are drawn electrophoretically into the matrix at a rate that

increases exponentially as the electrical potential increases. Two types of proton "slips" have also been identified (Pietrobon et al., 1983, 1986; Pietrobon and Caplan, 1986 a and b; Pietrobon, 1986; Zoratti et al., 1983, 1986). Protons may flow through F_0 without inducing ATP release or may traverse the protein domain of a redox pump without stimulating reverse electron transfer. As discussed above, a substantial transmembrane proton flux is associated with the electroneutral uptake of various substrates. Decreases in coupling are also attributable to occasional errors in the redox pumps, which may transfer electrons without extruding protons from the matrix (Pietrobon and Caplan, 1985). These leaks and slips lead to underestimates of H+/O ratios by increasing the amount of oxygen that must be consumed in order to produce a pH gradient of a given magnitude.

In view of the complications inherent to any analysis of proton flow, it comes as no surprise that a broad range of proton and ATP stoichiometries have been reported. Estimated values for the H+/O ratio of electron flow coupled to NADH oxidation in rat liver mitochondria include 6 (Mitchell and Moyle, 1967), 8 (Brand, 1979), 10 (Hinkle and Yu, 1979), 11 (Beavis and Lehninger, 1986 a and b; Beavis, 1987a and b), 12 (Vercesi et al., 1978; Lehninger, 1984), and 13 (Lemasters, 1984). As an example, in the 13 proton model of Lemasters (1984), the proton stoichiometries for complexes I, III, and IV are 5, 4, and 4, respectively (Freedman and Lemasters, 1984; Lemasters and Fleischman, 1987). It is now widely accepted that the H+/ATP ratio is equal to 3 (Brand et al., 1976). This value increases to 4 if the proton required for P_i transport is taken into account (Alexandre et al., 1978). From knowledge of the H+/O and ATP/H+ ratios, it is possible to estimate the ATP/O stoichiometry

for oxidative phosphorylation. The variable proton pumping stoichiometries obtained by different workers using different experimental methodologies underlies the widespread controversy raging over this issue. ATP/O ratios obtained during the oxidation of NADH-linked substrates are believed to equal 2.0 (Hinkle and Yu, 1979), 2.75 (Beavis and Lehninger, 1986b), and 3.25 (Lemasters, 1984; see also Lemasters et al., 1984). It is important to note here that the values reported in the latter two works are theoretical, not empirical. Due to high rates of respiration in the absence of ADP, these workers had to apply sophisticated mathematical corrections to their data in order to obtain estimates for what the mechanistic stoichiometry of oxidative phosphorylation could be if the mitochondria used had been perfectly coupled. Whatever the final verdict on these stoichiometries will be, it must be kept in mind that the amount of ATP that can be produced is thermodynamically limited by the degree to which mitochondria can couple the oxidation of substrates to the phosphorylation of ADP. This limitation is described by the relationship:

$$-\Delta G_{ox}/\Delta G_{P} \geq \text{ATP/O}. \qquad (6)$$

VII. ALTERNATIVE COUPLING SCHEMES

There are an increasing number of findings which suggest that the chemiosmotic mechanism as first envisioned by Mitchell (1961) may not correctly describe the path followed by protons during oxidative phosphorylation. It is generally accepted that protons are transported out of the matrix during respiration and that the gradient formed is likely the sole and obligatory intermediate of oxidative phosphorylation. In addition to the studies discussed above, this conclusion is supported by

the observations that an artifically generated $\Delta\mu_{H+}$ can drive ATP synthesis (Thayer and Hinkle, 1975) and that the $\Delta\psi$ and ΔpH components of $\Delta\mu_{H+}$ are kinetically equivalent in that either can drive ATP synthesis independent of the other (Jensen et al., 1986). The chemiosmotic theory cannot, however, accomodate certain properties of the $\Delta\mu_{H+}$ generated across mitochondrial membranes (reviews: Fillingame, 1980; Ferguson and Sorgato, 1982; Ferguson and Parsonage, 1984; Westerhoff et al., 1983, 1984a). First, the rates of electron transport and of ADP phosphorylation are relatively insensitive to the magnitude of $\Delta\mu_{H+}$ (Padan and Rottenberg, 1973; Azzone et al., 1978; Branca et al., 1981; Duszynski et al., 1984; for especially lucid demonstrations of this, see Sorgato et al., 1978 and Zoratti et al., 1982). Second, at equilibrium, ΔG_P is not proportional to $\Delta\mu_{H+}$ (Westerhoff et al., 1981 and references therein). The ratio of these thermodynamic potentials defines the proton stoichiometry of the F_1F_0-ATP synthetase:

$$\Delta G_P/\Delta\mu_{H+} = n_H^{ATP} \qquad (7)$$

That ΔG_P varies independent of $\Delta\mu_{H+}$ suggests that the proton stoichiometry of the ATP synthetase varies as a function of the magnitude of the proton electrochemical potential gradient. This is considered to be unlikely. Third, a variety of inhibitor studies (reviewed in Westerhoff et al., 1984b) suggest that the proton pumps of the electron transport chain and the ATP synthetase are directly coupled.

The explanation most often used to account for these anomalies is to postulate that protons are not transported from one bulk aqueous phase (the matrix) to another (the intermembrane space). Although practically untestable, Williams (1961; 1984; review: 1978) suggests that the high

energy intermediate of oxidative phosphorylation is an ensemble of energized protons contained within the highly controlled environment of the mitochondrial inner membrane. Kell (1979) has recently proposed that protons are pumped by the electron transport chain into the Gouy-Chapman unstirred layer along the outer face of the inner membrane. A somewhat refined version of this theory is that advanced by Westerhoff et al. (1984 b and c). These authors argue that the three anomalies discussed above may be reconciled with a chemiosmotic-type mechanism if it is assumed that a primary and secondary proton pump are spatially closely situated so as to create a functional proton compartment or "proton domain." Thus, protons would be transferred directly from a redox pump to the F_0 channel of the ATP synthetase. Because the total volume of the proton domain would be small, very few protons would have to be transported within the coupling unit in order to attain an electrochemical potential sufficient to drive ATP synthesis. A convincing statistical mechanical analysis of such a coupling scheme has been presented (Westerhoff and Chen, 1985). Seen in this light, the bulk phase $\Delta\mu_{H+}$ would therefore be essentially irrelevant to the coupling of oxidation to phosphorylation. The theme common to these three theories is that "energized" protons are localized either within or on the membrane. In contrast, according to Mitchell's theory, protons are delocalized because they are allowed to equilibrate with the bulk aqueous phase of a given compartment. Precise, convincing identification of the locale into which the electron transport chain pumps protons is still an unresolved problem. If proton translocation is indeed localized, it will be of interest to determine if this requires further revision in the experimentally determined stoichiometries of oxidative phosphorylation.

VIII. THE CONTROL OF RESPIRATION

Consistent with the notion that the teleological aim of oxidative phosphorylation is to maximize the efficiency with which free energy intrinsic to metabolites can be released and conserved, the rate of respiration must be a highly controlled process. By control is meant the capacity of mitochondria to limit the rate of electron transport in the absence of ADP. If this were not the case, large amounts of energy would simply be released into the environment in the form of heat. This type of free energy dissipation has been shown with the use of such uncouplers as lipophilic protonophores (Westerhoff and Kell, 1985) and a transmembrane protein in brown fat mitochondria known as thermogenin (Nicholls, 1979). Under normal metabolic conditions, however, it is of the essence that organisms commit the free energy of substrate oxidation to the synthesis of ATP. The reason for this is especially clear in a tissue such as myocardium: ATP is absolutely required to mediate cross-link formation between actin and myosin, and for the regulation of cytosolic cation concentrations by the Ca(II)- and Na^+/K^+-ATPases located in the sarcoplasmic reticulum and the plasmalemma, respectively.

A considerable amount of experimental work indicates that ADP is the metabolite primarily responsible for regulating the rate of respiration. Early work (Lardy and Wellman, 1952; Chance and Williams, 1956) on isolated mitochondria shows that maximal rates of respiration could only be elicited with the addition of ADP. In the absence of ADP, mitochondria respire at a much lower "resting" rate. In order to quantify the relative stimulation of respiration by ADP, Chance and Williams (1956) formulated the respiratory control ratio. State 3 respiration is the rate of respiration obtained in the presence of ADP, P_i, and

oxidizable substrate, whereas state 4 is the respiratory rate observed in the presence of P_i and oxidizable substrate only. The definition of states 1, 2, and 5 may be found in the monograph by Nicholls (1982). The respiratory control ratio (RCR) is the quotient of state 3 to state 4 respiration. It follows that the higher the magnitude of the RCR, the greater is the extent of control by ADP on the rate of respiration. Low RCR values suggest that the mitochondria are poorly coupled since rates of respiration are high in the absence of added ADP. The theory that ADP controls respiratory rate has been substantiated *in vivo* in skeletal muscle using ^{31}P-nuclear magnetic resonance spectroscopy (Chance *et al.*, 1982, 1985, and 1986). Similar experiments in working heart muscle, however, do not support this conclusion (Balaban *et al.*, 1986). Instead, these authors conclude that the redox poise ($NADH/NAD^+$) regulates respiratory rate. Consistent with the latter view are the findings of Hansford (1980) and of Denton and McCormack (1985) which indicate that since Ca(II) and ADP regulate the activity of the sub-strate dehydrogenases, these enzymes limit respiration by controlling rates of NADH production.

Additional theories describing the molecular basis for respiratory control have been advanced. The work of Wilson and colleagues (Wilson *et al.*, 1974 *a* and *b*; Holian *et al.*, 1977; Erecinska *et al.*, 1977; Forman and Wilson, 1983; review: Erecinska and Wilson, 1982) suggests that the rate of respiration varies with the magnitude of ΔG_p. According to this theory, the first two coupling sites of the respiratory chain (complexes I and III) are at near equilibrium with ΔG_p, such that $\Delta G_{ox}^{I,III} \approx \Delta G_p$. As the magnitude of ΔG_p changes, the rate of respiration will either increase or decrease depending upon what the system must do in order to reestablish equilibrium (for a theoretical treatment

of this, see Bohnensack, 1981). Other workers (Slater et al., 1973; Davis and Lumeng, 1975; Kuster et al., 1976) suggest that the rate of respiration depends on the ATP/ADP ratio because ATP is a competitive inhibitor of ADP binding sites on the adenine nucleotide translocase, the enzyme believed by some (Lemasters and Sowers, 1979; Klingenberg, 1980) to limit the rate of oxidative phosphorylation. The notion that the adenylate "energy charge" (Atkinson, 1971) regulates the rate of respiration was entertained for some time, but was later disproved (Erecinska et al., 1977). Clearly, because they are the substrates of the ATP synthetase and are used to regulate the activity of a number of mitochondrial enzymes, the adenine nucleotides and P_i must play a role in the regulation of respiratory rate. However, whether this control is kinetic, thermodynamic, or incorporates elements of both is an issue that is yet to be resolved.

In addition to the adenine nucleotide translocase and the substrate dehydrogenases, other enzymes have been shown to play a role in the phenomenon of respiratory control. Among these are NADH dehydrogenase (complex I) and the F_1F_0-ATP synthetase (Doussiere et al., 1984), cytochrome oxidase (complex IV) via allosteric modulation (Kadenbach, 1986), and the P_i transport protein (Mazat et al., 1986). Given the fact that mitochondria are extremely complex both kinetically and thermodynamically, it is entirely possible that some or all of these different mechanisms of control are utilized by these organelles in order to attain the necessary level of responsiveness to rapid, fluctuational demands for energy in the cytosol. By applying the principles of control theory (Kacser and Burns, 1973; Heinrich and Rapoport, 1974 a and b; Groen, 1984), it has been possible to show that the control of respiration is distributed

among a number of enzymes and transport proteins. In control theory, inhibitors are used to quantitate the control exerted by different enzymes over the rate at which mitochondria respire in either the presence or absence of ADP. When mitochondria oxidize succinate during state 3, the rate of respiration appears to be limited by the activity of the dicarboxylate carrier, bc_1 complex, cytochrome oxidase, and the adenine nucleotide translocase (Groen et al., 1982; Tager et al., 1983). Though not determined by control theory, it has been shown that during state 4 the rate of respiration is limited by proton leaks (Groen et al., 1982; Bohnensack et al., 1982), Ca(II) cycling (Stucki and Ineichen, 1974), and the rate at which ADP is regenerated from ATP by ATPases in the extramitochondrial space (Masini et al., 1983 a and b; 1984).

It is obvious from the number of different theories put forth that much remains to be elucidated about the nature of respiratory control. A likely reason for the diversity of results obtained is that there are many differences in experimental design. For instance, depending upon which substrate is used to drive respiration and at what concentration it is available, different enzymes will play roles in respiratory control. Moreover, it is not yet known whether all of the enzymes involved in energy transduction have been identified. It is also unknown whether all of the relevant forms of enzyme regulation have been elucidated. Before these gaps are filled in, no unifying and comprehensive model of respiratory control can be formulated.

REFERENCES

Alexandre, A., Reynafarje, B., and Lehninger, A.L. (1978) Proc. Nat. Acad. Sci. (U.S.A.) 75: 5296-5300.

Alfonzo, M., and Racker, E. (1979) Can. J. Biochem. 57: 1351-1358.

Atkinson, D.E. (1971) In Metabolic Pathways (ed. G.M. Grrenberg) 3rd ed., Vol. V, pp. 1-21. Academic Press, New York.

Azzone, G.F., Pozzan, T., Masari, S., and Bragadin, M. (1978) Biochim. Biophys. Acta 501: 296-306.

Baird, B.A., and Hammes, G.G. (1979) Biochim. Biophys. Acta 549: 31-53.

Balaban, R.S., Kantor, H.L., Katz, L.A., and Briggs, R.W. (1986) Science (Washington, D.C.) 232: 1121-1123.

Beavis, A.D., and Lehninger, A.L. (1986a) Eur. J. Biochem. 158: 307-314.

Beavis, A.D., and Lehninger, A.L. (1986b) Eur. J. Biochem. 158: 315-322.

Beavis, A.D. (1987a) J. Biol. Chem. 262: 6165-6173.

Beavis, A.D. (1987b) J. Biol. Chem. 262: 6174-6181.

Bertina, R.M., Steenstra, J.A., and Slater, E.C. (1974) Biochim. Biophys. Acta 368: 279-297.

Bohnensack, R. (1981) Biochim. Biophys. Acta 634: 203-218.

Bohnensack, R., Kuster, U., and Letko, G. (1982) Biochim. Biophys. Acta 680: 271-280.

Boyer, P.D. (1975) FEBS Lett. 50: 91-94.

Boyer, P.D., Chance, B., Ernster, L., Mitchell, P., Racker, E., and Slater, E.C. (1977) Ann. Rev. Biochem. 46: 955-1026.

Boyer, P.D. (1979) In Membrane Bioenergetics (Lee, C.P., Schatz, G., and Ernster, L., eds) pp. 461-479. Addison-Wesley, Reading, Massachusetts.

Boyer, P.D. (1984) Symposium on Frontiers in Bio-Organic Chemistry and Molecular Biology (Ovchinnikov, Y.A.), pp. 1-8. Elsevier, Amsterdam.

Branca, D., Ferguson, S.J., and Sorgato, C.M. (1981) Eur. J. Biochem. 116: 341-346.

Brand, M.D., Reynafarje, B., and Lehninger, A.L. (1976) Proc. Nat. Acad. Sci. (U.S.A.) 73: 437-441.

Campo, M.L., Zhang, C.J., and Tedeschi, H. (1984) Biochem. Soc. Trans. 12: 384-386.

Chance, B., and Williams, G.R. (1956) Adv. Enzymol. 17: 65-134.

Chance, B. (1961) J. Biol. Chem. 236: 1534-1543.

Chance, B., and Hollunger, G. (1961) J. Biol. Chem. 236: 1577-1584.

Chance, B. (1967) Biochem. J. 103: 1-18.

Chance, B. (1972) FEBS Lett. 23: 3-20.

Chance, B., Eleff, S., Bank, W., Leigh, J.S., and Warnell, R. (1982) Proc. Nat. Acad. Sci. (U.S.A.) 79: 7714-7718.

Chance, B., Leigh, J.S., Clark, B.J., Maris, J., Kent, J., Nioka, S., and Smith, D. (1985) Proc. Nat. Acad. Sci. (U.S.A.) 82: 8384-8388.

Chance, B., Leigh, J.S., Kent, J., McCully, K., Nioka, S., Clark, B.J., Maris, J.M., and Graham, T. (1986) Proc. Nat. Acad. Sci. (U.S.A.) 83: 9458-9462.

Chen, R.F., and Plaut, G.W.E. (1963) Biochemistry 2: 1023-1032.

Denton, R.M., and McCormack, J.G. (1985) Am J. Physiol. 249: E453-E554.

Doussiere, J., Ligeti, E., Brandolin, G., and Vignais, P.V. (1984) Biochim. Biophys. Acta 766: 492-500.

Duszynski, J., Bogucka, K., and Wojtczak, L. (1984) Biochim. Biophys. Acta 767: 540-547.

Dutton, P.L., Wilson, D.F., and Lee, C.P. (1970) Biochemistry 9: 5077-5082.

Eisenberg, E., and Hill, T.L. (1985) Science (Washington, D.C.) 227: 999-1006.

Erecinska, M., Veech, R.L., and Wilson, D.F. (1974) Arch. Biochem. Biophys. 160: 412-424.

Erecinska, M., Stubbs, M., Miyata, Y., Ditre, C.M., and Wilson, D.F. (1977) Biochim. Biophys. Acta 462: 20-35.

Erecinska, M., and Wilson, D.F. (1982) J. Membrane Biol. 70: 1-14.

Ernster, L., and Schatz, G. (1981) J. Cell Biol. 91: 227s-255s.

Ferguson, S.J., and Parsonage, D. (1982) Biochem. Soc. Trans. 12: 416-420.

Ferguson, S.J., and Sorgato, C. (1982) Ann. Rev. Biochem. 51: 185-217.

Ferguson, S.J. (1985) Biochim. Biophys. Acta 811: 47-95.

Freedman, J.A., and Lemasters, J.J. (1984) Biochem. Biophys. Res. Comm. 125: 8-13.

Fillingame, R.H. (1980) Ann. Rev. Biochem. 49: 1079-1113.

Forman, N.G., and Wilson, D.F. (1983) J. Biol. Chem. 258: 8649-8655.

Goebell, H., and Klingenberg, M. (1964) Biochem. Z. 340: 441-464.

Groen, A.K., Wanders, R.J., Westerhoff, H.V., van der Meer, R., and Tager, J.M. (1982) J. Biol. Chem. 257: 2754-2757.

Groen, A.K. (1984) Dissertation, University of Amsterdam.

Grubmeyer, C., Cross, R.L., and Penefsky, H.S. (1982) J. Biol. Chem. 257: 12,092-12,100.

Hansford, R.G. (1976) J. Biol. Chem. 251: 5483-5489.

Hansford, R.G. (1980) Curr. Top. Bioenergetics 10: 217-278.

Hansford, R.G., and Castro, F. (1981) Biochem. J. 198: 525-533.

Hansford, R.G. (1985) Rev. Physiol. Biochem. Pharmacol. 102: 2-72.

Hassinen, I.E., and Hiltunen, K. (1975) Biochim. Biophys. Acta 408: 319-330.

Hassinen, I.E. (1986) Biochim. Biophys. Acta 853: 135-151.

Hatefi, Y. (1985) Ann. Rev. Biochem. 54: 1015-1069.

Haworth, R.A., Hunter, D.R., and Berkoff, H.A. (1980) FEBS Lett. 110: 216-218.

Heinrich, R., Rapoport, T.A. (1974a) Eur. J. Biochem. 42: 89-95.

Heinrich, R., Rapoport, T.A. (1974b) Eur. J. Biochem. 42: 97-105.

Hoek, J.B., and Njogu, R.M. (1976) FEBS Lett. 71: 341-346.

Hinkle, P.C., and Yu, M.L. (1979) J. Biol. Chem. 254: 2450-2455.

Holian, A., Owen, C.S., and Wilson, D.F. (1977) Arch. Biochem. Biophys. 181: 164-171.

Hill, T.L., and Eisenberg, E. (1981) Quart. Rev. Biophys. 14: 463-511.

Hucho, F., Randall, D.D., Roche, T.E., Burgett, M.W., Pelley, J.W., and Reed, L.J. (1972) Arch. Biochem. Biophys. 159: 328-340.

Hutton, R.L., and Boyer, P.D. (1979) J. Biol. Chem. 254: 9990-9993.

Jencks, W.P. (1975) Adv. Enzymol. 51: 75-106.

Kacser, H., and Burns, J.A. (1979) Biochem. Soc. Trans. 7: 1149-1160.

Kadenbach, B. (1986) J. Bioenerg. Biomemb. 18: 39-54.

Kagawa, Y. (1978) Biochim. Biophys. Acta 505: 45-93.

Kauppinen, R.A., Hiltunen, J.K., and Hassinen, I.E. (1983) Biochim. Biophys. Acta 725: 425-433.

Kauppinen, R.A., Hiltunen, J.K., and Hassinen, I.E. (1986) In Myocardial and Skeletal Muscle Bioenergetics (Bratbar, N., ed), pp. 331-341. Plenum, New York.

Kell, D.B. (1979) Biochim. Biophys. Acta 549: 55-99.

Kerbey, A.L., Randle, P.J., Cooper, R.H., Whitehouse, S., Pask, H.T., Denton, R.M. (1976) Biochem. J. 154: 327-348.

Klingenberg, M., and Rottenberg, H. (1977) Eur. J. Biochem. 73: 125-130.

Klingenberg, M. (1980) J. Membr. Biol. 56: 97-105.

Klingenberg, M. (1984) Biochem. Soc. Trans. 12: 390-393.

Kramer, R.S., and Pearlstein, R.D. (1983) Proc. Nat. Acad. Sci. (U.S.A.) 80: 5807-5811.

Kramer, R., and Klingenberg, M. (1982) Biochemistry 21: 1082-1089.

Kuster, U., Bohnensack, R., and Kunz, W. (1976) Biochim. Biophys. Acta 440: 391-402.

LaNoue, K.F., and Schoolwerth, A.C. (1979) Ann. Rev. Biochem. 48: 871-922.

Latipaa, P.M., Peuhkurinen, K.J., Hiltunen, J.K., and Hassinen, I.E. (1985) J. Mol. Cell. Cardiol. 17: 1161-1171.

Lehninger, A.L., Vercessi, A., and Bababunmi, E.A. (1978) Proc. Nat. Acad. Sci. (U.S.A.) 75: 1690-1694.

Lehninger, A.L. (1984) Biochem. Soc. Trans. 12: 386-388.

Lemasters, J.J., and Sowers, A.E. (1979) J. Biol. Chem. 254: 1248-1251.

Lemasters, J.J., Grunwald, R., and Emaus, R.K. (1984) J. Biol. Chem. 259: 3058-3063.

Lemasters, J.J. (1984) J. Biol. Chem. 259: 13,123-13,130.

Lemasters, J.J., and Fleishman, K.E. (1987) Biochem. Biophys. Res. Comm. 142: 176-182.

Linn, T.C., Pettit, F.H., and Reed, L.J. (1969a) Proc. Nat. Acad. Sci. (U.S.A.) 62: 234-241.

Linn, T.C., Pettit, F.H., Hucho, F., and Reed, L.J. (1969b) Proc. Nat. Acad. Sci. (U.S.A.) 64: 227-234.

Lotscher, H., Winterhalter, K.H., Carafoli, E., and Richter, C. (1980) Eur. J. Biochem. 110: 211-216.

Lowenstein, J.M. (1967) In Metabolic Pathways (ed. G.M. Greenberg) 3rd ed., vol I, pp. 146-270. Academic Press, New York.

Lysiak, W., Toth, P.P., Suelter, C.H., and Bieber, L.L. (1986) J. Biol. Chem. 261: 13,698-13,703.

Lysiak, W., Lilly, K., DiLisa, F., Toth, P.P., and Bieber, L.L. (1988) J. Biol. Chem. 263: 1151-1156.

Malmstrom, B.G. (1985) Biochim. Biophys. Acta 811: 1-12.

Maloney, P.C. (1982) J. Membr. Biol. 67: 1-12.

Malpress, F.H. (1982) Biochem. Soc. Trans. 12: 399-401.

Margoliash, E., and Bosshard, H.R. (1983) Trends Biochem. Sci. 8: 316-320.

Martin, W.H., Beavis, A.D., and Garlid, K.D. (1984) J. Biol. Chem. 259: 2062-2065.

Masini, A., Ceccarelli-Stanzani, D., and Musctatello, U. (1983a) FEBS Lett. 160: 137-140.

Masini, A., Ceccarelli-Stanzani, D., and Musctatello, U. (1983b) Biochim. Biophys. Acta 724: 251-257.

Masini, A., Ceccarelli-Stanzani, D., and Musctatello, U. (1984) Biochim. Biophys. Acta 767: 130-137.

Mazat, J.P., Jean-Bart, E., Rigoulet, M., and Guerin, B. (1986) Biochim. Biophys. Acta 849: 7-15.

McCormack, J.G., and Denton, R.M. (1979) Biochem. J. 180: 533-544.

McCormack, J.G., and Denton, R.M. (1981) Biochem. J. 196: 619-624.

Meijer, A.J., and Van Dam, K. (1981) In Membrane Transport (Bonting, S.L., and de Pont, J.J., eds) pp. 235-256. Elsevier/North Holland

Biomedical Press.

Mela-Riker, L.M., and Bukoski, R.D. (1985) Ann. Rev. Physiol. 47: 645-663.

Mitchell, P. (1961) Nature (London) 191: 144-148.

Mitchell, P., and Moyle, J. (1967) Biochem. J. 105: 1147-1162.

Mitchell, P. (1975) FEBS Lett. 50: 95-97.

Mitchell, P. (1976) J. Theor. Biol. 62: 327-376.

Mitchell, P. (1979a) Science (Washington, D.C.) 206: 1148-1159.

Mitchell, P. (1979b) Eur. J. Biochem. 95: 1-20.

Mitchell, P. (1985) J. Biochem. 97: 1-18.

Nakashima, R.A., Dordick, R.S., and Garlid, K.D. (1982) J. Biol. Chem. 257: 12,540-12,545.

Nicholls, D.G. (1979) Biochim. Biophys. Acta 549: 1-29.

Nicholls, D.G. (1982) Bioenergetics: An Introduction to the Chemiosmotic Theory. Academic Press, New York.

O'Neal, C.C., and Boyer, P.D. (1984) J. Biol. Chem. 259: 5761-5767.

Oram, J.F., Bennetch, S.L., and Neely, J.R. (1973) J. Biol. Chem. 248: 5299-5309.

Padan, E., and Rottenberg, H. (1973) Eur. J. Biochem. 40: 431-437.

Palmieri, F., Quagliariello, E., and Klingenberg, M. (1970) Eur. J. Biochem. 17: 230-238.

Palmieri, F., Prezioso, G., Quaglieriello, E., and Klingenberg, M. (1971) Eur. J. Biochem. 22: 66-74.

Pande, S.V., and Parvin, R. (1978) J. Biol. Chem. 253: 1565-1573.

Papa, S., Francavilla, A., Pardies, G., and Meduri, B. (1971) FEBS Lett. 12: 285-288.

Papa, S. (1976) Biochim. Biophys. Acta 456: 39-84.

Pedersen, P.L., and Carafoli, E. (1987a) Trends Biochem. Sci. 12: 146-150.

Pedersen, P.L., and Carafoli, E. (1987b) Trends Biochem. Sci. 12: 186-189.

Penefsky, H.S. (1985) Proc. Nat. Acad. Sci. (U.S.A.) 82: 1589-1593.

Peuhkurinen, K.J. (1984) J. Mol. Cell. Cardiol. 16: 487-495.

Pettit, F.H., Pelley, J.W., and Reed, L.J. (1975) Biochem. Biophys. Res. Comm. 65: 575-582.

Pfaff, E., and Klingenberg, M. (1968) Eur. J. Biochem. 6: 66-79.

Pfaff, E., Heldt, H.W., and Klingenberg, M. (1969) Eur. J. Biochem. 10: 484-493.

Pietrobon, D., Zoratti, M., and Azzone, G.F. (1983) Biochim. Biophys. Acta 723: 317-321.

Pietrobon, D., and Caplan, S.R. (1985) Biochemistry 24: 5764-5770.

Pietrobon, D. (1986) Bioeletrochem. Bioenerg. 15: 193-209.

Pietrobon, D., Zoratti, M., Azzone, G.F., Caplan, S.R. (1986) Biochemistry 25: 767-775.

Pietrobon, D., and Caplan, S.R. (1986a) Biochemistry 25: 7682-7690.

Pietrobon, D., and Caplan, S.R. (1986b) Biochemistry 25: 7690-7696.

Puskin, J.S., Gunter, T.E., Gunter, K.K., and Russell, P.R. (1976) Biochemistry 15: 3834-3842.

Racker, E. (1968) Scientific American 218: 32-39.

Reed, L.J. (1974) Acc. Chem. Res. 7: 40-56.

Riley, W.W., and Pfeiffer, D.R. (1986) J. Biol. Chem. 261: 28-31.

Rottenberg, H., and Scarpa, A. (1974) Biochemistry 13: 4811-4819.

Rottenberg, H. (1986) Methods Enzymol. 125: 3-15.

Schwerzmann, K., and Pedersen, P.L. (1986) Arch. Biochem. Biophys. 250: 1-18.

Senior, A.E. (1979) Methods Enzymol. 55: 391-397.

Seren, S., Caporin, G., Galiazzo, F., Lippe, G., Ferguson, S.J., and Sorgato, M.C. (1985) Eur. J. Biochem. 152: 373-379.

Slater, E.C., Rosing, J., and Mol, A. (1973) Biochim. Biophys. Acta 292: 534-553.

Sorgato, M.C., Ferguson, S.J., Kell, D.B., and John, P. (1978) Biochem. J. 174: 237-256.

Stucki, J.W. (1976) Eur. J. Biochem. 68: 551-562.

Tager, J.M., Wanders, R.J.A., Groen, A.K., Kunz, W., Bohnensack, R., Kuster, U., Letko, G., Bohme, G., Duszynski, J., and Wojtczak, L. (1983) FEBS Lett. 151: 1-9.

Tanford, C. (1983) Ann. Rev. Biochem. 52: 379-409.

Thayer, W.S., and Hinkle, P.C. (1975) J. Biol. Chem. 250: 5330-5335.

Van Dam, K., Westerhoff, H.V., Rutgers, M., Bode, J.A., de Jonge, P.C., Bos, M.M., and van den Berg, G. (1981) In Vectorial Reactions in Electron and Ion Transport in Mitochondria and Bacteria (Palmieri, F., ed), pp. 389-397. Elsevier/North Holland Biomedical Press.

Van Dam, K., Woelders, H., Colen, A.M., and Westerhoff, H.V. (1984) Biochem. Soc. Trans. 12:401-402.

Van Der Bend, R.L., Petersen, J., Berden, J.A., Van Dam, K., and Westerhoff, H.V. (1985) Biochem. J. 230: 543-549.

Vary, T.C., Reibel, D.K., and Neely, J.R. (1981) Ann. Rev. Physiol. 43: 419-430.

Vercesi, A., Reynafarje, B., and Lehninger, A.L. (1978) J. Biol. Chem. 253: 6379-6385.

Wang, J.H. (1983) Ann. Rev. Biophys. Bioeng. 12: 21-34.

Westerhoff, H.V., Simonetti, A.L.M., and Van Dam, K. (1981) Biochem. J. 200: 193-202.

Westerhoff, H.V., Colen, A.M., and Van Dam, K. (1984a) Biochem. Soc. Trans. 11: 81-85.

Westerhoff, H.V., Melandri, B.A., Venturoli, G., Azzone, G.F., and Kell, D.B. (1984b) FEBS Lett. 165: 1-5.

Westerhoff, H.V., Melandri, B.A., Venturoli, G., Azzone, G.F., and Kell, D.B. (1984c) Biochim. Biophys. Acta 768: 257-292.

Westerhoff, H.V., and Chen, Y.D. (1985) Proc. Nat. Acad. Sci. (U.S.A.) 82: 3222-3226.

Westheimer, F.H. (1987) Science (Washington, D.C.) 235: 1173-1178.

Wikstrom, M. (1977) Nature (London) 266: 271-273.

Wikstrom, M., and Krab, K. (1979) Biochim. Biophys. Acta 549: 177-222.

Wikstrom, M., Krab, K., and Saraste, M. (1981) Ann. Rev. Biochem. 50: 623-655.

Williams, R.J.P. (1961) J. Theor. Biol. 1: 1-17.

Williams, R.J.P. (1973) Biochem. Soc. Trans. 1: 1-26.

Williams, R.J.P. (1978) Biochim. Biophys. Acta 505: 1-44.

Williams, R.J.P. (1984) Biochem. Soc. Trans. 12: 396-399.

Williamson, J.R. (1974) Biochem. J. 93: 97-106.

Williamson, J.R. (1979) Ann. Rev. Physiol. 41: 485-506.

Williamson, J.R., and Cooper, R.H. (1980) FEBS Lett. 117: K73-K85.

Wilson, D.F., Stubbs, M., Veech, R.L., Erecinska, M., and Krebs, H. (1974) Biochem. J. 140: 57-64.

Wilson, D.F., Stubbs, M., Oshino, N., and Erecinska, M. (1974) Biochemistry 13: 5305-5311.

Yeaman, S.J., Hutchinson, E.T., Roche, T.E., Pettit, F.H., Brown, J.R., Reed, L.J., Watson, D.C., and Dixon, G.A. (1978) Biochemistry 17: 2364-2370.

Zoratti, M., Pietrobon, D., and Azzone, G.F. (1983) Biochim. Biophys. Acta 723: 59-70.

Zoratti, M., Favaron, M., Pietrobon, D., and Azzone, G.F. (1986) Biochemistry 25: 760-767.

Chapter 2

ISOLATION OF HIGHLY COUPLED HEART MITOCHONDRIA IN HIGH YIELD USING A BACTERIAL COLLAGENASE

INTRODUCTION

Numerous methods have been developed for the isolation of mitochondria from a variety of tissues (Nedergaard and Cannon, 1979; Ernster and Schatz, 1981). These procedures usually involve homogenization of the tissue by mechanical means, and separation of the mitochondria containing fraction by differential centrifugation. The consistency of myocardium often necessitates the use of a nonspecific proteinase such as Nagarse to facilitate the disruption process (Chance and Hagihara, 1963; Palmer et al., 1977; Mela and Seitz, 1979). However, as demonstrated by Pande and Blanchaer (1970), mitochondria isolated in the presence of Nagarse lose all long chain fatty-acyl-CoA synthase (E.C. 2.3.1.86) activity, and likely lose other outer membrane associated enzymes as well. Heart mitochondria isolated according to currently available methods also show significant rates of state 4 respiration. Although oligomycin (Masini et al., 1983a, 1984) and an enzymatic ADP sink (Bishop and Atkinson, 1984) have been successfully used to reduce the rate of oxygen consumption in state 4, it is recognized that state 4 respiration is phenomenologically complex (Stucki and Ineichen, 1974; Nicholls, 1974; Stucki, 1976; Lemasters and Hackenbrock, 1980; Groen et al., 1982; Masini et al., 1983b) and difficult to suppress or eliminate.

Because collagen plays a major role in the organization of cells into tissues (Hay, 1981), and myocardium is known to have a highly developed collagen infrastructure (Caulfield and Borg, 1979), a collagenase (clostridiopeptidase A, E.C. 3.4.24.3) was selected to facilitate

degradation of intercellular connections. The use of collagenase in place of a nonspecific proteinase has the advantage of being specific toward collagen, with no activity toward other proteins (Seifter and Harper, 1971). One potential obstacle to the use of collagenase for the disruption of heart muscle is that the enzyme has an absolute requirement for Ca(II) (Bond and Van Wart, 1984), and addition of this divalent cation might be expected to damage the mitochondria (Lehninger et al., 1967; Lehninger, 1970; Stadhouders, 1981). This difficulty is circumvented by allowing the collagenase to be activated by endogenous Ca(II) released from the myocardium during homogenization.

In this chapter a collagenase-facilitated isolation of highly coupled heart mitochondria in high yield with morphologically intact inner and outer membranes is described.

EXPERIMENTAL PROCEDURES

I. MATERIALS

Water. In order to isolate highly coupled mitochondria, it is critical that the water used be as pure as possible. For the preparations described herein, water is glass distilled, deionized by passage over a column of Dowex MR-3 (Sigma Chemical Co., St. Louis, MO) mixed bed ion-exchange resin, and then redistilled from alkaline permanganate. The last step of this procedure may be essential for removing organic contaminants.

Collagenase. Collagenase (Sigma type VII, lot 33F-6819) is reconstituted prior to each experiment in a solution containing 0.225 M mannitol and 0.075 M sucrose.

Reagents. The following substances were reagent grade or better, used without further purification, and obtained from the sources noted: sucrose (RNase free), coenzyme A (CoA; type III-L), fatty acid free bovine serum albumin (BSA; fraction V), ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetra-acetic acid (EGTA), 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazo-lium chloride (INT), EDTA, rotenone, and the sodium salts of ADP and pyruvic, L-malic, L-glutamic, DL-β-hydroxybutyric, α-ketoglutaric, and succinic acids (Sigma); Tris (Boehringer Mannheim Biochemicals, Indianapolis, IN); mannitol, potassium permanganate, and phosphoric acid (Mallinckrodt, Paris, KY); palmitic acid (Nutritional Biochemical Corp., Cleveland, OH); and L-carnitine (gift of L.L. Bieber, Michigan State University). 7-Deoxycholic acid (Sigma, grade III) was

recrystallized three times from 80% acetone. Cytochrome c (horse heart, Sigma type VI) was purified and reduced as described by Thompson and Ferguson-Miller (1983).

Animals. Fertile single comb white leghorn eggs were purchased from a local farm and allowed to hatch in incubators at Michigan State University's Department of Animal Science. Chicks were maintained on a diet of Chick GO125 feed (Kent Feeds, Inc., Muscatine, IA) and were not starved prior to sacrifice.

Glassware. It is critical that all of the glassware used be as clean as possible. As described by Mela and Seitz (1979), the glassware used to isolate mitochondria must never be washed with detergent. Rather, glassware is cleaned with absolute ethanol and rinsed exhaustively with water distilled from permanganate. Departures from this regimen ensure that the mitochondria one isolates will have average to poor coupling parameters.

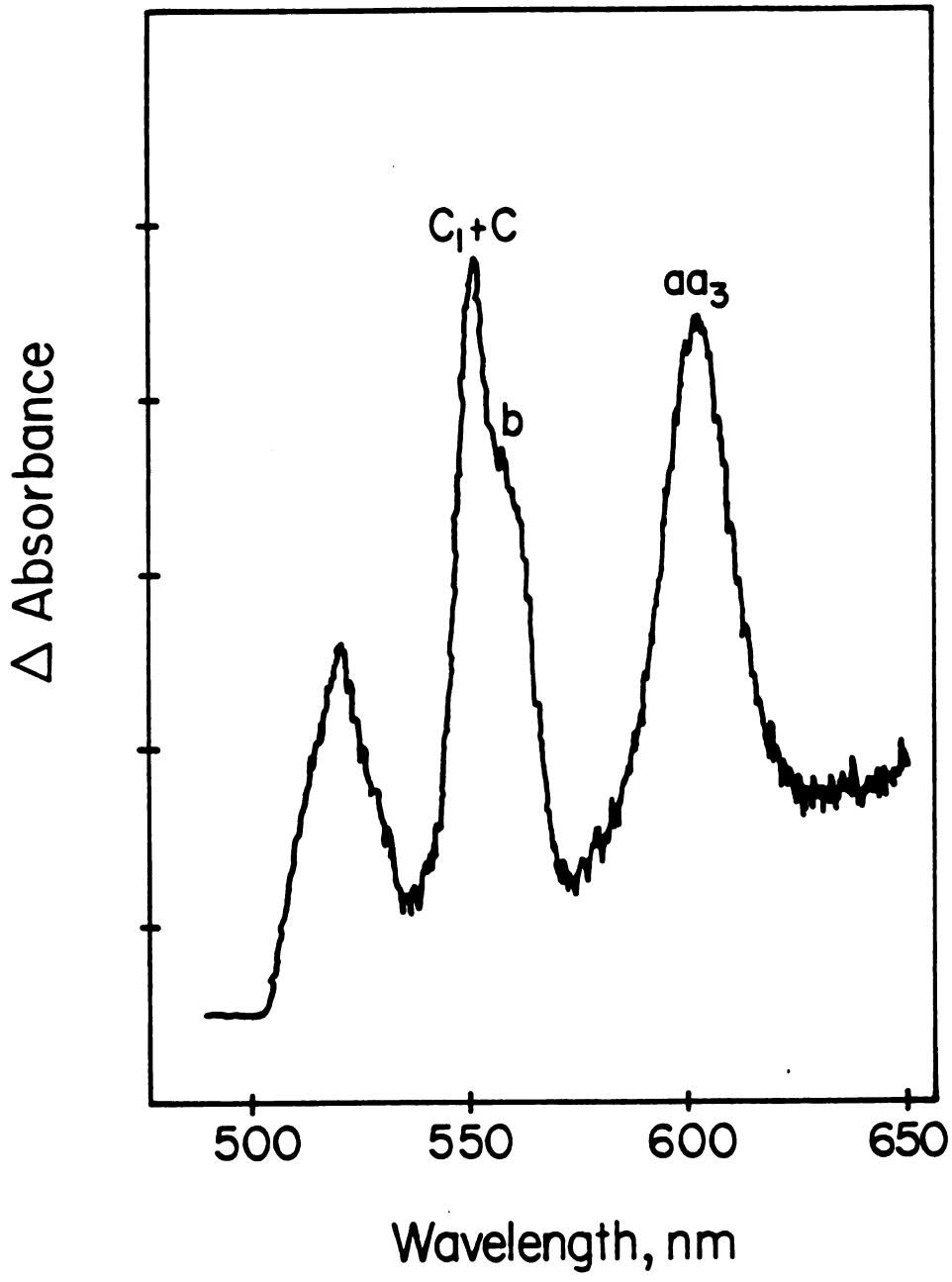
II. ENZYME ASSAYS

Cytochrome c oxidase (cyt aa_3, E.C. 1.9.3.1) concentrations are quantitated from difference spectra (Fig 1) of solubilized mitochondria (dithionite reduced minus ferricyanide oxidized) obtained with a Perkin-Elmer 559-A spectrophotometer. Mitochondria (≈2 mg/ml) are solubilized in a buffer containing 40 mM sodium phosphate, pH 7.8, 1% (w/v) deoxycholate. A $\Delta\varepsilon$(605-630 nm) = 24 mM^{-1} cm^{-1} is used (von Jagow and Klingenberg, 1972).

Succinate dehydrogenase (succinate:INT reductase, E.C. 1.3.99.1) is quantitated by the endpoint assay of Pennington (1961) after 7-10 min incubations at 37°C. The percentage yield of mitochondria is estimated

Figure 1. Difference Spectrum Showing the α Bands of Chick Heart Mitochondrial Cytochromes

Samples of solubilized mitochondria were reduced with excess ferricyanide or oxidized with dithionite. The absorbance of cyt aa_3 was used to quantitate mitochondria in respiration experiments.


C_1+C
aa_3
b
Δ Absorbance
500
550
600
650
Wavelength, nm

from the fraction of succinate:INT reductase activity recovered from the crude homogenate. One unit of succinate:INT reductase catalyzes the synthesis of one μmol of INT-formazan per min at 37°C.

Hexokinase (E.C. 2.7.1.1) is assayed according to the method of Polakis and Wilson (1982). The hexokinase activity remaining in the mitochondrial fraction after five rinses with 0.225 M mannitol/0.075 M sucrose is solubilized by incubation in 0.1 M sodium phosphate, pH 6.5, 1% Triton X-100 for 20 min. One unit of hexokinase catalyzes the synthesis of one μmol of glucose 6-phosphate per min at 30°C.

Long Chain Fatty-Acyl-CoA Synthase. To test for the presence of this enzyme, mitochondria (≈0.78 nmol cyt aa_3) are incubated at 30.5°C in a 0.225 M mannitol/0.075 M sucrose solution (1.75 ml) containing 450 μM CoA, 5 mM ATP, 5 mM magnesium acetate, 50 μM palmitate, 2 mM L-carnitine, and 10 mM Tris-buffered P_i, pH 7.4. In addition, 0.2% (w/v) fatty acid free BSA is included in the assay medium in order to control the concentration of free fatty acid in the mitochondrial suspension by creating an equilibrium between the free and bound forms (Spector et al., 1971), thereby safeguarding against the uncoupling effect of fatty acid (Van den Bergh, 1967), the detergent effect of acylcarnitine derivatives (L.L. Bieber, personal communication), and inhibition of the adenine nucleotide translocase (Woldegiorgis et al., 1982). β-oxidation of palmitoylcarnitine is monitored with an oxygen polarograph.

III. TRANSMISSION ELECTRON MICROSCOPY

Mitochondria were embedded in 4% (w/v) warm Bacto-Agar (Difco Laboratories, Detroit, MI), and fixed at 4°C for 1 hr in a buffer that contained 4% (v/v) glutaraldehyde and 0.1 M sodium phosphate, pH 7.2.

After two washes in a 0.1 M sodium phosphate buffer, mitochondria were post-fixed in 1% (w/v) OsO_4 at room temperature for 1 hr. Samples were dehydrated in a graded series of ethanol and embedded in a mixture of epon-araldite-Spurrs resin. Ultra-thin sections (70-80 nm) made with an MT-2 Ultramicrotome were stained with aqueous 2% (w/v) uranyl acetate and then counterstained with Reynolds lead (Dawes, 1971).

IV. SCANNING ELECTRON MICROSCOPY

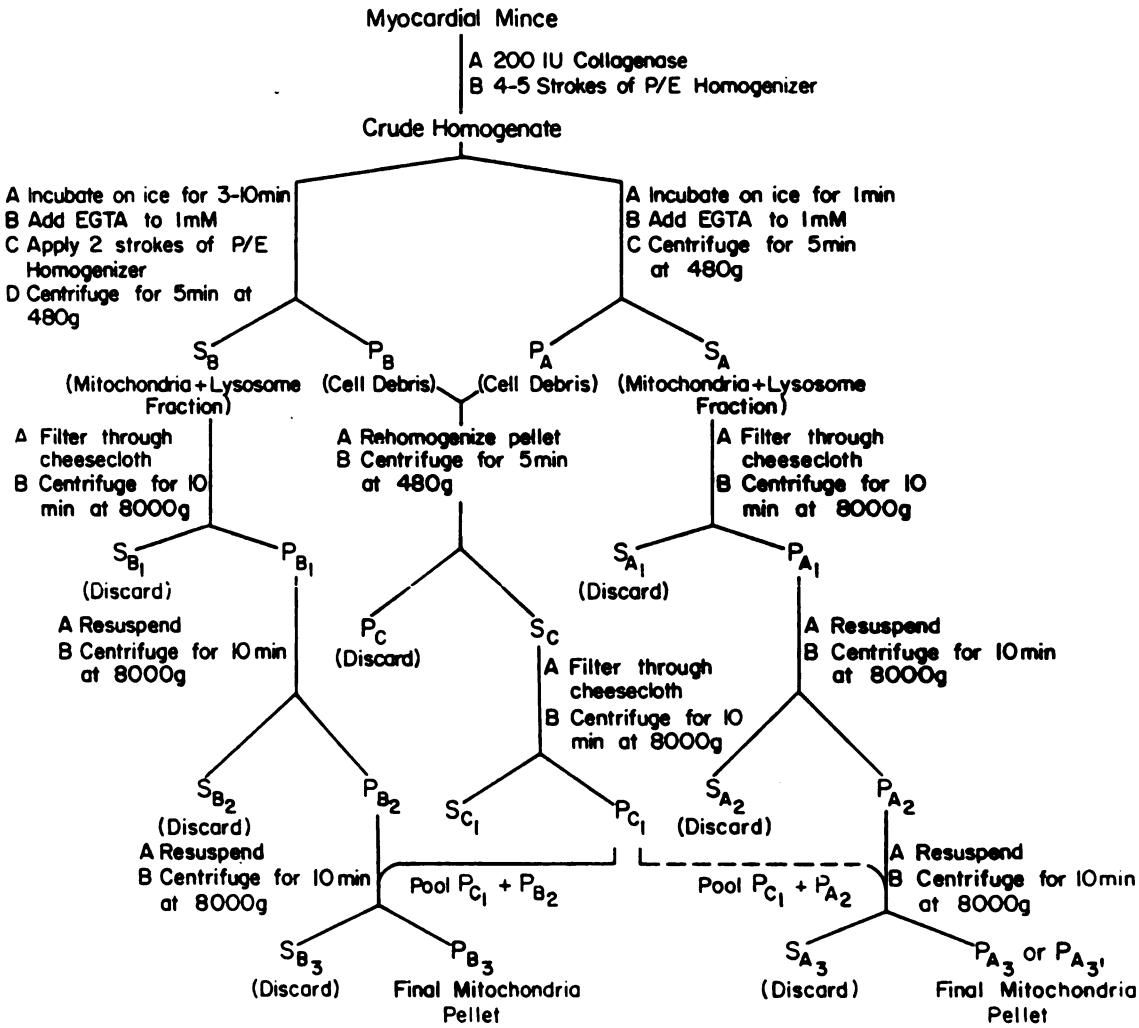
Mitochondria were fixed in 4% (v/v) glutaraldehyde buffered with 0.1 M sodium phosphate, pH 7.2. Fixed organelles were then mounted on a coverslip covered with 1% (w/v) poly-L-lysine hydrobromide (Sigma, type 1-B). The mounted organelles were washed three times with deionized water and dehydrated in a graded series of ethanol. The mitochondria were then critical point dried, sputter-coated with gold, and examined with a Jeol JSM-35C Scanning Electron Microscope operated at 20 kV.

V. ISOLATION OF MITOCHONDRIA

Mitochondria were isolated from ventricular myocardium according to the scheme shown in Fig 2. For a typical isolation of mitochondria, hearts were removed from three, 14-20 day old chickens. The pericardium, major vessels, and atria were excised, and the ventricles of each heart were immediately minced with scissors into 5 ml of fresh ice-cold osmotic support medium containing 0.225 M mannitol, 0.075 M sucrose, and 0.2% (w/v) fatty acid free BSA (MSB medium). In order to maintain optimal mitochondrial respiratory control, it is critical that the heart tissue from each animal be isolated and minced within 30 sec after decapitation and that all subsequent operations be performed at 0-4°C.

Figure 2. Flow Diagram for Collagenase Facilitated Isolation of Heart Mitochondria

Designations are as described under "Experimental Procedures."


Myocardial Mince
A 200 IU Collagenase
B 4-5 Strokes of P/E Homogenizer
Crude Homogenate
A Incubate on ice for 3-10min
B Add EGTA to 1mM
C Apply 2 strokes of P/E Homogenizer
D Centrifuge for 5min at 480g
A Incubate on ice for 1min
B Add EGTA to 1mM
C Centrifuge for 5min at 480g
S_B (Mitochondria+Lysosome Fraction)
P_B (Cell Debris)
P_A (Cell Debris)
S_A (Mitochondria+Lysosome Fraction)
A Filter through cheesecloth
B Centrifuge for 10 min at 8000g
A Rehomogenize pellet
B Centrifuge for 5min at 480g
A Filter through cheesecloth
B Centrifuge for 10 min at 8000g
S_{B_1} (Discard)
P_{B_1}
P_C (Discard)
S_C
S_{A_1} (Discard)
P_{A_1}
A Resuspend
B Centrifuge for 10min at 8000g
A Filter through cheesecloth
B Centrifuge for 10 min at 8000g
A Resuspend
B Centrifuge for 10min at 8000g
S_{B_2} (Discard)
P_{B_2}
S_{C_1}
P_{C_1}
S_{A_2} (Discard)
P_{A_2}
A Resuspend
B Centrifuge for 10min at 8000g
Pool P_{C_1} + P_{B_2}
Pool P_{C_1} + P_{A_2}
A Resuspend
B Centrifuge for 10min at 8000g
S_{B_3} (Discard)
P_{B_3} Final Mitochondria Pellet
S_{A_3} (Discard)
P_{A_3} or $P_{A_3'}$ Final Mitochondria Pellet

The combined mince is washed with three 30 ml volumes of MSB medium, resuspended in approximately 10 ml of MSB per gram of myocardium, and 200 I.U. of collagenase is added. Homogenization was performed in a glass vessel with four to five up and down strokes of a motor driven Potter-Elvejhem (P/E) pestle set at 500 rpm with a clearance of 0.117 mm (caliper measurement). The homogenate is allowed to incubate on ice for 1 additional min before EGTA is added to a final concentration of 1 mM to halt collagenolysis and prevent mitochondrial uptake of Ca(II). The volume of the homogenate was measured and a sample (0.5 ml) for yield determination withdrawn.

The homogenate was centrifuged at 480g for 5 min in 15 ml Corex tubes with a Sorvall SS-34 rotor in order to sediment cellular debris. The supernatant liquid was filtered through cheesecloth and centrifuged at 8,000g for 10 min to sediment the mitochondria. The pelleted mitochondria were gently resuspended with a Pipetman (Gilson Medical Instruments, Middleton, WI) in MSB medium containing 1 mM EGTA (MSBE medium). The mitochondria were washed twice in 10 ml of MSBE by centrifuging at 8,000g for 10 min and resuspended in this medium at a final concentration of 15-20 mg/ml. The above protocol should take no more than 1 hr to complete. The resulting mitochondrial pellet is designated P_{A_3}.

If enhanced yields are needed the following protocol should be followed. For a 10-15% increase in yield, the pellet resulting from low-speed (480g) centrifugation is rehomogenized, centrifuged once again at 480g, and then subjected to high speed (8,000g) centrifugation. The resulting mitochondrial pellet is washed once and pooled with the first pellet. This mitochondrial suspension corresponds to P_{A_3},. For maximal

yield, the crude homogenate is allowed to incubate on ice for 3 min in the presence of collagenase, made 1 mM in EGTA, further homogenized with two strokes of the P/E pestle, and centrifuged at 480g for 5 min. The mitochondria in the supernatant liquid are pelleted and washed and then pooled with mitochondria recovered from the crude pelleted material. The combined pellet ($^{P}B_{3}$) is washed and resuspended as above in MSBE medium.

VI. OXYGEN CONSUMPTION ASSAYS

Mitochondrial coupling parameters were assessed at 30.5°C with a Gilson model K-IC oxygen polarograph (Gilson Medical Electronics, Middleton, WI), equipped with a Yellow Springs Instruments (Yellow Springs, OH) Clark oxygen electrode (Model 5331) and a water-jacketed, 1.75 ml glass reaction chamber. Constant temperature was maintained with a Haake (Saddle Springs, NJ) circulating water bath. Mitochondrial respiration experiments were conducted in an assay medium containing 0.225 M mannitol, 0.075 M sucrose, and 20 mM Tris-P_i, pH 7.4. State 3 respiration was induced with approximately 400 nmoles of ADP after a 1 min incubation with oxidizable substrate. Respiratory control ratios were calculated as previously described (Chance and Williams, 1956; Estabrook, 1967). Mitochondria and all reaction components were added to the assay medium with Hamilton glass syringes (Hamilton Co., Reno, NE). Oxygen back diffusion into the assay medium at 30.5°C was estimated across a range of oxygen saturation levels. Oxygen was displaced by bubbling the assay medium with argon gas.

RESULTS

I. MITOCHONDRIA RECOVERIES

The percent recoveries of succinate:INT reductase for the indicated number of preparations are shown in Table I. Compared to control samples, the yield of heart mitochondria is enhanced nearly two-fold when isolated in the presence of collagenase. Yields are enhanced an additional 10-15% when the crude pelleted cell debris are rehomogenized and the remaining mitochondria are recovered. Subjecting the crude homogenate to further mechanical disruption after it has been incubated with collagenase for 3 min and recovering mitochondria lost in the crude pelleted material gives yields for $^{P}B_{3}$ of 70 ± 8%. Importantly, the mitochondria in pellets $^{P}A_{3}$ and $^{P}B_{3}$ have comparable respiratory parameters (data not shown).

II. OUTER MEMBRANE ENZYMES

Chick heart mitochondria isolated in the presence of collagenase retain the activity of outer membrane enzymes. Considerable long chain fatty acyl CoA synthetase activity remains associated with mitochondria (Fig 3A). Adding L(-)carnitine to mitochondria incubated with CoA, ATP, and palmitic acid stimulates state 3 respiration, indicating that free palmitic acid is converted to its CoA thioester and is available for transport by carnitine palmitoyl-transferase I into the mitochondrial matrix for β-oxidation. The ADP necessary to stimulate state 3 respiration is formed by the adenylate kinase-catalyzed transphosphorylation of

TABLE I.

YIELD OF HEART MITOCHONDRIA ISOLATED WITH OR WITHOUT COLLAGENASE.

Chicken heart mitochondria were isolated with collagenase as described under "Experimental Procedures." The designations for mitochondrial pellets correspond to those described in the text. Yields were determined from the percent recovery of succinate INT reductase from the crude homogenate. The values are the mean ± S.D. of the indicated number of preparations.

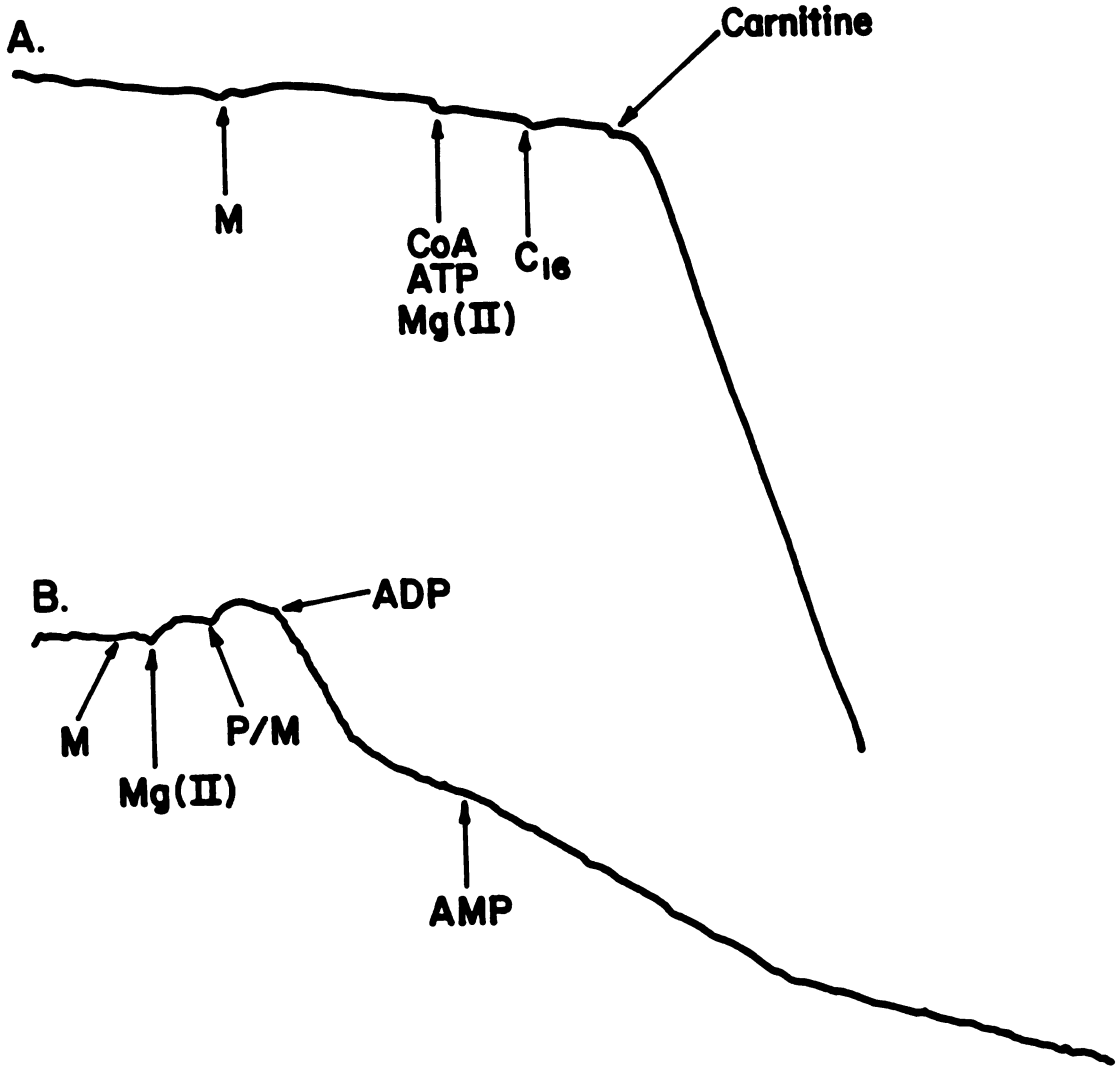
Mitochondria Pellet	Collagenase	Yield	No. Preps
		%	
P_{A3}	-	17 ± 4	7
P_{A3}	+	31 ± 8	29
$P_{A3'}$	+	41 ± 3	5
P_{B3}	+	70 ± 8	4

Figure 3. Oxygen Electrode Tracings used for Monitoring Enzyme Activities

(A) Activity of long chain fatty acyl CoA synthetase associated with chick heart mitochondria isolated with collagenase. Assay conditions were as described under "Experimental Procedures." The rate of state 3 respiration subsequent to the addition of 2 mM L-carnitine was 235 ng-atom O min^{-1} (nmol cyt aa_3)$^{-1}$.

(B) Activity of adenylate kinase. 0.069 nmol cyt aa_3 $(ml)^{-1}$ were assayed for respiration in 0.225 M mannitol, 0.075 M sucrose, 5 mM Mg(II), and 10 mM Tris-buffered P_i, pH 7.4, at 30.5 °C. After the phosphorylation of 400 nmol ADP, 200 nmol of AMP was added. The rate of state 3^{ADP} was 678 ng-atom O min^{-1} (nmol cyt aa_3)$^{-1}$ and that for state $3^{ATP+AMP}$ was 239 ng-atom O min^{-1} (nmol cyt aa_3)$^{-1}$. The total oxygen consumed was 127 and 146 ng-atom O following the addition of ADP and AMP, respectively. Oxygen consumption is approximately consistent with the reaction

$$AMP + ATP \longrightarrow 2ADP.$$


A.
Carnitine
M
CoA
ATP
Mg(II)
C16
B.
ADP
M
Mg(II)
P/M
AMP

AMP, which is produced during the fatty acid activation reaction. That these mitochondria contain the adenylate kinase necessary to produce ADP is demonstrated in Fig 3B. The intactness of outer membrane enzymes is further supported by the observation that over 50% of the hexokinase, whose reversible binding to the mitochondrial outer membrane is metabolically regulated (Wilson, 1980), remains with the mitochondrial fraction after exposure to collagenase (Table II). The I.U. of hexokinase per mg of mitochondrial protein is in good agreement with studies on rabbit heart mitochondria (Aubert-Foucher et al., 1984).

III. MEMBRANE INTACTNESS

Heart mitochondria isolated in the presence of collagenase have intact inner and outer membranes as evidenced by the inability of exogenous 1 mM NADH to support the phosphorylation of ADP and by the lack of respiratory stimulation in the presence of 2 mM reduced cytochrome c (data not shown). Scanning and transmission electron microscopy also show intact mitochondrial membranes (Fig 4 A and C). As shown in Fig 4C, chick heart mitochondria are comprised of a highly convoluted matrix and have easily distinguishable inner and outer membranes separated by an inter-membrane space. Approximately 3-3.5 hrs after the isolated mitochondria are suspended, the membranes become perforated (Fig 4B) and begin to extrude matrix material (Fig 4D). The disintegration of mitochondrial membranes coincides temporally with progressive increases in state 4 rates of respiration and decreases in state 3 rates (data not shown). The cause of this structural disintegration was not investigated, although the activation of proteases endogenous to the mitochondria is one possibility (Dean, 1983). Most preparations of chick

TABLE II.

RECOVERY OF HEXOKINASE IN HEART MITOCHONDRIA ISOLATED WITH COLLAGENASE.

Heart mitochondria isolated in the presence of collagenase were washed five times and assayed for hexokinase activity as described under "Experimental Procedures."

Trial	Crude Homogenate	Mitochondria	Recovery	Specific Activity Mitochondrial Hexokinase
	units[a]	units[b]	%	units mg^{-1}
1	11.1	6.0	54.1	0.092
2	8.6	5.0	58.1	0.10

[a] Indicates total units in the crude homogenate of a preparation.

[b] The units of hexokinase activity in the mitochondrial fraction were corrected for the yield of mitochondria.

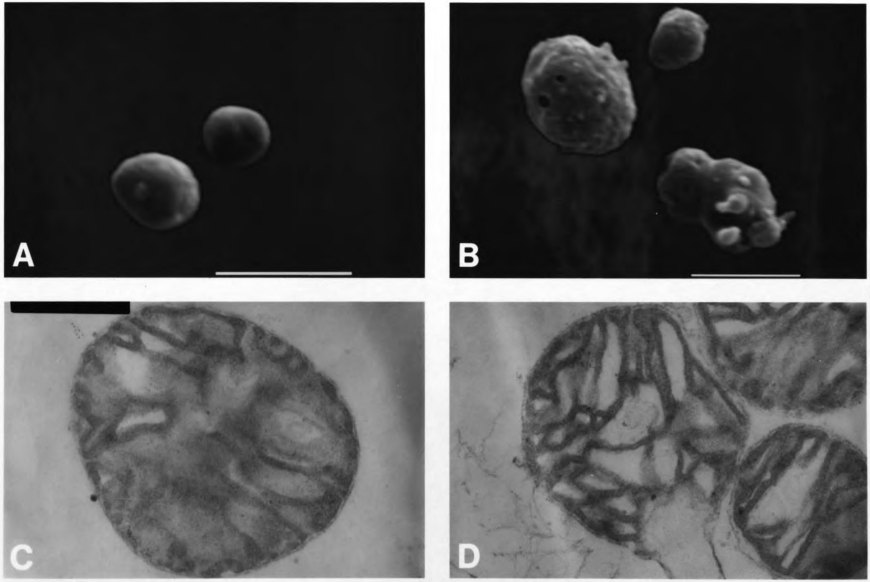
Figure 4. Representative Scanning and Transmission Electron Micrographs of Chick Heart Mitochondria Isolated with Collagenase

(A) Scanning electron micrograph (24,000x) of mitochondria at the time of suspending the final pellet in M/S medium. Note smooth, undisrupted membrane surfaces. The bar denotes 1 micron.

(B) Scanning electron micrograph (24,000x) of mitochondria 5 hrs after isolation. Perforated membranes are a common feature of the mitochondria at this time. The bar denotes 1 micron.

(C) Transmission electron micrograph (99,360x) of mitochondria immediately after suspending the final pellet. Mitochondria present with smooth inner and outer membranes, an intermembrane space, and a highly convoluted matrix.

(D) Transmission electron micrograph (54,000x) of mitochondria 5 hrs after isolation. Broken membranes and matrix extrusion are widespread at this time.


A
B
C
D

heart mitochondria maintain optimal RCR and ADP:O ratios for 3-3.5 hrs.

IV. RESPIRATORY CONTROL RATIOS

The respiratory control ratio (RCR) of chick heart mitochondria is strikingly dependent on the concentration of cyt aa_3 (Fig 5). The basis for this behavior is discussed in detail in Chapter II. For all preparations tested there is a narrow range of mitochondria concentration (0.050 ± 0.005 nmoles cyt aa_3/ml) where respiratory control is highest. Figure 6 is an example of an oxygen electrode tracing obtained at this concentration of mitochondria. ADP_1, ADP_2, and ADP_3 designate the first, second, and third addition of 400 nmoles of ADP, respectively. These mitochondria typically have a significant state 4 rate of respiration after phosphorylating ADP_1. However, after phosphorylating ADP_2 and ADP_3, state 4 rates approach and even attain zero. This demonstrates that chick heart mitochondria can exert extraordinary control over the rate at which electrons are transferred from the respiratory chain to oxygen in the absence of ADP. In keeping with the definition of RCR values (i.e., the ratio of the rates of state 3 and state 4 respiration), a zero rate of state 4 implies that these mitochondria, when optimized for concentration, can respire with infinite respiratory control.

V. BACK DIFFUSION OF OXYGEN

Because state 4 rates frequently approach zero and near-zero values, it was of interest to determine whether the diffusion of atmospheric oxygen into the respiration chamber could artifically depress state 4 rates. Oxygen back-diffusion measurements were made by recording the change in oxygen concentrations as a function of time at different

Figure 5. Respiratory Control Ratios Measured as a Function of Mitochondria Concentration

Increasing concentrations of mitochondria (expressed as nmoles cyt aa_3/1.75 ml) were assayed for respiratory control ratios using a Gilson oxygen polarograph as described under "Experimental Procedures." RCR_1 and RCR_2 values were calculated from the rates of state 3 and state 4 respiration following two sequential additions of 400 nmoles of ADP. Mitochondria oxidized 5 mM pyruvate/2.5 mM malate in the presence of 20 mM Tris-P_i, pH 7.4, at 30.5°C.

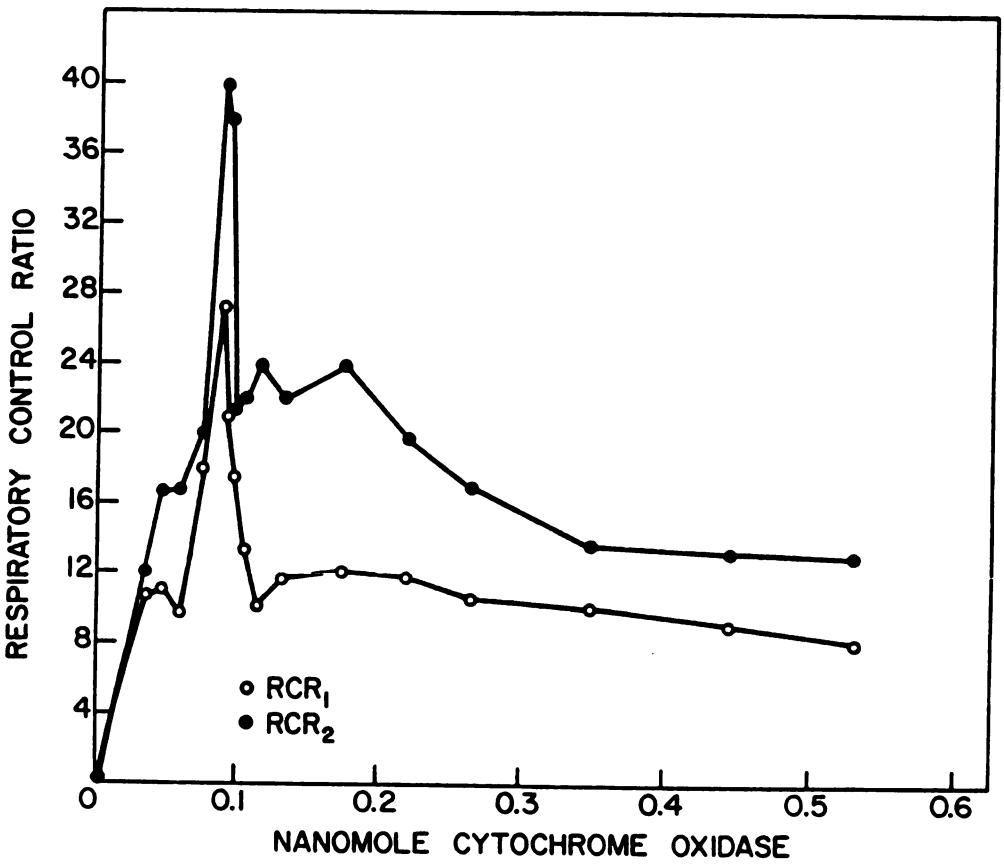
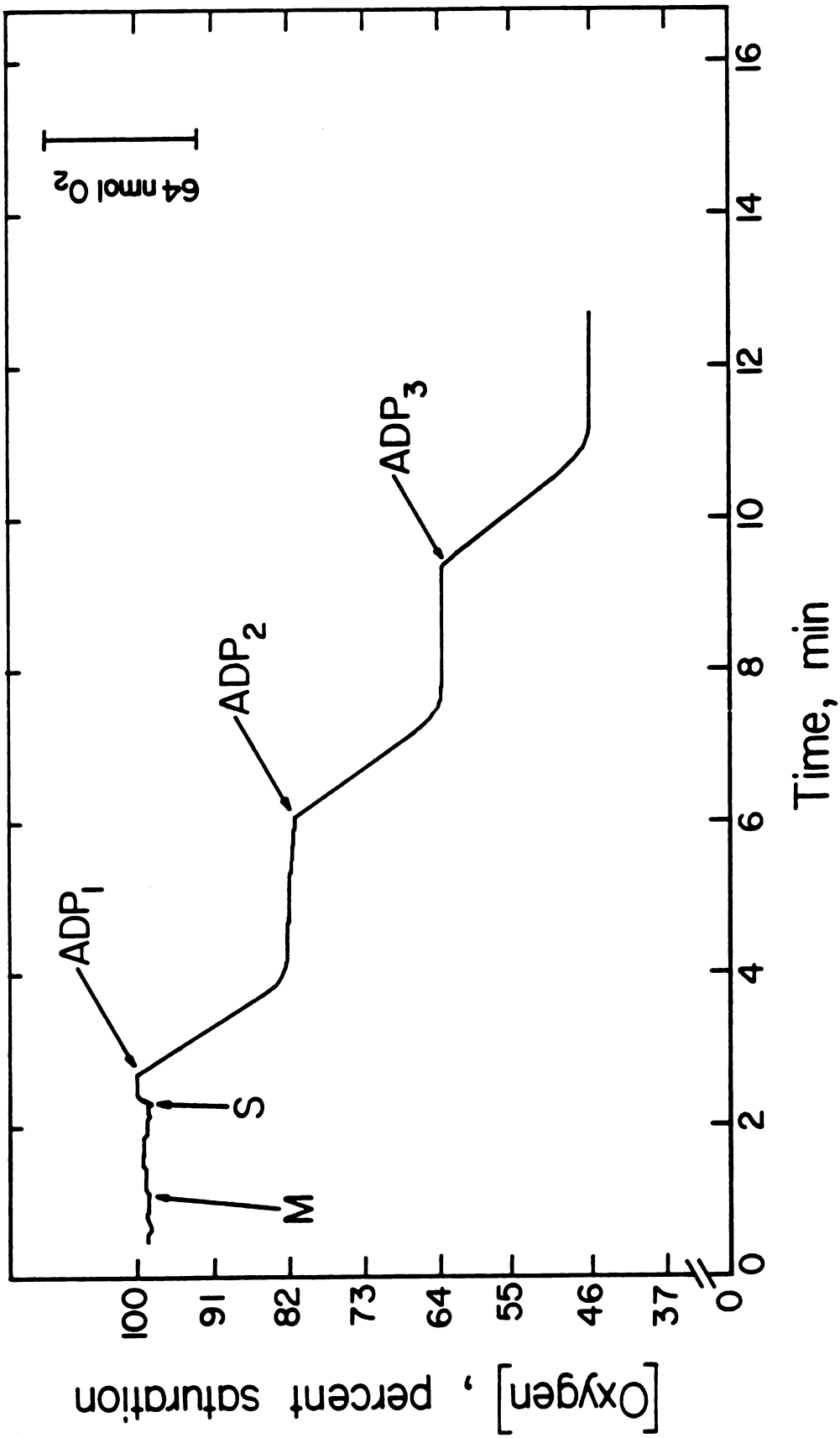

RESPIRATORY CONTROL RATIO
40
36
32
28
24
20
16
12
8
4
0
0.1
0.2
0.3
0.4
0.5
0.6
NANOMOLE CYTOCHROME OXIDASE
RCR1
RCR2

Figure 6. Oxygen Consumption Behavior of Chick Heart Mitochondria Isolated with Collagenase

Three state 3 to state 4 transitions at the critical mitochondrial concentration are shown. M, addition of mitochondria at 0.050 nmol cyt aa_3 ml^{-1} to M/S medium containing 20 mM Tris-buffered P_i, pH 7.4, at 30.5°C; P/M, addition of 5 mM pyruvate/2.5 mM malate; ADP_{1-3}, sequential additions of 400 nmoles of ADP.


[Oxygen], percent saturation
100
91
82
73
64
55
46
37
0
0
2
4
6
8
10
12
14
16
Time, min
ADP1
ADP2
ADP3
S
M
64 nmol O2

concentrations of oxygen. The rate of change of oxygen concentration in the respiration chamber depends on the oxygen concentration in the oxygen polarograph's assay vessel (Fig 7). At 40% saturation there is no change in oxygen concentration. Above 40% saturation there is a net decrease of oxygen presumably because the Clark electrode reduces oxygen to water at a rate that is more rapid than that at which oxygen diffuses back into the respiration chamber. Below 40% saturation diffusion into the respiration chamber is more rapid than uptake by the electrode. Since the changes in respiration induced by ADP_{1-3} were monitored at oxygen concentrations above 40% saturation, it is concluded that the rates of state 4 respiration reported herein are not artificially low. No corrections in rates of respiration were made for oxygen consumption by the Clark electrode.

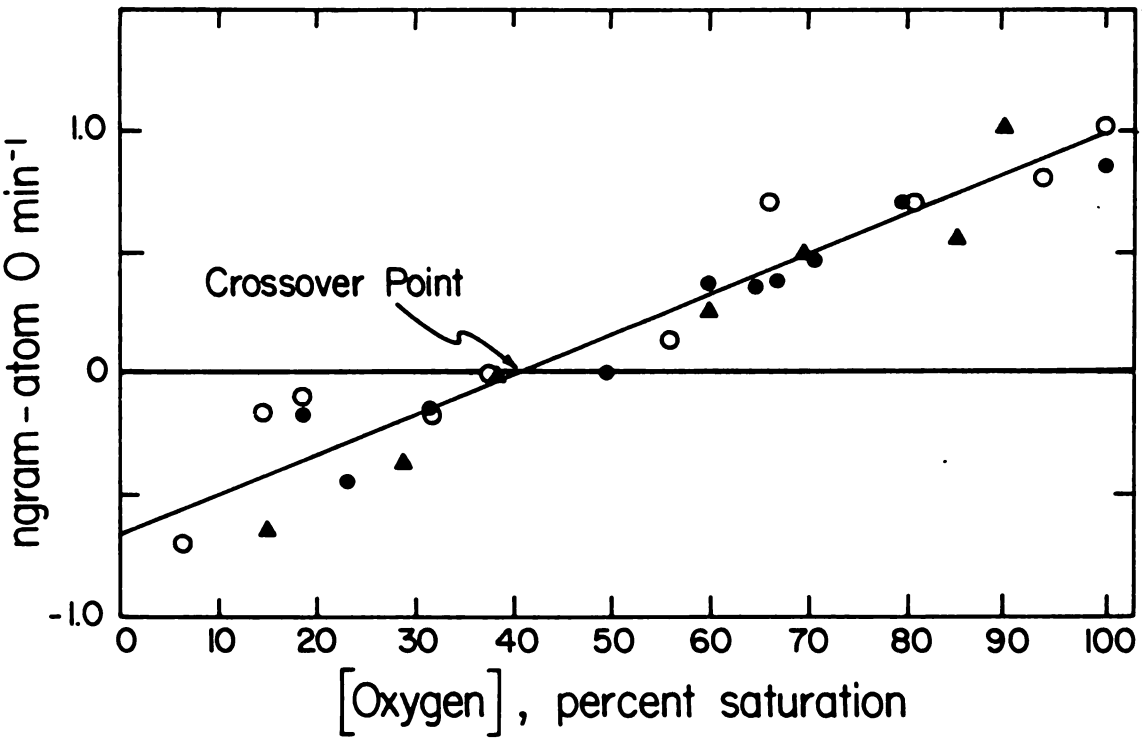
VI. GENERAL COMMENTS

At the concentration of mitochondria where optimum respiratory control is observed, a zero or very low rate of state 4 respiration subsequent to ADP_2 is observed between 22 and 33°C. Above 33°C, the rate of state 4 respiration increases as a function of temperature.

The respiratory control parameters of heart mitochondria isolated according to this method are extremely sensitive to micromolar concentrations of Mg(II), Mn(II), and Ca(II). The rate of state 4 respiration is increased and ADP:O ratios are decreased by these cations as a function of concentration. Mn(II) and Ca(II) also decrease the rate of state 3 respiration, indicating that chick heart mitochondria undergo some degree of damage subsequent to exposure to these cations. Consequently, these divalent cations should not be constituents of the assay medium if

Figure 7. Quantitation of Oxygen Consumption by the Clarke Electrode and Back Diffusion of Atmospheric Oxygen into the Oxygraph Assay Vessel

1.75 ml samples of M/S medium containing 20 mM Tris-buffered P_i were degassed to varying oxygen tensions by bubbling with argon gas. Oxygen consumption and back diffusion were measured using either a Yellow Springs "standard" (●,○) or "high sensitivity" (▲) Teflon membrane stretched very tightly over the Clark electrode. Samples were allowed to run for 20-30 min so as to enhance the accuracy of estimated rates. The line drawn through the data was calculated by least squares linear regression analysis.


1.0
0
-1.0
ngram - atom O min⁻¹
Crossover Point
0
10
20
30
40
50
60
70
80
90
100
[Oxygen], percent saturation

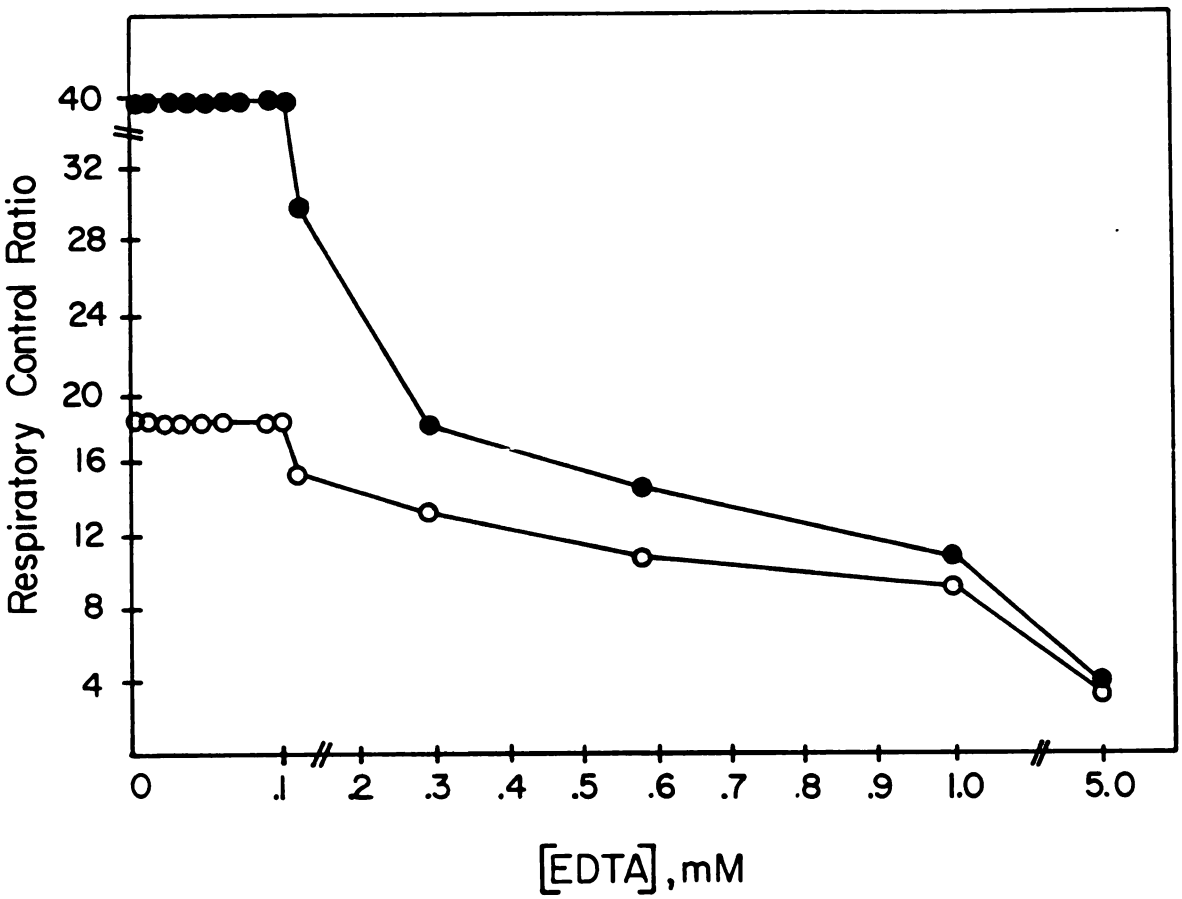
optimal respiratory parameters are desired. Concentrations of EDTA in excess of 100 μM decrease RCR values (Fig 8). This chelating agent stimulates state 4 respiration but has no effect on state 3 respiration. In contrast, EGTA concentrations up to 5 mM stimulate neither state 3 nor state 4 rates of respiration. However, as to be discussed more fully in Chapter 4, EGTA can be used to decrease state 4 rates of respiration by chelating divalent cations released from the mitochondria. This prevents the regeneration of ADP from ATP by divalent cation sensitive ATPases which cosediment with the mitochondria. The age of the chicks from which mitochondria are isolated exerts a critical influence over the magnitude of respiratory control that one may expect to observe. RCR values greater than 50 are only found with mitochondria isolated from chicks less than 20 days old (ex ovo). The RCRs of heart mitochondria from older chicks progressively decrease as a function of age.

All solutions must be kept rigorously free of microbial contamination. For optimum respiratory control, mannitol/sucrose solutions should be freshly made for each mitochondria preparation and stock solutions of P_i and substrate should be made every 7-10 days. Of all the respiratory substrates studied (5 mM pyruvate/2.5 mM malate, 5 mM glutamate/2.5 mM malate, 5 mM α-ketoglutarate/2.5 mM malate, 5 mM β-hydroxybutyrate, 5 mM succinate/5μM rotenone), the combination of pyruvate and malate support the highest state 3 rates and the lowest state 4 rates when the concentration of P_i is held fixed at 20 mM.

Collagenase can also be used to facilitate the isolation of highly coupled heart mitochondria from 14-20 day old Sprague-Dawley rats. For optimal respiratory control, 0.12 M KCl should be used as the osmotic support medium in both the isolation and assay media.

Figure 8. Chick Heart Mitochondrial Respiratory Control Measured as a Function of EDTA Concentration

Mitochondria were suspended at 0.074 nmoles cyt aa_3/ml in an osmotic support medium comprised of 0.225 M mannitol, 0.075 M sucrose, and 10 mM Tris-P_i, pH 7.4. EDTA was added to the indicated concentrations, and respiratory control ratios were measured. (○), RCR_1; (●), RCR_2.


Respiratory Control Ratio
40
32
28
24
20
16
12
8
4
0
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
5.0
[EDTA], mM

VII. CONCLUSIONS

The results of this investigation indicate that collagenase facilitates the release of mitochondria from myocardium. The resulting mitochondria are highly coupled, have enzymatically intact outer membranes, morphologically intact inner and outer membranes, and can be isolated in high yield.

REFERENCES

Aubert-Foucher, A., Font, B., and Gautheron, D.C. (1984) Arch. Biochem. Biophys. 232: 391-399.

Bishop, P.D., and Atkinson, D.E. (1984) Arch. Biochem. Biophys. 230: 335-344.

Bond, M.D., and Van Wart, H.E. (1984) Biochemistry 23: 3085-3091.

Caulfield, J.B., and Borg, T.K. (1979) Lab. Invest. 40: 364.

Chance, B., and Hagihara, B. (1963) Proc. Int. Congr. Biochem. 5: 3-37.

Chance, B., and Williams, G.R. (1956) Adv. Enzymol. 17: 65-134.

Chou, A.C., and Wilson, J.E. (1972) Arch. Biochem. Biophys. 151: 48-55.

Dean, B. (1983) Arch. Biochem. Biophys. 227: 154-163.

Ernster, L., and Schatz, G. (1981) J. Cell Biol. 91: 227s-255s.

Estabrook, R.W. (1967) Methods Enzymol. 10: 41-47.

Groen, A.K., Wanders, R.J.A., Westerhoff, H.V., van der Meer, R., and Tager, J.M. (1982) J. Biol. Chem. 257: 2754-2757.

Hay, E.D. (1981) J. Cell Biol. 91: 205s-223s.

Lehninger, A.L., Carafoli, E., and Rossi, C.S. (1967) Adv. Enzymol. 29: 259-320.

Lehninger, A.L. Biochem. J. 119: 129-138.

Lemasters, J.J., and Hackenbrock, C.B. (1980) J. Biol. Chem. 255: 5674-5680.

Markwell, M.A.K., Haas, S.M., Tolbert, N.E., and Bieber, L.L. (1981) Methods Enzymol 72: 296-303.

Masini, A., Ceccarelli-Stanzani, D., Muscatello, U. (1983a) FEBS Lett. 160: 137-143.

Masini, A., Ceccarelli-Stanzani, D., Muscatello, U. (1983b) Biochim. Biophys. Acta 724: 251-257.

Masini, A., Ceccarelli-Stanzani, D., Muscatello, U. (1984) Biochim. Biophys. Acta 767: 130-137.

Mela, L., and Seitz, S. (1979) Methods Enzymol. 55: 39-46.

Nedergaard, J., and Cannon, B. (1979) Methods Enzymol. 55: 5-28.

Nicholls, D. (1974) Eur. J. Biochem. 50: 305-315.

Palmer, J.W., Tandler, B., and Hoppel, C.H. (1977) J. Biol. Chem. 252: 8731-8739.

Pande, S.V., and Blanchaer, M.C. (1970) Biochim. Biophys. Acta 202: 43-48.

Pennington, R.J. (1961) Biochem. J. 80: 649-654.

Polakis, P.G., and Wilson, J.E. (1982) Biochem. Biophys. Res. Comm. 107: 937-943.

Spector, A.A., Fletcher, J.E., and Ashbrook, J.D. (1971) Biochemistry 10: 3229-3232.

Stadhouders, A.M. (1981) In Mitochondria and Muscular Diseases. (H.F.M. Busch, F.G.I. Jennekens, and H.R. Scholte, eds), p.77. Mefar b.v. Beetswerg, The Netherlands.

Stucki, J.W., and Ineichen, E.A. (1974) Eur. J. Biochem. 48: 365-375.

Stucki, J.W. (1976) Eur. J. Biochem. 68: 551- 562.

Thompson, D.A., and Ferguson-Miller, S.M. (1983) Biochemistry 22: 3178-3187.

von Jagow, G., and Klingenberg, M. (1972) FEBS Lett. 24: 278-282.

Van den Bergh, S.G. (1967) Methods Enzymol. 10: 749-755.

Wilson, J.E. (1980) Curr. Top. Cell. Regul. 16: 2-54.

Woldegiorgis, G., Yousufzai, S.Y.K., and Shrago, E. (1982) J. Biol. Chem. 257: 14,783-14,787.

Chapter 3

STUDIES OF THE FACTORS AFFECTING THE RESPIRATORY CONTROL AND ADP:O COUPLING RATIOS OF ISOLATED CHICK HEART MITOCHONDRIA

INTRODUCTION

Mitochondrial substrate oxidation, electron transfer, and ATP synthesis together comprise an integrated metabolic process designed to sensitively maintain cellular energy balance. The overall reaction of oxidative phosphorylation may be summarized with the following equation:

$$nADP + nP_i + NADH\ (FADH_2) + H^+ + 1/2O_2 \rightarrow nATP + NAD^+\ (FAD^+) + H_2O. \quad (1)$$

In general, it is believed that mitochondria couple substrate oxidation to the phosphorylation of ADP through an electrochemical potential gradient of protons (Mitchell, 1979). Protons are electrogenically ejected from the mitochondrial matrix into the intermembrane space by redox pumps located at the three coupling sites of the respiratory chain: NADH-ubiquinone oxidoreductase (site I), ubiquinol-cytochrome c oxidoreductase (site II), and cytochrome c oxidase (site III). The loss of energy from proton extrusion is coupled to a decrease in the oxidation-reduction potential of electrons as they pass through each of the coupling sites (Chance, 1977; Wilson, 1980).

How mitochondria regulate the rate of respiration is the subject of much study. The classic investigations of Chance and Williams (1956) show that ADP exerts predominant control over the extent to which respiration is stimulated in isolated mitochondria. More recent studies, however, have demonstrated that the control of respiration depends upon a number of other components of the mitochondrial matrix. Evidence shows that rates of respiration are modulated by changes in the activity of

various substrate dehydrogenases (Denton and McCormack, 1985; Hansford and Castro, 1981), electron transfer chain components (Forman and Wilson, 1982; Erecinska and Wilson, 1982; Chance, 1972), and inner mitochondrial membrane transport proteins (Lemasters and Sowers, 1979; Klingenberg, 1980; Doussiere et al., 1984; Groen et al., 1982). The widespread distribution of control insures that respiration is poised to rapidly respond to changes in metabolite transport, thermodynamic potentials, and dehydrogenase activities.

Respiratory control is a measure of the mitochondrion's capacity to limit the rate of electron transport in both the presence and absence of the phosphoryl group acceptor ADP. The ratio of the rate of oxidation during ATP synthesis (state 3) to that during "resting" (state 4) respiration is the respiratory control ratio (RCR; Chance and Williams, 1956). RCR values are regarded as an important index of mitochondrial function in vitro and it is known that mitochondria demonstrate respiratory control in vivo (Hassinen and Hiltunen, 1975; Wilson et al., 1974). Differences in RCR values among heart mitochondria preparations arise largely from differences in state 4 respiration which, from inspection of the literature, may be quite substantial. A variable percentage of the respiration during state 4 arises from residual ATP synthesis (Lemasters and Hackenbrock, 1980; Masini et al., 1983). The remaining respiration is due to the oxidation of pyridine and flavin nucleotides in the absence of phosphorylation (i.e., the respiration is uncoupled). This uncoupled respiration is accounted for in chemiosmotic terms by two mechanisms. The first assumes that protons are able to permeate the mitochondrial inner membrane via non-specific leak pathways. This constitutes a non-ohmic conductance of protons (Nicholls,

1974; Pietrobon et al., 1983). The second proposes that the redox pumps and the F_0 moiety of the ATP synthase are intrinsically uncoupled (Pietrobon and Caplan, 1985; Zoratti et al., 1986; Pietrobon et al., 1986). These proteins are believed to "slip", whereby the redox pump may transfer electrons without ejecting a proton and the F_0 protein may conduct protons without concomitantly inducing the release of ATP from F_1. The depletion of endogenous substrate in rabbit heart mitochondria decreases respiratory control (Tarjan and von Korff, 1967), while ATP loading in human placental mitochondria increases respiratory control (Illsley et al., 1985). In both cases, changes in rates of state 3 respiration accounted for changes in RCR values; state 4 rates were unaffected. Apart from complete inhibition of the ATP synthase (Masini et al., 1983, 1984) or the addition of an enzyme that traps ADP generated from ATP in the extramitochondrial space (Bishop and Atkinson, 1984), few means for decreasing state 4 rates of respiration have been elucidated.

The efficiency with which mitochondria transduce redox free energy into the free energy of hydrolysis of ATP is assessed from the ADP:O ratio (corresponding to n of equation 1). For many years it was believed that one ADP molecule is phosphorylated at each of the coupling sites per pair of electrons oxidized (Ernster, 1977). Such a stoichiometry would yield whole number ADP:O ratios of 3.0 for mitochondria oxidizing NAD-linked substrates. However, because currently debated models of chemiosmosis (Mitchell, 1979; Westerhoff et al., 1984) regard the proton as the intermediate between oxidation and phosphorylation, fractional ADP:O ratios are also acceptable stoichiometries. A careful reexamination of oxidative phosphorylation in rat liver mitochondria by Lemasters

et al. (1984) showed that ATP/site stoichiometries are fractional and can yield an ideal upper limit for the ADP:O ratio of 3.37 when β-hydroxybutyrate is used as substrate. This stoichiometry is in sharp contrast to those reported by others (2.0, Hinkle and Yu, 1979; 2.75, Beavis and Lehninger, 1986; reviews: Lemasters et al., 1984; Flatt et al., 1984; Ferguson, 1985; Beavis, 1988). Clearly, although ADP:O ratios likely vary as a function of the tissue source and the actual degree to which the mitochondria are coupled, there is significant disagreement as to what the values can and should equal in isolated mitochondria phosphorylating nmole pulses of ADP.

The focus of the present investigation was to characterize the factors affecting the respiratory parameters of isolated chick heart mitochondria. Because of so many other such studies performed in the past, such an undertaking may appear mundane. However, this study is of particular interest for two reasons. First, these mitochondria demonstrate an extraordinary capacity to control the rate of respiration in the absence of ADP. Practical consequences of such exquisite coupling are that these mitochondria have RCR values approaching infinity and ADP:O stoichiometries that equal the theoretical limits proposed by Lemasters et al. (1984). Second, a number of factors which regulate the rate of state 4 respiration are identified and characterized. The control of these factors makes it possible to systematically vary the respiratory parameters of chick heart mitochondria to fit the needs of particular types of experiments. Preliminary accounts of this work have been presented (Toth et al., 1985, 1986, and 1988).

EXPERIMENTAL PROCEDURES

I. MATERIALS

The water used for these experiments was purified as previously described (Toth et al., 1986). Mitochondria were isolated from the hearts of 14-21 day old chicks with collagenase according to the method of Toth et al. (1986). Corn mitochondria were isolated by the method of Day and Hanson (1977). Antisera and Arsenazo III were kindly provided by Dr. John Wang (Michigan State University) and Dr. Antonio Scarpa (Case Western Reserve University), respectively. Lactate dehydrogenase (type II), pyruvate kinase (type III), and collagenase (lots 27F-6824 and 47F-6829) were from Sigma (St. Louis, MO). All of the chemicals used were reagent grade or better and used without further purification.

II. POLYACRYLAMIDE GEL ELECTROPHORESIS

SDS-polyacrylamide gel electrophoresis was performed using the discontinuous buffer system of Laemmli (1970) in slab gels (16 cm x 18 cm x 0.75 mm). The resolving gel was 8% acrylamide/0.21% bisacrylamide, and was polymerized by adding 0.05% (w/v) ammonium persulfate and 0.071 M TEMED. The stacking gel (1 cm) contained 3% acrylamide/0.08% bisacrylamide. This gel was polymerized with 0.05% (w/v) ammonium persulfate and 0.014 M TEMED. Samples were solubilized in sample buffer (0.125 M Tris, pH 6.8, 4% $NaDodSo_4$ (w/v), 20% glycerol (v/v), 0.1 M DL-dithiothreitol, and 0.002% bromophenol blue (w/v)) and incubated in a boiling water bath for 5 min. Gels were run for 1 hr at 100 V, and then for 3.5 hrs at

300 V. The electrophoresis apparatus was cooled with tap water during the entire course of a run. Gels were fixed overnight in a fixative solution comprised of 50% methanol, 40% water, and 10% glacial acetic acid (v/v). Gels were stained for 3-4 hrs in fixative that contained 0.25% (w/v) Coomassie brilliant blue R250, and destained with two changes of a solvent that was 5% methanol, 7.5% glacial acetic acid, and 87.5% water (v/v).

Plots of log M_r *versus* R_F for the molecular weight standards used were non-linear. Data were fitted to the following relation (Peterson and Hokin, 1981):

$$\log R_F = \log a - m \log(1 + M_r/c). \qquad (2)$$

using the iterative least-squares linear regression methods specified by Bates and MaCallister (1974). Once the constants (a, c, and m) for the standard curve were calculated, the molecular weight of other bands were estimated from knowledge of their relative mobility. For the gel shown in Figure 15, a = 1.47, m = 2.96, and c = 121,461. In calculating the relationship between M_r and R_F, it was consistently found that BSA migrated anomalously. The error of curve fitting was reduced markedly by excluding BSA from the analysis. Consequently, this was routinely done.

III. IMMUNOBLOTTING

Actin and myosin were identified by immunoblotting. Samples were electrophoresed in $NaDodSO_4$-polyacrylamide gels as described above. Proteins were transferred from the gel and bound to nitrocellulose membranes as described by Towbin *et al.* (1979), for 1120 mA-hrs in transfer buffer (0.025 M Tris, 0.19 M glycine, pH 8.3, and 20% methanol

(v/v)). Nitro-cellulose membranes were then blocked in 20 mM Tris, pH 7.5, 0.5 M NaCl, 3% BSA for 6 hrs on a shaker platform. Excess BSA was removed by washing membranes twice in 20 mM Tris, pH 7.5, 500 mM NaCl, 0.05% Tween 20 (TTBS) for 10 min. Membranes were then incubated overnight with one of two antisera: (a) rabbit anti-calf thymus actin, or (b) rabbit anti-chicken gizzard myosin. Antisera were diluted 1:200 with 20 mM Tris, pH 7.5, 500 mM NaCl (TBS) prior to use. Excess antibody was removed by washing membranes twice for 10 min in TTBS. The membrane was incubated with a 1:3000 dilution of goat anti-rabbit IgG alkaline phosphatase conjugate for 1 hr (Bio Rad), and washed twice with TTBS and once with TBS. Blots were stained in 0.3 mg/ml nitro blue tetrazolium (NBT; Sigma, grade III), 0.15 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Sigma, p-toluidine salt), 0.1 M Tris, pH 9.5, 0.1 M NaCl, and 2 mM $MgCl_2$. Purified rabbit skeletal muscle actin and chick heart myosin heavy chain were used as positive controls.

IV. ISOLATION OF CHICK MYOCARDIAL MYOSIN

Myosin was isolated from homogenized chick ventricular myocardium using the method of Wikman-Coffelt et al. (1973). After ammonium sulfate precipitation (35-42% saturation), the myosin fraction was suspended in 50 mM Tris-HCl, pH 7.5, 1 mM DL-dithiothreitol, and 0.5 M KCl. The myosin was then dialyzed overnight against two changes of this buffer, made 50% (v/v) in glycerol, and stored at -20°C.

V. ATPase ASSAYS

(A). Myosin ATPase activity. 100 µl (0.36 or 0.4 mg/ml) of the myosin preparation was suspended in 0.9 ml of assay buffer (0.225 M mannitol,

0.075 M sucrose, 20 mM Tris-HCl, pH 7.4, varying concentrations of either Ca(II) or Mg(II), and 25 mM KCl). Reactions were initiated with the addition of 840 nmols of ATP and allowed to proceed for 8 min at 30.5°C. The kinetic parameters for the inhibition of myosin with Mg(II) were estimated by a least squares non-linear regression of the data fitted to the following equation assuming a constant absolute error:

$$v_0 = b - \frac{V_{max}[Mg(II)]}{K_{50} + [Mg(II)]} \qquad (3)$$

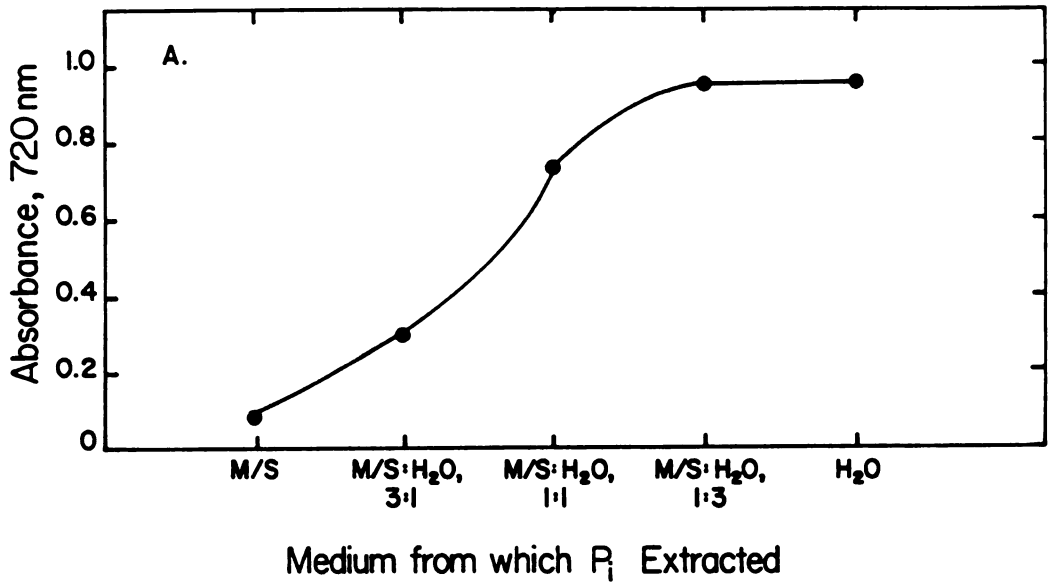
In equation 3, b represents the rate of ATP hydrolysis in the absence of Mg(II), and the other terms have their usual meaning. The amount of P_i released during the course of an incubation was assayed by the method of Pollard and Korn (1973), with one minor modification. Prior to denaturing protein, the assay mixture was diluted 3:1 with water. Diluting the assay 3:1 with water increased the efficiency of extracting the phosphomolybdate complex by 90% (Fig 1A). It is likely that dilution is necessary in order to decrease the concentration of mannitol, a compound known to have a high affinity for phosphomolybdate (Hagihara and Lardy, 1960). The amount of ATP hydrolyzed was estimated from the standard curve shown in Fig 1B.

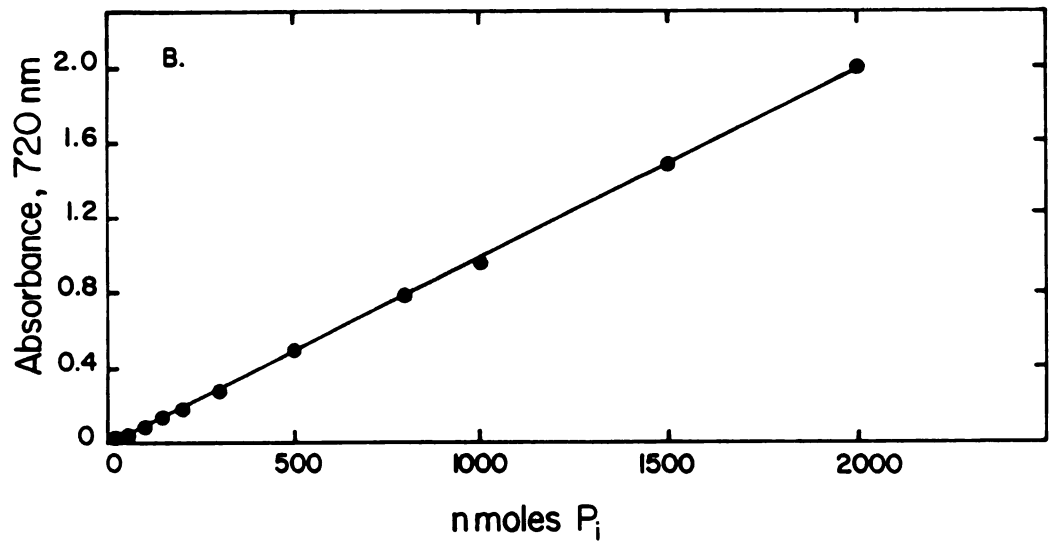
(B). Mitochondrial ATPase activity. The conditions for assaying mitochondrial ATPase activity are described in the legends to the pertinent Figures. The inorganic phosphate produced was assayed as described above. N-ethylmaleimide, an inhibitor of the P_i transport protein (Kaplan et al., 1986), was included in the assay medium to prevent mitochondria from internalizing P_i released during ATP hydrolysis. Electron flow was blocked with rotenone in order to inhibit the uptake

Figure 1. Extraction of Inorganic Phosphate from Suspensions of Chick Heart Mitochondria Hydrolyzing ATP

(A) Optimization of conditions for the extraction of P_i from suspensions of chick heart mitochondria. 940 nmols of P_i were added to the indicated solvents. The P_i was then extracted with an acidified isobutanol-benzene mixture. The relative amount of P_i in samples was determined by the method of Pollard and Korn (1973). M/S indicates 0.225 M mannitol and 0.075 M sucrose.

(B) Standard curve used for estimating the amounts of P_i released during ATPase assays. Calibrated amounts of P_i were added to M/S medium, diluted 3:1 with water, and then extracted and assayed as described in (A).


A.
Absorbance, 720 nm
1.0
0.8
0.6
0.4
0.2
0
M/S
M/S:H2O, 3:1
M/S:H2O, 1:1
M/S:H2O, 1:3
H2O
Medium from which Pi Extracted


B.
Absorbance, 720 nm
2.0
1.6
1.2
0.8
0.4
0
0
500
1000
1500
2000
n moles Pi

of Ca(II) and the phosphorylation of ADP.

VI. OXYGEN CONSUMPTION ASSAYS

Mitochondrial coupling parameters were assessed at 30.5°C with a Gilson model K-IC oxygen polarograph (Gilson Medical Electronics, Middleton, WI), equipped with a Yellow Springs Instruments (Yellow Springs, OH) 0.5 cm diameter Clark oxygen electrode (Model 5331) and a water-jacketed, 1.75 ml glass reaction chamber. Constant temperature was maintained with a Haake (Saddle Springs, NJ) circulating water bath. Reaction mixtures were stirred with a Teflon-coated metal bar (0.9 x 0.3 cm) and a Micro V (Cole Parmer) magnetic stirring device. The magnetic stir bar was operated at 600 rpm. Mitochondrial respiration experiments were conducted in an assay medium containing 0.225 M mannitol, 0.075 M sucrose, and 20 mM Tris-P_i, pH 7.4. State 3 respiration was induced with approximately 400 nmoles of ADP after a 1 min incubation with oxidizable substrate. State 4 respiration was typically allowed to proceed for 2 min after the completion of state 3 respiration. Respiratory control ratios were calculated as previously described (Chance and Williams, 1956; Estabrook, 1967). ADP:O ratios were calculated using "total" oxygen as described by Lemasters (1984).

The concentration of oxygen atoms in the air-saturated assay medium at 30.5°C was 405 µM as determined by measuring the oxygen consumption of corn (Zea mays, cultivar W64ATMS) mitochondria respiring in the presence of a known amount of NADH. Unlike mammalian mitochondria, the inner membrane of corn mitochondria is freely permeable to exogenous NADH (Miller et al., 1970; Douce, 1985). The concentration of oxygen was calculated by assuming that the added NADH was completely oxidized and

that one atom of oxygen was consumed for each molecule of NADH oxidized. Mitochondria and all reaction components were added to the assay medium across the capillary (5.7 x 0.2 cm) of a glass stopper with Hamilton glass syringes (Hamilton Co., Reno, NE).

VII. NUCLEOTIDE ASSAYS

ADP concentrations were assayed in a 1.0 ml reaction mixture containing 25 mM MOPS, pH 6.8, 5 mM magnesium acetate, 1 mM EDTA, 1.8 mM phosphoenolpyruvate, 0.32 mM NADH, 44 I.U. lactate dehydrogenase, and 12 I.U. pyruvate kinase. NADH concentrations were assayed in a 1.0 ml mixture containing 50 mM sodium acetate, pH 5.0, 3 mM pyruvate, and an excess of lactate dehydrogenase. For both assays, the oxidation of NADH was measured at 340 nm using a Beckman DU spectrophotometer. A molar extinction coefficient for NADH of $\Delta\varepsilon(340\ nm) = 6.23 \times 10^3\ M^{-1}\ cm^{-1}$ was used.

In order to ascertain whether the mitochondria described herein converted all exogenous ADP to ATP, both intra- and extra-mitochondrial ADP remaining after oxidative phosphorylation were assayed using the lactate dehydrogenase-pyruvate kinase coupled enzyme system noted above. Samples of mitochondria at the critical mitochondrial concentration (0.05 nmol cyt aa_3/ml) after phosphorylating three sequential additions of 400 nmoles of ADP using α-ketoglutarate as substrate were acidified with 0.5 ml of 2.0 N perchloric acid and allowed to incubate on ice for 10 min. Denatured samples were neutralized with 0.4 ml of 3.0 M sodium carbonate and centrifuged at 8,000g in an Eppendorf 3200 microcentrifuge for 10 min. The resulting supernatant liquid was assayed for ADP.

The capacity of pyruvate kinase to prevent Mg(II)-induced stimula-

tion of respiration during state 4 was assayed in a reaction mixture containing 50 mM KCl, 150 mM mannitol, 50 mM sucrose, 20 mM Tris-P_i, pH 7.4, 1 mM phosphoenolpyruvate, 1 mM Mg(II), and 5 mM pyruvate/2.5 mM malate. The inhibition of respiration during state 4 was monitored using an oxygen polarograph.

VIII. OTHER ASSAYS

Cytochrome oxidase was quantitated as previously described (Toth et al., 1986). The P_i contained in the assay medium used for oxygen consumption assays was determined by the method of Bencini et al. (1983), using potassium phosphate as the standard. Mitochondrial protein was determined by the modified Lowry method of Markwell et al. (1981), using BSA as the standard. For the experiments described herein mitochondria concentrations were expressed in terms of the amount of cyt aa_3 in oxygen consumption experiments and by protein in ATPase assays. The former was done in order to minimize the error in estimating respiratory parameters due to contaminating protein. ATPase activities were quantitated on a per mg protein basis because these enzymes are not associated with respiration per se.

IX. CALCULATIONS AND STATISTICAL ANALYSES

Hyperbolic Michaelis-Menten type kinetic data were analyzed for apparent V_{max} and K_m values using the computer program Wilman IV (Brooks and Suelter, 1986). The kinetic data fitted to equation II were analyzed using a nonlinear regression program provided by Dr. Stephen Brooks (Carleton University, Ottawa, Canada). Data were analyzed assuming a constant absolute error as specified by Wilkinson (1961), and were

assumed to fit the specified kinetic model if the residuals of points from the calculated regression line were randomly distributed (Mannervik, 1982). The least significant differences at the $p < 0.01$ and $p < 0.05$ levels between the respiratory parameters associated with successive additions of ADP were calculated by performing an analysis of variance using the F-test with software provided by Dr. Stanley Ries (Michigan State University).

RESULTS

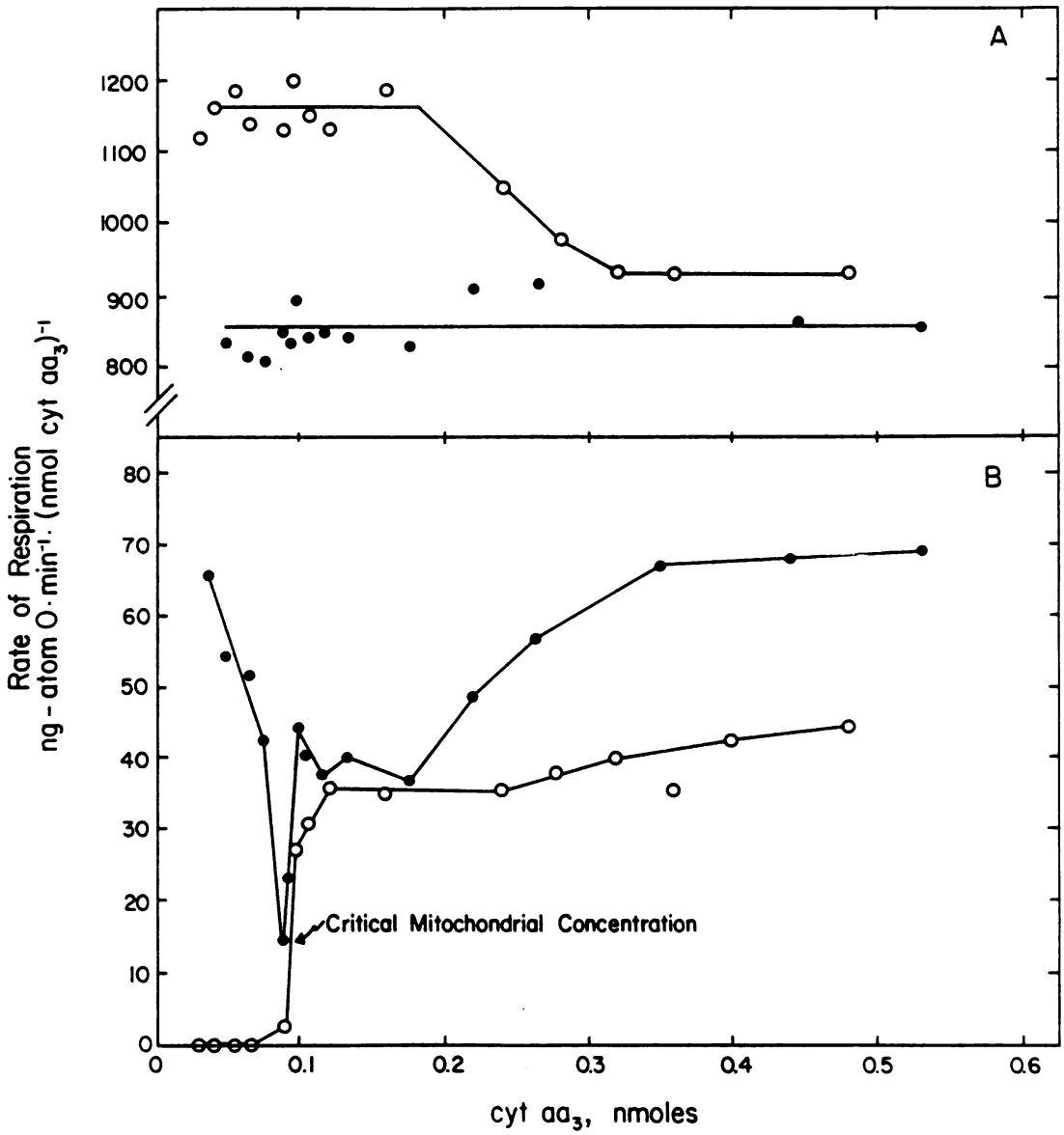
I. RATES OF RESPIRATION VARY WITH MITOCHONDRIA CONCENTRATION

Rates of mitochondrial oxygen consumption vary with the concentration of mitochondria in a complex manner (Fig 2). For the experiments shown in Fig 2, mitochondria oxidized 5 mM pyruvate and 2.5 mM malate. The state 3 rates of respiration for chick heart mitochondria isolated with collagenase are independent of mitochondria concentration. In contrast, state 4 rates initially decrease toward a minimum value [$\leq$15 ng-atom O min^{-1} (nmol cyt aa_3)$^{-1}$] and then increase toward a higher approximately constant value [70 ng-atom O min^{-1} (nmol cyt aa_3)$^{-1}$] as the concentration of mitochondria is increased. The amount of mitochondria necessary to minimize state 4 rates corresponds to 0.05 $\pm$ 0.005 nmoles of cyt aa_3/ml (n = 30). The highest RCR values for a given mitochondria preparation are obtained at this concentration of mitochondria (see Fig. 5 of Chapter 1). It will, therefore, be referred to as the "critical mitochondrial concentration."

The concentration dependence of state 4 respiration is striking. Insight into the basis for this relationship is provided by repeating these measurements with chick heart mitochondria isolated in the presence of the non-specific protease Nagarse. For mitochondria isolated by this method, state 4 respiration at or below the critical mitochondrial concentration (CMC) is completely abolished. In this concentration range the availability of ADP becomes rate-limiting for respiration by Nagarse treated mitochondria. As mitochondria concentrations are increased above the CMC, state 4 rates of respiration rapidly increase and reach a plateau.

Figure 2. Rates of State 3 and State 4 Respiration of Mitochondria Measured as a Function of Mitochondria Concentration

Rates of state 3 (A) and state 4 (B) respiration were measured for mitochondria isolated with either collagenase (●) or Nagarse (◯). Mitochondria were quantitated on the basis of cytochrome oxidase concentrations. All rates were obtained subsequent to the addition of ADP_2. The substrates for respiration were 5 mM pyruvate/2.5 mM malate and 20 mM Tris-buffered P_i, pH 7.4. Other assay conditions were as described under "Experimental Procedures."


A
B
1200
1100
1000
900
800
80
70
60
50
40
30
20
10
0
0
0.1
0.2
0.3
0.4
0.5
0.6
Critical Mitochondrial Concentration
Rate of Respiration
ng-atom O·min⁻¹·(nmol cyt aa₃)⁻¹
cyt aa₃, nmoles

Mitochondria isolated with Nagarse respire at significantly higher rates during state 3 respiration than mitochondria isolated with collagenase. At the CMC, Nagarse and collagenase treated mitochondria respire at 1144 ± 75 (n = 10) and 789 ± 93 (n = 30) ng-atom O min^{-1} (nmol cyt aa_3)$^{-1}$, respectively. Above the CMC, rates of state 3 respiration decrease toward values approximating those for collagenase mitochondria. These results suggest that Nagarse destroys a factor that stimulates state 4 respiration. In addition, limited Nagarse digestion appears to release some type of control over the V_{max} of oxidative phosphorylation.

II. ADP:O RATIOS ALSO VARY WITH MITOCHONDRIA CONCENTRATION

The empirical ADP:O ratios of chick heart mitochondria oxidizing pyridine nucleotide linked substrates also vary as a function of mitochondria concentration (Fig 3). ADP:O ratios are highest at or below the CMC. At the CMC, ADP:O ratios range from 3.2-3.5 when 5 mM pyruvate/2.5 mM malate (P/M), 5 mM β-hydroxybutyrate (βHB), or 5 mM α-ketoglutarate (αKG) are oxidized during the phosphorylation of a second addition of approximately 400 nmoles of ADP (ADP_2). As the concentration of mitochondria is increased, ADP:O ratios decrease to approximately 3.0. No difference is observed in the magnitude nor in the progressive fall in ADP:O ratios when mitochondria isolated with collagenase are compared with those isolated with Nagarse.

III. MAGNITUDE OF RESPIRATORY PARAMETERS AT THE CMC.

The respiratory parameters of chick heart mitochondria at the CMC oxidizing a variety of substrates are summarized in Table I. ADP:O ratios increase with each successive addition of ADP during the oxida-

Figure 3. Variation of ADP:O Ratios as a Function of Mitochondrial Concentration

The substrate(s) oxidized and the enzyme used to facilitate mitochondrial isolation were as follows:

(A) 5 mM pyruvate/2.5 mM malate, collagenase.

(B) 5 mM pyruvate/2.5 mM malate, Nagarse.

(C) 5 mM β-hydroxybutyrate, collagenase.

(D) 5 mM α-ketoglutarate, collagenase.

These ADP:O ratios were obtained after a second addition of 418 nmoles of ADP. The concentration of ADP and oxygen atoms in these assays was determined as described under "Experimental Procedures."

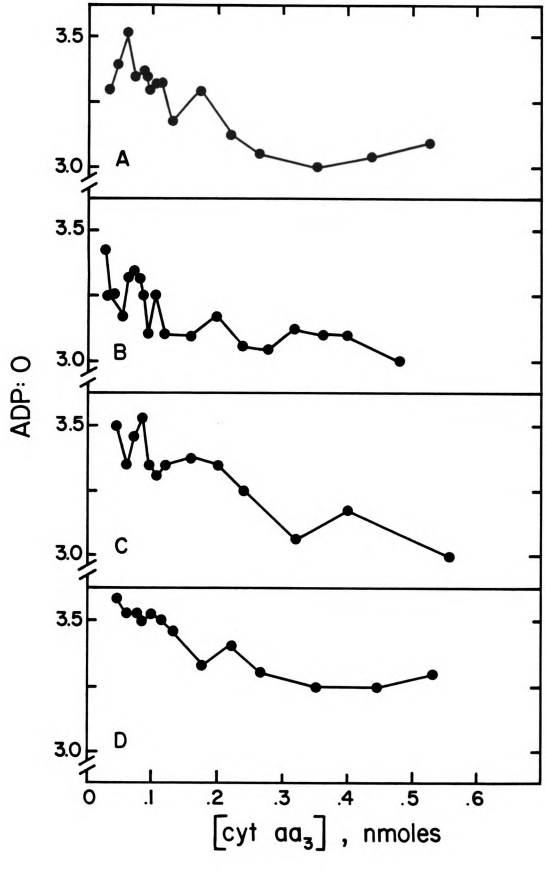

3.5
3.0
A
B
C
D
ADP: O
0
.1
.2
.3
.4
.5
.6
[cyt aa3] , nmoles

TABLE I

RESPIRATORY PARAMETERS OF CHICK HEART MITOCHONDRIA ISOLATED WITH COLLAGENASE.[a]

Substrate(s)	ADP[b]	Rate of Respiration[c] ngram-atom O/min/(nmol cyt aa_3) State 3	Rate of Respiration[c] ngram-atom O/min/(nmol cyt aa_3) State 4	RCR[d]	ADP:O[c,e]	n
5 mM Pyruvate/ 2.5 mM Malate	1	889 ± 97.9	54.9 ± 20.2	18.1 ± 5.4	3.32 ± 0.14	30
	2	789 ± 93.1*	9.2 ± 7.7*	86	3.46 ± 0.13*	
	3	722 ± 94.4	7.8 ± 9.0	93	3.53 ± 0.15*	
5 mM α-Ketoglutarate	1	607 ± 84.6	53.7 ± 4.6	11.3 ± 1.4	3.12 ± 0.10	6
	2	495 ± 53.0*	29.7 ± 14.7*	29.4 ± 34.9	3.27 ± 0.17#	
5 mM β-Hydroxybutyrate	1	618 ± 105	57.6 ± 11.3	10.7 ± 1.9	3.07 ± 0.10	6
	2	371 ± 77*	34.4 ± 10.6*	12.3 ± 6.5	3.33 ± 0.11*	
5 mM Glutamate/ 2.5 mM Malate	1	944 ± 173	77.8 ± 21.5	12.4 ± 1.5	2.74 ± 0.12	5
	2	808 ± 186*	7.9 ± 9.9*	16.8 ± 1.2	2.88 ± 0.02#	
5 mM Succinate/[f] 5 μM Rotenone	1	458	202	2.4	1.91	2
	2	411	178	2.5	1.89	

[a] Mitochondria oxidizing various substrates were assayed for respiration in a mannitol/sucrose osmotic support medium containing 20 mM P_i. Values shown are the means ± S.E. for the indicated number of preparations. The mean mitochondrial concentration in these assays was 0.050 ± 0.005 nmol cyt aa_3/ml (n = 30).

[b] Two to three successive additions of equimolar amounts of ADP (≈ 418 nmoles; ADP_{1-3}) were made after incubating mitochondria with substrate for 1 min.

[c] Differences between respiratory parameters following successive additions of ADP are significant at $p < 0.01$ (*) or $p < 0.05$ (#).

[d] The average respiratory control ratios for ADP_2 and ADP_3 when pyruvate and malate are substrates were calculated by taking the ratio of the mean state 3 and state 4 rates. This was done because the state 4 rates following the phosphorylation of these aliquots of ADP were frequently zero (RCR = infinity), thereby making an arithmetic average meaningless.

[e] Values of ADP:O ratios represent the nmoles of ADP phosphorylated per ngram-atom of oxygen consumed.

[f] Rotenone was included in the assay medium in order to block the oxidation of NADH produced subsequent to the oxidation of succinate.

tion of all of the NAD-linked substrates. These increases are significant at the $p < 0.01$ and $p < 0.05$ levels, as indicated. The data show that the ADP:O ratios are fractional and greater than 3.0 when P/M, βHB, or αKG support oxidative phosphorylation. These coupling ratios are not artifically inflated since: (a) both ADP and oxygen concentrations were enzymatically determined, and (b) after three sequential additions of ADP to mitochondria oxidizing αKG, no residual ADP is enzymatically assayable. ADP:O ratios are less than 3.0 when 5 mM glutamate and 2.5 mM malate (G/M) are used as oxidizable substrates. ADP:O ratios obtained during the oxidation of 5 mM succinate approximate to the long accepted value of 2.0.

The exogenous substrates are oxidized at different rates during states 3 and 4 when P_i concentrations are held fixed at 20 mM. The oxidation of P/M or G/M supports similar state 3 rates. The oxidation of αKG and βHB appears to generate reducing equivalents at equal rates, though more slowly than either P/M or G/M. When succinate is used as substrate, the rate of respiration is one-half that obtained with P/M. During the state 4 respiration following the first addition of ADP, the NAD-linked substrates are oxidized at comparable rates. Subsequent to the phosphorylation of ADP_2, state 4 rates of respiration decrease 2- to 10-fold, depending upon the substrate. The highest RCRs are obtained during the oxidation of P/M. As shown in Fig. 4A, the oxidation of P/M by chick heart mitochondria is subject to adenine nucleotide control. Under conditions in which the phosphorylation of ADP by the F_1F_0-ATP synthase is blocked by oligomycin, maximal rates of uncoupled respiration are only obtained following the addition of ADP. Thus, ADP must regulate one (e.g., isocitrate dehydrogenase) or more catalytic steps

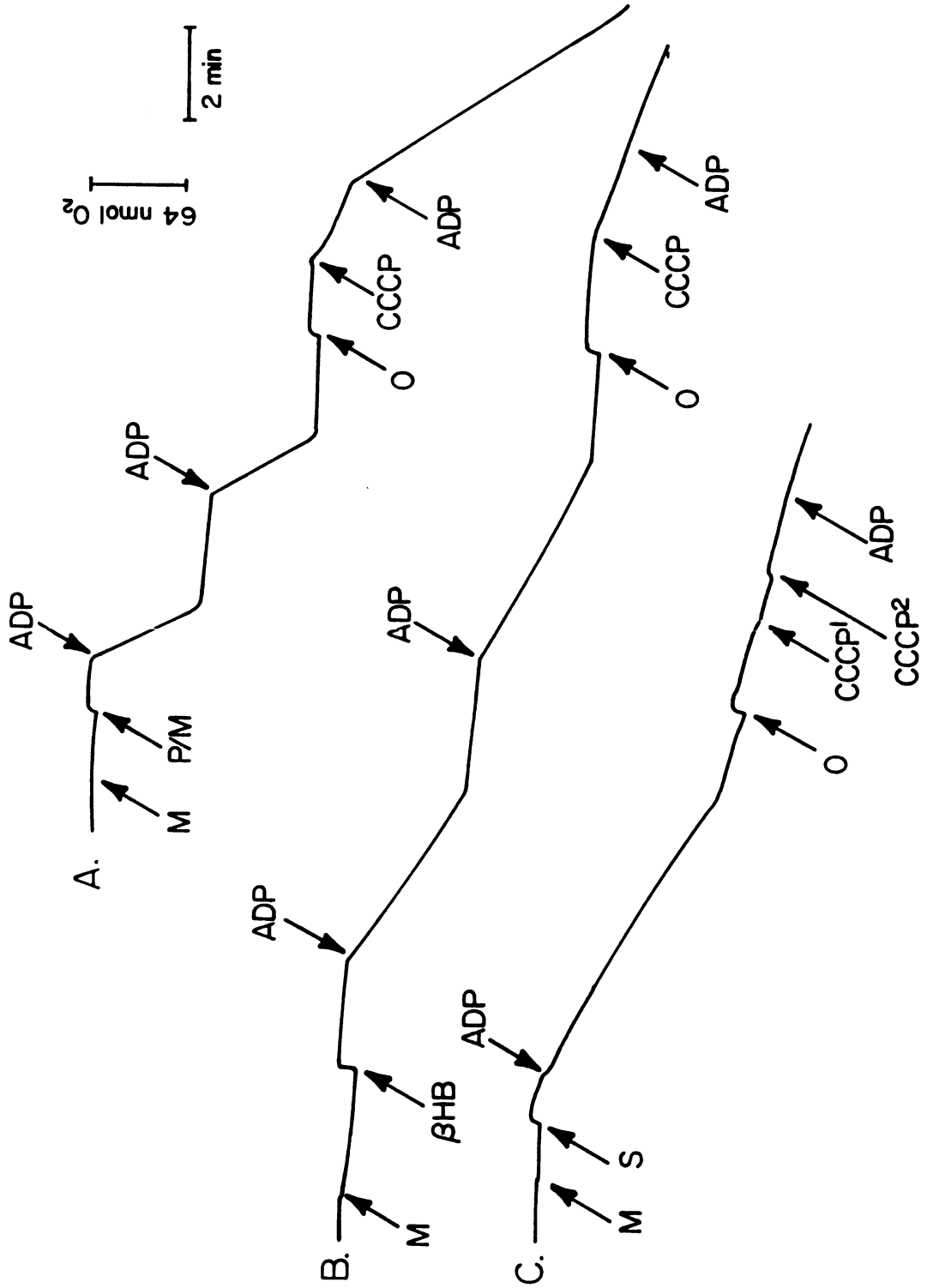
Figure 4. Effect of ADP on the Degree of Uncoupling Induced by CCCP when Mitochondria Oxidize Different Substrates

Mitochondria at the critical mitochondrial concentration were suspended in an assay medium comprised of 0.225 M mannitol, 0.075 M sucrose, and 20 mM Tris-P_i, pH 7.4. Rates of oxygen consumption were measured with an oxygen polarograph as described under "Experimental Procedures." The abbreviations used are as follows: M, mitochondria; ADP, 420 nmoles per addition; O, 1.7 μg oligomycin A/ml; CCCP, 2.9 nmoles of this uncoupler/ml per addition. The substrate(s) used to support respiration were:

(A) 5 mM Pyruvate/2.5 mM Malate

(B) 5 mM β-Hydroxybutyrate

(C) 5 mM Succinate


64 nmol O2
2 min
A.
M
P/M
ADP
ADP
O
CCCP
ADP
B.
M
βHB
ADP
ADP
O
CCCP
ADP
C.
M
S
ADP
O
CCCP1
CCCP2
ADP

of the tricarboxylic acid cycle in chick heart mitochondria. During the oxidation of βHB, ADP does not increase rates of uncoupled respiration (Fig. 4B). The addition of an uncoupler to mitochondria oxidizing succinate during state 4 has no effect on the rate of respiration (Fig. 4C). This result suggests that succinate oxidation is already very poorly coupled to the phosphorylation of ADP. These observations correlate well with the substrate-dependent RCR values and state 4 rates shown in Table I, in that chick heart mitochondria exert greatest control over respiration with P/M and least control with succinate.

IV. EFFECT OF PRE-INCUBATING MITOCHONDRIA WITH SUBSTRATE ON RESPIRATION

As indicated in Table I, the rates of both state 3 and state 4 respiration decrease subsequent to each sequential addition of ADP. The decreases in rates of respiration are all statistically significant at the $p < 0.01$ level. It was of interest to investigate the cause of this behavior in greater detail. The rate of state 4 respiration following the addition of 840 nmoles of ADP [35-55 ng-atom O min^{-1} (nmole cyt $aa_3)^{-1}$] does not correspond to that observed if two separate additions of 420 nmoles of ADP are interposed by a 2 min period of state 4 respiration [<10 ng-atom O min^{-1} (nmole cyt $aa_3)^{-1}$]. Since the state 4 period of respiration prior to the addition of ADP_2 is necessary in order to observe a high value for RCR_2, it was reasoned that during state 4 some kinetic or thermodynamic pressure was established which would limit the rate of mitochondrial substrate oxidation in the absence of ADP. Because prolonged exposure of mitochondria to P_i in the absence of substrate is deleterious to the structure and function of these organelles (see Chapter 5) it was possible that continued exposure of the mitochondria

to substrate during state 4 was causing the decreases in respiratory rates. Indeed, as the time of pre-incubation with P/M prior to the initiation of oxidative phosphorylation with ADP is increased, the rate of state 3 respiration decreases linearly by 30 ng-atom O min^{-1} (nmole cyt aa_3)$^{-1}$/min (Fig 5). Similarly, the rate of uncoupled state 4 respiration also decreases, but it does so in a non-linear manner. On a percentage basis the rate of change in state 4 respiration is much greater than that for state 3; consequently, the capacity to control respiration becomes quite high (Fig 5, Inset).

V. OLIGOMYCIN SENSITIVITY OF STATE 4 RESPIRATION

In an effort to discern whether ADP is regenerated from ATP during state 4, increasing concentrations of mitochondria were treated with an amount of oligomycin A sufficient to completely inhibit ATP synthesis. Below the CMC the state 4 respiration of chick heart mitochondria is unaffected by oligomycin (Fig 6), indicating that the protease-sensitive factor is not an ATPase. Above the CMC state 4 respiration becomes increasingly sensitive to oligomycin in both collagenase and Nagarse preparations of mitochondria. Up to 45% of the total respiration at high mitochondria concentrations (>0.3 nmol cyt aa_3/ml) is inhibitable by oligomycin. Consequently, at concentrations exceeding the CMC, a percentage of state 4 respiration is due to continuous ATP synthesis.

The steady-state concentrations of ADP established during state 4 with different mitochondria concentrations were inferred from the above oligomycin inhibition studies. By assuming that the ADP requirements of oxidative phosphorylation conform to hyperbolic-type saturation kinetics, the following substitutions were made in the Michaelis-Menten equation:

Figure 5. Rates of State 3 and State 4 Respiration at the Critical Mitochondrial Concentration Measured as a Function of Time of Preincubation with 5 mM Pyruvate/2.5 mM Malate Prior to the Addition of ADP

Respiration was monitored at 30.5 °C in an assay medium that contained 0.225 M mannitol, 0.075 M sucrose, and 20 mM Tris-buffered P_i. Least squares regression analysis was used to fit a line through the points for state 3 respiration. Insert: Respiratory control ratios measured as a function of time of preincubation of mitochondria with 5 mM pyruvate/2.5 mM malate.

State 3 Respiration
ng-atom O · min⁻¹·(nmol cyt aa₃)⁻¹
State 4 Respiration
ng-atom O · min⁻¹·(nmol cyt aa₃)⁻¹
TIME OF PREINCUBATION, sec
State 3
State 4
RESPIRATORY CONTROL RATIO
TIME OF PREINCUBATION, sec

Figure 6. Sensitivity of State 4 Respiration to Oligomycin A Measured as a Function of Mitochondria Concentration

Increasing concentrations of chick heart mitochondria isolated with either collagenase (A) or Nagarse (B) were treated with 0.86 μg oligomycin A ml^{-1} during the period of state 4 respiration subsequent to ADP_2. (●), respiration before the addition of oligomycin; (○), respiration after the addition of oligomycin.

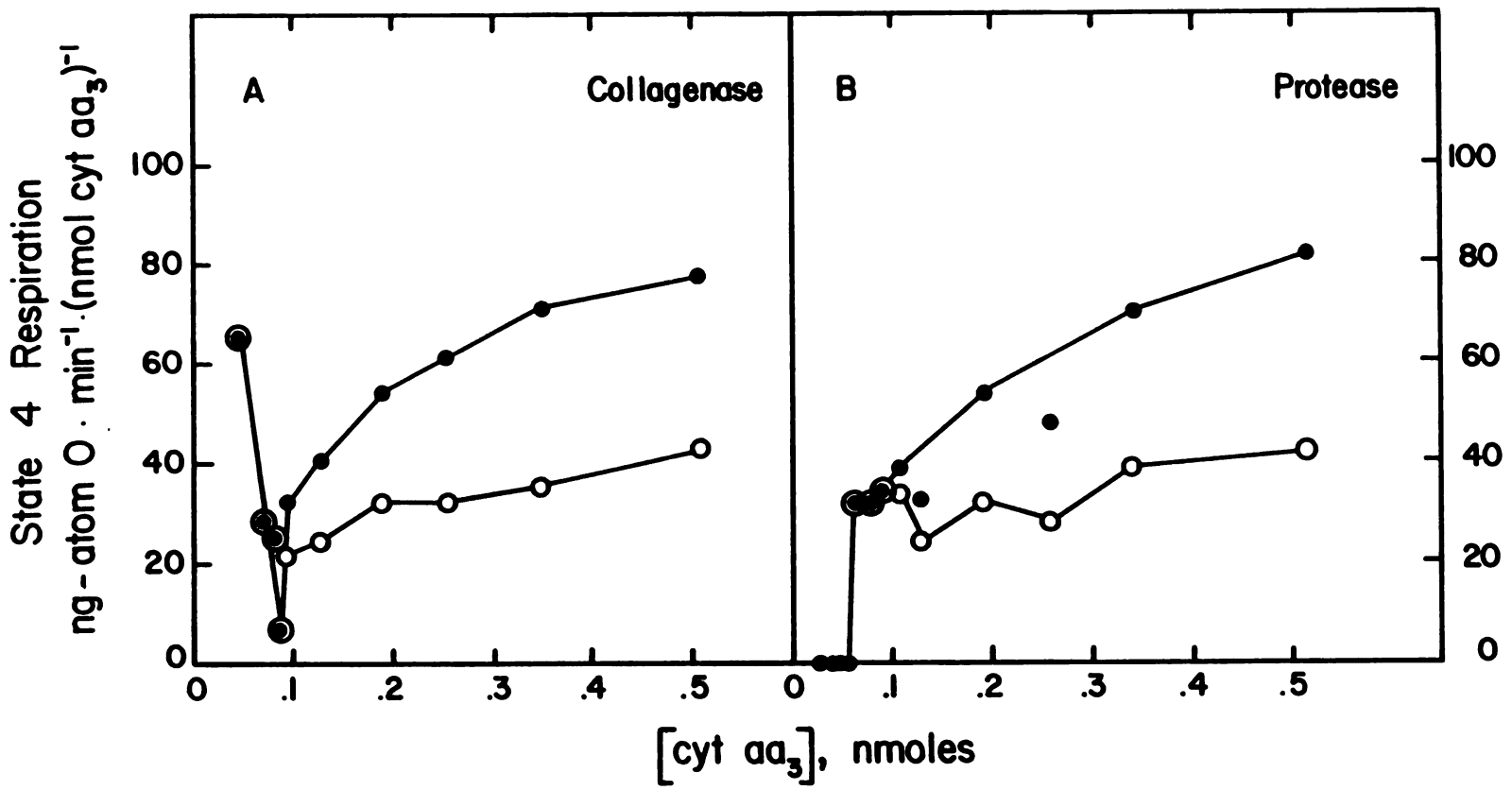
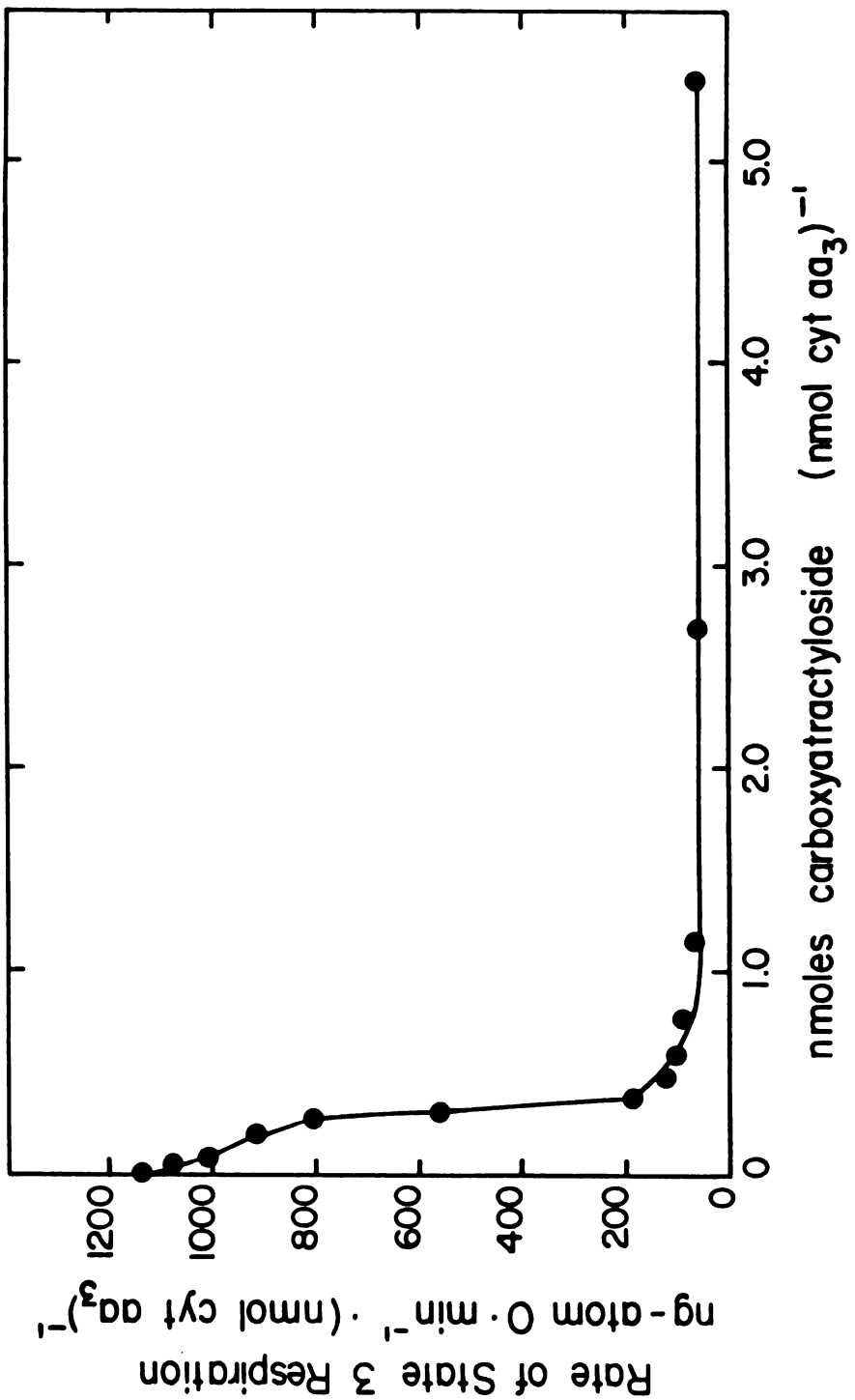

A
Collagenase
B
Protease
State 4 Respiration
ng-atom O · min⁻¹·(nmol cyt aa₃)⁻¹
100
80
60
40
20
0
0
.1
.2
.3
.4
.5
[cyt aa₃], nmoles

Figure 7. Inhibition of Chick Heart Mitochondrial State 3 Respiration with Increasing Concentrations of Carboxyatractyloside

Mitochondria (0.074 nmols cyt aa_3/ml) suspended in M/S oxidized 5 mM pyruvate and 2.5 mM malate in the presence of 20 mM Tris-buffered P_i, pH 7.4. State 3 respiration was initiated by the addition of 2.4 μmoles of ADP. Mitochondria were allowed to phosphorylate ADP for 1 min. Varying concentrations of carboxyatractyloside were added, and the state 3 rate subsequent to the binding of inhibitor was measured.


Rate of State 3 Respiration
ng-atom O · min⁻¹ · (nmol cyt aa₃)⁻¹
0
200
400
600
800
1000
1200
0
1.0
2.0
3.0
4.0
5.0
nmoles carboxyatractyloside (nmol cyt aa₃)⁻¹

$$(\text{state 4 rate})(\text{ADP:O}) = \frac{[(\text{state 3 rate})(\text{ADP:O})][\text{ADP}]}{K_m + [\text{ADP}]} \qquad (4)$$

where the product of respiratory rate and the ADP:O ratio is the rate of ATP synthesis, and K_m is the Michaelis constant of the chick heart mitochondrial adenine nucleotide translocase for ADP (20 μM, Dr. S. Brooks, personal communication). After taking the reciprocal of both sides and rearranging, equation 4 reduces to:

$$[\text{ADP}]_{ss} = K_m \Big/ \frac{J_o^{3}}{J_o^{4,olig}} - 1 \qquad (5)$$

where J_o^{3} is the oxidative flux during state 3 respiration and $J_o^{4,olig}$ is the oligomycin-sensitive oxidative flux during state 4. In this experiment, the adenine nucleotide translocase and ATPase(s) operate as a coupled enzyme system. Steady-state ADP concentrations could thus be maintained if the rate of ATP hydrolysis is equal to the rate of ATP synthesis. Because the measured parameter is an increase in the rate of respiration, it must be established that the translocase can be rate-limiting for oxidative phosphorylation. As shown in Fig 7, this appears to be the case. Although the plot shows slight sigmoidal character, carboxyatractyloside inhibits a percentage of state 3 respiration at all concentrations tested (K_I^{app} = 3 μM). State 3 respiration is inhibited by over 99% when the molar ratio of carboxyatractyloside to cyt aa_3 is equal to 1.0. Since the adenine nucleotide translocase and cyt aa_3 are present in the mitochondrial inner membrane at equimolar concentrations, carboxyatractyloside is clearly a very effective inhibitor. The calcu-

lations presented in Fig 8 show that the steady-state concentrations of ADP increase linearly as a function of mitochondria concentration. Moreover, mitochondria isolated with either collagenase or Nagarse maintain approximately the same steady-state concentrations of ADP during "state 4" respiration, being 2.5 and 2.0 μM ADP (nmole cyt aa_3)$^{-1}$, respectively.

VI. THE EFFECT OF EGTA ON RESPIRATORY PARAMETERS

The data in the inset of Fig 9 show that the oligomycin-sensitive compo-nent of state 4 respiration can also be inhibited with EGTA. The inhibition is instantaneous and decreases the rate of state 4 respiration over two-fold. Titrating a fixed concentration of mitochondria with increasing concentrations of EGTA shows significant initial inhibition below 250 μM, followed by a subsequent slower rate of inhibition (Fig 9). This result suggests that the EGTA binds two types of divalent cation which have different affinities for this chelating agent. For the preparation titrated, 17 mM EGTA completely inhibited the oligomycin-sensitive respiration. EGTA (20 mM) also prevents ADP:O ratios from decreasing as mitochondria concentrations are increased (data not shown).

VII. THE EFFECT OF Mg(II) ON RESPIRATORY PARAMETERS

A direct implication of the inhibition of state 4 respiration by EGTA is that divalent cations released from mitochondria stimulate oxygen consumption by facilitating the hydrolysis of ATP. As a test of the tenability of this hypothesis, chick heart mitochondria at the CMC were titrated with increasing concentrations of Mg(II). Mg(II) causes a precipitous drop in RCRs from 200 down to 4 (Fig 10). State 3 rates are unaffected by Mg(II) (Fig 10, Inset). The decreases in respiratory

Figure 8. Steady-State Concentrations of ADP Maintained During State 4 Respiration

The concentrations of ADP regenerated by increasing concentrations of mitochondria isolated with either collagenase (◯) or Nagarse (●) were inferred from the oligomycin sensitivity of state 4 respiration using equation 3. The lines through the data were calculated by least squares linear regression.

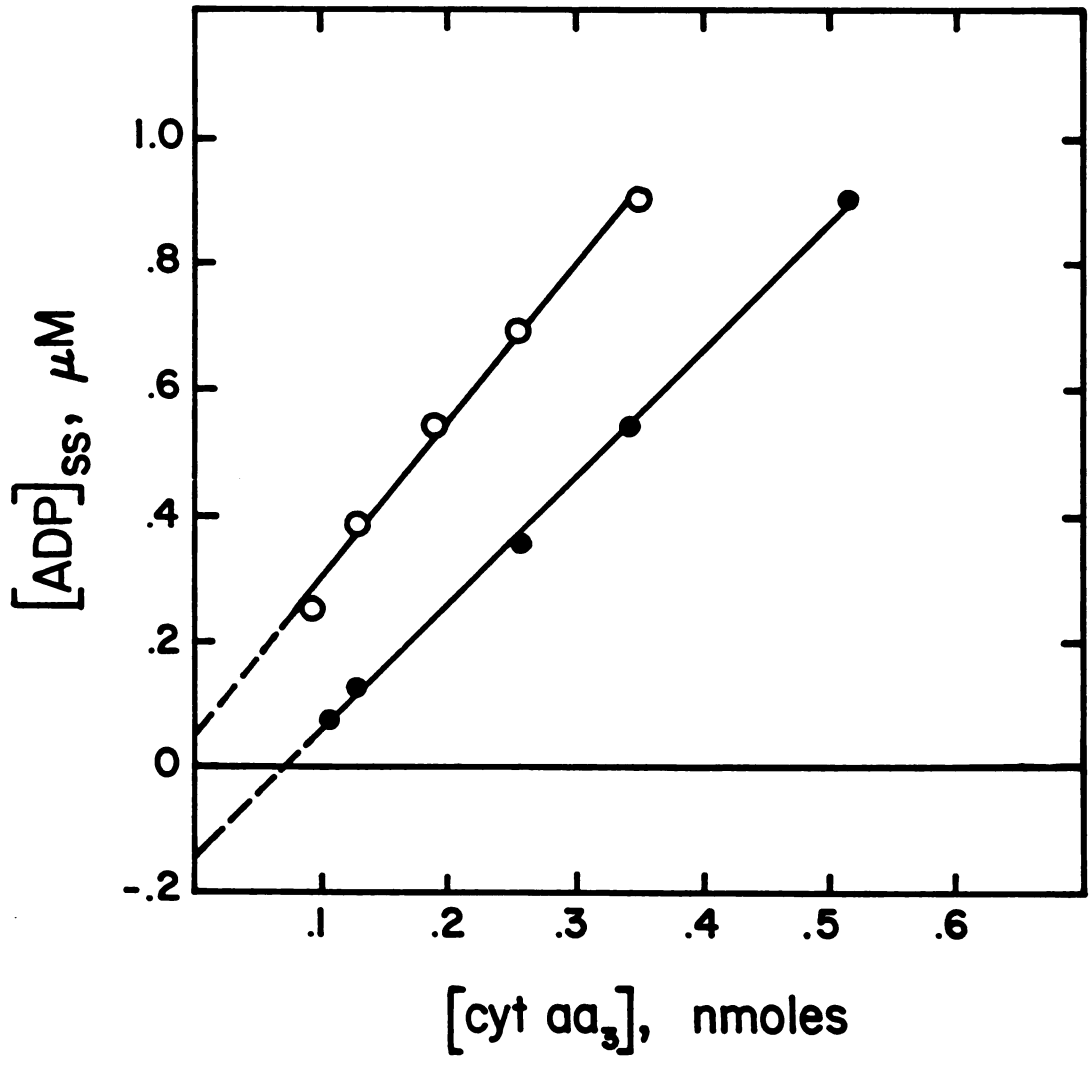

1.0
.8
.6
.4
.2
0
-.2
.1
.2
.3
.4
.5
.6
[ADP]ss, μM
[cyt aa3], nmoles

Figure 9. Inhibition of State 4 Respiration by Increasing Concentrations of EGTA

Mitochondria above the critical mitochondrial concentration (0.2 nmol cyt aa_3 ml^{-1}) oxidized 5 mM pyruvate/2.5 mM malate in M/S medium that contained 20 mM Tris-buffered P_i, pH 7.4. The plot shows the percent inhibition of state 4 respiration by increasing concentrations of EGTA following a single addition of 334 nmoles of ADP. Insert: Oxygen polarograph trace showing that EGTA inhibits all of the oligomycin-sensitive respiration during state "4". M, mitochondria at 0.14 nmol cyt aa_3 ml^{-1}; ADP, 334 nmoles; EGTA, 27 mM; O, oligomycin A at 1.7 μg ml^{-1}. Rates of state 4 respiration shown are expressed as ng-atom O min^{-1} (nmol cyt aa_3)$^{-1}$.

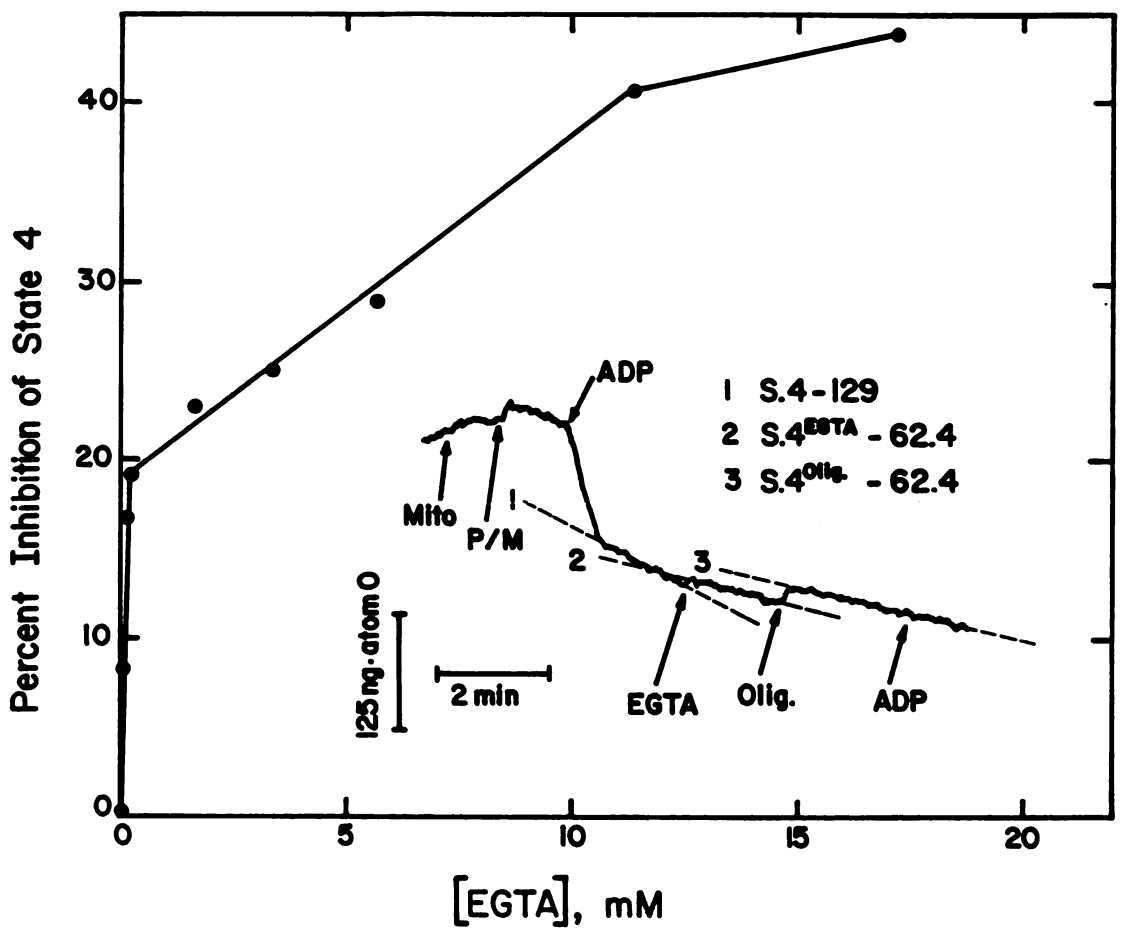
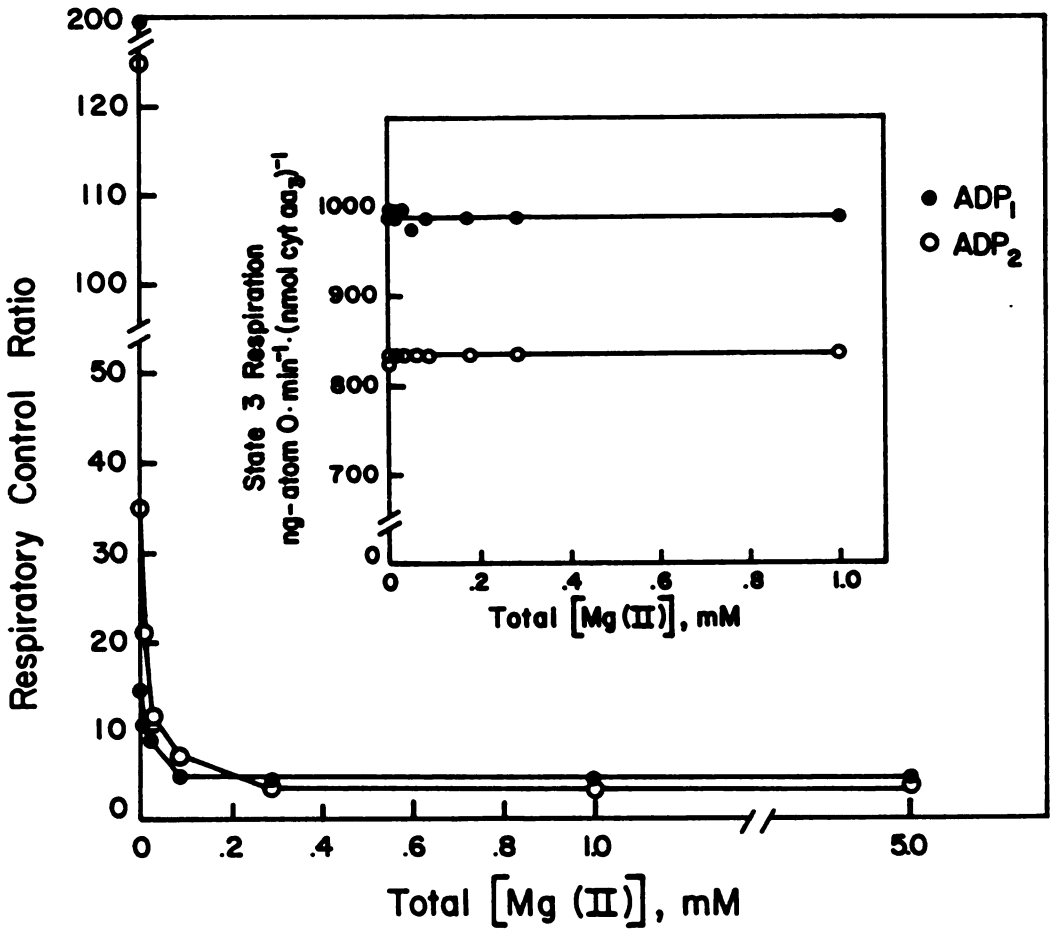

Percent Inhibition of State 4
40
30
20
10
0
0
5
10
15
20
[EGTA], mM
ADP
1 S.4-129
2 S.4EGTA - 62.4
3 S.4Olig. - 62.4
Mito
P/M
1
2
3
125 ng·atom O
2 min
EGTA
Olig.
ADP

Figure 10. Effect of Mg(II) on Chick Heart Mitochondrial Respiratory Control

The critical mitochondrial concentration was titrated with increasing concentrations of Mg(II) under conditions described in "Experimental Procedures." (●), RCRs secondary to ADP_1; (◯), RCRs secondary to ADP_2. Insert: Effect of Mg(II) on state 3 respiration. (●), state 3 induced by ADP_1; (◯), state 3 induced by ADP_2.


Respiratory Control Ratio
200
120
110
100
50
40
30
20
10
0
0
.2
.4
.6
.8
1.0
5.0
Total [Mg (II)], mM
State 3 Respiration
ng-atom O·min⁻¹·(nmol cyt aa₃)⁻¹
1000
900
800
700
0
0
.2
.4
.6
.8
1.0
Total [Mg(II)], mM
● ADP₁
○ ADP₂

control are due entirely to changes in state 4 respiration (Fig 11B). The manner by which Mg(II) stimulates state 4 respiration is clarified by the following experiments. First, the respiration stimulated by Mg(II) is completely inhibited by oligomycin, indicating that Mg(II) stimulates the hydrolysis of ATP synthesized during state 3 (data not shown). Second, ADP is regenerated extramitochondrially, as evidenced by the ability of pyruvate kinase to inhibit the respiration stimulated by Mg(II) (data not shown). Between 0 and 90 μM, the stimulation of ATP hydrolysis by Mg(II) is linear (Fig 11B). Above 90 μM Mg(II) no further stimulation is observed, suggesting that the ATPase(s) are saturated. The concentration of Mg(II) which gives half-maximal stimulation is approximately 34 μM. When the ATPase activity is saturated with Mg(II), oxygen consumption is stimulated to 20% of the V_{max} of state 3 respiration. As to be shown in Chapter 5, the Mg(II) stimulated ATPase activity is not due to an ion-motive transmembrane Mg(II)-ATPase.

Mitochondrial ADP:O ratios are also depressed by Mg(II) (Fig 11A). For example, the ADP:O ratio decreases from 3.10 to 2.54 following the addition of 1 mM Mg(II). In parallel with the above findings, ADP:O ratios are depressed because Mg(II) is stimulating the hydrolysis of ATP during state 3. This keeps the enzymes (adenine nucleotide translocase and F_1F_0-ATP synthase) regulating oxidative phosphorylation supplied with ADP for longer periods of time, thereby decreasing the empirical ADP:O ratio but not the "true" coupling of these organelles. Data presented in Chapter 5 show that chick heart mitochondria release Mg(II) and Ca(II). Divalent cations so released into the extramitochondrial space will stimulate ATPase activity. Increasing the concentration of mitochondria in an assay therefore stimulates ATPase activity by

Figure 11. Effect of Mg(II) on ADP:O ratios and State 4 Respiration

The critical mitochondrial concentration was titrated with increasing concentrations of Mg(II) and the effect on ADP:O ratios (A) and state 4 rates of respiration (B) was quantitated. All data points were obtained subsequent to the phosphorylation of ADP_1.

A
ADP: O
3.0
2.75
2.5
0
.2
.4
.6
.8
1.0
[Mg(II)], mM
B
State 4 Respiration
ng - atom O·min⁻¹·(nmol cyt aa₃)⁻¹
200
150
100
50
0
.2
.4
.6
.8
1.0
[Mg(II)], mM

increasing the concentration of divalent cations. This results in progressive increases in the rates of state 4 respiration and decreases in ADP:O ratios, as observed. ATPase activity has no effect on state 3 respiration because phosphorylation is proceeding at a rate that closely approximates the maximal velocity for this process.

VIII. QUANTITATION OF ATPASE ACTIVITY

In view of the effect of ATPase activity on mitochondrial respiratory parameters, it was of interest to quantify and identify the ATPase activities present in suspensions of chick heart mitochondria. Approximately 82% of the total ATPase activity is inhibitable with oligomycin (Fig 12A). One of the ATPases must, therefore, be the F_1F_0-ATP synthase associated with mitochondria whose inner membranes are disrupted. It is well known that the ATP synthase of broken mitochondria operates in the hydrolysis mode (Boyer, 1979). Under conditions in which the ATP synthase molecules of broken mitochondria are completely inhibited with oligomycin, a second ATPase hydrolyzes ATP at a constant rate of 11.3 nmoles min^{-1} $(mg\ mitochondria)^{-1}$ when the Mg(II) concentration is 50 μM (Fig 12B).

The collective hydrolytic activity of these ATPases in a suspension of mitochondria was measured in the presence of a constant concentration of either Mg(II) or Ca(II) (Fig 13). For these end-point assays, oligomycin was added at the indicated times after hydrolysis was initiated with the addition of ATP. The amount of ATP hydrolyzed by the F_1F_0-ATP synthase was the variable. The amount of ATP hydrolyzed by the other ATPase was constant for all the samples of a given preparation of mitochondria and corresponds to points at "0" time. Both Mg(II) and Ca(II) stimulate the two ATPases. In the presence of either cation the rate of

Figure 12. Sensitivity of Chick Heart Mitochondrial ATPase Activity to Oligomycin A

(A) Titration of chick heart mitochondrial ATPase with oligomycin A. Mitochondria (0.56 mg/ml) were incubated for 5 min at 30.5°C in an M/S medium that contained 50 μM Mg(II), 50 μM rotenone, 1 mM MalNEt, 20 mM Tris, pH 7.4, and the indicated amount of oligomycin A. Suspensions were made 2 mM in ATP, and ATP hydrolysis was allowed to proceed for 15 min. P_i released from ATP was assayed as described under "Experimental Procedures."

(B) Rate of oligomycin A-insensitive ATPase activity associated with isolated chick heart mitochondria. Mitochondria (0.73 mg/ml) were incubated in the assay medium described in (A) for 5 min. The concentration of oligomycin A, however, was held fixed at 20 μg/mg mitochondria. This concentration of oligomycin was sufficient to completely inhibit the hydrolysis of ATP by the ATP synthase molecules of broken mitochondria. Samples were then incubated with 2 mM ATP for 15 min. The line through the data was estimated by least squares linear regression (cc: 0.9941).

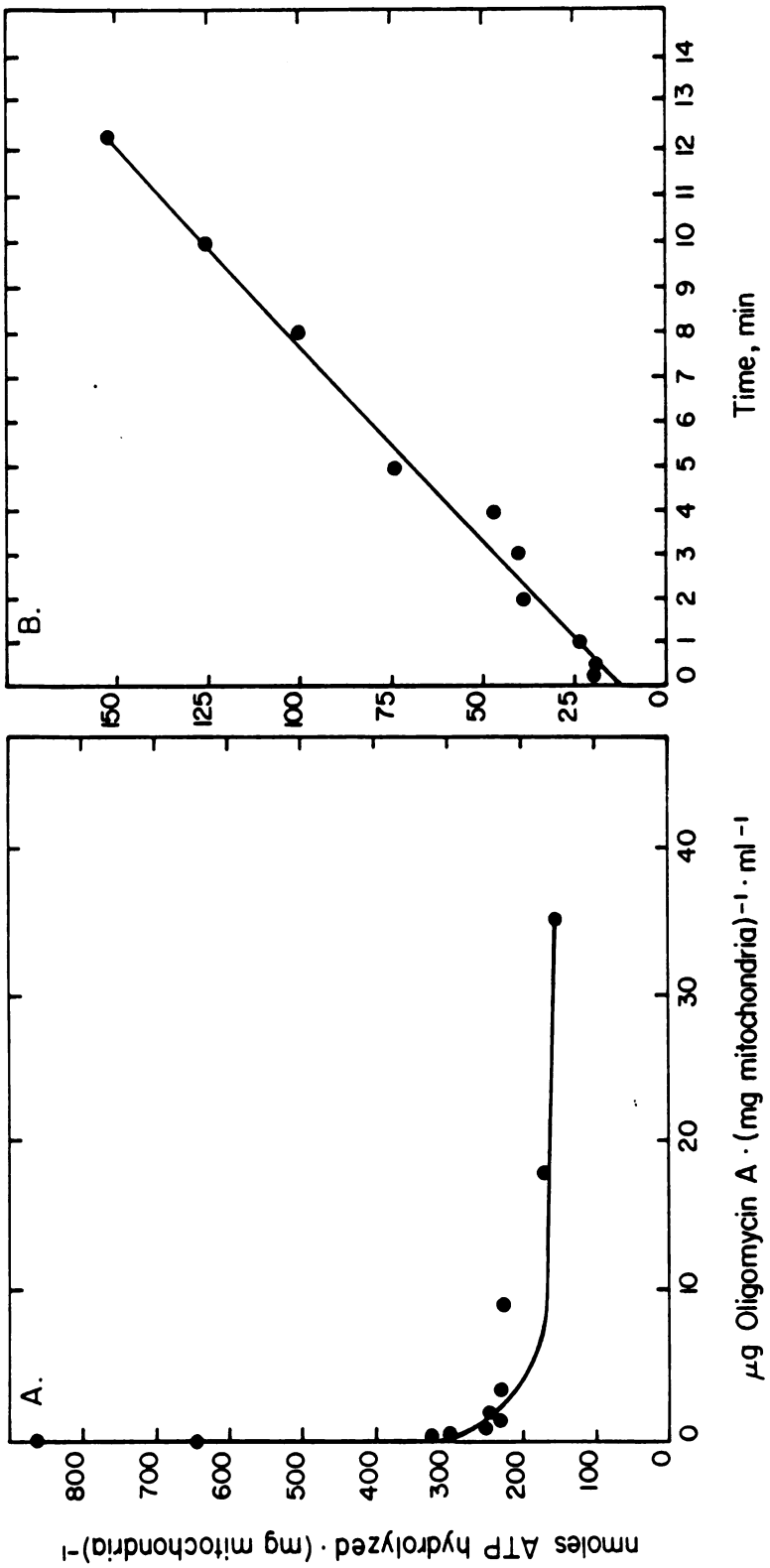
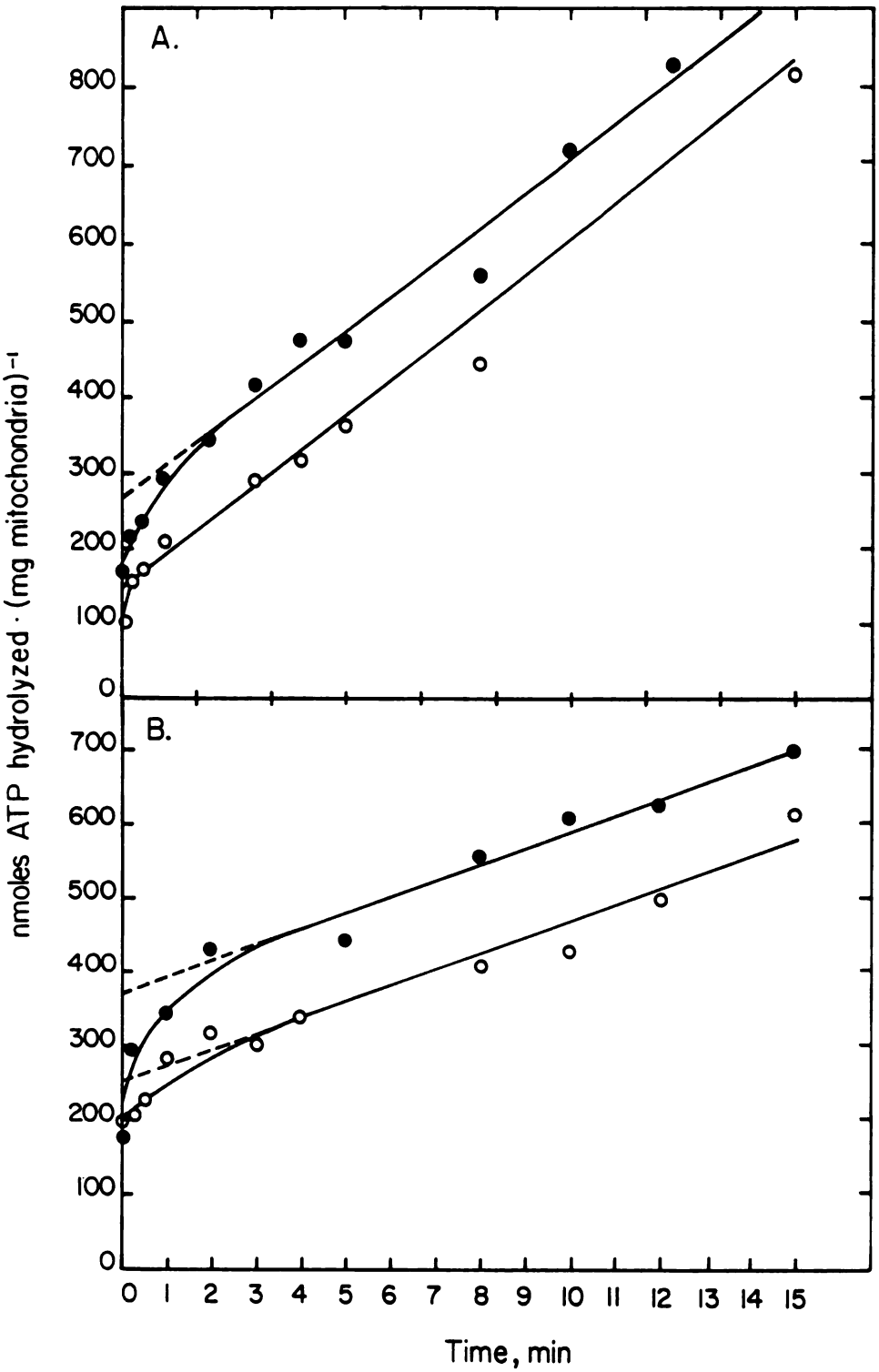

A.
B.
nmoles ATP hydrolyzed · (mg mitochondria)-1
μg Oligomycin A · (mg mitochondria)-1 · ml-1
Time, min

Figure 13. Quantitation of Total Divalent Cation-Sensitive ATPase Activity Associated with Isolated Chick Heart Mitochondria

(A) Mitochondria (0.49 mg/ml (●), 0.52 mg/ml (○)) were suspended in the M/S medium described in the legend to Figure 12, except that 20 µg oligomycin A/mg was added at the indicated times. The zero time point was obtained by preincubating the mitochondria with oligomycin for 5 min and then adding ATP. After initiating the reaction with the addition of ATP, mixtures were allowed to incubate at 30.5°C for 15 min. The amount of P_i released was corrected for P_i endogenous to the mitochondria and for that in the ATP solution. The divalent cation was 50 µM Mg(II).

(B) Mitochondria (0.34 mg/ml (●), 0.72 mg/ml (○)) were treated and incubated as described in (A). The divalent cation was 50 µM Ca(II). The linear portion of all data sets was estimated by least squares linear regression.


A.
B.
nmoles ATP hydrolyzed · (mg mitochondria)⁻¹
Time, min
0
100
200
300
400
500
600
700
800
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

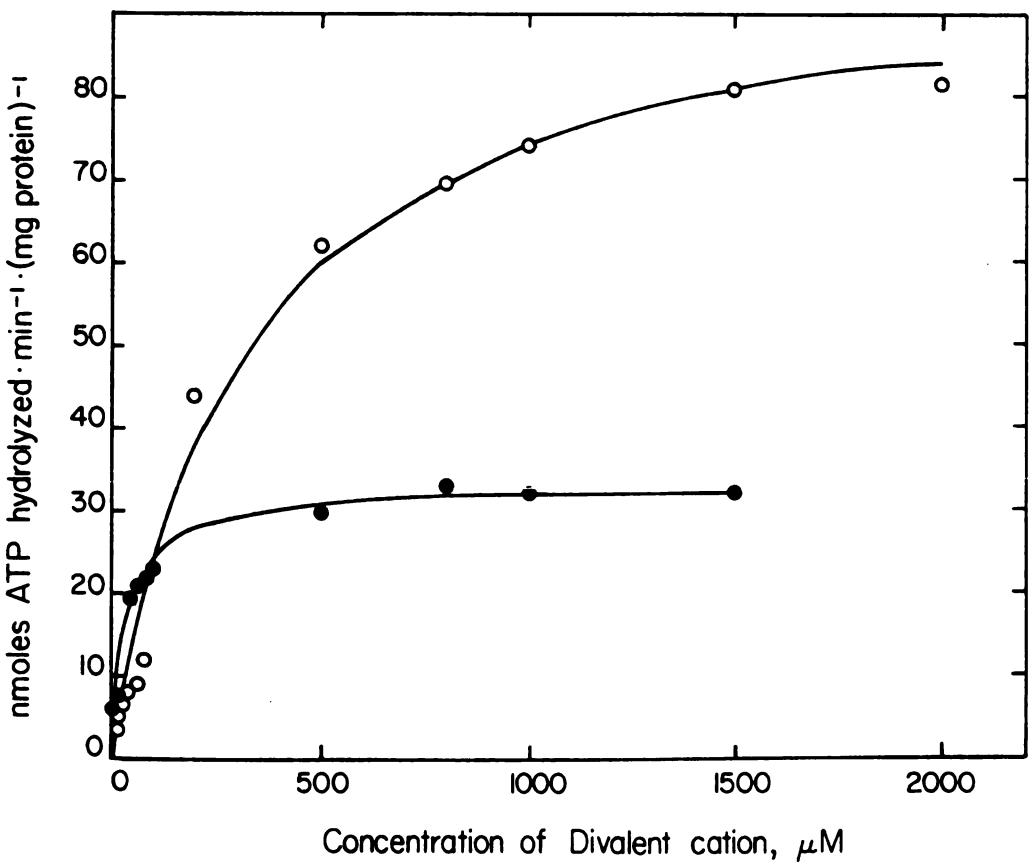
ATP hydrolysis by the F_1F_0-ATP synthase decreases during the first 2 min of the reaction. After 2 min, the rate of hydrolysis by this ATPase is constant. This suggests that the ATP synthase has a attained some type of steady-state. In the presence of 50 μM Mg(II), the rate of ATP hydrolysis in the steady-state is 45.1 ± 0.92 nmoles min^{-1} (mg mitochondria)$^{-1}$ for the two preparations shown (Fig 13A). The rate of ATP hydrolysis during this period with 50 μM Ca(II) is two-fold lower, being 21.6 ± 0.42 nmoles min^{-1} (mg mitochondria)$^{-1}$ for the preparations shown (Fig 13B). These data demonstrate that: (a) the ATPase activity co-sedimenting with mitochondria is quite consistent from preparation to preparation; (b) Mg(II) has a higher affinity for the F_1F_0-ATP synthase than does Ca(II); and (c) that two ATPases are responsible for regenerating ADP from ATP in the extramatrix space of isolated chick heart mitochondria.

The kinetic parameters for the activation of total ATPase activity by Ca(II) and Mg(II) were determined by titrating aliquots of a suspension of isolated chick heart mitochondria with these cations. As shown in Fig 14, the stimulation of ATPase activity by Ca(II) and Mg(II) obeys hyperbolic Michaelis-Menten type kinetics. The apparent K_m and V_{max} values for hydrolysis are presented in Table II. Mg(II) has a ten-fold higher affinity for at least one, and possibly both, of the ATPases as compared to Ca(II). This estimate (35 μM) for the apparent K_m of the ATPases for Mg(II) is in excellent agreement with the estimate for this parameter obtained from oxygen consumption measurements (34 μM). Interestingly, however, the V_{max} for Ca(II)-stimulated ATPase activity is three-fold higher than for that stimulated by Mg(II).

IX. IDENTIFICATION OF THE NON-MITOCHONDRIAL ATPASE

Figure 14. Titration of Chick Heart Mitochondrial Divalent Cation-Sensitive ATPase Activities with Either Mg(II) or Ca(II)

Mitochondria were suspended at 0.47 mg/ml (●) or 0.54 mg/ml (○) in a medium that contained 0.225 M mannitol, 0.075 M sucrose, and 20 mM Tris, pH 7.4. These organelles were then titrated with increasing concentrations of either Mg(II) (●) or Ca(II) (○). Other conditions were as described in "Experimental Procedures." The curves through the data were calculated with the Michaelis-Menten equation by weighted linear regression.


nmoles ATP hydrolyzed · min⁻¹ · (mg protein)⁻¹
0
10
20
30
40
50
60
70
80
0
500
1000
1500
2000
Concentration of Divalent cation, μM

For many years it was taken for granted that the ATPase activity associated with isolated heart mitochondria is attributable to a myosin contaminant. This has not, however, been demonstrated. In an effort to identify the unknown ATPase, chick heart myosin was isolated and its kinetic parameters characterized. The banding pattern of the isolated myosin preparation following $NaDodSO_4$-polyacrylamide gel electrophoresis is shown in Fig 15A. Coomassie staining reveals two major protein bands having molecular masses of 178.5 and 45.8 kDa. These bands were immunoblotted with antisera raised against purified actin and myosin. The low molecular weight band reacts with actin antibody (Fig 16A, lane 2), while the high molecular weight band reacts with myosin antibody (Fig 16B, lane 1). This immunoreactivity identifies these protein bands as actin and, based on the molecular weight estimate, myosin heavy chain. The myosin/actin mixture was titrated with increasing concentrations of divalent cations and the hydrolysis of ATP was quantitated. Consistent with the findings obtained with myosins from other species (Pollard, 1982), Mg(II) inhibits myosin ATPase activity (Fig 17; for kinetic parameters see Table I). In contrast, Ca(II) stimulates ATPase activity in the myosin/actin mixture (Fig 18). There is an approximately 100-fold difference in the affinity of these two cations for myosin/actin (Table II). In a suspension of mitochondria, the extra-matrix space contains both Ca(II) and Mg(II). It was of interest to ascertain whether a mixture of Ca(II) and Mg(II) would result in a stimulation of ATPase activity in the myosin preparation. When Mg(II) concentrations are increased in the presence of 50 μM Ca(II), inhibition of the ATPase is still observed. The slight increase in the K_I for this experiment likely reflects competition between Ca(II) and Mg(II) for the cation binding

Figure 15. SDS-Polyacrylamide Gel Electrophoresis of the Chick Heart Myosin Preparation used in ATPase Kinetic Studies

Proteins were electrophoresed through a 3% polyacrylamide stacking gel and an 8% resolving gel using the discontinuous buffer system of Laemmli (1970) on a Bio-Rad vertical slab gel apparatus. Both the stacking and resolving gels contained 0.1% SDS.

(A) Coomassie Blue R250-stained gel.

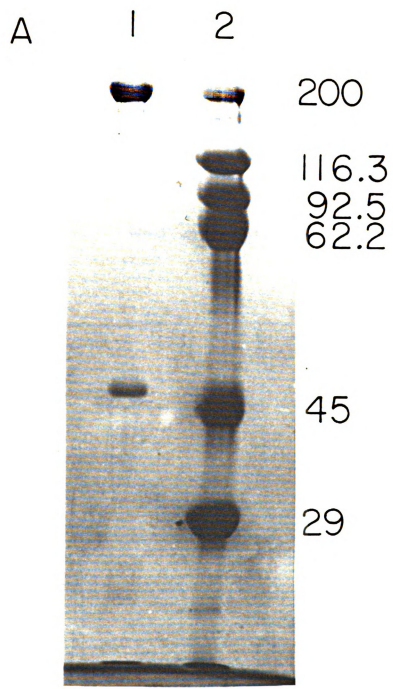
Lane 1, 8.4 µg of the myosin preparation.
Lane 2, 2 µg of each Bio-Rad high molecular weight standard protein: myosin (200,000); β-galactosidase (116,250); phosphorylase b (92,500); bovine serum albumin (62,200); ovalbumin (45,000). This preparation was enriched with 3 µg of carbonic anhydrase (Sigma; 29,000).

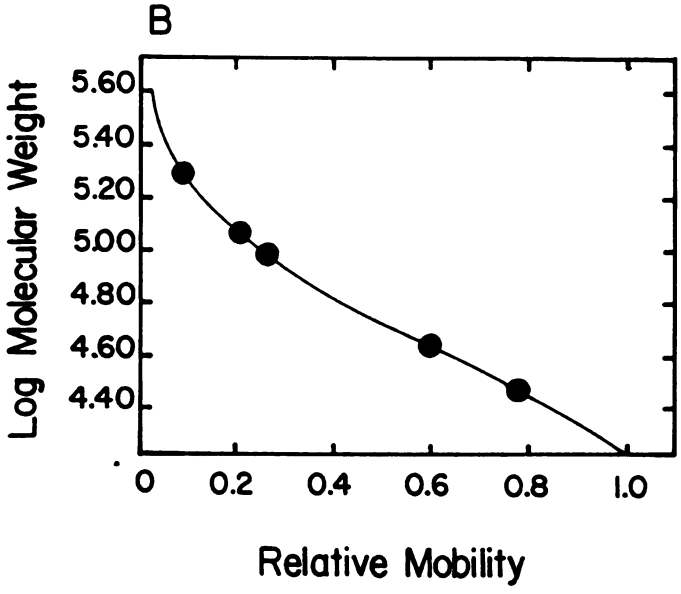
(B) A plot of the M_r of the molecular weight standards listed in (A) versus relative mobility. Relative mobilities were measured by using the top of the resolving gel and the trailing schlieren line at the bottom as reference points. The curve drawn through the data is theoretical using the quantitative methods described by Peterson and Hokin (1981) and outlined under "Experimental Procedures."

(C) Densitometric scans of gel lanes 1 and 2 in (A).

i.) Trace of the molecular weight standards. Impurities are designated as I and II.

ii.) Trace of the myosin preparation.


A
1
2
200
116.3
92.5
62.2
45
29


B
Log Molecular Weight
5.60
5.40
5.20
5.00
4.80
4.60
4.40
0
0.2
0.4
0.6
0.8
1.0
Relative Mobility

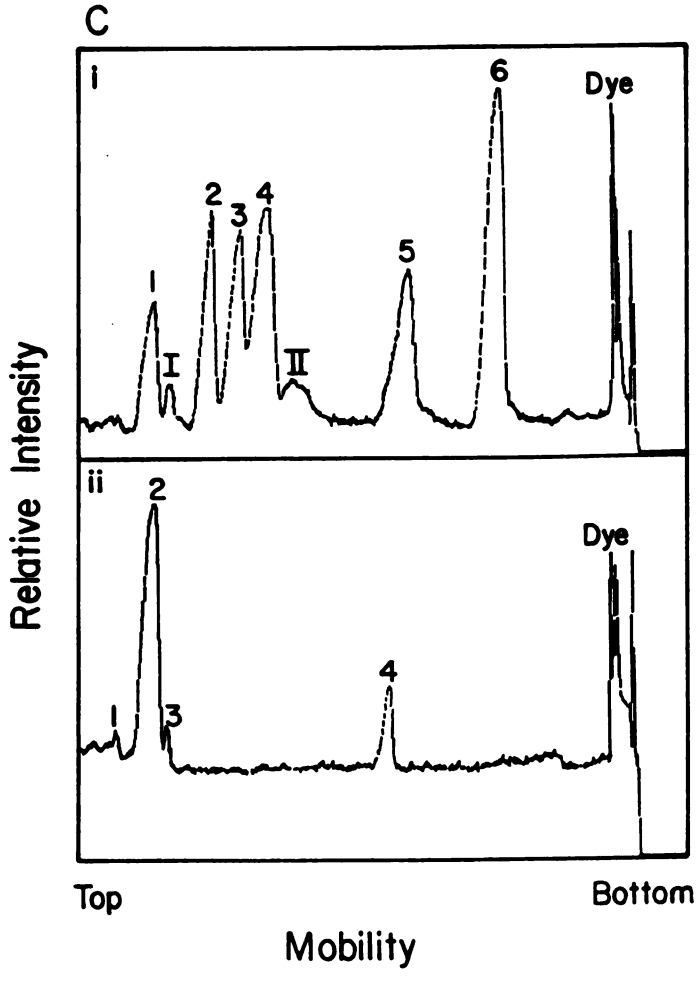

C
i
ii
Dye
Relative Intensity
Top
Bottom
Mobility

Figure 16. Immunoblotting of Chick Myocardial Actin and Myosin

Chick heart mitochondria and an aliquot of the myosin preparation were solubilized in sample buffer, incubated in a boiling water bath, and electrophoresed as described under "Experimental Procedures." The separated proteins were transferred to nitrocellulose filters as described by Towbin et al. (1979), and immunoblotted with antisera directed against actin and myosin. The protein bands capable of binding antibody were visualized using the Bio-Rad BCIP/NBT alkaline phosphatase color development system.

(A) Lane 1, 220 µg of chick heart mitochondria.
Lane 2, 12.5 µg of the myosin preparation.
Lane 3, 6.4 µg of purified rabbit skeletal muscle actin.

(B) Lane 1, 13.6 µg of the myosin preparation.
Lane 2, 310 µg of chick heart mitochondria.

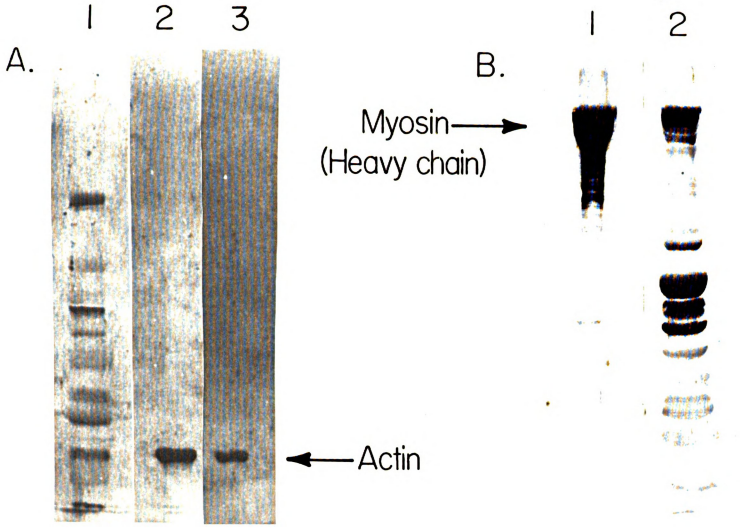

A.
1
2
3
Actin
B.
1
2
Myosin
(Heavy chain)

Figure 17. Titration of Chick Heart Myosin with Mg(II) in Either the Absence or Presence of Ca(II)

Assays were performed as described under "Experimental Procedures." The curves through the data are theoretical, calculated with kinetic constants estimated by a least-squares non-linear regression of the data fitted to equation 1. The protein concentration in these assays was 0.4 mg/ml (◯, +50 μM Ca(II)) and 0.36 mg/ml (●, no Ca(II)).

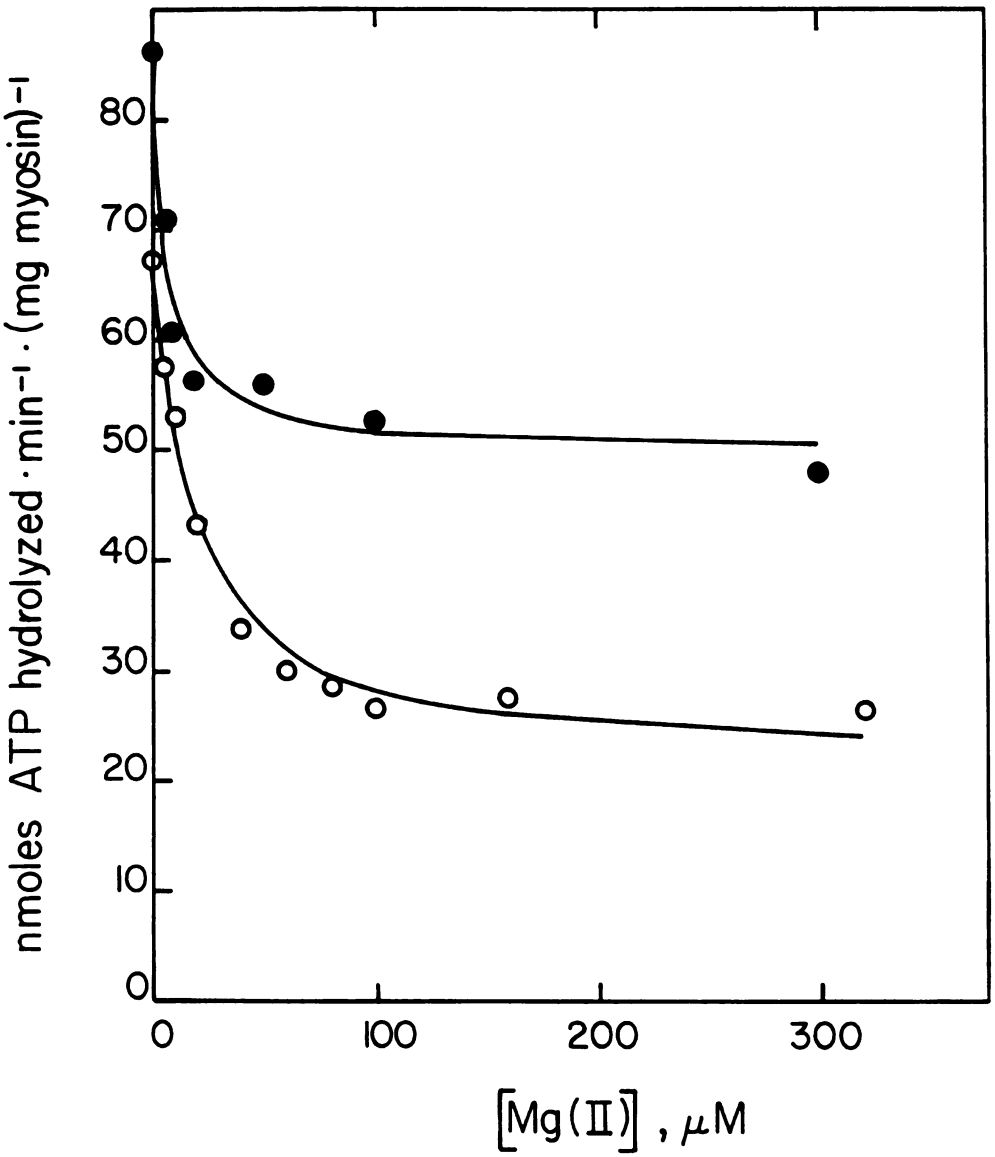

nmoles ATP hydrolyzed · min⁻¹ · (mg myosin)⁻¹
80
70
60
50
40
30
20
10
0
0
100
200
300
[Mg(II)] , μM

Figure 18. Titration of Chick Heart Myosin with Ca(II)

Myosin was suspended at 0.36 mg/ml and assayed as described under "Experimental Procedures." The theoretical curve drawn through the data was calculated with the kinetic constants estimated by a non-linear regression of the data fitted to the Michaelis-Menten equation.

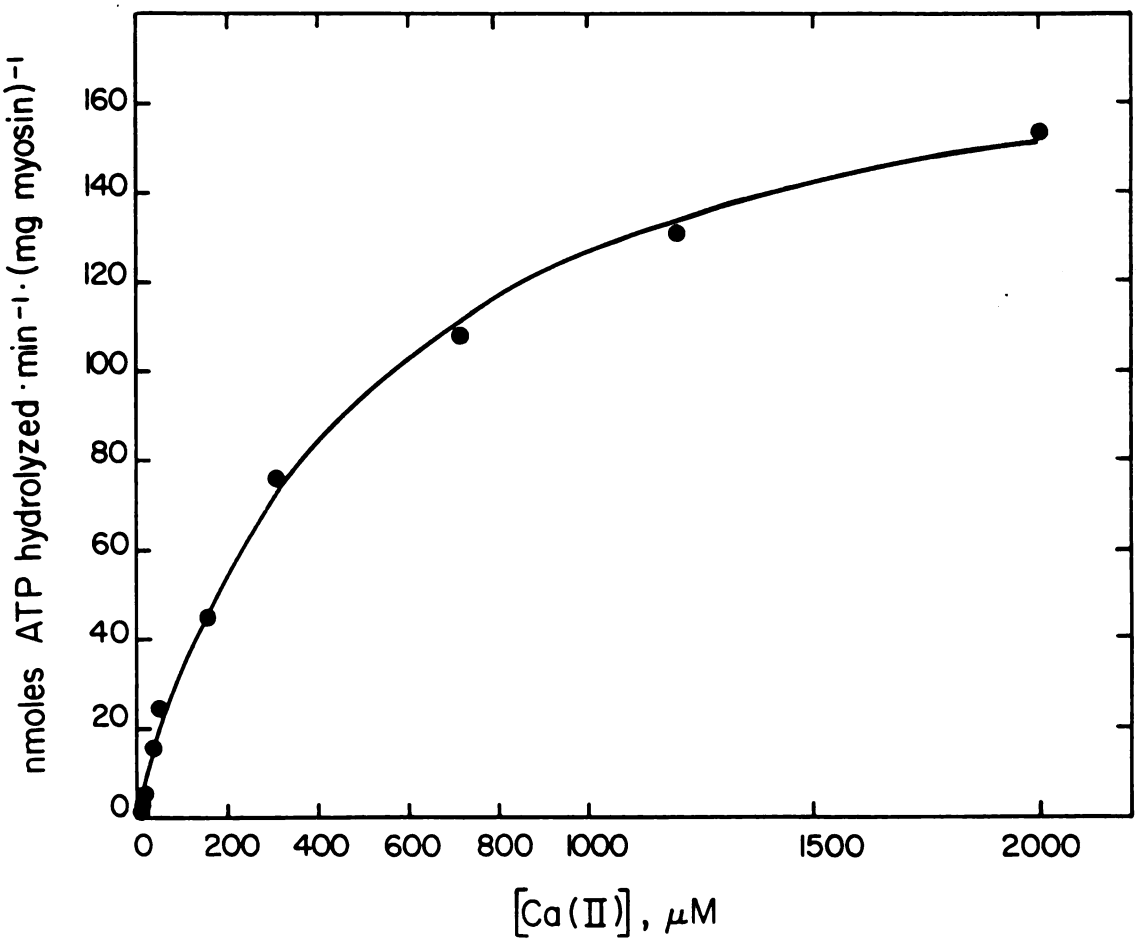

nmoles ATP hydrolyzed · min⁻¹ · (mg myosin)⁻¹
160
140
120
100
80
60
40
20
0
0
200
400
600
800
1000
1500
2000
[Ca(II)], μM

TABLE II.

APPARENT KINETIC CONSTANTS OF CHICK HEART DIVALENT CATION STIMULATED ATPases

ATPase[a]	DIVALENT CATION(S)	K_m[b] or K_I[c]	V_{max}[d]
		μM	
Mitochondria	Mg(II)	35 ± 5.4 [b]	33 ± 1.2
	Ca(II)	315 ± 61.8[b]	97 ± 5.8
Myosin	Mg(II)	6 ± 1.4 [c]	37 ± 2.6
	Mg(II) + 50 μM Ca(II)	17 ± 2.8 [c]	47 ± 1.9
	Ca(II)	493 ± 32.1[b]	187 ± 4.5

[a] As described under "Results," the term "ATPase" designates the preparation in which ATPase activity resides. It does not necessarily identify the ATPase(s) operating in the assay system.

[b] Concentration of divalent cation which gives half-maximal stimulation of total ATPase activity in the preparation assayed.

[c] Concentration of divalent cation which gives half-maximal inhibition of total ATPase activity in the preparation assayed.

[d] Maximal rates are expressed as nanomoles of ATP hydrolyzed/min/(mg of ATPase preparation).

site on myosin. Because the affinity of Mg(II) is so much higher than Ca(II), Ca(II) only weakly interferes with the binding of Mg(II) to myosin.

The kinetic data alone do not provide strong support for the hypothesis that myosin is the second ATPase in these mitochondria preparations. The Mg(II) titrations for the two systems (mitochondria and myosin/actin) show different behavior. The ATPase activity of the mitochondria preparation is stimulated by Mg(II), whereas myosin ATPase activity is inhibited by this cation. Some correlation is seen between the Ca(II) titrations for the two systems. Both are stimulated by Ca(II) (Fig's 17 and 18) and the apparent K_m's are similar (Table II). Immunoblotting afforded a more direct means of resolving this issue. As depicted in Fig 16, both actin and myosin are present in suspensions of mitochondria that were isolated with collagenase. This provides an unequivocal demonstration of the identity of the ATPases which contaminate suspensions of isolated heart mitochondria. The discrepancies between the kinetic data may be explained by differences in the degree to which the actin and myosin were associated in the samples of myosin and mitochondria that were tested.

X. Ca(II) UPTAKE BY CHICK HEART MITOCHONDRIA

Like most other mitochondria (Nicholls and Crompton, 1980), chick heart mitochondria transport Ca(II) from the extramitochondrial space into the matrix. As reported by the Ca(II) indicator Arsenazo III, uptake requires substrate and oxygen (Fig 19A). When the oxygen dissolved in the assay medium is exhausted, the Ca(II) is released back into the extramitochondrial space. In this experiment ADP was added to

Figure 19. Kinetics of Ca(II) Uptake and Release by Isolated Chick Heart Mitochondria

The reaction mixture contained 0.225 M mannitol, 0.075 M sucrose, 20 mM Tris-buffered P_i, pH 7.4, and 100 μM arsenazo III. Ca(II) transients were monitored by measuring the absorbance changes of arsenazo III at 685-675 nm with a dual wavelength spectrophotometer.

(A) M, mitochondria (0.25 mg/ml); P/M, 5 mM pyruvate/ 2.5 mM malate; ADP, 400 nmoles; R, 50 μM rotenone.

(B) M, mitochondria (0.25 mg/ml); P/M, 5 mM pyruvate/ 2.5 mM malate; R, 50 μM rotenone; S, 5 mM succinate.

ensure that anaerobiosis was achieved. Ca(II) release is unaffected by rotenone. The uptake of this cation, however, is inhibited by rotenone when P/M is used as oxidizable substrate (Fig 19B). By bypassing the rotenone-block with succinate, Ca(II) uptake is reinitiated. It is noted that in these experiments the mitochondria transported much more Ca(II) than they would in a typical oxygen consumption experiment because the commercial preparation of Arsenazo III used was contaminated with Ca(II) (A. Scarpa, personal communication).

DISCUSSION

I. UNCOUPLED RESPIRATION OF CHICK HEART MITOCHONDRIA

Respiration that is not coupled to the phosphorylation of ADP is stimulated by Ca(II) and by a Nagarse-sensitive factor presumably bound to the outer membrane of chick heart mitochondria (for a description of some of the properties of this factor, see Chapter 7). From the data in Table I it is clear that there are significant decreases in both state 3 and state 4 rates of respiration subsequent to the phosphorylation of each sequential equimolar addition of ADP. The likely basis for this behavior is that the Ca(II) in the extramitochondrial space is taken-up into the mitochondrial matrix in an energy-dependent manner. As the extramitochondrial concentration of Ca(II) decreases, a greater percentage of $\Delta\mu_{H+}$ may be committed to ATP synthesis and, consequently, the rate of substrate oxidation is decreased. In addition, it is possible that as the concentration of ATP and Ca(II) in the extramitochondrial space increase and decrease, respectively, the Nagarse-sensitive uncoupling factor dissociates from the outer membrane. This suggestion makes two pre-suppositions: (a) that the binding of this factor to mitochondrial membranes is a regulated phenomenon; and (b) that ATP and Ca(II) may play a role in regulating the strength with which the uncoupling factor binds to mitochondrial membranes. Additional experiments must be performed in order to validate the latter two points.

The stimulation of uncoupled respiration is attributable to other phenomena as well. The uptake of P_i, pyruvate, β-hydroxybutyrate, and

glutamate are energy-dependent processes. It is probable that until the concentrations of these solutes in the intra- and extramitochondrial spaces are equal, uptake into the matrix will continue. These substrate internalization processes constitute an energy drain. Differences in the rates of oxidative phosphorylation supported by different substrates are likely due to differences in the rates at which the dehydrogenases for these substrates can produce reducing equivalents. However, differences in the rates of uncoupled respiration during state 4 (assuming that they are corrected for Ca(II) transport and Nagarse-sensitive uncoupling activity) are probably due to two other factors: (a) the rate at which different substrates can be transported into the matrix; and (b) the degree to which different dehydrogenase activities can be regulated. Point (b) is supported by the experiments shown in Fig 4, which indicate that succinate oxidation is a very poorly controlled reaction; in contrast, the oxidation of pyruvate is subject to regulation by ADP.

II. ATPase ACTIVITY IN THE EXTRA-MITOCHONDRIAL SPACE

These studies suggest that the Ca(II) and Mg(II) stimulated ATPase activity of isolated chick heart mitochondria is due to a myosin contaminant and to the F_1F_0-ATPase molecules of broken mitochondria. The divalent cation sensitivity of respiration in isolated heart mitochondria is a well known phenomenon. By inhibiting part of the Mg(II)-stimulated ATPase activity of isolated bovine heart mitochondria with the F_1F_0-ATPase inhibitor protein (Cintron and Pedersen, 1979; Amzel and Pedersen, 1983), Barbour et al. (1984) concluded that their preparation was contaminated with a small population of broken mitochondria. Myosin contamination of many heart mitochondria preparations has been assumed but never demonstrated.

Though not quantitated in this study, it is also possible that the spontaneous hydrolysis of ATP induced by the binding of Mg(II) to this nucleotide contributes to the total ATPase activity of this mitochondria preparation (Terada et al., 1984).

The divalent cation-stimulated ATPase activity is an important cause of the concentration dependence of chick heart mitochondrial ADP:O ratios and state 4 rates of respiration. The ATPases and the adenine nucleotide translocase operate as a coupled enzyme system. Increases in ATP cleavage rates result from increases in the concentrations of Ca(II), Mg(II), and of the ATPases themselves. Ca(II) is released from these mitochondria in the absence of exogenous substrate; Mg(II) is released during P_i uptake (see Chapter 5 for experimental details). As the concentration of mitochondria is increased during an oxygen consumption assay, increasing amounts of all three of these components will be present. Thus, the concentration of ATP that is hydrolyzed to ADP during state 4 will increase as the concentration of mitochondria is increased. Increasing rates of ATP hydrolysis will lead to progressive decreases in ADP:O ratios and increases in state 4 rates of respiration. In most studies characterizing the respiratory parameters of mitochondria, high concentrations ($\geq$1.0 mg/ml) of these organelles are used. This will, particularly with heart mitochondria, lead to severe underestimates of ADP:O ratios and overestimates of state 4 rates. Depending upon the relative affinity of Ca(II) for the ATPases and the Ca(II) carrier systems of the mitochondria, ADP:O ratios would be expected to increase with each successive addition of ADP as the Ca(II) is cleared from the extramitochondrial space. The data reported herein are consistent with this hypothesis.

III. THE CRITICAL MITOCHONDRIAL CONCENTRATION

A plot relating state 4 rates of respiration to chick heart mitochondria concentration is complex. When mitochondria are isolated with collagenase, there is a highly reproducible concentration of mitochondria at which state 4 rates of respiration are at a minimum. Below the cmc, all of the state 4 respiration is due to uncoupled respiration (Ca(II) and substrate transport, uncoupling factor activity). Above the cmc state 4 respiration is due to both uncoupled respiration and respiration induced by the regeneration of ADP. It is of interest that oligomycin-sensitivity of state 4 respiration is only observable above the cmc. At the cmc, ADP will be present in the steady-state at approximately 0.2 μM. This is $0.01K_m$ of the adenine nucleotide translocase for ADP. Thus, at or below the cmc, respiration is probably stimulated by ADP to a degree that is below the sensitivity threshold of the oxygen polarograph.

To our knowledge, these are the lowest state 4 rates of respiration yet reported for any preparation of mitochondria. Under the conditions of these experiments, the diffusion of atmospheric oxygen back into the oxygraph vessel does not artificially depress the measured state 4 rates of chick heart mitochondria (Toth et al., 1986; see Fig 7 of Chapter 1). With respiratory control ratios approaching infinity during the oxidation of pyruvate and malate, it is apparent that these mitochondria must: (a) have an extremely low rate of flow through intrinsic proton leak pathways, and/or (b) some dehydrogenases can be rate-limiting for respiration under particular state 4 conditions. These data also suggest that at the cmc under the conditions prevailing during the period of

state 4 respiration subsequent to ADP_2 (i.e., when ADP, bound uncoupling factor, and extra-matrix Ca(II) concentrations are presumably minimal), the electron transport chain can come very close to equilibrium.

IV. ADP:O STOICHIOMETRIES FOR OXIDATIVE PHOSPHORYLATION

There is continued disagreement over the ADP:O (or ATP:O) stoichiometries of heart and liver mitochondria. In the following analysis it is assumed that, like rat liver mitochondria (Lemasters, 1984), the H+/ATP ratio of chick heart mitochondria is equal to 4. In the vast majority of studies, isolated mitochondria respire with RCRs ranging from 3-10. This indicates that the mitochondria are not well coupled. Therefore, in order to estimate the true mechanistic stoichiometry of oxidative phosphorylation, some workers have applied mathematical corrections to their data. These corrections take into account the degree of coupling (Lemasters, 1984) or allow for an extrapolation when the RCR is assumed to be infinite (Beavis and Lehninger, 1986). During the oxidation of pyruvate and malate, the RCRs of chick heart mitochondria at the cmc closely approach infinity (i.e., state 4 rates of respiration $\rightarrow$ 0). This indicates that, under these conditions, these mitochondria are almost perfectly coupled. No correction of the data should be necessary. The empirically obtained ADP:O ratios approximate to 3.5 during the phosphorylation of ADP_2 and ADP_3 (see Table I). Because ADP:O ratios approximate to 2.0 during succinate oxidation, this suggests that the H+/O stoichiometry of complex I in chick heart mitochondria is 6. The total H+/O stoichiometry for complexes III and IV would approximate to 8. Thus, based on a comparison of the ADP:O ratios obtained during the oxidation of pyruvate/ malate and of succinate, the data support a 14

proton model for oxidative phosphorylation.

Substrate-level phosphorylation is not taken into account by these studies. Therefore, it may be argued that the ADP:O ratios obtained with pyruvate/malate are inflated due to the phosphorylation of ADP by nucleoside diphosphokinase. β-Hydroxybutyrate is an NAD-linked substrate whose oxidation does not involve substrate-level phosphorylation. During the oxidation of this substrate, the average ADP:O ratio obtained subsequent to the addition of ADP_2 is 3.33. This ratio would be consistent with a 13 proton model for oxidative phosphorylation (5 for complex I and 8 for complexes III and IV), and is in excellent agreement with the studies of Lemasters (1984). Of interest is that the oxidation of α-ketoglutarate also results in ADP:O ratios consistent with a 13 proton model. The contribution of substrate-level phosphorylation to ATP synthesis during α-ketoglutarate and pyruvate/malate oxidation cannot be discerned from these data. Although 1-5 mM malonate is often included in the assay media used by other workers as a means of inhibiting substrate-level phosphorylation, assays on chick heart mitochondria show that even at 100 mM this compound does not completely inhibit succinyl CoA oxidation.

A surprising result of these studies is that coupling is relatively insensitive to RCRs. The RCRs obtained during the oxidation of glutamate/malate, α-ketoglutarate, and β-hydroxybutyrate are approximately equal. Yet, the ADP:O ratios obtained with glutamate/malate (2.8-3.0) are significantly lower than those obtained with α-ketoglutarate or β-hydroxybutyrate (≈3.3). The reason for this is not immediately apparent. One point that can be made is that glutamate oxidation results in a lower yield of ATP per atom of oxygen consumed when compared with the other pyridine nucleotide-linked substrates. It is possible that the stoichi-

ometry for oxidative phosphorylation is depressed by the ammonia produced during the glutamate dehydrogenase reaction. This assumes that ammonia, if present at sufficient concentrations, is moderately toxic to mitochondria. Substantiation of this possibility will require additional experimentation. Even though chick heart mitochondria are very poorly coupled during succinate oxidation, the empirical ADP:O ratios obtained with this substrate conform closely to the classically accepted value of 2.0.

V. CONCLUSIONS

These studies show that: (a) The ADP:O ratios and state 4 rates of respiration are highly dependent upon the concentration of mitochondria used to assay these parameters. (b) The isolated mitochondria are contaminated with divalent cation-stimulated ATPases identified as myosin and the F_1F_0-ATPase of broken mitochondria. These ATPases are activated by the Ca(II) and Mg(II) released from mitochondria. They are completely inhibitable by EGTA. (c) Uncoupled state 4 respiration can be abolished by treating the mitochondria with Nagarse and by pre-incubating mitochondria with substrate. The rate of uncoupled respiration is highly dependent upon the substrates used to drive respiration. For chick heart mitochondria oxidation of the substrate couple pyruvate/malate results in optimal respiratory parameters. (d) RCR values approaching infinity can be obtained under certain conditions. (e) The ADP:O ratios obtained during the oxidation of pyruvate/malate, α-ketoglutarate, and β-hydroxybutyrate are fractional and significantly greater than 3.0. These ATP/O stoichiometries suggest that the H+/O stoichiometry of chick heart mitochondrial oxidative phosphorylation is at least 13 and possibly as

high as 14. The ADP:O ratios obtained with glutamate and succinate conform closely to the classically accepted values of 3.0 and 2.0, respectively.

REFERENCES

Amzel, L.M., and Pedersen, P.L. (1983) Ann. Rev. Biochem. 52: 801-824.

Barbour, R.L., Ribaudo, J., and Chan, S.P. (1984) J. Biol. Chem. 259: 8246-8251.

Beavis, A.D., and Lehninger, A.L. (1986) Eur. J. Biochem. 158: 315-322.

Beavis, A.D. (1987) J. Biol. Chem. 262: 6165-6173.

Bencini, D.A., Wild, J.R., and O'Donovan, G.A. (1983) Anal. Biochem. 132: 254-258.

Bishop, P.D., and Atkinson, D.E. (1984) Arch. Biochem. Biophys. 230: 335-343.

Bogucka, K., and Wojtczak, L. (1971) Biochem. Biophys. Res. Comm. 44: 1330-1337.

Boyer, P.D. (1979) In Membrane Bioenergetics (Lee, C.P., Schatz, G., and Ernster, L., eds) pp. 461-479, Addison-Wesley, Reading, Massachusetts.

Brooks, S.P.J., and Suelter, C.H. (1986) Int. J. Biomedical Computing 19: 89-99.

Chance, B., and Williams, G.R. (1956) Adv. Enzymol. 17: 65-134.

Chance, B. (1972) FEBS Lett. 23: 3-20.

Chance, B. (1977) Ann. Rev. Biochem. 46: 967-980.

Cintron, N.M., and Pedersen, P.L. (1979) Methods Enzymol. 55: 408-414.

Day, D.A., and Hanson, J.B. (1977) Plant Sci. Lett. 11: 99-104.

Denton, R.M., and McCormack, J.G. (1985) Am. J. Physiol. 249: E453-E554.

Douce, R. (1985) In Mitochondria in Higher Plants: Structure, Function, and Biogenesis. Academic Press, New York.

Doussiere, J., Ligeti, E., Brandolin, G., and Vignais, P.V. (1984) Biochim. Biophys. Acta 766: 492-500.

Dow, D.S. (1967) Biochemistry 6: 2915-2922.

Erecinska, M., and Wilson, D.F. (1982) J. Membr. Biol. 70: 1-14.

Ernster, L. (1977) Ann. Rev. Biochem. 46: 981-994.

Estabrook, R.W. (1967) Methods Enzymol. 10: 41-47.

Ferguson, S.J. (1985) Biochim. Biophys. Acta 811: 47-95.

Flatt, J.P., Pahud, P., Ravussin, E., and Jequier, E. (1984) Trends Biochem. Sci. 5: 466-468.

Forman, N.G., and Wilson, D.F. (1983) J. Biol. Chem. 258: 8649-8655.

Goren, A.K., Wanders, R.J., Westerhoff, H.V., van der Meer, R., and Tager, J.M. (1982) J. Biol. Chem. 257: 2754-2757.

Hagihara, B., and Lardy, H.A. (1960) J. Biol. Chem. 235: 889-894.

Hansford, R.G., and Castro, F. (1981) Biochem. J. 198: 525-533.

Hassinen, I.E., and Hiltunen, K. (1975) Biochim. Biophys. Acta 408: 319-333.

Hinkle, P.C., and Yu, M.L. (1979) J. Biol. Chem. 254: 2450-2455.

Illsley, N.P., Coade, S.B., and Harkness, R.A. (1985) Placenta 6: 187-198.

Kaplan, R.S., Pratt, R.D., and Pedersen, P.L. (1986) J. Biol. Chem. 261: 12,767-12,773.

Klingenberg, M. (1980) J. Membr. Biol. 56: 97-105.

Laemmli, U.K. (1970) Nature (London) 227: 680-685.

Lemasters, J.J., and Sowers, A.E. (1979) J. Biol. Chem. 254: 1248-1251.

Lemasters, J.J., Grunwald, R., and Emaus, R.K. (1984) J. Biol. Chem. 259: 3058-3063.

Lemasters, J.J. (1984) J. Biol. Chem. 259: 13,123-13,130.

Mannervik, B. (1982) Methods Enzymol. 87: 370-390.

Markwell, M.A.K., Haas, S.M., Tolbert, N.E., and Bieber, L.L. (1981) Methods Enzymol. 72: 296-303.

Masini, A., Ceccarelli-Stanzani, D., and Muscatello, U. (1983a) J. Bioenergetics Biomembranes 15: 217-234.

Masini, A., Ceccarelli-Stanzani, D., and Muscatello, U. (1983b) Biochim. Biophys. Acta 724: 251-257.

Masini, A., Ceccarelli-Stanzani, D., and Muscatello, U. (1984) Biochim. Biophys. Acta 767: 130-137.

Miller, R.J., Dumford, S.W., Koeppe, D.E., and Hanson, J.B. (1970)

Plant Physiol. 45: 649-653.

Mitchell, P. (1979) Science (Washington, D.C.) 206: 1148-1159.

Nicholls, D.G. (1974) Eur. J. Biochem. 50: 305-315.

Nicholls, D.G., and Crompton, M. (1980) FEBS Lett. 111: 261-268.

Peterson, G.L., and Hokin, L.E. (1981) J. Biol. Chem. 256: 3751-3761.

Pietrobon, D., Zoratti, M., and Azzone, G.F. (1983) Biochim. Biophys. Acta 723: 317-321.

Pietrobon, D., and Caplan, S.R. (1985) Biochemistry 24: 5764-5770.

Pietrobon, D., Zoratti, M., Azzone, G.F., and Caplan, S.R. (1986) Biochemistry 25: 767-775.

Pollard, T.D., and Korn, E.D. (1973) J. Biol. Chem. 248: 4682-4690.

Pollard, T.D. (1982) Methods Cell Biol. 24: 333-371.

Pullman, M.E., and Monroy, G.C. (1963) J. Biol. Chem. 238: 3762-3769.

Robertson, J.G., Thomas, J.E., Gowing, L.R., Boland, M.J. (1984) Arch. Biochem. Biophys. 232: 337-347.

Tarjan, E.M., and Von Korff, R.W. (1967) J. Biol. Chem. 242: 318-324.

Terada, H., Ikuno, M., Shinohara, Y., and Yoshikawa, K. (1984) Biochim. Biophys. Acta 767: 648-650.

Toth, P.P., Ferguson-Miller, S.M., and Suelter, C.H. (1985) Proc. 13th Intn'l Cong. Biochem., vol 5, p.767.

Toth, P.P., Ferguson-Miller, S.M., and Suelter, C.H. (1986) Fed. Proc. 45(6): 1922.

Toth, P.P., Sumerix, K.J., Ferguson-Miller, S.M., and Suelter, C.H. (1988) FASEB J. 2(5): A1122.

Toth, P.P., Ferguson-Miller, S., and Suelter, C.H. (1986) Methods Enzymol. 125: 16-25.

Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Nat. Acad. Sci. U.S.A. 76: 4350-4354.

Tsien, R.Y. (1980) Biochemistry 19: 2396-2404.

Webb, M.R., and Trentham, D.R. (1980) J. Biol. Chem. 255: 8629-8632.

Westerhoff, H.V., Melandri, B.A., Venturoli, G., Azzone, G.F., and Kell, D.B. (1984) Biochim. Biophys. Acta 768: 257-292.

Wikman-Coffelt, J., Zelis, R., Fenner, C., and Mason, D.T. (1973) Prep. Biochem. 3: 439-449.

Wilkinson, G.N. (1961) Biochem. J. 80: 324-332.

Wilson, D.F., Stubbs, M., Veech, R.L., Erecinska, M., and Krebs, H. (1974) Biochem. J. 140: 57-64.

Zoratti, M., Favaron, M., Pietrobon, D., and Azzone, G.F. (1986) Biochemistry 25: 760-767.

Chapter 4

THE ADVANTAGES AND LIMITATIONS OF USING 90° LIGHT SCATTER TO MONITOR CHANGES IN THE VOLUME AND PYRIDINE NUCLEOTIDE CONTENT OF THE CHICK HEART MITOCHONDRIAL MATRIX

INTRODUCTION

Like many other membrane-enclosed compartments, mitochondria are highly responsive to the osmolality of the environment in which they are maintained. These organelles are able to rapidly adjust their volume as osmotic conditions change because they: (a) contain a large number of transport proteins capable of mediating the transmembrane flux of many different ions (LaNoue and Schoolwerth, 1985); (b) are comprised of a highly convoluted inner membrane which may contract or unfold, depending upon the activity of water in the matrix space; and (c) can accomodate significant matrix swelling without bursting their outer membrane due to the presence of an inter-membrane space. A large number of studies (reviews: Lehninger, 1962; Brierly, 1973; Garlid and Beavis, 1987) have demonstrated that mitochondria obey the Boyle-van't Hoff law (Tedeschi and Harris, 1958; Beavis et al., 1985) and thus are well-behaved osmometers.

The intensity of light that a suspension of mitochondria scatters is related to matrix volume (Tedeschi and Harris, 1955; Koch, 1961). For example, as the matrix shrinks, the intensity of light that is scattered away from the linear axis of observation (i.e., 0°) increases. This change is a consequence of an increase in the refractive index of the matrix as it becomes more compact. Because the transport of water across the mitochondrial inner membrane is essentially instantaneous (Beavis et al., 1985), light scattering measurements have been used for some time to conveniently and continuously monitor the apparent transport rates of

a variety of electrolytes and non-electrolytes (Raaflaub, 1953; Harris and Tedeschi, 1955; Packer, 1960, 1969; Jung et al., 1977). Garlid and Beavis (1985) have recently refined light scatter methodology as applied to mitochondria by making these measurements more quantitative and by correcting for structure-dependent changes in apparent transport as measured by swelling rates. However, because these authors monitored changes at 0° (optical density), they concluded that the method was insensitive to the measurement of solute transport in isotonic media.

The classical descriptions of light scatter by Rayleigh (1867), Mie (1909), and Debye (1947) have facilitated analysis of a number of physical systems. These and other more recent mathematical theories of light scatter phenomena (Bier, 1957; Koch, 1961; Melikhov et al., 1981; Twersky, 1983; Perrin and Chiapetta, 1985; Perrin and Lamy, 1986; Singham and Bohren, 1987), are highly sophisticated, but difficult to apply in meaningful ways to large biological systems such as mitochondria. There are three major reasons for this failure:

i.) Mitochondria are larger than the wavelengths of light with which they are typically irradiated during scatter experiments.

ii.) Mitochondria are not opaque, ideal spheres.

iii.) Concentrations of mitochondria used in experiments are far greater than those mathematically modeled in theoretically ideal situations.

Two techniques are most often used to measure changes in mitochondrial light scattering intensities. The first uses an optical system similar to that used to measure absorbance and views the intensity of light transmitted through the sample at a 0° angle (i.e., directly in-line with the collimated beam). This approach is called turbidimetry and

is a well-documented and popular method. The second technique allows one to view the intensity of light scattered at a 90° angle relative to the incident beam. This method is known as nephelometry. In this investigation, the intensity of light scattered by mitochondria is measured at both 0° and 90°. Since the optical properties of mitochondrial suspensions makes them unamenable to analysis using classical theory, a more direct empirical theory for light scattering measurements is outlined. This empirical approach makes the following assumptions:

i.) Light scattering and absorption can both be defined as the loss of photons from a collimated beam as it traverses the sample.

ii.) Measuring the scatter of light at an observation angle of 0° is similar to the process of measuring absorbance, except that in the former case there is a finite probability of recovering photons that at some point were deflected from the collimated beam. Clearly, in the case of absorbance, photons lost from the incident beam cannot be recovered.

iii.) Measurements of light scattered at 0° or 90° have been typically used for different applications; however, they are dependent upon the same phenomena. Consequently, light scatter measurements at the two angles are coupled.

In addition, it is shown that light scattering measurements at 90° are much more sensitive to changes in mitochondrial volume than those taken at 0°. The ability to obtain meaningful ratios of I and I_0 (i.e., absorbance) using the 0° detector makes turbidimetric measurements reproducible and accurate. However, these measurements have a limited range (10^3 or A ≈ 3.0) and their sensitivity is limited by the reference beam intensity. Nephelometric measurements, on the other hand, are open-ended and have a wide range (10^6, not normalized). At 90° one starts from virtual darkness in the absence of scattering particles, thereby enabling a measurement of much greater sensitivity. This results in

kinetic measurements of increased signal-to-noise ratios.

EXPERIMENTAL PROCEDURES

I. INSTRUMENTATION

The instrument used for the spectroscopic investigations reported herein is a computerized spectrofluorometer (utilizing the optical components of a Perkin-Elmer Model 512) capable of performing simultaneous fluoresence and absorption measurements and of automatically correcting for the artifacts of fluorescence measurement. The system configuration is shown in Fig 1. Two photovoltaic cells (PVC, Hamamatsu Type S1337-1010BQ) were added to the cell compartment in order to measure the intensity of the incident beam of light before entering (R, reference) and after passing through (S, sample) the cuvette. A quartz plate is used to reflect approximately 4% of the light from a xenon lamp source toward the R detector. A concave mirror is employed to focus transmitted light onto the S detector. A photomultiplier tube (Hamamatsu R-446) is located at 90° to the collimated light source. The two PVC's and the photomultiplier tube provide the necessary means of detection in order to measure light intensities at 0° and 90°. In addition, the absorbance [log(R/S)] can be monitored simultaneously. Neutral density filters (Turner, 1% and 10% T) were used to adjust the intensities of light when required.

A PDP/8e computer is dedicated to system control and data collection, processing, and outputting. The three detector outputs are amplified using a FET operational amplifier (Texas Instruments, TL081C), filtered, directed through a four channel CMOS mutliplexer (Datel Systems, MXD-

Figure 1. Design Features of the Integrated Spectrofluorometer/ Spectrometer

(A). System configuration of the integrated spectrofluorometer/ spectrometer used for light scattering measurements.

(B). Optical diagram of instrumental components. Key:

1, xenon lamp/excitation source;

2, excitation monochromator;

3, quartz plate;

4, sample cell;

5, front-surface concave focussing mirror;

6, emission monochromator;

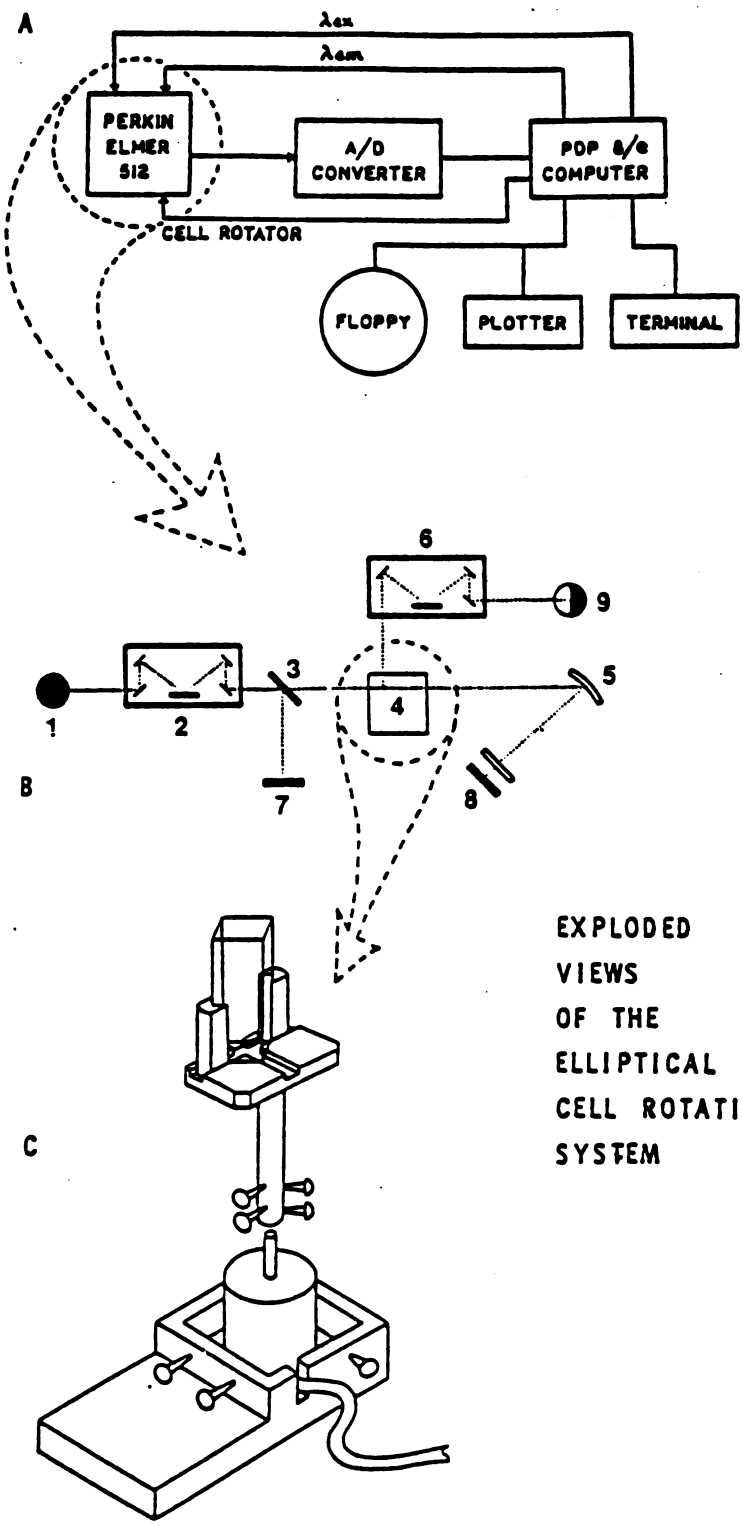
7, reference beam detector;

8, sample beam detector (or "S" detector);

9, fluorescence beam detector (photomultiplier tube).

For light scattering measurements, component 8 is used to detect 0° scattering and component 9 is used to detect 90° scattering with components 2 and 6 set at the same wavelength.

(C). Off-center sample cell rotator device employed for absorption corrected fluorescence and scattering measurements.



EXPLODED VIEWS OF THE ELLIPTICAL CELL ROTATION SYSTEM

409), and sent to an A/D converter (Analogic, MP2112) for processing under computer control. Each reading represents the average of 40 points collected over a 250 msec period in order to minimize noise. A calibration table is utilized to correct for any imbalance in the optical system and to convert measured light intensities into quanta. Real time output is achieved using a Tektronix 2000A graphics terminal. Data storage is accomplished by a floppy disk drive system (Data Systems, SA800/801), and a hard copy is obtained by a Houston Instruments plotter. A rotating cell pedestal is mounted in the spectrofluorometer's sample chamber. The design details of the system are described elsewhere (Adamsons, 1985). The off-axis rotation of the cell-rotator produces variations in the pathlength through which the incident beam of radiation must pass.

II. ISOLATION OF MITOCHONDRIA

Heart mitochondria were isolated from 14-21 day-old chicks using Nagarse, as previously described (Chance and Hagihara, 1961). Chick heart ventricular myocardium was minced into an ice-cold isotonic isolation medium that contained 225 mM mannitol, 75 mM sucrose, and 20 mM Tris, pH 7.4 (MST). The myocardium was then homogenized with five up-and-down strokes of a Potter-Elvejhem pestle (operated at 500 r.p.m.) in the presence of approximately 0.5 I.U. Nagarse/g muscle. The homogenized tissue was allowed to incubate on ice for 3 min, and was then subjected to two additional up-and-down strokes of the pestle. The crude homogenate was centrifuged for 5 min at 800g. The resulting supernatant liquid containing the mitochondria was filtered through two layers of cheesecloth and centrifuged at 8,000g for 10 min. The mitochondria were then rinsed twice by resuspending these organelles in 7-8 ml of MST and

centrifuging at 8,000g for 10 min. The final washed mitochondrial pellet was resuspended in MST at 15-20 mg/ml. All experiments were completed within 4-5 hrs after isolation of mitochondria.

THEORETICAL CONSIDERATIONS

I. THE EFFECT OF PHOTON RECOVERY ON LIGHT SCATTERING MEASUREMENTS

One may consider the efficiency of scatter to be related to the efficiency of absorption (absorptivity) and the probability of a photon encountering a surface from which it may be scattered. If one assumes that in very dilute solutions of the scattering species no multiple scatter encounters occur, then even in the absence of absorption it would be reasonable to expect that when measured at 0° the scattering species obeys the Beer-Lambert law, expressed as:

$$\log(I_0/I) = \alpha bc \qquad (1)$$

where I_0 is the intensity of the incident beam, I is the intensity of the transmitted beam, c is the concentration of the scattering species, α is the absorptivity of the scattering species, and b is the pathlength of the sample through which the light must pass. However, as the concentration of the scattering species is increased, the likelihood for scattered photons to undergo sequential internal reflections and refractions also increases. This creates a probability of recovery whereby a fraction of the scattered radiation is redirected toward, and eventually reaches, the detector. Herein lies the major difference between absorption and scatter measurements. In the former case only one type of encounter occurs, namely the absorption and loss of a photon from the collimated beam, while in the latter sequential scattering events may take place and photons lost from the beam may be recovered. Thus, the actual

intensity of light measured by the detector in a mitochondrial light scattering experiment is greater than what would be expected from the Beer-Lambert relationship because of the recovery of increasing amounts of scattered light as the concentration of mitochondria is increased.

For the purpose of the following considerations, it is assumed that the sample pathlength is equal to 1 cm and, therefore, b will be omitted from the equations which follow. The Beer-Lambert law (eqn 1) holds for dilute suspensions of scattering particles. However, as the concentration of the scattering species is increased, the intensity of the transmitted beam increasingly deviates from the theoretical intensity that would satisfy the Beer-Lambert law. This deviation may be expressed as follows:

$$I_s = I \times (I_0/I_s)^{\alpha cR} \qquad (2)$$

where I_s is the intensity of the observed beam in the presence of scattering, I is the theoretical intensity of light expected if beam attenuation is due to a single encounter only, I_0 is the intensity of the incident beam, α is the probability of scatter, and R represents the probability of recovering photons. The value of R is a concentration-dependent factor but extrapolates to a constant at extremely high concentrations of mitochondria. The ratio I_0/I_s may be interpreted as the reciprocal of the light transmitted by the sample. For samples in which photons may have only one possible type of interaction with the suspended particle (i.e., absorption or a single scatter event), R = 0 and eqn 2 reduces to the Beer-Lambert law.

Solving for I in equation 2 we obtain

$$I = I_s \times (I_0/I_s)^{-\alpha cR}. \qquad (3)$$

This expression may be substituted into equation 1:

$$\log \frac{I_0}{I_s(I_0/I_s)^{-\alpha cR}} = \alpha c. \qquad (4)$$

Equation 4 may be rearranged to the following:

$$\log(I_0/I_s) + \alpha cR \times \log(I_0/I_s) = \alpha c. \qquad (5)$$

By substituting the measured absorbance, A, for $\log(I_0/I_s)$, the above may be rewritten as

$$A + \alpha cRA = \alpha c. \qquad (6)$$

After factoring out A and taking the reciprocal of both sides, absorbance can be related to the recovery coefficient as specified by equation 7:

$$1/A = 1/\alpha c + R \qquad (7)$$

Thus, in situations where absorption by the scattering species is negligible, α and R may be inferred from the slope and y-intercept, respectively, of the double reciprocal plot relating the concentration of mitochondria (mg/ml) to changes in optical density.

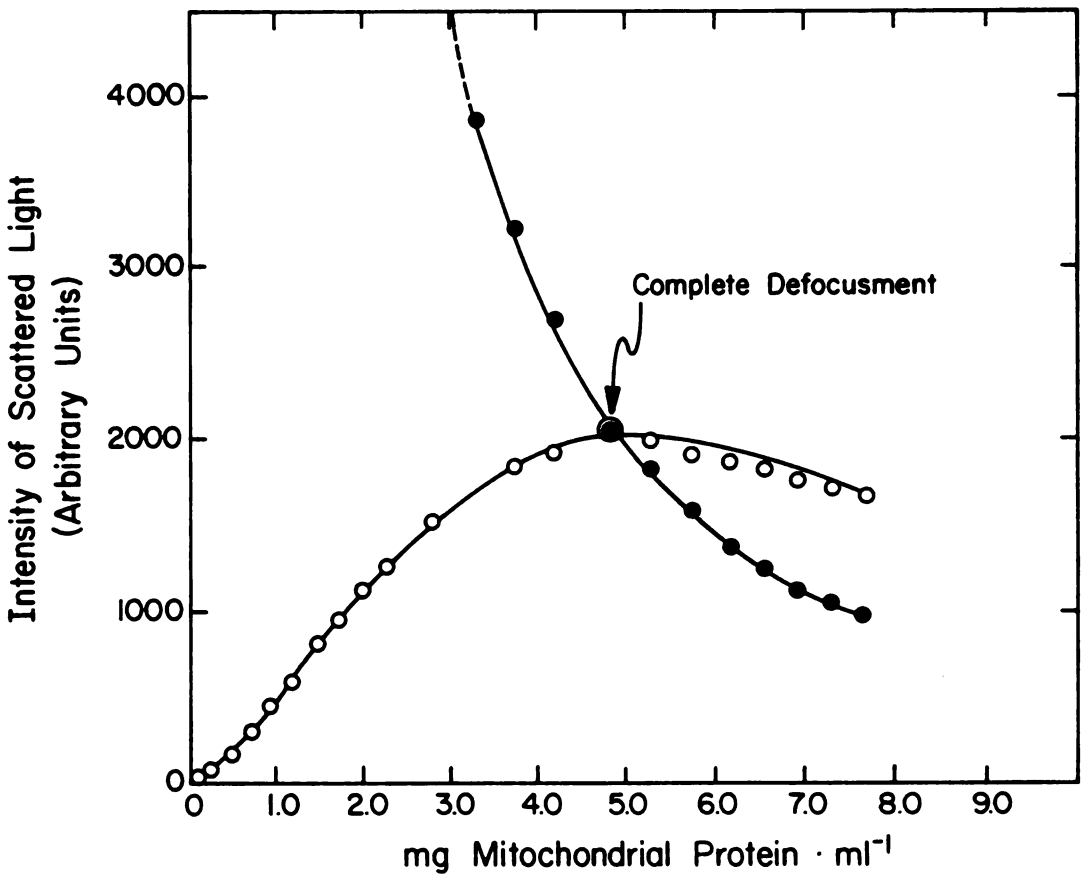
II. CORRELATION OF THE INTENSITY OF LIGHT SCATTERED AT 0° AND 90°

Since the detectors situated at 0° and 90° are measuring changes in the same scattering processes, one expects that the rate of photon recep-

tion for each will vary as a function of the concentration of scattering particles. Assuming no absorption occurs, the limits within which we can consider the scattering phenomena are from: (a) the completely focused beam, maintained only in the absence of scattering particles; and (b) the completely defocused beam, in which sequential internal reflections and refractions result in the uniform scattering of photons throughout the spherical dimensions of the observation compartment. It follows that the intensity of light reaching the 0° detector will be at a maximum when there are no scattering particles in the cuvette. As the concentration of scattering particles increases, increasing amounts of light will be diverted from the focused beam. All of the scattered light will not reach the detector; hence, the detector sees less and less light (Fig 2). As the population of scattering particles increases, the probability for multiple sequential scattering of photons increases since the light, after the initial scattering, will encounter a greater and greater number of surfaces prior to exiting the cuvette. As this occurs a finite probability exists for a photon originally scattered from the focused beam to be redirected toward the 0° detector. Consequently, all of the photons removed from the focused incident beam are not lost, since a fraction of them will be recovered when sequential scattering processes occur. Eventually, a concentration of scattering particles is attained in which all of the light in the incident beam is subject to multiple scattering events. At this point the beam is completely defocused. In the ideal case, the light in all directions of the observation system will be of equal intensity. This means that the fraction of the scattered light striking the 0° detector can be calculated from the following relationship:

Figure 2. Intensity of Scattered Light at 0° and 90° Measured as a Function of Mitochondria Concentration

Measurements at 0° (●) represent changes in the intensity of transmitted light or, simply, changes in optical density. Measurements at 90° (◯) represent changes in the intensity of scattered light. For this experiment the sensitivity of the S detector and the photomultiplier tube were set equal. Samples were irradiated with light having a wavelength of 500 nm.


Intensity of Scattered Light
(Arbitrary Units)
4000
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Complete Defocusment
0
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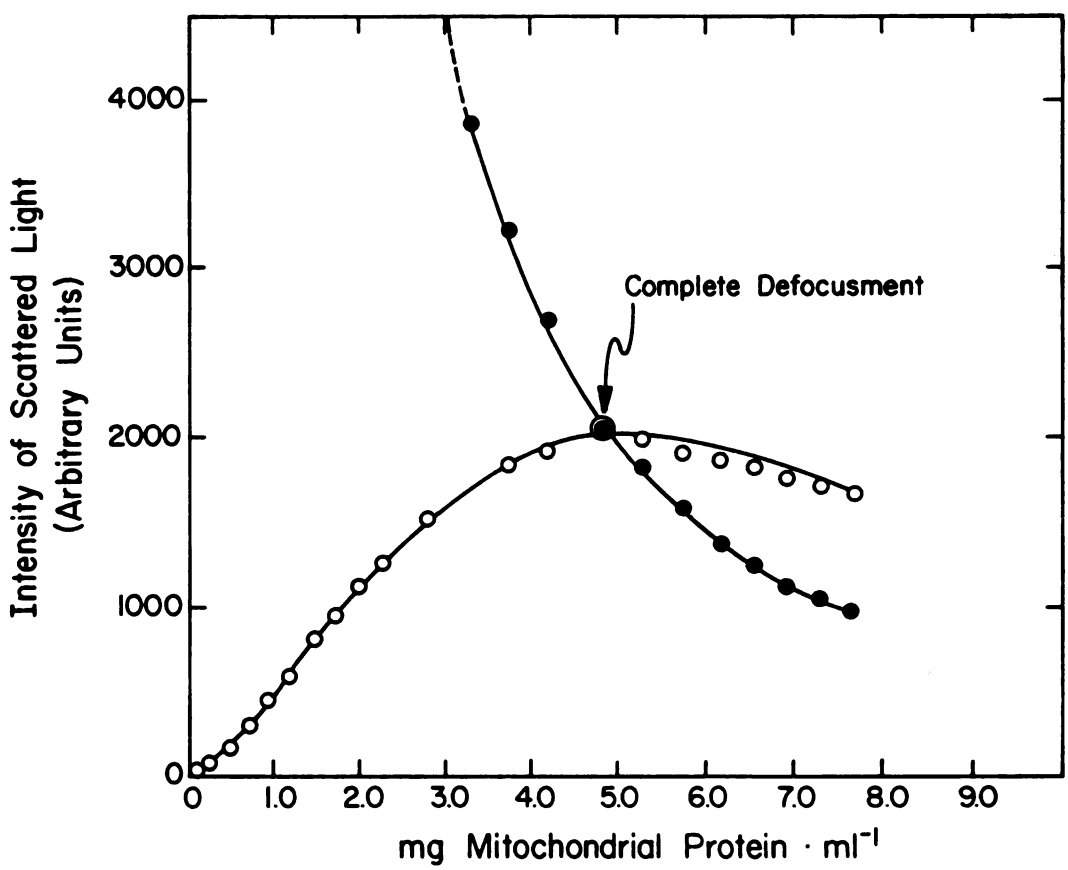
8.0
9.0
mg Mitochondrial Protein · ml⁻¹


Intensity of Scattered Light
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4000
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Complete Defocusment
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4.0
5.0
6.0
7.0
8.0
9.0
mg Mitochondrial Protein · ml⁻¹

$$I = I_0 \frac{A_d}{4/3\pi r^2} \qquad (8)$$

where I is the intensity of the detected radiation (photons per unit time), I_0 is the intensity of the incident beam, A_d is the area of the detector, and r is the distance from the detector to the center of the observation sphere.

The scenario for the 90° detector will be opposite to that of the 0° detector. In the absence of scattering particles there is virtually no light striking the detector. As the concentration of scattering particles increases, increasing amounts of light will be directed to the detector. When measured as a function of mitochondria concentration, the intensity of light scattered at 90° is non-linear (Fig 2). Above 0.5 mg mitochondria/ml, there is an abrupt increase in the slope of the light scatter function. In this concentration range, multiple sequential scattering becomes significant, increasing the overall scattering efficiency. As for scatter measured at 0°, the probability of photon recovery increases with increasing concentrations of the scattering particles until a concentration of scattering particles is reached at which the incident beam is completely defocused. This concentration corresponds to the point where the intensity of light striking the detectors at 0° and 90° are equal. In the ideal case, as the concentration of scattering particles is further increased, the light intensity would remain the same for both detectors because the beam is completely defocused and additional scattering has no effect on the fraction of light striking either detector. In the real case this does not occur for two reasons:

i.) The scattering particles absorb a fraction of the incident beam. (Very small absorbances can become significant with multiple sequential scattering as each scattering event represents an additional opportunity for photons to be absorbed.)

ii.) Back scatter of the incident radiation.

As shown by the data presented in Fig 2, the light intensity corresponding to complete defocusing is not maintained with increasing concentrations of the scattering particle. As higher concentrations of mitochondria are added to the system, the probability of encounter between mitochondria and photons in the incident beam greatly increases, thereby reducing the mean distance into the solution that the individual photons travel prior to their first encounter with a scattering surface. This gives rise to a back scatter phenomenon which begins to redirect the light into the path of least resistance, i.e. in the direction of the light source. In the extreme, if sufficient mitochondria concentrations were attained, the wave-fronts of light would reflect off of mitochondria as from a plane surface, in accordance with Snell's law. An analysis of back scatter phenomena predicts that it would manifest itself first in the data received from the 0° detector and, subsequently, in the data received from the 90° detector. This is intuitively obvious from the geometrical relationship between the light source, the detector at 90°, and the detector at 0° (i.e., the incident light can travel a smaller mean distance in order to reach the 90° detector as compared to the one at 0°). Thus, as back scatter becomes significant, it will attenuate the intensity of light reaching the 0° detector more than that reaching the 90° detector. Careful consideration of Fig 2 indicates that this appears to occur in the actual experiment. What is useful information

from these data, however, is identification of the point of complete beam defocusment. This point is chosen as the maximum in the response curve of the 90° detector. This point can be a well-defined empirical quantity delineating the upper limit of a calibration scheme for light scatter measurements of dense suspensions.

RESULTS AND DISCUSSION

I. RELATIONSHIP BETWEEN LIGHT SCATTER INTENSITY AND MEDIUM OSMOLALITY

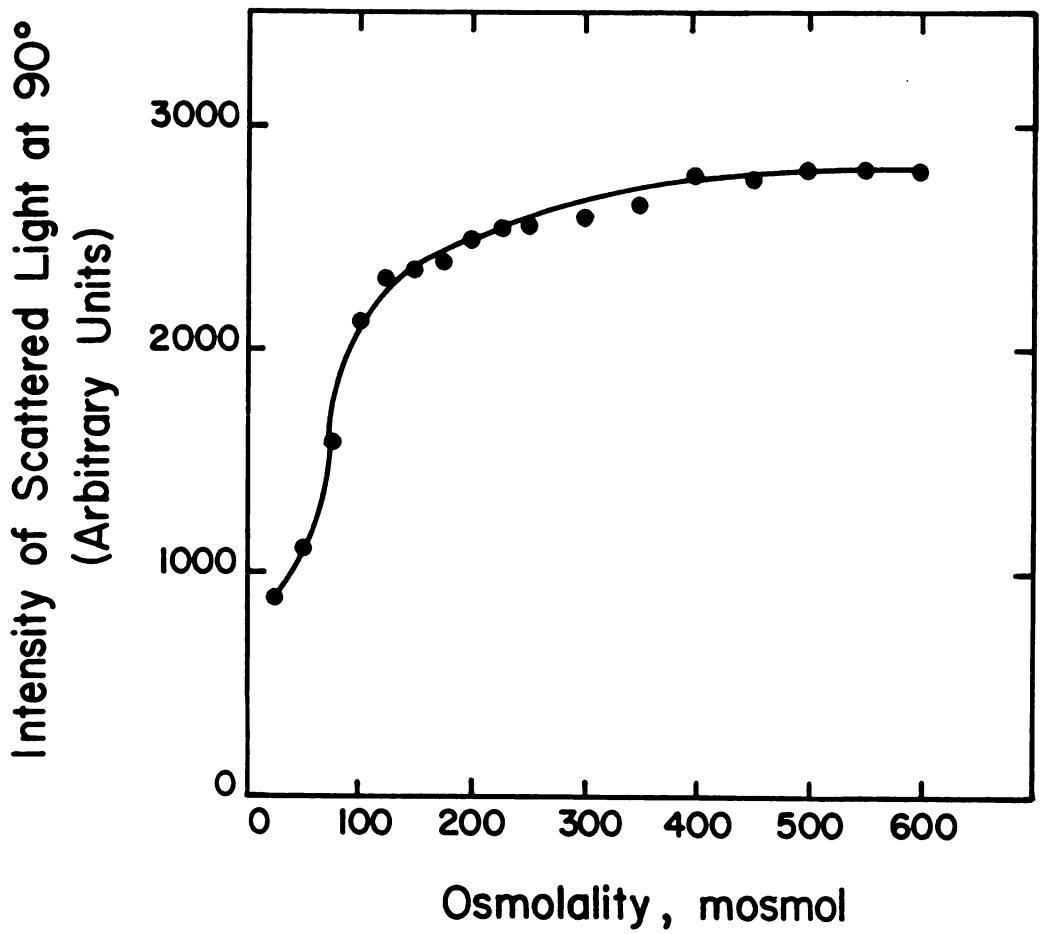
The intensity of light that chick heart mitochondria scatter depends upon the osmolality of the medium in which they are suspended (Fig 3). As the medium osmolality is decreased from 600 to 125 mosmol, mitochondrial matrix volume increases as indicated by a decrease in the intensity of scattered light. However, as the medium osmolality is decreased below approximately 125 mosmol, the extent of mitochondrial swelling increases sharply. Below 70 mosmol the amount of change in matrix volume induced by increasingly lower osmolalities decreases. These data are in close agreement with the findings of Beavis et al. (1985) which show that between 68 and 115 mosmol mitochondria undergo an irreversible structural transition. This transition involves rupture of the outer membrane and unfolding of the inner membrane. Thus, the volume changes above 115 mosmol correspond to matrix swelling proceeding within the constraints of the outer membrane; those occurring below ≈70 mosmol correspond to matrix swelling unrestricted by the outer membrane.

II. RELATIONSHIP BETWEEN LIGHT SCATTER INTENSITY AND WAVELENGTH OF LIGHT

The efficiency with which chick heart mitochondria scatter light varies with the wavelength of light used to irradiate these organelles. In order to measure this scattering efficiency, mitochondria were sus pended in a solution that contained 0.225 M mannitol and 0.075 M sucrose. The excitation and emission monochromators of the computerized spectro-

Figure 3. Responsiveness of the Chick Heart Mitochondrial Matrix to Changes in External Osmolality

Mitochondria (0.50 mg/ml) were suspended in 2.0 ml of M/S media of the indicated osmolality. The molar ratio of mannitol:sucrose was held constant at 3:1 in all assays. Once the photomultiplier tube output signal stabilized (indicating that the mitochondrial matrix volume had come to osmotic equilibrium), the intensity of light (500 nm) scattered at 90° was measured.


Intensity of Scattered Light at 90°
(Arbitrary Units)
3000
2000
1000
0
0
100
200
300
400
500
600
Osmolality, mosmol

fluorometer were coupled, and the intensity of light scattered at 90° was measured. Between 500 and 700 nm, the intensity of 90° light scattered by these mitochondria varies exponentially with wavelength (Fig 4). Wavelengths smaller than 500 nm were not used because mitochondrial cytochromes and other components absorb significant amounts of such light.

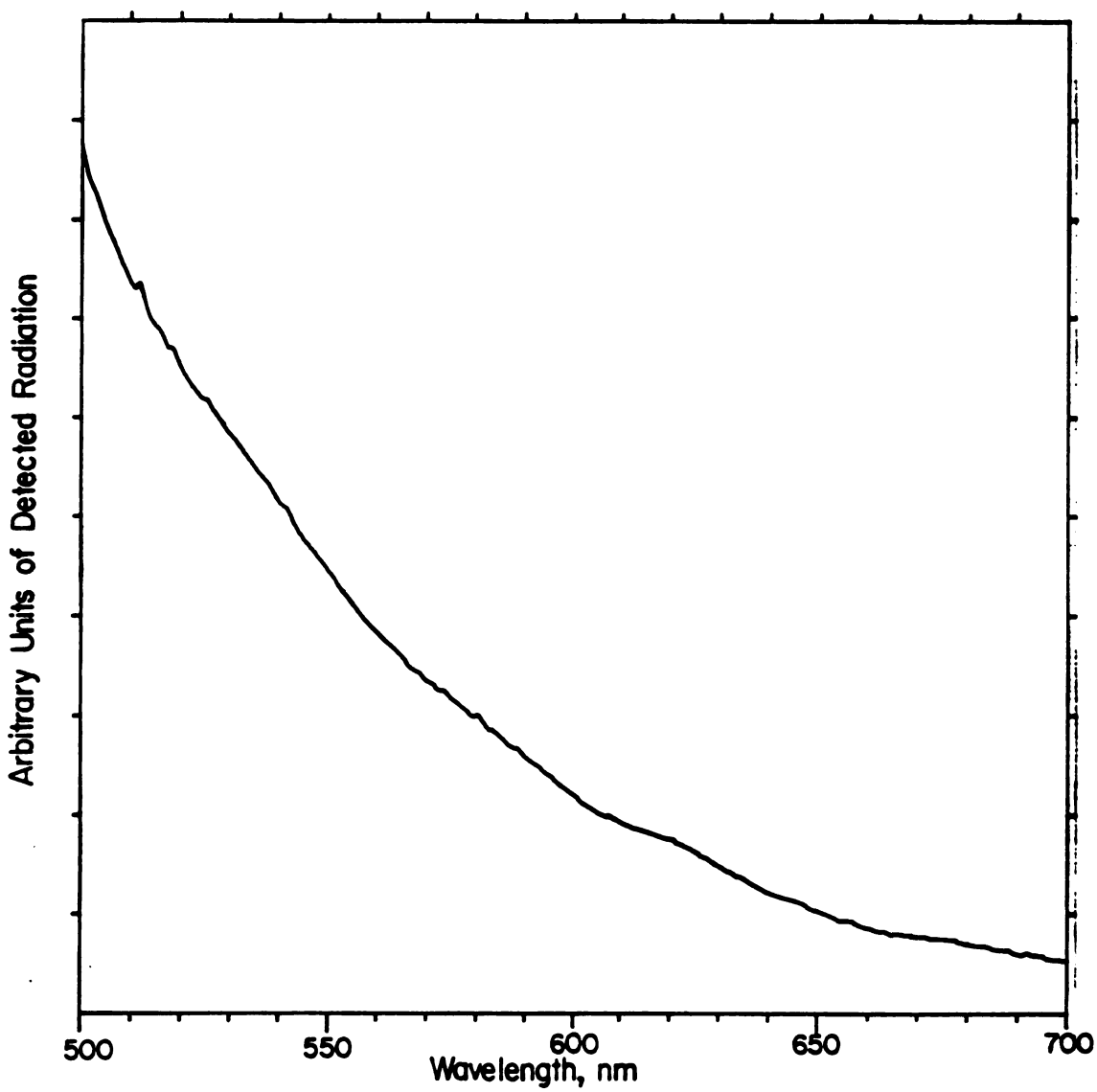
III. MEASUREMENT OF CHANGES IN MATRIX NADH LEVELS

As demonstrated by Chance and Williams (1955), dual wavelength spectroscopy can be used to continuously monitor relative changes in mitochondrial matrix NADH levels. In this technique mitochondria are irradiated with two wavelengths of light: 340 nm (the absorption maximum of NADH) and 374 nm (the reference wavelength). Changes in the intensity of transmitted light result from increases or decreases in absorbance and the scattering of light. Clearly, the technique derives its sensitivity from being able to accurately measure changes in absorbance as matrix NADH concentrations change. If the intensity of light scatter remains uniform as the metabolic state of mitochondria is varied, then dual wavelength spectroscopy will give reliable measurements of NADH levels. However, because mitochondria swell or shrink in response to various treatments, the efficiency with which these organelles scatter light will change at the same time NADH concentrations are changing. This combination of effects makes the interpretation of data obtained with dual wavelength measurements problematic.

In most types of mitochondria yet tested, pyridine nucleotides undergo net oxidation during state 3 respiration. A notable exception to this is the blowfly flight muscle mitochondrion in which pyridine

Figure 4. Intensity of Light Scattered by Chick Heart Mitochondria Measured as a Function of Wavelength

Mitochondria were suspended in 2.0 ml of an osmotic support medium comprised of 0.225 M mannitol and 0.075 sucrose at 0.35 mg/ml. The wavelength of light with which these organelles were irradiated was varied from 500-700 nm, and the intensity of light scattered at 90° was measured. The emission and excitation monochromators were coupled (i.e., they were continuously adjusted in synchrony).


Arbitrary Units of Detected Radiation
500
550
600
650
700
Wavelength, nm

nucleotides undergo net reduction after the addition of ADP (Hansford and Chappell, 1968; Hansford, 1972). As measured by dualwavelength spectrophotometry (340-374 nm), chick heart mitochondrial pyridine nucleotides undergo an apparent net reduction during state 3 respiration when oxidizing a variety of NAD-linked substrates (Fig 5A). After the addition of ADP, absorbance at 340 nm relative to that at 374 nm increases. Once the ADP is phosphorylated to ATP, the differential absorbance decreases. As a control NAD was reduced with excess borohydride. As expected this reaction results in an upward deflection of the trace (Fig 5B). In contrast to chick heart mitochondria, rat liver mitochondria experience a significant decrease in the apparent redox state of matrix pyridine nucleotides during state 3 (Fig 6). Because this difference in NADH production and utilization could have important metabolic consequences for chick heart muscle, additional experiments were conducted to affirm whether the information obtained with dual wavelength spectrometry could be interpreted in a straightforward manner.

The computerized spectrofluorometer was used to differentiate between absorbance and any artifacts due to changes in the intensity of light scatter. In order to do this, three types of measurements were made. The first was conventional absorbance of the excitation radiation or "A1". The two other measurements exploit the capability of this instrument to rotate the sample cell between three different viewing positions (Fig 7A). Rotation of the sample cell allows one to vary the solution thickness through which the incident radiation must pass. By measuring the emission of a sample at positions 1 and 4 (Fig 7B), it is possible to measure the amount of light that is lost, due to absorption

Figure 5. Apparent Changes in the Absorbance of Chick Heart Mitochondrial Matrix Pyridine Nucleotides During State 3 to State 4 Transitions

Mitochondria (0.1 mg/ml) were suspended in 2.0 ml of a buffer that contained 0.225 M mannitol, 0.075 M sucrose, 20 mM Tris-buffered P_i, and the substrate(s) indicated below. 418 nmols of ADP were added at the times shown. Absorbance changes at 340-374 nm were measured with a dual beam spectrophotometer. Reduction is indicated by an upward deflection in the trace.

(A). 5 mM pyruvate and 2.5 mM malate

(B). 5 mM α-ketoglutarate

(C). 5 mM β-hydroxybutyrate

(D). 5 mM glutamate and 2.5 mM malate

The lower panel shows a control reduction of 1.2 μmoles of NAD with excess sodium borohydride in the absence of mitochondria.

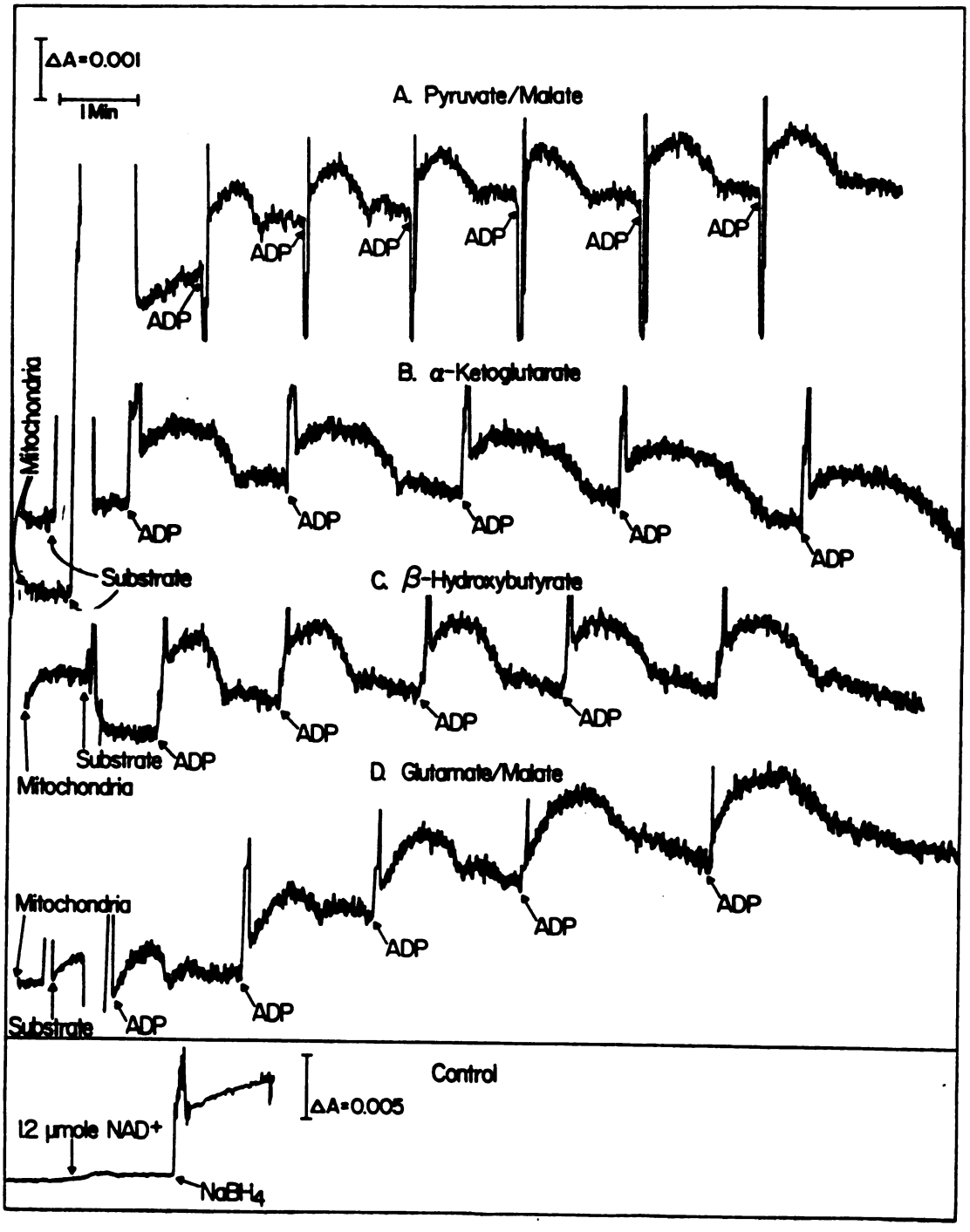

ΔA=0.001
1 Min
A. Pyruvate/Malate
ADP
ADP
ADP
ADP
ADP
ADP
Mitochondria
B. α-Ketoglutarate
ADP
ADP
ADP
ADP
ADP
Substrate
C. β-Hydroxybutyrate
ADP
ADP
ADP
Substrate
ADP
Mitochondria
D. Glutamate/Malate
Mitochondria
ADP
ADP
ADP
ADP
Substrate
ADP
ADP
Control
ΔA=0.005
12 µmole NAD+
NaBH4

Figure 6. Apparent Changes in the Absorbance of Rat Liver Mitochondrial Matrix Pyridine Nucleotides During State 3 to State 4 Transitions

Experimental conditions were as described in the legend to Fig 5. Oxidation is indicated by a downward deflection in the trace. The substrate(s) used are as indicated below.

(A). 5 mM glutamate and 2.5 mM malate

(B). 5 mM succinate

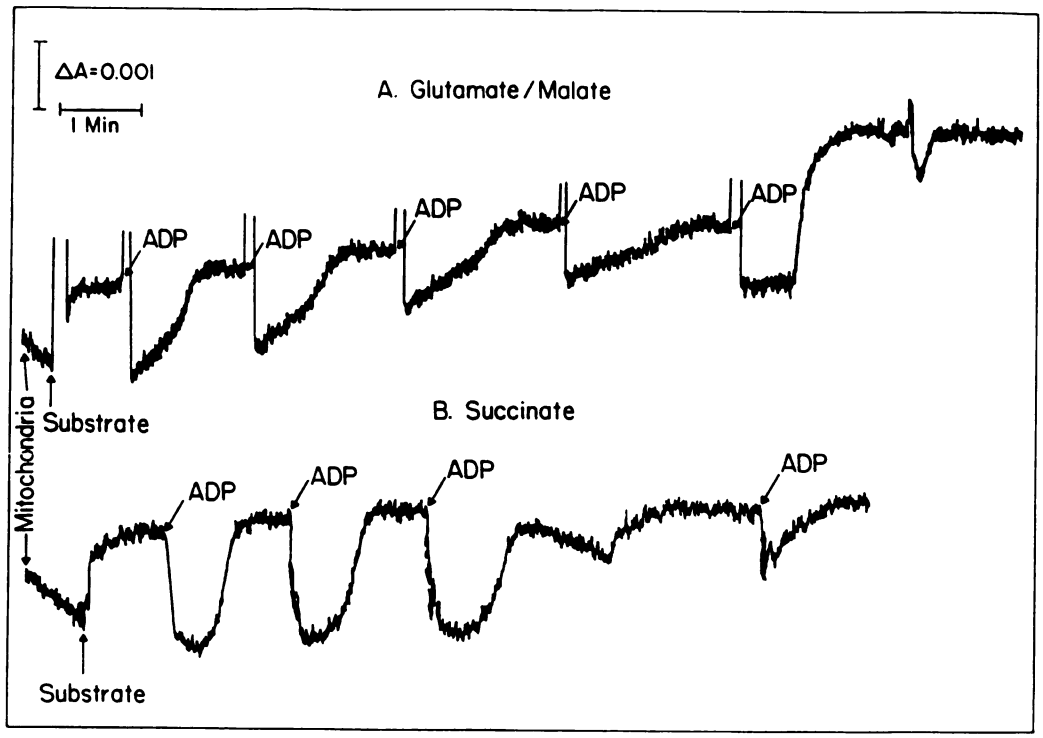
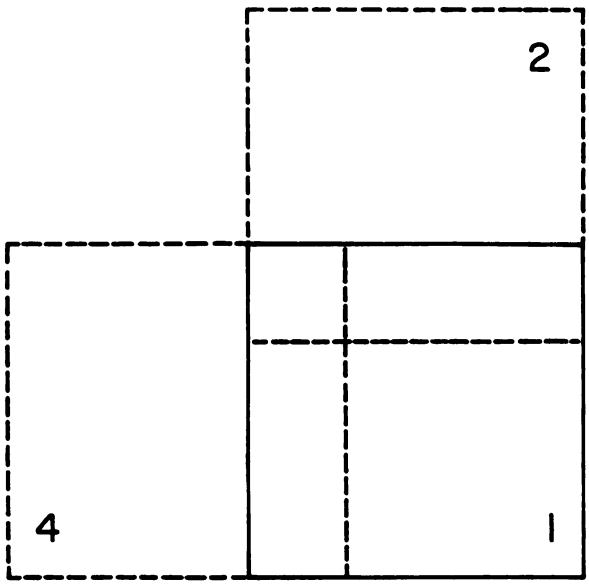

ΔA=0.001
1 Min
A. Glutamate / Malate
ADP
ADP
ADP
ADP
ADP
Mitochondria
Substrate
B. Succinate
ADP
ADP
ADP
ADP
Substrate

Figure 7. Off-Center Sample Cell Rotation

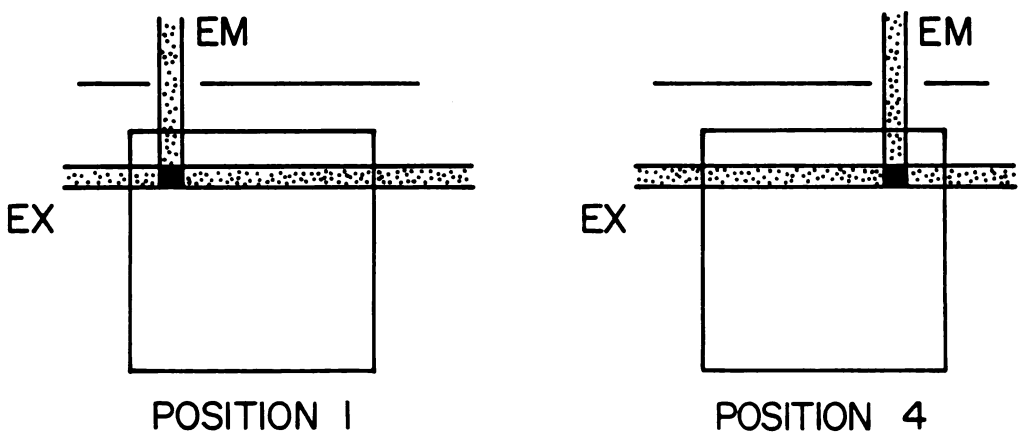
(A). Positions 1, 2, and 4 of the off-center sample cell rotation system.

(B). Schematic depiction of sample penetration by the incident beam of light in positions 1 and 4. The beam denoted "EX" is the excitation or incident light; the beam denoted "EM" is the sample emission or scattered light.

A.



B.



or scatter, between the two viewing positions. The latter measurement, designated "A2", is thus based on fluorescence and is described by the following relationship:

$$A2 = G3\log(F1/F4) \qquad (9)$$

where F1 and F4 are the fluorescence intensities at positions 1 and 4, respectively, and G3 is a geometric factor defined as $b/(\phi_2 - \phi_1)$, where b is the pathlength of the sample cuvette and the ϕ values are the centers of the fluorescence observation windows. The third type of measurement was simply relative fluorescence intensity at position 1 (F1).

The instrumental outputs of these functions are shown in Fig 8A-C. In agreement with results obtained with differential absorption spectrometry, there is an increase in A1 after the addition of ADP to chick heart mitochondria pre-incubated with P_i and pyruvate/malate (Fig 8A). This indicates that the mitochondria absorb a greater amount of 340 nm light during state 3 when compared to state 4. The F1 (Fig 8B) and A2 (Fig 8C) measurements both show, however, that the emission of 450 nm light decreases as the mitochondria undergo a state 4 to state 3 transition. Fluorescence measurements therefore suggest that matrix NADH concentrations decrease during state 3 respiration. The question arises: Why do the results obtained with absorbance and fluorescence measurements conflict with one another? The lack of correlation is due to significant changes in the intensity of light scatter during a state 4 to state 3 transition. Changes in the intensity of light scatter were monitored by setting the excitation and emission monochromators at 500 nm. The addition of ADP to mitochondria incubated as in Fig 9 results in a large increase in light scattered by the suspension. This is consistent with

Figure 8. Fluorescence and Absorbance Measurements of Chick Heart Mitochondrial Matrix Pyridine Nucleotides

Mitochondria (0.1 mg/ml) were suspended in 2.0 ml of M/S osmotic support medium. The following reaction components were added to this suspension: P_i, 20 mM (Tris-buffered, pH 7.4); P/M, 5 mM pyruvate/2.5 mM malate; ADP, 930 nmols per addition. λ_{ex} = 340 nm; λ_{em} = 450 nm. Slit widths were set at 20 nm.

(A). A1, changes in conventional absorbance (optical density).

(B). 1F, fluorescence at 450 nm.

(C). A2, fluorescence-based absorbance.

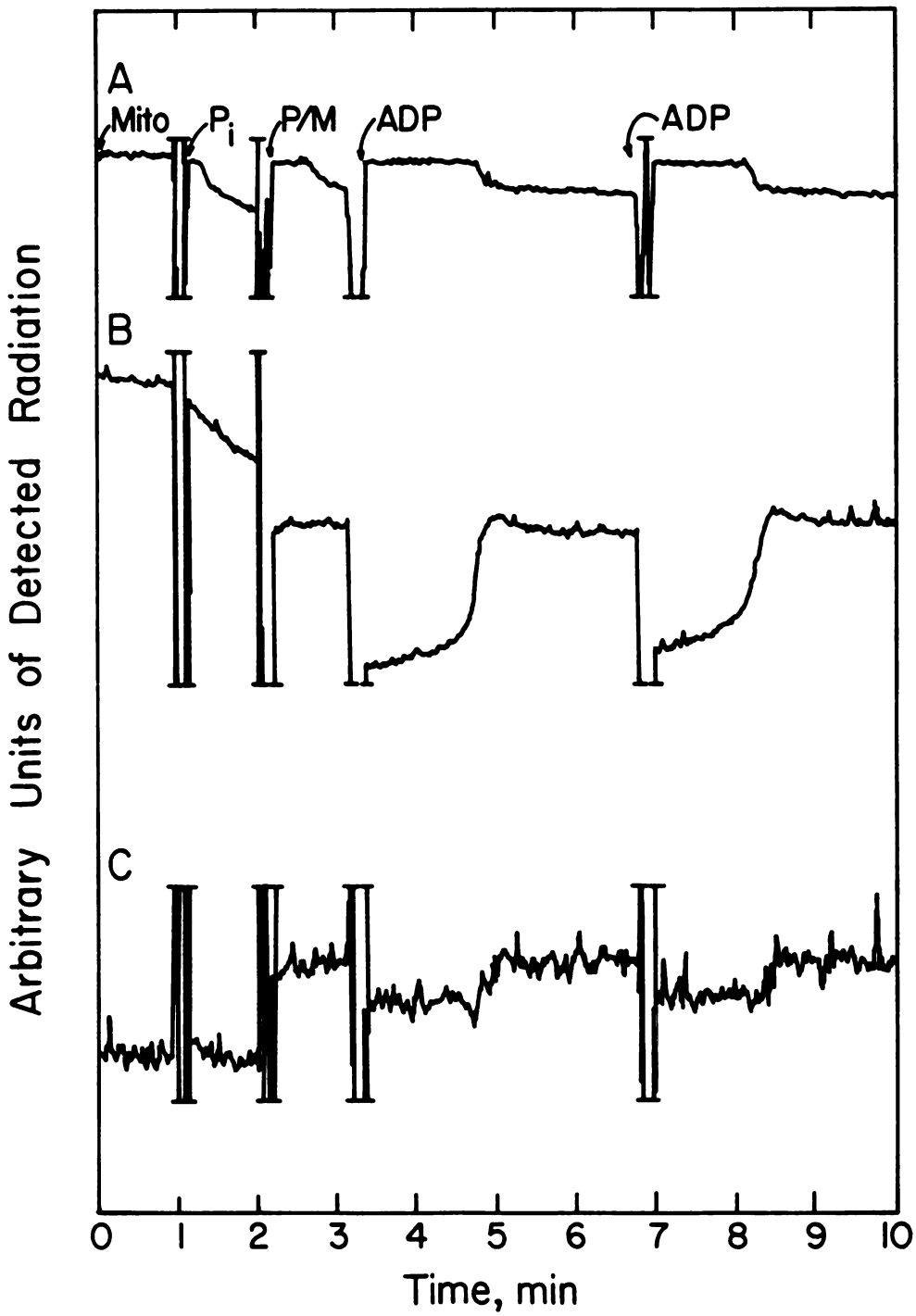
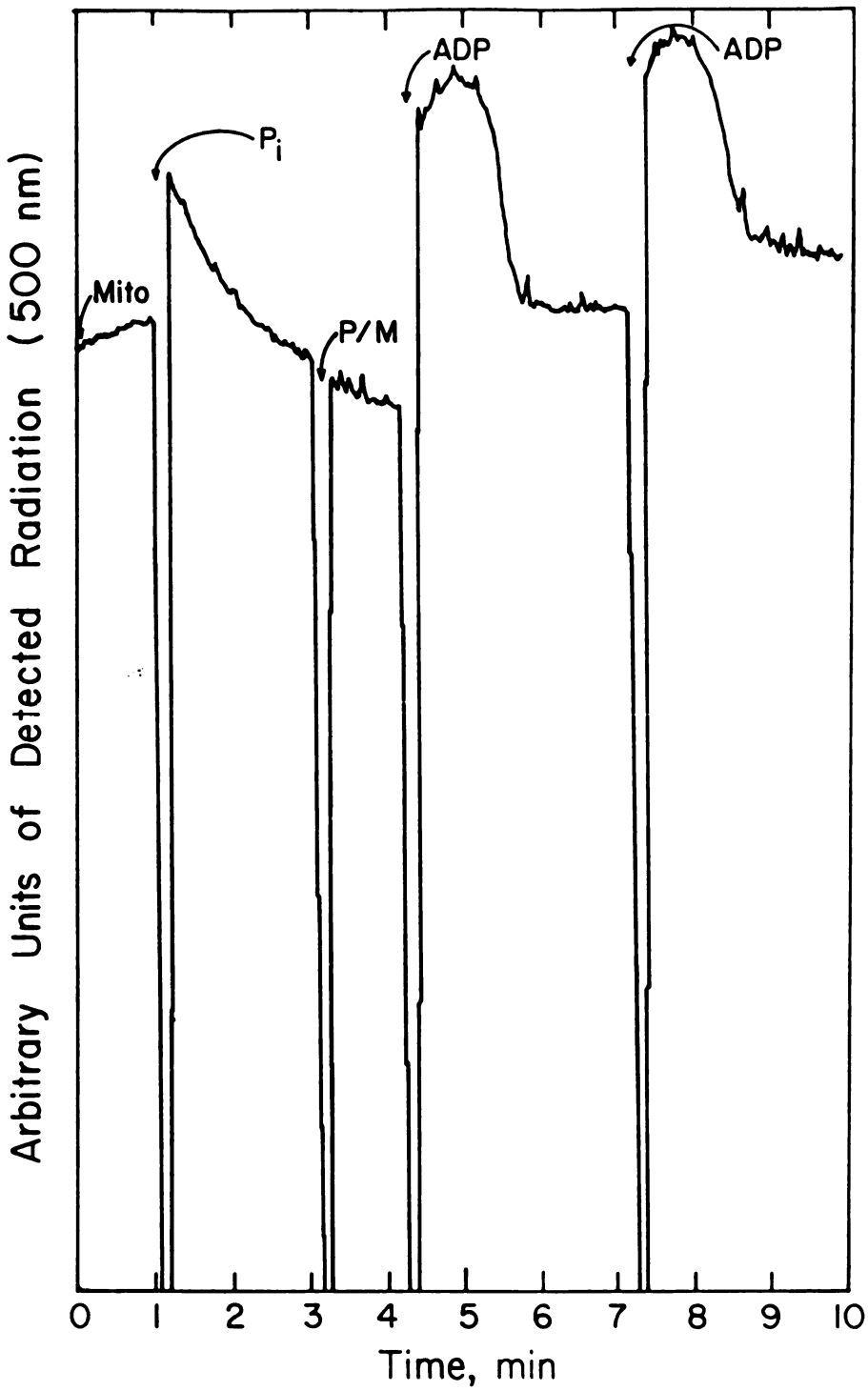

A
Mito
P_i
P/M
ADP
ADP
B
C
Arbitrary Units of Detected Radiation
0
1
2
3
4
5
6
7
8
9
10
Time, min

Figure 9. Changes in the Intensity of Light Scattered at 90° Subsequent to the Addition of Pi, Substrate, and ADP

Mitochondria were suspended in 2.0 ml of an osmotic support medium that contained 0.225 M mannitol and 0.075 M sucrose at 0.80 mg/ml The following additions were made: P_i, 20 mM; P/M, 5 mM pyruvate, 2.5 mM malate; ADP, 400 nmoles per addition. The excitation and emission monochromators of the integrated spectrofluormeter/spectrometer were set at 500 nm.


Arbitrary Units of Detected Radiation (500 nm)
Mito
Pi
P/M
ADP
ADP
0
1
2
3
4
5
6
7
8
9
10
Time, min

the findings of Hackenbrock (1966) which showed that ADP induces a contraction of the mitochondrial matrix. Therefore, both conventional absorbance and differential absorption spectrometry register artifactual increases subsequent to the addition of ADP because the mitochondria become more efficient scattering particles (i.e., the matrix becomes more dense). With these measurements, changes in scattering obviate the possibility of accurately monitoring true changes in the absorbance of matrix pyridine nucleotides. In conclusion, chick heart mitochondrial pyridine nucleotides undergo net oxidation during state 3. It is likely that the redox state of rat liver mitochondrial pyridine nucleotides may be inferred from differential absorption measurements because these mitochondria do not contract to the same degree as chick heart mitochondria subsequent to the addition of ADP.

IV. SENSITIVITY OF 0° and 90° LIGHT SCATTER TO CHANGES IN MATRIX VOLUME

The major application of light scatter methodology to studies of mitochondria is to continuously monitor the uptake of various solutes into the matrix. The following properties of mitochondria make this possible:

i.) Mitochondria are near perfect osmometers.

ii.) The matrix undergoes rapid swelling/shrinking in response to perturbations in extramitochondrial osmolarity.

iii.) As matrix configuration changes, the intensity of scattered light changes.

iv.) As osmolarity ↑, matrix volume ↓, and light scatter ↑.

v.) As osmolarity ↓, matrix volume ↑, and light scatter ↓.

vi.) Net inward transport (uptake) of a solute correlates

with matrix swelling.

The sensitivity of 0° and 90° light scatter to P_i-induced swelling of the chick heart mitochondrial matrix is compared in Fig 10. Clearly, for kinetic measurements, nephelometry is the method of choice due to its much higher sensitivity and larger signal-to-noise ratio. The calculation of kinetic constants from the type of data presented in Fig 10B is discussed in Chapter 5 of this dissertation. It is of importance to note here that little if any quantitative information about the transport of P_i can be obtained from turbidimetric measurements alone.

Turbidimetric measurements do, however, yield information about the magnitude of R and α. Double reciprocal plots relating the optical density of increasing concentrations of mitochondria before and after swelling is induced with the addition of 20 mM P_i are shown in Fig 11. The values for α are 4.07 and 3.24 before and after P_i-induced swelling, respectively. These values are reasonable since one would expect swollen mitochondria to scatter light less efficiently than those which are at osmotic equilibrium with an isotonic support medium. Finally, although a slight difference in R values for the two conditions may be discerned from the plots, these are not significant. Small variations in R are observed in different preparations of mitochondria most likely due to errors in the determination of mitochondrial concentration. On a theoretical basis R would be expected to be a constant since it depends on the geometry of the observation system and the number of scattering surfaces per unit volume, not their structural density.

Figure 10. Relative Sensitivity of 0° and 90° Light Scatter to Pi-Induced Changes in Chick Heart Mitochondrial Matrix Volume

Mitochondria were suspended in 0.225 M mannitol, 0.075 M sucrose at 0.85 mg/ml. Inorganic phosphate (20 mM) and pyruvate (1 mM)/malate (0.5 mM) were added simultaneously at the indicated time to the mitochondrial suspension. Mitochondrial swelling is indicated by a downward deflection in the traces.

(A). Trace showing the change in absorbance (optical density) as a function of time.

(B). Trace showing the change in 90° light scatter intensity as a function of time. The initial drop in light intensity subsequent to the addition of Pi and substrate is due to a small dilution of the mitochondria and to the swelling which occurs during the time (2-4 sec) it takes to manually mix the sample.

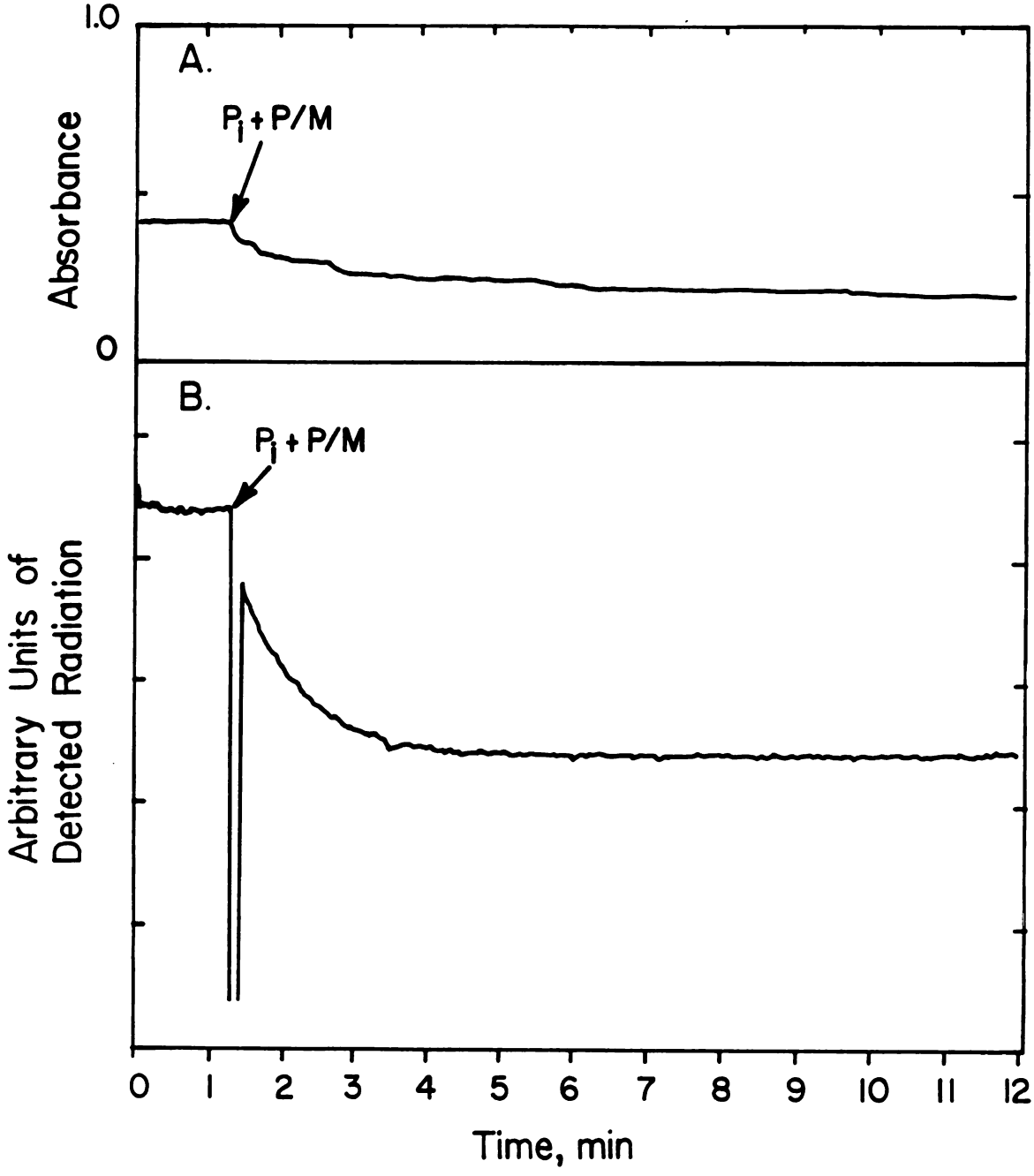
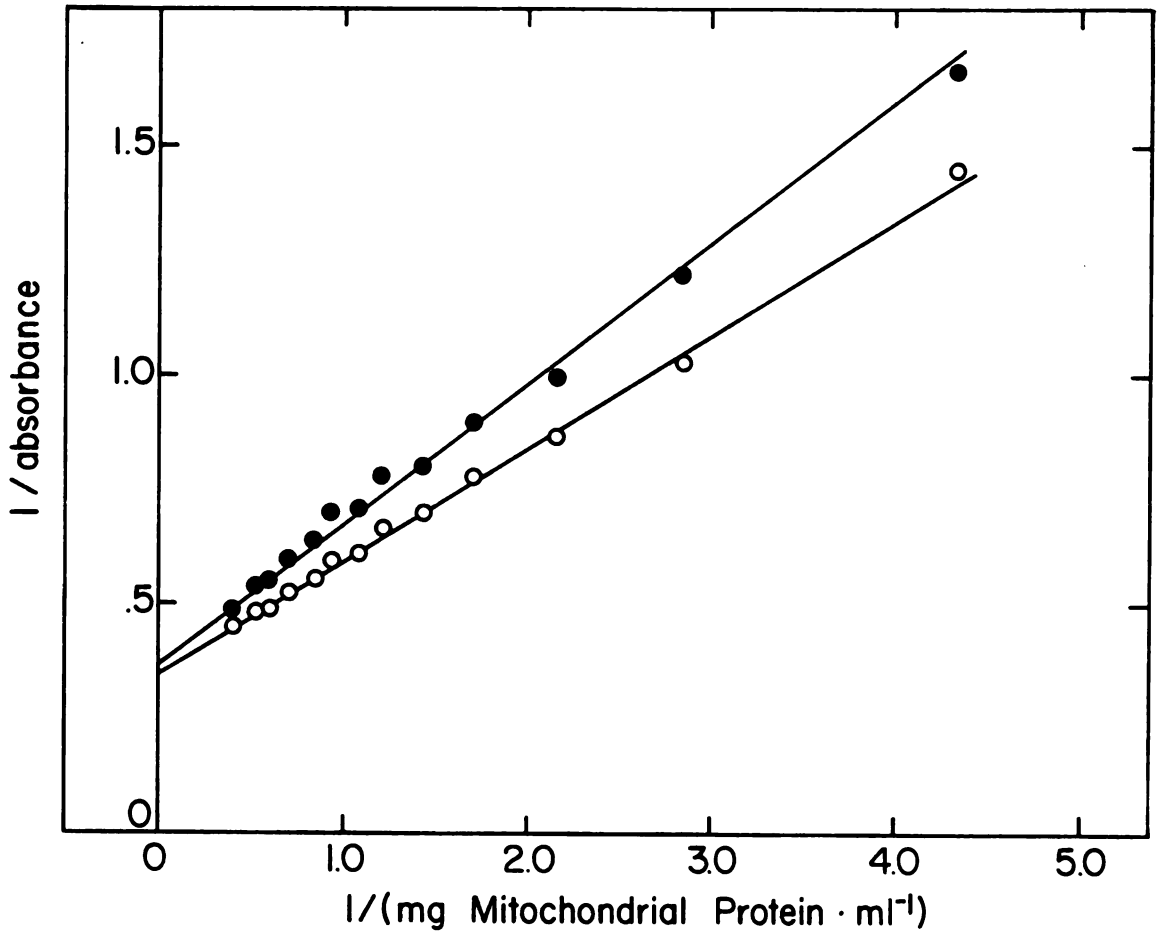

1.0
A.
P_i + P/M
Absorbance
0
B.
P_i + P/M
Arbitrary Units of
Detected Radiation
0 1 2 3 4 5 6 7 8 9 10 11 12
Time, min

Figure 11. Double Reciprocal Plot Relating Changes in Optical Density to Increases in the Concentration of Mitochondria

Changes in optical density were monitored continuously from before the addition of 20 mM Tris-buffered P_i (●) to after mitochondrial volume had stabilized secondary to P_i uptake (○). Mitochondria concentration was varied ten-fold, from 0.23 to 2.30 mg/ml. All assays were performed in M/S medium. Samples were irradiated with light having a wavelength of 500 nm.


1.5
1.0
.5
0
1 / absorbance
0
1.0
2.0
3.0
4.0
5.0
1/(mg Mitochondrial Protein · ml⁻¹)

V. CONCLUSIONS

The experiments and theoretical considerations presented in this study show that: (a) The deviation of mitochondrial suspensions from Beer's Law can be explained by assuming that much scattered light is recovered by the incident beam of light prior to striking the 0° detector; (b) Nephelometric measurements of mitochondrial P_i transport are much more sensitive than those performed with turbidometric methods; (c) Turbidimetric measurements, although relatively insensitive for monitoring continuous changes in mitochondrial volume, are of value in that the magnitude of R (recovery coefficient) and α (scattering efficiency) can be estimated from equilibrium measurements of mitochondrial suspensions. (d) Chick heart mitochondria can withstand large variations in external osmolarity (150-600 mosmolal) without undergoing appreciable apparent structural alteration or damage. (e) Dual wavelength spectrophotometry yields artifactual information concerning chick heart mitochondrial matrix pyridine nucleotide content during a state 4 to state 3 transition due to concomitant changes in the light scattering efficiency of these organelles. Fluorescence and fluorescence-based absorbance measurements provide much more accurate data on relative changes in the matrix pyridine nucleotide content.

REFERENCES

Adamsons, K. (1985) Dissertation. Michigan State University.

Beavis, A.D., Brannan, R.D., and Garlid, K.D. (1985) J. Biol. Chem. 260: 13424-13433.

Bier, M. (1957) Methods Enzymol. 4: 147-166.

Chance, B., and Williams, G.R. (1955) J. Biol. Chem. 217: 409-428.

Debye, P. (1947) J. Phys. Colloid Chem. 51: 18-30.

Garlid, K.D., and Beavis, A.D. (1985) J. Biol. Chem. 260: 13434-13441.

Garlid, K.D., and Beavis, A.D. (1987) Biochim. Biophys. Acta 853: 187-204.

Hackenbrock, C.R. (1966) J. Cell Biol. 30: 269-297.

Halestrap, A.P., and Quinlan, P.T. (1983) Biochem. J. 214: 387-393.

Hansford, R.G., and Chappell, J.B. (1968) Biochem. Biophys. Res. Comm. 30: 643-648.

Hansford, R.G. (1972) Biochem. J. 127: 271-283.

Jung, D.W., Chavez, E., and Brierly, G.P. (1977) Arch. Biochem. Biophys. 183: 452-459.

Koch, A.L. (1961) Biochim. Biophys. Acta 51: 429-441.

Melikhov, A.A., Storonkin, B.A., and Kuni, F.M. (1981) Opt. Spectrosc. (U.S.S.R.) 49: 321-324.

Packer, L. (1960) J. Biol. Chem. 236: 214-220.

Perrin, J.M., and Chiapetta, P. (1985) Optica Acta 32: 907-921.

Perrin, J.M., and Lamy, P.L. (1986) Optica Acta 33: 1001-1022.

Quinlan, P.T., Thomas, A.P., Armston, A.E., and Halestrap, A.P. (1983) Biochem. J. 214: 395-404.

Raaflaub, T. (1953) Helv. Physiol. Pharmacol. Acta 11: 142-156.

Rayleigh, J.W. (1871) Phil. Mag. 41: 107, 274.

Singham, S.B., and Bohren, C.F. (1987) Optics Lett. 12: 10-12.

Tedeschi, H., and Harris, D.L. (1955) Arch. Biochem. Biophys. 58: 52-67.

Tedeschi, H., and Harris, D.L. (1958) Biochim. Biophys. Acta 28: 392-402.

Twersky, V. (1983) J. Opt. Soc. Am. 73: 313-320.

Chapter 5

INTERACTION OF INORGANIC PHOSPHATE WITH CHICK HEART MITOCHONDRIA

I. CHANGES IN VOLUME AND ION COMPOSITION OF THE MATRIX

INTRODUCTION

Mitochondria have evolved an elaborate system of carriers responsible for mediating the flux of inorganic phosphate across the inner membrane. Net inward transport and P_i-P_i transmembrane exchange is catalyzed by the P_i transport protein (Coty and Pederson, 1974; Ligeti et al., 1985). Transport of P_i is a proton-coupled process (McGivan and Klingenberg, 1971), and at equilibrium P_i is distributed between compartments according to the following relation (Palmieri et al., 1970):

$$\log [P_i^-]_m/[P_i^-]_c = \Delta pH \qquad (1)$$

where $[P_i^-]_m$ and $[P_i^-]_c$ are the concentrations of monovalent P_i in the matrix and cytosol, respectively. The P_i transport protein has been isolated from bovine heart (Wohlrab, 1980) and rat liver mitochondria (Kaplan et al., 1986), and molecular details concerning the actual transport mechanism of this carrier are beginning to emerge (Wohlrab, 1986). In addition to meeting the substrate requirements for oxidative phosphorylation, the inward transport of P_i compensates the charge imbalance resulting from the electrogenic exchange of ADP^{3-} for ATP^{4-} on the adenine nucleotide translocase (McGivan et al., 1971). P_i is transported out of the matrix by the dicarboxylate carrier (Forman and Wilson, 1982) and the P_i transport protein (Coty and Pederson, 1975). In addition, other evidence suggests that P_i efflux may be catalyzed by the adenine nucleotide translocase (Reynafarje and Lehninger, 1978) and an as yet undefined mechanism mediating a carboxyatractyloside-insensitive flux of P_i and adenine nucleotides (Austin and Aprille, 1984).

Under conditions in which ATP is hydrolyzed in the matrix, the efflux of P_i has been attributed solely to the P_i transport protein (Tyler, 1980) or to the P_i transport protein and another carrier that is not the P_i/dicarboxylate exchanger (Fonyo and Vignais, 1979; Kaplan and Pedersen, 1983). It has also been proposed that mitochondria contain an anion porter that conducts P_i out of the matrix when these organelles are osmotically stressed (Garlid and Beavis, 1987). It is apparent from these studies that mitochondria are highly responsive, both chemically and osmotically, to the intra- and extramatrix concentrations of P_i.

Although criticized for its limitations (LaNoue and Schoolwerth, 1979), light scatter measurements have been used extensively to measure transmembrane fluxes in mitochondria (for reviews: Lehninger, 1962; Garlid and Beavis, 1987). This technique can be used to monitor mitochondrial swelling changes induced by a variety of agents which include P_i (Packer, 1961), Ca(II) (Chappell and Crofts, 1965; Crofts and Chappell, 1965), K^+ (Jung et al., 1977; Chavez et al., 1977; Martin et al., 1984), and such hormones as thyroxine (Tedeschi, 1961), glucagon (Armston et al., 1982), and α-agonists (Halestrap et al., 1986), among others. Light scattering measurements can be made at both 0° and 90°. The efficacy of 0° light scatter measurements was recently reevaluated and its quantitative applications expanded (Beavis et al., 1985; Garlid and Beavis, 1985). It has been known for some time (Lehninger, 1962; see also Chapter 4), however, that light scattering measurements performed at 90° are more sensitive than those performed at 0°.

Considering the fact that mitochondria swell during myocardial ischemia (Jennings and Ganote, 1976), studies of the ability of P_i to induce mitochondrial swelling in various media (Izzard and Tedeschi,

1973; Jung et al., 1977 and references therein) are taking on increasing relevance. Previous studies suggest that P_i-induced swelling of heart mitochondria is the result of a Ca(II)-dependent membrane transition (Hunter et al., 1976; Hunter and Haworth, 1979a). The rate of P_i-induced swelling has also been found to be dependent on the concentration of P_i (Izzard and Tedeschi, 1973; Vaghy et al., 1981). As part of our investigation of the nature by which P_i affects chick heart mitochondrial structure and function, we have examined the following: First, the effects of P_i on mitochondrial volume; second, the perturbation of matrix solute content subsequent to P_i uptake; third, whether Ca(II) is an obligatory component for swelling subsequent to the addition of P_i; and, fourth, the kinetic constants characterizing the P_i-induced swelling changes. The latter measurements also yielded an indirect estimate for the apparent K_m of the P_i transport protein which is much less than previous estimates of this parameter.

EXPERIMENTAL PROCEDURES

I. MATERIALS

Water was purified first by passage through a standard reverse osmosis system (Continental Millipore, Detroit, MI) which removes contaminants by carbon adsorption, microporous membrane filtration, and ion-exchange, and then by distillation from alkaline permanganate. The following substances were reagent grade or better, used without purification, and obtained from the sources noted: sucrose (RNase-free), carbonyl cyanide m-chlorophenyl hydrazone (CCCP), N-ethylmaleimide (MalNEt), ADP (grade X), and the sodium salts of pyruvic and malic acids (Sigma Chemical Co.); mannitol (Fisher Scientific); strontium chloride and toluene (J.T. Baker Chemical Co.); potassium permanganate and phosphoric acid (Mallinckrodt); Tris (Boehringer-Mannheim); carrier-free [$^{32}P_i$]-orthophosphoric acid and [$^{14}C(U)$]-sucrose (671 mCi/mmol) were from New England Nuclear; 2,5-diphenyloxazole and Triton-X100 (Research Products International). Nagarse (Sigma type XXVII, lot 97F-0218) was reconstituted prior to each isolation in a solution containing 225 mM mannitol and 75 mM sucrose (M/S). Rotenone and CCCP were dissolved in absolute ethanol (AAPER Alcohol and Chemical Co.). Phosphate solutions were prepared by titrating phosphoric acid with Tris to pH 7.4. Single comb white leghorn chicks were purchased from Michigan State University's Department of Animal Science. Chicks were fed Chick G0125 feed (Kent Feeds, Inc., Muscatine, IA) and were not starved prior to sacrifice.

II. ISOLATION OF MITOCHONDRIA

Heart mitochondria were isolated from 14-21 day-old chicks using Nagarse, as previously described (Chance and Hagihara, 1961). Chick heart ventricular myocardium was minced into an ice-cold isotonic isolation medium that contained 225 mM mannitol, 75 mM sucrose, and 20 mM Tris, pH 7.4 (MST). The myocardium was then homogenized with five up-and-down strokes of a Potter-Elvejhem pestle (operated at 500 r.p.m.) in the presence of approximately 0.5 I.U. Nagarse/g muscle. The homogenized tissue was allowed to incubate on ice for 3 min, and was then subjected to two additional up-and-down strokes of the pestle. The crude homogenate was centrifuged for 5 min at 800g. The resulting supernatant liquid containing the mitochondria was filtered through two layers of cheesecloth and centrifuged at 8,000g for 10 min. The mitochondria were then rinsed twice by resuspending these organelles in 7-8 ml of MST and centrifuging at 8,000g for 10 min. The final washed mitochondrial pellet was resuspended in MST at 15-20 mg/ml. All experiments were completed within 4-5 hrs after isolation of mitochondria. EGTA was not added to the isolation or assay media, unless specified otherwise, because this chelating agent would be expected to interfere with divalent metal cation fluxes across the mitochondrial inner membrane. Bovine serum albumin was also excluded from these solutions because this protein is known to scatter light and has some capacity to bind cations. Preliminary studies show that Nagarse destroys a soluble protein factor that stimulates uncoupled respiration (i.e., oligomycin-insensitive) in chick heart mitochondria (Toth et al., 1985). As described in greater detail in Chapter 3 of this dissertation, under optimal conditions (0.050 nmol cyt aa_3/ml, 20 mM P_i, and 5 mM pyruvate/2.5 mM malate), these mitochondria are highly coupled, as evidenced by respiratory control ratios

routinely ≥ 100 and ADP:O ratios of 3.42 ± 0.12 (n=30).

III. LIGHT SCATTERING MEASUREMENTS

A computerized spectrometer/fluorometer was used to characterize the kinetics of mitochondrial swelling secondary to the addition of Tris-buffered P_i (pH 7.4). A Perkin-Elmer Model 512 spectrofluorometer was modified to the single beam mode. A quartz fluorescence cuvette with a 1 cm pathlength was mounted on an off-center cell rotation device (Adamsons et al., 1982). Two photovoltaic cells (PVC; Hamamatsu, S1377-1010BQ) were added to the cell compartment in order to measure the intensity of light incident to and transmitted from the sample. The xenon lamp source and excitation and emission grating monochromators of the instrument were retained. The photomultiplier tube (PMT; fluorescence detector) output along with the two PVC outputs were amplified and then multiplexed into an analog/digital converter for processing by a PDP 8/e Digital computer.

Rates of phosphate-induced mitochondrial swelling were continuously monitored by measuring changes in 500 nm light scattering intensities at 0° and/or 90°. No attenuation of the light intensity was observed at this wavelength. The intrinsic gain of the PMT, the intensity of the 150 W xenon lamp, and the cell geometry resulted in a higher signal-to-noise ratio and a greater dynamic range for 90° light scattering (vs 0°). After initial mixing of reactants (elapsed time of 2-3 sec), readings were collected. Each stored reading represents an average of 40 data points collected over a 200 msec interval in order to minimize 60 Hz line noise. Slit widths for the excitation and emission windows were set at 20 nm. All light scattering measurements were performed at room

temperature (22-23°C). First-order rate constants for P_i-induced swelling were calculated with the following equation:

$$k = \ln \frac{L.S._{max} - L.S._{min}}{L.S._t - L.S._{min}} \Big/ t \qquad (2)$$

where $L.S._{max}$ is the intensity of scattered light immediately after the addition of P_i, $L.S._t$ is the intensity of scattered light at each time t (measured in 4 sec intervals), and $L.S._{min}$ is the intensity of scattered light once the maximal volume for a swelling phase has been attained. Experimental conditions are described in the legends to Figures 1 and 3.

IV. DETERMINATION OF INTRAMITOCHONDRIAL $^{32}P_i$.

Mitochondria (1.9 mg) were suspended in a 1.0 ml final volume containing 225 mM mannitol, 75 mM sucrose, 20 mM Tris (MST; pH 7.4), 5 mM pyruvate/2.5 mM malate, and increasing concentrations of Tris-buffered P_i (pH 7.4) that was enriched with carrier-free $^{32}P_i$. Mitochondria were allowed to incubate with P_i for 10 min. The mitochondria were then pelleted by centrifuging the suspension at 15,600g for 4 min in an Eppendorf 5414 Microcentrifuge. Duplicate 100 µl aliquots of the supernatant liquid were withdrawn and added to 10 ml of a scintillation cocktail composed of toluene, Triton-X100, and 2,5-diphenyloxazole. The remaining supernatant liquid was discarded, and the wall of the centrifuge tube was swabbed and carefully wiped. The mitochondrial pellet was resuspended in 0.3 ml of MST. The mitochondria were then denatured so as to release internalized $^{32}P_i$ by adding 0.7 ml of 2.0 M perchloric acid. The denatured mitochondria were pelleted by centri-

fuging the sample at 15,600g for 4 min. Duplicate 100 µl aliquots of the supernatant liquid were withdrawn and added to 10 ml of the scintillation cocktail described above. Liquid scintillation counting was performed on a Packard TriCarb 300 CD counter. $^{32}P_i$ was counted for 10 min with a window setting of 5-1700 KeV.

The amount of $^{32}P_i$ in the mitochondria was corrected for the amount of $^{32}P_i$ in the extramitochondrial water co-sedimenting with the mitochondrial pellet. The amount of non-matrix water in the pellet was calculated from data obtained in an experiment in which 0.1 µCi (149 pmol) of [^{14}C(U)]-sucrose was incubated with mitochondria for 1 min before sedimenting. Non-matrix water associated with the mitochondrial pellets was calculated with the following equation (Jensen et al., 1986):

$$^{ex}V_P = \frac{(^{14}C_P)(V_S)}{^{14}C_S} \qquad (3)$$

where $^{ex}V_P$ is the volume of extra-matrix water, V_S is the volume of the suspension, and $^{14}C_P$ and $^{14}C_S$ are the dpm of [^{14}C(U)]-sucrose in the pellet and supernatant fractions, respectively. The insert to Fig. 6 shows that, subsequent to a 10 min pre-incubation with P_i, the volume of non-matrix water co-sedimenting with the mitochondria is independent of P_i concentration (mean: 9.31 ± 1.25 µl H_2O/mg mitochondria). For that particular experiment, mitochondria were sedimented by centrifuging at 9,000g for 4 min in a Fisher 59A Microcentrifuge. By centrifuging these organelles at 15,600g for 4 min, the amount of extramitochondrial water was decreased over 2-fold to 4.10 ± 0.31 µl H_2O/mg mitochondria for a typical experiment run in triplicate. [^{14}C(U)]-sucrose samples were

counted for 10 min with a window setting of 0-156 KeV in the scintillation cocktail noted earlier. Cpm were converted to dpm for each isotope by counting a series of ten acetone-quenched standards. The resulting quench curves (quench index vs counting efficiency) were stored in the scintillation counter's memory and dpm were automatically calculated.

V. QUANTITATION OF TRANSMEMBRANE IONIC FLUXES

The concentrations of Ca(II), Mg(II), and K^+ were quantitated from absorbance measurements made at 422.7, 285.2, and 766.5 nm, respectively, with an Instrumentation Laboratory 951 atomic absorption spectrophotometer using an air-acetylene mixture as fuel and a spectral bandpass of 1.0 nm. Ca(II), Mg(II), and K^+ standards were obtained from Varian Techtron (Palo Alto, CA). Incubation conditions are described in the legends to Figures 7-10. Reactions were terminated by pelleting mitochondria with a Fisher Model 59A Microcentrifuge operated at 9,000g for 3 min. When assaying for Ca(II) or Mg(II), resulting supernatant liquids were acidified with one drop of 10 N HCl and made 0.1 M in Sr(III) in order to control for interfering absorption by P_i. Acidification of these samples prevented formation of a strontium phosphate precipitate. The addition of a releasing agent to K^+-containing samples was unnecessary because P_i does not interfere with the measurement of this cation. Total mitochondrial content of these ions was determined by assaying the supernatant liquids of sedimented perchloric acid precipitates. Data were corrected for nonmitochondrial Ca(II), Mg(II), and K^+ arising from impurities in incubation medium constituents.

VI. OTHER ASSAYS

P_i concentrations were determined by the method of Bencini et al. (1983) using potassium phosphate as standard. Mitochondrial protein was determined by the modified Lowry method of Markwell et al. (1981) using BSA as the protein standard.

RESULTS

I. VOLUME CHANGES INDUCED BY INORGANIC PHOSPHATE ARE COMPLEX

Representative traces revealing changes in mitochondrial matrix volume secondary to P_i uptake under a variety of conditions are shown in Fig 1. The simultaneous addition of P_i and the substrate couple pyruvate/malate (P/M) induces a volume change (Fig 1A) that obeys first-order kinetics through 90% of the reaction (Fig 2). It is clear that P_i uptake causes swelling since the intensity of scattered light is decreasing. Analysis of the data linearized according to equation I (Fig. 2) shows that swelling proceeds with a half-time of 40.8 ± 4.0 sec and a first-order rate constant (k) equal to 1.08 ± 0.1 min^{-1} (n=20). The final volume attained is stable for approximately 10 min. After this time the mitochondria appear to lose water. In agreement with Hackenbrock (1966), adding ADP triggers a conformational change in the mitochondrial matrix, the so-called "orthodox to condensed" transition. The ability of mitochondrial suspensions to undergo this transition was used as an index of structural intactness. Swelling induced by P_i in the presence of P/M leaves mitochondria morphologically intact, as evidenced by the immediate contraction of the mitochondrial matrix subsequent to the addition of ADP (Fig 1A). Once the ADP is nearly all phosphorylated, mitochondria reassume the volume, and presumably the morphology, observed prior to the initiation of state 3 respiration. In the presence of a large exogenous energy source (millimolar concentrations of P/M), P_i-induced swelling of mitochondria must be a highly controlled process because the structure and function of the organelles is not

Figure 1. Perturbations in 90° Light Scattering Subsequent to the Addition of Pi to Suspensions of Chick Heart Mitochondria

Mitochondria were suspended at 0.66 mg/ml in an osmotic support buffer that was 0.225 M mannitol, 0.075 M sucrose, and 20 mM Tris, pH 7.4. The suspension was then made 20 mM in Tris (pH 7.4) buffered P_i under a variety of conditions. Intensities of scattered light were measured as described under "Experimental Procedures." The sudden drop in light scatter intensity secondary to the addition of P_i or P_i and substrate is due to a small dilution of the sample and the loss of data during manual mixing (2-4 sec) of the mitochondrial suspension. The break in the time axis is used to indicate a variable (30-60 sec) lag in the time after which monitoring of the sample was reinitiated. Monitoring was briefly interrupted because the instrument was set up to collect data continuously for 11 min periods.

(A). 1 mM pyruvate/0.2 mM malate added simultaneously with P_i. Once the perturbation induced by P_i went to completion, 2.1 μmoles of ADP were added.

(B). Addition of 20 mM P_i without exogenous substrate.

(C). P_i added to mitochondria suspended in M/S medium that contained 5 mM EGTA. No exogenous substrate was added.

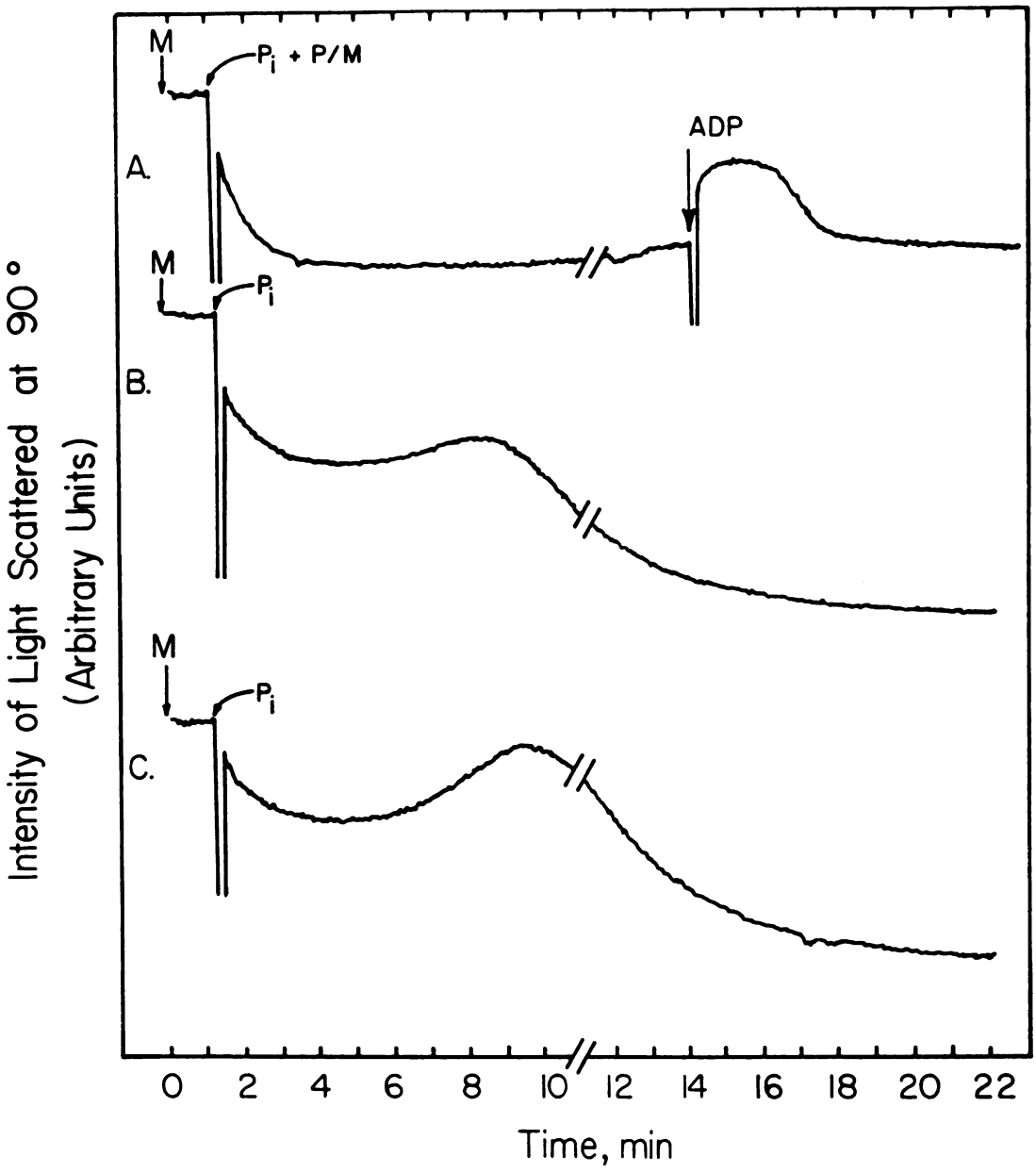
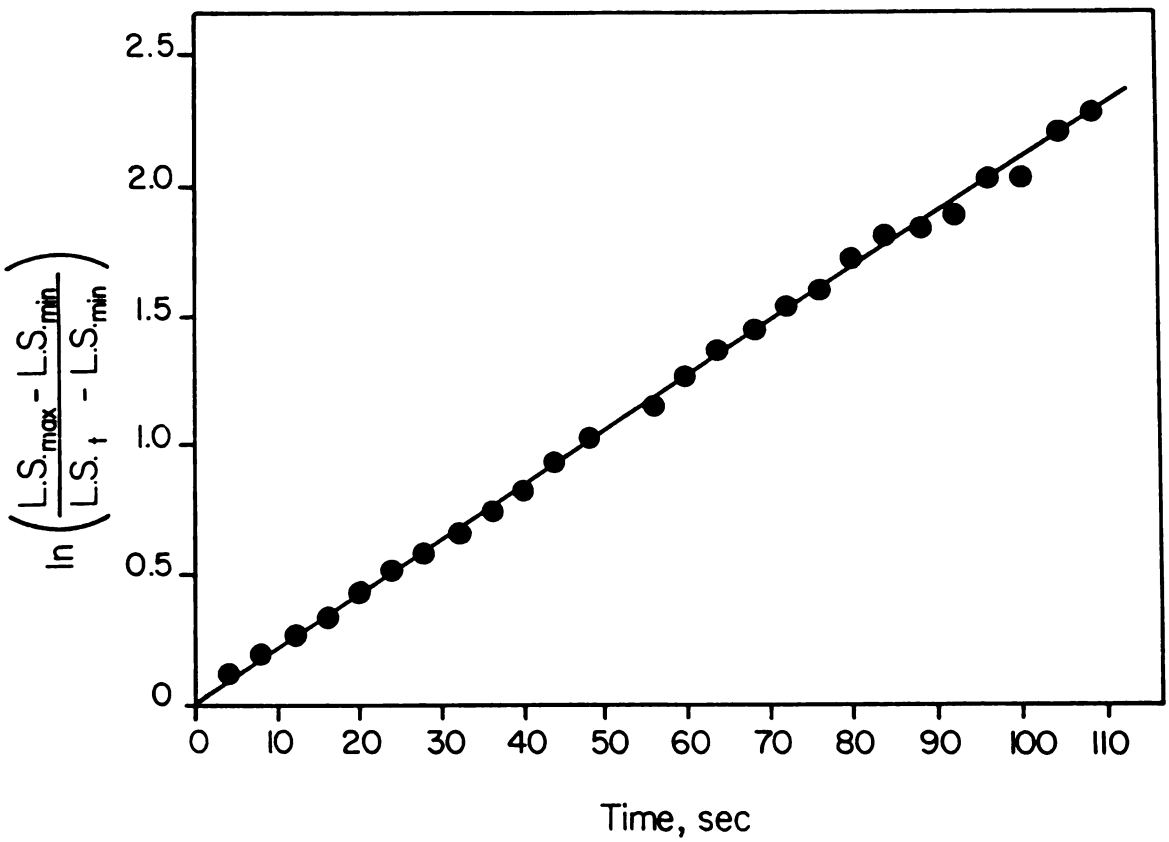

M
P_i + P/M
ADP
A.
M
P_i
B.
M
P_i
C.
Intensity of Light Scattered at 90°
(Arbitrary Units)
0
2
4
6
8
10
12
14
16
18
20
22
Time, min

Figure 2. Determination of the First-Order Kinetic Constants of Mitochondrial Swelling Changes Induced by Pi

Data obtained from nephelometric measurements were linearized using equation 1. The first-order rate constant (k) is equal to the slope of the plot. The half-time for the swelling process was calculated with the relation $t_{0.5}$ = ln 2/k. For the experiment shown, mitochondria were suspended in 2.0 ml of M/S/T at 0.90 mg/ml. Swelling was induced with the simultaneous addition of 18 mM Tris-P_i and 1 mM pyruvate/0.5 mM malate. The line through the data was calculated using least-squares linear regression analysis (cc: 0.9990). k = 1.26 min^{-1} and $t_{0.5}$ = 33.2 sec.


2.5
2.0
1.5
1.0
0.5
0
0
10
20
30
40
50
60
70
80
90
100
110
Time, sec
$\ln\left(\frac{L.S._{max} - L.S._{min}}{L.S._{t} - L.S._{min}}\right)$

adversely affected.

When P_i-induced swelling proceeds in the absence of exogenous substrate, a different sequence of structural alterations is initiated (Fig 1B). The initial swelling phase is again first-order described by the constants noted above. However, following a 3 min lag (measured from the time P_i is added), the mitochondria shrink and then undergo a large second phase of swelling. Subsequent to the second swelling phase, no conformational transition is observed upon the addition of P/M and ADP, indicating that these mitochondria have undergone extensive structural damage (data not shown).

It has been suggested by others (Hunter and Haworth, 1979 a and b; Garlid and Beavis, 1987) that P_i-induced mitochondrial swelling is the result of Ca(II)-dependent changes in inner membrane permeability. The trace shown in Fig 1C is not consistent with such a conclusion. In the presence of the Ca(II) chelating agent EGTA (5 mM), P_i still induces two phases of matrix swelling. Moreover, the shrinking phase is exacerbated. The kinetics of the first swelling phase are unaffected by EGTA.

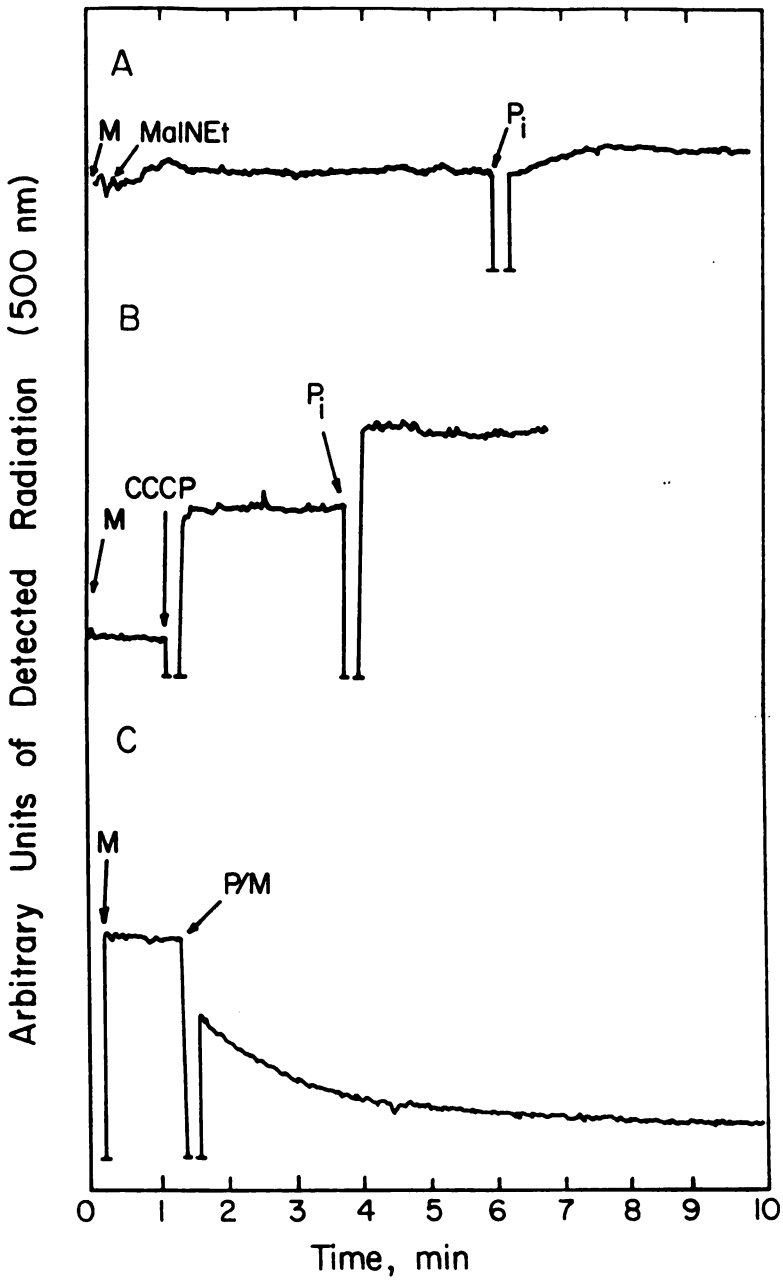
Considerable evidence indicates that the first swelling phase is coupled to P_i uptake. MalNEt is known to inhibit the mitochondrial P_i transport protein by alkylating thiol groups essential for activity (Fonyo and Ligeti, 1984; Klingenberg et al., 1974). Preincubating mitochondria with MalNEt completely inhibits swelling subsequent to the administration of P_i (Fig. 3A). Uptake catalyzed by the P_i transport protein is believed to be a proton-coupled process (Hoek et al., 1971; McGivan and Klingenberg, 1971; Coty and Pederson, 1974). Therefore, the addition of a protonophore should inhibit P_i-induced swelling if the volume change is associated with catalytic P_i inter-

Figure 3. Characterization of some of the Factors Affecting the Pi-Induced Change in Mitochondrial Matrix Volume

(A). Inhibition of light scattering changes secondary to the addition of 20 mM P_i by preincubating chick heart mitochondria (M, 0.87 mg ml^{-1}) with N-ethylmaleimide (NEM, 1.4 mM) for 6 min.

(B). Inhibition of mitochondrial swelling secondary to the addition of 20 mM P_i by preincubating chick heart mitochondria (0.22 mg ml^{-1}) with 50 μM of the uncoupler CCCP.

(C). Perturbation in chick heart mitochondrial light scatter secondary to the addition of 1 mM pyruvate and 0.5 mM malate. Mitochondria were suspended in 2.0 ml of M/S media at 0.97 mg ml^{-1}.


A
M
MalNEt
Pi
B
Pi
CCCP
M
C
M
P/M
Arbitrary Units of Detected Radiation (500 nm)
0
1
2
3
4
5
6
7
8
9
10
Time, min

nalization. Consistent with this rationale, CCCP (Fig 3B) inhibits the initial volume change. In addition, the first-order rate constant for the first swelling phase (1.08 ± 0.1 min^{-1}) is conspicuously close to the k determined for the purified P_i transporter of rat liver mitochondria (0.85 min^{-1}; Kaplan et al., 1986). Taken together, these data strongly suggest that the swelling observed immediately after P_i addition reflects properties of the P_i transport protein and, thus, is a measure of the rate of P_i uptake.

In the absence of exogenous substrate, the matrix of these mitochondria cannot undergo ADP-induced contraction after the second swelling phase. The most reasonable explanation for this lack of response is that the mitochondria become subject to such severe stress that the outer membrane is lysed, thereby allowing the inner membrane to completely unfold. As discussed by Beavis et al. (1985), the latter process is irreversible and would obviate any possibility of ordered inner membrane transitions from taking place.

II. LIMITATIONS ON THE RATE AND EXTENT OF SWELLING

The above experimental evidence supports the assumption that P_i-induced swelling proceeds at a rate that is limited by the P_i transporter. Accordingly, light scatter measurements were used to estimate the K_m of the P_i transporter for P_i. By adding increasing concentrations of P_i to a constant concentration of mitochondria, two types of measurements of the first swelling phase were made. The first was $\Delta L.S./\Delta t$, the dependence of the rate of swelling on P_i concentration; and, second, the total change in light scatter ($\Delta L.S._{Total} = L.S._{max} - L.S._{min}$). The latter values provide information on the relative change in matrix volume as a

function of P_i concentration. Uptake was allowed to proceed in the presence of endogenous substrate only, because the uptake of pyruvate and malate is also accompanied by mitochondrial swelling (Fig 3C).

The first-order rate constant of P_i-induced swelling is independent of P_i concentrations over a ninety-fold range (0.5-45 mM; Fig 4). These kinetic data indicate that the chick heart mitochondrial P_i transporter has a K_m for P_i less than or equal to 50 μM. $\Delta L.S._{Total}$ varies linearly as a function of P_i up to approximately 18 mM (Fig 5). From 18-45 mM, no further increase in $\Delta L.S._{Total}$ is observed, suggesting that the mitochondrial matrix has attained its maximal volume while still subject to the morphological constraints exerted by the outer membrane.

III. FACTORS REGULATING THE AMOUNT OF TRANSPORTED INTO THE MATRIX

Since the results shown in Fig 5 imply that matrix volume could limit the amount of P_i that mitochondria transport in, the following experiment was designed. Mitochondria were coincubated with increasing concentrations of $^{32}P_i$ and 5 mM pyruvate/2.5 mM malate for 10 min. Exogenous substrate was provided so as to insure structural integrity of mitochondria for the entire course of an incubation (see Fig 1A). It is important to note that mitochondria coincubated with P_i and P/M also attain maximal volume at concentrations of $P_i \geq 18$ mM. The amount of P_i internalized under these conditions increases linearly from 3-100 mM P_i (Fig. 6). Clearly, net uptake continues above 18 mM P_i. Once mitochondria reach their maximal matrix volume and water can no longer flow in, some other matrix solute(s) must continuously flow out so as to compensate for osmotic imbalances that may develop as P_i continues to be transported in. If this were not the case, the light scatter signal subsequent to

Figure 4. Kinetic Constants of the First Pi-Induced Swelling Phase Measured as a Function of Pi Concentration

Changes in the intensity of scattered light were measured as described under "Experimental Procedures." First-order kinetic constants were calculated as described in the legend to Figure 2. All measurements were performed in the absence of exogenous substrate.

(A). Half-time ($t_{0.5}$, in seconds).

(B). First-order rate constant (k, min^{-1}).

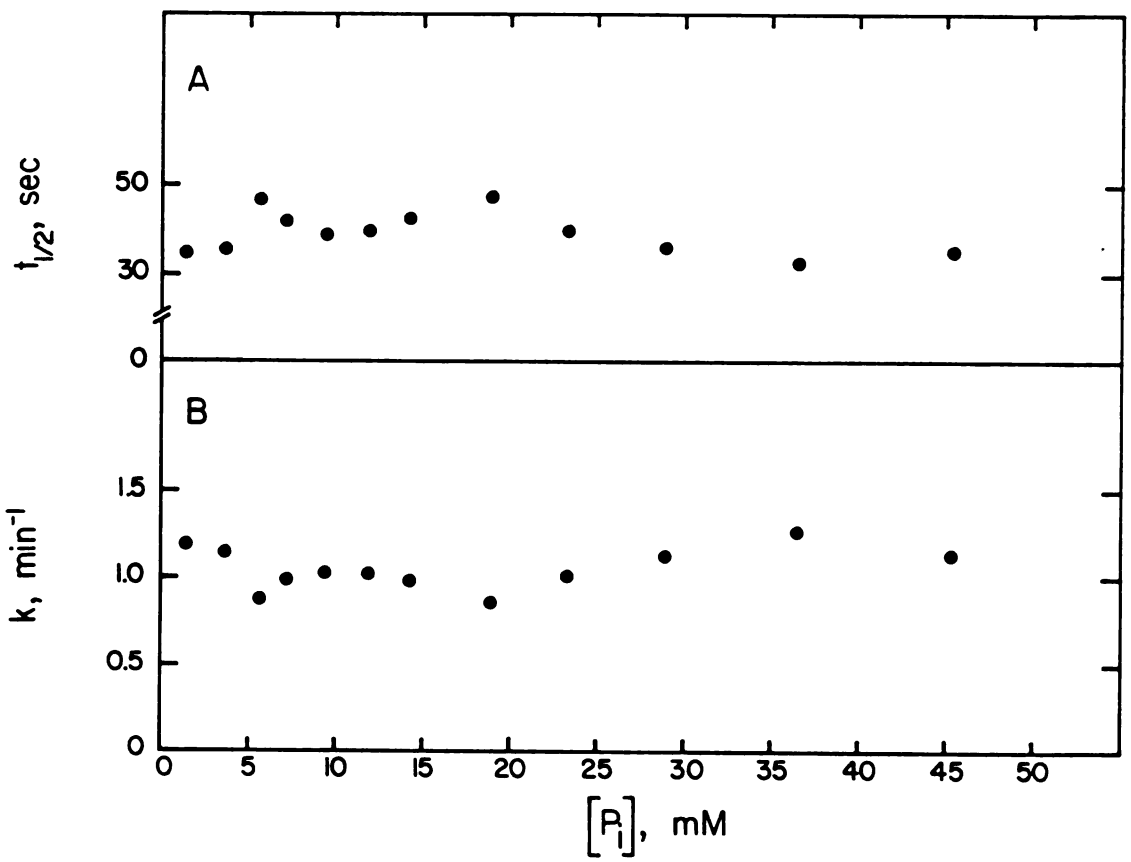

A
B
$t_{1/2}$, sec
k, min^{-1}
50
30
0
1.5
1.0
0.5
0
0
5
10
15
20
25
30
35
40
45
50
$[P_i]$, mM

Figure 5. Total Change in Chick Heart Mitochondrial Light Scattering Measured as a Function of Initial Extramitochondrial Pi Concentration

Mitochondria (0.8 mg ml^{-1}) were irradiated with green light (500 nm) and changes in 90° light scattering were monitored continuously from before the addition of P_i to the end of the first swelling phase.

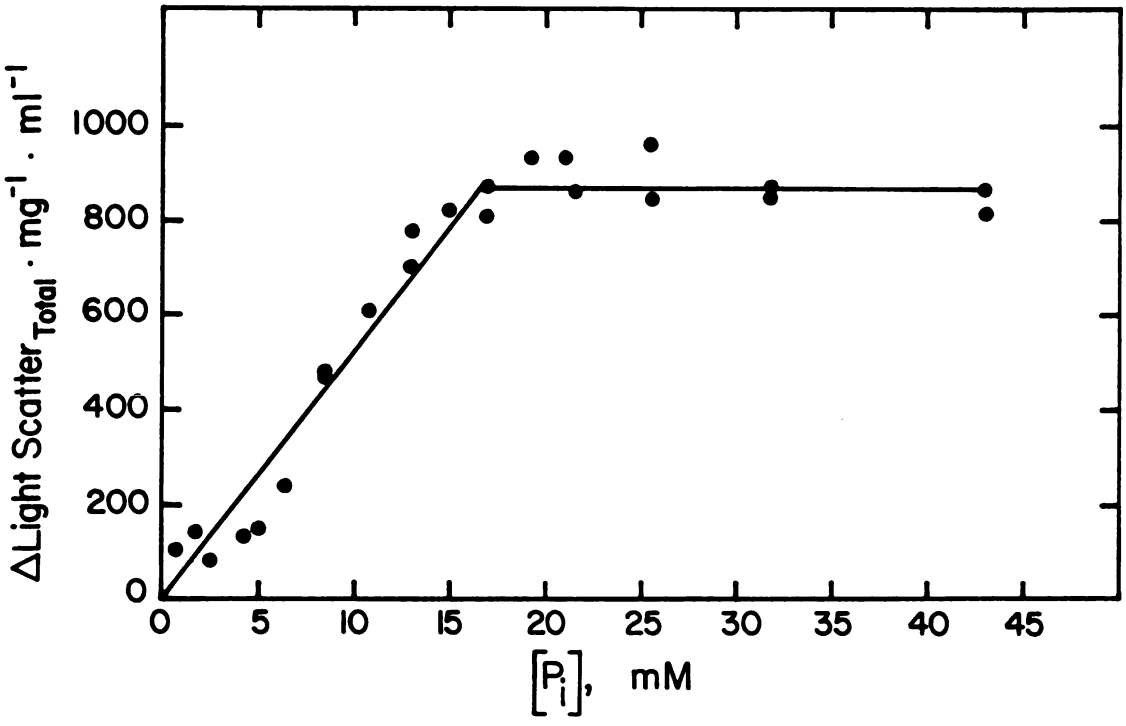
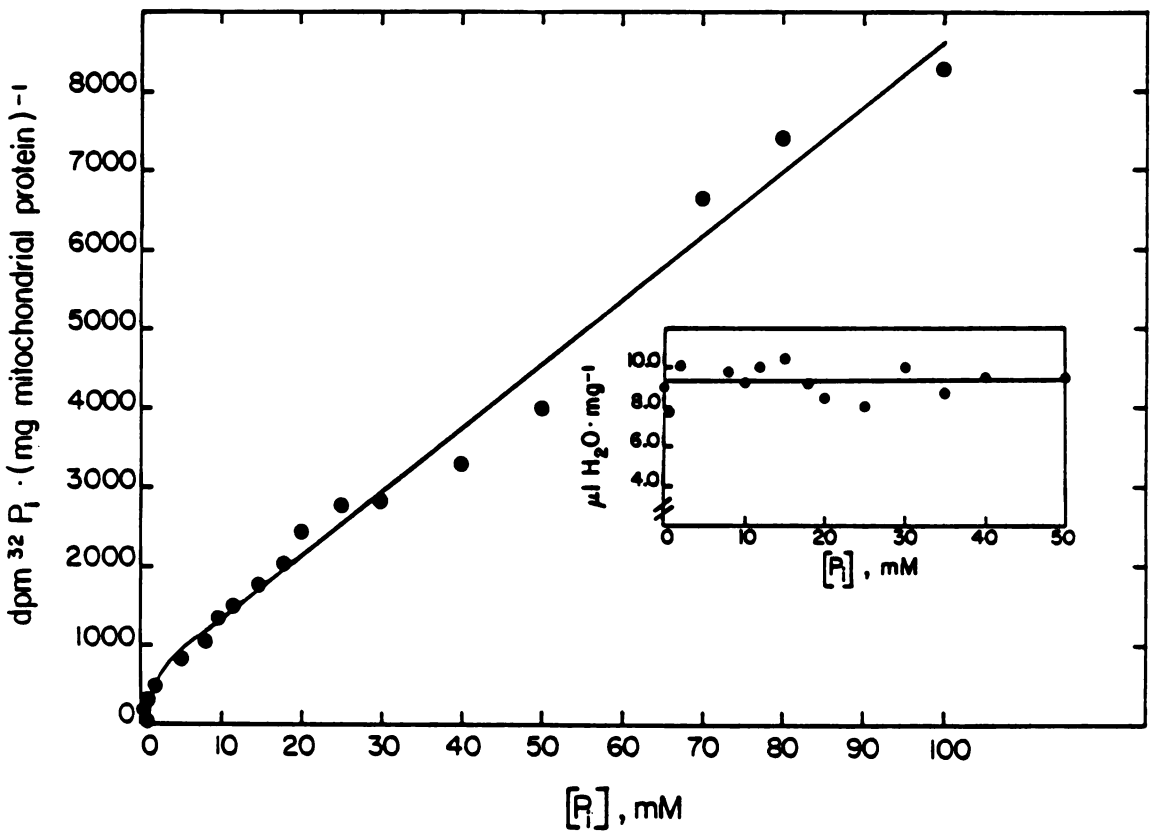

ΔLight Scatter Total · mg-1 · ml-1
1000
800
600
400
200
0
0
5
10
15
20
25
30
35
40
45
[Pi], mM

Figure 6. Mitochondrial Uptake of ^{32}Pi Measured as a Function of Pi Concentration

Mitochondria were incubated with and assayed for $^{32}P_i$ as described under "Experimental Procedures." All points represent the mean of two determinations. The inset shows the volume of extra-mitochondrial water that co-sediments with mitochondria that have been pre-incubated with the indicated concentrations of P_i.


dpm $^{32}P_i$ · (mg mitochondrial protein)$^{-1}$
8000
7000
6000
5000
4000
3000
2000
1000
0
0
10
20
30
40
50
60
70
80
90
100
[P_i], mM
μl H_2O · mg^{-1}
10.0
8.0
6.0
4.0
0
10
20
30
40
50
[P_i], mM

the swelling phase could not remain stable (Fig. 1A).

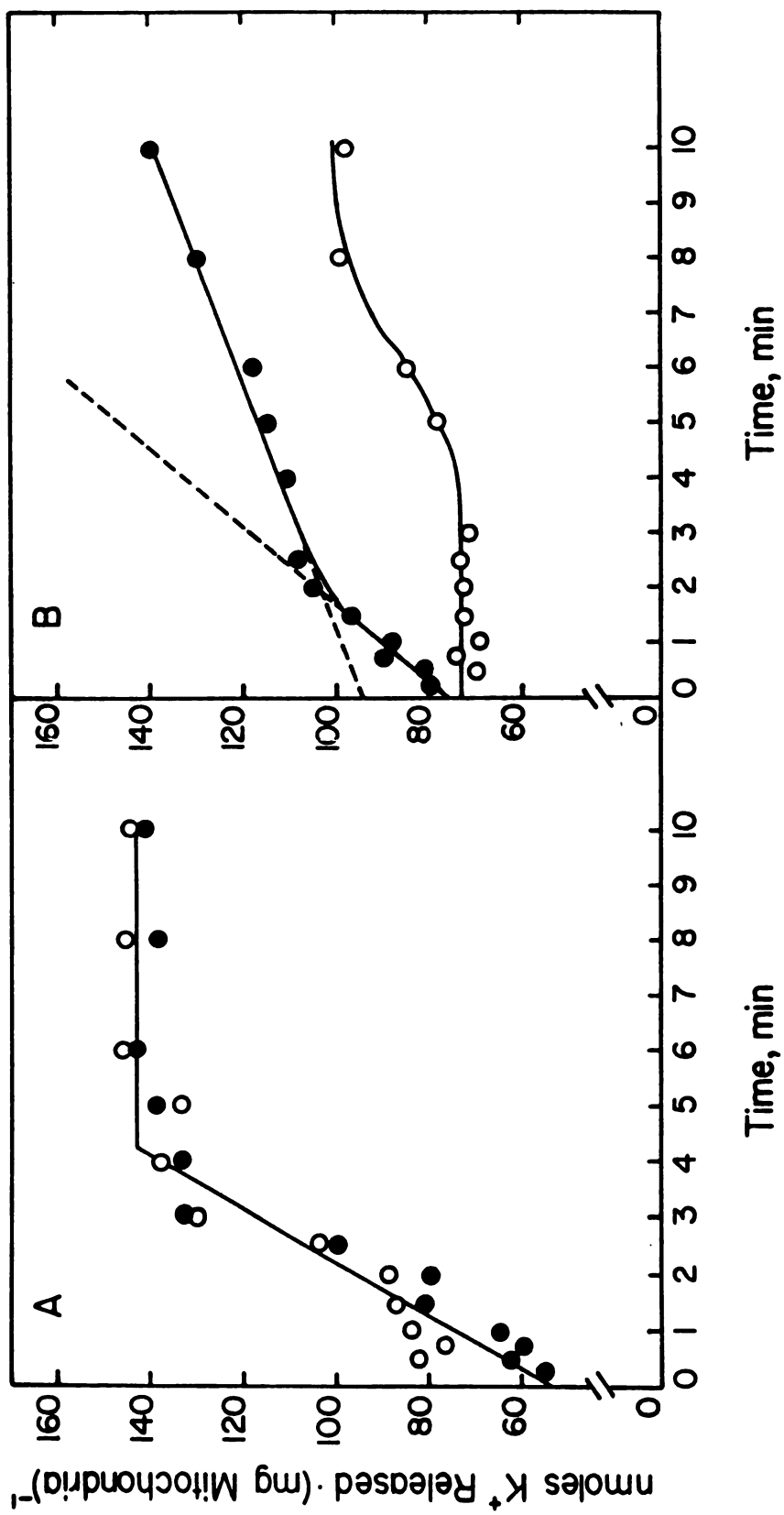
Considering the sheer number of inner membrane substrate transporters (Lanoue and Schoolwerth, 1986), as well as the complex interrelations possible between them, the variety of electrolytes that may participate to some degree in precise osmotic adjustments is large. In order to simplify the analysis, transmembrane fluxes of K^+, Mg(II), and Ca(II) were examined because changes in the matrix concentrations of these cations are known to influence mitochondrial volume significantly (for review, see Garlid and Beavis, 1987). In the absence of exogenous substrate, both diluting mitochondria into a larger volume of isotonic M/S medium or adding the organelles to M/S made 20 mM in P_i (M/S/P_i) elicits the extrusion of K^+ into the extramitochondrial space (Fig 7A). The response in the two cases is identical. K^+ is released linearly as a function of time at a rate of 21.1 ± 2.1 nmoles/min/mg mitochondria for 4 min. Between 4 and 10 min, no further release of K^+ is observed. After reaching the plateau, these mitochondria release 61-65% of their endogenous K^+. With excess P/M added as an energy source, the kinetics of K^+ extrusion in both the presence and absence of 20 mM P_i are dramatically changed (Fig 7B). With exogenous substrate only, K^+ efflux proceeds after a 4 min lag. Subsequent to the lag, the mitochondria release approximately 25.6 nmoles K^+/mg over an additional 4 min period. When mitochondria are simultaneously presented with P/M and P_i, K^+ efflux is biphasic with k_1 = 14.1 ± 1.6 and k_2 = 4.4 ± 0.34 nmoles/min/mg mitochondria.

The difference in K^+ efflux kinetics during P_i transport proceeding with and without exogenous P/M is striking. In the former case, mitochondria undergo a single swelling phase and remain structurally intact. The

Figure 7. The Effect of Pi and Exogenous Substrate on the Rate of K+ Efflux from Chick Heart Mitochondria

(A). K^+ concentration in the extramitochondrial space measured as a function of time of incubation in either M/S (◯) or M/S/P_i (●). Mitochondria at 0.56 mg ml^{-1} were suspended in a final volume of 1.75 ml and incubated at 30.5°C.

(B). Time course for K^+ efflux in M/S/T containing 5 mM pyruvate and 2.5 mM malate (P/M; ◯) or M/S/T enriched with both P/M and 20 mM Tris-P_i (●). Mitochondria at 0.65 mg ml^{-1} were suspended in a final volume of 1.76 ml and incubated at 30.5°C.


nmoles K+ Released · (mg Mitochondria)-1
A
B
Time, min
Time, min

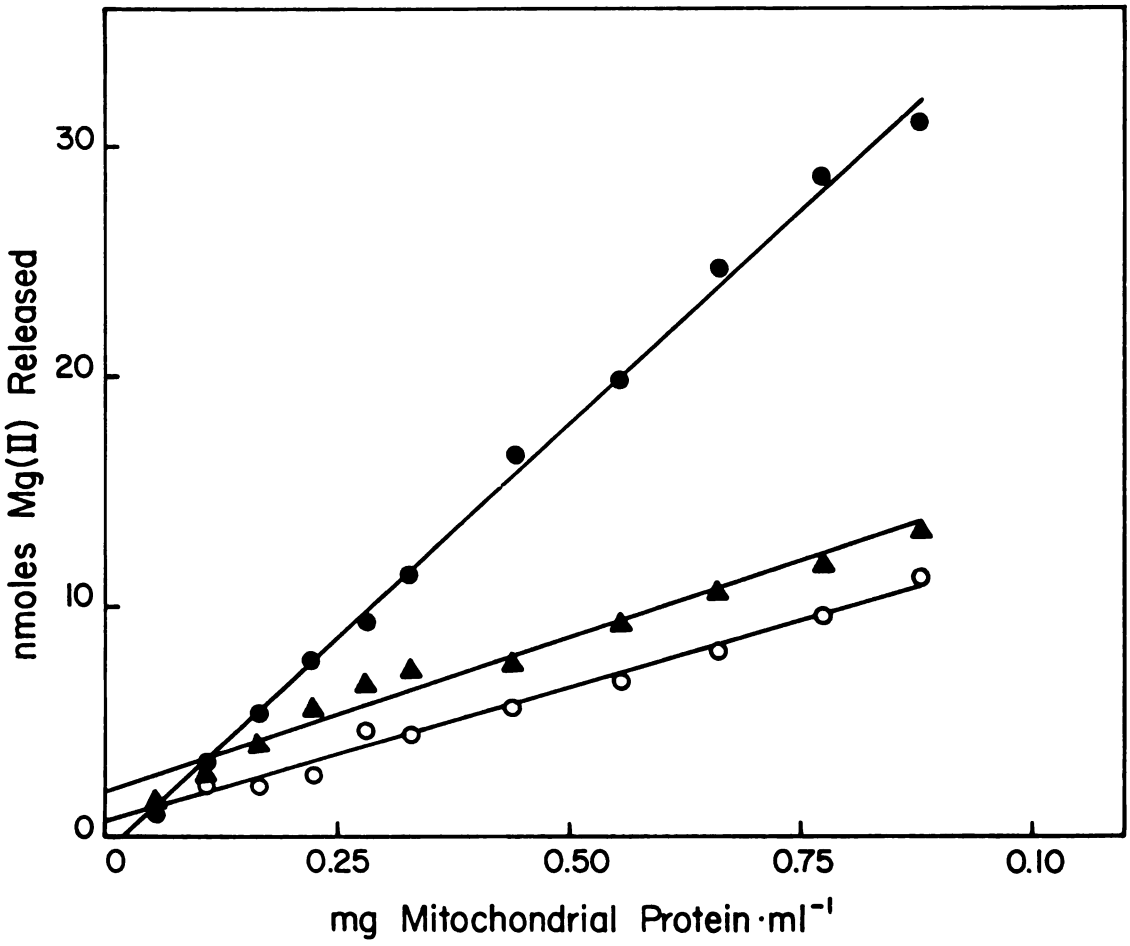
first, faster phase of K^+ efflux proceeds until P_i induced swelling is 90% complete. The rate of K^+ efflux becomes three-fold slower as swelling nears and finally attains completion. At this point mitochondria maintain a constant volume and take up P_i independent of water by closely matching the efflux of K^+ and likely other ions with P_i influx. In the absence of added P/M, K^+ loss is not coupled to P_i uptake. Consequently, once swollen to their maximal volume, the mitochondria become stressed osmotically because K^+ is no longer used to balance solute pressure on both sides of the inner membrane. If P_i uptake continues without osmotic control, the outer membrane ruptures and the inner membrane unfolds.

The following major points can be drawn from these data: (a) K^+ efflux occurs concomitantly with P_i influx; (b) P_i itself does not induce K^+ extrusion; rather, K^+ is used to relieve part of the osmotic stress resulting from the disruption of osmotic balance across the inner membrane; (c) exogenous substrate limits non-specific loss of K^+ and appears to provide mitochondria with the energy necessary to continuously maintain osmotic balance as P_i and water are transported into the matrix.

Fluxes of Mg(II) also participate in the volume changes induced by P_i. The amount of Mg(II) released by chick heart mitochondria incubated in 20 mM P_i also depends on whether or not the incubation medium contains exogenous substrate (Fig 8). In the absence of exogenous substrate, these mitochondria release 36.9 ± 0.63 nmoles Mg(II)/mg over a 20 min period. This decreases to 11.5 ± 0.54 and 13.1 ± 0.79 nmoles Mg(II)/mg when the mitochondria are co-incubated with pyruvate/malate or succinate, respectively. These results suggest that in the presence of substrate the mitochondria either release less Mg(II) during

Figure 8. Mg(II) Concentration in the Extra-Mitochondrial Space Measured as a Function of Mitochondrial Concentration

Mitochondria at the indicated concentration were allowed to incubate at 30.5°C for 20 min in 225 mM mannitol, 75 mM sucrose, and 20 mM Tris-P_i, pH 7.4. Suspensions were centrifuged and the supernatants were treated and assayed as described under "Experimental Procedures." Incubations conducted with: no substrate (●); 5 mM pyruvate/2.5 mM malate (▲); or 5 mM succinate (◯).


nmoles Mg(II) Released
30
20
10
0
0
0.25
0.50
0.75
0.10
mg Mitochondrial Protein·ml⁻¹

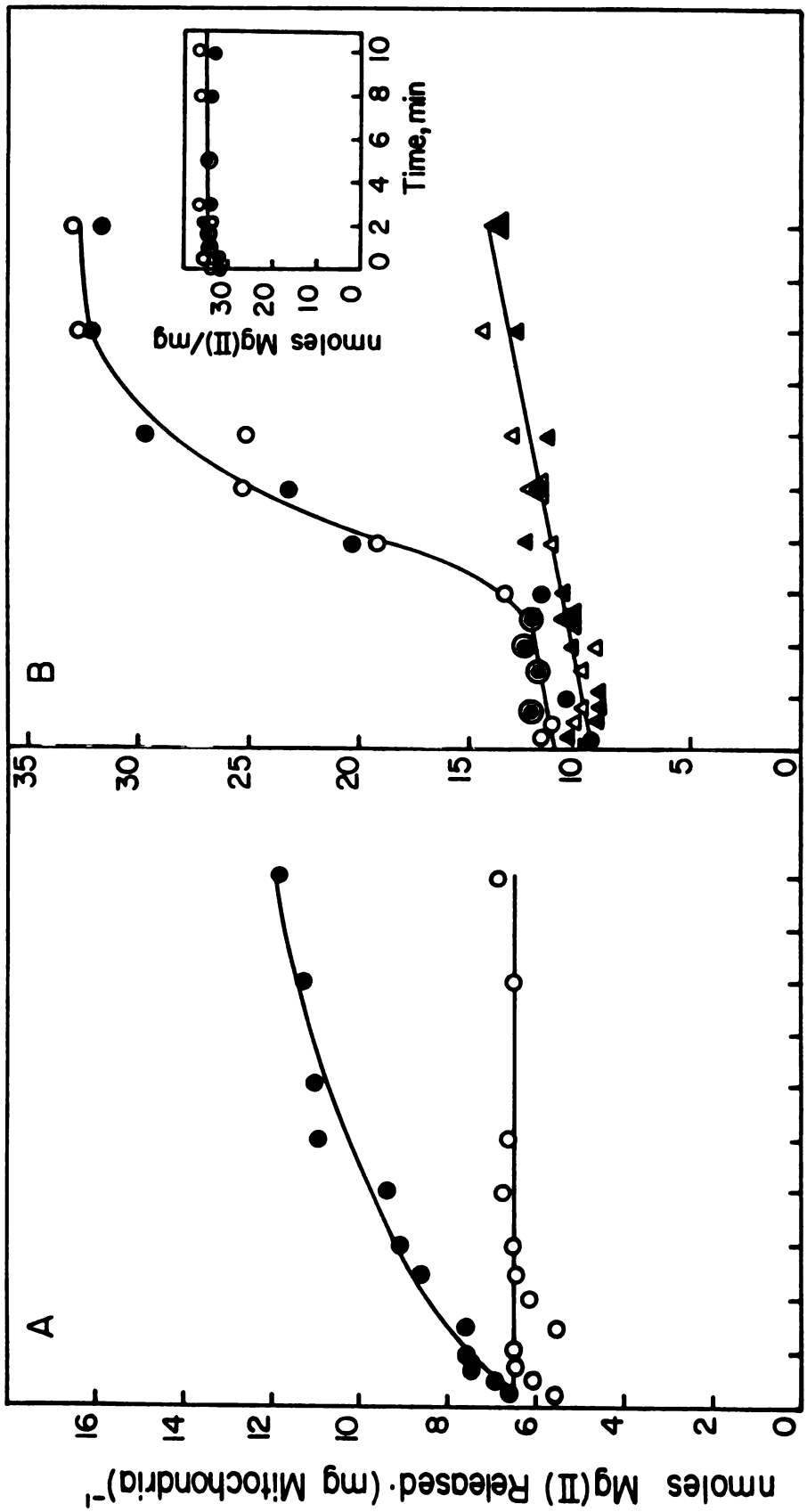
P_i uptake or that the released Mg(II) can be reinternalized in an energy-dependent manner. In order to distinguish between these possibilities, the following experiments were performed. Fig 9A shows the time course for Mg(II) release when mitochondria are incubated with or without P_i in the presence of P/M. In M/S medium enriched with P/M only, the amount of extramitochondrial Mg(II) remains constant at 6.4 ± 0.47 nmoles/mg mitochondria. This represents the amount of Mg(II) lost during isolation. As mitochondria take up P_i when suspended in M/S/P_i containing P/M, Mg(II) is released at a steadily decreasing rate. This result suggests that Mg(II) efflux is also coupled to osmotic stabilization of the matrix as water and P_i are transported in. In the absence of exogenous P/M and P_i, Mg(II) effluxes at a constant rate of 0.49 ± 0.021 nmoles/min/mg mitochondria (Fig 9B). Maintaining a constant matrix Mg(II) concentration thus requires energy. After an initial 3 min lag, the P_i-induced swelling process gives rise to a large increase in the rate of Mg(II) efflux from chick heart mitochondria metabolizing endogenous substrate only (Fig 9B). This results in the loss of 85% of endogenous Mg(II).

The timing of this massive release of Mg(II) coincides with the transient matrix contraction which occurs subsequent to P_i-induced swelling (Fig 1B). It is not possible to discern from these data whether the contraction detected by light scatter measurements represents water loss coupled to Mg(II) efflux or some as yet undefined change in inner membrane configuration triggered by the loss of Mg(II). One point favoring the former possibility is that both Mg(II) efflux and the mitochondrial contraction occur in synchrony, in that both begin after a 3 min lag and take 5 min to go to completion. The Mg(II) so released cannot be reinternalized under either standard state 3 or state 4

Figure 9. The Effect of Pi and Exogenous Substrate on the Rate of Mg(II) Efflux from Chick Heart Mitochondria

(A). Mg(II) concentration in the extramitochondrial space measured as a function of time of incubation in either 225 mM mannitol, 75 mM sucrose, 5 mM pyruvate/2.5 mM malate, 20 mM Tris, pH 7.4 (◯) or this assay medium enriched with 20 mM Tris-P_i (●). Mitochondria (0.65 mg ml^{-1}) were suspended in a final volume of 1.76 ml and incubated at 30.5°C.

(B). Mg(II) concentration in the extramitochondrial space measured as a function of time of incubation in either M/S (▲) or M/S/P_i (●). The data represent two separate experiments, differentiated by open and closed symbols. The insert shows the concentration of Mg(II) in the extra-mitochondrial space as a function of time of incubation with 5 mM pyruvate/2.5 mM malate (state 4, ◯) or 5 mM pyruvate/2.5 mM malate and 4.2 µmoles of ADP (state 3, ●) after mitochondria (0.61 mg ml^{-1}) were incubated for 20 min at 30.5°C in M/S/P_i.


nmoles Mg(II) Released · (mg Mitochondria)⁻¹
A
B
Time, min
Time, min
nmoles Mg(II)/mg
Time, min

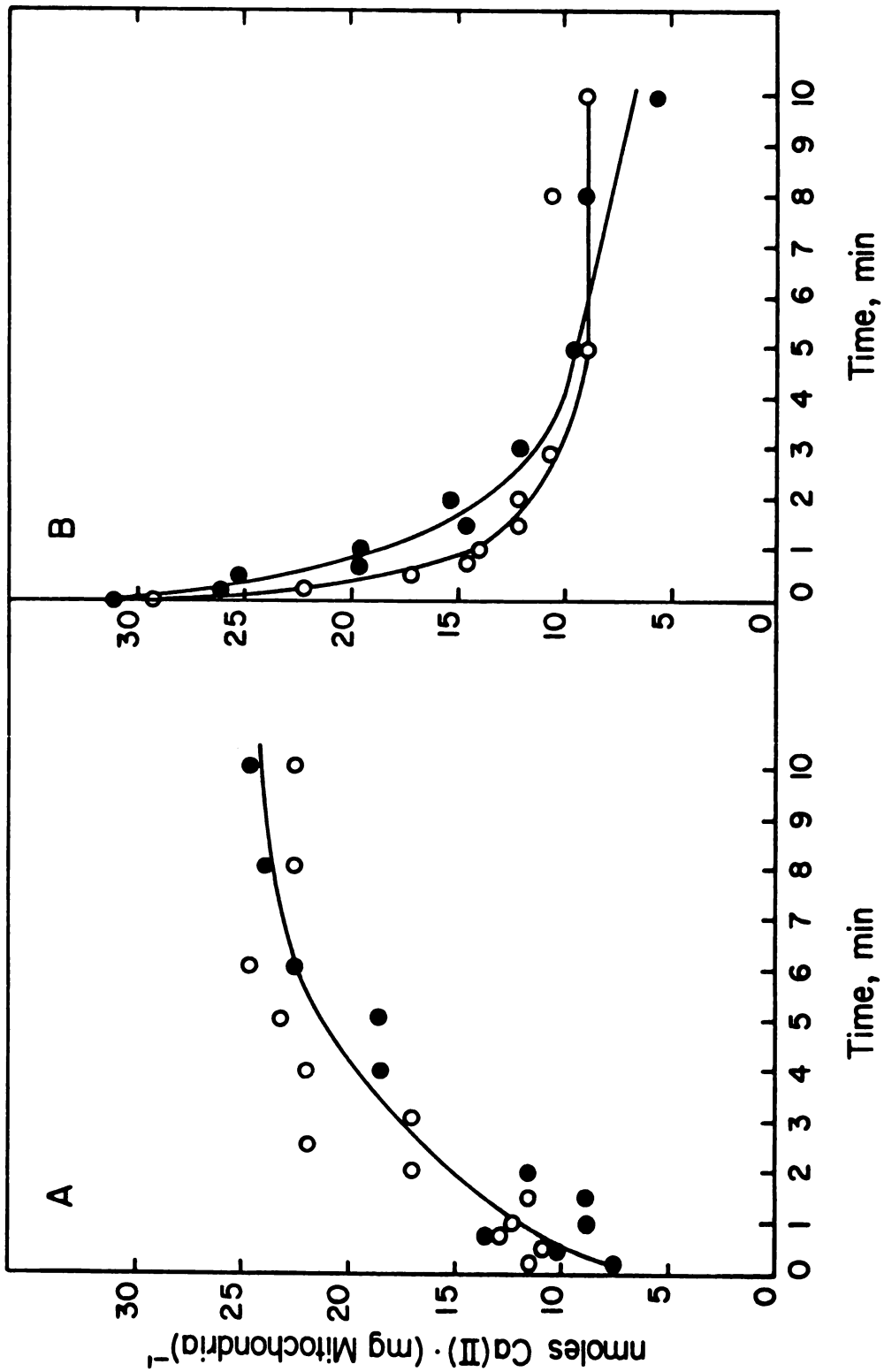
conditions (Insert, Fig 9B).

During the course of P_i uptake, Ca(II) fluxes are not involved in preventing the development of osmotic stress in any specific way. Chick heart mitochondria release 92% of endogenous Ca(II) over a 10 min period when added to a larger volume of M/S or M/S/P_i media (Fig 10A). If provided with exogenous P/M after the second swelling phase (Fig 1B), these mitochondria transport Ca(II) back into the matrix with an approximate half-time of 35 sec (Fig 10B). In M/S or M/S/P_i media enriched with exogenous P/M, extra-matrix Ca(II) content remains essentially constant at 7.1 ± 1.2 and 7.0 ± 1.7 nmoles/mg mitochondria, respectively. These results show that: (a) P_i uptake does not induce Ca(II) efflux. Whether Ca(II) efflux occurs depends, under these conditions, solely upon the mitochondrial energy poise. (b) The mitochondrial inner membrane is intact and respiration-competent subsequent to the second swelling phase that occurs when P_i uptake proceeds in the absence of exogenous substrate. This conclusion also implies that the outer membrane is ruptured but not stripped away, because cytochrome c cannot be lost to the bulk medium if respiration is to occur. (c) When metabolizing endogenous substrate only, energy is preferentially committed to the uptake of P_i rather than that of Ca(II). The functional and mechanistic basis for such preferential energy utilization is yet to be elucidated.

Figure 10. The Effect of Pi and Exogenous Substrate on Transmembrane Ca(II) Fluxes in Chick Heart Mitochondria

(A). Ca(II) concentration in the extra-mitochondrial space measured as a function of time of incubation in either M/S (○) or M/S/P_i (●). Mitochondria were suspended in a final volume of 1.75 ml at 0.73 mg ml^{-1}.

(B). Internalization of Ca(II) by chick heart mitochondria. Mitochondria were incubated in M/S/P_i at 30.5°C for 20 min to allow for Ca(II) efflux. Ca(II) concentrations in the extra-mitochondrial space are shown as a function of time of incubation with 5 mM pyruvate/2.5 mM malate (state 4, ●) or 5 mM pyruvate/2.5 mM malate and 4.2 μmoles of ADP (state 3, ○). Mitochondria (0.61 mg/ml) were suspended in a final volume of 1.75 ml.


nmoles Ca(II) · (mg Mitochondria)⁻¹
A
B
Time, min
Time, min
0
5
10
15
20
25
30
0 1 2 3 4 5 6 7 8 9 10

DISCUSSION

I. Ca(II) IS NOT REQUIRED FOR Pi-INDUCED SWELLING OF CHICK HEART MITOCHONDRIA

A variety of studies have suggested that P_i-induced swelling is the result of an interaction between internalized Ca(II) and the mitochondrial inner membrane. The "Ca(II)-induced membrane transition" (Hunter et al., 1976, 1979 a and b; Haworth and Hunter, 1979) or the "Ca(II)-induced hole" (Garlid and Beavis, 1987) is believed to facilitate P_i permeation due to an increase in the permeability of the inner membrane to both charged and uncharged solutes. In this model the initiation of P_i-induced swelling depends absolutely on Ca(II) that is transported from the extramitochondrial space into the matrix. Consistent with this, Garlid and Beavis (1987) found that, in the absence of exogenous Ca(II), rat liver mitochondria oxidizing succinate do not swell secondary to the addition of P_i. The Ca(II)-dependent increase in membrane permeability is accompanied by increases in mitochondrial 1-acyllysophospholipid content, indicating that once inside the matrix, Ca(II) activates phospholipase A_2 (Beatrice et al., 1980). Of interest is that in all of these studies, swelling in the presence of P_i proceeds after a lag time of approximately one to several min.

For the swelling studies detailed here, no lag between the addition of P_i to mitochondrial suspensions and the initiation of swelling is seen. Moreover, when swelling was monitored in the absence of exogenous P/M, Ca(II) was lost to the extramitochondrial space (Fig 10A). Under

these conditions no Ca(II) internalization took place during the course of a series of volume changes (see Fig 1B). That a generalized permeability change could not be facilitating P_i uptake is further evidenced by the facts that the rate of Mg(II) efflux is unaffected during the initial swelling phase (Fig 9B) and this swelling occurs in the presence of a high concentration of EGTA (Fig 1C). When presented with exogenous P/M, chick heart mitochondria readily take up extramitochondrial Ca(II) (see Fig 10B of this Chapter, and Fig 19 of Chapter 3). Yet, as shown in Fig 1A, this did not lead to the type of large-scale, massive swelling to be expected subsequent to a Ca(II)-induced membrane transition. Rather, the mitochondria were able to maintain precise osmotic control over matrix volume after the initial swelling phase reached steady-state. Taken together, these results show that in chick heart mitochondria, the Ca(II)-dependent membrane transition is not requisite to P_i-induced swelling. The swelling arises from P_i internalization catalyzed by the P_i transport protein as well as concomitant cationic fluxes. The discrepency between these studies and those of the authors cited above is difficult to account for. It is possible that chick heart mitochondria simply lack the biochemical apparatus underlying the Ca(II) induced membrane transition observed in rat liver (Garlid and Beavis, 1987) and bovine heart (Hunter and Haworth, 1979 a and b) mitochondria.

II. K^+ AND Mg(II) FLUXES SECONDARY TO Pi ADDITION

K^+ and Mg(II) appear to participate directly in osmotic stabilization of the mitochondrial matrix during the steady-state subsequent to P_i-induced swelling (see Fig's 7 and 9). It may be argued that K^+ and Mg(II) are lost because: (a) they are passively diffusing down their

respective concentration gradients; or (b) the binding of $Tris^+$ to mitochondrial membranes displaces these metal cations from negatively charged phospholipid head groups. Both of these possibilities are negated by the data shown in Fig's 7B and 9A. Neither K^+ nor Mg(II) is continuously lost when incubated in a Tris-buffered osmotic support medium containing exogenous P/M. In the case of Mg(II), for instance, matrix stores of this ion remain constant. It follows that when mitochondria are suspended in a Tris-buffered medium containing P/M and P_i-induced swelling is initiated, changes in matrix Mg(II) must be coupled to the uptake of P_i. Consistent with the findings of others (Crompton et al., 1976), the control of Mg(II) efflux during P_i uptake proceeding in the presence of excess substrate appears to be absolutely dependent on respiration. The degree of control over K^+ extrusion and the apparent coupling of this process to mitochondrial volume changes (Fig. 7B) are striking. Although not discernible from these data, it is possible that the putative mitochondrial inner membrane K^+/H^+ antiporter (Nakashima and Garlid, 1982; Martin et al., 1984) is responsible for controlling the rate of K^+ efflux. In the steady-state (2-10 min after the addition of P_i), these, and likely other additional (e.g., electrophoretic Tris internalization and P_i export via malate exchange on the dicarboxylate carrier) processes operate to ensure that $[solutes]_{in} = [solutes]_{out}$. If this were not true the mitochondria would rapidly lyse subsequent to the continued uptake of P_i. That the mitochondria remain functionally, as well as structurally, intact after P_i uptake in the presence of exogenous P/M is further evidenced by the observation that respiratory control increases as a function of time of pre-incubation with these substrates prior to the initiation of state 3 respiration

with ADP (see Fig 5 of Chapter 3).

It is apparent that if mitochondrial P_i uptake proceeds in the absence of sufficient substrate, structural damage of the organelle occurs. Under these conditions, the contraction following P_i-induced swelling (Fig. 1B) is clearly coupled to the loss of Mg(II) (Fig. 9B). Depletion of matrix Mg(II) destabilizes the inner membrane (Binet and Volfin, 1974; Coelho and Vercesi, 1980) and increases the permeability of mitochondria to Ca(II) (Zoccarato et al., 1981) and K^+ (Jung and Brierly, 1986). Mg(II) can only stabilize the inner membrane when membrane thiol groups are reduced; Mg(II) is released to the extra-mitochondrial space when these thiols become oxidized (Siliprandi et al., 1975, 1979). In the absence of exogenous substrate, the loss of Mg(II) does not appear to be coupled to P_i internalization. Rather, as matrix substrate is depleted subsequent to the uptake of P_i, the energy requisite to the maintinence of matrix Mg(II) concentrations is no longer available. This loss of Mg(II) would thus be expected to proceed independent of respiration. Considering the distribution of Mg(II) between the outer membrane, inter-membrane space, inner membrane, and matrix (Bogucka and Wojtczak, 1971), the massive loss (86%) of this cation occurs in a surprisingly concerted fashion (i.e., it is mono-phasic). The efflux of Mg(II) is accompanied by a large loss of water. This phase cannot be due to the disruption of membranes because no additional K^+ is lost (see Fig. 7A). As the process of Mg(II) efflux goes to completion, another swelling phase is initiated which leads to extensive disruption of mitochondrial morphology. The trigger for this transition appears to be the loss of Mg(II). The second swelling phase leads to unfolding of the inner membrane and, very likely, rupture of

the outer membrane. Although Mg(II) in the extramitochondrial space was not reinternalized after the second swelling phase, this does not imply that these mitochondria cannot transport Mg(II) in because net influx requires concentrations of Mg(II) in excess of 5 millimolar (Brierly et al., 1963; Schuster and Olson, 1974). The second swelling phase does not lead to complete lysis of these organelles since Ca(II) was rapidly internalized when exogenous substrate was provided (Fig 10B). In order for the mitochondria to be able to clear extra-matrix Ca(II) they must have had: (a) intact inner membranes; and (b) a capacity to transfer electrons to cytochrome oxidase (i.e., cytochrome c could not have been lost to the extramitochondrial space). It can be inferred from the data presented herein that substrate depletion leads to a breakdown in mitochondrial morphology because Mg(II) is lost and the mitochondria are no longer able to match solute fluxes in and out of the matrix via energy dependent ion-exchange reactions.

III. IMPLICATIONS FOR MYOCARDIAL ISCHEMIA

The effects of myocardial ischemia on mitochondrial structure and function is an area currently under intensive investigation. P_i accumulates in the cell during ischemia as the magnitude of the cytosolic phosphorylation potential decreases. Heart mitochondria swell in ischemic myocardium (Jennings and Ganote, 1976). P_i is believed to participate in the induction of this swelling (Matlib et al., 1983). P_i-induced swelling of heart mitochondria has been reported to inhibit oxidative phosphorylation in the presence of exogenous substrate (Vaghy et al., 1981; Lange et al., 1984). Both groups invoke Ca(II) as a participant in the inhibition process. The results of experiments reported here do

not support these findings. In the presence of exogenous substrate P_i induces swelling but compromises neither structure nor function. Moreover, Ca(II), which is actively taken-up under these conditions, exerts no detectable deleterious effects. During periods of severe ischemic distress, respiration will be inhibited due to a lack of oxygen. Protons will not be pumped, the flux through proton-coupled ion permeation pathways will be negligible, and thus the capability of mitochondria to make precise osmotic adjustments using K^+, Mg(II), or other ions will be depressed. This situation is akin to that shown in Fig. 1B, where subsequent to P_i internalization substrate is depleted. The loss of Mg(II) and possibly other matrix components leads to the collapse of membrane organization and a loss in the capacity of mitochondria to phosphorylate ADP. The swelling induced by P_i does not per se lead to compromise; it is the energy deficiency that does so.

IV. USE OF LIGHT SCATTER METHODOLOGY TO MEASURE Pi UPTAKE KINETICS

By continuously monitoring changes in the intensity of light that is scattered by a suspension of chick heart mitochondria, it is possible to extract valuable information about the kinetic properties of the P_i transport protein and to ascertain the effect of P_i on mitochondrial structure and function. These measurements do not provide information about the specific transport rate of P_i (i.e., nmoles P_i/min/mg mitochondria). However, by assuming that the rate of swelling is limited by the rate of P_i transport (Garlid and Beavis, 1985), it is possible to calculate an apparent rate constant for, and to identify the order of, the P_i translocation reaction. P_i-induced swelling revealed, in close agreement with the more direct studies of Kaplan et al. (1986), that P_i

transport is a first-order process with a k equal to approximately 1.0 min^{-1}.

Error in k may arise from ionic fluxes that proceed concomitantly with P_i uptake. For instance, when P_i-induced swelling is monitored in the presence of exogenous substrate, Ca(II) will be transported in while K^+ and Mg(II) are transported out. These fluxes will influence matrix volume. The extent of this effect depends on the degree to which these fluxes cancel one another. This cannot be estimated from the data reported in this communication. However, based on the inhibition of P_i-induced swelling changes with MalNEt and CCCP, it can be stated with confidence that P_i-uptake is the transport process limiting the rate of mitochondrial swelling under the conditions used in these experiments. Based on comparison with other published studies, 90° light scatter measurements are more sensitive to alterations in mitochondrial volume than monitoring changes in absorbance (0° scatter). To our knowledge, this is the first report showing that P_i induces a triphasic, not monophasic, change in mitochondrial volume. That others have not detected this sequence of changes is probably due to a lack of sensitivity in the 0° light scatter instrumentation employed.

V. ROLE OF THE Pi TRANSPORT PROTEIN IN RESPIRATORY CONTROL

In recent years much attention has been directed toward identifying the rate limiting step or steps of oxidative phosphorylation (Lemasters and Sowers, 1979; Wilson, 1980; Groen et al., 1982). The P_i transporter is believed to exert significant control over the rate of ATP synthesis in yeast mitochondria (Mazat et al., 1986). Although swelling studies do not constitute direct kinetic assays of P_i transport, the fact that the

rate of P_i-induced swelling is independent of P_i concentration in the range of 0.5-45 mM suggests that the P_i transport protein is nearly saturated at these concentrations. At pH 7.4, one would expect that the total P_i in solution will consist of an equilibrium between the mono- and divalent forms of this anion. Assuming that 90% saturation of an enzyme with its substrate is equal to $10K_m$, then it is estimated that the K_m of the chick heart mitochondrial P_i transport protein for P_i is no higher than 25-50 μM. This value is in sharp contrast with that published for the rat liver P_i transport protein (4.21 mM; Coty and Pederson, 1974). The basis for the difference in the affinity of these mitochondria for P_i may in part be attributed to the specific energy requirements of heart muscle and liver cells. The metabolic focus of mitochondria in these two tissues is very different. Heart mitochondria may require greater efficiency in binding and transporting P_i into the matrix because of the constant beat-to-beat energy demands of myocardium. Under physiological conditions the P_i transporter in heart would thus be expected to be very nearly saturated at all times. This would preclude a role for the P_i transporter in the control of oxidative phosphorylation.

VI. CONCLUSIONS

The studies described herein demonstrate that: (a) P_i induces respiration-dependent swelling of chick heart mitochondria. Significantly, this process does not require a Ca(II) triggered alteration in mitochondrial membrane permeability. (b) In the presence of excess oxidizable substrate, the volume change is monophasic and native mitochondrial morphology does not appear to be disrupted. (c) When mitochondria oxidize endogenous substrate only, P_i induces a triphasic change in mitochondrial

volume, resulting in extensive morphological damage. (d) Complex K^+ and Mg(II) fluxes accompany the volume changes resulting from P_i uptake. (e) The rate of swelling observed secondary to the addition of P_i reflects the kinetic properties of the mitochondrial inner membrane P_i transport protein.

REFERENCES

Adamsons, K., Sell, J.E., Holland, J.F., and Timnick, A. (1982) Anal. Biochem. 54: 2186-2190.

Armston, A.E., Halestrap, A.P., and Scott, R.D. (1982) Biochim. Biophys. Acta 681: 429-439.

Austin, J., and Aprille, J.R. (1984) J. Biol. Chem. 259: 154-160.

Beatrice, M.C., Palmer, J.W., and Pfeiffer, D.R. (1980) J. Biol. Chem. 255: 8663-8671.

Beavis, A.D., Brannan, R.D., and Garlid, K.D. (1985) J. Biol. Chem. 260: 13,424-13,433.

Bencini, D.A., Wild, J.R., and O'Donovan, G.A. (1983) Anal. Biochem. 132: 254-258.

Binet, A., and Volfin, P. (1974) Arch. Biochem. Biophys. 164: 756-764.

Brierly, G., Murer, E., Bachmann, E., and Green, D.E. (1963) J. Biol. Chem. 238: 3482-3489.

Brierly, G.P., Jurkowitz, M., Scott, K.M., and Merola, A.J. (1971) Arch. Biochem. Biophys. 147: 545-556.

Chance, B., and Hagihara, B. (1963) Proc. 5th int. Congr. Biochem. 5: 3-37.

Chappell, J.B., and Crofts, A.R. (1965) Biochem. J. 95: 378-386.

Chavez, E., Jung, D.W., and Brierly, G.P. (1977) Arch. Biochem. Biophys. 183: 460-470.

Coelhi, J.C., and Vercesi, A.E. (1980) Arch. Biochem. Biophys. 204: 141-147.

Coty, W.A., and Pederson, P.L. (1974) J. Biol. Chem. 249: 2593-2598.

Crofts, A.R., and Chappell, J.B. (1965) Biochem. J. 95: 387-392.

Crompton, M., Capano, M., and Carafoli, E. (1976) Biochem. J. 154: 735-742.

Fonyo, A., and Vignais, P.V. (1979) FEBS Lett. 102: 301-305.

Garlid, K.D., and Beavis, A.D. (1985) J. Biol. Chem. 260: 13,434-13,441.

Garlid, K.D., and Beavis, A.D. (1987) Biochem. Biophys. Acta 853: 187-204.

Greenbaum, N.L., and Wilson, D.F. (1985) J. Biol. Chem. 260: 873-879.

Groen, A.K., Wanders, R.J.A., Westerhoff, H.V., Van der Meer, R., and Tager, J.M. (1982) J. Biol. Chem. 257: 2754-2757.

Hackenbrock, C.R. (1966) J. Cell Biol. 30: 269-297.

Halestrap, A.P., Quinlan, P.T., Whipps, D.E., and Armston, A.E. (1986) Biochem. J. 236: 779-787.

Haworth, R.A., Hunter, D.R. (1979) Arch. Biochem. Biophys. 195: 460-467.

Hoek, J.B., Lofrumento, N.E., Meyer, A.J., and Tager, J.M. (1971) Biochem. Biophys. Acta 226: 297-308.

Hunter, D.R., Haworth, R.A., and Southard, J.H. (1976) J. Biol. Chem. 251: 5069-5077.

Hunter, D.R., and Haworth, R.A. (1979) Arch. Biochem. Biophys. 195: 453-459.

Hunter, D.R., and Haworth, R.A. (1979) Arch. Biochem. Biophys. 195: 468-477.

Izzard, S., and Tedeschi, H. (1973) Arch. Biochem. Biophys. 154: 527-539.

Jennings, R.B., and Ganote, C.E. (1976) Circ. Res. 38: 80-91.

Jung, D.W., Chavez, E., and Brierly, G.P. (1977) Arch. Biochem. Biophys. 183: 452-459.

Kaplan, R.S., and Pederson, P.L. (1983) Biochem. J. 212: 279-288.

Kaplan, R.S., Pratt, R.D., and Pedersen, P.L. (1986) J. Biol. Chem. 261: 12,767-12,773.

Klingenberg, M., Durand, R., and Guerin, B. (1974) Eur. J. Biochem. 42: 135-150.

Lange, L.G., Hartman, M., and Sobel., B.E. (1984) J. Clin. Invest. 73: 1046-1052.

LaNoue, K.F., and Schoolwerth, A.C. (1979) Ann. Rev. Biochem. 48: 871-922.

Lehninger, A.L. (1962) Physiol. Rev. 42: 467-517.

Lemasters, J.J., and Sowers, A.E. (1979) J. Biol. Chem. 254: 1248-1251.

Ligeti, E., and Fonyo, A. (1984) Eur. J. Biochem. 159: 279-285.

Lotscher, H., Winterhalter, K.H., Carafoli, E., and Richter, C. (1980) Eur. J. Biochem. 110: 211-216.

Markwell, M.A.K., Haas, S.M., Tolbert, N.E., and Bieber, L.L. (1981) Methods Enzymol. 72: 296-303.

Martin, W.H., Beavis, A.D., and Garlid, K.D. (1984) J. Biol. Chem. 259: 2062-2065

Matlib, M.A., Vaghy, P.L., Epps, D.E., and Schwartz, A. (1983) Biochem. Pharmacol. 32: 2622-2625.

Mazat, J., Jean-Bart, E., Rigoulet, M., and Guerin, B. (1986) Biochem. J. 849: 7-15.

McGivan, J.D., and Klingenberg, M. (1971) Eur. J. Biochem. 20: 292-399.

McGivan, J.D., Grebe, K., and Klingenberg, M. (1971) Biochem. Biophys. Res. Comm. 45: 1533-1541.

Nakashima, R.A., and Garlid, K.D. (1982) J. Biol. Chem. 9252-9254.

Nicholls, D.G., and Crompton, M. (1980) FEBS Lett. 111: 261-268.

Packer, L. (1961) J. Biol. Chem. 236: 214-220.

Palmieri, F., Quagliariello, E., and Klingenberg, M. (1970) Eur. J. Biochem. 17: 230-238.

Pozzan, T., Bragadin, M., and Azzone, G.F. (1977) Biochemistry 16: 5618-5625.

Puskin, J.S., Gunter, T.E., Gunter, K.K., and Russell, P.R. (1976) Biochemistry 15: 3834-3842.

Schuster, S.M., and Olson, M.S. (1974) J. Biol. Chem. 249: 7151-7158.

Siliprandi, D., Toninello, A., Zoccarato, F., Rugolo, M., and Siliprandi, N. (1975) Biochem. Biophys. Res. Comm. 66: 956-961.

Siliprandi, D., Rugolo, M., Zoccarato, F., Toninello, A., and Siliprandi, N. 1979) Biochem. Biophys. Res. Comm. 88: 388-394.

Tedeschi, H. (1961) Biochim. Biophys. Acta 46: 159-169.

Tyler, D.D. (1980) Biochem. J. 192: 821-828.

Vaghy, P.L., Matlib, M.A., and Schwartz, A. (1981) Biochem. Biophys. Res. Comm. 100: 37-44.

Wilson, D.F. (1980) In Membrane Structure and Function (E.E. Bittar, ed.), pp. 153-195. J. Wiley Press, New York.

Wohlrab, H. (1986) Biochim. Biophys. Acta 853: 115-134.

Zoccarato, F., Rugolo, M., Siliprandi D., and Siliprandi, N. (1981) Eur. J. Biochem 114: 195-199.

Chapter 6

INTERACTION OF INORGANIC PHOSPHATE WITH CHICK HEART MITOCHONDRIA. II. SUBSTRATE-DEPENDENT MODULATION OF STATE 3 AND STATE 4 RATES OF RESPIRATION.

INTRODUCTION

Inorganic phosphate (P_i) is a functionally versatile anion (Westheimer, 1987) which regulates the flux through numerous key metabolic pathways. Mitochondrial oxidative phosphorylation is one such pathway. However, the explicit kinetic and thermodynamic mechanisms by which P_i regulates the rate of mitochondrial respiration are unclear. A number of theories concerning this issue have been elaborated in recent years. Wilson and coworkers (Wilson et al., 1974; Holian et al., 1977) showed that the rate of mitochondrial respiration depends on the extramitochondrial phosphorylation potential, defined as:

$$\Delta G_P = \Delta G^{\circ}_P + RT \ln([ATP]/[ADP][P_i]) \qquad (1)$$

where all terms have their usual meaning. According to this theory, increased P_i concentrations activate respiration because the phosphorylation potential (ΔG_P) would no longer be at equilibrium with the redox state of the electron transfer chain components involved in energy conservation. Lemasters and Sowers (1979) postulated that increasing P_i concentrations would stimulate the rate of oxidative phosphorylation by raising the magnitude of the $\Delta\psi$ component of protonmotive force, thereby accelerating the rate at which ADP^{3-} exchanges with ATP^{4-} across the mitochondrial inner membrane. In contrast, other workers claim that the rate of respiration is independent of P_i and depends instead on the ratio of ATP to ADP concentrations, primarily because ATP competes with the binding of ADP to the adenine nucleotide translocase (Slater et al.,

1973; Davis and Lumeng, 1975; Kuster et al., 1976). Early work by Chance and Williams (1955, 1956) with isolated mitochondria suggested that ADP is the primary regulator of respiratory rate. This regulation has been substantiated in intact skeletal muscle under steady-state conditions using phosphorous magnetic resonance spectroscopy (Chance et al., 1985, 1986). In those models which attribute primary rate control to ADP, P_i may be rate-limiting if the P_i requirements for near maximal activity of the ATP synthase are not met. Thus, in kinetic models P_i apparently modulates the rate of respiration because it is a substrate of oxidative phosphorylation with a characteristic affinity for the F_1F_0-ATP synthetase.

Integrative models describing the relative contribution of P_i to respiratory control must account for all possible ways that P_i regulates the rates of mitochondrial substrate oxidation and ADP phosphorylation. The P_i carrier protein catalyzes the transport of P_i into the mitochondrial matrix via P_i/H^+ symport or P_i/OH^- antiport (Hoek et al., 1971; Greenbaum and Wilson, 1985). If the K_m of this protein for P_i is in the millimolar range (Coty and Pedersen, 1974; Ligeti et al., 1979), then it too could exert some control over the rate of oxidative phosphorylation. However, as shown in Chapter 5, in chick heart mitochondria the K_m of this protein for P_i appears to be less than or equal to 50 μM. Since P_i concentrations in vivo approximate to 1-2 mM (Chance et al., 1986), the P_i transport protein of these heart mitochondria is very likely nearly saturated under normal conditions and thus would not be expected to exert significant control over rates of respiration. Another possibility for regulation by P_i is that this anion may positively or negatively affect the kinetics of matrix dehydrogenases. In this case P_i would

limit respiration by modulating matrix concentrations of NADH.

The following investigations were performed to characterize the kinetics of chick heart mitochondrial P_i utilization in an effort to contribute toward a more comprehensive model of how P_i regulates respiration. The data show that P_i regulates the rate of mitochondrial respiration in several ways. It is, first, an effector of the dehydrogenases for α-ketoglutarate and β-hydroxybutyrate during state 3 respiration; second, a substrate for the ATP synthetase; and third, an inhibitor of state 4 respiration when the dehydrogenases for pyruvate and α-ketoglutarate are rate-limiting. The data support the arguments that P_i regulates respiration through kinetic rather than thermodynamic means and that mitochondrial matrix dehydrogenases participate in the phenomenon of respiratory control.

EXPERIMENTAL PROCEDURES

I. MATERIALS

The water used for these experiments was purified as previously described in Chapter 3 of this dissertation. The following substances were reagent grade or better, used without further purification, and obtained from the sources noted: ADP (grade X), GDP (type I), IDP, NAD (grade III-C), NADH (grade II), rotenone, phosphoenolpyruvate, thiamine pyrophosphate, INT, EGTA, fatty acid free BSA, Tris, sucrose (RNase-free), β-mercaptoethanol, lactate dehydrogenase (type II), pyruvate kinase (type III), collagenase (type VII, lot 47F-6829), lipoamide dehydrogenase (type III), and the sodium salts of α-ketoglutaric, pyruvic, β-hydroxybutyric, malic, and glutamic acids (Sigma Chemical Co., St. Louis, MO); HEPES (Boehringer-Mannheim Biochemicals, Indianapolis, IN); and mannitol (Fisher Scientific). Collagenase was reconstituted prior to each isolation in a solution containing 225 mM mannitol and 75 mM sucrose. Rotenone was dissolved in absolute ethanol (AAPER Alcohol and Chemical Co.) and stored at -20°C. Phosphate solutions were prepared by titrating a 1.0 M solution of phosphoric acid (Mallinckrodt) with either Tris or HEPES, as indicated, to pH 7.4. The lipoamide dehydrogenase was dialyzed at 5°C against two changes of a 20 mM HEPES, pH 7.4, buffer prior to use in order to remove the ammonium sulfate in which it was suspended. Single comb white leghorn chicks were obtained as described in Chapter 3.

II. ISOLATION AND PREPARATION OF MITOCHONDRIA

For oxygen consumption experiments, highly coupled heart mitochondria were isolated from 14-21 day-old chicks using collagenase (Toth et al., 1986). The final mitochondrial pellet was resuspended in a medium that contained 225 mM mannitol, 75 mM sucrose, 1 mM EGTA, and 0.2% (w/v) BSA. Mitochondria used in matrix dehydrogenase assays were isolated as specified by Hinman and Blass (1981), with minor modifications. Chick ventricular myocardium was homogenized in a buffer that contained 220 mM mannitol, 70 mM sucrose, 1 mM β-mercaptoethanol, 1 mM EGTA, 0.05% (w/v) BSA, and 20 mM HEPES, pH 7.4. Mitochondria obtained after the first high speed centrifugation step (8,000xg) were washed six times (8,000xg for 20 min) in a hypotonic buffer comprised of 15 mM KCl, 1 mM EGTA, 1 mM β-mercaptoethanol, and 20 mM HEPES, pH 7.4. The resulting swollen mitochondria were resuspended at approximately 15 mg ml^{-1} and frozen in 1.5 ml Eppendorf centrifuge tubes at -105°C for 48 hrs. Prior to use mitochondria were broken by thawing for 15 min in a water bath adjusted to 37°C.

III. ASSAYS

Mitochondrial oxygen consumption was assayed at the critical mitochondrial concentration as detailed in Chapter 3. NADH production by mitochondrial matrix dehydrogenases was assayed according to the method of Hinman and Blass (1981). In this assay, NADH production is coupled to the reduction of INT using lipoamide dehydrogenase. Assays were performed at 500 nm and a molar absorptivity of 12,400 M^{-1} cm^{-1} for reduced INT was assumed (Owens and King, 1975). Broken mitochondria were suspended in 1.0 ml of an assay medium that contained 2.5 mM NAD, 2 mM

EGTA, 0.6 mM INT, 0.2 mM thiamin pyrophosphate, 0.3 mM DL-dithiothreitol, 1 mM $MgCl_2$, 0.1% (w/v) BSA, 20 mM HEPES, pH 7.4, 10 μM rotenone, and an excess of lipoamide dehydrogenase. In addition, the assay medium contained oxidizable substrate (5 mM), as well as P_i and a nucleotide diphosphate at concentrations indicated in Tables 2 and 3 and in the legend to Figure 5. In order to ensure that the activiation of dehydrogenase activity by P_i and nucleotide diphosphates was specific, EGTA was included in the assay medium because Ca(II) is known to activate the dehydrogenases for pyruvate and α-ketoglutarate (Denton and McCormack, 1986; McCormack and Denton, 1979). Rotenone was included in the assay medium so as to prevent the oxidation of NADH by site I of the electron transfer chain.

ADP concentrations were assayed using the pyruvate kinase/lactate dehydrogenase assay detailed elsewhere (Toth et al., 1988a). GDP and IDP concentrations were determined spectrophotometrically in 1.0 ml of a 20 mM Tris, pH 7.4, buffer. The extinction coefficients used for GDP and IDP were ε(252 nm) = 13,700 M^{-1} cm^{-1} (Bock et al., 1956) and ε(265 nm) = 23,600 M^{-1} cm^{-1} (C.H. Suelter, personal communication), respectively.

Other assays and statistical analyses were performed using the methods described in Chapter 3.

RESULTS

I. THE INORGANIC PHOSPHATE REQUIREMENTS OF STATE 3 RESPIRATION

The exogenous P_i requirements of chick heart mitochondrial state 3 respiration vary with the carboxylic acid substrates (Fig's 1 and 2). In these experiments, rates of state 3 respiration supported by endogenous P_i were substracted from rates measured at each concentration of added P_i. Therefore, the rates represent respiration stimulated by exogenous P_i. The kinetic parameters for P_i saturation of state 3 respiration are summarized in Table I. The K_{50}'s for P_i saturation of state 3 respiration in the presence of pyruvate/malate (P/M) and glutamate/malate (G/M) (Fig 1) are very similar and are in close agreement with the K_{50} of bovine heart ATP synthase for P_i (Matsuno-Yagi and Hatefi, 1986). Therefore, the K_{50} obtained during the oxidation of these substrate pairs probably reflects directly the P_i requirements of the chick heart ATP synthase, and no other site of interaction for P_i in the matrix is apparent.

Similar titrations with P_i when α-ketoglutarate (αKG; Fig 1) or β-hydroxybutyrate (βHB; Fig 2) are oxidized suggest, however, the existence of more than one binding site for P_i. The kinetics of stimulating the rate of state 3 respiration with P_i during the oxidation of α-ketoglutarate are sigmoidal. Consistent with sigmoid-type kinetics found with other enzymes (Monod et al., 1965; Koshland et al., 1966), it is possible that P_i binds cooperatively to the dehydrogenase for α-ketoglutarate. It is reasonable to assume that the affinity of the ATP synthase for P_i will not vary as the source of reducing equivalents for electron

Figure 1. State 3 Rates of Respiration Measured as a Function of Pi Concentration When Mitochondria Oxidize Pyruvate/Malate, Glutamate/Malate, or α-Ketoglutarate

Mitochondria phosphorylating 400 nmols of ADP oxidized either 5 mM pyruvate/2.5 mM malate (●), 5 mM glutamate/ 2.5 mM malate (▲), or 5 mM α-ketoglutarate (■). The hyperbolic curves were calculated with the Michaelis-Menten equation by weighted non-linear regression. The sigmoid curve was calculated by fitting data to the following modified Michaelis-Menten equation using a non-linear least squares regression program:

$$v_0 = \frac{V_{max}\,[P_i]^n}{K_{50}{}^n + [P_i]^n}.$$

Where "n" is equal to the Hill coefficient and all the other terms have their usual meaning. The insert shows a Klotz (1946) plot of the data normalized for n (equal to 1.0 for P_i titrations performed while mitochondria oxidize pyruvate/malate and glutamate/malate) and K_{50} values. Note that P_i concentrations vary from 10-90% saturation of the enzyme system.

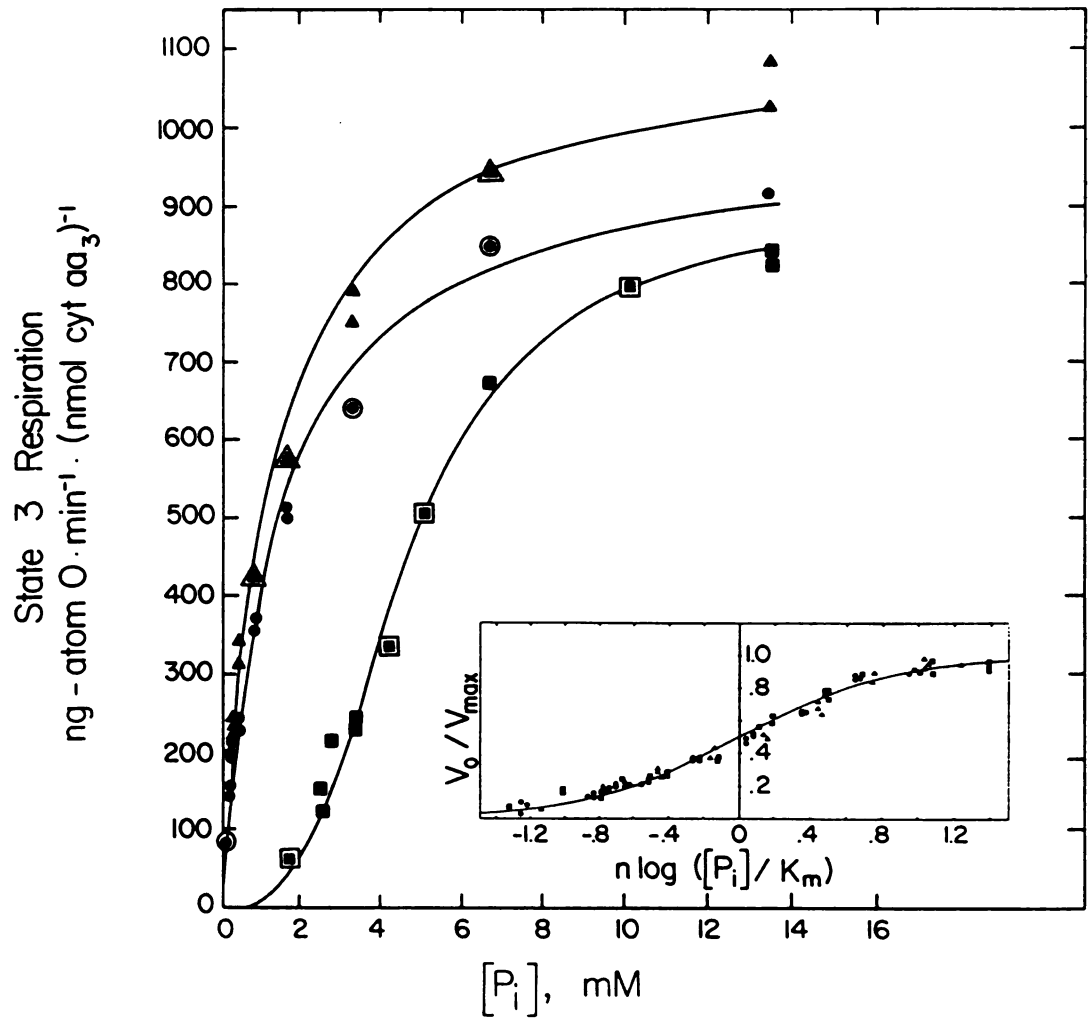

State 3 Respiration
ng-atom O·min⁻¹·(nmol cyt aa₃)⁻¹
1100
1000
900
800
700
600
500
400
300
200
100
0
0
2
4
6
8
10
12
14
16
[Pi], mM
V₀ / Vmax
1.0
.8
.4
.2
-1.2
-.8
-.4
0
.4
.8
1.2
n log ([Pi] / Km)

TABLE I

EFFECT OF SUBSTRATES ON THE KINETIC PARAMETERS FOR THE STIMULATION OF STATE 3 RESPIRATION BY INORGANIC PHOSPHATE[a]

Carboxylic Acid(s)[b]	Kinetics	K_{50}[c]	V_{max}
		mM	ng-atom O min^{-1} (nmol cyt aa_3)$^{-1}$
Pyruvate/Malate	Hyperbolic	1.24 ± 0.11	1110 ± 31.8
Glutamate/Malate	Hyperbolic	1.41 ± 0.12	990 ± 29.8
α-Ketoglutarate	Sigmoidal (n_H = 2.9)[d]	4.68 ± 0.14	881 ± 24.3
β-Hydroxybutyrate	Biphasic	0.42 ± 0.10 28.8 ± 6.1	325 ± 40.8 560 ± 42.9[e] (Σ = 885)

[a] The stimulation of oxygen consumption by Pi was monitored with an oxygen polarograph, as described under "Experimental Procedures." In these experiments the rate of state 3 respiration supported by mitochondria in the absence of exogenous Pi was subtracted from the rate observed subsequent to the addition of Pi. Using pyruvate/malate, glutamate/malate, and β-hydroxybutyrate as substrates, the rates of state 3 respiration with endogenous Pi were 178, 200, and 101 ng-atom O/min/(nmole cytochrome oxidase), respectively. With α-keto-glutarate as substrate, no state 3 respiration is observed without the addition of exogenous P_i.

[b] Substrate concentrations were as follows: pyruvate, glutamate, β-ketoglutarate, and β-hydroxybutyrate were 5 mM; when coincubated with pyruvate and glutamate, malate was present at 2.5 mM.

[c] Because of the uncertainty of the rate limiting step in state 3 respiration, these values are called K_{50}'s instead of K_m's.

[d] n_H is the Hill coefficient.

[e] This value represents the sum of the maximal velocities for the two phases of respiratory stimulation by P_i when mitochondria oxidize β-hydroxybutyrate.

transfer is varied (i.e., using different exogenous substrates should have no effect on the P_i requirements of the ATP synthase). The concentration of P_i which gives half-maximal stimulation of state 3 rates of respiration during α-kg oxidation is 3-4 fold higher when compared with the K_{50}'s observed during the oxidation of P/M and G/M (see Table I). Thus, P_i is rate-limiting for α-kg oxidation. However, it cannot be discerned from these data whether this rate-limitation is direct or indirect. The P_i requirement for state 3 respiration is biphasic when β-hydroxybutyrate is used as oxidizable substrate. Of interest with this substrate is that the K_{50}'s for both phases are significantly different from the K_{50} of the ATP synthase for P_i. It is possible that βHB dehydrogenase has two mutually exclusive binding sites for P_i. Alternatively, P_i may function as a positive effector of βHB dehydrogenase and some other matrix enzyme that is coupled to the further catabolism of acetoacetate. The phosphorylation of ADP during the oxidation of βHB, then, appears to involve at least two and possibly three binding sites for P_i. These oxygen consumption studies cannot provide information on the precise identity of P_i binding sites in the mitochondrial matrix.

II. THE INORGANIC PHOSPHATE REQUIREMENTS OF STATE 4 RESPIRATION

Chick heart mitochondrial state 4 respiration also shows clear differences in its response to increasing P_i concentrations as substrates are varied. With either P/M or αKG, increasing P_i concentrations inhibit uncoupled (i.e., oligomycin-insensitive) state 4 respiration in a hyperbolic, saturable manner (Fig 3). The $K_I(50)$ values are 1.5 ± 0.28 mM and 6.6 ± 4.4 mM in the presence of P/M and αKG, respectively. On the other hand, P_i has little or no effect on state 4 respiration when G/M or βHB

Figure 2. State 3 Rates of Respiration Measured as a Function of Pi Concentration when Mitochondria Oxidize 5 mM β-Hydroxybutyrate

The biphasic curve is theoretical, calculated with the kinetic constants obtained by a non-linear regression of the data fitted to the equation:

$$v_0 = \frac{V_{max}^{1}[P_i]}{K_m^{1} + [P_i]} + \frac{V_{max}^{2}[P_i]}{K_m^{2} + [P_i]}$$

where the superscripts 1 and 2 distinguish kinetic parameters for the two phases of respiratory stimulation by P_i. The insert is an Eadie-Hofstee plot of the same data to emphasize their biphasic nature. The dotted lines are theoretical, based on the estimated K_{50} and V_{max} values for the two phases.

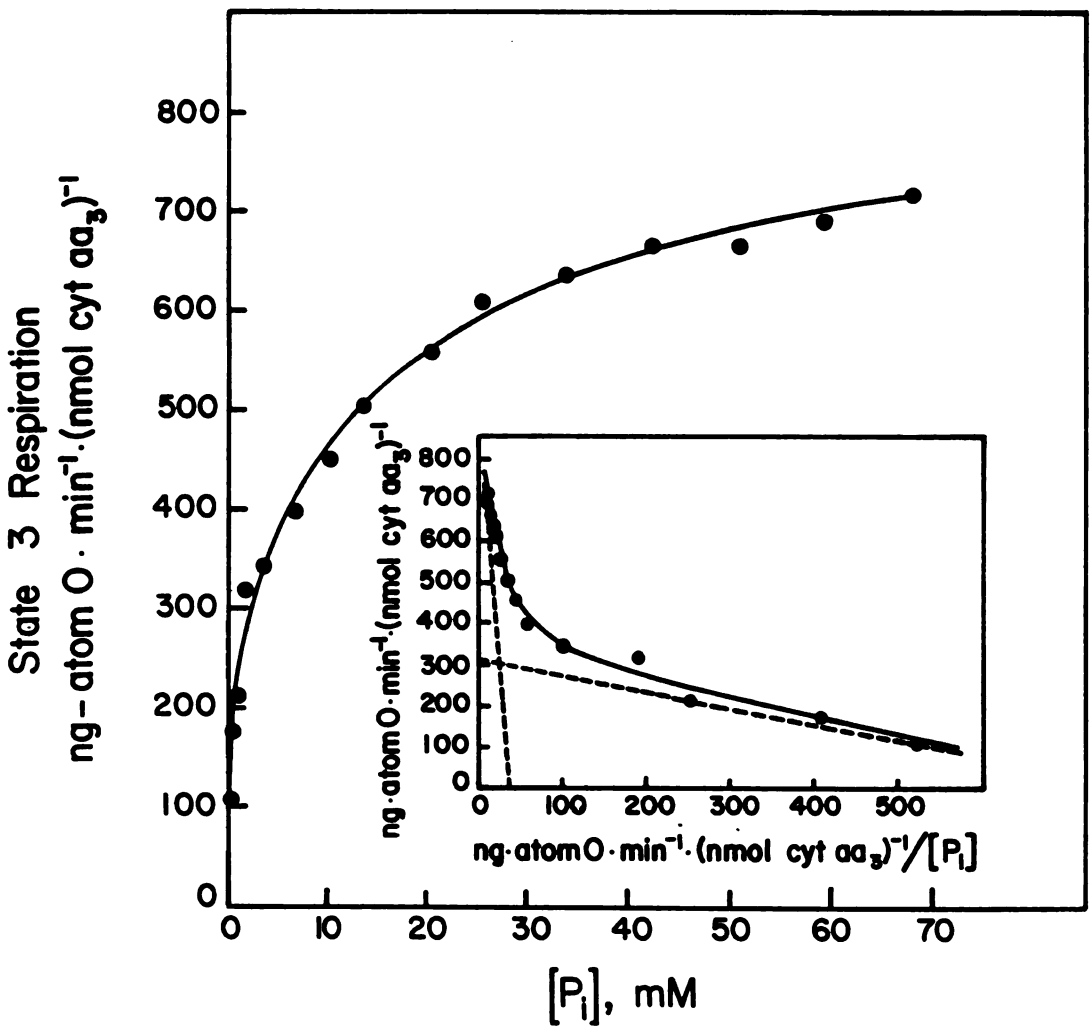
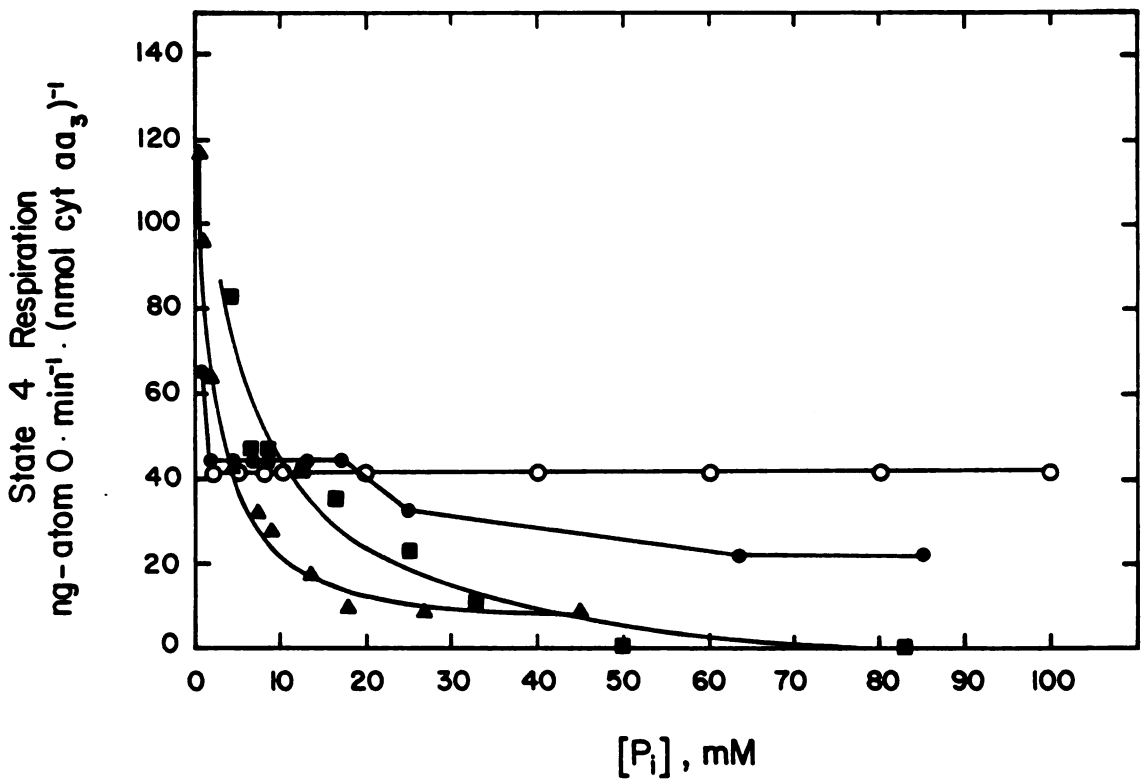

State 3 Respiration
ng-atom O · min⁻¹ · (nmol cyt aa₃)⁻¹
800
700
600
500
400
300
200
100
0
0
10
20
30
40
50
60
70
[Pi], mM
ng·atom O·min⁻¹·(nmol cyt aa₃)⁻¹
ng·atom O·min⁻¹·(nmol cyt aa₃)⁻¹/[Pi]

Figure 3. State 4 Rates of Respiration Measured as a Function of Pi Concentration When Mitochondria Oxidize Pyruvate/Malate, Glutamate/Malate, α-Ketoglutarate, or β-Hydroxybutyrate

Mitochondria were titrated with increasing P_i concentrations in the presence of either 5 mM pyruvate/2.5 mM malate (▲), 5 mM glutamate/2.5 mM malate (●), 5 mM α-ketoglutarate (■), or 5 mM β-hydroxybutyrate (○). The theoretical curves for data obtained with pyruvate/malate and α-ketoglutarate were calculated by a least squares non-linear regression of the data fitted to the following equation assuming a constant absolute error:

$$v_o = b - \frac{V_{max}[P_i]}{K_I50 + [P_i]}$$

where b is the y-intercept (respiration at 0 P_i) and K_I50 is the concentration of P_i which gives half maximal inhibition of state 4 respiration.


State 4 Respiration
ng-atom O · min⁻¹ · (nmol cyt aa₃)⁻¹
140
120
100
80
60
40
20
0
0
10
20
30
40
50
60
70
80
90
100
[Pi], mM

are used as substrates (Fig 3). These data indicate that in the absence of ADP certain enzymes in, or coupled to, the tricarboxylic acid cycle can be rate-limiting for respiration.

III. ACTIVATION OF SUBSTRATE DEHYDROGENASE ACTIVITIES BY P_i

In an effort to probe possible sites of interaction for P_i more directly, disrupted mitochondria were titrated with P_i in the presence of a variety of substrates. Rates of NADH production were inferred from the rate at which lipoamide dehydrogenase transferred reducing equivalents from pyridine nucleotides to the redox-sensitive dye INT. HEPES was used as the buffer in this assay system because Tris interferes with the transfer efficiency (Fig 4A). The efficiency of transferring reducing equivalents from NADH to INT is 100% (i.e., the slope of a plot of nmoles INT reduced measured as a function of nmoles of NADH added is 1.0) when the assay medium is buffered with 20 mM HEPES. In contrast, the efficiency drops to only 67% when the system is buffered with Tris. As shown in Fig 4B, the transfer efficiency remains uncompromised regardless of HEPES-P_i concentration. Thus, for the following experiments, mitochondrial matrix substrate dehydrogenases were titrated with HEPES-P_i.

The kinetic constants obtained for the substrate-dependent activation of dehydrogenase activity by P_i are summarized in Table 2. ADP (1 mM) was included in the assay medium in order to simulate state 3 conditions. When glutamate or α-ketoglutarate are used as substrates (Fig 5), P_i stimulates NADH production in a hyperbolic manner. With a much lower ADP concentration (40 μM) the activation by P_i remains hyperbolic. In agreement with the data of McCormack and Denton (1986) on other mitochondria, Ca(II) also stimulates the α-ketoglutarate dehydrogenase acti-

Figure 4. Efficacy of HEPES in Buffering the INT Reduction Assay System

(A) Effect of buffers on the efficiency with which lipoamide dehdyrogenase transfers reducing equivalents from NADH to INT. Calibrated amounts of NADH were added to a solution of 0.6 mM INT buffered with either 20 mM HEPES (○) or 20 mM Tris (●). The final absorbance was measured and the amount of reduced INT formed was calculated. The lines through the data were calculated by least-squares linear regression. The slopes of these lines were 1.02 and 0.67 for the assays performed in HEPES and Tris, respectively.

(B) Effect of HEPES-P_i on the efficiency with which lipoamide dehydrogenase transfers reducing equivalents from a constant concentration of NADH to INT. 68 nmoles of NADH were added to 1.0 ml of a 0.6 mM solution of INT which contained the indicated concentrations of HEPES-P_i. The final absorbance was measured and the amount of reduced INT formed was calculated. The line was drawn through the mean value of the data.

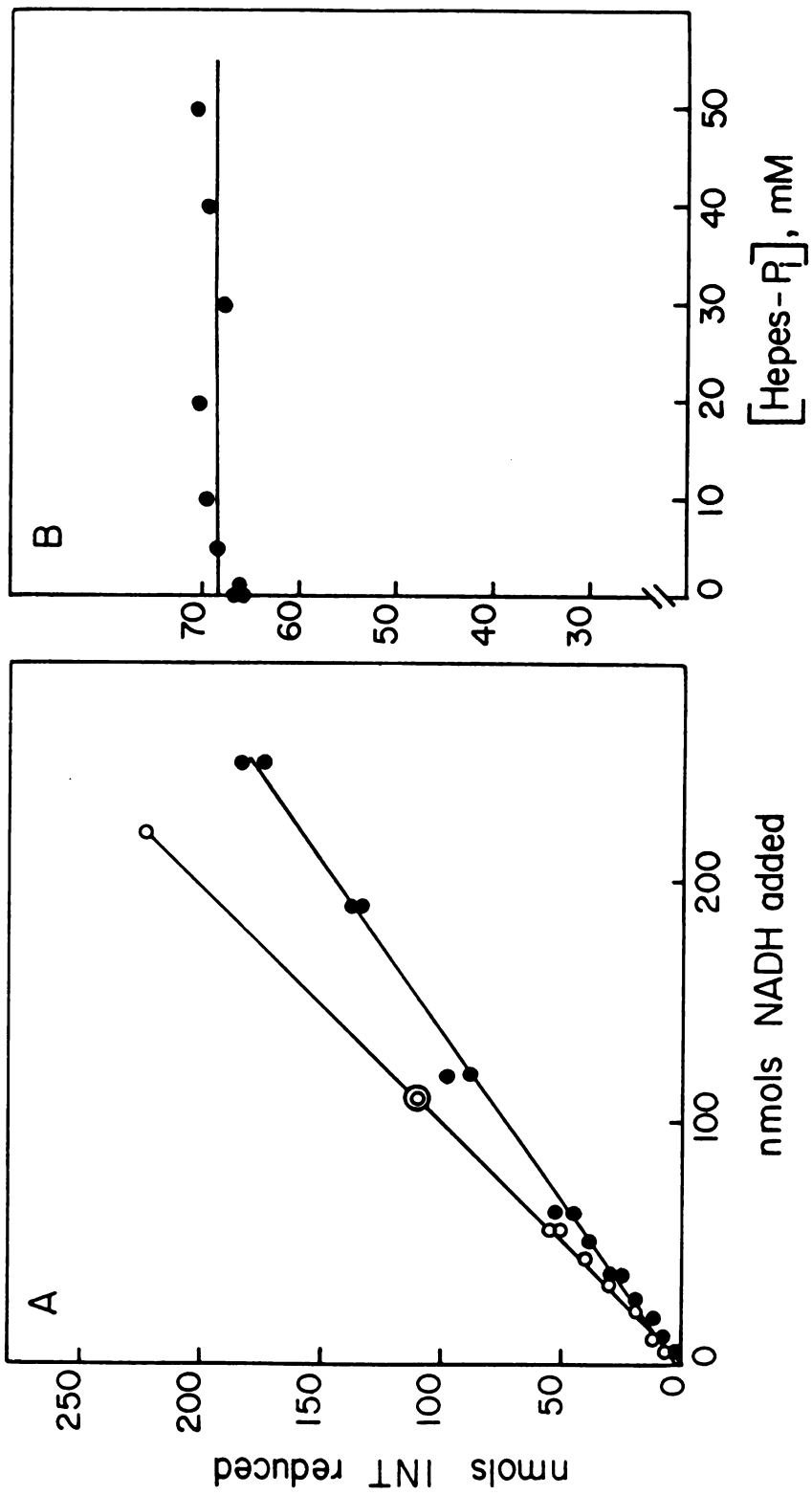

A
B
nmols INT reduced
nmols NADH added
[Hepes-Pi], mM
0
50
100
150
200
250
30
40
50
60
70
0
100
200
0
10
20
30
40
50

Figure 5. Titration of Mitochondrial Dehydrogenase Activity with Pi using either α-Ketoglutarate or Glutamate as Substrates

The rate of NADH production was inferred from the rate at which INT was reduced, as described under "Experimental Procedures." The assays were performed in the presence of either 5 mM α-ketoglutarate (●) or 5 mM glutamate (○) using broken chick heart mitochondria suspended at 0.15 mg/ml. For each point, the rate of NADH production measured in the absence of P_i was subtracted from the rate measured after the addition of P_i. The curves drawn through the data are theoretical, calculated with the K_m and V_{max} values estimated by a non-linear regression of the data fitted to the Michaelis-Menten equation.

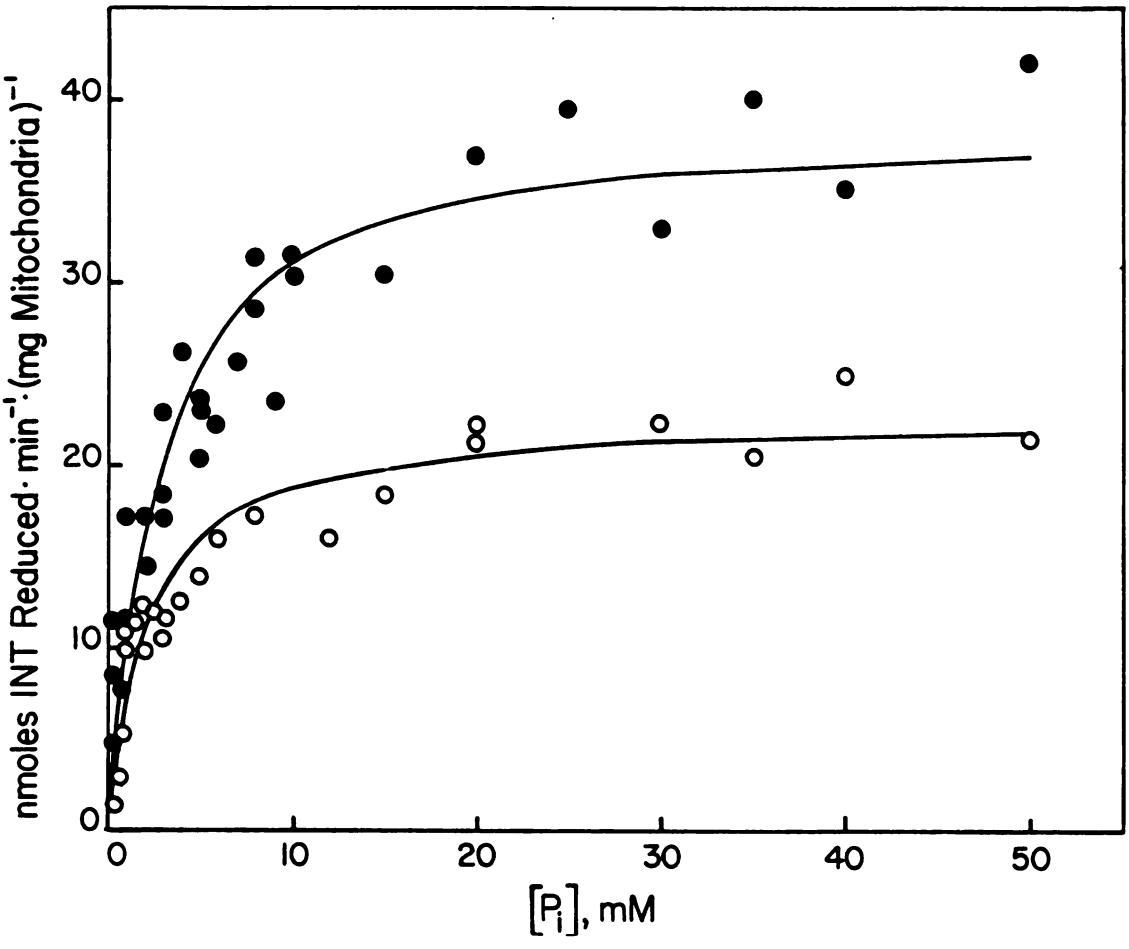

nmoles INT Reduced · min⁻¹ · (mg Mitochondria)⁻¹
0
10
20
30
40
0
10
20
30
40
50
[Pi], mM

TABLE II

APPARENT KINETIC CONSTANTS FOR THE STIMULATION OF CHICK HEART MITOCHONDRIAL MATRIX DEHYDROGENASES BY INORGANIC PHOSPHATE[a]

CARBOXYLIC ACID(S)	NDP[b]	K_m	V_{max}[c]
		mM	
5 mM Pyruvate/2.5 mM Malate	ADP	75.9 ± 24.4	468 ± 110
5 mM Glutamate	ADP	2.0 ± 0.29	22.6 ± 0.86
5 mM α-Ketoglutarate	ADP	2.6 ± 0.37	39.0 ± 1.5
5 mM α-Ketoglutarate	GDP	4.7 ± 0.68	37.6 ± 1.9
5 mM α-Ketoglutarate	IDP	7.4 ± 1.4	34.7 ± 2.4

[a] The stimulation of the reduction of INT by Pi was monitored using the method of Hinman and Blass (1981), as described under "Experimental Procedures." The rate of reduced INT production in the presence of substrate and nucleotide diphosphate was subtracted from all rates observed after the addition of P_i.

[b] NDP, nucleotide diphosphate. The designated NDPs were present at 1 mM during titration of the enzyme system with inorganic phosphate.

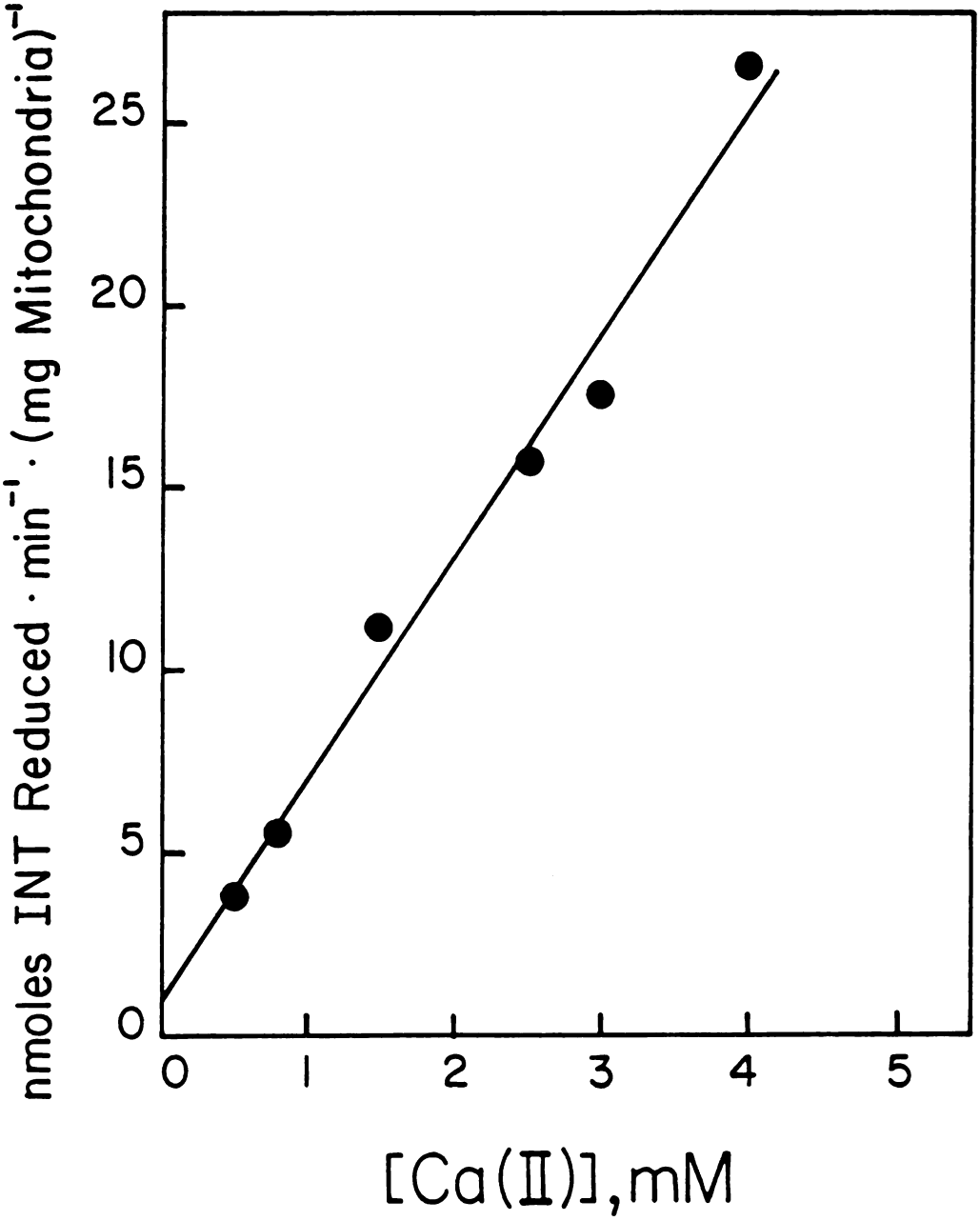
[c] Maximal rates are expressed as nmoles of INT reduced/min/(mg mitochondria).

vity in chick heart mitochondria (data not shown). A further question arises: Does Ca(II) alter the interaction of P_i with the dehydrogenase for α-ketoglutarate? In the absence of EGTA, P_i stimulates α-ketoglutarate oxidation in a hyperbolic manner with a K_{50} = 1.12 ± 0.16 mM (data not shown). These data show that P_i does not bind cooperatively to α-ketoglutarate dehydrogenase. The K_{50} for the stimulation of dehydrogenase activity by P_i in the presence of either glutamate or α-ketoglutarate is approximately 2 mM. This is a physiologically attainable concentration of P_i. When P_i titrations are performed using α-ketoglutarate as substrate in the presence of IDP or GDP, the apparent kinetic parameters for activation are identical, within error, to those obtained in the presence of ADP (Table 2). The apparent K_{50} for P_i activation of NADH production when pyruvate and malate are oxidized is 76 mM (Table 2). In addition, the V_{max} for NADH production from the substrate couple pyruvate/malate is approximately 10-fold higher than that observed with either α-ketoglutarate or glutamate.

The following experiments were performed to localize the site at which P_i induces an increase in the rate of α-ketoglutarate oxidation. It was of interest to determine whether α-ketoglutarate was catabolized beyond succinyl CoA in these assays. In a reaction mixture containing all the standard components except for INT, formation of the CoA thioester was monitored at 230 nm (Bridger, Ramaley, and Boyer, 1969). While the absorbance of the sample increased at 340 nm (i.e., NADH was being produced), no change in absorbance at 230 nm was observed (data not shown). These results indicate that although α-ketoglutarate was being oxidized, succinyl CoA was not accumulating. Because the concentration of succinyl CoA appears to remain constant, the CoA available to

Figure 6. Titration of Mitochondrial Dehydrogenase Activity with Ca(II) using β-Hydroxybutyrate as Substrate

Rates of NADH production were inferred from the rate at which INT was reduced in the assay medium described under "Experimental Procedures." Mitochondria were suspended at 0.16 mg/ml of the assay medium which contained, in addition to the usual constituents, 1 mM ADP, 5 mM β-hydroxybutyrate, and 20 mM Hepes-P_i, and were titrated with the indicated concentrations of Ca(II). The assay medium did not contain EGTA. The rates shown represent rates of NADH production stimulated by Ca(II).


nmoles INT Reduced · min⁻¹ · (mg Mitochondria)⁻¹
0
5
10
15
20
25
0
1
2
3
4
5
[Ca(II)], mM

the system will cycle (see Fig 8 for schematic depiction). This is confirmed by the observation that even when NADH production from α-ketoglutarate is monitored in the absence of exogenous CoA, the rate of INT reduction remains constant for the entire time course over which the assay is monitored (> 5 min). Thus, even in disrupted mitochondria, α-ketoglutarate dehydrogenase and succinyl thiokinase are still highly coupled. Therefore, one obvious explanation for the P_i requirement during α-ketoglutarate oxidation is that succinate thiokinase must have a source of this anion if substrate-level phosphorylation is to proceed. In support of this hypothesis are the apparent affinities of the various nucleotide diphosphates for the reaction activated by P_i (Table 3). The K_{50} of the reaction for GDP is 4 μM, in excellent agreement with the K_{50} of pig heart succinyl thiokinase (3 μM; Cha and Parks, 1964). It is known that this enzyme has a K_{50} for IDP that is very similar to that for GDP (Mazumder et al., 1960). The data summarized in Table 3 are consistent with this. The apparent affinity of ADP for the P_i activated reaction is 10-fold lower. It is possible that this K_{50} represents the affinity of nucleoside diphosphokinase for ADP. In support of this suggestion, most of the nucleoside diphosphokinases yet studied have Michaelis constants for ADP ranging from 40-50 μM (review: Parks and Agarwal, 1973). Activation by ADP is to be expected since the trans-phosphorylation reaction will insure that GDP is rapidly recycled within the system (see Fig. 8).

P_i has no effect on the rate of NADH production when β-hydroxybutyrate is oxidized (data not shown). This result is not consistent with the hypothesis presented above which assumes that P_i interacts with β-hydroxybutyrate dehydrogenase. In order to examine this problem in

TABLE III

APPARENT K_m's FOR THE STIMULATION OF CHICK HEART MITOCHONDRIAL NADH PRODUCTION BY NUCLEOTIDE DIPHOSPHATES WHEN α-KETOGLUTARATE IS OXIDIZED

NDP[b]	K_m
	μM
ADP	41.5 ± 3.4
GDP	4.0 ± 1.8
IDP	1.9 ± 0.9

[a] The stimulation of the reduction of INT by the various NDP's was monitored using the method of Hinman and Blass (1981), as described under "Experimental Procedures." The rate of reduced INT production in the presence of 5 mM α-ketoglutarate and 20 mM HEPES-phosphate was subtracted from all rates observed after the addition of the respective NDP.

[b] NDP denotes the nucleoside diphosphate with which the system was titrated.

greater detail, it was reasoned that P_i may not stimulate β-hydroxybutyrate dehydrogenase directly. Rather, P_i may stimulate this enzyme by activating another enzyme that is closely coupled to it. One candidate for this enzyme would be α-ketoglutarate dehydrogenase. Succinyl CoA is a necessary co-substrate in the formation of acetoacetyl CoA via β-oxoacid CoA transferase (Fig 8). As shown in Fig 5, P_i limits the oxidation of α-ketoglutarate. At low P_i concentrations, only small amounts of succinyl CoA can be produced. It is possible that this would limit the rate of β-hydroxybutyrate dehydrogenase due to the accumulation of acetoacetate. Therefore, another P_i titration was performed in the presence of 5 mM β-hydroxybutyrate and 5 mM malate. By including malate in the assay medium, the acetyl units produced from acetoacetyl CoA could be used to produce citrate, and this in turn could be oxidized to succinyl CoA. In the foregoing it was assumed that some succinyl CoA endogenous to the mitochondria would be available to initiate the transferase reaction. P_i once again had no effect on the rate of NADH production (data not shown). In the course of these experiments, it was found that β-hydroxybutyrate could be oxidized at a faster rate when EGTA was excluded from the assay medium. A titration with Ca(II) shows that this cation stimulates the rate at which NADH is produced from β-hydroxybutyrate (Fig 6). The possibility that activation by P_i is a Ca(II)-dependent process was explored. Broken chick heart mitochondria oxidizing β-hydroxybutyrate in the presence of 1 mM Ca(II) were titrated once again with P_i. No effect by P_i could be discerned (data not shown).

IV. INHIBITION OF SUBSTRATE DEHYDROGENASE ACTIVITIES WITH P_i

The method of Hinman and Blass (1981) was also used to monitor NADH production during the oxidation of pyruvate and α-ketoglutarate in the absence of ADP (i.e., state 4). In agreement with the respiration experiments (Fig 3), increasing concentrations of P_i inhibit the the rate at which the dehydrogenases for these substrates produce NADH (Fig 7). Much higher concentrations of P_i must be used in order to obtain half-maximal inhibition with the dehydrogenases contained in the preparation of disrupted mitochondria. This probably reflects (a) changes in the chemical and physical environment of the enzymes subsequent to lysis of the mitochondria, or (b) that the concentration of P_i inside the matrix of intact mitochondria is greater than that in the extramitochondrial space.

Figure 7. Titration of Mitochondrial Dehydrogenase Activity with Pi in the Absence of ADP using Pyruvate/Malate and α-Ketoglutarate as Substrates

Rates of NADH production were measured as described in the legend to Figure 6. The disrupted mitochondria were incubated in the assay medium described under "Experimental Procedures," except it did not contain ADP. The mitochondria were suspended at 0.35 and 0.41 mg/ml when oxidizing 5 mM pyruvate/2.5 mM malate (●) and 5 mM α-ketoglutarate (◯), respectively.

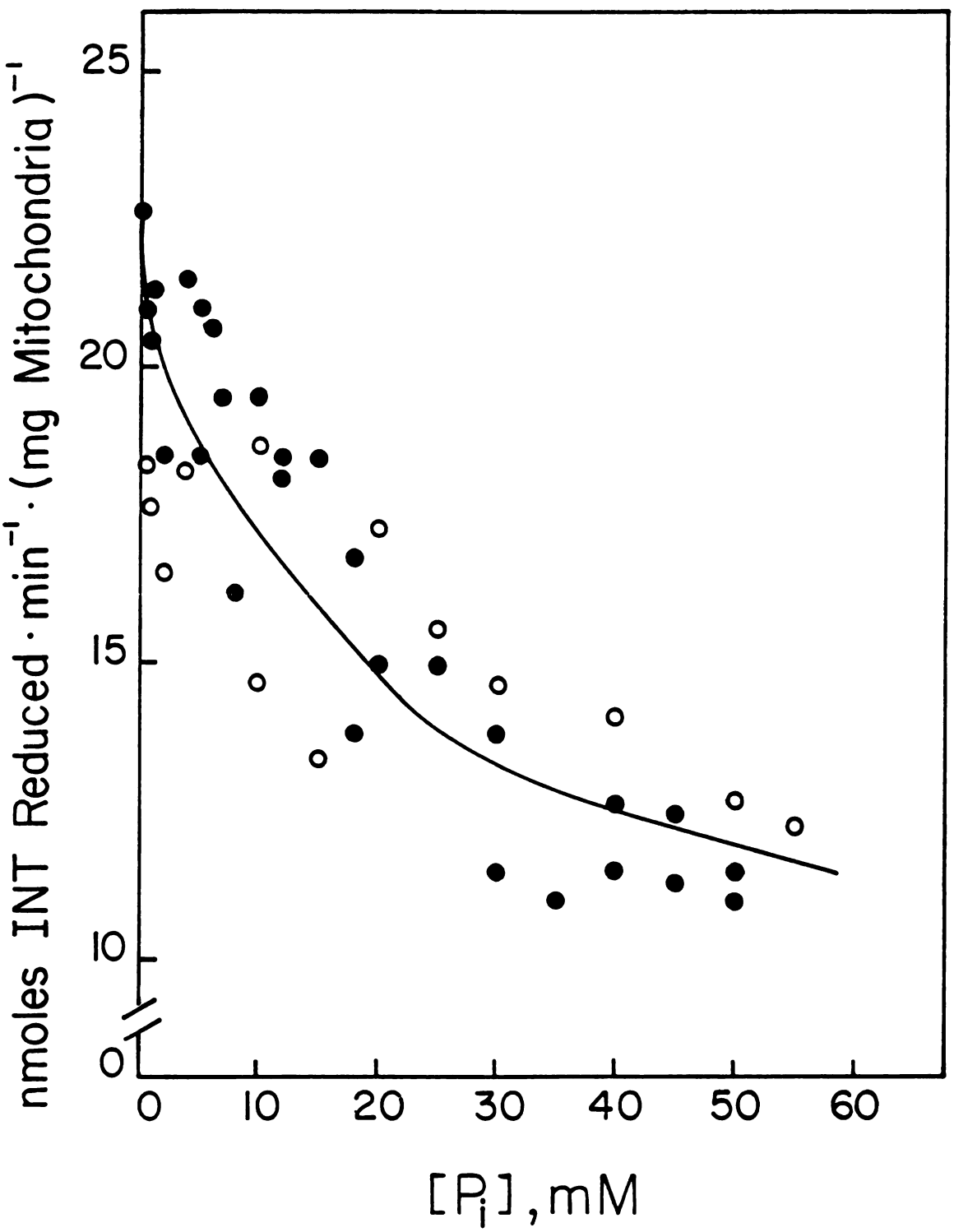
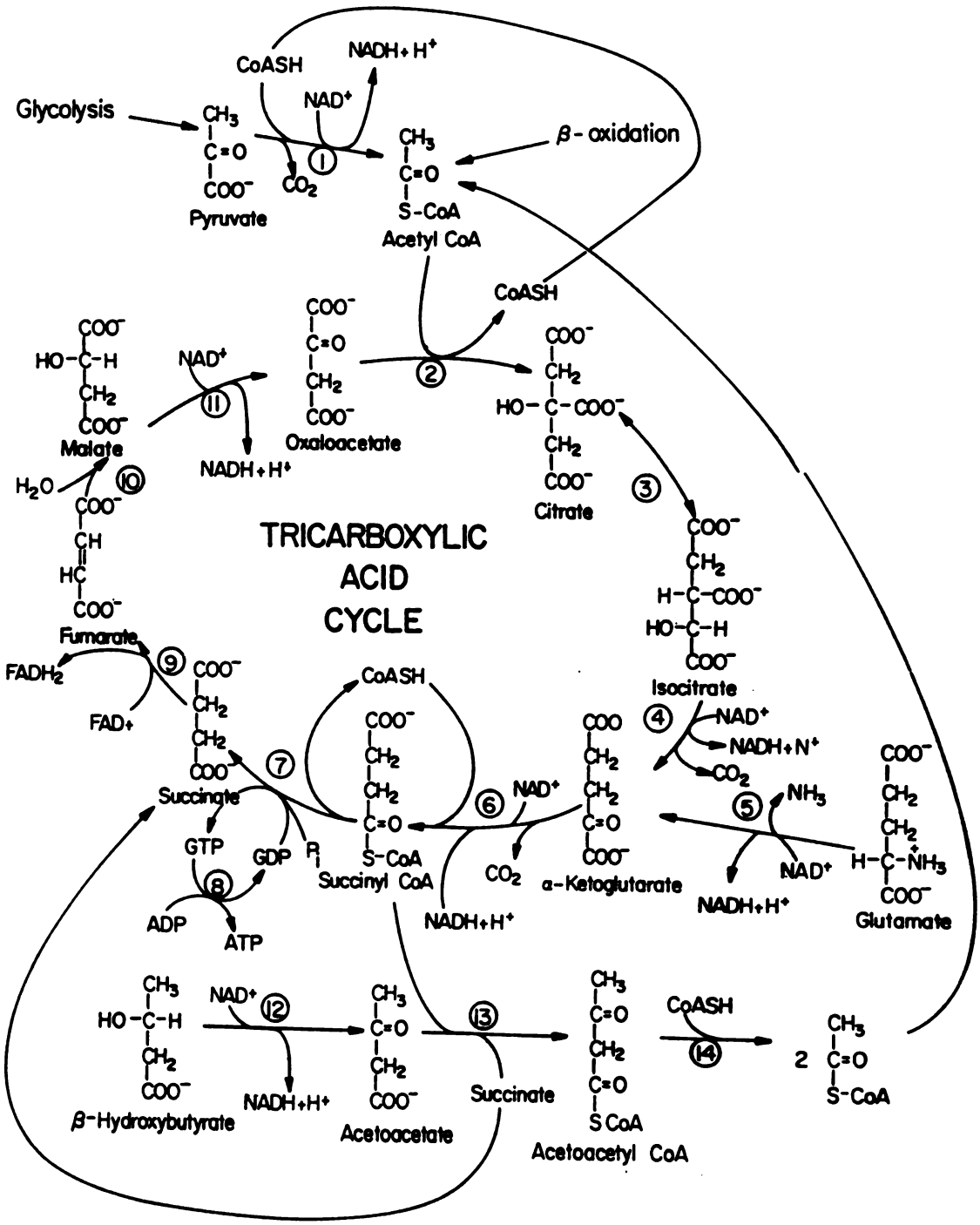

nmoles INT Reduced · min⁻¹ · (mg Mitochondria)⁻¹
25
20
15
10
0
0
10
20
30
40
50
60
[Pi], mM

Figure 8. Integrative Scheme Depicting the Reactions in and Coupled to the Tricarboxylic Acid Cycle

The numbers between intermediates are used to designate the identity of the following enzymes:

1. Pyruvate Dehydrogenase Complex
2. Citrate Synthase
3. Aconitate Hydratase
4. Isocitrate Dehydrogenase
5. Glutamate Dehydrogenase
6. α-Ketoglutarate Dehydrogenase Complex
7. Succinyl-CoA Synthetase
8. Nucleoside Diphosphokinase
9. Succinate Dehydrogenase
10. Fumarate Hydratase
11. Malate Dehydrogenase
12. β-Hydroxybutyrate Dehydrogenase
13. β-Oxoacid CoA-Transferase
14. Acetoacetyl-CoA Thiolase


TRICARBOXYLIC
ACID
CYCLE
Glycolysis
Pyruvate
CoASH
NADH+H+
NAD+
CO2
β-oxidation
Acetyl CoA
Oxaloacetate
Citrate
Isocitrate
α-Ketoglutarate
Succinyl CoA
Succinate
Fumarate
Malate
H2O
FADH2
FAD+
GTP
GDP
Pi
ADP
ATP
NH3
Glutamate
β-Hydroxybutyrate
Acetoacetate
Acetoacetyl CoA
2

DISCUSSION

I. PHOSPHATE CONTROL OF DEHYDROGENASES DURING STATE 3 RESPIRATION

The oxygen consumption experiments reported in this study indicate that the regulation of state 3 rates of respiration by P_i is complex in the sense that a number of binding sites for this anion are implicated. The sigmoidal and biphasic saturation kinetics observed during the oxidation of α-ketoglutarate and β-hydroxybutyrate, respectively, suggested that P_i is a direct effector of the dehydrogenases for these substrates. Measurement of the rates of NADH production by these dehydrogenases do not, however, support this hypothesis.

In broken mitochondria, P_i control over rates of α-ketoglutarate oxidation is due to the P_i requirement of the substrate-level phosphorylation catalyzed by succinyl CoA synthetase. The rate of glutamate oxidation is stimulated by P_i because α-ketoglutarate is the product of the glutamate dehydrogenase reaction. Thus, for this substrate, P_i simply stimulates catabolism beyond α-ketoglutarate. The sigmoidal saturation kinetics of state 3 respiration during α-ketoglutarate oxidation does not arise from cooperative binding of P_i to the dehydrogenase for this substrate. Instead, it is likely that this sigmoidicity arises from the substrate requirements for α-ketoglutarate transport across the inner membrane. The dicarboxylate carrier can catalyze the antiport of such dicarboxylic acids as succinate, malate, and α-ketoglutarate (Palmieri et al., 1971). In addition, this carrier can also catalyze the import of dicarboxylates via exchange with matrix P_i. Since there would not be

sufficient concentrations of endogenous dicarboxylic acids to drive the import of large amounts of exogenous α-ketoglutarate, the sigmoidal saturation of state 3 respiration is a probable result of P_i limiting the rate at which α-ketoglutarate can be transported into the mitochondrial matrix via the dicarboxylate carrier. Seen in this light, the discrepancy between the K_ms for P_i activation of α-ketoglutarate oxidation (1.2 mM) and state 3 respiration when α-ketoglutarate is used as substrate (4.7 mM) reflects differences in the affinity of succinyl CoA synthetase and the dicarboxylate carrier for P_i.

The manner by which P_i stimulates state 3 respiration during β-hydroxybutyrate oxidation is not apparent. β-Hydroxybutyrate dehydrogenase exists as a dimer and is associated with the matrix side of the mitochondrial inner membrane (McIntyre et al., 1978). The oxygen consumption data suggest that this enzyme experiences a complex biphasic interaction with P_i during state 3 respiration. Direct measurement of rates of NADH production by matrix enzymes in the presence of β-hydroxybutyrate revealed no stimulation by P_i. It is possible that when the mitochondria were disrupted, β-hydroxybutyrate dehydrogenase was irreversibly damaged. It is also possible that by removing the enzyme from its native environment (i.e., disrupting mitochondrial membranes and destroying the environment unique to the matrix), all sensitivity to P_i is lost. Because this substrate is transported into the matrix by the pyruvate carrier (Parvin and Pande, 1979), it is unlikely that the biphasic activation of respiration by P_i is a transport phenomenon since (a) this carrier does not transport P_i and (b) it does not diplay biphasic saturation kinetics. At the least, these data show that state 3 respiration driven by β-hydroxybutyrate displays two separate affinities for P_i that are significan-

tly different from the P_i affinities of the F_1F_0-ATP synthetase and succinyl CoA synthetase. Whether the two binding sites for P_i exist on the dehydrogenase for β-hydroxybutyrate cannot be discerned from these data.

The P_i saturation kinetics of state 3 respiration when pyruvate/malate and glutamate/malate are used as substrates indicate that this anion is largely committed to ATP synthesis. P_i stimulates NADH production in the presence of pyruvate and malate with a high K_m (76 mM). This is physiologically untenable and may reflect an ionic strength effect. P_i does stimulate NADH production when glutamate and malate are used as substrates. However, one molecule of NADH is produced during the glutamate dehydrogenase reaction. Thus, even if α-ketoglutarate oxidation is rate-limited by P_i, it is apparent that NADH concentrations sufficient to drive oxidative phosphorylation are produced by glutamate dehydrogenase within intact mitochondria. Finally, the nearly identical V_{max} values for oxygen consumption at infinite P_i and high substrate concentrations suggest that the rate of oxidative phosphorylation is limited by a reaction common to all of the substrates.

II. PHOSPHATE CONTROL OF DEHYDROGENASES DURING STATE 4 RESPIRATION

Based on the data shown in Fig's 3 and 7, the dehydrogenases for pyruvate and α-ketoglutarate are inhibited by P_i during state 4. As evidenced by its insensitivity to inhibition by oligomycin, all of the state 4 respiration in these experiments was due to uncoupled respiration. It is unlikely that P_i inhibits enzymes of the electron transfer chain under these conditions. All of the substrates tested produce NADH. If P_i acted at the level of NADH dehydrogenase (complex I) or the cytochromes, one would expect uniform inhibition of respiration independent of the

source of reducing equivalents. This is not observed.

These data indicate that in the absence of ADP the enzymes catalyzing two of the rate-limiting steps in or coupled to the tricarboxylic acid cycle can regulate the rate of respiration. As P_i concentrations are increased during the oxidation of either pyruvate or α-ketoglutarate, the rate of oxygen consumption during state 4 approaches zero. This is in sharp contrast to what one would expect if the near-equilibrium hypothesis (Wilson et al., 1974; Erecinska et al., 1977) for the control of respiration were true for chick heart mitochondria. As P_i concentrations are increased without compensatory alterations in extramitochondrial ATP concentrations, this theory suggests that the rate of respiration should increase since the magnitude of the cytosolic phosphorylation potential will decrease. The data reported herein support the notion advanced in recent years (Hansford, 1977, 1980, 1985; Hansford and Castro, 1981) that under some conditions the matrix dehydrogenases catalyzing non-equilibrium reactions in the matrix control the rate of respiration. The inhibition of a significant percentage of uncoupled respiration by P_i during the oxidation of pyruvate/malate and α-ketoglutarate is attainable by physiological concentrations of P_i. Consequently, as cytosolic ADP concentrations change during variations in cardiac contraction rates, P_i may play a significant role in the transient regulation of dehydrogenase activity in the mitochondrial matrix. The actual mechanism by which this inhibition of the dehydrogenases for pyruvate and α-ketoglutarate is effected is yet to be elucidated. However, it may be stated that these data support a model in which P_i exerts kinetic rather than thermodynamic control over the rate of respiration. In addition, these data show that P_i can be used as a non-toxic inhibitor of uncoupled

respiration. Thus, at the cmc, when P_i concentrations are optimized, the mitochondria become highly coupled and respire with respiratory control ratios that approach infinity.

III. CONCLUSIONS

The experiments reported in this study show that: (a) The P_i saturation kinetics of state 3 respiration vary with the nature of the carboxylic acid(s) used as oxidizable substrate; (b) The control of state 3 respiration by P_i is distributed among a number of enzymes: the F_1F_0-ATP synthetase, succinyl CoA synthetase, and possibly β-hydroxybutyrate dehydrogenase as well as the dicarboxylate carrier during α-ketoglutarate oxidation; (c) The cytosolic phosphorylation potential does not regulate the rate of respiration; (d) P_i inhibits the oxidation of pyruvate and α-ketoglutarate in the absence of ADP; and (e) The data are consistent with the notion that particular mitochondrial matrix dehydrogenases participate in the phenomenon of respiratory control via a mechanism that is independent of changes in the ratios of NADH/NAD, ATP/ADP, and succinyl (or acetyl) CoA/CoA.

REFERENCES

Bencini, D.A., Wild, J.R., and O'Donovan, G.A. (1983) Anal. Biochem. 132: 254-258.

Bock, R.M., Ling, N.S., Morell, S.A., and Lipton, S.H. (1956) Arch. Biochem. Biophys. 62: 253-

Bridger, W.A., Ramaley, R.F., and Boyer, P.D. (1969) Methods Enzymol. 13: 70-75.

Bridger, W.A. (1974) The Enzymes (3rd ed.) 10: 581-606.

Brooks, S.P.J., and Suelter, C.H. (1987) Int. J. Bio-Med. Comp. 19: 89-99.

Bulos, B.A., Thomas, B.J., Shukla, S.P., and Sacktor, B. (1984) Arch. Biochem. Biophys. 234: 382-393.

Cha, S., and Parks, R.E. (1964) J. Biol. Chem. 239: 1961-1967.

Cha, S. (1969) Methods Enzymol. 13: 62-69.

Chance, B., and Williams, J.R. (1955) J. Biol. Chem. 217: 383-393.

Chance, B., Leigh, J.S., Clark, B.J., Maris, J., Kent, J., Nioka, S., and Smith, D. (1985) Proc. Nat. Acad. Sci. (U.S.A.) 82: 8384-8388.

Chance, B., Leigh, J.S., Kent, J., McCully, K., Nioka, S., Clark, B.J., Maris, J.M., and Graham, T. (1986) Proc. Nat. Acad. Sci. (U.S.A.) 83: 9458-9462.

Coty, W.A., and Pedersen. P.L. (1974) J. Biol. Chem. 250: 2593-2598.

Davis, E.J., and Lumeng, L. (1975) J. Biol. Chem. 250: 2275-2282.

Denton, R.M., and McCormack, J.G. (1986) In Hormones and Cell Regulation (Nunez, J., et al., eds) Vol. 139, pp. 249-259, John Libbey Eurotext Ltd.

Erecinska, M., Stubbs, M., Miyata, Y., Ditre, C.M., and Wilson, D.F. (1977) Biochim. Biophys. Acta 462: 20-35.

Greenbaum, N.L., and Wilson, D.F. (1985) J. Biol. Chem. 260: 873-879.

Hansford, R.G. (1976) J. Biol. Chem. 251: 5483-5489.

Hansford, R.G. (1980) Current Topics in Bioenergetics 10: 217-278.

Hansford, R.G., and Castro, F. (1981) Biochem. J. 198: 525-533.

Hansford, R.G. (1985) Rev. Physiol. Biochem. Pharmacol. 102: 1-72.

Hinman, L.M., and Blass, J.P. (1981) J. Biol. Chem. 256: 6583-6586.

Holian, A., Owen, C.S., and Wilson, D.F. (1977) Arch. Biochem. Biophys. 181: 164-171.

Klotz, I.M. (1946) Arch. Biochem. Biophys. 9: 109-117.

Koshland, D.E., Nemethy, G., and Filmer, D. (1966) Biochemistry 5: 365-385.

Kuster, U., Bohnensack, R., and Kunz, W. (1976) Biochim. Biophys. Acta 440: 391-402.

Lemasters, J.J., and Sowers, A.E. (1979) J. Biol. Chem. 254: 1248-1251.

Mannervik, B. (1982) Methods Enzymol. 87: 370-390.

Matsuno-Yagi, A., and Hatefi, Y. (1986) J. Biol. Chem. 261: 14,031-14,038.

Mazumder, R., Sanadi, D.R., and Rodwell, V.W. (1960) J. Biol. Chem. 235: 2546-2550.

McIntyre, J.O., Bock, H.O., and Fleischer, S. (1978) Biochim. Biophys. Acta 513: 255-267.

McCormack, J.G., and Denton, R.M. (1979) Biochem. J. 180: 533-544.

Monod, J., Wyman, J., and Changeux, J.P. (1965) J. Mol. Biol. 12: 88-118.

Nishimura, J.S., and Grinnell, F. (1972) Adv. Enzymol. 36: 183-202.

Owens, T.G., and King, F.D. (1975) Mar. Biol. 30: 27-36.

Palmieri, F., Prezioso, G., Quagliarello, E., and Klingenberg, M. (1971) Eur. J. Biochem. 22: 66-74.

Parks, R.E., and Agarwal, R.P. (1973) The Enzymes (3rd ed) 8: 307-333.

Parvin, R., and Pande, S.V. (1979) J. Biol. Chem. 254: 5423-5429.

Rigoulet, M., Velours, J., and Guerin, B. (1985) Eur. J. Biochem. 153: 601-607.

Slater, E.C., Rosing, J., and Mol, A. (1973) Biochim. Biophys. Acta 292: 534-553.

Toth, P.P., Ferguson-Miller, S.M., and Suelter, C.H. (1986) Methods Enzymol. 125: 16-25.

Westheimer, F.H. (1987) Science (Washington, D.C.) 235:1173-1178.

Wilkinson, G.N. (1961) Biochem. J. 80: 324-332.

Wilson, D.F., Stubbs, M., Veech, R.L., Erecinska, M., and Krebs, H.A. (1974) Biochem. J. 140: 57-64.

Chapter 7

IDENTIFICATION OF A SOLUBLE FACTOR THAT UNCOUPLES THE OXIDATION OF SUBSTRATES FROM THE PHOSPHORYLATION OF ADP IN CHICK MYOCARDIUM

INTRODUCTION

The mitochondria of most tissues have evolved both structurally and functionally in ways which maximize the potential of these organelles to commit the free energy of substrate oxidation toward the synthesis of ATP. An exception to this are the mitochondria of brown fat adipocytes. These mitochondria are able to release the free energy of fatty acid oxidation in the form of heat (Nicholls et al., 1972; Cannon et al., 1984). This nonshivering thermogenesis is induced by the activity of a 32,000-M_r uncoupling protein located in the mitochondrial inner membrane (review: Nicholls, 1979). The function of the uncoupler protein is to provide protons with a means of reentering the matrix without flowing through the F_0 moiety of the ATP synthetase. The conductance of the proton channel formed by this uncoupler protein is subject to regulation by purine nucleotide di- and triphosphates and by fatty acids (Nicholls, 1984; Klingenberg, 1984). The uncoupler protein from rat (Bouillaud et al., 1986) and hamster (Aquila et al., 1985) brown fat has been isolated, purified, and its amino acid sequence determined. The uncoupling of substrate oxidation from the phosphorylation of ADP can also be achieved by subjecting mitochondria to lipophilic protonophores (Kell and Westerhoff, 1985), low oxygen tensions (Kramer and Pearlstein, 1983), and large Ca(II) loads (Lehninger et al., 1967). Significantly, however, no protein has yet been found to uncouple mitochondria from tissues other than mammalian brown fat.

The purpose of this brief report is to present prliminary evidence

for the existence of a soluble uncoupler protein in chick myocardium. The protein is small (≤15 kD), binds with high apparent affinity to chick heart mitochondria, and its activity is insensitive to oligomycin and EGTA, agents that would inhibit respiration attributable to the phosphorylation of ADP or the uptake of Ca(II), respectively. Phenomenologically, the protein exists; however, the teleological basis for its existence is not clear. As shown in this dissertation, isolated chick heart mitochondria are extraordinarily highly coupled. It is apparent from the work to be described herein that, due to the presence of this uncoupling protein, it is unlikely this degree of coupling is actually available to myocardium in vivo. One possible explanation for the existence of this protein is that its controlled expression may regulate rates of cardiac thermogenesis. In contrast, its function could be related to a range of novel kinetic and/or thermodynamic mechanisms regulating the complex processes responsible for cardiac energy transduction.

EXPERIMENTAL PROCEDURES

I. MATERIALS

Water was purified as described in Chapter 3. The following substances were reagent grade or better, used without further purification, and obtained from the sources noted: essentially fatty acid free BSA (fraction V), EGTA, glycine, mannitol, sucrose, DL-dithiothreitol, ammonium sulfate, bromophenol blue, rotenone, oligomycin A, Dowex MR-3, Tris, collagenase (type VII), Nagarse, (type VII), and the sodium salts of ADP and pyruvic and malic acids (Sigma, St. Louis, MO); urea, glycerol, glacial acetic acid, silver nitrate, formaldehyde, and phosphoric acid (Mallinckrodt, Paris, KY); acrylamide (Serva, West Germany); N,N'-methylenebisacrylamide (Miles Laboratories); TEMED (Merck Laboratories); sodium dodecyl sulfate (Matheson Coleman and Belle); absolute methanol and ammonium hydroxide (J.T. Baker Chemical). Single comb white leghorn chicks were obtained from the Department of Animal Science at Michigan State University. Ammonium sulfate (enzyme grade, Mallinckrodt) was recrystallyzed from an aqueous 1 mM EGTA (pH 7.4) solution. Dialysis tubing (3500 molecular weight cutoff, Spectrapore) was soaked for 1 hr at 60° in a solution that contained 1% (w/v) ammonium carbonate and 0.1% (w/v) $NaDodSO_4$. The soaked dialysis tubing was subsequently rinsed exhaustively with ultra-pure water.

II. ISOLATION OF MITOCHONDRIA

Mitochondria were isolated from chick heart muscle using either collagenase (Toth et al., 1986) or Nagarse (Chance and Hagihara, 1963).

These methods are described in detail in Chapters 2 and 4, respectively.

III. COLLECTION OF CRUDE FRACTION CONTAINING THE UNCOUPLER PROTEIN

A crude fraction of the uncoupler protein is prepared from the supernatant liquid overlying the first mitochondrial pellet obtained after centrifuging these organelles at 8,000g for 10 min. Henceforth, this supernatant liquid will be referred to as "S8000." To remove particulate matter, S8000 is filtered through a double layer of cheesecloth and centrifuged at 40,000g for 20 min. The S8000 is once again filtered through a double layer of cheesecloth and loaded into dialysis tubing. The S8000 is then concentrated by placing the dialysis tubing over cheesecloth and covering it with crystalline sucrose for 12 hrs at 4°C. The resulting concentrate is centrifuged at 10,000g for 20min. The clear supernatant liquid is collected and stored at 4°C. The uncoupling activity of this concentrated S8000 (cS8000) is stable for at least two weeks.

IV. STABILIZATION OF CONCENTRATED S8000 IN GLYCEROL

Because sucrose promotes rapid bacterial growth in the stored S8000, another agent that stabilizes the uncoupling activity was sought. In order to test the stability of the coupling activity in glycerol, aliquots of cS8000 were dialyzed against 1.5 L of the following buffers for 21 hrs at 4°C: (a) 10% glycerol, 20 mM Tris, 2 mM EGTA, pH 7.4; (b) 25% glycerol, 20 mM Tris, 2 mM EGTA, pH 7.4; (c) 50% glycerol, 20 Mm Tris, 2 mM EGTA, pH 7.4. After the dialysis step, these cS8000 aliquots were assayed for uncoupling activity.

V. AMMONIUM SULFATE PRECIPITATION OF THE UNCOUPLING ACTIVITY IN S8000

In an attempt to partially purify the uncoupling activity, the cS8000 was subjected to a series of ammonium sulfate precipitation steps. 5 ml of the cS8000 was dialyzed at 4°C against 1.5 liters of a buffer that contained 50% glycerol (v/v), 20 mM Tris, pH 7.4, and 1 mM EGTA (GTE). This buffer was adjusted to either 50, 60, 70, 80, or 90% saturation with ammonium sulfate. After the various ammonium sulfate cuts, samples were centrifuged at 40,000g for 15 min. The supernatant liquids were withdrawn, and precipitates were redissolved in minimal volumes of GTE. In order to remove the ammonium sulfate, the precipitates and the supernatant liquids were dialyzed for 12 hrs at 4°C against 1.5 liters of a buffer that contained 50% glycerol (v/v), 20 mM Tris, pH 7.4, and 2 mM EGTA. The dialyzed samples were then concentrated against solid crystalline sucrose as described above.

VI. ASSAY FOR UNCOUPLING ACTIVITY

The activity of this protein was measured by determining its effect on the state 4 respiration of intact chick heart mitochondria isolated with collagenase. One unit of uncoupling activity is that amount of crude protein/mg mitochondria that stimulates mitochondrial oxygen consumption by 100 ng atom O $(min)^{-1}$ $(mg\ mitochondria)^{-1}$. Oxygen consumption experiments were performed as described in Chapter 3. The assay medium was comprised of 0.225 M mannitol, 0.075 M sucrose, 20 mM Tris-P_i, pH 7.4, and 5 mM pyruvate/2.5 mM malate. Other experimental conditions were as described in the legend to Fig 1.

VII. POLYACRYLAMIDE GEL ELECTROPHORESIS

The protein banding profiles of cS8000 and of mitochondria isolated with either collagenase or Nagarse were compared using $NaDodSO_4$-polyacrylamide gel electrophoresis. Electrophoresis was performed using the discontinuous buffer system of Laemmli (1970) in slab gels (16cm x 18cm x 0.75 mm). The resolving gel contained 15% (w/v) acrylamide/0.40% bisacrylamide and 3.75 M urea. The stacking gel (typically 1-1.5 cm) contained 3% acrylamide/0.08% bisacrylamide and 0.95 M urea. Prior to mixing, acrylamide solutions were swirled over a bed of Dowex MR-3 ion exchange resin (Sigma) in order to remove contaminating acrylic acid. Samples were solubilized in boiling sample buffer (0.125 M Tris, pH 6.8, 4% (w/v) $NaDodSO_4$ (w/v), 20% (v/v) glycerol, 0.1 M DL-dithiothreitol, 3.75 M urea, and 0.002% (w/v) bromophenol blue) and incubated in a boiling water bath for 5 min. It was important when working with mitochondria isolated with Nagarse that boiling sample buffer be used; if not, residual Nagarse that could not be removed from mitochondria by rinsing would completely hydrolyze the solubilized protein. The electrophoresis apparatus was not cooled with tap water during the course of a run because the urea crystallizes out of the gel. Gels were run for 1.5 hrs at 100 V, and then for 10-12 hrs at 300 V. The protein standards were those obtained in the Sigma Dalton Mark VI kit: bovine serum albumin (66,000); ovalbumin (45,000); porcine pepsin (34,700); bovine trypsinogen (24,000); bovine β-lactoglobulin (18,400); and lysozyme (14,300). Gels were fixed and then stained with silver as described by Giulian et al. (1983).

VII. OTHER ASSAYS

Protein concentrations were determined by the modified Lowry method

of Markwell et al. (1981) using BSA as standard. P_i concentrations were assayed using the method of Bencini et al. (1983) using potassium orthophosphate as standard. Cytochrome oxidase was quantitated as described in Chapter 2.

RESULTS

I. STABILITY OF UNCOUPLER ACTIVITY

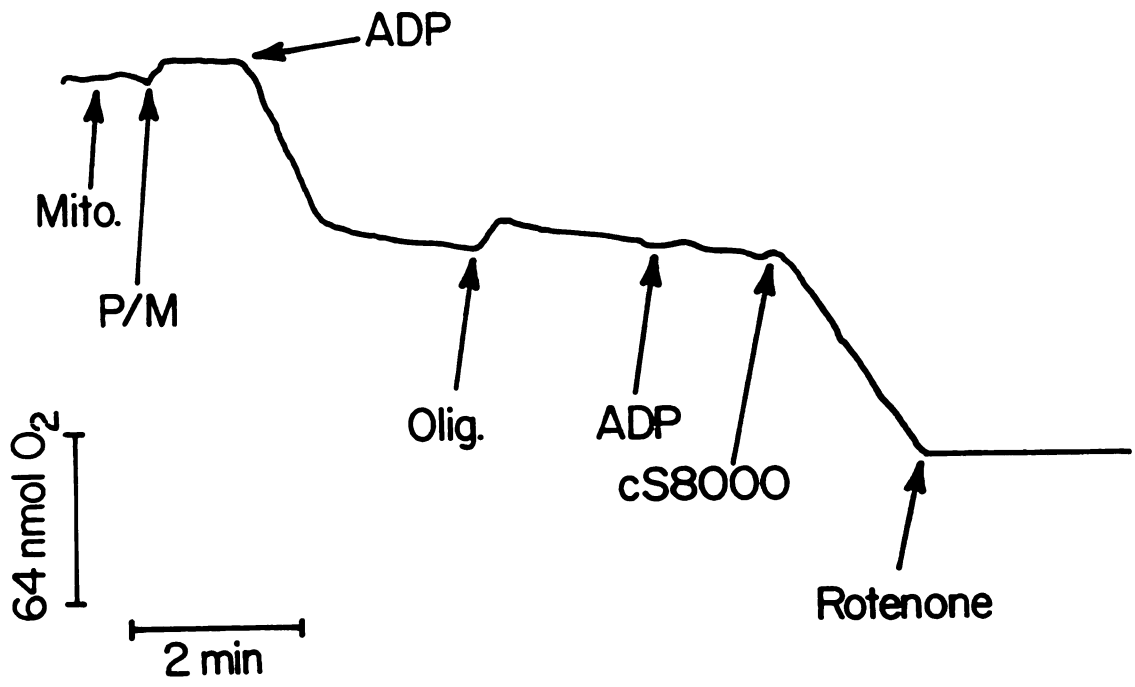
The uncoupler activity of the supernatant liquid overlying the first mitochondrial pellet is unstable in the 0.225 M mannitol/0.075 M sucrose medium used to isolate mitochondria. Within 24 hrs little if any activity remains. In the 10 and 25% glycerol buffers, most of the uncoupling activity is lost within 2-3 days. However, if the cS8000 is dissolved in either the 50% glycerol buffer or in concentrated sucrose, the uncoupling activity is stable for up to two weeks. Longer storage periods were not attempted. Consequently, the maximal storage time could well be in excess of two weeks.

II. STIMULATION OF RESPIRATION

The addition of an aliquot of cS8000 stimulates uncoupled respiration in chick heart mitochondria (Fig 1). A number of observations strongly suggest that the stimulation of oxygen consumption is indeed attributable to an uncoupler protein. First, the cS8000 stimulates mitochondrial respiration in the presence of a concentration of oligomycin sufficient to completely inhibit the stimulation of respiration by ADP. Therefore, the stimulation of respiration is not due to the activity of an ATPase. Second, respiration is not being stimulated by the addition of contaminating Ca(II), since the rate of uncoupled respiration stimulated by cS8000 is unaffected by the presence of 10 mM EGTA. Third, the uncoupling activity is completely abolished by treating S8000 with the nonspecific protease Nagarse or by incubating this crude protein mixture

Figure 1. The Stimulation of Chick Heart Mitochondrial Oxygen Consumption by cS8000

Mitochondria isolated with collagenase were suspended in 1.75 ml of an assay medium that contained 0.225 M mannitol, 0.075 M sucrose, 10 mM EGTA, and 20 mM Tris-buffered P_i, pH 7.4. Oxygen consumption was measured with a Clark electrode at 30.5°C. The following additions to the reaction mixture were made: M, mitochondria (0.091 nmoles cyt aa_3/ml); S, substrate (5mM pyruvate/ 2.5 mM malate); ADP, 400 nmoles per addition; O, oligomycin A, 66 µg/nmole of cyt aa_3; cS8000, crude mixture of protein containing the uncoupling factor, 3.4 mg/ml; and rotenone, 10 µM.


ADP
Mito.
P/M
Olig.
ADP
cS8000
Rotenone
64 nmol O_2
2 min

in a boiling water bath for 5 min.

Titrating chick heart mitochondria with increasing amounts of cS8000 suggests that the uncoupler protein has an apparent high affinity for these organelles (Fig 2). From this plot it is discerned that one unit of uncoupling activity corresponds to 0.35 mg crude protein/mg mitochondria. Maximal uncoupling is obtained subsequent to the addition of 9.1 units of crude protein/mg mitochondria. The fact that the stimulation of oxygen consumption is linear with rapid saturation suggests that this protein binds to mitochondria stoichiometrically. In the experiment shown, cS8000 at saturating concentrations stimulates oxygen consumption at a rate equal to 70% of the V_{max} for state 3 respiration.

III. AMMONIUM SULFATE PRECIPITATION OF THE UNCOUPLER PROTEIN

Additional evidence suggesting that the uncoupling factor is a protein is that it can be salted-out of solution with ammonium sulfate (Fig 3). The uncoupler begins to precipitate above 60% saturation with ammonium sulfate, as indicated by a steadily decreasing uncoupling activity in the GTE buffer. Importantly, the uncoupling activity lost during precipitation can be recovered by removing ammonium sulfate and resuspending the precipitate in GTE buffer.

IV. MOLECULAR WEIGHT ESTIMATE FOR THE UNCOUPLER PROTEIN

Experiments in which dialysis tubing of different pore sizes were used indicated that the uncoupler protein has an apparent molecular weight of 6-15 x 10^3 (i.e., if dialysis tubing with a molecular weight cutoff greater than 15,000 was used, the uncoupling activity was lost to the dialyzing buffer). Treatment of chick heart mitochondria with

Figure 2. Effect of Increasing Concentrations of cS8000 on the Rate of Oxygen Consumption by Chick Heart Mitochondria

Assay conditions were as described in the legend to Figure 1. cS8000 was prepared as described under "Experimental Procedures."

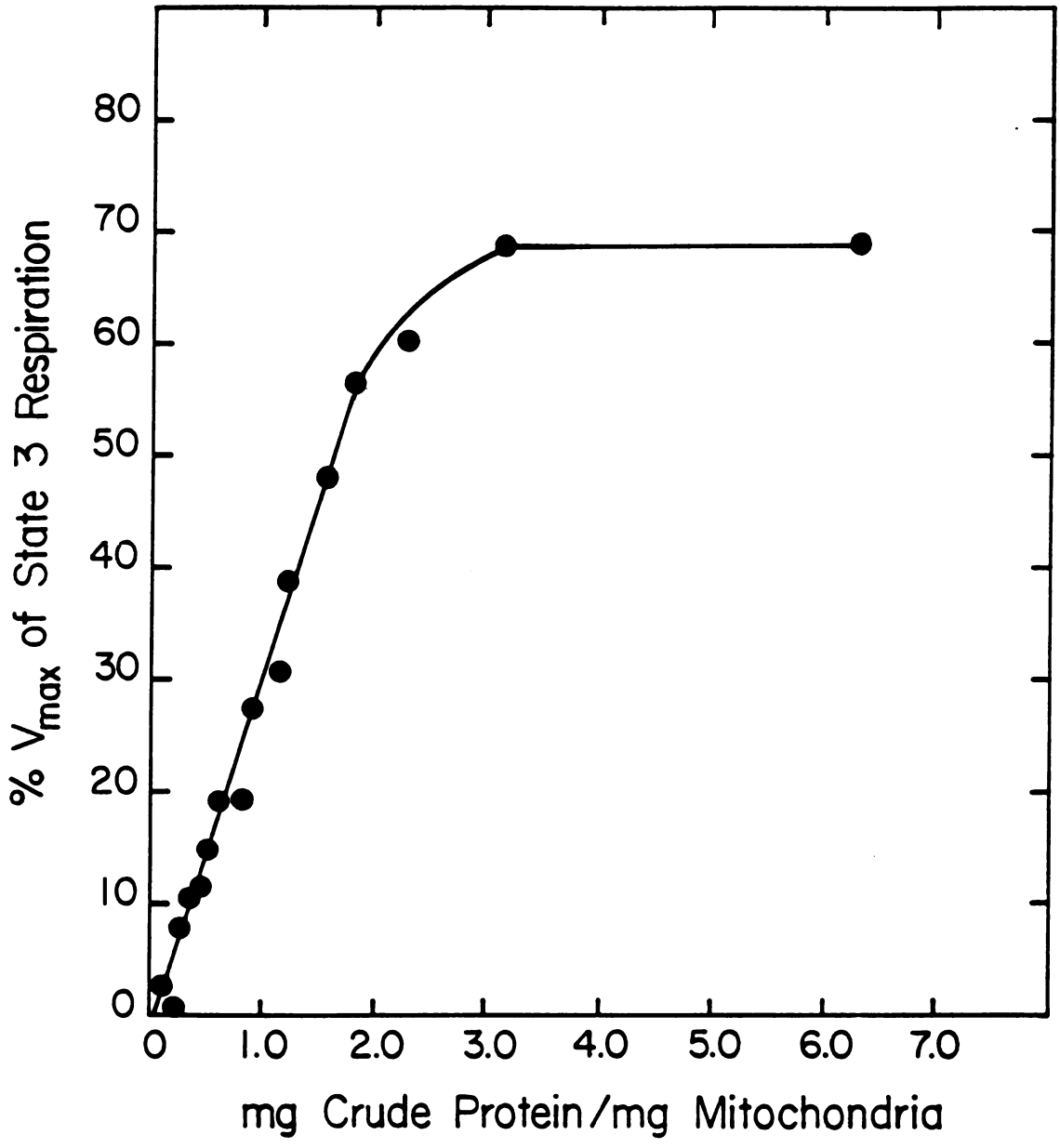
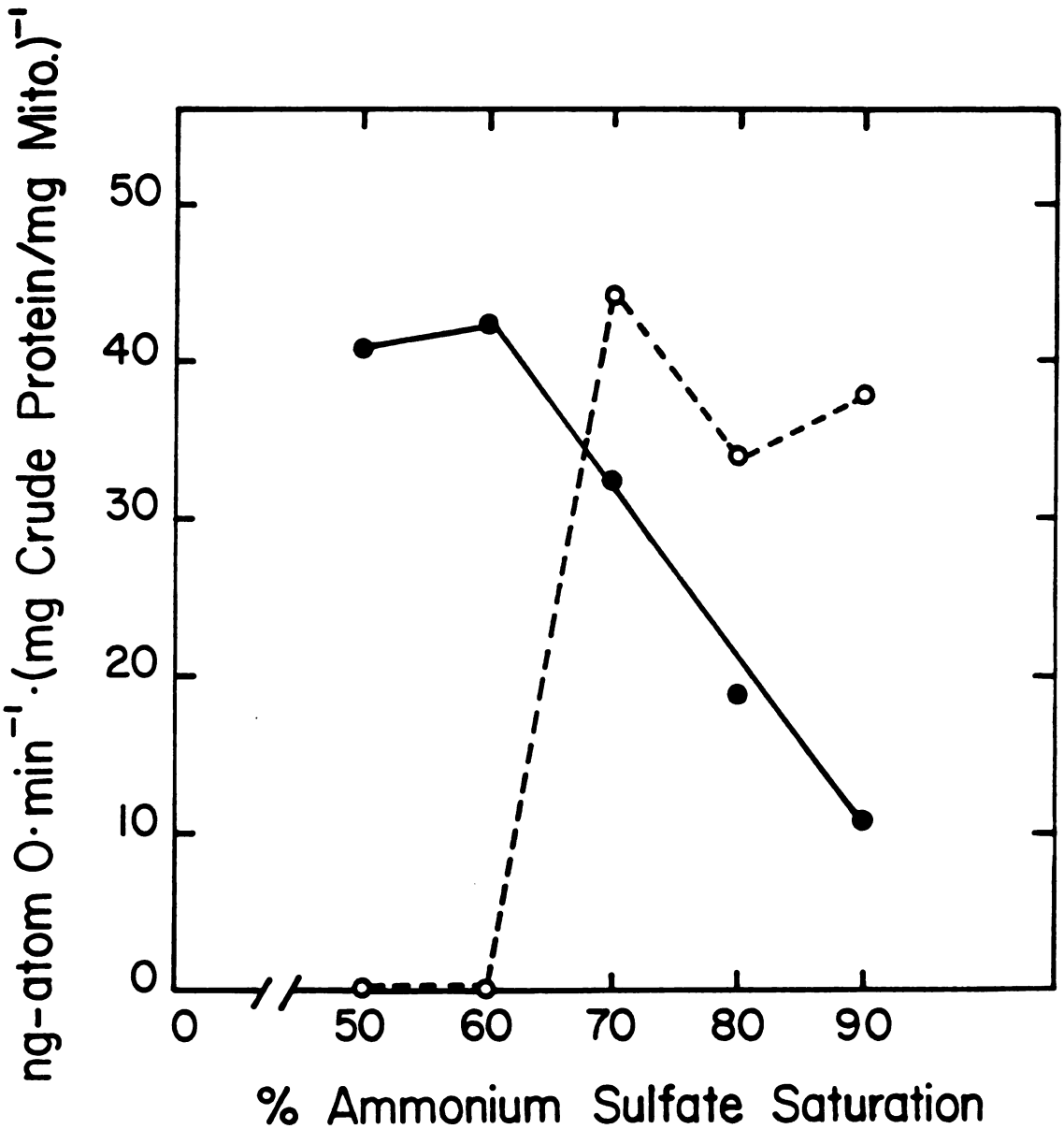

% V_{max} of State 3 Respiration
0
10
20
30
40
50
60
70
80
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
mg Crude Protein/mg Mitochondria

Figure 3. Activity of the Uncoupler Protein in the Ammonium Sulfate Cuts of cS8000

Aliquots of the supernatant liquids (●) and the precipitates (◯) resulting from treatment of cS8000 with the indicated concentrations of ammonium sulfate were added to 0.38 mg mitochondria/ml. Oxygen consumption assays were performed as described in the legend to Figure 1.


ng-atom O·min⁻¹·(mg Crude Protein/mg Mito.)⁻¹
50
40
30
20
10
0
0
50
60
70
80
90
% Ammonium Sulfate Saturation

Nagarse produces a substantial increase in their respiratory control ratio (see Chapter 3). It was of interest to determine whether Nagarse destoys a protein with a molecular weight in the range estimated by the dialysis tubing experiments. Mitochondria isolated with either Nagarse or collagenase and cS8000 were loaded into separate lanes and electrophoresed through a denaturing polyacrylamide gel. Densitometric measurements reveal that over 50 protein bands are obtained in the lane that contained mitochondria isolated with collagenase (data not shown). After comparing the banding patterns in the three lanes, a band with an apparent M_r of 14,300 found in the lanes for cS8000 and collagenase mitochondria is missing from the lane containing mitochondria isolated with Nagarse (data not shown). The two experiments are thus consistent. However, much work must still be done in order to establish the identity of the 14.3 kD band that is missing. It is possible that it is the uncoupling protein. At the least, these experiments provide a reasonably good candidate with which to begin the search for the uncoupling protein.

DISCUSSION

The data reported herein provide strong suggestive evidence for the existence of a relatively small soluble protein that uncouples mitochondrial electron transport from the synthesis of ATP in chick heart muscle. Because it is soluble and is found in a tissue other than mammalian brown fat, it is the first such protein to be discovered. These data establish that the uncoupling factor is a protein since it can be inactivated by treatment with heat and protease digestion and it can be precipitated by ammonium sulfate. The activity of the protein can be stabilized in highly viscous solvents containing either sucrose or glycerol. It is also shown that the stimulation of respiration is not attributable to Ca(II) or ADP contaminants in the cS8000. Future work will be aimed at purifying the uncoupler protein and characterizing its structure, function, and the mechanism by which it stimulates electron transport. The sensitivity of this protein to Nagarse indicates that it binds to mitochondria on the outer membrane. Questions concerning whether the protein binds to porin or some other receptor, and the nature of the regulatory mechanisms (if any) regulating its activity and the strength of its binding to mitochondria will all be of considerable interest to elucidate.

At this point one can only speculate about the function of this protein <u>in vivo</u>. Chick myocardium has evolved a cytosolic factor that appears to compromise the intrinsic high degree of coupling of the mitochondria which it contains. This is somewhat astonishing for two

reasons. First, a fundamental struggle in nature has always seemed to center around the development of more and more efficient means of procuring and distributing energy within an organism. Second, heart muscle mitochondria have long been assumed to be particularly specialized for ATP synthesis (i.e., other mitochondrial functions such as urea metabolism and erythropoietic heme biosynthesis are performed in non-cardiac tissues). The uncoupler protein provides myocardium with a molecule capable of inducing mitochondria to increase the rate of respiration independent of changes in cytosolic ADP or Ca(II) concentrations. Instead of ATP, heat presumably will be produced. At this point it is not possible to say why or under what conditions heart muscle would uncouple its mitochondria, but it is important to know that it can and, based on findings with isolated heart mitochondria, does.

Further work on this protein will provide novel insight into the means by which heart muscle regulates mitochondrial coupling and respiratory control. It will also be of interest to determine whether this protein plays a role in any forms of cardiac dysfunction. For instance, if a patient suffers from cardiac ischemia, an uncoupling protein, if it exists in the human heart, will only exacerbate the oxygen deficit. On the other hand, the controlled clinical use of an organic uncoupler could be beneficial, since it may well promote rapid weight loss if it can be used to uncouple the mitochondria of adipose tissue in obese persons.

REFERENCES

Aquila, H., Link, T.A., and Klingenberg, M. (1985) EMBO J. 4: 2369-2376.

Bencini, D.A., Wild, J.R., and O'Donovan, G.A. (1983) Anal. Biochem. 132: 254-258.

Bouillaud, F., Weissenbach, J., and Ricquier, D. (1986) J. Biol. Chem. 261: 1487-1490.

Cannon, B., Bernson, V.S., and Nedergaard, J. (1984) Biochim. Biophys. Acta 766: 483-491.

Guilian, G.G., Moss, R.L., and Greaser, M. (1983) Anal. Biochem. 129: 277-287.

Kell, D.B., and Westerhoff, H.V. (1985) In Oragnized Mutlienzyme Complexes: Catalytic Properties (Welch, R., ed.), pp. 63-139. Academic Press, New York.

Klingenberg, M. (1984) Biochem. Soc. Trans. 12: 390-393.

Kramer, R.S., and Pearlstein, R.D. (1983) Proc. Nat. Acad. Sci. (U.S.A.) 80: 5807-5811.

Laemmli, U.K. (1970) Nature (London) 227: 680-685.

Markwell, M.A.K., Haas, S.M., Tolbert, N.E., and Bieber, L.L. (1981) Methods Enzymol. 72: 296-303.

Nicholls, D.G., Grav, H.J., and Lindberg, O. (1972) Eur. J. Biochem. 31: 526-533.

Nicholls, D.G. (1979) Biochim. Biophys. Acta 549: 1-29.

Nicholls, D.G., Snelling, R., and Rial, E. (1984) Biochem. Soc. Trans. 12: 388-390.

APPENDIX

BIBLIOGRAPHY

Published Papers

1. Toth, P.P. (1981) Transfer ribonucleic acid tertiary structure stabilization by cation binding measured as a function of terbium fluorescence behavior. Undergraduate Thesis. 119 pp.

2. Toth, P.P., Ferguson-Miller, S.M., and Suelter, C.H. (1986) The isolation of highly coupled heart mitochondria in high yield using a bacterial collagenase. Methods Enzymol. 125: 16-27.

3. Lysiak, W., Toth, P.P., Suelter, C.H., and Bieber, L.L. (1986) Quantitation of the efflux of acylcarnitines from rat heart, brain, and liver mitochondria. J. Biol. Chem. 261: 13698-13703.

4. Lysiak, W., Lilly, K., DiLisa, F., Toth, P.P., and Bieber, L.L. (1988) Quantitation of the effect of L-carnitine on the levels of acid soluble short chain acyl-CoA and CoASH in rat heart and liver mitochondria. J. Biol. Chem. 263: 1151-1156.

5. Lysiak, W., Lilly, K., Toth, P.P., and Bieber, L.L. (1988) Effect of the concentration of carnitine on acetylcarnitine production by rat heart mitochondria oxidizing pyruvate. Nutrition 4: 214-219.

6. Toth, P.P., Chance, B., Sell, J.E., Holland, J.F., Ferguson-Miller, S., and Suelter, C.H. (1988) The kinetics of inorganic phosphate uptake and utilization by chick heart mitochondria. In Integration of Mitochondrial Function (Lemasters, J.J., Hackenbrock, C.R., Thurman, R.G., and Westerhoff, H.V., eds). Plenum Press, New York. In press.

7. Toth, P.P., Sell, J.E., Holland, J.F., Ferguson-Miller, S., and Suelter, C.H. (1988) Interaction of inorganic phosphate with chick heart mitochondria. I. Changes in volume and ion content of the matrix. To be submitted to J. Biol. Chem.

8. Toth, P.P., Ferguson-Miller, S., and Suelter, C.H. (1988) Interaction of inorganic phosphate with chick heart mitochondria. II. Substrate-dependent modulation of state 3 and state 4 rates of respiration. To be submitted to J. Biol. Chem.

9. Toth, P.P., Sumerix, K., Ferguson-Miller, S., and Suelter, C.H. (1988) Characterization of the factors affecting the respiratory control and ADP:O coupling ratios of isolated chick heart mitochondria. To be submitted to J. Biol. Chem.

10. Toth, P.P., Sell, J.E., Ferguson-Miller, S., Suelter, C.H., and Holland, J.F. (1988) Simultaneous measurement of absorbance, fluorescence, and light scattering intensities to determine the kinetic parameters of carrier-mediated transport processes in heart mitochondria. To be submitted to Anal. Biochem.

Abstracts

1. Toth, P.P., Ferguson-Miller, S.M., and Suelter, C.H. (1984) Heart mitochondria with high respiratory control isolated in high yield using collagenase. Fed Proc. 43(7): 2001.

2. Toth, P.P., Ferguson-Miller, S.M., and Suelter, C.H. (1985) Characteristics of highly coupled heart mitochondria. Proc. 13th Intn'l Cong. Biochem., vol 5, p. 767.

3. Lilly, K., Lysiak, W., Toth, P.P., and Bieber, L.L. (1986) Effect of carnitine concentration on the efflux of specific short-chain acylcarnitines from rat heart mitochondria. 16th Annual Michigan Cardiovascular Research Forum, Detroit, Michigan.

4. Lysiak, W., Toth, P.P., Suelter, C.H., and Bieber, L.L. (1986) Quantitation of the efflux of short-chain acylcarnitines from rat mitochondria. In Clinical Aspects of Human Carnitine Deficiency (Borum, P., ed.), Pergamon Press, New York, p. 41.

5. Toth, P.P., Ferguson-Miller, S.M., and Suelter, C.H. (1986) Coupling and respiratory control of chick heart mitochondria. Fed. Proc. 45(6): 1922.

6. Lilly, K., Lysiak, W., Toth, P.P., and Bieber, L.L. (1987) Regulation of the acyl CoA/CoAsh ratio in rat heart and liver mitochondria by carnitine. Fed. Proc. 46(3): 1013.

7. Toth, P.P., Ferguson-Miller, S.M., and Suelter, C.H. (1987) The kinetic parameters for phosphate saturation of chick heart mitochondrial state 3 respiration vary with the carboxylic acid substrates. Fed. Proc. 46(6): 1931.

8. Toth, P.P., Chance, B., Sell, J.E., Holland, J.F., Ferguson-Miller, S.M., and Suelter, C.H. (1987) Kinetics of phosphate uptake and utilization by chick heart mitochondria. International Conference on the Integration of Mitochondrial Function, 4-7 June 1987, Chapel Hill, North Carolina.

9. Toth, P.P., Chance, B., Sell, J.E., Ferguson-Miller, S.M., Suelter, C.H., and Holland, J.F. (1987) Kinetic studies of carrier-mediated uptake processes in heart mitochondria by light scattering measurements. Federation of Analytical Chemistry and Spectroscopic Societies, 4-9 Oct 1987, Detroit, Michigan. p. 116.

10. Toth, P.P., Sumerix, K., Ferguson-Miller, S.M., and Suelter, C.H. (1987) The role of Ca(II) and Mg(II) in the uncoupled and oligomycin-sensitive components of state 4 respiration in isolated chick heart mitochondria. 7th Annual Michigan Cardiovascular Research Forum, East Lansing, Michigan.

11. Toth, P.P., Ferguson-Miller, S.M., and Suelter, C.H. (1987) ADP:O

stoichiometries of chick heart mitochondria. 17th Annual Michigan Cardiovascular Research Forum, East Lansing, Michigan.

12. Toth, P.P., Ferguson-Miller, S.M., and Suelter, C.H. (1987) Kinetics of phosphate utilization during chick heart mitochondrial state 3 and state 4 respiration. 17th Annual Michigan Cardiovascular Research Forum, East Lansing, Michigan.

13. Toth, P.P., Sell, J.E., Suelter, C.H., and Holland, J.F. (1987) Characterization and use of chick heart mitochondrial light scattering properties to measure phosphate uptake kinetics. 17th Annual Michigan Cardiovascular Research Forum, East Lansing, Michigan.

14. Lilly, K., Lysiak, W., DiLisa, F., Toth, P.P., and Bieber, L.L. (1987) The effect of L-carnitine on the short-chain acyl-CoA/CoASH ratio in rat heart mitochondria. 17th Annual Michigan Cardiovascular Research Forum, East Lansing, Michigan.

15. Suelter, C.H., Toth, P.P., Bertsch, R.A., and Ferguson-Miller, S.M. (1988) Inorganic phosphate is a complex activator of chick heart mitochondrial α-ketoglutarate dehydrogenase. FASEB J. 2(4): A554.

16. Toth, P.P., Sumerix, K., Ferguson-Miller, S., and Suelter, C.H. (1988) ADP:O stoichiometries of chick heart mitochondria. FASEB J. 2(5): A1122.

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