# THE ROLE OF CALCIUM IN THE REGULATION OF NADH OXIDATION IN JERUSALEM ARTICHOKE TUBERS

by

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## ABSTRACT

The EGTA-inhibition of exogenous NADH oxidation in Jerusalem artichoke (*Helianthus tuberosus*) mitochondria can be reversed by $Ca^{2+}$ (Coleman & Palmer, 1971). The purpose of this thesis is to investigate the role which $Ca^{2+}$ may play in the regulation of exogenous NADH oxidation. A wide range of chelators was found to inhibit NADH oxidation. $Ca^{2+}$ reversed the inhibition in most cases (analogues of EGTA, citrate, solochrome) supporting (but not proving) the involvement of $Ca^{2+}$. However, in other cases, $Ca^{2+}$ either had no effect on the inhibition (calcichrome) or it actually enhanced the inhibition (DHPE, HSN, fluorescein complexone).

Extensive attempts were made to obtain $Ca^{2+}$-dependent exogenous NADH oxidation by washing mitochondria with various EGTA media, supplemented with A23187 (a divalent cation ionophore) or NADH or divalent cations. These procedures were successful at reducing the mitochondrial $Ca^{2+}$ content and at rendering NADH oxidation much more sensitive to subsequent exposure to EGTA.

The inhibited rate of NADH oxidation was found to depend on whether NADH oxidation is initiated before or after EGTA is added; these observations gave rise to speculations on the nature of the interaction of $Ca^{2+}$ with the dehydrogenase.

A wide range of cations of various valencies, inorganic and organic, were found to stimulate the rate of NADH oxidation. Fluorescence of 9-aminoacridine (9-AA) could be used to monitor the surface potential of the mitochondrial membranes under low salt conditions. Since there is good correlation between the general cation stimulation of NADH oxidation and the decrease in the surface potential, the general response to

cations (trivalent, most effective; monovalent, least effective) may be interpreted as being due to the screening of fixed negative charges associated with the lipid and protein components of the membranes. Screening of the charges decreases the electrostatic repulsion of the negatively charged substrate, NADH, resulting in an apparent increase in affinity between the enzyme and substrate.

Independent of the general screening effect, a specific $Ca^{2+}$-requirement for exogenous NADH oxidation has been demonstrated.

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## ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| A/A | antimycin A |
| A23187 | monocarboxylic antibiotic, $C_{29}H_{37}N_3O_6$, Mol. wt. 523 |
| ADP/O | adenosine diphosphate:oxygen ratio |
| 9AA | 9-aminoacridine monohydrochloride |
| BSA | bovine serum albumin |
| $C^{n+}$ | cation |
| DAD | 2,3,5,6-tetramethyldiaminobenzene |
| DHPE | di-(*o*-hydroxyphenylimino)ethane |
| $(DM)Br_2$ | decamethylene-bis-trimethylammonium bromide |
| DMSO | dimethyl sulphoxide |
| DPTA | diethylenetriaminepentacetic acid |
| EGTA | ethyleneglycol-bis-(β-aminoethyl ether)-N,N'-tetraacetic acid |
| FCCP | carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone |
| GOT | glutamate-oxaloacetate transaminase |
| HEDTA | N-(2-hydroxyethyl)ethylenediamine-N,N',N'-triacetic acid |
| HSN | 2-hydroxy-1-(2-hydroxy-4-sulpho-1-naphthylazo)-3-naphthoic acid |
| Km | substrate concentration at which velocity is half maximal |
| lys-lys | lysyl lysine.2HCl |
| $M^{n+}$ | metal cation |
| MOPS | 3-(N-morpholino)propanesulphonic acid |
| pmf | protonmotive force |
| P/A | piericidin A |
| RCR | respiratory control ratio |
| SHAM | salicylhydroxamic acid |
| stability constant | $K = \frac{[M^{n+}][\text{chelator}]}{[\text{M:chelator complex}]}$ |

| | |
|---|---|
| $(TEC)Cl_3$ | tris-(ethylenediamine)cobalt (III) chloride |
| TES | N-((trishydroxymethyl)methyl)-2-aminoethanesulphonic acid |
| TMPD | N,N,N',N'-tetramethyl-*p*-phenylenediamine |
| TPP | thiamin pyrophosphate |
| $V_{max}$ | maximum velocity |
| Z | 2.3RT/F |
| $-Z\Delta pH$ | chemical potential difference |
| $\Delta p$ | protonmotive force |
| $\Delta\psi$ | electrical potential difference |

## LIST OF ENZYMES

| Enzyme Commission Number | Trivial name | Systematic name |
|---|---|---|
| 1.1.1.37 | malate dehydrogenase | L-malate:$NAD^+$ oxidoreductase |
| 1.1.1.39 | malic enzyme ($NAD^+$-requiring) | L-malate:$NAD^+$ oxidoreductase (decarboxylating) |
| 1.2.4.1 | pyruvate dehydrogenase | pyruvate:lipoate oxidoreductase (decarboxylating and acceptor-acetylating) |
| 1.3.99.1 | succinate dehydrogenase | succinate:(acceptor) oxidoreductase |
| 1.4.1.2 | glutamic acid dehydrogenase | L-glutamic:$NAD^+$ oxidoreductase (deaminating) |
| 1.6.99.3 | NADH dehydrogenase | NADH:(acceptor) oxidoreductase |
| 2.6.1.1 | glutamate-oxaloacetate transaminase | L-aspartate:2-oxoglutarate aminotransferase |
| 3.1.1.4 | phospholipase $A_2$ | phosphatide 2-acylhydrolase |
| 3.1.4.4 | phospholipase D | phosphatidyl choline phosphatidohydrolase |
| 3.4.24.11 | kidney brush border neutral proteinase | |
| 3.6.1.3 | proton translocating reversible ATPase | ATP phosphohydrolase |

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## 1. INTRODUCTION

Earlier investigations into the structural and functional properties of mitochondria led to the traditional view that the mitochondrion was the powerhouse of the cell, whose principle function was to synthesize ATP, the intermediate on which the energy economy of the cell depended. The synthesis of ATP is now generally accepted to be coupled to a linear respiratory chain by a proton motive force as proposed in the chemiosmotic theory presented by Mitchell (1966). The majority of early investigations used mitochondria from mammalian tissues, especially rat-liver. Relatively few investigations used mitochondria from plant tissues and those which did concluded that mitochondria from plant and animal tissue were fundamentally similar in structure and function (Hackett, 1959; Mercer, 1960; Crane, 1961; Bonner, 1965; Lieberman and Baker, 1965; Hanson and Hodges, 1967; Pullman and Schatz, 1967; Chance *et al.*, 1968; Lardy and Ferguson, 1969; Racker, 1970; Van Dam and Meyer, 1971; Ikuma, 1972; Palmer and Hall, 1972; and Öpik, 1974). Those features of plant mitochondria which make them different from rat-liver mitochondria, i.e. the ability of plant mitochondria to oxidize exogenously added NADH, the ability to oxidize malate and a different sensitivity to various inhibitors of electron transport have attracted relatively little attention. However in response to data accumulated over recent years, a reappraisal of the function of plant mitochondria has been undertaken by various groups, whose work collectively suggest that the considerably more complex respiratory chain of plant mitochondria may enable plant mitochondria to fulfil a more complex metabolic function in the cell than is the case with mammalian mitochondria.

## 1.1 Isolation of plant mitochondria

Relatively few investigations of mitochondrial structure and function used plant tissues due partly, perhaps, to the greater problems encountered in the isolation of plant mitochondria compared with the isolation of mammalian mitochondria. Plant mitochondria were considered more difficult to isolate than mammalian mitochondria for three main reasons, (a) the need to break the tough cell wall without also damaging the integrity of the mitochondrial membranes, (b) the need to maintain a relatively constant pH environment, which is made difficult by the release of large quantities of organic acids frequently stored in the vacuoles of many types of plant cells, and (c) the need to control mitochondrial contamination by potentially harmful chemicals, such as certain metal ions, and oxidation products of polyphenols liberated upon homogenization. Such difficulties contributed to the assertion that many of the results obtained with plant mitochondria were due to artefacts induced by isolation (Packer *et al.*, 1970). However, as methods for the isolation of plant mitochondria evolved, research progressed using mitochondria from a wide range of plant material. Unlike research in animal mitochondria, where there is in the ubiquitous laboratory rat a source of mitochondria which is easily isolated and which behaves in a consistent manner, in plant mitochondria research there is no 'ideal' material which is cheaply available all year round, in the same metabolic state and which all workers agree yields acceptable mitochondria. In the absence of an obvious choice of plant material as a source of mitochondria, different groups have developed research programmes using plant mitochondria from different tissues and this, together with the evolution of independent isolation media, has limited the usefulness of comparing data from different laboratories.

## 1.2 Assessment of integrity and purity of mitochondrial preparations

So that comparisons of different preparations of plant mitochondria may be made with confidence, it is clearly desirable that a set of criteria is established to assess the quality of various preparations. In the case of mammalian mitochondria, which tend to behave in a consistent manner, a set of criteria has been enumerated to define the level of organelle integrity and purity. Basically these criteria are: (a) negligible endogenous respiration; (b) slow state 4 respiration (metabolic states referred to in this study are as defined by Chance and Williams, 1956); (c) rapid state 3 rate; (d) high pyridine nucleotide: cytochrome c ratio; (e) no response of malate oxidation upon the addition of exogenous $NAD^+$. In general these criteria are easily met in preparations of mammalian mitochondria. There has been a trend to apply these criteria when assessing the integrity and purity of preparations of plant mitochondria (Chance *et al.*, 1968) but it is questionable whether these criteria are useful in defining preparations of plant mitochondria. In his review, Palmer (1976) criticised the acceptability of criteria (a)-(e) and concluded that they do not form a satisfactory or adequate means of assessing the quality of a preparation of plant mitochondria. For example, criterion (a) may be satisfied by adequate washing of the preparation. Criteria (b) and (c) are relative terms and it would be more useful to use the respiratory control ratio of the state 3 rate to the state 4 rate to define the preparation. The respiratory control ratio measures the degree of control that the phosphorylating system may exert over the electron transport system. The application of the respiratory control ratio as a parameter for the integrity of the preparation depends upon the electron carriers being coupled to oxidative phosphorylation, as is the case in the mammalian

system. But in the higher plant system the occurrence of non-phosphorylating electron transport pathways found in a variety of higher plant tissues challenges the validity of the respiratory control ratio as a parameter for the integrity of the preparation. For example, Bahr and Bonner (1973a, 1973b) have estimated that in mung-bean mitochondria electron flow is mediated by both the cyanide-sensitive oxidase and the cyanide-insensitive oxidase. Similar observations have been made using mitochondria from the fungus *Neurospora crassa* (Edwards *et al.*, 1974). So under certain metabolic conditions in mitochondria from various sources, part of state 4 respiration is also mediated by a pathway not participating in state 3 respiration.

A fascinating feature of the state 4 respiration rate in plant mitochondria is that it varies depending upon which substrate is used. For example, Ikuma and Bonner (1967) have found that the state 4 rate using malate as substrate is slower than the state 4 rate using succinate. In state 4, the rate is defined by Chance and Williams (1956) as being limited by the lack of phosphate acceptor in the presence of substrate. However in plant mitochondria, it seems likely that often under conditions when phosphate acceptor is sub-optimal, the rate of supply of electrons may also be limited by another component. For example, Ikuma and Bonner (1967) found that the state 4 rate using malate as substrate is not linear. After the phosphorylation of an addition of ADP, at the onset of state 4, the rate is slow but progressively increases in velocity. This change in rate is related to the disappearance of oxaloacetate (the accumulation of which causes an inhibition of malate oxidation; Lance and Bonner, 1968; Hobson, 1970; Palmer, 1980) rather than the availability of phosphate acceptor. Thus under such conditions of malate oxidation it is strictly incorrect

to describe such a rate as the state 4 rate.

Criteria (d) and (e) are designed to check that no loss of $NAD^+$ has been incurred. It is known with some confidence that in mammalian mitochondria the inner membrane is impermeable to pyridine nucleotides (Lehninger, 1951, 1955; Chance and Williams, 1955; Pullman and Racker, 1956; Klingenberg and Pfaff, 1966). When applying criteria (d) and (e) to higher plant mitochondria it is assumed that the inner membranes are also impermeable to pyridine nucleotides although there is no direct experimental evidence to verify this assumption. In the case of mitochondria from *Saccharomyces carlsbergensis*, the inner membrane is impermeable to pyridine nucleotides (von Jagow and Klingenberg, 1970). On the other hand however, Neuberger and Douce (1978) have suggested that higher plant mitochondria (mung bean and white potato) may be permeable to pyridine nucleotides.

Criterion (e), lack of response of malate oxidation to exogenous $NAD^+$ may be of less use in estimating the integrity of a preparation of plant mitochondria because it assumes that all malate oxidation in the mitochondrial fraction occurs within the matrix. This contention is controversial because there is evidence to support the view that there may be an externally located malic enzyme (Macrae and Moorhouse, 1970; Macrae, 1971a; Coleman and Palmer, 1972; Brunton and Palmer, 1973; Day and Wiskich, 1974; Palmer and Arron, 1976; Palmer, 1980) and an externally located malate dehydrogenase (Palmer, 1980). It has been suggested that external malate oxidation may simply be an artefact indicating the level of membrane integrity. However in a mitochondrial preparation which, using succinate:cytochrome c oxidoreductase activity as a parameter of membrane integrity, often appears to be over 97% intact, malate oxidation in the presence of exogenous $NAD^+$ accounts for

from 3% broken mitochondria
a greater rate of oxidation than expected. Thus, if it is valid to assign some of the malic enzyme and/or malate dehydrogenase activity to the intermembrane space, such extra-matrix malate oxidation, together with the activity of the externally located, rotenone-insensitive NADH dehydrogenase (Palmer and Passam, 1971; Douce *et al.*, 1973) provides a hypothetical pathway for the complete oxidation of external reducing equivalents in an intact mitochondrial preparation. Therefore lack of malate oxidation in the presence of exogenous $NAD^+$ may not be definitive in assessing membrane integrity. Such a pathway for the complete oxidation of external reducing equivalents in plant mitochondria is of great significance as it provides a mechanism for highly undesirable equilibration of the respective characteristic redox poise of matrix and intermembrane space (= cytosolic) compartments (Chance, 1959; Chance and Thorrell, 1959; von Jagow and Klingenberg, 1970) and would certainly require regulation to preserve the integrity of various compartments and to prevent inefficient use of metabolic pathways.

The possibility that microsomal contamination of the mitochondrial preparation occurs unavoidably as a result of differential centrifugal has led to a recommendation that further purification of the mitochondrial preparation be carried out using sucrose density gradients (Douce *et al.*, 1973; Bonner, 1974; Lambowitz and Bonner, 1974) although earlier reports expressed confidence in the purity of preparations isolated simply by differential centrifugation (Bonner, 1965; Bonner and Plesnicar, 1967). Douce *et al.* (1973) found an enhancement of the biochemical activity of their preparation (mung-bean mitochondria) but it is frequently found that the biochemical character of the preparation after purification on a sucrose density gradient is little changed (Baker *et al.*, 1968; Pomeroy, 1975).

Methods for determining the integrity of a mitochondrial preparation are based on measuring the impermeability of the mitochondrial al membranes to various agents. One method offering a high level of sensitivity is the determination of the activity of succinate-cytochrome c oxidoreductase (Douce *et al.*, 1973) which is negligible in intact mitochondria but which may increase up to 100-fold as the preparation becomes osmotically damaged. Although the rate of reduction of exogenous cytochrome c is held to be limited by its permeation through the outer membrane (Douce *et al.*, 1972 a,b), Palmer and Kirk (1974) suggest that the rate of reduction of cytochrome c is more likely to be limited by the inability of exogenous cytochrome c to react with the endogenous cytochrome c bound to the inner membrane. However, although the rate-limiting step of the reduction of cytochrome c remains in dispute, the simplicity and sensitivity of this method recommends itself. In addition, the permeability of the inner membrane to NADH may be estimated as a function of osmotic damage by measuring the rate of oxidation of exogenous NADH by oxaloacetate catalysed by the matrix malate dehydrogenase (Palmer and Kirk, 1974), while another method by the same authors measures the permeability of the inner membrane to ferricyanide by determining the antimycin A-insensitivity of the succinate-ferricyanide reductase, which will increase as membrane permeability to ferricyanide increases.

### 1.3 Oxidation of exogenous NADH by a calcium-stimulated, rotenone-insensitive pathway

One of the major differences between plant and mammalian (animal) mitochondria is the ability of the former to readily oxidize exogenous NADH (in the absence of added cytochrome c and antimycin A). In contrast it had been found that exogenously added NADH was not oxidized by

rat-liver mitochondria (Lehninger, 1951; Chance and Williams, 1955; Pullman and Racker, 1956; Devlin and Bedell, 1960). However, oxidation of oxogenous NADH could be induced in rat-liver mitochondria if they were subjected to hypotonic treatment (Lehninger, 1955) or if mitochondria were treated with chaotropic agents likely to alter membrane permeability characteristics (Hogeboom and Schneider, 1953; Ernster and Navazio, 1956). From these observations it was suggested that in the mammalian system exogenous pyridine nucleotides could not interact with the mitochondrial electron transport chain directly and that the inner mitochondrial membrane presented a barrier to the free passage of pyridine nucleotides between the matrix pool of pyridine nucleotides and a cytosolic pool of pyridine nucleotides. Cells are the sites of an enormous range of diverse activities, which may be described as either anabolic (i.e. synthetic) or catabolic (i.e. oxidative) in nature. The concept of subcellular compartments was developed to explain how reactions which are essentially incompatible in nature could proceed under optimal conditions (Lehninger, 1955; Chance and Williams, 1956; Chance and Thorell, 1959; Klingenberg, 1963a,b). Such discrete compartments may be maintained by membranes of selective permeability and would permit the adoption of different redox poises, favouring particular reactions. The unchecked activity of the exogenous NADH dehydrogenase would lead to the attainment of redox equilibrium between the pools of pyridine nucleotide in the mitochondrial matrix and the cytosol. The destruction of discrete pools of pyridine nucleotide of characteristic redox poise would lead to the loss of regulation of cellular metabolism and a biologically unstable state would prevail, resulting in cell death.

Compartmentalisation is therefore well illustrated in rat-liver

mitochondria in the inability of exogenously added (i.e. analagous to cytosolic) NADH to react with the electron transport chain (Lehninger, 1955) until the permeability characteristics of the mitochondrial membranes had been altered. Isotopic studies have shown that external pyridine nucleotides readily penetrated the outer mitochondrial membrane (Greenspan and Purvis, 1965; Klingenberg and Pfaff, 1966) but exchanged only very slowly with the intramitochondrial pyridine nucleotide pool. Similar permeability characteristics have been established for mitochondria from *Saccharomyces carlsbergensis* (von Jagow and Klingenberg, 1970) and from *Neurospora crassa* (Weiss *et al.*, 1970).

The ability of carefully isolated plant mitochondria to oxidize exogenous NADH by an antimycin A-, KCN-sensitive pathway was first reported by Humphreys and Conn in 1956 using lupin mitochondria. Oxidation of exogenous NADH was found to be coupled to phosphorylation in particles isolated from spinach leaves and in mitochondria isolated from sweet potato and corn root, respectively (Zelitch and Barber, 1960; Baker and Lieberman, 1962; Cunningham, 1964). The phenomenon of respiratory control was demonstrated to accompany the oxidation of exogenous NADH in mitochondria from cauliflower, sweet potato, apple fruit, mung bean, wheat seedling, corn root, Jerusalem artichoke tuber and broad bean, respectively (Bonner and Voss, 1961; Wiskich and Bonner, 1963; Jones *et al.*, 1964; Ikuma and Bonner, 1967; Sarkissian and Srivastava, 1968; Wilson and Hanson, 1969; Palmer and Passam, 1971; Matlib *et al.*, 1971). In higher plants, exogenous NADH oxidation by a rotenone-insensitive, antimycin A-sensitive pathway has been found lacking only in the case of beetroot mitochondria (Day *et al.*, 1976); however exogenous NADH oxidation by a rotenone-insensitive, antimycin A-sensitive pathway may be induced if slices of beetroot are aged in

$CaSO_4$ solution. Mitochondrial oxidation of exogenous NADH has also been observed in several species of fungi (Ohnishi *et al.*, 1966a,b; von Jagow and Klingenberg, 1970).

Reports of exogenous NADH oxidation by higher plant mitochondria have tended to be regarded by some authors with diffidence until relatively recently; it was felt that the oxidation of exogenous NADH in higher plant mitochondria was due to the activity of the internal dehydrogenase (Hackett, 1961; Lieberman and Baker, 1965; Hodges and Hanson, 1965; Kenefick and Hanson, 1966a). NADH was believed to have crossed the inner mitochondrial membrane, possibly via 'minute ruptures'. The term 'leaky' was introduced to describe such mitochondria (Ikuma, 1972), considered by some authors to be caused by an artefact of isolation (Hanson and Hodges, 1967; Packer *et al.*, 1970). However, it was difficult to reconcile the concept of 'leaky membranes' with the observations of Cunningham (1964) who reported that the use of exogenous NADH led to the measurement of a lower P:O ratio than that obtained using a $NAD^+$-linked substrate. He suggested that exogenous NADH was oxidized by a phosphorylating pathway which did not involve the dehydrogenase responsible for the oxidation of endogenously generated NADH.

Many observations on the oxidation of exogenous NADH in higher plant mitochondria have led gradually to an acceptance of the existence of a flavoprotein, capable of oxidizing exogenous NADH and located on the outer face of the inner membrane. Divalent cations, especially calcium, have been noted to stimulate the oxidation of exogenous NADH yet have little effect on the oxidation of $NAD^+$-linked substrates. Hackett first reported such a stimulation in 1961 and attributed the stimulation to a facilitated transport of NADH into the matrix space where he suggested oxidation occurred via the internal NADH dehydrogenase.

An alternative suggestion was that divalent cations released an unknown rate-limiting step in the pathway of NADH oxidation (Hackett, 1961; Miller *et al.*, 1970; Miller and Koeppe, 1971). However, Coleman and Palmer (1971) found that the oxidation of exogenous NADH was specifically inhibited by chelators of divalent cations, e.g., EDTA, EGTA and HEDTA. Because EGTA does not have access to the matrix space (Reed and Bygrave, 1974) EGTA appeared to inhibit the oxidation of exogenous NADH at a site on the outer face of the inner membrane, providing evidence to support the external location of the calcium-stimulated, rotenone-insensitive, antimycin A-sensitive NADH dehydrogenase. The complete reversal of the EGTA inhibition by divalent ions (calcium, strontium and manganese; Coleman and Palmer, 1971) suggested that there is a divalent cation requirement for the oxidation of exogenous NADH which is not found in the oxidation of $NAD^+$-linked substrates or succinate. In their review, Palmer and Coleman (1974) presented further evidence for the external location of the dehydrogenase. They showed that calcium stimulation of exogenous NADH occurred under conditions when calcium uptake was prevented by the addition of lanthanum. Thus the accumulation of calcium into the matrix was not necessary to stimulate the oxidation of exogenous NADH and this observation was consistent with the external siting of the dehydrogenase.

Other evidence which strongly supports the existence of a separate flavoprotein for the oxidation of exogenous NADH comes from data using amytal, rotenone and piericidin A. In intact higher plant mitochondria, the oxidation of exogenous NADH is completely insensitive to treatment by these compounds (Wilson and Hanson, 1969; Palmer and Passam, 1971; Coleman and Palmer, 1971; Palmer and Coleman, 1974). In sharp contrast, these compounds are powerful inhibitors of the oxidation of

internally generated NADH in mammalian mitochondria, but have no effect on succinate oxidation (Ernster *et al.*, 1963; Jeng *et al.*, 1968). Intact mammalian mitochondria do not readily oxidise exogenously added NADH (Lehninger, 1951, 1955) but the matrix-facing NADH dehydrogenase can be made to oxidise exogenous NADH if a preparation of these mitochondria is sonicated to produce sub-mitochondrial particles (smps; vesicles formed from the inner mitochondrial membranes; Lee and Ernster, 1966). Smps readily oxidise exogenous NADH because they are formed from inverted inner membranes, with the matrix surface of the membrane facing outwards. The dehydrogenase oxidizing NADH generated by matrix $NAD^+$-linked substrates is closely bound to the inner membrane with its active centre facing the matrix (Lee and Ernster, 1966; Singer, 1968). Therefore in smps the active centre of the NADH dehydrogenase may only be approached from outside the vesicle. These vesicles contain all the electron transport carriers when prepared in the presence of magnesium and ATP and have the capacity to carry out oxidative phosphorylation at three sites of ATP synthesis. However, unlike the oxidation of exogenous NADH in higher plant mitochondria, the oxidation of exogenous NADH in mammalian smps is fully rotenone-sensitive (Low and Vallin, 1963). Amytal, rotenone and piericidin A appear to bind at the same site, on the oxygen side of the iron-sulphur centres associated with the NADH dehydrogenase bound to the matrix side of the inner membrane (Singer and Gutman, 1971).

Thus, in the case of higher plant mitochondria, in order to interpret data which show that exogenous NADH is readily oxidized by intact organelles with an associated ADP:O ratio of approximately 2, it is necessary to postulate that such an oxidation occurs via an NADH dehydrogenase located on the outer face of the inner membrane, whose

existence is supported by several indirect observations. At the same time it is also necessary to postulate that such an oxidation occurs in a rigorously controlled fashion. It has been suggested that strict regulation of the activity of the exogenous NADH dehydrogenase may be attained by varying the concentration of calcium ions (Coleman and Palmer, 1971) because it was found that divalent cation chelators inhibit the oxidation of exogenous NADH. This study is concerned with the elucidation of the means by which the rate of oxidation of exogenous NADH is regulated and of the role which calcium ions may play in this regulation.

## 1.4 Rotenone-insensitive oxidation of internally generated NADH in higher plant mitochondria

In higher plant mitochondria, it seems likely that a rotenone-sensitive pathway coupled to three sites of ATP synthesis is present for the oxidation of internally generated NADH, identical to the mammalian pathway. However in higher plant mitochondria oxidizing malate, the addition of rotenone leads to a massive transient inhibition which appears to initiate a rotenone-insensitive pathway, bypassing the site of rotenone inhibition and concurrently causing a drop in the ADP:O ratio of approximately one unit, which suggests that in higher plant mitochondria, the site of rotenone inhibition is intimately associated with the first site of phosphorylation (Brunton and Palmer, 1973). In the case of the other $NAD^+$-linked substrates, the addition of piericidin A or rotenone led to the immediate onset of a partial inhibition (Brunton and Palmer, 1973).

## 1.5 Properties of the exogenous NADH dehydrogenase on the outer face of the inner membrane

Hypotonic treatment of purified mung-bean mitochondria caused the release of a flavoprotein into the supernatant (Douce *et al.*, 1973). This enzyme exhibits β-stereospecificity to tritiated NADH as do the energy-linked NADH dehydrogenases (Ernster *et al.*, 1965; von Jagow and Klingenberg, 1970). The flavoprotein solubilised by hypotonic treatment was different (Douce *et al.*, 1973) from the one bound to the inner face of the inner membrane. This latter dehydrogenase required drastic treatment for release from the inner membrane (*eg.*, King and Howard, 1962; Galente and Hatefi, 1979) using mammalian mitochondria. Moreover the flavoprotein solubilised by hypotonic treatment was probably identical to the NADH dehydrogenase located on the outer face of the inner mitochondrial membrane in Jerusalem artichoke mitochondria (Palmer and Passam, 1971; Coleman and Palmer, 1971) and in *Saccharomyces cerevisiae* mitochondria (von Jagow and Klingenberg, 1970).

A preliminary attempt to isolate a calcium-stimulated NADH dehydrogenase has been made (Ramakrishnan and Hanson, 1974). Purified corn mitochondria were fractionated using the method of Sottocasa *et al.*, (1967). A soluble fraction was obtained which exhibited enzymatic activity, both calcium-stimulated antimycin A-insensitive NADH:cytochrome c-oxidoreductase and calcium-insensitive cyanide-sensitive cytochrome c-oxidoreductase activity. Ramakrishnan and Hanson compared the NADH oxidase activity of their soluble fraction with enzyme systems isolated by various workers (King and Howard, 1962; Sottocasa *et al.*, 1967; von Jagow and Klingenberg, 1970; Coleman and Palmer, 1971; Douce *et al.*, 1973). It seems probable that such a comparison was invalid since it is strongly suggested that their soluble fraction was a crude preparation

containing fragments of both inner and outer mitochondrial membranes in order to account for the observed enzyme activity.

No other attempts have been made to isolate and define the flavoprotein which appears to be located on the outer face of the inner membrane, and its activity is thought to account for the rapid oxidation of added NADH in intact mitochondria in a rotenone-insensitive, antimycin A- and chelator-sensitive manner, yielding an ADP:0 ratio of approximately 2.

## 1.6 Calcium in plant mitochondria

The purpose of this thesis is to indicate the nature of the role which calcium may play in the oxidation of exogenously added NADH. In order to do this it is necessary first to examine the role of calcium in mitochondria in general.

### 1.6.1 The subcellular role of calcium

The general disorganisation of cells and tissues suffering calcium deficiency suggests that the role of calcium in maintaining membranes in a functional state may be one of its key functions. This is so because cellular organization is to a considerable extent a matter of compartmentation and metabolic pools, and all compartments such as the protoplast, vacuole, nucleus and other organelles are delimited by membranes.

Evidence for the paramount role of calcium in maintaining the structure of membranes has been obtained through electron microscopy. Marinos (1962) studied cells in the shoot apex of barley affected by calcium deficiency. In contrast to the clearly organized cytoplasm of normal cells, that of calcium-deficient cells had structureless areas, fragmented membranes, various vesicles and amorphous inclusions.

$Mg^{2+}$ deficiency produced no such features (Marinos, 1963). Marschner and Günther (1964) and Marschner *et al.* (1966) have reported similar findings for $Ca^{2+}$-deficient cells of barley and corn root tips respectively. The ultrastructural alteration of plant plasma membranes induced by auxin and calcium ions has been described by Morré and Bracker (1976). Calcium deficiency leads to a decrease in total mitochondrial yield and a decrease in the mitochondrial protein concentration (Florell, 1956; Lindblad, 1959). Chloroplasts are also affected by calcium deficiency (Vesk *et al.*, 1966). Chloroplasts, isolated by the non-aqueous method to prevent loss by leaching from tobacco and bean leaves were found to contain 60% of the total leaf calcium, and it was concluded that chloroplasts act as sites of calcium accumulation (Stocking and Ongun, 1962; Larkum, 1968). Energy-dependent calcium transport in chloroplasts has been described by Nobel (1967, 1969).

Sorokin and Sommer (1929, 1940) showed that severe lack of calcium in pea roots caused failure of normal mitosis, leading to incomplete separation of chromosomes, spindle abnormality, aggregation of chromatin, failure to produce the cell plate for new cell wall formation thus producing binucleate cells. Differentiation was suppressed but vacuolation was enhanced. As little as 0.06 ppm (1.5 μM) calcium permitted mitotic division but the axis was abnormally orientated so that lateral swelling rather than axial extension occurred. At this concentration of calcium other irregularities in mitosis still persisted in lateral primordia. Calcium deficiency appears to induce fairly consistent and similar cytological and mitotic abnormalities in several species but the concentration at which such effects occur differ considerably between species.

Brewbacker and Kwack (1963) found that calcium was indispensable

for the germination of pollen and growth of the pollen tube in plants from numerous families. They attributed the importance of calcium to its involvement in cell wall synthesis but a role in synthesis of the plasmalemma is equally plausable.

A long established view (Mangin, 1892; Hansteen, 1910) is that calcium in plants combines with pectic acid to form pectate bridges with non-methylated carboxyl groups in the middle lamella region of the cell wall. The observation by Bennett-Clark (1955) that EDTA promotes cell extension, as does ammonium acetate, is consistent with the view that calcium pectate limits the plasticity of the cell wall. In addition it was suggested that a $Ca^{2+}$-stimulated lecithinase system (phospholipase A, E.C. 3.1.1.4) may be involved in ion uptake and transport (Bennett-Clark, 1955; Hewitt and Smith, 1975).

Adamson (1962) showed that the processes of cell division and expansion in Jerusalem artichoke tubers could be controlled relative to each other by calcium ion concentration whereas magnesium was much less effective. It was considered that the formation of calcium-uronic acid salts, making the cell wall less plastic, best explained the observations.

Bishop *et al.* (1958) investigated the chemical constitution of the primary cell walls of *Avena* coleoptile. Kohn and Luknár (1977) have described calcium binding to membrane constituent polyuronates, polygalacturonate and polyguluronate. Bonner *et al.* (1973) reported that divalent cations protect the outer mitochondrial membranes from rupture whereas chelators such as EDTA and EGTA weaken the mitochondrial membranes. Calcium is commonly the major cation of the middle lamella of cell walls, of which calcium pectate is a principle constituent. Calcium salts therefore contribute a component of mechanical strength

to plant tissues (Tagawa and Bonner, 1957; Cleland, 1960; Ito and Fugiwara, 1967; Rasmussen, 1967). In algae, other calcium and magnesium polysaccharides are important compounds of the cell wall. In some plants, including algae (Johnson, 1961; Lewin, 1962) calcium salts occur as crystals; calcium oxalate and carbonate are common (Pobeguin, 1954) while calcium phosphate and sulphate are occasionally found. In tobacco rattle virus particles, Robinson and Raschké (1977) have suggested a stabilising role for divalent metal ions following virus inactivation due to EDTA treatment, while Ingebretson and Sanner (1976) have reported the activation of NADP-specific isocitrate dehydrogenase by chelating agents.

Calcium thus appears to have many functions in plant cells, having been implicated in the formation of the middle lamella of cell walls, in the integrity of plasmalemma and organelle membranes, nuclear substructure and as a base equivalent for inorganic and organic anions of the cell.

### 1.6.2 Mobility of calcium in the plant

Biddulph *et al.* (1958) reported that calcium did not recirculate following its initial delivery via the transpiration stream. However, Biddulph *et al.* (1959) concluded that foliar applied calcium could be exported from the leaf via the phloem in the ratio of 1 Ca : 100 P but that the flow was very small.

Bell and Biddulph (1963) reported that if calcium translocation were due to mass flow, one would expect various tissues to acquire nutrient ions in proportion to their transpirational rates, but observed that various tissues acquire nutrient ions in proportion to their metabolic needs. They obtained data which supported the view that calcium translocation was by a process of exchange, in controversy with the

classical concept of mass flow.

Millikan and Hanger (1965) investigated the mobility of foliar applied $^{45}Ca$ in various beans by injection or foliar application. Calcium mobility depended on (i) calcium status, (ii) inclusion of certain cations or chelating agents and (iii) total calcium dose. If the total amount of calcium was small, little calcium translocation was detected. If excess protons or chelating agents (EDTA, citric acid) or both were added with the calcium, then calcium was transported out in an acropetal direction. When EDTA alone was added toxic symptoms were noted in broad beans and stocks, and the extent of leaf damage indicated that EDTA was mobile in the plant in the transpiration stream. It was suggested that the toxic effects were due to the chelation of essential metal ion activators. It is thought that calcium in leaf tissue is fixed to negatively charged sites of absorption. When excess protons are present with calcium, competition for sites occurs, with protons being bound leaving calcium free for translocation. Their observations confirm those of Biddulph *et al.* (1959) who concluded that immobility in bean leaves of foliar applied calcium was not due to blockage in conductive tissue but to the tissue having a 'high fixation power' which left no calcium free to spill over into the phloem stream.

Biddulph (1967) reported on the distribution of $^{45}Ca$ in intact bean root, noting that $^{45}Ca$ was particularly associated with cell walls. The endodermis did not appear to be a diffusion barrier. Small parenchyma cells associated with conducting elements acquired a high calcium concentration and therefore appeared to be implicated in absorption and transfer to xylem. The region between 30-80 mm from the root tip appeared to participate in calcium uptake and transfer to xylem. Deposition of $^{45}Ca$ in oxalate crystals represented an almost complete

immobilisation of calcium. The observations were in accord with the suggestion that the first phase of calcium uptake is an exchange process, exchange sites being in the cell wall (Bell and Biddulph, 1963). and, according to Jansen *et al.* (1960), the amount of pectic substances in the cell walls of living tissues can qualitatively account for the total exchange capacity of the cell. The data suggest that excess calcium ions which could not be accommodated by exchange sites in the actively growing root are carried in the transpiration stream to sites in the growing stem and leaves, agreeing with data of Bell and Biddulph (1963).

In trees, prior to leaf abscission, calcium is one of the elements not withdrawn from the leaf and is therefore subsequently lost to the plant.

### 1.6.3 Calcium levels in biological tissues

Calcium and magnesium are the two most common divalent cations found in biological material (Epstein, 1972). Common calcium salts found in plant tissues include calcium pectate, phytate, carbonate and oxalate. The presence of large amounts of insoluble calcium salts of organic acids in many plants suggests that calcium may have a role in regulating the acidity of cell sap. On the other hand, acid production could be specifically to precipitate surplus calcium ions.

Azzi and Chance (1969) reported that isolated rat-liver mitochondria contain significant amounts of bound calcium and magnesium even if isolated in media containing EDTA or EGTA. They found that the nanomolar concentration of mitochondrial calcium is in a highly dynamic state using aequorin, a bioluminescent jellyfish protein which measures free calcium. In the absence of chelators, the calcium and magnesium content of rat-lung mitochondria was 213 and 102 nmol/mg

protein respectively; use of EDTA in the isolation medium reduced the calcium and magnesium content to 28 and 23 nmol/mg protein respectively while rat-liver mitochondria were found by a similar method to have 7 nmol calcium/mg protein (Fisher *et al.*, 1973).

The free calcium concentration of the matrix space is estimated to be about 100 μM (Carafoli *et al.*, 1977). On the basis of the Nernst equation, assuming a membrane potential of 180 mV, the concentration of free calcium in the cytosol would reach levels as low as 0.1 nM if electrophoretic systems of calcium uptake actually reached equilibrium (Rottenberg and Scarpa, 1974). However this would be incompatible with cytosol metabolism and is negated by indirect estimates of free calcium concentration in the matrix and cytosol (based on assays of calcium-regulated enzymes) and therefore it is evident that efficient return of calcium to the cytosol from the matrix must occur (Carafoli *et al.*, 1977).

Other estimates suggest that the free calcium concentration of the matrix space could be about 10 μM (Denton, 1977); discrepancies such as this of one order of magnitude are attributed to assumptions made in calculations (Carafoli *et al.*, 1977; Denton, 1977).

Chen and Lehninger (1973) found that the endogenous calcium content of isolated plant mitochondria varied widely. They used 5 mM EDTA in the isolation medium (except in the case of seedlings, when 0.5 mM EDTA was used) and subsequently treated the mitochondria twice with chelator-free wash medium, so that the calcium remaining in these mitochondria represents tightly bound calcium not removed by a single exposure to EDTA. Mitochondria from roots such as sweet potato, beet, carrot and turnip were found to have 0.43, 1.58, 2.0 and 4.53 nmol calcium/mg protein respectively. Mitochondria from stems such as

white potato and yellow onion were found to have 0.1 and 0.58 nmol calcium/mg protein respectively; cabbage leaf mitochondria were found to have 0.2 nmol calcium/mg protein; artichoke bud mitochondria had 0.53 nmol calcium/mg protein and mitochondria from seedlings of corn and mung bean were found to have 0.02 and 0.05 nmol calcium/mg protein respectively.

The amount of $^{45}Ca^{2+}$ which binds to corn mitochondria in the presence and absence of EGTA has been investigated (Earnshaw, 1975); depending on KCl concentration, 15-25 nmol $Ca^{2+}$/mg protein was bound externally (Earnshaw, 1975) while $^{45}Ca^{2+}$ bound in the presence of EGTA was assumed to be inside the inner membrane, which EDTA cannot cross (Reed and Bygrave, 1974).

Oursel *et al.* (1973) made some observations regarding lipids and passive calcium fixation. They reported that in *Lupinus luteus*, a calcifuge species (intolerant of calcium), 8% of total lipids were phosphatidic acid; other acidic phospholipids account for another 30% and in general, Lupinus lipids are rich in linolenic acid, whereas in *Vicia faba*, a calcicole species (tolerant of calcium) only 2% of total lipids are phosphatidic acid, only 20% of remaining phospholipids are acidic and Vicia lipids are richer in linolenic acid. When a species is intolerant of calcium, they suggested that there is a relationship between the amount of phospholipids in the membrane and the passive fixation of calcium and that lipids are present which are capable of passively fixing calcium and taking it out of free solution. Dupont (1976) has reported a form of calcium and phosphate accumulation localised in the lipid phase of cauliflower mitochondria which did not require a source of energy.

Bonner *et al.* (1973) have reported that divalent cations protect

the outer mitochondrial membrane from rupture, whereas chelators such as EDTA and EGTA weaken the mitochondrial membranes. There is a correlation between the uronic acid content of plant outer mitochondrial membranes and osmotic stability of the outer membranes (Mannella and Bonner, 1975). These results implicated calcium in the mode of binding the negatively charged polysaccharides to these membranes and suggested that the observed increased fragility of the outer membranes following EDTA treatment was due to the removal of divalent cations (calcium) from the membranes. They found that up to 10% of the total membrane mass was made up by carbohydrates while a negligible carbohydrate content for liver outer mitochondrial membranes was reported by Parsons *et al.* (1967). Mannella and Bonner (1975) also discussed the possibility that the weakening effect which EDTA exerts on plant outer membranes might involve interactions between divalent cations with lipid and/or protein moieties. Calcium is known to have a condensing or rigidifying effect on lipid mono- or bilayers, such effects being most pronounced with a preponderance of negatively charged lipids such as phosphatidic acid. Since overnight dialysis of outer membranes against 5 mM EDTA did not significantly decrease the protein content of these membranes it was considered unlikely that EDTA affected the binding of outer membrane protein.

Calcium levels from various tissues are collated in table 11 (p 119) along with calcium determinations for Jerusalem artichoke mitochondria made in this thesis.

### 1.6.4 Cations, mitochondria and ion uptake

The main interest of this thesis is the role which calcium may play in regulating the activity of exogenous NADH dehydrogenase. Experimentally, the use of FCCP to dissipate the protonmotive force,

$\Delta p$, has abolished any action which calcium or any other cation used in this study may have in energy-linked ion transport processes. However a brief description of some additional actions of calcium and other cations in plant mitochondria is relevant here.

For example, there is non-specific binding to negatively-charged membrane sites of both low (Kd = 600 μM) and high (Kd = 50 μM) affinity (Earnshaw, 1978; Earnshaw and Cooke, 1981); low affinity sites may be concerned with contraction of mitochondrial membranes, perhaps representing conformational changes (Earnshaw, 1978).

Cations have long been implicated in a structural role, as evidenced by chelator-induced damage to outer membranes, which is reduced by divalent cations (Marinos, 1962; Stoner and Hanson, 1966; Bonner *et al.*, 1973; Mannella and Bonner, 1975; Hanson and Day, 1980).

1.6.4.1 Calcium and mitochondrial ion uptake

The electrochemical gradient resulting from electron transport may be utilised for energy-requiring ion transport processes in addition to the synthesis of ATP.

Early experiments showed that under various conditions plants do indeed accumulate ions (Robertson *et al.*, 1955; Honda and Robertson, 1956; Kahn and Hanson, 1959) and ion uptake was demonstrated in mitochondria (Shean and Levitt, 1959). Beetroot mitochondria showing respiratory control with ADP accumulated magnesium in the presence of phosphate during substrate oxidation (Millard *et al.*, 1964, 1965) and corn mitochondria showing respiratory control with ADP accumulated calcium and magnesium in the presence of phosphate during either substrate oxidation or ATP hydrolysis (in the presence of magnesium, Hanson *et al.*, 1965; Hodges and Hanson, 1965); substrate supported ion transport in both cases was oligomycin-insensitive.

Electron transport in intact mitochondria is a process resulting in proton extrusion across the inner membrane, thus causing the matrix to become negative with respect to the external environment. The inner membrane is considered to be impermeable to the backflow of the extruded protons except in a controlled fashion through enzyme systems or in association with lipid soluble proton-conducting uncouplers (Mitchell, 1966).

Proton extrusion is electrogenic and produces an electrochemical gradient of protons or protonmotive force ($\Delta p$). Expressed in millivolts, $\Delta p = \Delta\psi - Z\Delta pH$, where $\Delta\psi$ is the electrical potential difference across the membrane (also called membrane potential) and $-Z\Delta pH$ is the chemical potential difference ($Z = 2.3RT/F$) (Mitchell, 1966). Liver mitochondria in state 4 respiration (limited by phosphate acceptor) have $\Delta p$ values of 228 mV, with $\Delta\psi$ contributing about 2/3 depending on ion species in the medium (Nicholls, 1974). Plant mitochondria show $\Delta p$ values in the range of 150-160 mV, with a $\Delta\psi$ contributing 75-80% (Moore *et al.*, 1978). Blocking the respiratory chain with antimycin A causes a drop in $\Delta p$ and a complete reversal of the pH gradient when succinate is the substrate, but not with malate. Stability with malate is attributed to electron transport through complex 1 and the alternative oxidase (Moore *et al.*, 1978).

The electrochemical gradient of protons or protonmotive force ($\Delta p$) can be coupled to chemiosmotic work in a number of ways.
(1) Backflow of protons through the coupling ATPase to form ATP; this is a reversible process, and hydrolysis of ATP can drive $H^+$ efflux.
(2) Influx or efflux of salts can be coupled to $\Delta p$ through carrier proteins or 'porters'. For salt influx, the electrical potential difference, $\Delta\psi$, drives an electrophoretic influx of the cation via a

uniport, while the chemical gradient, $\Delta pH$, carries out a neutral exchange of $OH^-$ for the anion via the antiport. For salt efflux the $H^+$ enters in exchange for the cation, and it is the anion which fluxes down the electrical gradient. The polarisation of the process lies not with the porters, which can function in either direction, but with electrogenic $H^+$ efflux. (3) Protons can be carried in by undissociated weak acids diffusing through lipid domains of the membrane. The pH gradient is thus discharged by acid influx. Acetate salts are rapidly taken up by this mechanism; there is little discrimination as to cation (Wilson *et al.*, 1969).

1.6.4.2 Ion and substrate transport

Guidelines were built up from extensive work done first with mammalian mitochondria; ion transport is at the expense of a high energy intermediate of oxidative phosphorylation (now widely accepted as $\Delta p$, protonmotive force). In addition, it was suggested that in 'energised mitochondria' the contracted state provided a potential for binding a small amount of calcium and for transporting this bound calcium into the matrix in the presence of phosphate (Lehninger, 1962, 1964; Ernster and Lee, 1964; Longo and Arrigoni, 1964; Chance, 1965; Sanadi, 1965; Rasmussen *et al.*, 1965; Chappell and Haarhoff, 1967; Lehninger *et al.*, 1967; Carafoli, 1976; Carafoli and Crompton, 1976; Bygrave, 1977, 1978; Nicholls and Crompton, 1980). There are differences between animal and plant mitochondria, most notable of which is the dramatic uncoupling of vertebrate mitochondria during active binding of small amounts of calcium (Robertson, 1960; Lehninger, 1970) whereas plant mitochondria actively absorb calcium only in connection with (anion) phosphate transport (Hanson and Hodges, 1967; Chen and Lehninger, 1973). In addition, plant mitochondria seem to be more permeable to

salts than vertebrate mitochondria (Hanson and Hodges, 1967). Principles of ion transport in mammalian mitochondria are now confirmed in many respects for plant mitochondria (Hanson and Hodges, 1967; Kenefick and Hanson, 1966a, b; Hanson and Koeppe, 1975; DeSantis *et al.*, 1976; Wiskich, 1977; Hanson and Day, 1980).

During oxidative phosphorylation there is an influx of pyruvate, phosphate, ADP and $O_2$, and efflux of $CO_2$, $H_2O$ and ATP. Fluxes of small neutral molecules, $O_2$, $CO_2$ and $H_2O$ are by diffusion through the inner membrane and their transport is not rate-limiting. But anions, having negative charge, face accumulation against an electrical gradient, and this is by-passed by neutral exchange. The anion generated for exchange is $OH^-$ and the primary exchanges are phosphate/$OH^-$ and pyruvate/$OH^-$. Since pyruvate is rapidly oxidised, it is phosphate transport at the expense of the pH gradient which is fundamental to establishing and maintaining the anion content of the matrix and $\Delta\psi$ (electrical potential difference) (Lehninger, 1974; Wiskich, 1977). Although other anions, such as acetate (Wilson *et al.*, 1969), can be transported *in vitro*, it is phosphate that plays the physiological role.

The $ADP^{3-}_{in}/ATP^{4-}_{out}$ exchange is electrogenic and is driven by $\Delta\psi$ (electrical potential difference) contributing to ATP formation and the high ATP:ADP ratio of the cytosol (Klingenberg, 1970). Hence the cumulative effect of the neutral phosphate/$OH^-$ exchange may be viewed as maintaining an electrical potential favourable to $ADP^{3-}_{in}/ATP^{4-}_{out}$ exchange and ATP formation. The processes are closely integrated; electrogenic ADP/ATP exchange can be conversely considered as compensated by phosphate influx (McGivan *et al.*, 1971).

Salt transport, driven by ATP hydrolysis or respiration, is

often accompanied by alternate swelling and contraction, and this phenomenon is observed in both plant and mammalian mitochondria (Chappell and Crofts, 1965a; Azzi and Azzone, 1967; Rottenberg and Solomon, 1969; Izzard and Tedeschi, 1970, 1973; Massari *et al.*, 1972).

1.6.4.3 Phosphate transport

Phosphate transport has been extensively studied in plant mitochondria (Kenefick and Hanson, 1966b; Truelove and Hanson, 1966; Hanson and Miller, 1967; Wilson *et al.*, 1969). The process is similar to the system in animal mitochondria, inhibited by the hydrophilic sulphydryl reagents, mersalyl and N-ethylmaleimide. In respiring mitochondria the kinetics of phosphate transport show a 2-phase absorption curve, with the first phase half saturated at about 0.25 mM phosphate (Hanson *et al.*, 1972). When respiration is inhibited or $\Delta p$ collapsed with uncoupler, rapid passive efflux of phosphate occurs and this is also inhibited by mersalyl (Hensley and Hanson, 1975; DeSantis *et al.*, 1975, 1976). From these studies it seems that phosphate transport is responsive to the phosphate gradient as well as $\Delta p$ and that high matrix phosphate content (and hence other ion content) is maintained only so long as respiration maintains $\Delta p$.

Arsenate uncoupling involves cyclic arsenate transport (in by the phosphate transporter, out as ADP-As on the AdN transporter) and mersalyl is as effective as oligomycin in blocking the arsenate uncoupling (Bertagnolli and Hanson, 1973).

The exchange studies of DeSantis *et al.* (1976) indicate that sulphate enters by the dicarboxylate transporter in exchange for phosphate or dicarboxylic acids. However, corn mitochondria oxidising NADH in the absence of phosphate or dicarboxylate accumulate potassium sulphate by a process inhibited by mersalyl (Kimpel and Hanson, 1978).

Sulphite exchanges for phosphate with the same inhibitor sensitivity as phosphate/phosphate exchange (DeSantis *et al.*, 1976). However DeSantis *et al.* (1976) suggest that $SO_3/OH^-$ occurs independently of the phosphate transporter.

1.6.4.4 Cation transport

Net accumulation of anions must be charge-compensated by uptake of cations and *in vivo* these are primarily $K^+$ and $Mg^{2+}$. It is now widely accepted in animal mitochondria that univalent cation uptake occurs via an electrophoretic uniport mechanism, while efflux is via a cation/$H^+$ antiporter (Brierley, 1976a, b; Fiskum and Lehninger, 1980). The same conclusion is drawn for plant mitochondria (Hanson and Koeppe, 1975).

For animal mitochondria it is questioned whether the uniport for electrophoretic entry of monovalent cations is a carrier protein, since there is little discrimination between cations during rapid energised flux of acetate or phosphate (Brierley, 1976a). This is also true for plant mitochondria (Wilson *et al.*, 1969). However, observations do not permit discrimination between a simple electrophoretic cation penetration of a hydrophilic pore in the lipid bilayer and an interaction with a broad spectrum cation carrier. The same applies to the electrophoretic efflux of anions during energy-linked salt extrusion.

In studies of respiration-linked potassium phosphate and acetate uptake by plant mitochondria, the uptake of potassium via the uniport appears to be rate limiting since addition of valinomycin or gramicidin (lipid soluble, potassium mobilising ionophores) greatly increases the rate and extent of salt uptake and osmotic swelling (Wilson *et al.*, 1972; Hanson *et al.*, 1972; Kirk and Hanson, 1973).

Unlike the cation uniport, the cation/$H^+$ antiport might be assumed to be a protein, but no specific inhibitor is known and no exchange carrier has been isolated. The evidence for the existence of the antiport is largely based on the energised osmotic shrinkage of salt-loaded mitochondria, generally followed by absorbancy changes. The assumptions here are supported by $K^+$ analyses (Kirk and Hanson, 1973) size changes by Coulter counter and ultrastructural changes (Pomeroy, 1977). It seems that morphology rather than size may be a dominant factor in absorbancy changes (Pomeroy, 1977).

The two cation transport processes, outlined above, may be the means by which mitochondrial volume is controlled *in vivo* (Izzard and Tedeschi 1970; Brierley, 1976b). In corn mitochondria, during steady state osmotic swelling in potassium phosphate there is cyclic salt transport (Hensley and Hanson, 1975).

Valinomycin does not always react to increase the rate and extent of energy linked salt influx. Corn mitochondria oxidising NADH in 5 mM potassium phosphate respond to valinomycin with additional salt uptake and swelling; if 5 mM potassium sulphate is substituted for potassium phosphate the initial rate of swelling is much slower and there is a rapid shrinkage upon the addition of valinomycin (Kimpel and Hanson, 1978). This sulphate response can be mimicked with phosphate if a limited amount of mersalyl is introduced to add a resistance to phosphate transport. It is concluded that the relative resistance of the anion/$OH^-$ and $K^+/H^+$ antiports governs whether there is net influx or efflux of salt (Kimpel and Hanson, 1978).

Divalent cation uptake (principally $Ca^{2+}$) is electrophoretic in mammalian mitochondria (Lehninger, 1974) probably by means of a lanthanum- and ruthenium red-sensitive $Ca^{2+}$ carrier (Carafoli, 1976).

Whether $Ca^{2+}$ is taken up as the cation or as a phosphate complex is not resolved (Moyle and Mitchell, 1977a, b; cf. Reynafarje and Lehninger, 1977). For plant mitochondria, the situation is less clear. In the case of mung bean (Moore and Bonner, 1977) no respiration-linked calcium transport was detected; however, calcium uptake has been reported as linked to oligomycin-insensitive phosphate uptake (Hanson and Hodges, 1967; Chen and Lehninger, 1973; Wilson and Graesser, 1976). Russell and Wilson (1978) have suggested that failure to observe calcium uptake in plant mitochondria (Bonner and Pressman, 1965; Moore and Bonner, 1977) may result if murexide is used, rather than Arsenazo 111, which is very much more sensitive to low calcium concentrations. Using Arsenazo 111, Russell and Wilson (1978) have reported low rates of calcium transport in plant mitochondria (20 nmol/min/mg protein; cf. 480 nmol/min/mg protein in animal mitochondria, Carafoli, 1976) in the presence of a permeant anion, supported by either electron transport or ATP hydrolysis and inhibited by ruthenium red and lanthanides.

Limited calcium transport in maize mitochondria, supported by respiration and ATP hydrolysis, occurs in the absence of phosphate, though for massive accumulation of calcium, phosphate is essential (Elzam and Hodges, 1968).

Strontium and barium are taken up as well as calcium (Miller *et al.*, 1970; Wilson and Minton, 1974) and magnesium uptake occurs in high magnesium concentrations (Millard *et al.*, 1965). Accumulation of strontium and calcium in the presence of phosphate produces electron dense precipitates in the matrix (Ramirez-Mitchell *et al.*, 1973; Peverley *et al.*, 1974). Weinbach and von Brand (1965) isolated and analysed the granules from rat liver mitochondria. Depending on the method of isolation the granules contained about 30-34% calcium,

14-19% phosphate, 3-4% magnesium and 3-4% carbonate on the basis of total residual weight after incineration. The granules as isolated were amorphous but incineration resulted in the loss of nitrogen and partial loss of carbonates and induced crystallization. The crystalline form contained calcium orthophosphate, mostly in the form of hydroxylapatite. The formation of the granules in heart mitochondria (Brierley and Slautterback, 1964) and in rat liver mitochondria (Greenawalt *et al.*, 1964) has been examined by electron microscopy. It seems that the granules, many of them 500 Å in diameter, are predominantly associated with the inner membrane. In plants the Ca/P accumulation ratio is 1.7 (Elzam and Hodges, 1968) and proton release accompanies $CaPO_4$ uptake with a $H^+/Ca^+$ ratio of 0.8, a result attributed to the formation of $CaPO_4$ precipitates (Earnshaw *et al.*, 1973).

In the absence of added phosphate, there is an energised uptake or binding of about 100 nmoles of $Ca^{2+}$/mg protein (Kenefick and Hanson, 1967; Hanson and Miller, 1967). The amount of binding depends on the level of endogenous phosphate (Earnshaw *et al.*, 1973; Day *et al.*, 1978). During calcium binding the endogenous phosphate moves from a readily leached to a leaching-resistant phase, with both the $Ca^{2+}$ and phosphate rapidly released when respiration ceases (Earnshaw *et al.*, 1973; Earnshaw and Hanson, 1973). Unlike the case in mammalian mitochondria, $Ca^{2+}$ is not actively taken up with acetate (Truelove and Hanson, 1966; Day *et al.*, 1978) and there is little or no evidence for high affinity binding sites (Chen and Lehninger, 1973; Day *et al.*, 1978). There is however $H^+$ release during $Ca^{2+}$ binding with an $H^+/Ca^{2+}$ ratio of 0.9 (Earnshaw *et al.*, 1973; Day *et al.*, 1978). It is believed that a calcium phosphate complex accounts for the $Ca^{2+}$ binding and that the complex is the vehicle of $Ca^{2+}$ transport during massive calcium

loading in the presence of phosphate (Wilson and Minton, 1974; Day *et al.*, 1978). Calcium and strontium phosphate uptake is competitive with ATP formation and is most active under state 4 conditions (Hanson and Miller, 1967; Johnson and Wilson, 1973).

### 1.6.5 Calcium activation of enzymes

Calcium is one of fifteen different metal cations found to activate various enzymes. The size of the ion is probably an important factor in enzyme activation, and it is found that most activating cations have similar ionic radii. However these cations are by no means randomly interchangeable; in some cases only one, but more frequently, two or more of these ions are specifically capable of activating a particular enzyme. For example, magnesium is the natural activator of the majority of enzymes which act on phosphorylated substrates (excluding the phosphorylases); in nearly all these cases, magnesium can be replaced by manganese but not usually by any other metal. This ability for manganese to replace magnesium would not be expected on chemical grounds. On the other hand, however, although sodium is chemically similar to potassium, it cannot replace potassium as an activator of pyruvate kinase (E.C. 2.7.1.40), though it can replace potassium in the case of ketohexokinase (E.C. 2.7.1.3). Much information about metal activation of enzymes has been collected by Lehninger (1950), Malmstrom and Rosenberg (1959), Williams (1953, 1970, 1974, 1976) and Dixon and Webb (1964, 1979).

A few enzymes are specifically activated by calcium, mainly lipases or phospholipases (Wills, 1965; Laties, 1974). For example, there are several hydrolases, such as glycerol ester hydrolase, i.e. triacyl glycerol lipase (E.C. 3.1.1.3), found in animal and plant tissues and moulds; phospholipase $A_2$ (E.C. 3.1.1.4), found in venoms

(Dawson, 1963); phosphatidylcholine phosphatidohydrolase, i.e. phospholipase D (E.C. 3.1.4.4), found in plant tissues (Quarles and Dawson, 1969; Galliard, 1974) and also activated by strontium and barium ions less efficiently. However, Clermont and Douce (1970) reported that mitochondria and plastids lack phospholipase activity when isolated from various plant tissues by methods which exclude contamination by other cell organelles.

There is also 1,4-glucan 4-glucanhydrolase, i.e. amylase (E.C. 3.2.1.1), found in animal and plant tissues, e.g. barley (*Hordeum vulgare*) and in bacteria, e.g. *Bacillus subtilis*, in which calcium is believed to be a constituent of the hydrolytic site, while zinc is separately involved in the aggregation of the subunits into the oligomeric form of the protein (Dixon and Webb, 1964; Hewitt and Smith, 1975).

A calcium-stimulated ATPase (apyrase, ATP:diphosphohydrolase, E.C. 3.6.1.5) has been isolated from potato tubers, whereas an ATPase (E.C. 3.6.1.3) from carrot and pea roots, stimulated by any of these monovalent cations, sodium, potassium, rubidium, lithium or ammonium, has a separate requirement for magnesium which may be replaced by calcium, ferrous or manganese ions with varying degrees of effectiveness (Hewitt and Smith, 1975).

Calcium-sensitive ATPase activity has been detected in the microsomal fraction of *Vicia* roots (a calcicole species); however, calcium-sensitive ATPase activity is absent from *Lupinus*, a standard calcifuge species (Monestiez-Lorenzini *et al.*, 1976).

The presence of calcium in plant glutamic acid dehydrogenase ($NAD^+$-linked, E.C. 1.4.1.2) causes the aggregation of the subunits of the enzyme with a consequent change in the substrate Km (Hewitt and Smith, 1975).

Blackwood and Miflin (1976) have described the effects of salts on $NAD^+$-linked malic dehydrogenase in maize and barley. The apparent Km of predominant isoenzymes at given salt concentrations was found to decrease as total malic dehydrogenase activity increased.

In *Lemna major*, the presence of calcium favours the aggregation of the malic dehydrogenase (E.C. 1.1.1.37). Other enzymes of *Lemna*, including $NAD^+$-linked glutamic acid dehydrogenase (E.C. 1.4.1.2), $NADP^+$-linked isocitric dehydrogenase (E.C. 1.1.1.42) and glucose 6-phosphate dehydrogenase (E.C. 1.1.1.49), were also reported to be subject to the effect of calcium ions with respect to their allosteric properties (Jefferies *et al.*, 1969).

Habig and Racusen (1974) reported that some of their observations in the formation of high molecular weight malic dehydrogenase (E.C. 1.1.1.37) indicate the presence of a salt-type or ionic interaction in high molecular weight malic dehydrogenase from bean leaves and speculate upon the possibility that a divalent cation such as calcium may be essential for the formation of high molecular weight malic dehydrogenase.

It has been suggested that the concentration of calcium may participate in the regulation of active (non-phosphorylated) and inactive (phosphorylated) forms of pyruvate dehydrogenase (E.C. 1.2.4.1) (Denton, 1977; Hansford, 1980).

Birnbaum *et al.* (1977) have described the calcium binding site in bovine chymotrypsin A (E.C. 3.4.21.1) where calcium ions stabilise chymotrypsin against autolysis and denaturation. On the other hand however, calcium ions are found to accelerate the formation of active trypsin from trypsinogen.

Cases of ion antagonism in enzyme activation are fairly common.

For instance, a number of magnesium-activated enzymes are inhibited by calcium ions (e.g. 5-nucleotidase, E.C. 3.1.3.5; inorganic pyrophosphatase, E.C. 3.6.1.1; magnesium-stimulated ATPase, E.C. 3.6.1.3), whereas the calcium-activated myosin ATPase (E.C. 3.6.1.3) is inhibited by magnesium ions. The antagonistic effects of magnesium and calcium ions in many of the phosphotransferase reactions are due to their competition to form the active or inactive complex respectively, whether it be metal-nucleotide or metal-enzyme (Kuby and Noltman, 1962; Mildvan and Cohn, 1965, 1966).

In several cases, two ions, usually of different valency, are required simultaneously for activation, though the reason for this is not clear. Activation by two ions simultaneously probably permits particularly sensitive control of enzyme activity (Bygrave, 1967). Frequently in such cases a double ionic antagonism is found, for instance, in the case of pantothenate synthetase (E.C. 6.3.2.1). This enzyme requires either magnesium or manganese ions in addition to either potassium or ammonium ions, whereas calcium or zinc compete with the activating divalent ion and sodium with the activating monovalent cation. Thus activation of this enzyme may be finely controlled by the relative concentrations of several different activating and inhibiting cations.

Ions which usually act as mutual antagonists such as calcium and magnesium do not invariably do so and may act on occasion as alternative activators of the same enzyme. Examples of enzymes which are activated by calcium or magnesium (and usually by one other cation) are aldehyde dehydrogenase (yeast, E.C. 1.2.1.4); oxaloacetate decarboxylase (E.C. 4.1.1.3); pyruvate decarboxylase (E.C. 4.1.1.1); and pantetheine kinase (E.C. 2.7.1.34; Dixon and Webb, 1979).

### 1.6.6 Proposed mechanism for metal activation of enzymes

#### 1.6.6.1 Metal ion may be constituent of active centre

The metal may form an essential part of the active centre of the enzyme. This is the most commonly assumed mechanism of enzyme activation. Kubowitz (1938) demonstrated in the case of the diphenol oxidases (E.C. 1.10.3.1 and 1.10.3.2), that copper ions are an integral part of the active centre of the enzyme. It was possible to remove copper ions from the enzyme thus inducing inactivation and to then re-incorporate copper, inducing specific activation. In this case copper acts as a redox carrier.

#### 1.6.6.2 Metal may bind enzyme and substrate

The metal may act as a binding link between the enzyme and substrate, combining with both and so holding the substrate at the active centre of the enzyme.

There is evidence to suggest that the role of the cation is primarily to form a ternary Michaelis complex with the enzyme and adenine nucleotide in phosphotransferase-catalysed reactions. e.g., Nordlie and Lardy (1962) suggest that this is the primary role of magnesium ions.

Often the metal combines first with the nucleotide to form the 'active' substrate, which then combines with the enzyme to form the ternary complex (Mildvan and Cohn, 1965, 1966).

(There are three possible cases according to the order of combination of metal, substrate and enzyme, related to the affinity of the metal for enzyme and substrate. In the complex of metal and substrate, the metal may merely act as a handle holding the substrate in the required position or it may itself be involved in the activation

of the enzyme.) There is also evidence that metal ions are capable of binding organic molecules to proteins. Klotz and Loh-Ming (1954) studied the influence of metal ions on the binding of azo-dyes by serum albumin and concluded that zinc ions markedly increased such binding in a manner which was strongly pH-dependent. They considered their results could be adequately explained by co-ordination without the necessity of assuming chelation, and regarded the zinc-stimulated binding of azo-dyes to serum albumin as a model for metal activation of enzymes.

1.6.6.3 Alteration of the equilibrium constant of the enzyme reaction

Another way in which metal ions can produce an activation is to alter the equilibrium constant of the enzyme reactivation. Such an apparent activation may take place in two ways. Where the true substrate is the metal complex, addition of the metal will increase the concentration of the metal complex, thus displacing the equilibrium in the desired direction. Alternatively, the metal may complex with the product thus removing it from the equilibrium. An example of this latter type of action is the general accelerating effect of calcium on the activity of lipases (e.g. E.C. 3.1.1.3), which is probably due to the formation of complexes or even gross product precipitation.

Metal ions may indirectly accelerate enzyme reactions by complexing with an inhibitor, or by the displacement of an ineffective metal ion from combination with the active centre of the enzyme or with the functional group of the substrate. Furthermore, a metal which does not itself activate the enzyme may produce an activation by displacing the true activating metal, already present in the enzyme preparation, from combination with groups other than the active site.

This may explain why partially purified arginase (E.C. 3.5.3.1) is activated by several ions, while the highly purified enzyme is activated only by manganese ions (Dixon and Webb, 1964).

# 2. MATERIALS AND METHODS

## 2.1 Plant material

Jerusalem artichoke tubers were obtained from the University of London Botanical Supply Unit in batches of about 50 kg in autumn through to early spring. The tubers were freed of surface soil and stored in plastic bags lined with adsorbent bench paper at 0-4°C in a cold room. Occasionally potatoes from the local market were obtained and treated as described for Jerusalem artichokes.

## 2.2 Chemicals and enzymes

Sucrose, succinic acid, $MgCl_2$, $CaCl_2$, $MgSO_4$, $K_3Fe(CN)_6$, KCN, KCl, $Na_2S_2O_4$, sodium citrate, NaCl, $Na_2CO_3$, NaOH, EDTA, HEDTA, calcichrome, fluorescein complexone, solochrome, murexide, Folin and Ciocalteu's phenol reagent and DMSO were obtained from BDH Chemicals, Poole, Dorset, U.K.

BSA, TPP, oligomycin, potassium glutamate, oxaloacetate, malate, pyruvate, sodium deoxycholate, EGTA, (DM)$Br_2$ and L-lysyl L-lysine.2HCl were obtained from Sigma London Chemical Co., Kingston-upon-Thames, Surrey, U.K.

$KH_2PO_4$, $Na_2S_2O_5$, $MnCl_2$, $LaCl_3$, DHPE, DPTA, HSN, TES and MOPS buffers were obtained from Hopkin and Williams, Ltd., Chadwell Heath, Essex, U.K.,

Enzymes, NADH, ADP, ATP and cytochrome c were obtained from the Boehringer Corporation (London) Ltd., Lewes, Sussex, U.K.

Antimycin A was obtained from Calbiochem Ltd., Bishops Stortford, Herts., U.K.

(TEC)$Cl_3$ was obtained from Alfa Products, Danvers, MA., USA (U.K. distributors Lancaster Synthesis Ltd., Lancaster).

SHAM was obtained from Aldrich Chemical Co. Ltd., Gillingham, Dorset, U.K.

9AA was obtained from Kock-Light Laboratories, Colnbrook, Bucks., U.K.

FCCP and piericidin A were generous gifts from Dr P.G. Heytler (Du Pont) and Professor T.P. Singer respectively.

A23187 was a generous gift from Lilly Research Centre Ltd., Erl Wood Manor, Windlesham, Surrey GU20 6PH, U.K.

The substrates were obtained as acids or as monosodium salts. For use in the studies they were dissolved in distilled water to make 1 M stock solutions, the pH of which were adjusted to 7.2 with KOH solution when necessary. FCCP and piericidin A were dissolved in absolute ethanol. A23187 was dissolved in DMSO.

Abbreviations are listed on p. 11.

## 2.3 Media used in the studies

### 2.3.1 Isolation media

Isolation media are presented in table 1 for mitochondria from Jerusalem artichoke tubers and white potato tubers. KOH solution was used to adjust the pH of the medium in each case.

### 2.3.2 Wash media

Wash media are presented in table 2.

### 2.3.3 Reaction media

Reaction media are presented in table 2, with the exception of the reaction medium for the assay of succinate-cytochrome c oxido-reductase, which is presented below.

Table 1 Isolation media

| Component | standard isolation medium (2.3.1.1) | EDTA-free isolation medium (2.3.1.2) | + EGTA isolation medium (2.3.1.3) | low salt isolation medium (2.3.1.4) |
|---|---|---|---|---|
| Sucrose, M | 0.5 | 0.5 | 0.5 | 0.5 |
| EDTA, mM[a,b] | 5.0 | - | - | - |
| EGTA, mM[b,c] | - | - | 5.0 | - |
| MOPS, mM | 10.0 | 20.0 | 10.0 | - |
| TES, mM[d,e] | - | - | - | 5.0 |
| BSA, % (w/v)[d,e,f,g] | 0.1 | 0.1 | 0.1 | 0.1 |
| $Na_2S_2O_5$, mM[a,h] | 2.0 | 2.0 | 2.0 | 2.0 |
| pH[j] | 7.8 | 7.8 | 7.8 | 7.8 |

(a) Tager, 1954

(b) Sillén and Martell, 1964; 1971

(c) Owen, 1976

(d) Good *et al.*, 1966

(e) Hobson, 1970

(f) Stinson and Spencer, 1968

(g) Schew *et al.*, 1974

(h) Stokes *et al.*, 1968

(j) Longo and Arrigoni, 1964.

KOH was used to adjust the pH of each medium

Table 2 Wash media and reaction media

| Component | Wash medium | | | | |
|---|---|---|---|---|---|
| | standard (2.3.2.1) | + EGTA (2.3.2.2) | | | low salt (2.3.2.3) |
| Sucrose, M | 0.4 | 0.4 | | | 0.4 |
| TES, mM | 10.0 | 10.0 | | | 2.0 |
| EGTA, mM | - | 2.0 | | | - |
| BSA, % (w/v) | 0.1 | 0.1 | | | 0.1 |
| pH | 7.2 | 7.2 | | | 7.2 |
| | Reaction medium | | | | |
| | standard (2.3.3.1) | + EGTA (2.3.3.2) | phosphate-free (2.3.3.3) | $Mg^{2+}$-free (2.3.3.4) | low salt (2.3.3.5) |
| Sucrose, M | 0.3[(a)] | 0.3 | 0.3 | 0.3 | 0.3 |
| $KH_2PO_4$, mM | 5.0 | 5.0 | - | 5.0 | - |
| TES, mM | 5.0 | 5.0 | 5.0 | 5.0 | 2.0 |
| $MgCl_2$, mM | 2.5 | 2.5 | 2.5 | - | - |
| EGTA, mM | - | 1.0 | - | - | - |
| pH | 7.2 | 7.2 | 7.2 | 7.2 | 7.2 |

(a) reduction of sucrose concentration improves quality and yield of mitochondria, Baker *et al.*, 1968.

KOH was used to adjust the pH of each medium

2.3.3.6 Reaction medium for assay of succinate-cytochrome c oxidoreductase

| | |
|---|---|
| Sucrose, M | 0.0-0.3 |
| $MgCl_2$, mM | 5.0 |
| KCl, mM | 10.0 |
| $KH_2PO_4$, mM | 10.0 |
| cytochrome c, μM | 50.0 |
| KCN, mM | 1.0 |
| ATP, μM | 200.0 |

## 2.4 Isolation of mitochondria from Jerusalem artichoke tubers

### 2.4.1 Typical small preparation using standard media

The temperature of centrifugation and of the solutions used during the isolation procedure was maintained at 0-4°C. This method was based on the method of Palmer and Kirk (1974). Peeled Jerusalem artichoke tubers (200 g) were finely grated in 300 ml standard isolation medium. Standard isolation medium (table 1) contains EDTA (Lund *et al.*, 1958; Knocke and Horner, 1970), sodium metabisulphite, a mild reducing agent in aqueous solution which helps protect mitochondria from damage by phenolic compounds released during tissue disruption (Stokes *et al.*, 1968) and BSA, which protects the mitochondria from the deleterious effects of free fatty acids released during the isolation procedure (Schew *et al.*, 1974). The suspension was filtered through two layers of muslin and the filtrate was centrifuged at 20,000 rpm for 2 min in a Sorvall RC-2B refrigerated centrifuge using the SS-34 rotor (8 x 50 ml capacity, 48,000 *g* at 20,000 rpm). The supernatant was carefully poured off and the pellets were gently suspended using a glass rod followed by gentle homogenization in a

10 ml glass homogenizer with a teflon pestle. The suspension was then diluted to 100 ml with standard wash medium (table 2) (which lacked EDTA, Lieberman and Biale, 1955). Large particles were removed by accelerating the centrifuge rotor from 0-10,000 rpm (12,000 *g*; 20 sec) and then bringing it to rest (3 min). The supernatant was carefully decanted and further centrifuged at 20,000 rpm (48,000 *g*) for 2 min to precipitate the mitochondria. The final mitochondrial pellets were resuspended and brought to 1 ml with the wash medium, giving a final protein concentration of about 20-25 mg/ml.

### 2.4.2 EDTA-free preparation

For some experiments it was necessary to prepare mitochondria in the absence of EDTA (or any chelator). The same general procedure was followed as outlined for the typical small preparation, with the substitution of an EDTA-free isolation medium, in which the concentration of buffer used was twice that used in standard isolation medium, to compensate for the loss of the buffering capacity of the chelator (table 1). The mitochondria were then washed once and suspended using standard wash medium as described before.

### 2.4.3 Preparation of mitochondria in the presence of EGTA

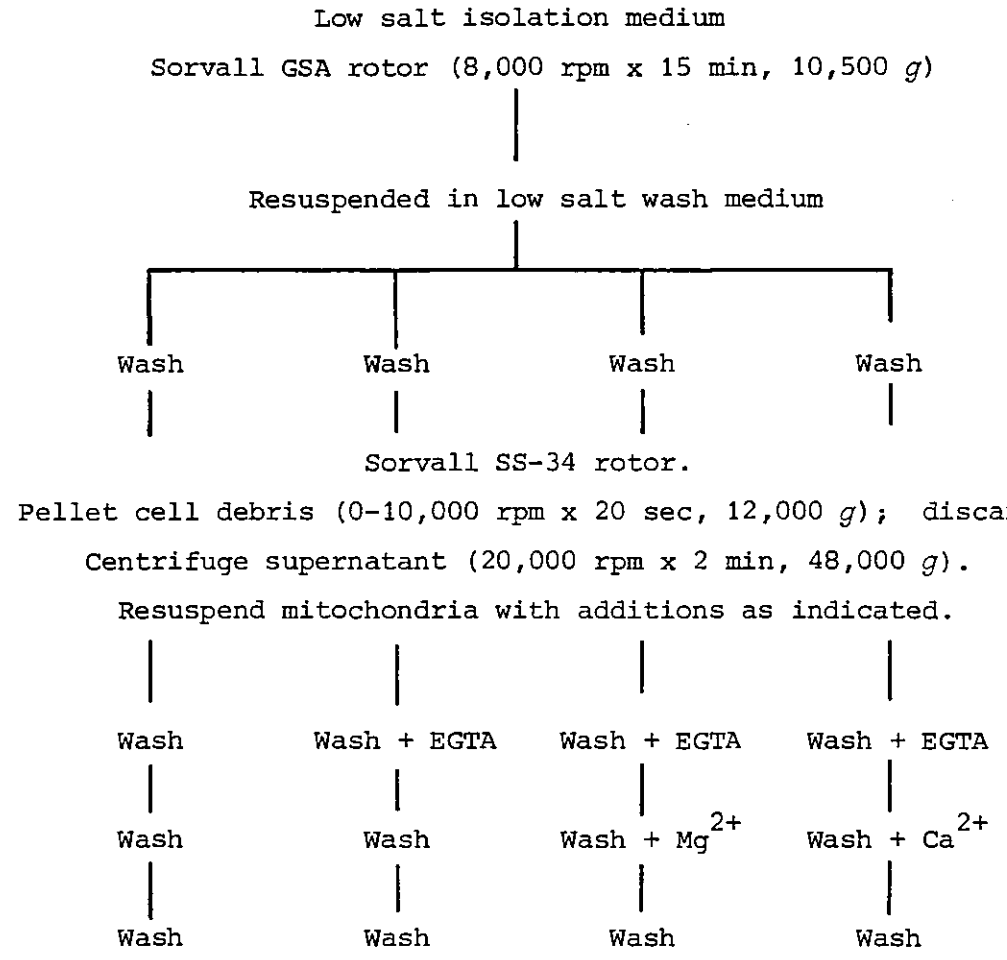
For some experiments it was necessary to prepare mitochondria in the presence of EGTA. The same general procedure was followed as outlined for the typical small preparation, with the substitution of EGTA-containing media at all stages of the preparation through to the final suspension (tables 1 and 2). Occasionally the mitochondria were given a final wash in and suspended in standard wash medium so that the effects of isolation in the presence of EGTA upon the oxidation of exogenous NADH could be studied in these mitochondria separately

from the effects of suspension in a medium containing EGTA.

### 2.4.4 Low salt preparation

A low salt preparation was made following the same general procedure for a typical small preparation but using media specially designed to contain as few added salts as possible without causing the mitochondria to be osmotically stressed. Buffer was added at low levels (2 mM) which was found to be sufficient for the oxidation of NADH in the presence of FCCP in a manner similar to that observed in the presence of buffer at concentrations normally used (5 mM). Therefore low salt isolation medium (table 1, i.e., containing no chelator and only half the usual concentration of buffer, but using TES buffer rather than MOPS or any other, Good *et al.*, 1966; Hobson, 1969) was used in the isolation of low salt mitochondria, which were then washed using low salt wash medium (table 2, i.e., containing half the usual concentration of buffer). Finally the mitochondria were suspended in approximately 1 ml of low salt wash medium, final concentration 15-18 mg/ml protein.

2.4.5 Flow diagram of a typical washing treatment




(N.B. Wash = wash with low salt wash medium, table 2; EGTA, 1 mM, $Mg^{2+}$ and $Ca^{2+}$, 2.5 mM.)

2.4.6 Large-scale preparation of Jerusalem artichoke mitochondria using standard media

For some experiments it was necessary to use 50-80 mg mitochondrial protein so a modified, large-scale procedure was developed. This procedure was also used to provide sufficient mitochondria for the preparation of submitochondrial particles.

Peeled tubers (1 kg) were cut into 1 cm cubes and placed in 1 litre of ice-cold standard isolation medium (table 1) and disinte-

grated with a Moulinex Robot Marinette salad maker to a slurry (about 50 sec). The homogenate was filtered through 2 layers of muslin and centrifuged for 15 min at 8,000 rpm (10,000 *g*) using the Sorvall GSA rotor. Each pellet was resuspended in 10 ml wash medium (table 2), homogenized gently using a small glass homogenizer with teflon pestel and diluted to 50 ml with standard washing medium. Cell debris and large particles were removed from the suspension by centrifugation in the SS-34 rotor as described earlier for small-scale preparations. The supernatant was centrifuged at 20,000 rpm for 2 min (48,000 *g*) and the washed mitochondria thus collected were resuspended in a standard wash medium to about 5 ml, giving a final protein concentration of about 18 mg/ml.

When a large-scale preparation of mitochondria using low salt media, or media containing EDTA or EGTA, was desired, the above procedure was followed substituting the appropriate media and including an extra washing step when appropriate.

## 2.5 Assays and analytical procedures

### 2.5.1 Determination of protein concentration

Mitochondrial protein concentration was determined by the method of Lowry *et al.* (1951) after first solubilising the protein with deoxycholate. Sodium deoxycholate (0.4 ml of 10% w/v solution) was added to a known volume of mitochondrial suspension (3-7 µl containing not more than 160 µg protein) in a test-tube. This was made up to 0.5 ml using distilled water. 5 ml of 2% $Na_2CO_3$ in 0.1 N NaOH solution was added, followed by 0.1 ml of an equal mixture of 1% $CuSO_4$ and 2% Na/K tartrate (freshly mixed). The contents of the test-tube were mixed thoroughly and left standing at room temperature for 10 min.

0.5 ml of diluted Folin and Ciocalteu's phenol reagent (1 part phenol reagent:2 parts distilled water) was added to the contents of the test-tube with vigorous mixing. After 30 min the absorbance of the coloured solution was read at 600 nm in a Unicam 500 spectrophotometer (Pye Unicam Ltd., Cambridge, U.K.), using the wash/suspending medium, treated in the same way, as the blank. The protein content in the test-tube could then be determined from a calibration curve prepared by using bovine serum albumin (0-180 μg) with the same batch of reagents, thus the concentration of mitochondrial protein in the original suspension could be calculated.

2.5.2 Polarographic assay of oxygen consumption

Polarographic assay of oxygen consumption was basically as described by Chappell (1961) and Hagihara (1961). A Rank oxygen electrode (10 mm internal diameter reaction chamber) (Rank Bros., Bottisham, Cambridge) was connected via a control box to a Servoscribe potentiometric recorder (type R.E.520.20). A teflon membrane (Yellow Springs Instrument Co., Ohio, USA) held in position by a rubber O-ring and permeable to molecular oxygen was used to cover saturated KCl solution which bathed the electrode surfaces. The current was proportional to the concentration of oxygen in the reaction medium. Rapid mixing of reagents was achieved by use of a magnetic stirrer. The reaction chamber had a capacity of 2 ml although the experiments were generally carried out using a final volume of 1 ml. The reaction chamber was surrounded by a water jacket which was maintained at a temperature of 25$^{o}$C by a thermostatic water heater and pump (Julabo Paratherm, Scienco Western, Wiltshire). A tightly fitting stopper, with an unstirred capillary through which reactants were introduced from

hypodermic syringes, prevented oxygen from dissolving into the reaction medium (table 2) from the air. The concentration of oxygen in air-saturated assay medium had previously been determined for this electrode to be 240 μM (Truesdale *et al.*, 1955; Cowley, 1977) and this value is used throughout the present study. The rates of oxygen consumption (nmol $O_2$/min/mg mitochondrial protein) were calculated from the oxygen electrode traces according to the method of Chance and Williams (1955).

At the beginning of each experiment, the oxygen electrode was calibrated at the polarizing voltage of 0.7 V and input for the recorder of 10 mV. The recorder pen was set at the 100% level on the chart paper when the reaction chamber contained only reaction medium. The zero-oxygen level was found by adding a few crystals of dithionite ($Na_2S_2O_4$). The zero-oxygen calibration knob was adjusted so that the pen was set at 0% on the chart paper and matched the zero current position.

### 2.5.3 Activation of succinate dehydrogenase

In isolated mitochondria, succinate dehydrogenase is often found to be inactive (Wiskich and Bonner, 1963; Oestreicher *et al.*, 1973). Mitochondrial succinate dehydrogenase was activated following the method described by Sotthibandhu (1977) and Cowley (1977). Mitochondria were incubated at 26°C for 4 min in 5 ml activating medium (0.4 M sucrose; ATP and $KH_2PO_4$, 1 μmol/mg protein; 5 mM $MgCl_2$). Following this treatment the mitochondria were kept in an ice bath; mitochondrial succinate dehydrogenase remained active for at least three hours.

2.5.4 Assay of succinate-cytochrome c oxidoreductase

The activity of succinate-cytochrome c oxidoreductase (E.C. 1.3.99.1) was assayed by the method of Palmer and Kirk (1974). The activity of succinate-cytochrome c oxidoreductase was measured in an Aminco DW-2 dual wavelength spectrophotometer at 550 nm with the reference wavelength at 540 nm. The assays were done in 3.0 ml portions of succinate-cytochrome c oxidoreductase medium (section 2.3.3.6) containing varying amounts of sucrose (0.0-0.3 M). The reaction was initiated by the addition of 10 mM succinate.

The activity of succinate-cytochrome c oxidoreductase is extremely low in small preparations of Jerusalem artichoke mitochondria isolated using standard media (tables 1 and 2) when assayed in 0.3 M sucrose. The activity of succinate-cytochrome c oxidoreductase may be used as a means of assessing the degree of integrity of plant mitochondria. It is agreed that low values of activity are associated with a high degree of integrity, whereas high values are associated with increased membrane permeability. There is controversy as to how increased membrane permeability leads to greater succinate-cytochrome c activity. Douce *et al.* (1972a) claim that the outer membrane of mung bean mitochondria is impermeable to cytochrome c and state that the activity of the succinate-cytochrome c oxidoreductase is limited by the permeability of the outer membrane. Palmer and Kirk (1974) suggest that it may not be possible to differentiate between this situation and one in which the extent of an interaction between exogenous cytochrome c and the electron carriers in the inner mitochondrial membrane is determined by the osmolarity of the suspending medium.

2.5.5 Estimation of total mitochondrial calcium using atomic absorption spectrophotometry

Total mitochondrial calcium was estimated using the method of acid extraction (see Carafoli and Lehninger, 1971) followed by atomic absorption spectrophotometry (AAS).

Mitochondria were prepared using the low salt isolation method (section 2.4.4) on a large scale (section 2.4.6). The preparation was divided into 4 aliquots, 3 of which underwent washing treatments, the 4th being retained as control. A flow diagram of a typical washing treatment is presented in section 2.4.5. After the final centrifugation, each sample was suspended in 0.5 N HCl, made up with deionized water containing 0.1% lanthanum chloride (to overcome suppression of the calcium absorption by phosphate ions) and placed in a boiling water bath for 20 min, then chilled in an ice bath. Each sample was then centrifuged (40,000 *g* x 2 min), the supernatant being retained for AAS and the pellet discarded.

Standards were prepared using $CaCl_2$ and deionized water such that the final concentration of buffer, HCl and lanthanum was the same as found in the test samples. Standard solutions covered the range of 0-10 ppm calcium, with 10 ppm $Ca^{2+}$ yielding a transmission reading of 51-51.5.

Before reading samples and standards the atomic absorption spectrophotometer, a Pye Unicam SP90 (Pye Unicam Ltd., York St., Cambridge, England) was zeroed using deionized water, wavelength setting 422.7 nm, slit width 0.08 mn, air/acetylene flame.

### 2.5.6 9AA fluorescence

9AA (9-aminoacridine, a fluorescent probe) is a weak amine with a pK of 10.0 (Schuldiner *et al.*, 1972). In an aqueous solution it shows a fluorescence emission maximum around 460 nm when excited at 398 nm (Searle *et al.*, 1977). As the concentration of 9AA in solution is raised above 0.1 mM a decrease in fluorescence yield (fluorescence at 456 nm/absorbance at 398 nm) is observed and at 0.6 mM the yield is reduced to 10% of that measured in the 0-100 μM range (Searle *et al.*, 1977).

#### 2.5.6.1 Use of 9AA to measure pH across biological membranes

9AA has been used extensively to measure ΔpH across biological membranes, e.g., in chloroplasts (Schuldiner *et al.*, 1972) and in smps with reversed polarity (Bashford and Thayer, 1977). In these investigations the fluorescence quenching of 9AA is used to measure the accumulation of 9AA in the low pH compartment. The pH difference across the membrane is then calculated on the basis of three assumptions, which are: (1) fluorescence quenching is complete in the acidic (low pH) compartment, i.e., the observed fluorescence is due only to 9AA in the bulk of the solution; (2) 9AA crosses the membrane in the uncharged form only; (3) 9AA is a univalent cation in both compartments (Schuldiner *et al.*, 1972; Haraux and de Kouchkovsky, 1980).

(N.B. when 9AA is used to measure ΔpH, high concentrations of cations are usually present (Schuldiner *et al.*, 1972; Bashford and Thayer, 1977). Under these conditions, very little 9AA will go into the diffuse layer.)

2.5.6.2 Use of 9AA to monitor surface potentials

9AA has been used to monitor the extent of electrostatic screening of fixed negative charges on the surface of thylakoid membranes under conditions where no pH gradient would be formed (Searle *et al.*, 1977; Chow and Barber, 1980a, 1980b). Under such conditions and in the presence of low concentrations of cations the fluorescence of 9AA is quenched upon the addition of thylakoid membranes due to an accumulation of the probe into the membrane-associated diffuse layer. The further addition of cations causes an increase in fluorescence as a result of an increased level of screening and a decreased surface potential. The relative efficiency of cations of different valencies ($C^{3+}>C^{2+}>C^{+}$), the general lack of chemical specificity and the independence of associated anion all conform to the predictions of the theory of the diffuse layer (Searle *et al.*, 1977). The fluorescence of 9AA is consistent with its behaviour as a good indicator of the surface potential of thylakoid membranes.

Compared with its fluorescence with thylakoid membranes, 9AA behaves in a similar fashion when interacting with other negatively charged surfaces such as carboxymethylcellulose, azolectin, phosphatidyl serine liposomes, red blood cells and EL4 tumour cells (Searle and Barber, 1978; Chow and Barber, 1980c).

Negatively charged lipids are known to be present in plant mitochondrial membranes, as are proteins, the net charge of which is unknown. Thus it is reasonable to believe that the membranes of plant mitochondria do carry a net negative charge, like thylakoid membranes; indeed this has been supported by some preliminary experiments using particle electrophoresis (unpublished data, S.P.J., I.M.M. & H.Y.N.). Therefore 9AA may be used as a useful tool in plant mitochondria in

investigating electrostatic screening of the membrane surfaces.

2.5.6.3 Measurement of 9AA fluorescence

The fluorescence of 9AA was measured at room temperature in a Perkin Elmer MPF-3 fluorescence spectrophotometer, as described by Searle *et al.* (1977). The excitation wavelength was 398 nm (slitwidth 2 nm) and the emission was measured at 456 nm (slitwidth 5 nm). At a sensitivity setting of 1.0 this gave a reading of 72-74% full scale deflection on the recorder with 20 μM 9AA. The medium (3.0 ml) was low salt reaction medium (table 2). 9AA (20 μM), mitochondria and aliquots of stock salt solutions were added consecutively and readings taken after each addition.

# 3. RESULTS

## 3.1 Effect of a range of divalent cation chelators on oxidation rates

### 3.1.1 The oxidation of NADH, malate and succinate

The oxidation of exogenously added NADH, malate and succinate was studied in mitochondria isolated in standard media (section 2.4.1) from Jerusalem artichoke tubers (*Helianthus tuberosus*). Rates of oxidation in state 3 and state 4 are presented in table 3. (States 3 and 4 refer to metabolic states as defined by Chance and Williams, 1956.) The respiratory control ratio, which is defined as the ratio of the state 3 to state 4 respiratory rates, is also presented in table 3 for each of the substrates studied and may be used as a parameter of the purity and integrity of the preparation; compared with other work, the preparations used in this study are of similar standard (Ikuma and Bonner, 1967; Douce *et al.*, 1972a). The respiratory control ratio obtained when malate was the substrate was lower than with other substrates tested. A possible explanation for this observation may be that it is often difficult to interpret the rate of oxidation of malate following the addition of ADP in terms of state 3 and state 4, because oxaloacetate accumulation during malate oxidation influences the rate of malate oxidation (Lance and Bonner, 1968; Hobson, 1970; Palmer, 1980). If malate oxidation is inhibited by oxaloacetate, true state 3 and state 4 rates of oxidation are not operational and the respiratory control ratio no longer applies.

The succinate-cytochrome c oxidoreductase activity, considered to be an acceptable measure of membrane integrity (Douce *et al.*, 1972a) was found to be low in the mitochondria used in this study (12 nmol cyt c reduced/min/mg protein) and stimulated greatly following disruption in medium of low osmolarity (291 nmol cyt c reduced/min/mg

Table 3 Substrate oxidation rates, respiratory control ratio and ADP/O ratio in Jerusalem artichoke mitochondria

| Substrate | Oxidation rates | | Respiratory control ratio | ADP/O ratio |
|---|---|---|---|---|
| | state 3 | state 4 | | mean value |
| NADH, 1 mM | 170 | 61 | 2.77 | 1.22 |
| Succinate, 20 mM | 139 | 63 | 2.20 | 1.24 |
| Malate, 10 mM, glutamate, 10 mM, glutamate-oxalo-acetate transaminase, 20 µg | 54 | 26 | 2.01 | 1.83 |

Oxidation is initiated by the addition of substrate.

Rates, nmol $O_2$/min/mg protein.

Oxidation in state 3 is caused by 125 nmol ADP. The state 3 rate is measured after the second addition of ADP.

Protein concentration: NADH oxidation, 0.2 mg/ml; succinate or malate oxidation, 0.4 mg/ml.

Figures are the mean values of three sets of data.

States 3 and 4 are terms defined by Chance and Williams (1956).

protein, i.e. 96% intact).
Each time isolation, washing and assay conditions were altered, the integrity of the mitochondria was checked and found to be acceptable (i.e. 96% intact).

Lower than theoretical ADP/O ratios could indicate the presence of a non-phosphorylating pathway (that is, oxidation mediated partly via the inhibitor-resistant alternative oxidase). However in this study, cyanide-resistant respiration does not account for a significant proportion of the observed rate of oxidation; using NADH, succinate and malate, rates of oxidation were 96, 94 and 97% inhibited respectively (data not presented).

3.1.1.1 The oxidation of exogenous NADH

The rate of oxidation of exogenously added NADH was approximately 170 nmol $O_2$/min/mg protein under state 3 conditions. ADP/O ratios obtained with exogenous NADH were consistent with electrons from NADH traversing two phosphorylation sites (table 3). Because exogenous NADH oxidation is insensitive to rotenone, an inhibitor of electron transport associated with phosphorylation site 1 (Coleman and Palmer, 1972), electrons from exogenous NADH appear to bypass site 1 and traverse sites 2 and 3. The electrons from exogenously added NADH probably join the main electron transport chain at the level of ubiquinone.

3.1.1.2 The effect of ADP and FCCP on NADH oxidation

The data in table 4 show that the rate of oxidation of exogenous NADH was 17% faster in the presence of FCCP, a weak acid uncoupler, than the state 3 rate of oxidation.

The effect of FCCP upon the rate of NADH oxidation may be explained using the chemiosmotic theory. According to the chemi-

Table 4 Stimulation of the rate of exogenous NADH oxidation by ADP or FCCP in Jerusalem artichoke mitochondria

| Additions | Rate of NADH oxidation |
|---|---|
| - | 59 ±4.83 |
| ADP, 125 nmol/ml | 154 ±5.01 |
| FCCP, 0.1 nmol/ml | 178 ±4.23 |

Additions of FCCP or ADP were made 1 min after the addition of NADH. Rates, nmol $O_2$/min/mg protein. Protein concentration, 0.23 mg/ml. Figures are the mean values of three sets of data.

osmotic theory proposed by Mitchell (1966, 1968), the redox activity of electron transport in mitochondria is accompanied by proton translocation outwards across the inner mitochondrial membrane which is relatively impermeable to ions and is the site of oxidative phosphorylation. The protonmotive force (the sum of the pH gradient and the membrane potential) thus generated brings about ATP synthesis in the presence of a phosphate acceptor, phosphate and the proton-translocating, reversible ATPase system. In tightly coupled mitochondria the rate of electron transport may be limited by the rate of transport of electrons from different substrates through the electron transport chain or by the concentration of phosphate acceptor or phosphate (i.e., the phosphate potential). FCCP appears to behave as a passive proton mediator and thus affects the permeability of the coupling membrane to protons by allowing the electrochemical force to collapse, thereby uncoupling oxidative phosphorylation from electron transport. FCCP has been shown to cause a rapid collapse of the respiration-supported pH gradient across the coupling membrane of rat-liver mitochondria (Mitchell and Moyle, 1967). The rate of electron transport is therefore unchecked by the phosphate potential in the presence of FCCP.

In this study the uncoupled rate of exogenous NADH was used for investigating the involvement of calcium in the operation of the exogenous NADH dehydrogenase unless otherwise stated.

3.1.1.3 Succinate oxidation

When succinate was added to mitochondria in the presence of ADP, the rate of oxidation gradually increased. The increase in rate may be explained by the activation of the succinate dehydrogenase which is often found to be inactivated in isolated mitochondria (Wiskich and Bonner, 1963). Activation of the succinate dehydrogenase in Jerusalem

artichoke mitochondria could be achieved by pre-incubating the mitochondria with ATP (see table 3, Wiskich and Bonner, 1963; Oestreicher *et al.*, 1973). The procedure for the activation of succinate dehydrogenase in isolated mitochondria is described in section 2.6.3.

3.1.1.4 Malate oxidation

Plant mitochondria are different from mammalian mitochondria in the respect that they readily oxidise malate. However in the absence of a system to remove oxaloacetate, the rate of malate oxidation (under state 3 conditions) gradually tails off, as oxaloacetate accumulates. This observation is consistent with the view that malate oxidation, under these conditions, is mainly brought about by the activity of the malate dehydrogenase, which is inhibited by the accumulation of oxaloacetate (Lance and Bonner, 1968; Hobson, 1970; Palmer, 1980). Malate oxidation is also brought about by an $NAD^+$-linked malic enzyme which produces pyruvate (Macrae and Moorhouse, 1970; Macrae, 1971a, 1971b, 1971c; Palmer, 1980); factors which determine the relative contributions of the malate dehydrogenase and malic enzyme to the overall rate of malate oxidation are very complex. In this study an oxaloacetate trapping systed was used for malate oxidation (see table 3).

3.1.2 Effect of various chelators and calcium on oxidation rates

The hypothesis is proposed that calcium is involved in the operation of the exogenous NADH dehydrogenase. This view is supported by data obtained using divalent cations and divalent cation chelators as detailed below.

Divalent cations have been found to stimulate the oxidation of exogenous NADH (Hackett, 1961; Miller *et al.*, 1970; Coleman and

Palmer, 1971; Miller and Koeppe, 1971; Earnshaw, 1975). The oxidation of exogenous NADH is inhibited by various divalent cation chelators, EDTA and its analogues, EGTA, DPTA and HEDTA (Coleman and Palmer, 1971). In this thesis EDTA and EGTA have been used extensively. EDTA is a divalent cation chelator with binding constants for calcium and magnesium, log $K_{metal-ligand}$, of 10.7 and 8.9 respectively (Sillén and Martell, 1964, 1971); the binding constant of EGTA for calcium is 11.0 (recently re-determined by Owen, 1976, using a newly developed calcium selective electrode, innovated by Brown *et al.*, 1976). However, the binding constant of EGTA for magnesium is 5.4, over 5 orders of magnitude smaller than the EDTA binding constant for calcium. Thus, EGTA may be used as a tool for removing calcium from the mitochondrial divalent cation pool, accessible to EGTA, by selectively complexing the calcium, leaving the magnesium pool intact (Schmid and Reilly, 1957; Settlemire *et al.*, 1968).

3.1.2.1 EDTA and EGTA

In considering the regulation of the NADH dehydrogenase, thought to be located on the outer face of the inner membrane (Coleman and Palmer, 1971) and in particular the implication that calcium may be involved in such regulation, it is clearly important to know where EDTA and EGTA and other chelators used in this study are likely to have their action in relation to the mitochondrial compartments.

The chelating agents, EDTA and EGTA, and their analogues, DPTA and HEDTA, are multidentate ligands of carboxylate donors (Hopkin and Williams, 1964). In the EDTA:Ca complex, EDTA and $Ca^{2+}$ are equimolar. This is because calcium can have a co-ordination number of 6-8 (Williams, 1976) and there are 6 ligand atoms in EDTA available to form co-ordination bonds with a divalent cation (Martell and Calvin,

1952; Bennett and Wise, 1956). There are also 6 ligand atoms in EGTA available to form co-ordination bonds such that free manganese binds EGTA in a 1:1 ratio (Chance, 1959). Thus it is reasonable to assume that calcium and EGTA are also equimolar in the EGTA:Ca complex. Magnesium invariably has a co-ordination number of 6 (Williams, 1976).

The EDTA:metal chelate complex is fairly stable under alkaline conditions but because protons are produced upon the formation of the chelate complex, the degree of ionization of EDTA and the effective concentration of its anions are determined by pH (as protons are a product of the reaction). If M = metal and Y = EDTA radical, then

$$M^{2+} + H_2Y^{2-} \rightleftharpoons MY^{2-} + 2H^+$$

(Sillén and Martell, 1964, 1971; Hopkin and Williams, 1964).

It is clearly of great importance to know whether the chelators used in this study have access to the mitochondrial matrix. Most of the data available refer to the chelators EDTA and EGTA, but are probably also applicable to their analogues DPTA and HEDTA. It is now widely agreed that EDTA and EGTA have no access to the mitochondrial matrix. For example, $Mn^{2+}$ accumulated into the mitochondrial matrix is inaccessible to EGTA (Chappell *et al.*, 1963; Chappell and Crofts, 1965b) and $^{14}$C-sucrose and $^{14}$C-EDTA have access to identical mitochondrial spaces, i.e. EDTA lacks access to the mitochondrial matrix (Harris and Van Dam, 1968). Mitochondrial swelling does not occur in isosmotic solutions of $NH_4$EDTA or $NH_4$EGTA, which supports the view that EDTA and EGTA act as impermeant anions (Reed and Bygrave, 1974). Data presented by Bygrave (1977) and Imedidze *et al.* (1978) were also consistent with EDTA and EGTA having action external to the mitochondrial matrix.

Under certain conditions, however, some reports indicate

that chelators are able to cross membranes. For example, the uptake of certain trivalent metal-chelate complexes by roots was reported by De Kock and Mitchell (1957), who attributed the observations to the charge on the chelated molecule; those with 2 negative charges were not taken up, while those with 1 (or none) were.

Settlemire *et al.* (1968) found that the inner mitochondrial membrane was impermeable to EDTA and EGTA at neutral pH but reported that under certain conditions EDTA in heart mitochondria had access to the matrix, i.e. at pH 8.1 (when removal of mitochondrial magnesium by EDTA is more effective) in the presence of an energy source, a transportable metal such as sodium, and a permeant anion. Wehrle *et al.* (1976) also found that EDTA had access to the matrix at pH > 8.0 in swollen heart mitochondria. In addition data presented by Blankenship and Sauer (1974) were interpreted as showing that EDTA readily crosses the thylakoid membrane in TRIS-washed chloroplasts.

Although there are data that show EDTA and EGTA may have access to the mitochondrial matrix under certain conditions, most of the experiments carried out in this study were performed under conditions which one can be confident did not permit the uptake of EDTA or EGTA into the mitochondrial matrix (in the presence of FCCP at pH 7.2 and 0.3 M sucrose). A few experiments (section 3.2.5) were conducted at pH 8.2 and these will be carefully considered later.

Thus EGTA and EDTA are considered to have access to mitochondrial calcium on the outer membrane, the outer face of the inner membrane (and in the intermembrane space) i.e., to "extra-matrix" calcium.

Divalent cations, especially calcium, have been implicated in the operation of the exogenous NADH dehydrogenase, section 3.1.2.

The next section tests this hypothesis, using a range of chelators of different chemical structures and various substrates; if calcium was indeed involved in the regulation of the exogenous NADH dehydrogenase, it was expected that chelators would inhibit the oxidation of exogenous NADH alone. $NAD^+$-linked substrate oxidation would not be affected.

3.1.2.2 Various chelators and exogenous NADH oxidation

The data in table 5 present the effect of mitochondrial pre-incubation with various calcium chelators upon the rate of oxidation of various substrates in Jerusalem artichoke mitochondria and the effect of added calcium upon chelator-treated mitochondria. The data in figure 1 present the relationship between the concentration of many of the chelators used in this study and the extent of inhibition of exogenous NADH oxidation. It can be seen that all the chelators tested except murexide inhibited NADH oxidation to some extent. These data supported the hypothesis that calcium was involved in the operation of the exogenous NADH dehydrogenase because the inhibition of NADH oxidation could be attributed to the removal of mitochondrial-associated calcium by the respective chelators. In the case of murexide, which lacks access to the matrix (Mela and Chance, 1968) and which is used to measure free calcium levels (Scarpa, 1972), a possible reason why it was unable to induce inhibition of exogenous NADH oxidation may be that the stability constant of murexide for calcium (2.68) is lower than that of the mitochondrial binding site for calcium (cf. range of stability constants, table 6).

3.1.2.3 Various chelators and malate and succinate oxidation

The concentration of chelators known to inhibit the oxidation of external NADH had no influence on the rate of malate or

Table 5 Addition of calcium to chelator-treated mitochondria oxidising various substrates

| Chelator | Inhibition of NADH oxidation, % | Effect of calcium upon chelator-treated NADH oxidation | Inhibition of NADH oxidation in the presence of chelator and calcium, % | Malate oxidation inhibited? | Succinate oxidation inhibited? |
|---|---|---|---|---|---|
| Murexide | 0 | N.D. | N.D. | No | No |
| EGTA | 92 | inhibition reversed | 0 | No | No |
| EDTA | 90 | inhibition reversed | 0 | No | No |
| DPTA | 85 | inhibition reversed | 0 | No | No |
| HEDTA | 80 | inhibition reversed | 0 | No | No |
| Citrate | 74 | inhibition reversed | 0 | No | No |
| Solochrome | 94 | inhibition partially reversed | 65 | No | No |
| DHPE | 43 | inhibition unchanged | 43 | slight (18%) | No |
| Fluorescein complexone | 55 | inhibition enhanced | 70 | N.D. | N.D. |
| HSN | 65 | inhibition enhanced | 74 | N.D. | N.D. |
| Calcichrome | 54 | inhibition enhanced | 65 | No | No |

N.D. = not done. Rate of oxidation, 182 nmol $O_2$/min/mg protein. Protein concentration, 0.26 mg/ml.

(EGTA conc. 1mM)

## Legend to figure 1

Mean rate of NADH oxidation, 172 nmol $O_2$/min/mg protein.

FCCP ($2 \times 10^{-7}$ M) was always added immediately after mitochondria unless otherwise stated. NADH concentration was 1 mM unless otherwise stated.

Mitochondria were isolated at pH 7.2 using standard media (tables 1 & 2) and standard procedure (section 2.4.1, i.e. exposed to EDTA).

Additions: standard ($+Mg^{2+}$, table 2) reaction medium, pH 7.2; mitochondria; chelator; FCCP; 30 sec later, NADH.

Key

| | chelator | mitochondrial protein concentration (mg/ml) |
|---|---|---|
| A | EGTA | 0.99 |
| B | solochrome | 0.28 |
| *C | fluorescein complexone | 0.35 |
| *D | HSN | 0.25 |
| *E | DHPE | 0.42 |
| *F | calcichrome | 0.19 |
| G | citrate | 0.26 |
| *H | murexide | 0.22 |

(*denotes limit of solubility of chelator in reaction medium)

Figure 1. The relationship between concentration of various chelators and % inhibition of exogenous NADH oxidation.

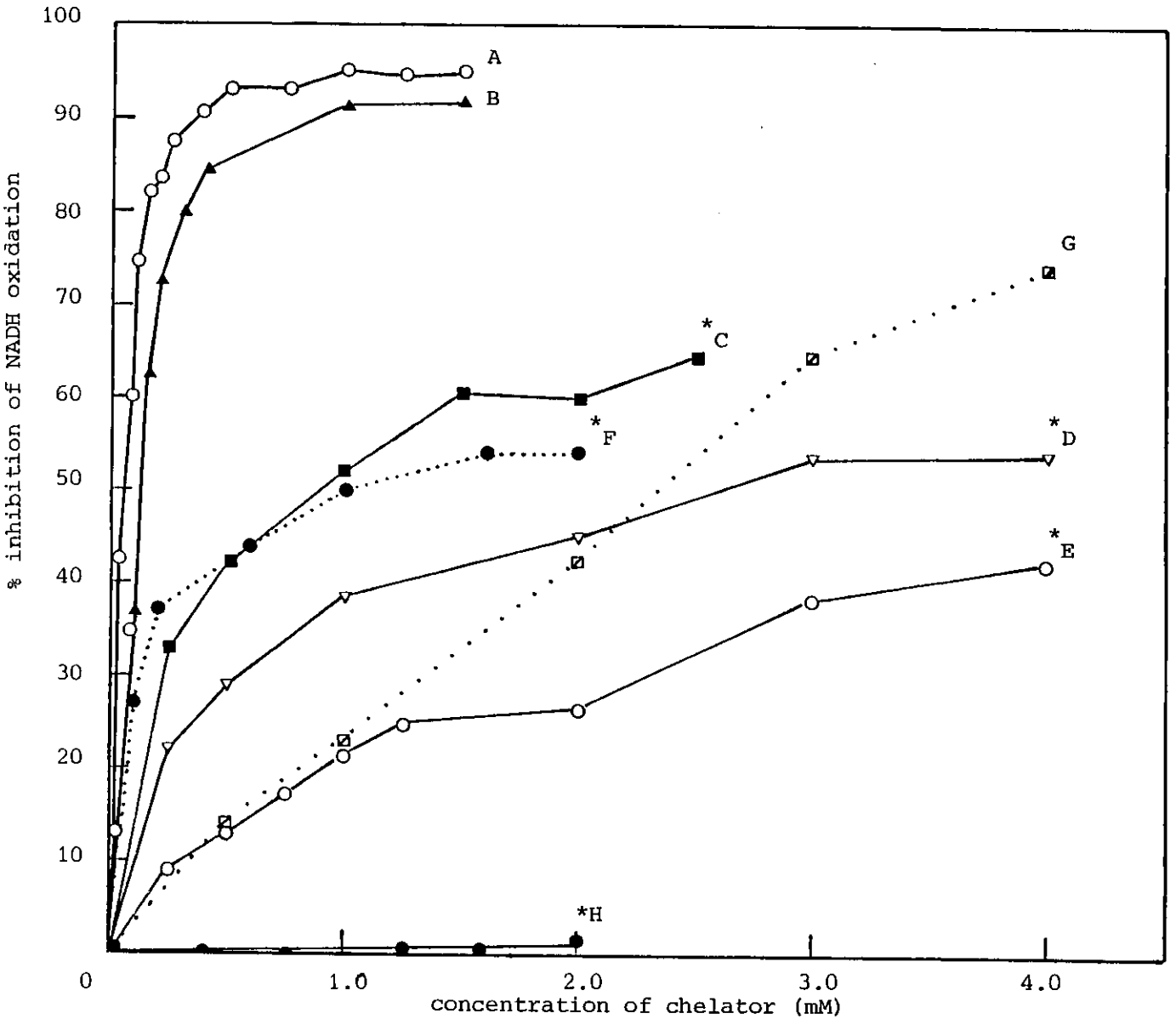


Table 6 Respective chelator stability constants for calcium, solvent solubility and chelator inhibition of NADH oxidation in the presence of calcium

| Chelator | % Inhibition of NADH oxidation in presence of chelator and calcium | Chelator stability constant ($K_1$) for calcium | Solubility of chelator |
|---|---|---|---|
| Murexide | N.D. | 2.68* | Not readily soluble in $H_2O$ |
| EGTA | 0 | 11.0§ | Water soluble |
| EDTA | 0 | 10.68* | Water soluble |
| DPTA | 0 | 10.6* | Water soluble |
| HEDTA | 0 | - | Water soluble |
| Citrate | 0 | 4.8× | Water soluble |
| Solochrome | 65 | 5.25+ | Not readily soluble in EtOH or $H_2O$ |
| DHPE | 43 | 4.3+ | EtOH soluble |
| Fluorescein complexone | 70 | 3.85+† | EtOH soluble |
| HSN | 74 | 5.0+ | EtOH soluble |
| Calcichrome | 65 | 26.45* | Not readily soluble in $H_2O$ |

* Sillén and Martell (1964, 1971)

§ Owen (1976)

× Williams (1976)

+ Bishop (1972)

† Stability constant for the chelator group of this fluorescent molecule.

N.D., not done.

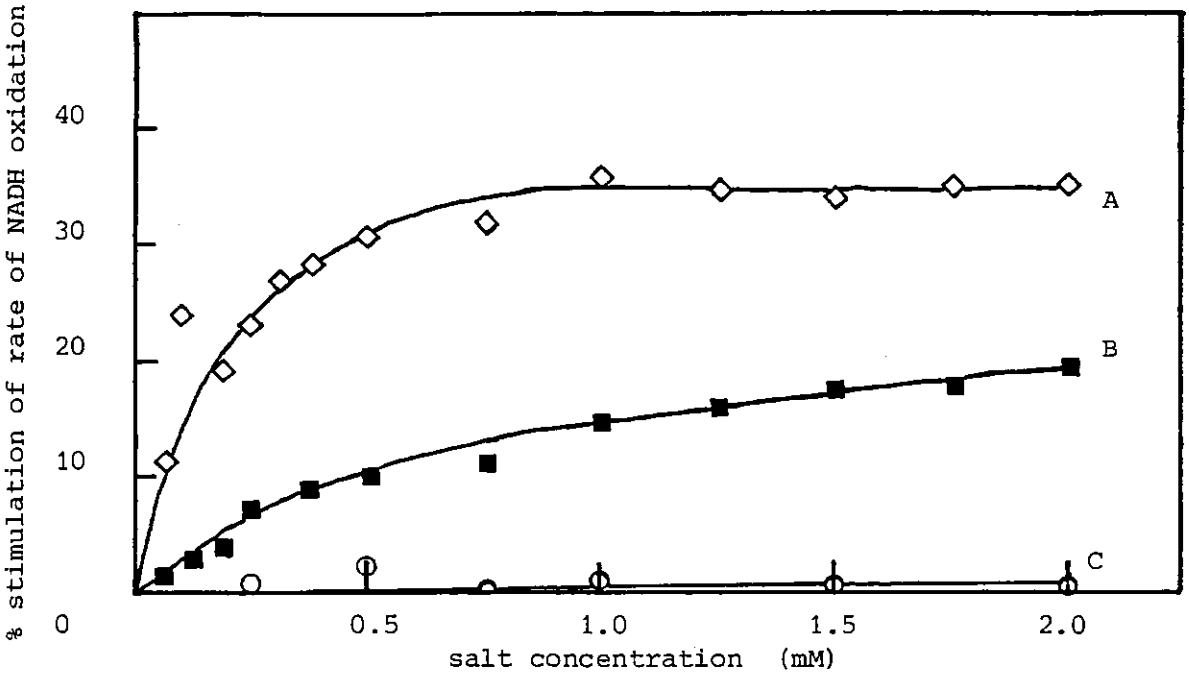
(EGTA conc. 1mM)

succinate oxidation (table 5). DHPE gave the only exception, causing a slight reduction in malate oxidation (table 5). Thus the influence of the chelators must be limited to the section of the respiratory chain between the flavoprotein and ubiquinone (Coleman and Palmer, 1971; Cowley and Palmer, 1978).

3.1.2.4 Metabolic state and stimulation of exogenous NADH oxidation

Calcium and other divalent cations have been reported to stimulate the oxidation of exogenous NADH in mitochondria from different tissues (section 3.1.2). In Jerusalem artichoke tubers, stimulation of oxidation rates varied seasonally. For example, in mitochondria prepared from tubers dug up from the ground in October 1975, NADH oxidation (+FCCP) was stimulated 35% by calcium (figure 2, trace A) but no stimulation was observed in mitochondria from tubers dug up in December 1975 (figure 2, trace C). (Average stimulation was 11% over a 4-month period; 8 preparations, data not shown.) Calcium stimulation of exogenous NADH oxidation was generally seen best in mitochondria prepared from tubers dug up from the ground around the month of October in both 1975 and 1976. This stimulation was unrelated to the ability of calcium to uncouple oxidative phosphorylation found in rat-liver mitochondria (Carafoli and Lehninger, 1971) because it occurred in the presence of uncoupler, FCCP (figure 3, trace A, 11% stimulation). The calcium stimulation of NADH oxidation was further indicated to be unrelated to an energy-linked process by the observation that in the absence of ADP or FCCP the addition of calcium to mitochondria treated with EGTA only restored the rate of NADH oxidation to a control value without an additional stimulation (figure 3, trace B).

Figure 2. Seasonal variation in the relative stimulation of exogenous NADH oxidation by calcium and manganese




Mean rate, 172 nmol $O_2$/min/mg protein

Isolation of mitochondria, see legend figure 1

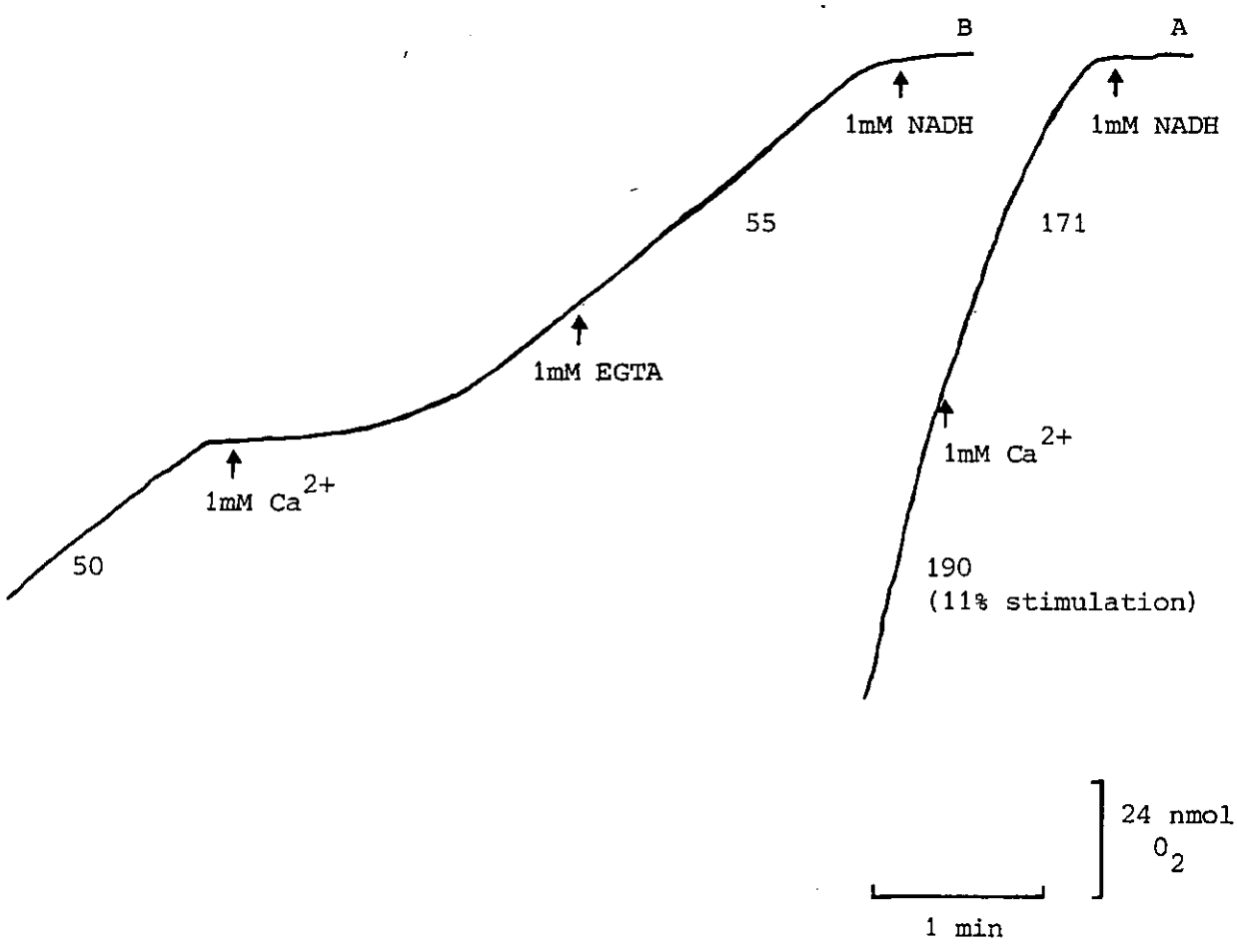
Additions, standard reaction medium, pH 7.2, mitochondria FCCP, salt, NADH (1mM)

Trace A, + $Ca^{2+}$, tubers removed from ground October, 1975. 0.39mg protein/ml

Trace B, + $Mn^{2+}$, tubers removed from ground October, 1975. 0.39mg protein/ml

Trace C, + $Ca^{2+}$, tubers removed from ground December, 1975. 0.30mg protein/ml.

Figure 3. Calcium stimulation of exogenous NADH oxidation under different metabolic conditions




Rates, nmol $O_2$/min/mg protein

Isolation of mitochondria, see legend figure 1

Additions: A, standard reaction medium, pH 7.2, mitochondria, FCCP, NADH, EGTA, $CaCl_2$

B, standard reaction medium, pH 7.2, mitochondria, NADH, EGTA, $CaCl_2$

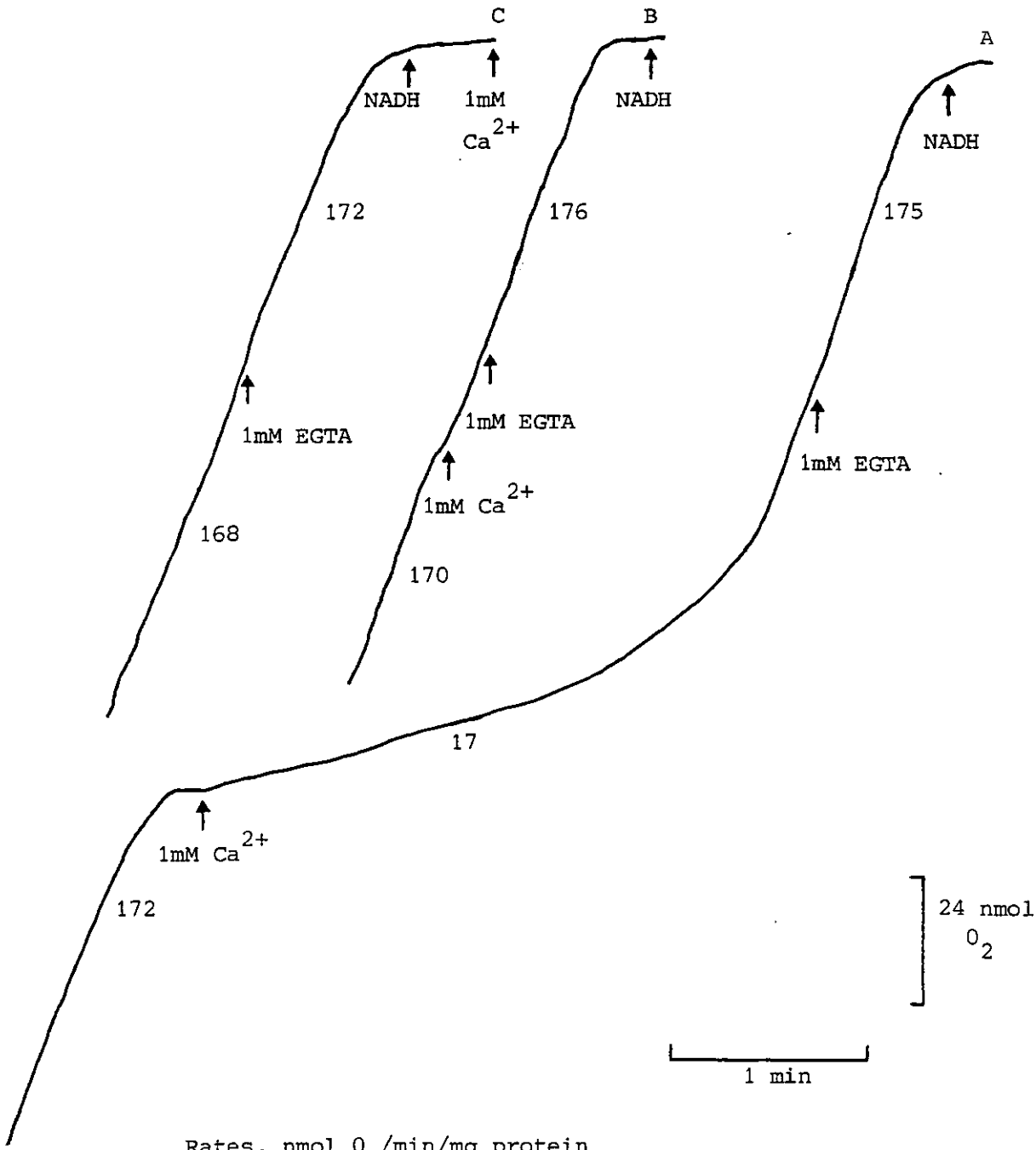
3.1.2.5 Sequence of addition of NADH, calcium and EGTA

The hypothesis in section 3.1.2 suggested that exogenous NADH oxidation is inhibited by chelators because they remove calcium essential for the oxidation. In this section the sequence of addition of NADH and EGTA (in the presence of FCCP) is shown to influence the development of the EGTA inhibition. Addition of the EGTA before the NADH is initially more effective at causing the inhibition than when the sequence is NADH-EGTA; this point is well illustrated by comparing the data in trace A of figures 4 and 5. These data suggest that when NADH is added first (figure 4) EGTA is initially less able to cause the inhibition because the calcium is bound more tightly to the membranes; when EGTA is added first (figure 5), essential calcium is more susceptible to chelation by EGTA. Data in trace A of figures 4 and 5 also show that the addition of calcium several minutes after EGTA had inhibited NADH oxidation resulted in a complete and rapid restoration of the oxidation rate (irrespective of sequence of addition of NADH and EGTA). Moreover, addition of calcium in rapid succession to (trace B) or before (trace C) NADH and EGTA prevented the inhibition of NADH oxidation and resulted in control oxidation rates.

As outlined in section 3.1.2.1, EGTA lacks access to the matrix (Reed and Bygrave, 1974) and its influence under these experimental conditions is considered to be limited by the inner mitochondrial membrane; the speed with which calcium reverses the EGTA-induced inhibition of NADH oxidation supports the view that calcium is also acting externally to the inner mitochondrial membrane. Calcium is therefore strongly implicated in the operation of the exogenous NADH dehydrogenase.

The EGTA-induced inhibition of NADH oxidation is reported

Figure 4. Addition of EGTA to mitochondria oxidising NADH and the influence of calcium on oxidation rates.



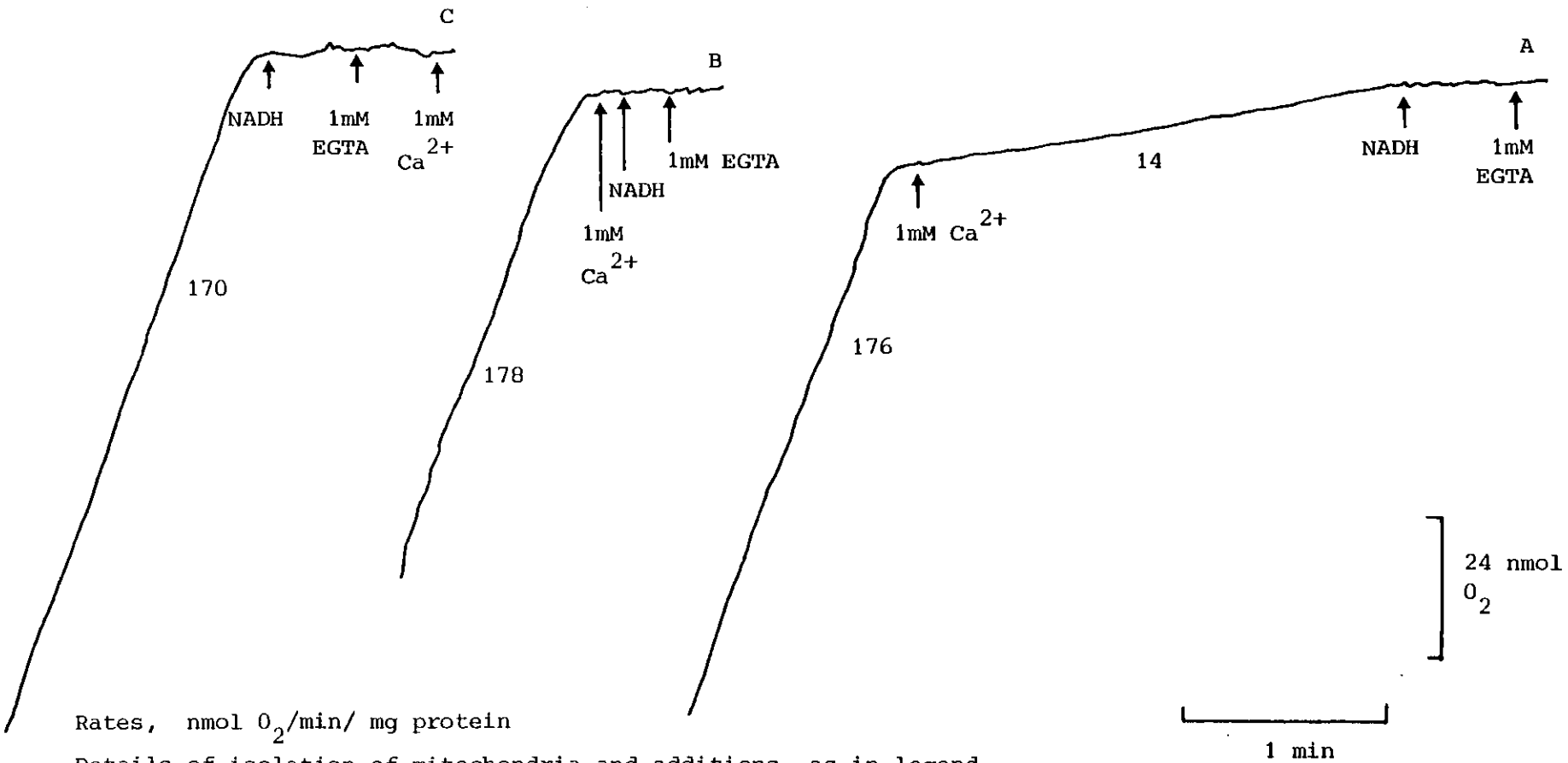

Rates, nmol $O_2$/min/mg protein

Isolation of mitochondria, see legend figure 1

Additions, standard reaction medium, pH 7.2, mitochondria, FCCP. Further additions of calcium, EGTA and NADH as indicated under traces A-C.

0.21 mg protein/ml

Figure 5. Incubation with EGTA before the addition of NADH and the influence of calcium on oxidation rates




Rates, nmol $O_2$/min/ mg protein

Details of isolation of mitochondria and additions, as in legend to figure 4 and as indicated under traces A-C, figure 5.

at greater length in sections 3.2 and 3.3.

### 3.1.3 Effect of divalent cations on chelator-inhibited NADH oxidation

#### 3.1.3.1 $Ca^{2+}$, $Mn^{2+}$ and $Sr^{2+}$ and EGTA-inhibited NADH oxidation

The data in table 7 show that when assayed in standard (section 2.3.4.1, $+Mg^{2+}$) reaction medium, the EGTA-inhibited rate of exogenous NADH oxidation could be stimulated by $Ca^{2+}$, $Mn^{2+}$ and $Sr^{2+}$; $Ca^{2+}$ was more effective than $Mn^{2+}$ or $Sr^{2+}$ (i.e., a lower concentration produced a faster rate of oxidation). It is noteworthy that 2.5 mM $Mg^{2+}$, a constituent of standard reaction medium, was ineffective at reversing or preventing EGTA inhibition of NADH oxidation, unlike $Ca^{2+}$, $Mn^{2+}$ and $Sr^{2+}$. This point will be discussed further in section 3.3.1.

#### 3.1.3.2 The addition of $Ca^{2+}$ to mitochondria treated with various chelators

Stimulation of NADH oxidation was tested using $Ca^{2+}$ in mitochondria treated with various chelators to inhibit NADH oxidation. Mitochondria were therefore suspended in standard ($+Mg^{2+}$) reaction medium and additions made in this order: FCCP, chelator, NADH and 2 minutes later, $Ca^{2+}$.

Calcium stimulates the rate of NADH oxidation back to a control value in mitochondria treated with EGTA and its analogues EDTA, DPTA and HEDTA and with citrate respectively (table 5) in agreement with published data (Coleman and Palmer, 1971). In addition, calcium partially (65%) stimulates the rate of NADH oxidation in solochrome-treated mitochondria (table 5). These data are therefore consistent with the inhibition of NADH oxidation being due to the removal of calcium.

Table 7 Relative stimulation of NADH oxidation in EGTA-treated mitochondria by divalent cations

| Salt | Rate of NADH oxidation<br>nmol $O_2$/min/mg protein | Stimulation of EGTA-treated rate<br>% |
|---|---|---|
| 0 | 18 | 0 |
| $Mn^{2+}$, 2 mM | 104 | 547 |
| $Sr^{2+}$, 2 mM | 106 | 504 |
| $Ca^{2+}$, 1 mM | 176 | 1257 |

Isolation of mitochondria, standard procedure (section 2.4.1), pH 7.2.

Additions: standard reaction medium, pH 7.2 (table 2), mitochondria, FCCP, chelator, NADH, salt.

Mean rate of NADH in standard reaction medium, 178 ±4 nmol $O_2$/min/mg protein.

(EGTA conc. 1mM)

However, in the case of mitochondria treated with DHPE, fluorescein complexone, HSN and calcichrome respectively, and in contrast to expectation, subsequent treatment with calcium either does not change the rate of NADH oxidation (DHPE) or actually enhances the inhibition of NADH oxidation (fluorescein complexone, HSN and calcichrome respectively)(table 5). Clearly these data are not as predicted if the inhibition of NADH oxidation is simply due to the removal of calcium.

It was intriguing that when the addition of calcium to chelator-treated mitochondria caused a restoration of the rate of NADH oxidation, that chelator tended to be readily water soluble (table 6) whereas when no change or enhancement of inhibition occurred, that chelator tended to be more soluble in organic solvents than in water. The combination of relatively low solubility in water plus relatively low stability constant for calcium (compared with other chelators used in this study, table 6) might have made DHPE, HSN, fluorescein complexone and calcichrome relatively poor calcium chelators when interacting with mitochondrial calcium.

However data obtained using EGTA and its analogues, EDTA, HEDTA and DPTA, citrate and solochrome (to a lesser extent) were consistent with the hypothesis that calcium was involved in the oxidation of exogenous NADH and, using EGTA, prompted a closer examination of the chelator-inhibited, calcium-stimulated oxidation of exogenous NADH.

## 3.2 Nature of the EGTA-inhibition of exogenous NADH oxidation

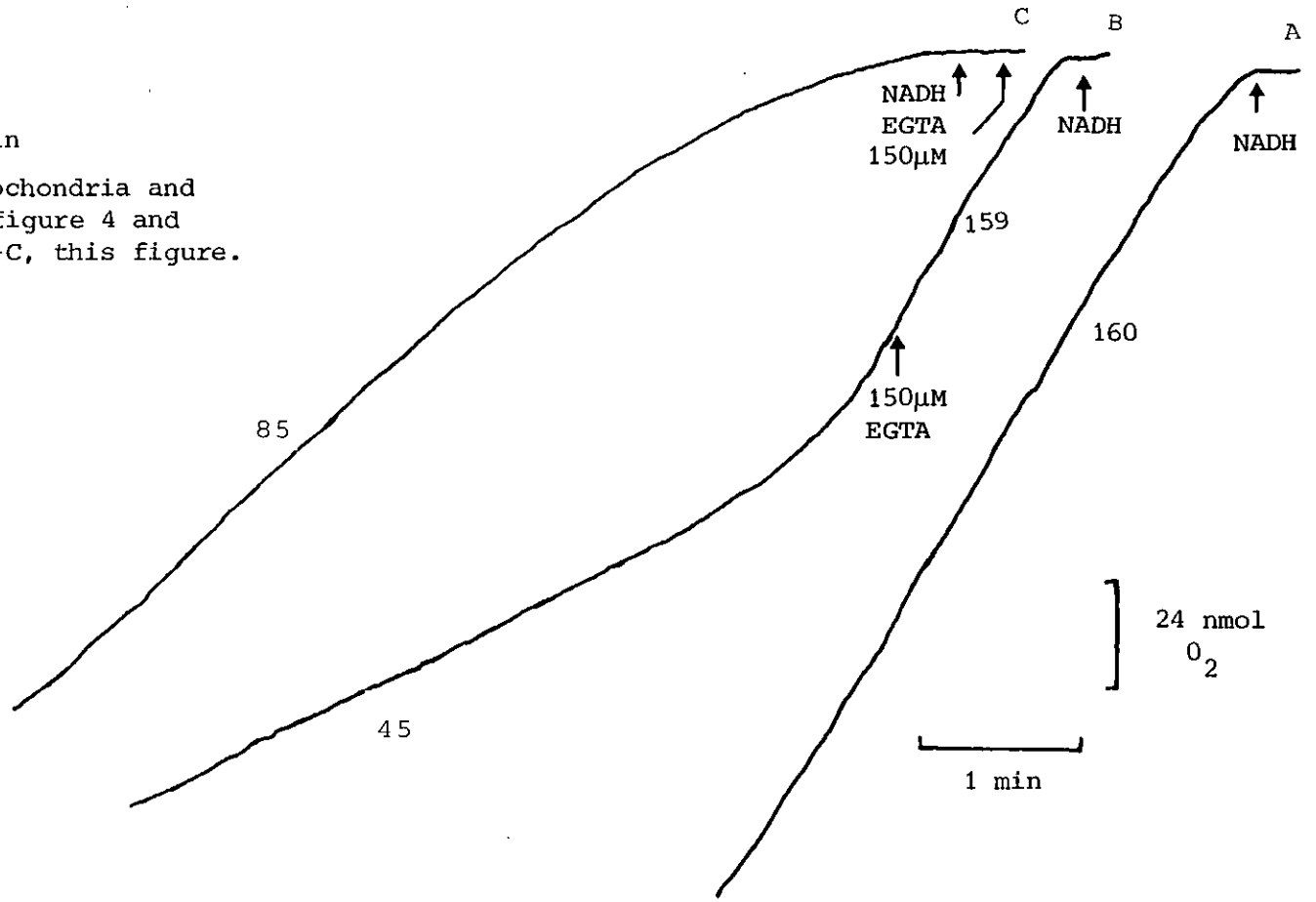
### 3.2.1 Sequence of addition of NADH and EGTA

When EGTA was added to mitochondria oxidising NADH a progressive inhibition of the rate occurred (figure 6, trace A). Experiments were done using much lower concentrations of EGTA than described in section 3.1.2.5. However, if

Figure 6. Effect of sequence of addition of EGTA and NADH on the onset of EGTA inhibition of NADH oxidation

Rates, nmol $O_2$/min/mg protein

Details of isolation of mitochondria and additions, as in legend to figure 4 and as indicated under traces A-C, this figure.



EGTA was added before NADH the inhibition was great at first but decreased progressively, suggesting that a gradual recovery from EGTA treatment was taking place (figure 6, trace B). After several minutes, when the sequence of addition was NADH-EGTA, the rate of oxidation was lower (45 nmol $O_2$/min/mg protein) than the partially recovered rate when the sequence was EGTA-NADH (85 nmol $O_2$/min/mg protein). Because the rate of NADH oxidation in the presence of EGTA progressively changed in velocity depending on whether the addition of EGTA was before or after NADH, there have been difficulties in evaluating the magnitude of the inhibition of exogenous NADH oxidation which may be related to how the inhibition is brought about.

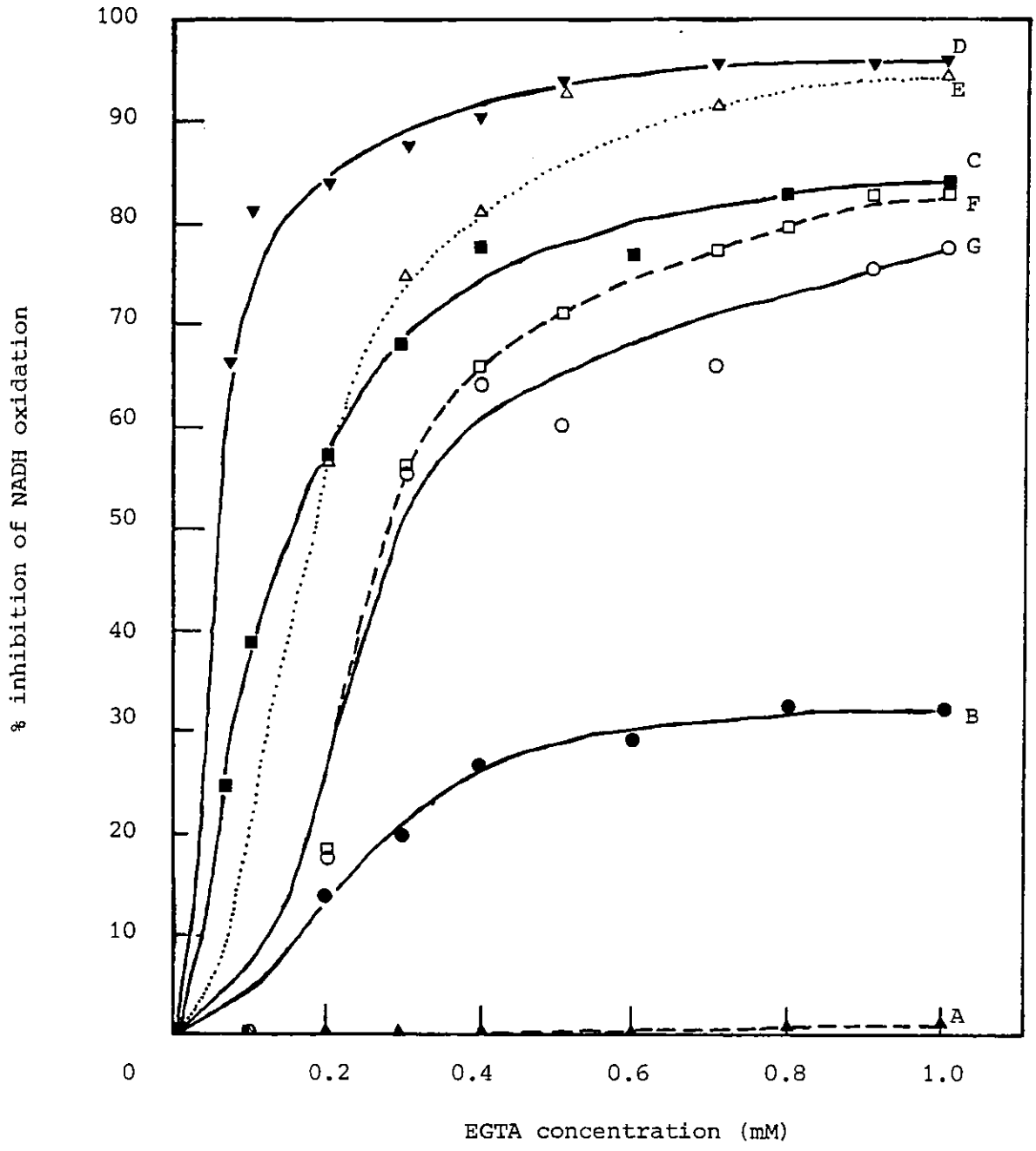
3.2.1.1 The addition of EGTA to mitochondria oxidising NADH

The data in figure 7 show how the inhibition of NADH oxidation caused by a range of EGTA concentrations varied with time when EGTA additions were made once NADH oxidation was initiated. The decrease in rate was greatest immediately after the addition of EGTA was made, thereafter the rate decreased by progressively smaller increments so that the rate eventually reached a constant value (or the change in rate was small enough to be considered negligible).

3.2.1.2 Pre-incubation with EGTA and substrate oxidation rates

Mitochondria were pre-incubated for 10 minutes in the presence and absence of 1.5 mM EGTA before the addition of NADH, malate and succinate respectively to find out if incubation with EGTA was harmful to the mitochondrial preparation. (It has been reported that corn mitochondria incubated with the chelators citrate or EDTA exhibited diminished membrane integrity, Stoner and Hanson, 1966.) Additions of ADP were made and respiratory control ratios were calculated and

Figure 7. Onset of EGTA inhibition of NADH oxidation




Sequence of additions, NADH-EGTA, traces A-C; EGTA-NADH, traces D-G

Mean rate of NADH oxidation, 190 nmol $O_2$/min/mg protein

Isolation of mitochondria at pH 7.2, EDTA-free procedure, section 2.4.2

Additions, standard reaction medium, pH 7.2, mitochondria, FCCP, then as appropriate, NADH, 1.5 min later, EGTA, traces A-C or EGTA, 30 sec later, NADH, traces D-G.

Key:
A initial rate
B rate at 1 min
C rate at 5 min
D initial rate
E rate at 1 min
F rate at 5 min
G rate at 10 min

compared. The data in table 8 show that pre-incubation with 1.5 mM EGTA caused a small decrease in R.C. ratios for all three substrates tested, but this was considered insignificant in comparison with the magnitude of the calcium stimulation of exogenous NADH oxidation.

The incubation period with EGTA was varied from 10.0-0.5 min but this caused no change in either the initial rates or the rates recorded 5 min after the onset of NADH oxidation (data not shown). Therefore recovery to EGTA treatment in the case of NADH oxidation does not appear to occur until NADH has been added. Consequently the incubation period normally used when EGTA was added before NADH was 0.5 min unless otherwise stated.

3.2.1.3 Incubation of mitochondria with EGTA

The data in figure 7 also show that pretreatment of mitochondria in EGTA caused an initial high level of inhibition of NADH oxidation, which was followed by a gradual increase in rate leading to a partial recovery.

The initial high level of inhibition is attributed to the removal of essential calcium, required for the oxidation of exogenous NADH. The gradual increase in the rate of oxidation is attributed to the re-activation of the dehydrogenase, thought to be due to the passage of calcium from the matrix (inaccessible to EGTA, Reed and Bygrave, 1974). Mitochondria from turnip and beet roots have 4.5 and 1.6 nmol calcium/mg protein respectively (Chen and Lehninger, 1973).

The final, more rapid rate is referred to as the recovered rate and has been used to compare different treatments in later experiments.

Table 8 Effect of incubating mitochondria for 10 min with EGTA on oxidation rates and respective RC ratios

| Substrate | Oxidation rate after 10 min preincubation period with EGTA | | | Control, minus EGTA. Oxidation rate after 10 min incubation period | | | Decrease in R.C.R. | Change in oxidation rate |
|---|---|---|---|---|---|---|---|---|
| | State 3 | State 4 | R.C.R. | State 3 | State 4 | R.C.R. | | |
| NADH, 1 mM | 22 | 8 | 2.63 | 170 | 61 | 2.77 | 7% | 87% inhibition |
| Succinate, 20 mM | 135 | 51 | 2.67 | 139 | 63 | 2.8 | 5% | 3% inhibition |
| Malate, 10 mM (+ glutamate, 10 mM, + glutamate-oxaloacetate-transaminase, 20 µg) | 60 | 27 | 2.2 | 54 | 26 | 2.01 | 9% | 11% stimulation |

Isolation of mitochondria, standard procedure (section 2.4.1), pH 7.2.

Additions: standard reaction medium (table 2), pH 7.2, mitochondria, FCCP, 1.5 mM EGTA as appropriate, followed by 10 min incubation period, substrate as appropriate.

Protein concentrations: NADH oxidation, 0.2 mg/ml; malate and succinate oxidation, 0.4 mg/ml.

Rates, nmol $O_2$/min/mg protein. R.C.R., respiratory control ratio. All figures mean values of 3 sets of data.

3.2.1.4 The concentration of EGTA causing 50% inhibition of NADH oxidation

When plotting the relationship between inhibition of rate of NADH oxidation and EGTA concentration, an S-shaped graph is obtained. Because 100% inhibition is not clearly defined (e.g. figure 7) it is convenient for the purpose of comparing different preparations to determine the concentration of EGTA which causes a clearly defined level of inhibition, arbitrarily chosen as 50% inhibition.

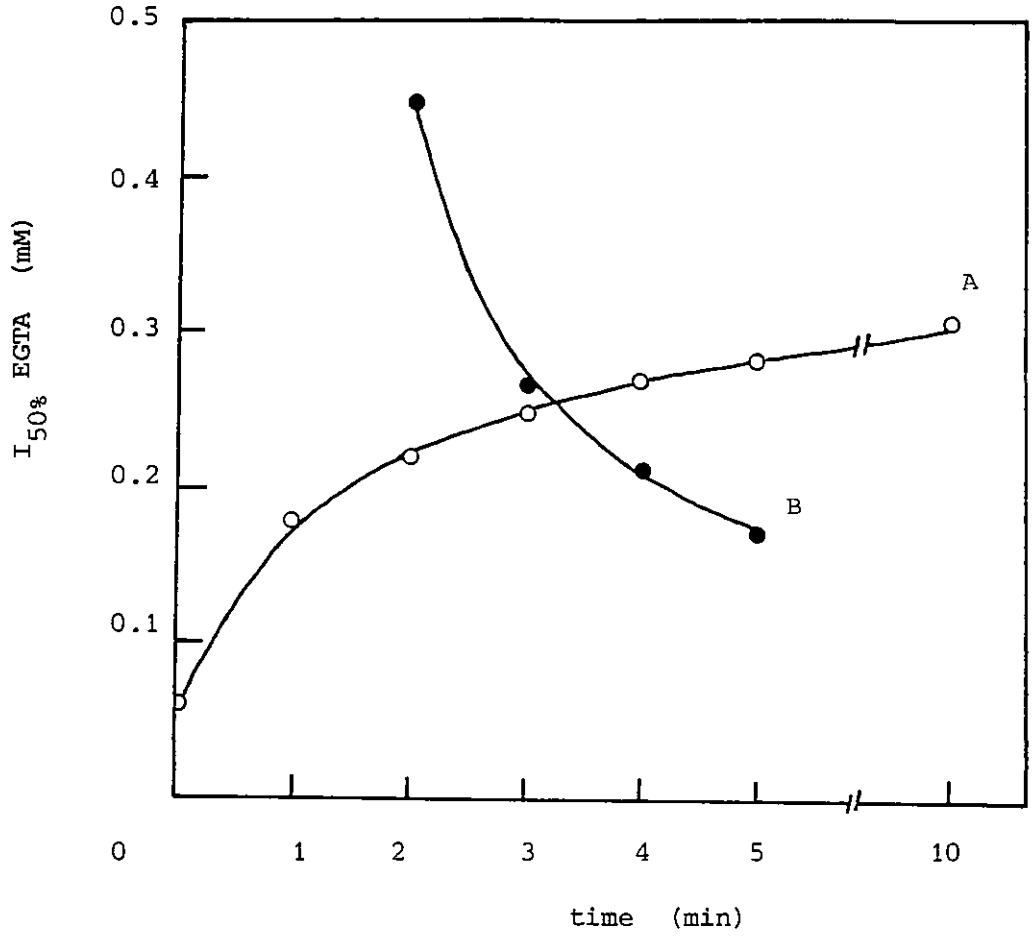
When the sequence of additions was NADH-EGTA, the concentration of EGTA causing 50% inhibition of NADH oxidation was initially very great but gradually decreased for a given preparation (figure 8). In contrast, in the same preparation, when the sequence of addition was EGTA-NADH, the concentration of EGTA causing 50% inhibition of NADH oxidation was initially very low but gradually increased (figure 8). (These observations depended upon the time of incubation.)

As indicated previously (figure 7) the data in figure 8 also illustrate how the sequence of addition of NADH and EGTA determines the final rate of NADH oxidation, i.e., when the sequence was NADH-EGTA, the inhibition of NADH oxidation caused by a given concentration of EGTA was finally greater than when the sequence was EGTA-NADH.

3.2.2 Variations in the concentration of EGTA causing 50% inhibition of NADH oxidation

Because this study is concerned with the elucidation of the role of calcium in the operation of the external NADH dehydrogenase, it was decided to examine exogenous NADH oxidation in mitochondria isolated using media which lacked chelators. EDTA is a usual constituent of mitochondrial extraction media, included so that liberated divalent cations, which may stimulate undesirable enzyme activity

Figure 8. Influence of sequence of addition of NADH and EGTA on the development of EGTA inhibition of NADH oxidation




Data derived from figure 7.

Sequence of additions, A, EGTA-NADH;
B, NADH-EGTA.

(e.g. $Ca^{2+}$-stimulated phospholipase, E.C. 3.1.1.4) can be rapidly removed from free solution (Tager, 1954). However, exposure to EDTA would also be expected to remove calcium from those mitochondrial surfaces to which it has access, i.e., both surfaces of the outer membrane and the outer face of the inner membrane (Reed and Bygrave, 1974); consequently an isolation procedure designed to leave the total mitochondrial calcium pool intact was expected to exert an effect on exogenous NADH oxidation. In addition, if the hypothesis that calcium is involved in exogenous NADH oxidation is true and the EGTA-inhibition of exogenous NADH oxidation is due to the chelation of mitochondrial calcium, one would predict a relationship to exist between mitochondrial protein concentration and the extent of inhibition of NADH oxidation caused by a given concentration of EGTA. Accordingly, the relationship between the concentration of mitochondrial protein and the inhibition of NADH oxidation was investigated.

3.2.2.1 Sensitivity of NADH oxidation to EGTA treatment

In mitochondria prepared using standard media, i.e., exposed to EDTA (5 mM) during isolation, the concentration of EGTA which caused 50% inhibition of NADH oxidation was found to be proportional to protein concentration, 38 nmol EGTA/mg protein (figure 9).

In contrast, in mitochondria prepared in the absence of chelators, the concentration of EGTA which caused 50% inhibition of NADH oxidation was very much greater, i.e., 730 nmol EGTA/mg protein, roughly an eighteen-fold increase (figure 10).

The difference in sensitivity to treatment with EGTA appeared to be related to the EDTA depletion of mitochondrial calcium during isolation. Thus it is reasonable to assume that sensitivity to EGTA during NADH oxidation reflects the level of mitochondrial calcium.

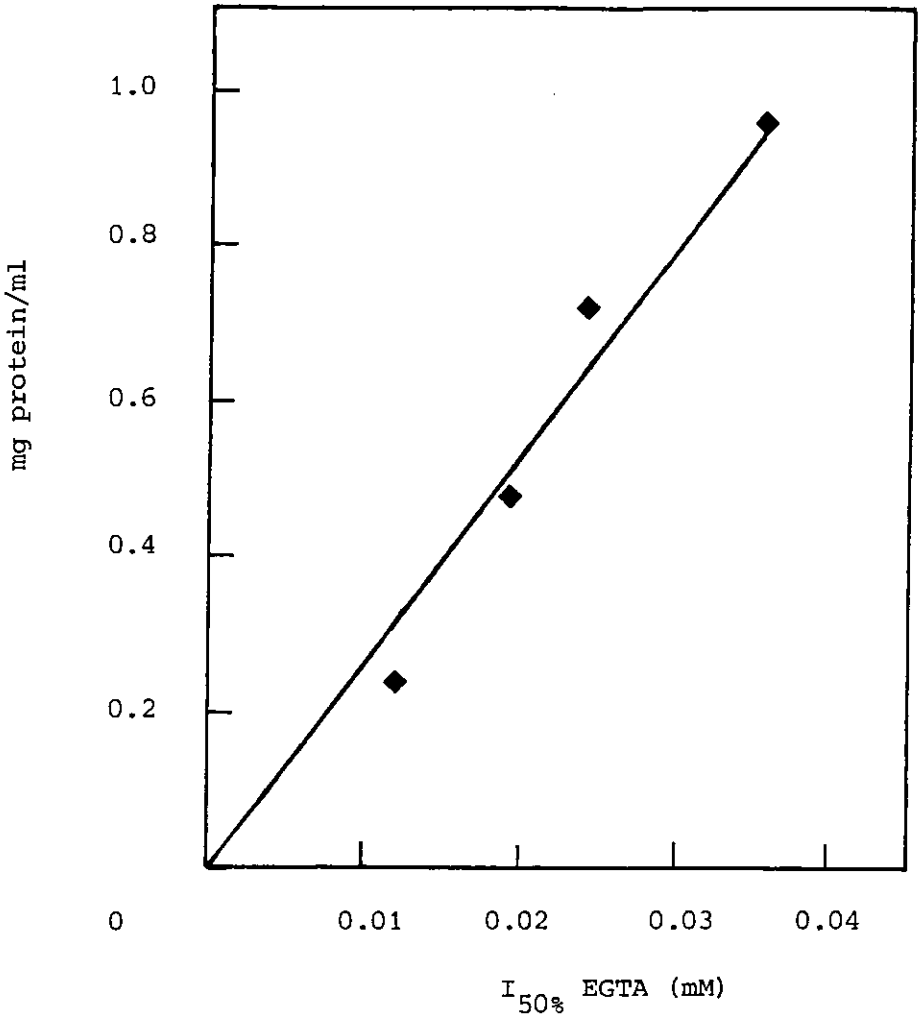


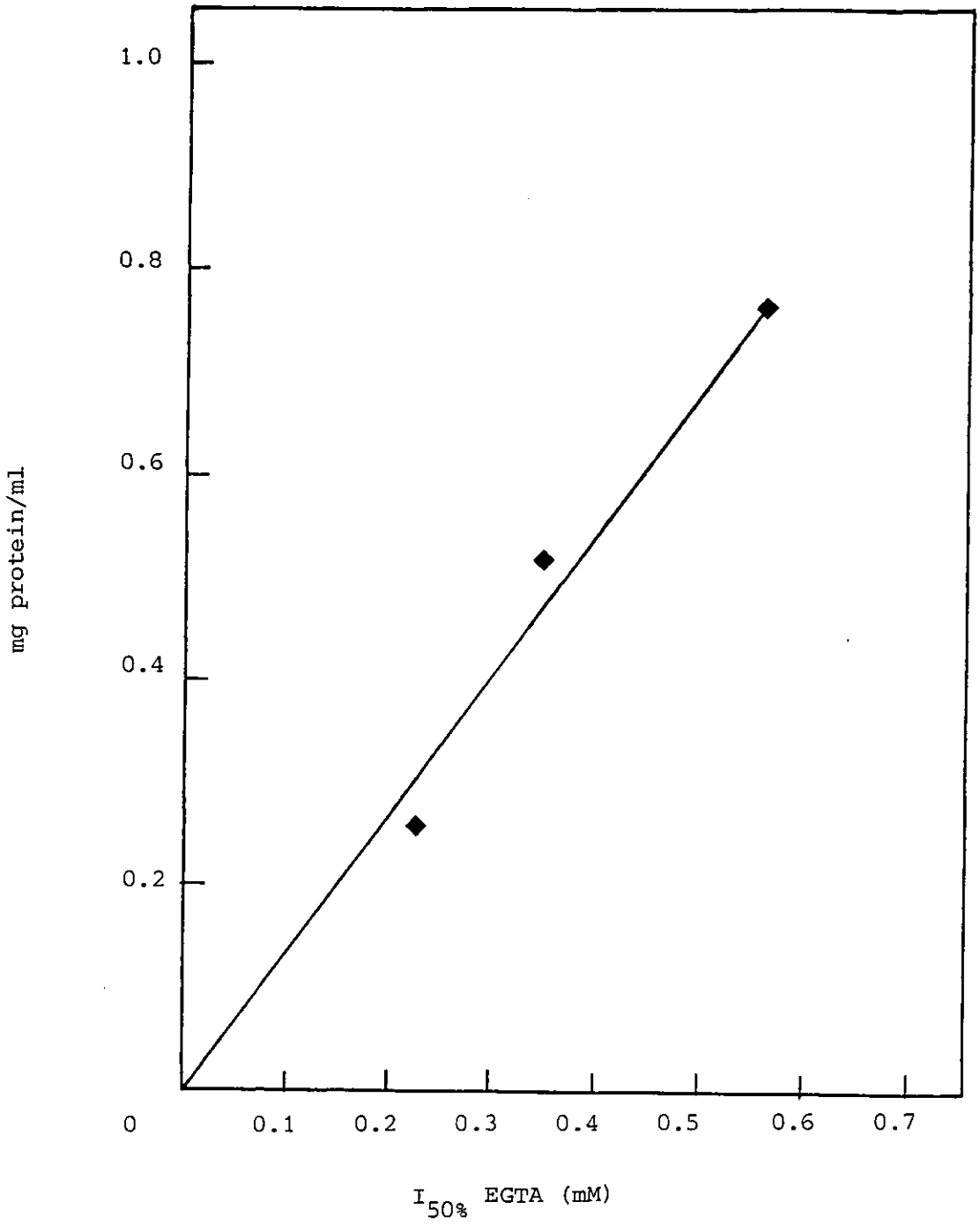

Figure 9. The relationship between the EGTA concentration causing 50% inhibition of NADH oxidation and the mitochondrial protein concentration

Rate of NADH oxidation, 199 nmol $O_2$/min/mg protein
Isolation of mitochondria, standard procedure, section 2.4.1

Additions, standard reaction medium, pH 7.2,
mitochondria, FCCP, EGTA, 30 sec later, NADH

The concentration of EGTA causing 50% inhibition of NADH was determined at several protein concentrations. By extrapolation, 38 nmol EGTA causes 50% inhibition per mg protein.

Figure 10. The relationship between the EGTA concentration causing 50% inhibition of NADH oxidation and the mitochondrial protein concentration (EDTA-free procedure).




Rate of NADH oxidation, 196 nmol $O_2$/min/mg protein.

Isolation of mitochondria, EDTA-free procedure at pH 7.2, section 2.4.2.

Additions as legend to figure 9.

The concentration of EGTA causing 50% inhibition of NADH oxidation was determined at several protein concentrations. By extrapolation, 730 nmol EGTA causes 50% inhibition/mg protein (cf. data in figure 9, standard preparation).

It might have been expected that the rate of NADH oxidation in the absence of EGTA would reflect the large discrepancy seen in the EGTA-sensitivity of NADH oxidation, depending on whether chelators were used during isolation or not, since this is probably related to mitochondrial calcium levels.

However, as the rate of NADH oxidation in the absence of EGTA was the same, whether isolated in the presence or absence of chelator (i.e., 173 and 175 nmol $O_2$/min/mg protein respectively) when assayed in standard (+2.5 mM $Mg^{2+}$) reaction medium, it is possible that the concentration of EDTA used in the isolation medium (5 mM) was insufficient to remove all the calcium associated with the exogenous NADH dehydrogenase and thereby produce a calcium-dependent NADH oxidation in the absence of a chelator. Assuming that calcium associated with exogenous NADH oxidation is located on the outer face of the inner membrane (i.e., is accessible to EDTA and EGTA, section 3.1.2.1), attempts were made to render NADH oxidation calcium-dependent using various washing procedures designed to fully deplete mitochondria of calcium.

### 3.2.3 Calcium depletion of mitochondria

#### 3.2.3.1 Use of EGTA washing medium

Mitochondria were prepared using chelator-free media, washed three times with media containing or lacking 5 mM EGTA as appropriate and finally washed free of chelator (see Methods).

No difference could be attributed to the presence or absence of chelator in the wash medium in the rate of NADH oxidation assayed in standard (+2.5 mM $Mg^{2+}$) reaction medium (170 and 172 nmol $O_2$/min/mg protein, washed plus and minus EGTA respectively). Moreover the

addition of calcium had no effect on the rate of NADH oxidation in mitochondria (3% stimulation, plus and minus EGTA respectively). Thus calcium associated with the operation of the exogenous NADH dehydrogenase did not appear to be available for chelation during EGTA-washing treatment.

However when the relationship between the rate of NADH oxidation and EGTA concentration causing 50% inhibition was examined, it was found that EGTA washing treatment caused a very considerable increase in sensitivity to EGTA treatment during NADH oxidation (figure 11). If the data presented in figure 11 for mitochondria washed with EGTA are expressed per mg protein (55 nmol EGTA causes 50% inhibition of NADH oxidation) they may be compared with data from mitochondria prepared using EDTA isolation medium (figure 9, section 3.2.2.1; 38 nmol EGTA/mg protein causes 50% inhibition of NADH oxidation). These values obtained using EDTA and EGTA are of a similar order of magnitude. In contrast, the data in figure 11 show that mitochondria isolated in the complete absence of chelator require approximately 13 times more chelator during NADH oxidation to effect 50% inhibition of the rate (715 nmol EGTA/mg protein).

As EGTA washing treatment causes a considerable decrease in the concentration of EGTA required to cause 50% inhibition of NADH oxidation it is reasonable to assume that the treatment is effective at removing some mitochondrial calcium; however, the depletion of calcium is not complete as NADH oxidation is not dependent on added calcium (in the absence of chelator).

3.2.3.2 Use of A23187 during NADH oxidation

EGTA washing treatment appeared to remove some mitochondrial calcium (previous section); further depletion of calcium was attempted

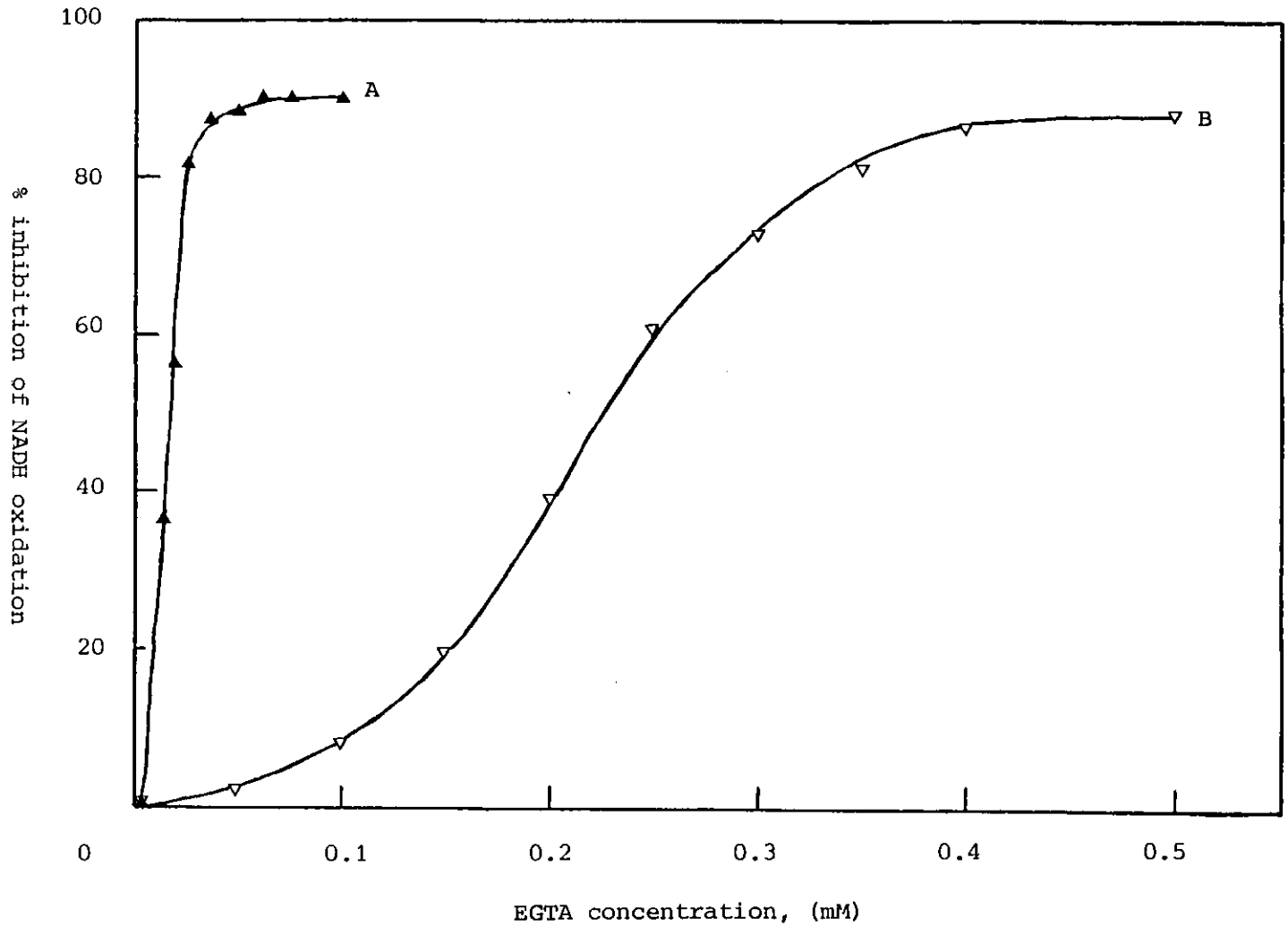
Legend to figure 11

Isolation of mitochondria, EDTA-free procedure, pH 7.2 (section 2.4.2).

A, Mitochondria washed 3 times with EGTA wash medium (table 2), followed by standard (EGTA-free) wash medium.
Rate of NADH oxidation, 185 nmol $O_2$/min/mg protein.
17.5 µM EGTA causes 50% inhibition in 0.32 mg protein/ml
(= 55 nmol EGTA/mg protein).

B, Control; mitochondria washed 4 times with standard wash mdeium (table 2).
Rate of NADH oxidation, 183 nmol $O_2$/min/mg protein.
225 µM EGTA cuases 50% inhibition in 0.28 mg protein/ml
(= 803 nmol EGTA/mg protein).

Figure 11. Relationship between EGTA concentration and inhibition of NADH oxidation in EGTA-washed mitochondria



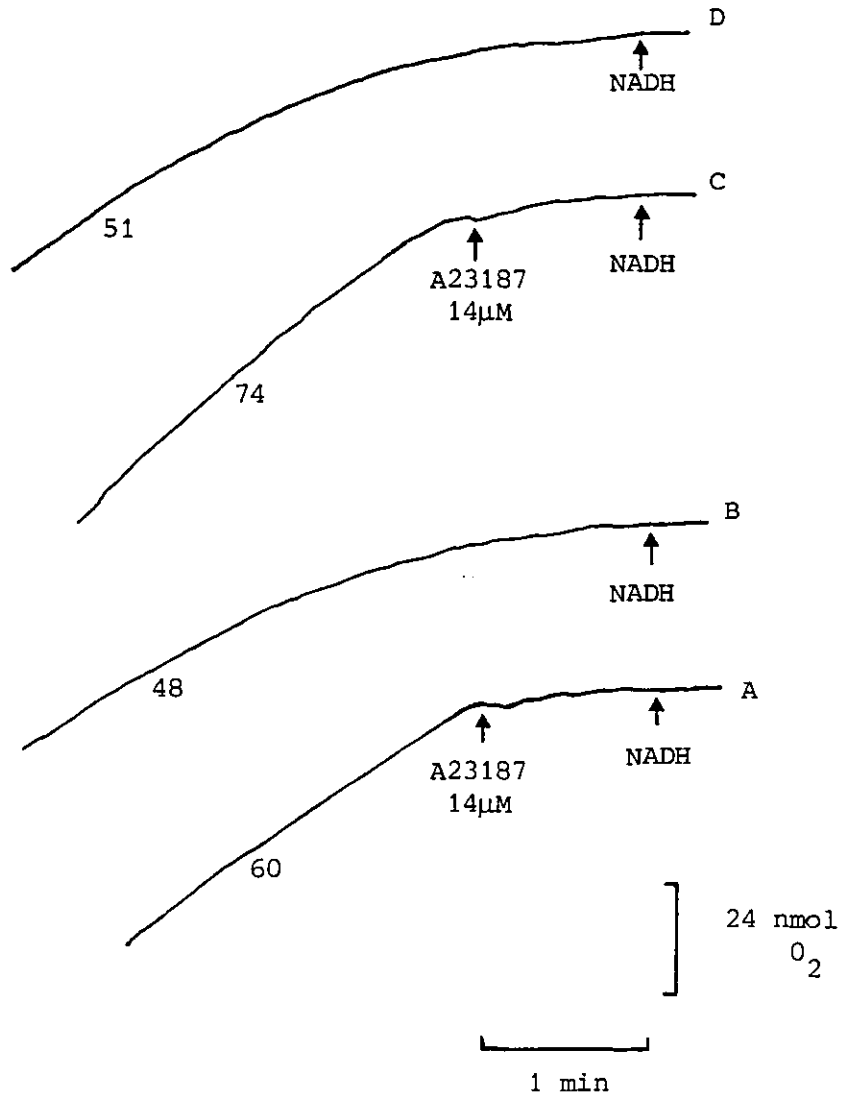
by adding A23187 to mitochondria oxidising NADH in the presence of EGTA.

As a divalent cation ionophore, A23187 is used to induce specific cation transport in mitochondria (Lardy *et al.*, 1967; Kafka and Holz, 1976; Reed and Lardy, 1972a; Pfeiffer and Lardy, 1976; Duszynski and Wojtczak, 1977; Gómez-Puyou and Gómez-Lojero, 1977). A23187 is considered to bind and transport calcium in preference to magnesium (Reed, 1972; Reed and Lardy, 1972a, 1972b; Truter, 1976).

Using standard (+2.5 mM $Mg^{2+}$) reaction medium, the effect of A23187 in the presence of EGTA on the rate of NADH oxidation was studied in mitochondria prepared using wash medium which contained or lacked EGTA as appropriate. It is found that A23187 increases the recovered rate of oxidation (figure 12). It is proposed that when mitochondria are treated with EGTA before NADH is added, the inhibition of NADH oxidation is due to the removal of essential calcium (section 3.2.1.3) from the membrane surfaces to which EGTA has access and furthermore that the observed recovery rate is due to calcium moving from the EGTA-inaccessible matrix. Therefore the increase in recovery rate observed when A23187 is present may be attributed to the additional movement of calcium mediated by A23187 from the matrix, replacing calcium removed by chelation with EGTA.

The concept that the recovery from EGTA pretreatment may only occur after the initiation of NADH oxidation was introduced earlier (section 3.2.1.2; table 8) and implies that there are further reserves of calcium inaccessible to EGTA during washing treatment which become available for activation of the exogenous NADH dehydrogenase. Support for this concept was supplied by the observation that in mitochondria pretreated with EGTA, the rate of NADH oxidation increased gradually

Figure 12. Effect of A23187 on the rate of NADH oxidation in mitochondria incubated with EGTA




Traces A & B.

Isolation of mitochondria, in presence of EGTA, at pH 7.2, section 2.4.3. Protein concentration, 0.18mg/ml.

Traces C & D.

Isolation of mitochondria, in absence of chelators, at pH 7.2, section 2.4.2. Protein concentration, 0.16mg/ml.

All traces, additions, standard reaction medium, pH 7.2, mitochondria, FCCP, EGTA (0.25mM, traces A & B; 1mM traces C & D); 30 sec later, NADH; 1 min later, 14μM A23187 (traces A & C).

(section 3.2.1.3) and was stimulated by A23187 (figure 12). Therefore in an attempt to demonstrate calcium involvement in exogenous NADH oxidation further washing treatments were devised using A23187 and NADH in addition to EGTA to induce increased calcium depletion.

3.2.3.3 Use of wash media containing EGTA, A23187 and/or NADH

A preparation of mitochondria was divided into five. One portion was washed twice with EGTA-only wash medium, while each of three further portions was washed twice with medium containing either A23187 or NADH or both in addition to EGTA; each of these four portions was then washed with standard wash medium while the control portion was washed three times with standard wash medium.

Data in table 9 show that none of the washing treatments significantly altered the rate of exogenous NADH oxidation when assayed in standard (+2.5 mM $Mg^{2+}$) reaction medium. It was noted that the rate of oxidation in mitochondria washed with 0.2 mM NADH during isolation was generally slightly faster (table 9) and in addition the maximum rate of oxidation was reached more rapidly. These observations suggested that the exogenous NADH dehydrogenase may have undergone activation or a change in its kinetics may have occurred during isolation.

The relationship between inhibition of exogenous NADH oxidation and EGTA concentration was also investigated. Data in figure 13 show that there is a great difference between the control and each treatment in the concentration of EGTA causing 50% inhibition of NADH oxidation, but essentially no difference between each of the four treatments tested. The concentration of EGTA causing 50% inhibition of exogenous NADH oxidation per mg control mitochondrial protein was 812 nmol compared with 42-50 nmol EGTA/mg protein in each of the treatments with

Table 9 Effect of EGTA and calcium on the rate of NADH oxidation in mitochondria treated with various wash media

| Treatment | Rate of NADH oxidation in absence of EGTA or calcium, nmol $O_2$/min/mg protein | EGTA concentration causing 50% inhibition, nmol/mg protein | Stimulation of rate of NADH oxidation by 1 mM calcium, % |
|---|---|---|---|
| EGTA wash medium (table 2) | 181 | 50 | 1.5 |
| + 0.2 mM NADH | 185 | 46 | 0 |
| + 0.1 mM A23187 | 179 | 48 | 0 |
| + 0.2 mM NADH + 0.1 mM A23187 | 190 | 42 | 1 |
| Control, standard wash medium only (section 2.3.2.1) | 178 | 812 | 0 |

Protein concentrations, 0.15 - 0.17 mg/ml.

A23187 concentration in wash medium, 200 nmol/mg protein.

All figures, mean values of 3 sets of data.

## Legend to figure 13

Rate of NADH oxidation, 190 nmol $O_2$/min/mg protein.

Isolation of mitochondria, EDTA-free procedure (section 2.4.2), pH 7.2.

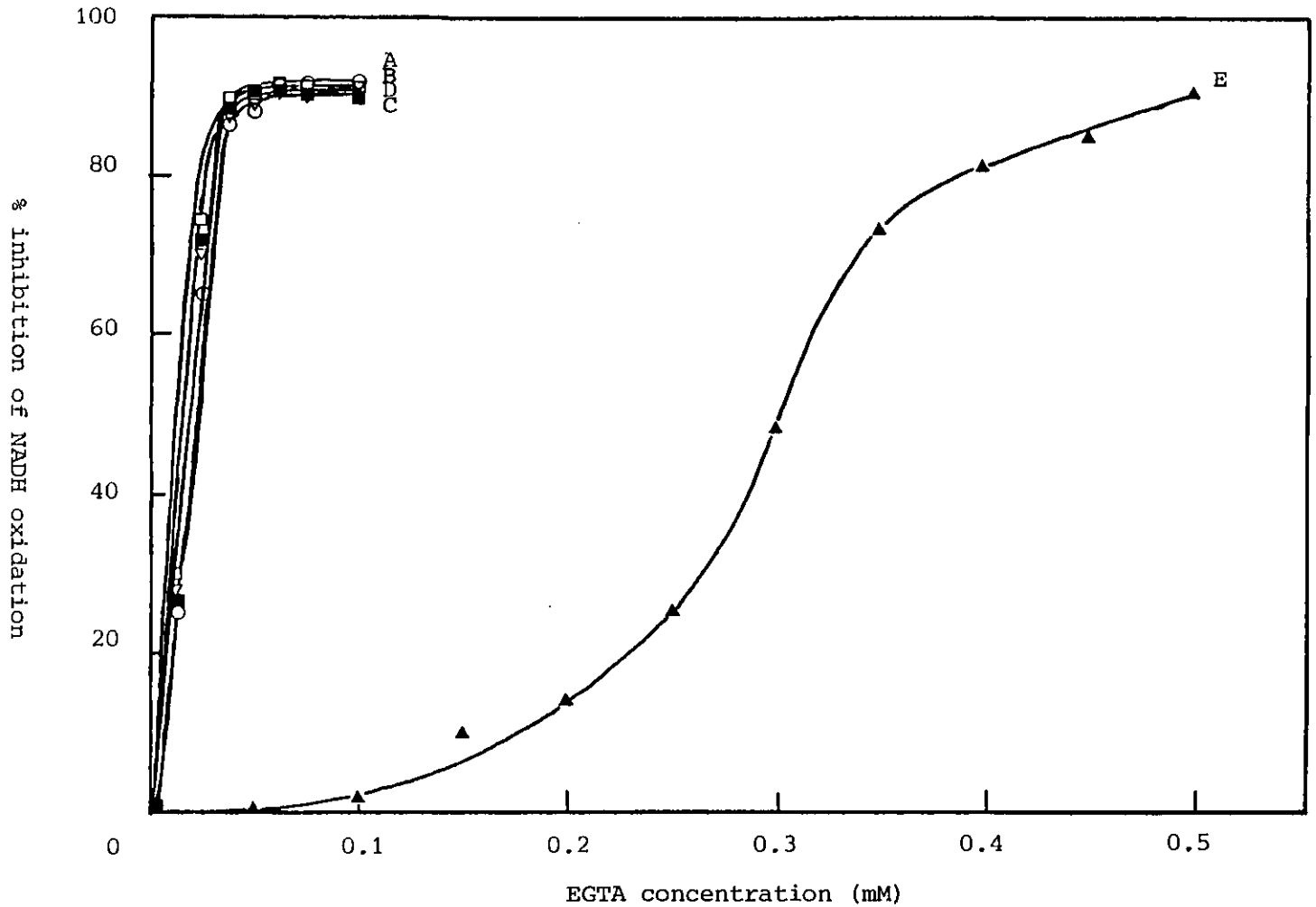
Each aliquot of mitochondria was washed with standard wash medium (table 2) plus the following additions as indicated. There was a final wash with standard wash medium.

Key

| | | Protein concentration (mg/ml) |
|---|---|---|
| A | + 2 mM EGTA | 0.41 |
| B | + 2 mM EGTA, 100 µM A23187 | 0.43 |
| C | + 2 mM EGTA, 0.2 mM NADH | 0.39 |
| D | + 2 mM EGTA, 100 µM A23187 + 0.2 mM NADH | 0.37 |
| E | control | 0.38 |

Additions: standard reaction medium (table 2), pH 7.2; mitochondria; FCCP; EGTA; 30 sec later, NADH.

Figure 13. Effect of various wash media on the rate of NADH oxidation in the presence of EGTA.



A23187, NADH or both in addition to EGTA. These values for the concentration of EGTA per mg protein to cause 50% inhibition of exogenous NADH oxidation may be compared with similar values obtained from mitochondria prepared both in the presence of EDTA and in the absence of any chelator (section 3.2.2.1).

The various washing media used in the experiments described above were unsuccessful in producing a preparation of mitochondria in which the oxidation of exogenous NADH was dependent upon the addition of calcium in the absence of EGTA. It was considered reasonable to interpret an increase in sensitivity to EGTA treatment as representing a loss of some mitochondrial calcium. It seemed therefore that a range of EGTA washing treatments was apparently effective at removing some mitochondrial calcium, yet because EGTA-washed mitochondria oxidised NADH at maximum rates in the absence of added calcium, this removal of calcium by EGTA washing treatments was limited; some calcium did not seem to be available for chelation but appeared to remain associated with the NADH dehydrogenase. It was not possible to elicit a calcium-dependent NADH oxidation in the absence of EGTA; however mitochondria subjected to washing treatments containing EGTA (±NADH, ±A23187) exhibited enhanced sensitivity to further EGTA treatment during NADH oxidation. Addition of A23187 and NADH to wash medium containing EGTA was unsuccessful at eliciting a significant increase in EGTA sensitivity or a calcium requirement for NADH oxidation. Only during NADH oxidation was EGTA treatment apparently effective at eliciting calcium-dependent NADH oxidation. In the next section an attempt is made to correlate the inhibition caused by EGTA in mitochondria prepared in various ways to the overall concentration of mitochondrial calcium.

### 3.2.4 The calcium concentration in Jerusalem artichoke mitochondria

Estimations of mitochondrial calcium content were made by acid extraction and atomic absorption spectroscopy (section 2.6.5) in mitochondria subjected to various washing programmes (as in section 3.2.3).

#### 3.2.4.1 Estimations of total calcium content by atomic absorption spectroscopy

Results are presented in table 8 which shows that mitochondria prepared in the absence of chelators were found to contain 188 nmol $Ca^{2+}$/mg protein. When assayed in standard (+ magnesium + phosphate) reaction medium, acceptable rates of NADH oxidation were obtained from mitochondria subjected to each washing treatment (185 ±12 nmol $O_2$/min/mg protein; data not presented).

EGTA treatment alone is effective at removing about 20% of the total mitochondrial calcium, suggesting that at least this proportion of the total calcium pool lies within the EGTA-accessible spaces of the mitochondria (outer membrane, outer face of inner membrane and intermembrane space) since EDTA and its analogues lack access to the matrix (Reed and Bygrave, 1974).

It was decided to investigate the effect of washing mitochondria with divalent cations in addition to chelator, to see whether this influenced the final overall calcium concentration. Any unbound divalent cation was removed using standard wash medium. Calcium and magnesium were used, being the most common inorganic divalent cations occurring in mitochondria. Decamethonium, $(DM)^{2+}$, an organic divalent cation was also used to investigate whether associated mitochondrial calcium could be displaced by a cation which can be expected to lack chemical specificity.

Washing mitochondria with chelator plus magnesium leads to

50% reduction (approximately) in the overall calcium concentration (table 10). These results indicate that magnesium plus chelator treatment is more effective at depleting mitochondria of calcium than is chelator treatment alone (50% cf. 20% depletion). Thus about half the total mitochondrial calcium appears to be in the extra-matrix spaces of the mitochondria.

Treatment with chelator plus calcium led to an elevation of calcium levels by approximately 40% whereas treatment with chelators plus the organic divalent cation $(DM)^{2+}$ leads to 50% reduction (approximately) in the overall calcium concentration, a similar reduction as caused by chelator plus magnesium treatment (table 10).

These results suggest that with chelators both inorganic and organic divalent cations are able to exchange with a pool of membrane-associated calcium, external to the matrix, part of which is apparently resistant to chelation using chelator alone. Moreover these results are consistent with the concept that the calcium is compartmentalised; about half the total mitochondrial calcium pool is apparently external to the matrix. It is possible to speculate that a proportion is associated with the polyuronide component of the outer membrane as suggested by Mannella and Bonner (1975).

3.2.4.2 The relationship between total mitochondrial calcium concentration and EGTA inhibition of NADH oxidation

Table 11 presents a collation of data on calcium levels in mitochondria from various tissues, both plant and animal sources. Mitochondria isolated in the absence of chelator from animal and plant sources may have similar levels of calcium (213 and 188 nmol calcium/mg protein, rat-lung and Jerusalem artichoke respectively). Following washing treatment with chelator however, there is considerable

Table 10 Calcium values in mitochondria subjected to different washing treatments

| Treatment | Washing treatments involving EGTA (nmol $Ca^{2+}$/mg protein) | Change in calcium levels | Washing treatments involving EDTA (nmol $Ca^{2+}$/mg protein) | Change in calcium levels |
|---|---|---|---|---|
| Control | 185 ±21 (4)<br>195 ±19 (2) | | 185 ±9 (2) | |
| + chelator | 131 ±18 (4) | 20-25% decrease | 204 ±15 (2) | |
| + chelator + magnesium | 96 ±5 (4)<br>119 ±16 (2) | = ca. 50% decrease | 88 ±8 (2) | = ca. 50% decrease |
| + chelator + calcium | 254 ±3 (4)<br>293 ±8 (2) | = ca. 40% increase | 246 ±11 (2) | = ca. 40% increase |
| + chelator + $(DM)^{2+}$ | 107 ±11 (2) | = ca. 50% decrease | N.D. | |

Isolation, low salt procedure (section 2.4.4). Typical washing treatment flow diagram, section 2.4.5.

Control, mean calcium concentration, 188 ±32 nmol/mg protein (18 replicates).

N.D., not done. Figures in brackets denote number of replicates.

Table 11 Calcium levels in mitochondria from various tissues

| Tissue | Isolation ± chelator | Calcium, nmol/mg protein | Magnesium, nmol/mg protein | Reference |
|---|---|---|---|---|
| rat-lung | - chelator | 213 | 102 | Fisher *et al.* (1973) |
| rat-lung | + EDTA | 28 | 23 | *ibid.* |
| rat-liver | + EDTA | 7 | N.D. | *ibid.* |
| rat-liver | + EDTA | 5 | N.D. | Carafoli & Lehninger (1971) |
| Jerusalem artichoke tuber | - chelator | 188 | N.D. | this thesis, table 10 |
| Jerusalem artichoke tuber | + EGTA | 131 | N.D. | *ibid.* |
| Jerusalem artichoke tuber | + EGTA + either $Mg^{2+}$ or $(DM)^{2+}$ | 107 | N.D. | *ibid.* |
| sweet potato | + EDTA | 0.43 | N.D. | Chen & Lehninger (1973) |
| turnip | + EDTA | 4.53 | N.D. | *ibid.* |

All values derived from atomic absorption spectroscopy.

variation in calcium levels of plant and animal mitochondria ranging from 0.43-131.00 nmol calcium/mg protein. The range of calcium values per mg protein indicates that the components of mitochondria from different tissues have differing affinity for calcium.

Considering only the pool of calcium apparently located externally to the matrix (i.e., extra-matrix) it is of interest to recall the concentration of EGTA causing 50% inhibition of NADH oxidation in both mitochondria isolated in the absence and presence of chelator (EDTA) i.e., 730 and 38 nmol EGTA/mg protein respectively (section 3.2.2.1). It is also clear from a comparison of these data that the relationship between the concentration of EGTA causing 50% inhibition of exogenous NADH oxidation and extra-matrix calcium per mg protein alters following washing treatment with EGTA. Mitochondria isolated in the absence of chelator (total calcium 188 nmol/mg protein) have roughly 94 nmol extra-matrix calcium/mg protein (section 3.2.4.1) and require 730 nmol EGTA/mg protein to cause 50% inhibition of NADH oxidation (9-fold excess). On the other hand, mitochondria isolated in the presence of EDTA alone (i.e., no divalent cations added during isolation) have roughly 37 nmol extra-matrix calcium/mg protein (section 3.2.4.1) but require roughly 38 nmol EGTA/mg protein to cause 50% inhibition of NADH oxidation (1.3-fold excess). These observations therefore suggest that mitochondria isolated in the absence of chelator have other divalent cations (probably magnesium) in addition to calcium associated with the membranes and available for chelation with EGTA. This is the simplest explanation to account for why approximately only 50 nmol calcium/mg protein (i.e., the difference in calcium concentration arising from the presence or absence of chelator) should be involved in the 50% inhibition of NADH oxidation brought about by

approximately 800 nmol EGTA/mg protein (i.e., the difference in EGTA concentration causing 50% inhibition of NADH oxidation arising from the presence and absence of chelator). Thus when isolated in the presence of chelator the mitochondria are relatively depleted of associated divalent cations and are therefore much more sensitive to treatment with EGTA.

3.2.5 The effect of pH 8.2 on EGTA inhibition of exogenous NADH oxidation

Previous attempts to obtain mitochondria in which exogenous NADH oxidation was calcium-dependent in the absence of chelator were ineffective (assayed in standard reaction medium, sections 3.2.3-3.2.3.3). Because EGTA chelates more effectively at alkaline pH (due to release of protons upon the formation of the metal-EGTA complex) it was decided to investigate the effect of EGTA at alkaline pH on exogenous NADH oxidation for if EGTA inhibition of NADH oxidation was enhanced, this would be consistent with the involvement of divalent cations in exogenous NADH oxidation.

As indicated in section 3.1.2.1, there are various reports which suggest that in the presence of an energy source, the inner mitochondrial membrane is permeable to EDTA (and presumably EGTA too) at alkaline pH (>8.0, Settlemire *et al.*, 1968; Wehrle *et al.*, 1976) in heart mitochondria. But as experiments investigating the effect of EDTA on NADH oxidation at pH 8.2 were always performed in the presence of FCCP, added immediately after the mitochondria were suspended in reaction medium, before any other additions were made, it is considered that even at alkaline pH, EGTA remained outside the inner membrane as a non-permeant anion. (As an uncoupling agent, FCCP dissipates the pmf across the inner membrane.)

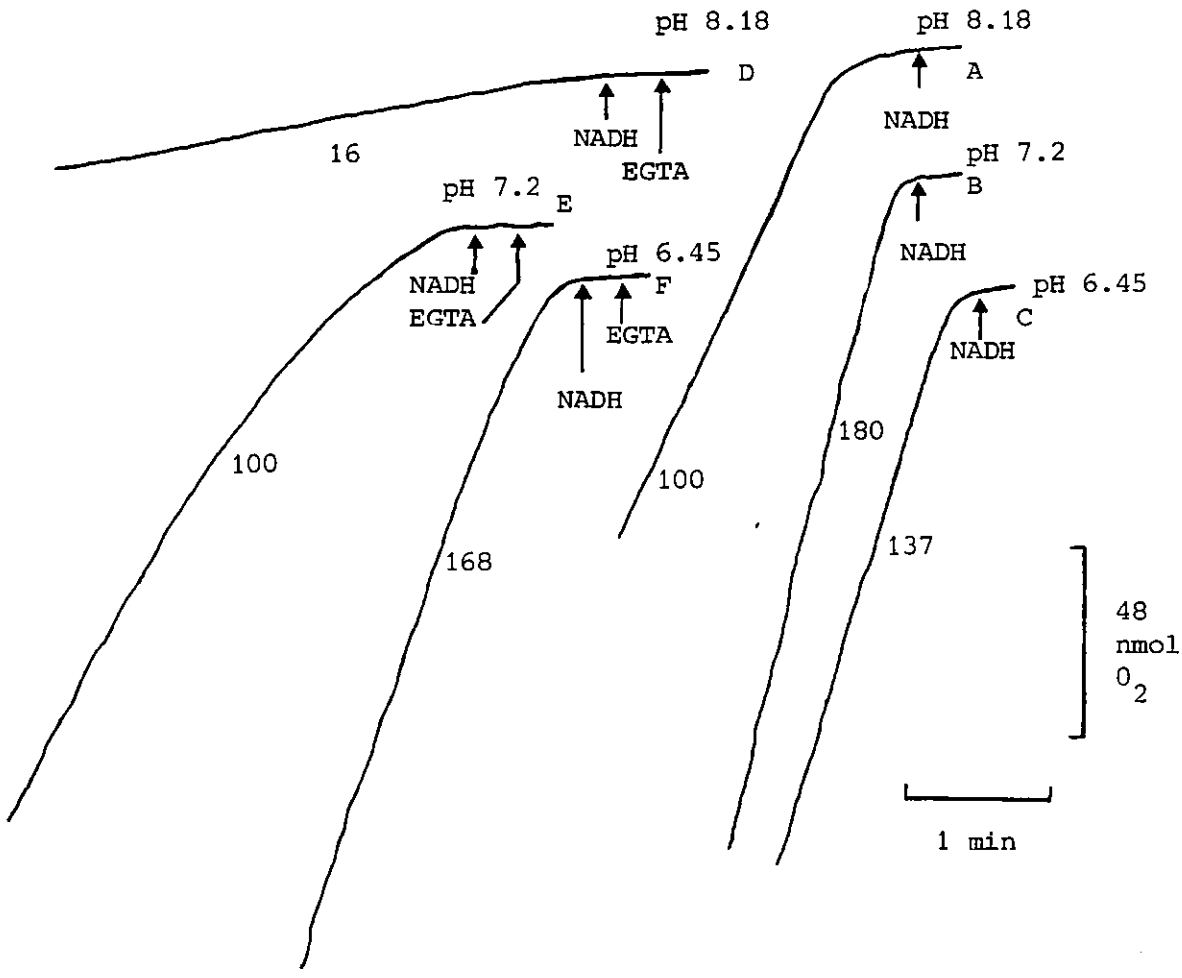
3.2.5.1 Influence of pH on the potency of EGTA as an inhibitor of exogenous NADH oxidation

Mitochondria were prepared in the absence of EDTA or EGTA. Rates of NADH oxidation were measured in mitochondria pre-incubated with EGTA at various pH values using standard reaction medium (i.e., in the presence of magnesium and phosphate) adjusted with potassium hydroxide solution. Several oxygen electrode traces are presented in figure 14 which show the rate of exogenous NADH oxidation at three pH values, 6.45,7.20 and 8.18, both in the absence of EGTA and after pre-incubation with 0.5 mM EGTA. The rate of NADH oxidation was constant at pH 6.45 and7.20 but at pH 8.18, after consumption of approximately 60% oxygen, the rate began to decrease slightly. Experiments at pH 8.18 were therefore carried out at between 100-40% oxygen saturation of the reaction medium. After pre-incubation with EGTA at pH 8.18 the chart recorder traces of EGTA-inhibited NADH oxidation were much less curved than similar traces obtained at pH 7.2. EGTA inhibition of exogenous NADH oxidation was greater at pH 8.2 than at pH 7.2 (figure 14).

Both the rate of NADH oxidation (±1 mM EGTA) and the % inhibition of NADH caused by EGTA vary with pH (figure 15). Expressed in nmol $O_2$/min/mg protein, at pH values of7.20, 6.45 and 8.2, the rate of exogenous NADH oxidation is 180, 128 (=29% inhibition cf. pH 7.2) and 109 (=40% inhibition cf. pH 7.2) respectively. With 1 mM EGTA there is no inhibition of NADH oxidation at pH 6.45 whereas at pH 8.2, inhibition is approximately 90%.

The extent of the EGTA inhibition of NADH oxidation was measured over a range of concentrations of EGTA at pH values of7.20, 6.45 and 8.2 respectively (figure 16). For example 1 mM EGTA caused

Figure 14. The oxidation of NADH in the presence and absence of EGTA at 3 pH values.



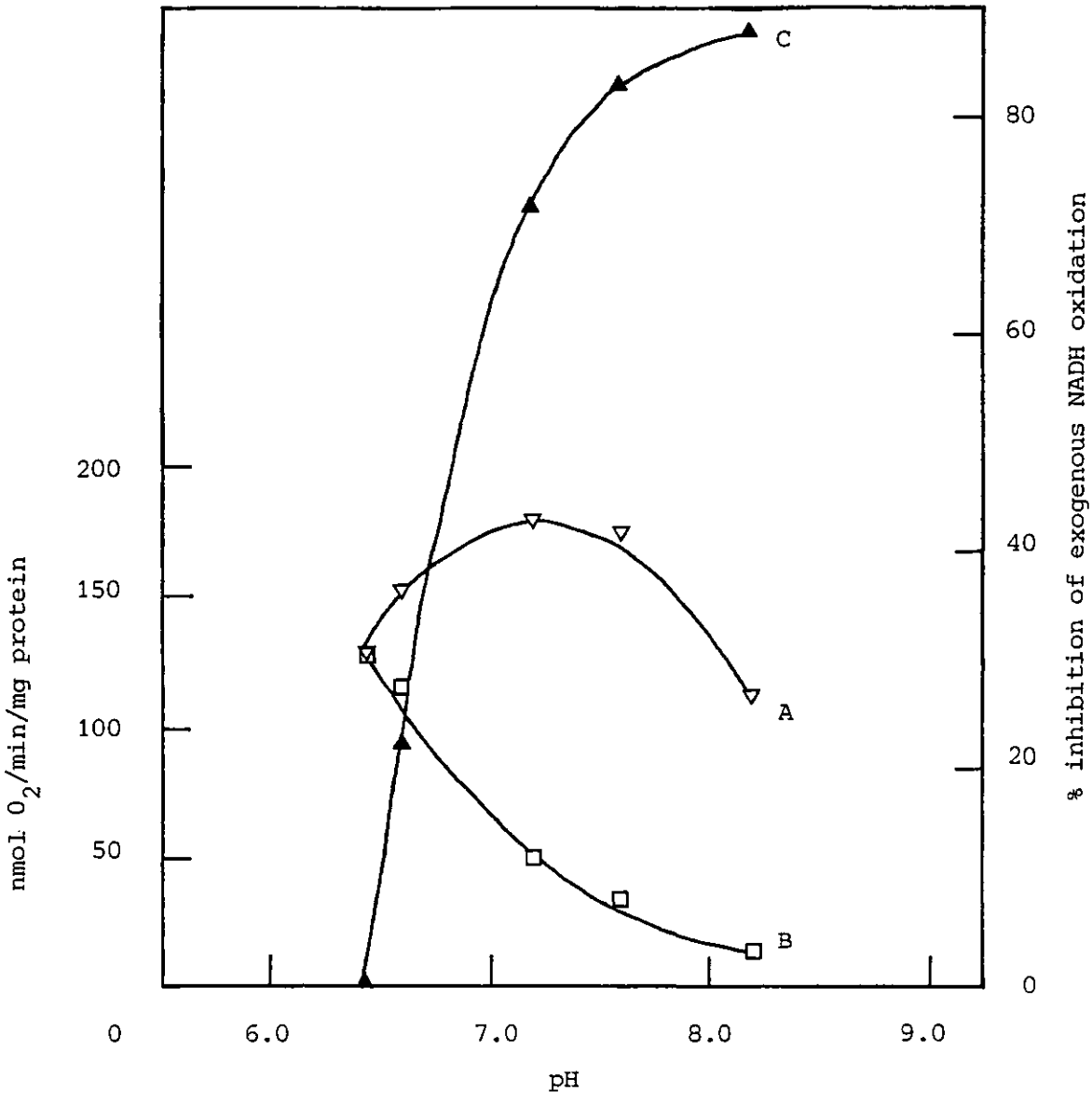

Rates, nmol $O_2$/min/mg protein.

Isolation of mitochondria, standard procedure, section 2.4.1.
Details as in legend figure 1.

Mitochondria were suspended in standard reaction media (table 2) adjusted using KOH solution to pH 8.18, 7.20 or 6.45 respectively.

Additions, standard reaction medium, mitochondria, FCCP, EGTA (0.5mM) (traces D,E & F only) NADH.

Figure 15. The influence of pH on the rate of NADH oxidation and EGTA inhibition



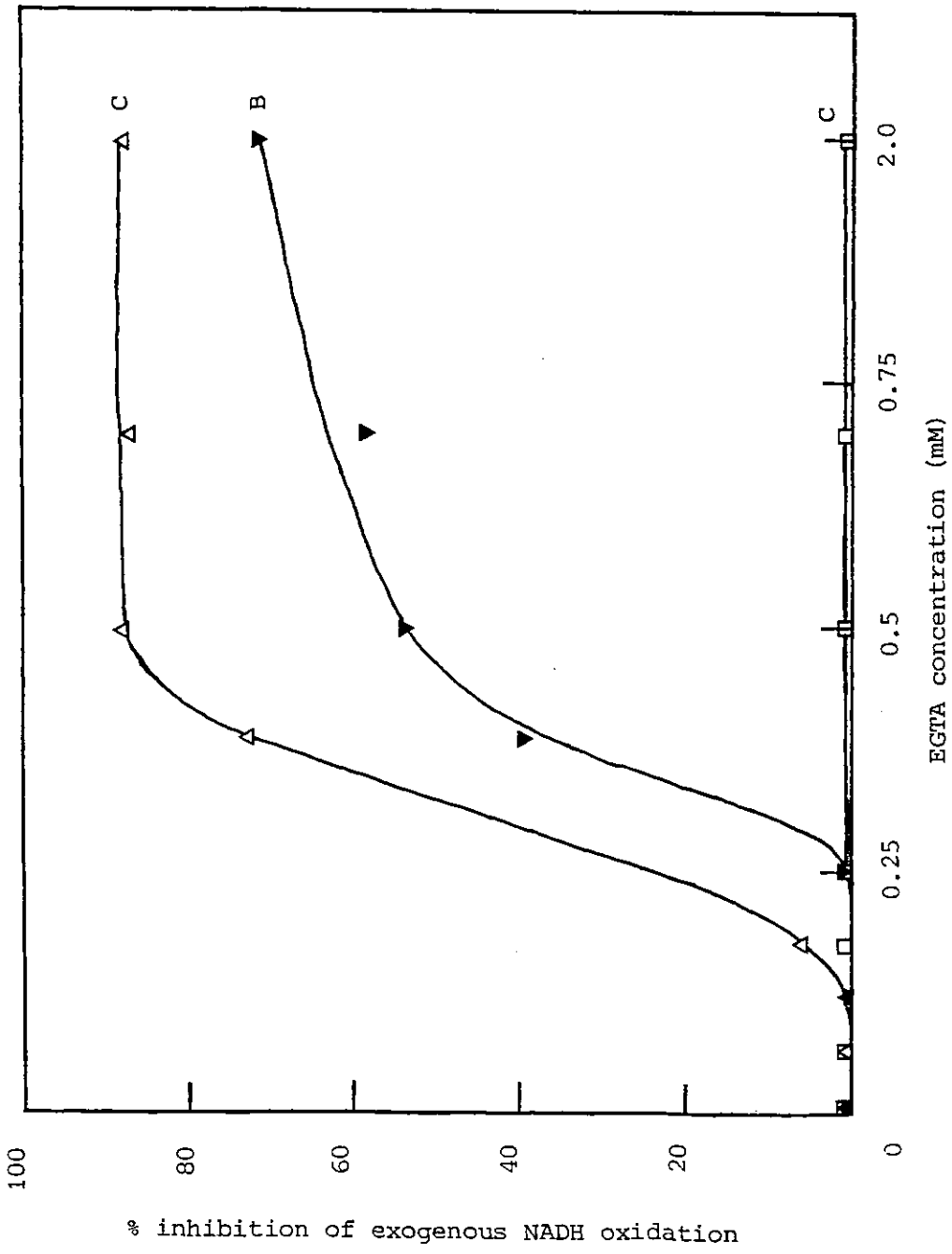

Mean rate of NADH oxidation at pH 7.2, 197 nmol $O_2$/min/mg protein
Isolation of mitochondria, pH 7.2, EDTA-free procedure, section 2.4.2.
Additions, standard reaction medium at specified pH values,
mitochondria, FCCP, EGTA, (when appropriate plus 30 sec lag)
NADH

Key: A influence of pH on NADH oxidation
B influence of pH on NADH oxidation + EGTA
C relationship between pH and EGTA-inhibition of NADH oxidation

Protein concentration, 0.74mg/ml (% inhibition)
(A&B nmol $O_2$/min/mg protein)

Figure 16. EGTA inhibition of NADH oxidation at 3 pH values

Rate of NADH oxidation at pH 7.2, 184 nmol $O_2$/min/mg protein
Isolation of mitochondria, pH 7.2, EDTA-free procedure, section 2.4.2.
Additions, standard reaction medium at specified pH values, mitochondria, FCCP, EGTA, 30 sec later, NADH.
Key: A pH 6.45
B pH 7.20
C pH 8.18



no inhibition of exogenous NADH oxidation at pH 6.45 compared with 0.5 mM EGTA causing 88% inhibition at pH 8.2. The results in figure 16 may be expressed as nmol EGTA causing 50% inhibition of NADH oxidation per mg protein, i.e., 0.43, 0.62 and >1.00 for pH 8.2, 7.2 and 6.45 respectively. The estimated total calcium concentration for mitochondria isolated in the absence of chelator is 188 nmol calcium/mg protein (section 3.2.4.1).

3.2.5.2 Effect of EGTA wash treatment at pH 8.2 on the rate of exogenous NADH oxidation at pH 7.2

A batch of mitochondria, prepared using chelator-free isolation medium at pH 7.2 was divided into two. One portion of mitochondria was washed twice with medium containing EGTA (+ A23187 + NADH) at pH 8.2 while the control portion was washed with medium at pH 7.2. (See tables 1&2). Both portions of mitochondria were then washed with standard (EGTA-free) wash medium and suspended in standard (+ magnesium + phosphate) reaction medium at pH 7.2. The rate of NADH oxidation was then measured in the presence of increasing concentrations of EGTA.

The data in figure 17 show that washing with EGTA at pH 8.2 did not alter the concentration of EGTA found to inhibit NADH oxidation by 50% at pH 7.2. The data in figure 17 can be expressed as 57 nmol EGTA/mg protein causing 50% inhibition of NADH oxidation in mitochondria washed with EGTA at both pH 7.2 and 8.2.

The results support the view that at pH 8.2 during wash treatment EGTA had access to the same calcium pool as found at pH 7.2.

3.2.5.3 pH and the relationship between protein concentration and inhibition of NADH oxidation

The relationship between protein concentration and the EGTA

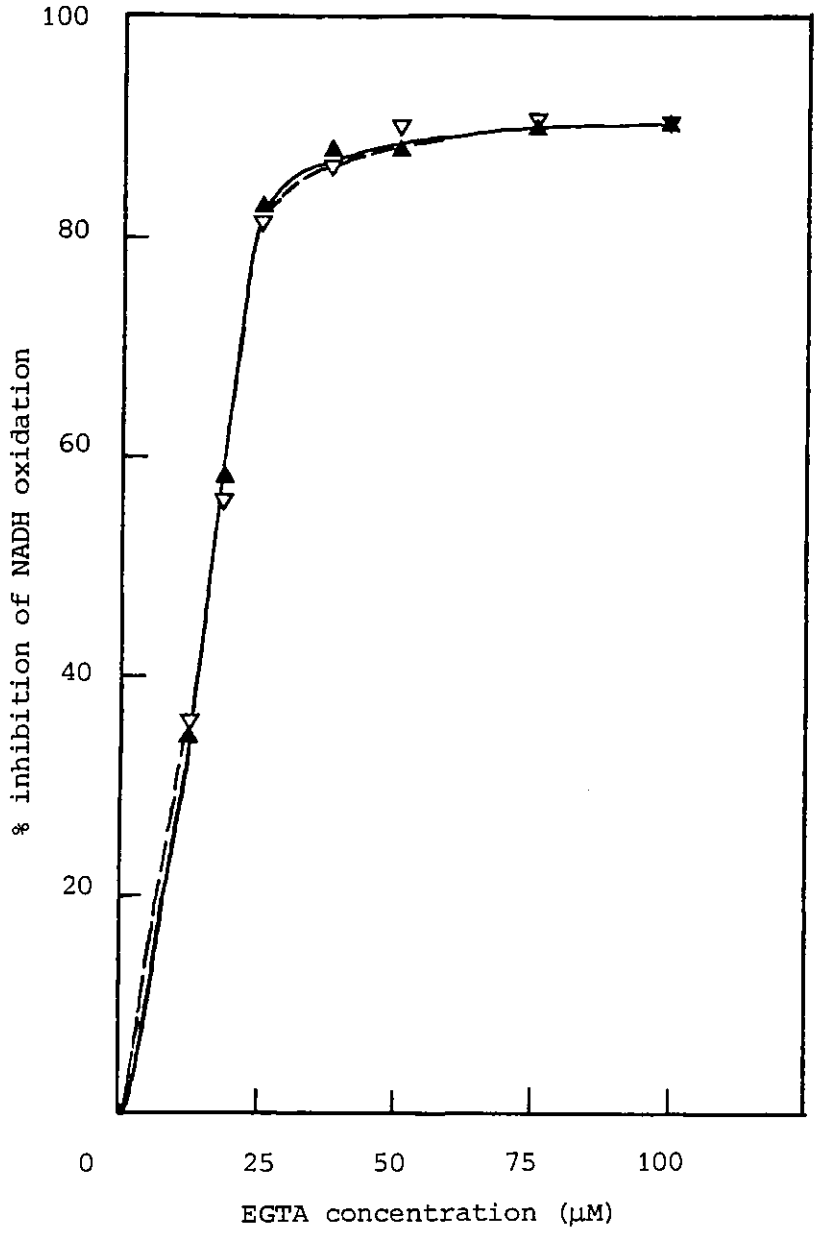



Figure 17. Influence of EGTA wash treatment at pH 8.18 on EGTA inhibition of NADH oxidation assayed at pH 7.2

Isolation of mitochondria, pH 7.2, EDTA-free procedure, section 2.4.2. Mitochondria were then washed at either pH 8.18 or 7.2 respectively with medium containing 2mM EGTA, 100μM A23187 and 0.2mM NADH.

Additions, standard reaction medium, pH 7.2, mitochondria, FCCP, EGTA, 30 sec later, NADH.

Key: A EGTA-washed at pH 8.18
B EGTA-washed at pH 7.2

16 nmol EGTA causes 50% inhibition of NADH oxidation/0.28mg protein (=57 nmol EGTA/mg protein).

concentration causing 50% inhibition in mitochondria pre-incubated with EGTA was investigated at pH 8.18 Would an increase in the efficiency of EGTA as a chelator alter the relationship which existed at pH 7.2 which indicated that mitochondrial divalent cation binding sites might be involved in the oxidation of exogenous NADH?

Mitochondria were isolated in the absence of chelators. Inhibition of NADH oxidation in mitochondria pre-incubated with EGTA at pH 8.18 was measured at three protein concentrations. The data in figure 18 show that the concentration of EGTA to cause 50% inhibition of NADH oxidation was directly related to mitochondrial protein concentration. This result supported the view that at pH 8.18 mitochondrial divalent cation binding sites are involved in the oxidation of NADH, as was found to be the case at pH 7.2. The concentration of EGTA causing 50% inhibition of NADH oxidation expressed per mg protein at pH 8.18 was found to be 360 nmol EGTA compared with 843 nmol EGTA at pH 7.2, showing that on average, EGTA treatment at pH 8.2 during exogenous NADH oxidation was more than twice as effective at inhibiting exogenous NADH oxidation. This observation is in agreement with the suggestion that there are several pools of mitochondrial calcium, one available for chelation with EGTA during washing treatments, another becoming available for chelation with EGTA with the onset of NADH oxidation (section 3.2.3.1) in the presence of standard (+ magnesium) reaction medium.

3.2.5.4 The effect of EGTA on NADH oxidation at pH 8.18

It is of interest to examine the influence of EGTA on NADH oxidation at pH 8.18 when the sequence of additions is NADH-EGTA because sequence of addition of EGTA and NADH determines the pattern

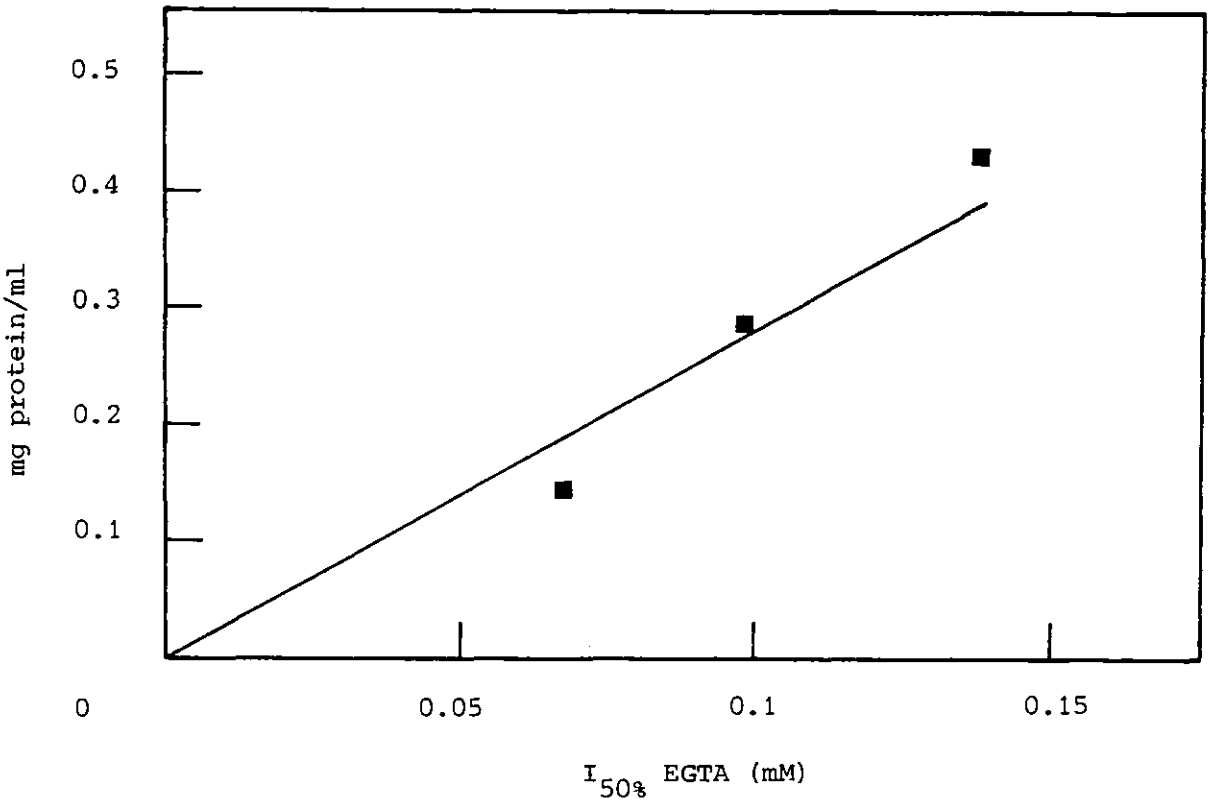



Figure 18. Relationship between the EGTA concentration causing 50% inhibition of NADH oxidation at pH 8.2 and the mitochondrial protein concentration (EDTA-free procedure).

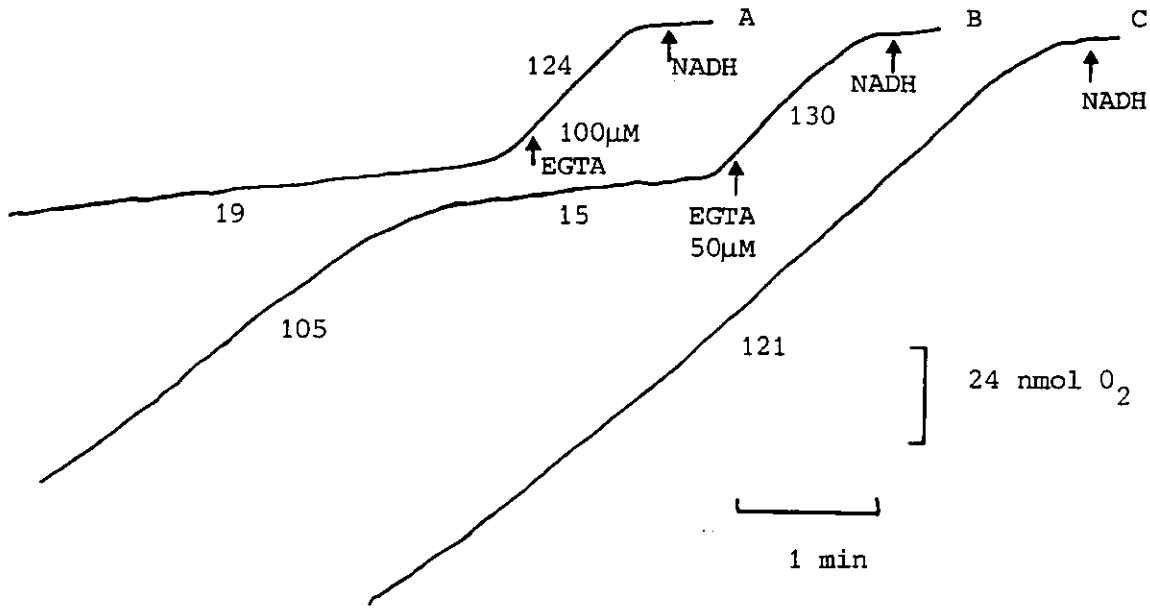
Rate of NADH oxidation, pH 8.2, 125 nmol $O_2$/min/mg protein.
Isolation of mitochondria, pH 7.2, EDTA-free procedure, section 2.4.2.
Additions, standard reaction medium, pH 8.2, mitochondria, FCCP, EGTA, NADH.
By extrapolation, 360 nmol EGTA cause 50% inhibition of NADH oxidation/mg protein at pH 8.2.

of inhibition at pH 7.2 (section 3.1.2.5).

Mitochondria were prepared in the absence of chelator and a comparison was made at pH 8.18 of the inhibition of NADH oxidation caused by EGTA pre-incubation (section 3.1.5.1, figures 14, 15 and 16) with the inhibition caused by the addition of EGTA to mitochondria already oxidising NADH. Typical oxygen electrode traces of NADH oxidation at pH 8.18 are presented in figure 19. The data in trace A show how 0.5 min after the addition of 0.1 mM EGTA to mitochondria oxidising NADH the rate of oxidation decreased to a constant value of 85% inhibition. This trace may be compared with trace A in figure 6 which shows that 1.75 min after the addition of 0.15 mM EGTA (at pH 7.2) the rate of NADH oxidation was just 70% inhibited (same protein concentration). Thus at pH 8.2, EGTA inhibition is greater and maximum inhibition is achieved more quickly. Because alkaline pH favours increased chelation of divalent cations by EGTA, especially calcium, these results are consistent with the hypothesis that calcium is involved in exogenous NADH oxidation.

However, when lower concentrations of EGTA are added during NADH oxidation at pH 8.18 an interesting phenomenon is observed and a typical example is presented in trace B of figure 19. When 50 μM EGTA is added to 0.18 mg protein/ml the rate of NADH oxidation decreases rapidly to a constant value of 85% inhibition. This level of inhibition lasts for approximately 2 min until the rate of oxidation rapidly increases to another constant level of 19% inhibition of NADH oxidation. The manifestation of two distinct levels of inhibition of NADH oxidation is only found at pH 8.18-8.2 when the sequence of additions is NADH-EGTA. Further investigations were made into these observations. The size of the second level of inhibition is found to vary with EGTA

Figure 19. Addition of EGTA at pH 8.18 to mitochondria oxidising NADH




Rates, nmol $O_2$/min/mg protein.

Isolation of mitochondria, pH 7.2, EDTA-free procedure, section 2.4.2.

Additions, standard reaction medium, pH 8.18, mitochondria, FCCP, NADH, EGTA as appropriate.

Protein concentration, 0.18mg/ml.

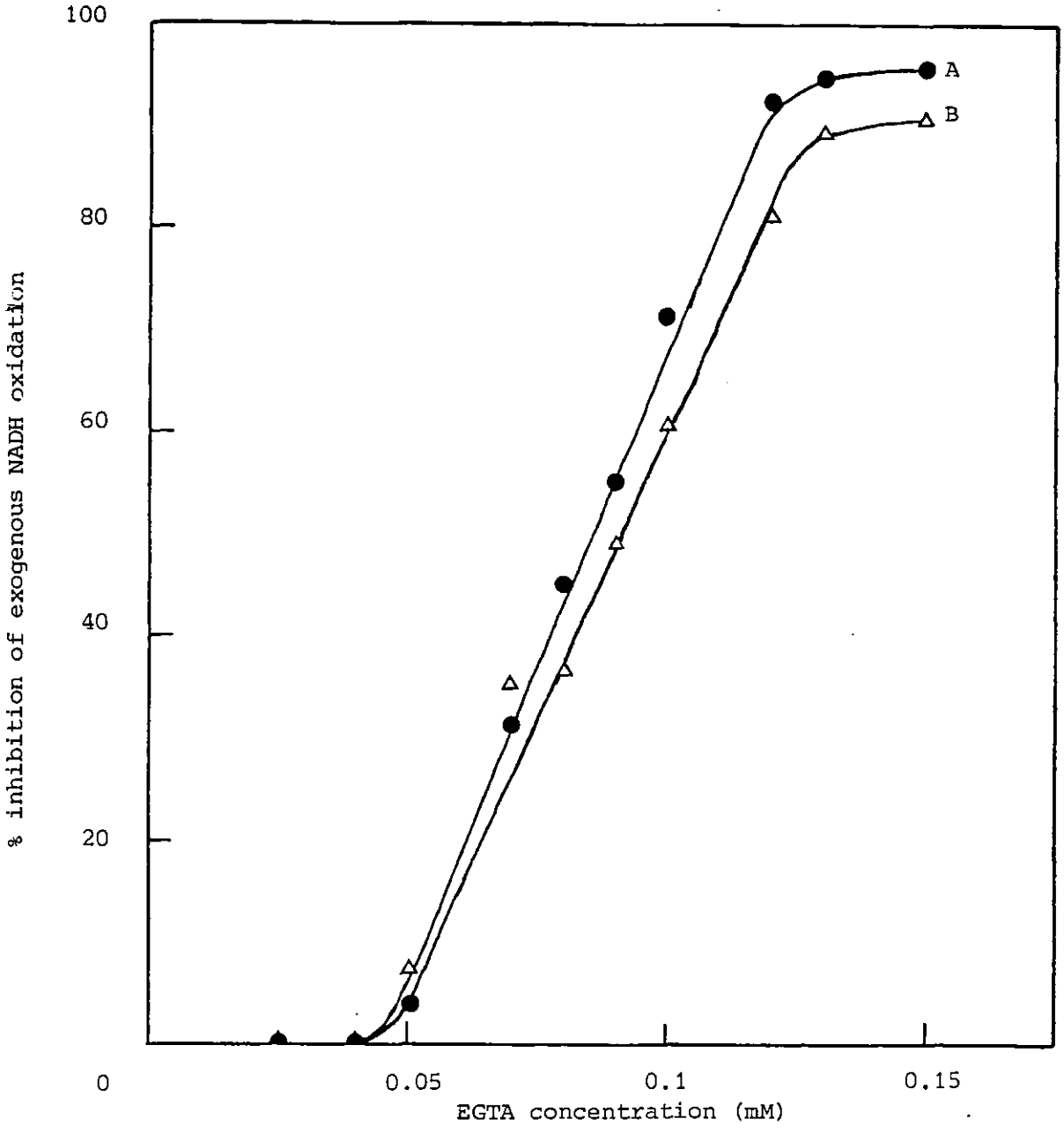
concentrations (figure 20). The first level of inhibition is constant in magnitude (figure 19, trace B) but its duration varies directly with the concentration of EGTA and this is further investigated in the following section.

3.2.5.5 Two levels of EGTA inhibition and A23187

The relationship between the duration of the first level of inhibition and EGTA concentration in mitochondria treated with EGTA when oxidising NADH at pH 8.18 was further examined at three protein concentrations. The duration of the first level of inhibition is taken to be from the intercept of the rates before and immediately after the addition of EGTA to the intercept of the first and second levels of inhibition (figure 19). The duration of the first level of inhibition depends on EGTA concentration (figure 21). This relationship varies with mitochondrial protein concentration, i.e., an increase in protein concentration leads to a decrease in the duration of the inhibition at a given EGTA concentration. Thus both the duration of the first level of inhibition and the magnitude of the second level of inhibition appears to depend on EGTA concentration and varies with protein concentration. As the concentration of EGTA is increased the duration of the first level of inhibition increases and the transition between the first and second levels of inhibition becomes less precise. The influence of protein concentration is consistent with the notion that a mitochondrial component such as calcium is responsible for the observed relationship.

What appears to occur when EGTA is added to mitochondria oxidising NADH at pH 8.2 is the removal by chelation of the EGTA-accessible calcium pool (i.e., extra-matrix calcium) including that which is associated with the exogenous NADH dehydrogenase. The first

Figure 20. EGTA-inhibition of NADH oxidation at pH 8.18.

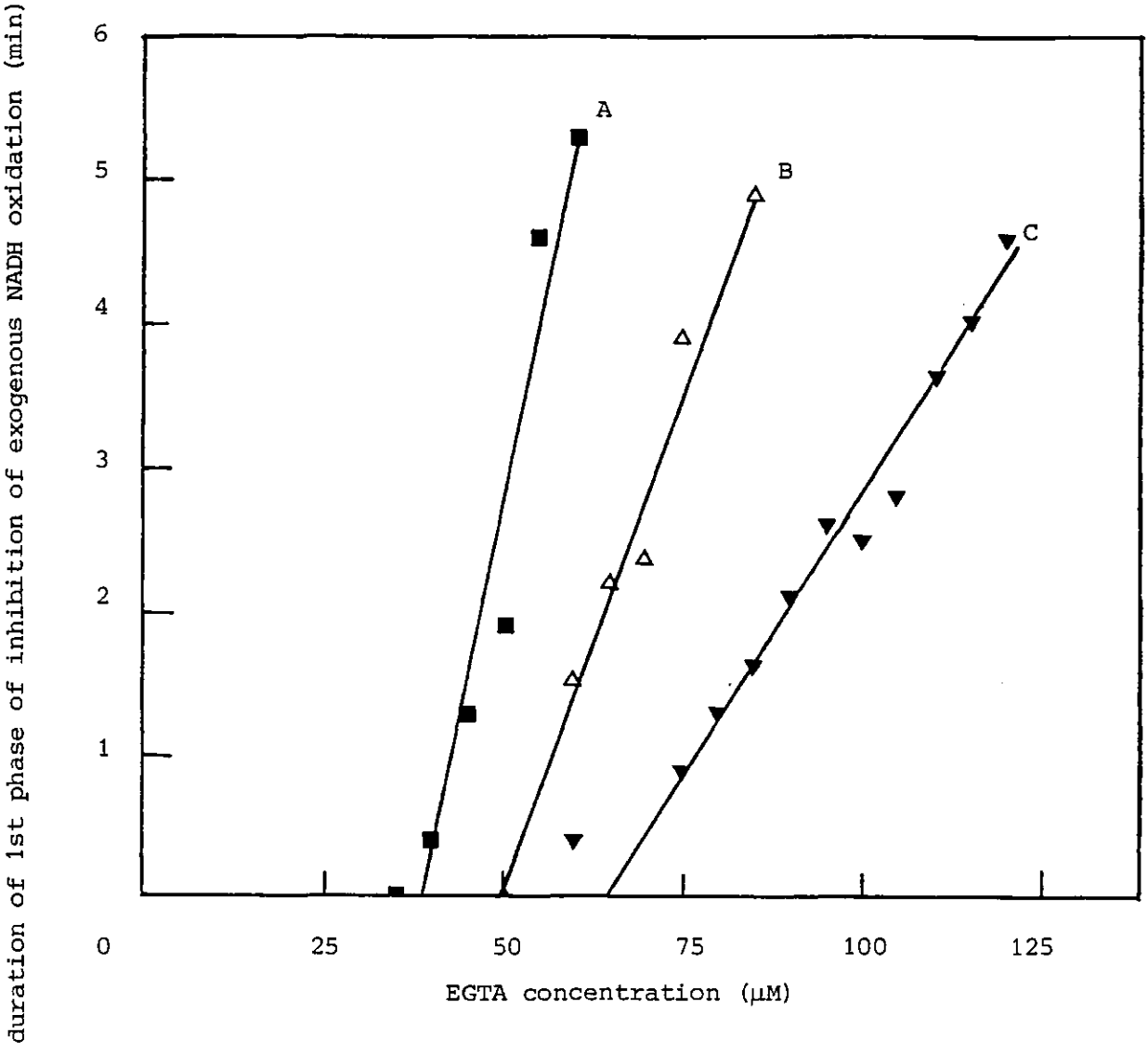



Rate of NADH oxidation, pH 8.18, 115 nmol $O_2$/min/mg protein.
Isolation of mitochondria, pH 7.2, EDTA-free procedure, section 2.4.2.
Additions, standard reaction medium, pH 8.18, mitochondria, FCCP, and sequence A, NADH-EGTA; sequence B, EGTA-NADH.

Key: A (rates measured after disappearance of the transient, massive inhibition.) 85 nmol EGTA causes 50% inhibition of NADH oxidation in 0.22 mg protein/ml (=386 nmol EGTA/ml)

B 92 nmol EGTA causes 50% inhibition of NADH oxidation in 0.22mg protein/ml (=418 nmol EGTA/ml).

Figure 21. Protein concentration and the duration of the transient, massive, first level of EGTA inhibition at pH 8.18.




Rate of NADH oxidation at pH 8.18, 112 nmol $O_2$/min/mg protein.
Mitochondria, isolated at pH 7.2, EDTA-free procedure, section 2.4.2.
Additions, standard reaction medium, pH 8.2, mitochondria, FCCP, NADH, EGTA.
The duration of the EGTA inhibition decreases as protein concentration is increased, for a given EGTA concentration.

| Key: | Protein concentration (mg/ml) |
|---|---|
| A | 0.14 |
| B | 0.29 |
| C | 0.43 |

level of inhibition is massive but time-dependent, suggesting the involvement of a transport process across a membrane. It is postulated that such a calcium movement from the matrix to the intermembrane space would complex EGTA until excess calcium becomes available to replace calcium at sites associated with the exogenous NADH dehydrogenase. Thus as excess calcium becomes available the rate of NADH oxidation increases to the second level of inhibition (i.e., partial recovery); however this explanation does not completely account for the observations because the inhibition is not completely reversed back to the control rate in time, i.e., the second level of inhibition remains proportional to the EGTA concentration.

Partial recovery from EGTA treatment does occur at pH 7.2 when NADH is added to mitochondria suspended with EGTA in reaction medium. However under these conditions the recovery is gradual and there are no signs of two clearly defined levels of EGTA inhibition, as found at pH 8.2.

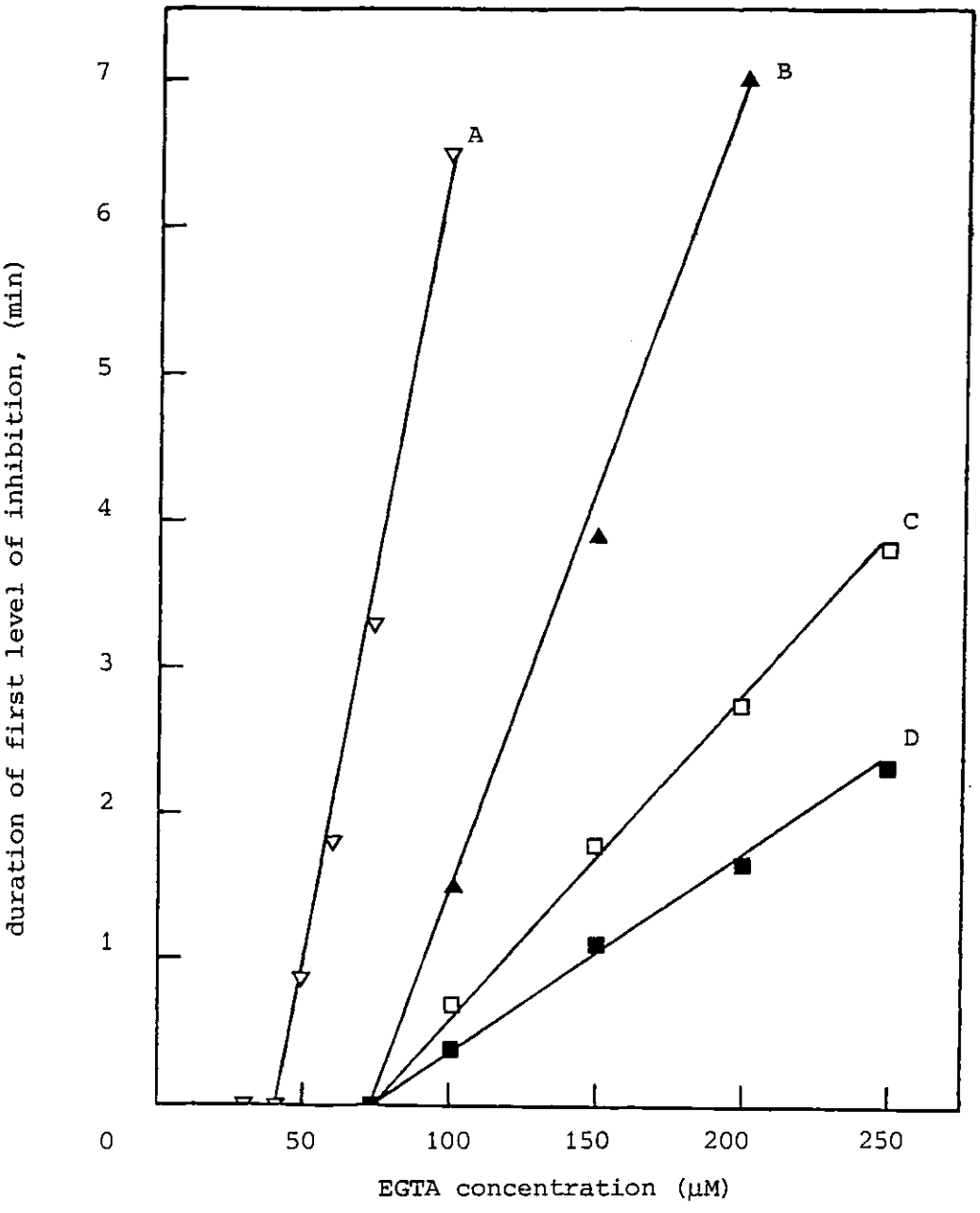
The apparent movement of calcium from the matrix to the EGTA-accessible space at pH 8.18-8.2 in mitochondria treated with EGTA while oxidising NADH was investigated further using the divalent cation ionophore A23187. A23187 promotes movement of calcium (and other divalent cations) across the inner mitochondrial membrane (section 3.2.3.2).

After preparation in the absence of chelator, mitochondria (uncoupled with FCCP) were incubated at pH 8.2 with increasing concentrations of A23187 for 30 sec before NADH was added. EGTA was added after a constant rate of oxidation was established. The duration and magnitude of the first level of inhibition and the magnitude of the second level of inhibition were measured as a function of EGTA concen-

tration at different A23187 concentrations. Increasing concentrations of A23187 on the one hand caused the duration of the first level of EGTA inhibition to diminish (figure 22) and on the other caused the size of the second level of EGTA inhibition to decrease (figure 23). These data suggest that the decrease in sensitivity to EGTA treatment observed in the presence of A23187 may be attributed to the A23187-mediated passage of calcium from the matrix to the intermembrane space until excess calcium becomes available to activate the exogenous NADH dehydrogenase.

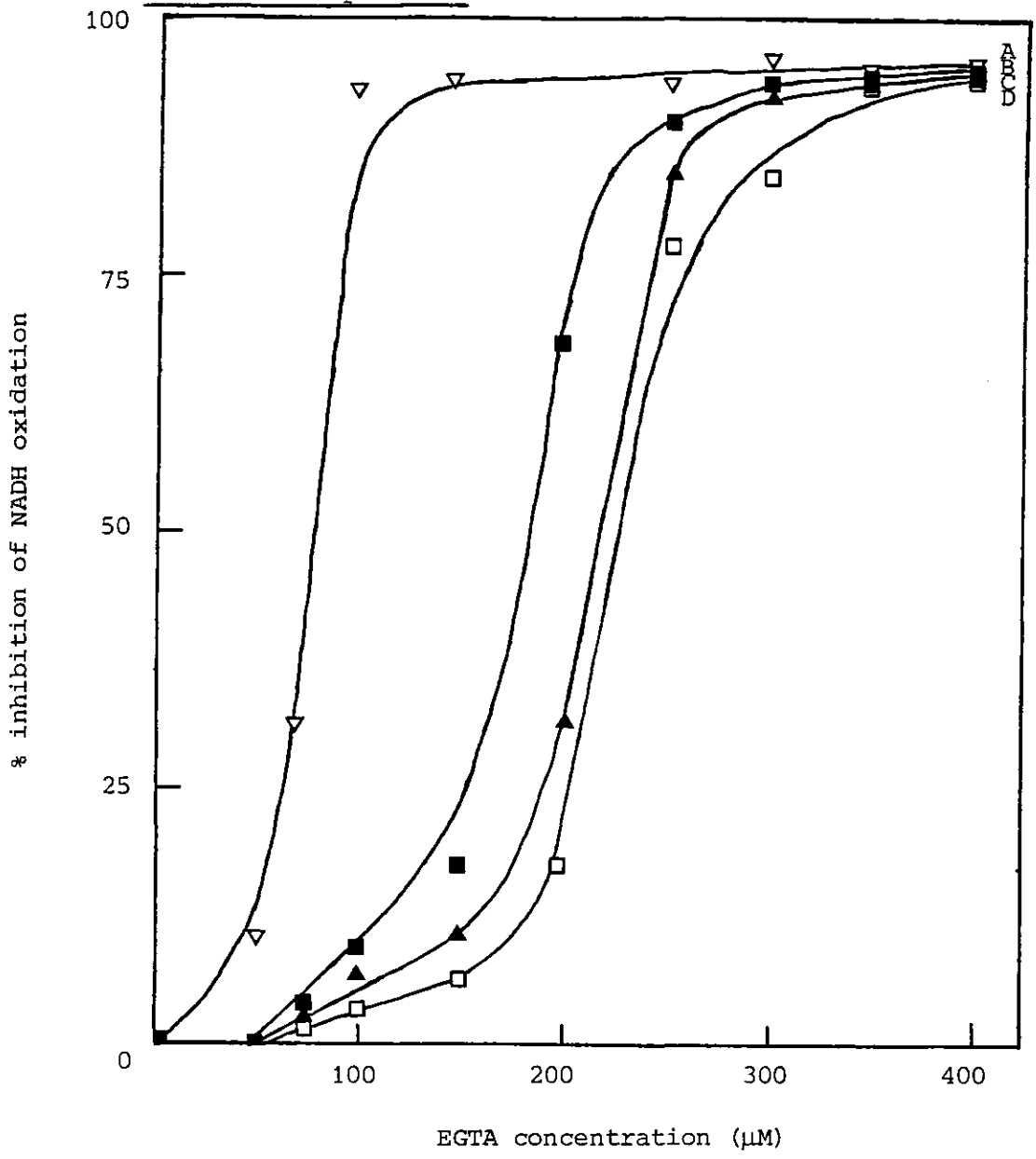
The manifestation of two levels of EGTA inhibition is consistent with the concept that mitochondrial calcium is distributed into at least two pools. One, EGTA-inaccessible, appears to be mainly located in the matrix while another is EGTA-accessible and is apparently located outside the inner membrane (i.e., the extra-matrix space). Recovery from EGTA treatment both at pH 7.2 and 8.2 seemed to suggest that under certain conditions calcium can move from the matrix to the extra-matrix pool. However, some EGTA-inaccessible calcium appears to be located in the extra-matrix space as it appears to be exchanged for magnesium using magnesium-EGTA washing medium (section 3.2.4.1). Other EGTA-inaccessible calcium may be bound to the inner face of the of the inner membrane, associated with the matrix proteins or laid down as deposits of insoluble calcium salts (Earnshaw, 1975) most probably as calcium hydroxylapatite (Weinbach and von Brand, 1965). Some calcium in the EGTA-accessible space is probably associated with the polyuronide component of the outer membrane as suggested by Mannella and Bonner (1975). In addition some calcium is presumed to be associated with the exogenous NADH dehydrogenase, on the outer face of the inner membrane.

Figure 22. A23187 and the duration of the transient, massive, first level of EGTA inhibition at pH 8.18.




Rate of NADH oxidation, pH 8.18, 115 nmol $O_2$/min/mg protein.
Isolation of mitochondria, pH 7.2, EDTA-free procedure, section 2.4.2
Additions, standard reaction medium, pH 8.2, mitochondria, FCCP, A23187, 30 sec later, NADH, 1.5 min later, EGTA.
Protein concentration, 0.39mg/ml
A23187 concentration, (nmol /mg protein); A, B, C and D: 0. 7, 14 and 21 respectively.

Figure 23. A23187 concentration and the second level of NADH oxidation at pH 8.18.




Isolation of mitochondria, pH 7.2, EDTA-free procedure, section 2.4.2.

Additions, standard reaction medium, pH 8.18, mitochondria, FCCP, A23187, 30 sec later, NADH, 1.5 - 2.0 min later, EGTA.

| Key: | A23187 concentration (nmol/mg protein) |
|---|---|
| A | 0 |
| B | 7 |
| C | 14 |
| D | 21 |

Recovery from EGTA treatment is considered to be most likely due to the release of matrix calcium because it is found to be the most effective cation at stimulating exogenous NADH oxidation in standard reaction medium (i.e., + magnesium, + phosphate) (section 3.1.3.1) and because in general it is one of the commonest alkali earth metals found in biological material (section 1.7.3). As stated earlier in this section though, it is not clear why, in the presence of A23187 as in its absence, the recovery to EGTA treatment should be limited, in the second level of EGTA inhibition, to a rate of NADH oxidation which remains proportional to EGTA concentration.

Further interpretation of the observations was made difficult because these experiments were carried out in mitochondria, isolated in either standard or chelator-free media and invariably assayed in standard reaction medium (i.e., containing magnesium and phosphate in addition to sucrose and buffer) in an attempt to mimic conditions *in vivo*.

Before assigning the observed EGTA inhibition of NADH oxidation as due simply to the removal of mitochondrial calcium by chelation with EGTA it became necessary to consider the effects of the other components of the standard reaction medium. In particular, although EGTA has a relatively low stability constant for magnesium (section 3.1.2) it was desirable to see what effect, if any, the removal of magnesium had.

## 3.3 Experiments with "low salt" mitochondria

Isolation and washing media were prepared containing only sucrose and low concentrations of buffer (i.e., minimum components for the maintenance of the osmotic integrity) for the isolation of "low salt" mitochondria (see Methods for details). Mitochondria

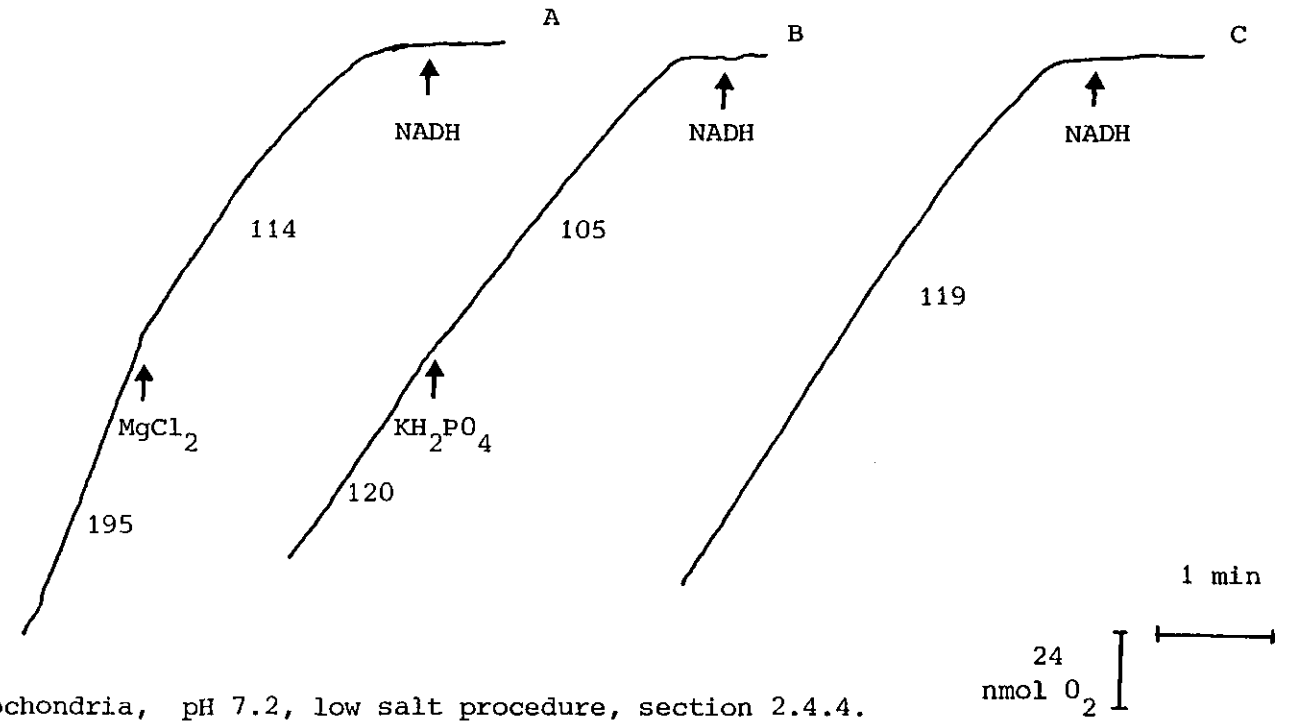
were variously treated with chelator to deplete mitochondrial divalent cations, or with chelator plus salts to induce loading of a particular cation as desired, using low salt wash medium with appropriate additions (1 mM chelator; 2.5 mM divalent cation). Reaction media were also prepared using 2 mM TES and 0.3 M sucrose in which various components were missing from the basic standard reaction medium, i.e. $MgCl_2$, $KH_4PO_4$ or both (see Methods).

3.3.1 Oxidation of NADH in "low salt" mitochondria

The rate of NADH oxidation of "low salt" mitochondria in low salt reaction medium (no magnesium; no phosphate: see Methods) is more curvilinear compared with that observed using standard reaction medium and in addition, the final constant rate is depressed by about one half (figure 24, trace A). The constituents of the standard reaction medium that are absent from the low salt reaction medium, i.e., $KH_2PO_4$ and $MgCl_2$ are respectively added to mitochondria oxidising NADH (figure 24, traces B and C). The addition of 5 mM $KH_2PO_4$ has little effect on NADH oxidation (figure 24, trace B), but addition of 2.5 mM $MgCl_2$ has an immediate effect on the rate of NADH oxidation (figure 24, trace C) and the rate is stimulated to a value expected in mitochondria isolated and suspended in standard media (i.e., 189 nmol $O_2$/min/mg protein, figure not presented). This result with magnesium was surprising because EGTA- or EDTA-inhibited NADH oxidation in previous experiments had not responded to the addition of magnesium; moreover, NADH oxidation was inhibited by EGTA in mitochondria suspended in standard (i.e., excess $Mg^{2+}$) reaction medium (section 3.1.3.1).

In considering these observations (that in the absence of EGTA, magnesium stimulated NADH oxidation), together with previous observations that even in the presence of chelator, calcium and, to a lesser extent, manganese and strontium were

Figure 24. Effect of two components of standard reaction medium on the rate of NADH oxidation




Isolation of mitochondria, pH 7.2, low salt procedure, section 2.4.4.

Additions, low salt reaction medium, pH 7.2, mitochondria, FCCP, NADH, 2.5mM $MgCl_2$ and 5mM $KH_2PO_4$ as appropriate.

Protein concentration, 0.42mg/ml.

also stimulatory, it became desirable to examine further the stimulation of exogenous NADH oxidation by various cations in "low salt" mitochondria. A series of salts, inorganic and organic, monovalent and polyvalent was drawn up; the ability to stimulate exogenous NADH oxidation of the various types of cation used would indicate how this stimulation was brought about. Was the cation stimulation due to a specific cation requirement or was non-specific cation screening of fixed electronegative charges on the mitochondrial membranes involved? The ability of organic cations to stimulate exogenous NADH oxidation would be significant because the organic cations used in this study, $(lys\text{-}lys)^{2+}$, $(DM)^{2+}$ and $(TEC)^{3+}$ are not thought to have any physiological role; their ability to stimulate NADH oxidation would tend to support the view that non-specific screening of electronegative charges accounts for any observed stimulation. Similarly, stimulation by cations of various valency would also tend to support this view.

Results are presented in table 12. The following salts enhanced the rate of NADH oxidation: KCl, NaCl, $CaCl_2$, $MgCl_2$, $MgSO_4$, $MnCl_2$, (lys-lys)-$Cl_2$, (DM)$Br_2$ and (TEC)$Cl_3$. The series of salts can be arranged by valency of the cation into three distinct groups which maximally stimulated NADH oxidation over different concentration ranges, i.e., salts of monovalent cations maximally stimulated NADH oxidation over a concentration range of 80-85 mM, salts of divalent cations, between 0.45-1.00 mM and (TEC)$Cl_3$ (organic trivalent) at 0.08 mM. In each case the uncoupler FCCP was added immediately after the mitochondria were suspended in the low salt reaction medium and before any further additions; thus the stimulation of exogenous NADH oxidation was considered to occur exclusively outside the inner

Table 12 Effect of various salts on the rate of NADH oxidation

| Salt | Salt concentration at which maximum stimulation or inhibition of NADH oxidation occurred, mM | Stimulation or inhibition | Rate of NADH oxidation in maximally-stimulated mitochondria |
|---|---|---|---|
| KCl | 80 | stimulation | 170 |
| NaCl | 85 | stimulation | 175 |
| $CaCl_2$ | 0.5 | stimulation | 195 |
| $MgCl_2$ | 0.45 | stimulation | 190 |
| $MgSO_4$ | 0.5 | stimulation | 204 |
| (lys.lys)$Cl_2$ | 0.7 | stimulation | 150 |
| (DM)$Br_2$ | 1 | stimulation | 162 |
| $MnCl_2$ | ca. 1 | stimulation | 147 |
| (TEC)$Cl_3$ | 0.08 | inhibition | 171 |
| $MnCl_2$ | ca. 10 | inhibition | 90 |
| $LaCl_3$ | 0.38 | inhibition | 24 |

Isolation, low salt procedure (section 2.4.4).

Rates, nmol $O_2$/min/mg protein.

Rate of NADH oxidation in the absence of salt, 130 nmol $O_2$/min/mg protein.

membrane.

$MgCl_2$ and $MgSO_4$ were equally stimulatory, suggesting that it is the cation of the salt which is effective in enhancing the rate of exogenous NADH oxidation. Normally the chloride salt was used, except in the case of $(DM)Br_2$, the chloride salt being unavailable. Typical curves for stimulation of NADH oxidation by mono-, di- and trivalent cations respectively are presented in figure 25.

Previously a range of divalent and monovalent cations was reported to stimulate exogenous NADH oxidation (Hackett, 1961); the stimulation was attributed to a postulated facilitated transport of NADH into the matrix space where the oxidation could occur via the internal NADH dehydrogenase. Later, stimulation of exogenous NADH oxidation by a range of cations was reported in corn mitochondria by Miller *et al.* (1970); there was good correlation between the concentration of salt to give maximum stimulation and its ionic radius. More recently Earnshaw (1975) reported that KCl stimulated the rate of NADH oxidation and suggested that this was due to an increase in free $Ca^{2+}$ concentration in the vicinity of the dehydrogenase.

### 3.3.2 Lanthanum- and manganese-inhibition of NADH oxidation

In the case of lanthanum (table 12; figure 26) inhibition of exogenous NADH oxidation is observed at the lowest concentrations of added lanthanum; maximum inhibition is 78% (rate with NADH alone, 125 nmol $O_2$/min/mg protein). Lanthanum inhibition of NADH oxidation is only seen in the absence of phosphate. In the presence of phosphate, lanthanum has little influence on the rate of NADH oxidation and a precipitate of lanthanum phosphate is formed. (Calcium is unable to reverse the lanthanum inhibition of exogenous NADH oxidation, indeed the addition of calcium serves to enhance the level of inhibition

Legend to figure 25

Rate of NADH oxidation in the absence of salt, 134 nmol $O_2$/min/mg protein.

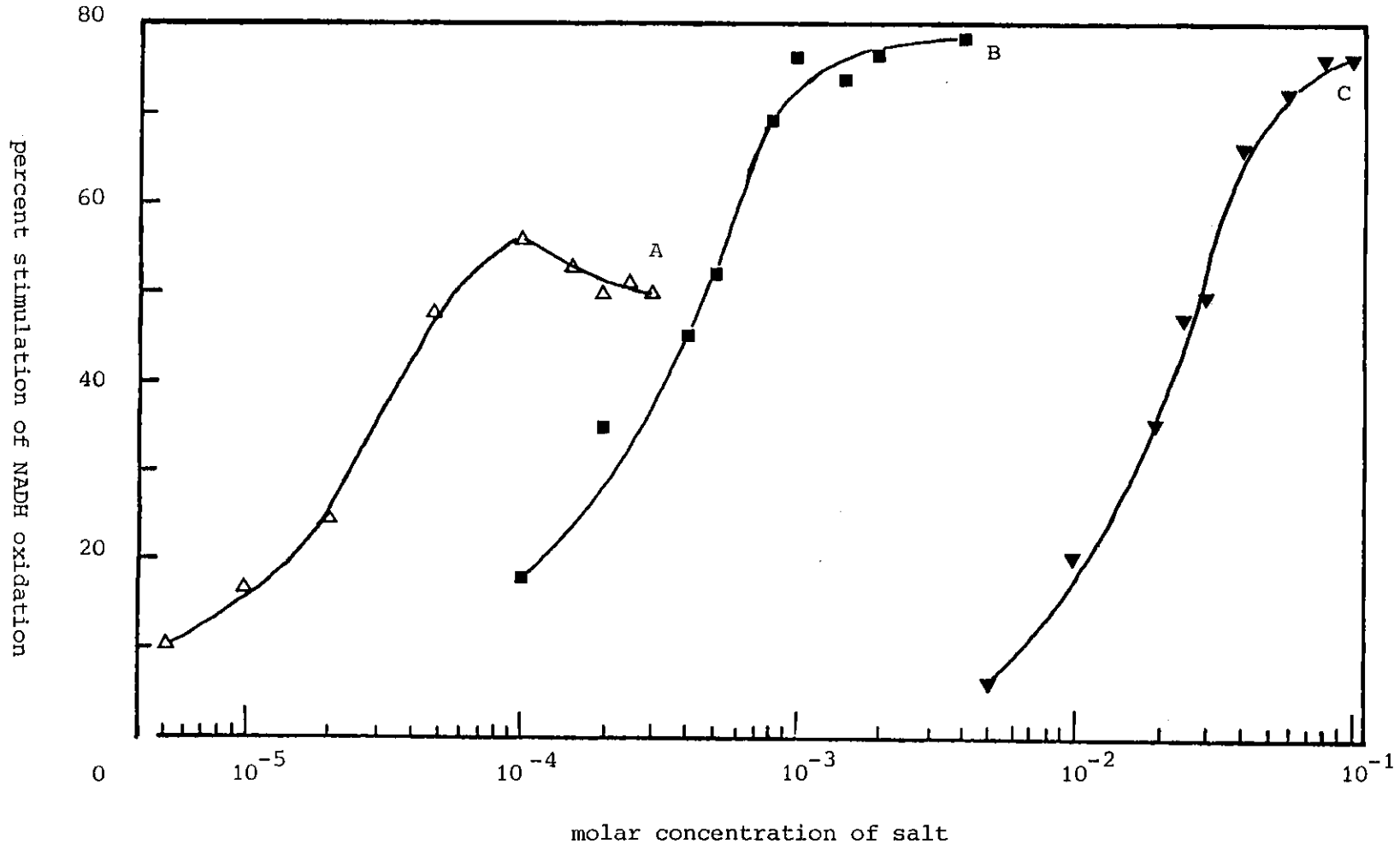
Isolation of mitochondria, pH 7.2, low salt procedure (section 2.4.4).

Additions: low salt reaction medium, pH 7.2; mitochondria; FCCP; salt as appropriate; NADH.

Protein concentration, 0.4 mg/ml.

The stimulation caused by (TEC)$Cl_3$ (A), (DM)$Br_2$ (B) and KCl (C) is expressed as the percent stimulation over the control containing no added salt.

Figure 25. The stimulation by salts of the oxidation of exogenous NADH.



Legend to figure 26

Rate of NADH oxidation in the absence of salt, 125 nmol $O_2$/min/mg protein.

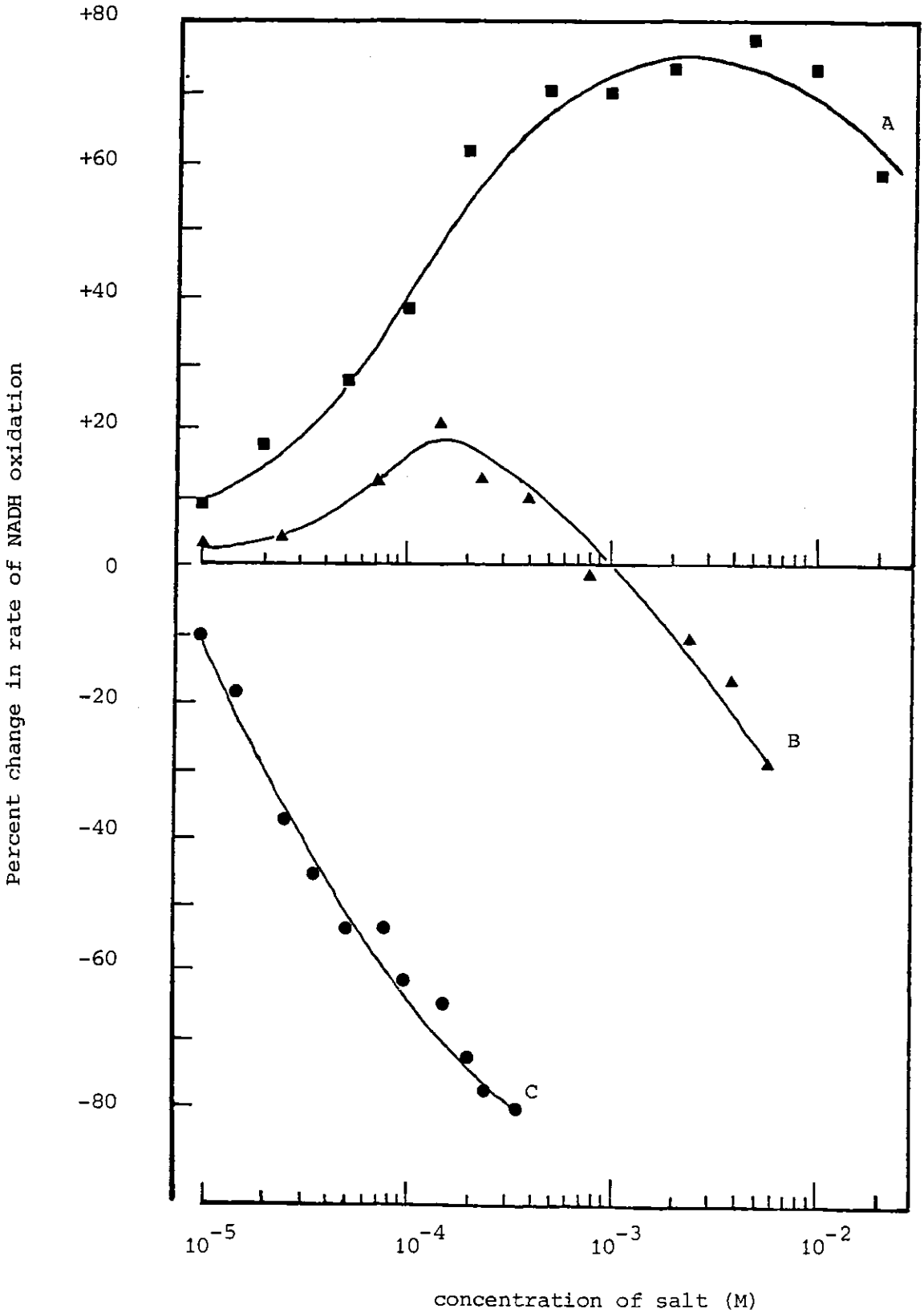
Isolation of mitochondria, pH 7.2, low salt procedure (section 2.4.4).

Additions: low salt reaction medium, pH 7.2; mitochondria; FCCP; salt as appropriate; NADH.

Protein concentration, 0.4 mg/ml.

The change in rate of oxidation of NADH cuased by $MgSO_4$ (A), $MnCl_2$ (B) and $LaCl_3$ (C) is expressed as a percent of the rate obtained in the absence of added salt.

Figure 26. The effect of magnesium, manganese and lanthanum upon the rate of oxidation of exogenous NADH



slightly; data not included.)

Also of interest is the observation that in $La^{3+}$-inhibited NADH oxidation EDTA and EGTA, slightly in excess of lanthanum, are capable of removing that inhibition (table 13). NADH oxidation in mitochondria thus treated responds to additions of calcium or magnesium with an increase in rate (table 13). However the final rate of oxidation is lower in the case of mitochondria treated with lanthanum, EGTA and magnesium than that obtained for the other combinations. An explanation for this is offered later in section 3.3.5. Thus it seems that the mitochondrial lanthanum binding site has a lower affinity for lanthanum than has EGTA or EDTA (15.6 and 15.4 respectively, Sillen and Martell, 1964, 1971). EGTA or EDTA may be used to fully reverse the lanthanum inhibition of NADH oxidation for even after such treatment NADH oxidation still responds to the addition of divalent cations with a stimulation in rate.

In the case of manganese (table 12 and figure 26) although very low concentrations (<0.1 mM) stimulate NADH oxidation (in the presence of FCCP) inhibition soon becomes apparent as the concentration is increased (>1.0 mM). These observations are consistent with manganese having two possible effects; low concentrations of manganese replace calcium at the dehydrogenase whereas at higher concentrations manganese also binds to another group thereby causing inhibition of NADH oxidation.

On the basis that mono-, di- and trivalent cations, both inorganic and organic enhance the rate of exogenous NADH oxidation, the stimulation apparently lacks chemical specificity towards the stimulating cation, in agreement with the observations of Hackett (1961) and Miller *et al.* (1970) who found that a selection of cations led to a

Table 13 Reversal of lanthanum-induced inhibition of NADH oxidation by addition of EDTA or EGTA

| Additions | Sequence of additions | | | |
|---|---|---|---|---|
| | Experiment 1 $La^{3+}$-EDTA-$Mg^{2+}$ | Experiment 2 $La^{3+}$-EDTA-$Ca^{2+}$ | Experiment 3 $La^{3+}$-EGTA-$Mg^{2+}$ | Experiment 4 $La^{3+}$-EGTA-$Ca^{2+}$ |
| 1. NADH | 130 | 130 | 124 | 123 |
| 2. $La^{3+}$, 250 µM | 30 | 32 | 27 | 31 |
| 3. chelator, 100 µM (1st addition) | 30 | 32 | 35 | 37 |
| 4. chelator, 100 µM (2nd addition) | 30 | 33 | 35 | 36 |
| 5. chelator, 100 µM (3rd addition) | 139 | 129 | 135 | 141 |
| 6. salt, 1.5 mM | 164 | 158 | 140 | 167 |

Isolation, low salt procedure (section 2.4.4).

Rates, nmol $O_2$/min/mg protein. Protein concentration, 0.22 mg/ml.

stimulation in the rate of exogenous NADH oxidation. The apparent lack of chemical specificity and the determination of effective concentration range by cation valency is good evidence that the ability of cations to stimulate NADH oxidation is due to an electrostatic screening of fixed negative charges associated with the mitochondrial membranes. The non-specific stimulation of exogenous NADH oxidation by various cations of differing valency could be explained by a reduction in the membrane surface potential, brought about by altering the cation composition of the diffuse layer. A reduction in the membrane surface potential would then decrease the repulsion of NADH, which at physiological pH is negatively charged, thereby allowing this substrate to approach the membrane surface and hence the NADH dehydrogenase more closely.

However, inconsistent with the view that the cation-stimulation of exogenous NADH oxidation simply lacks chemical specificity, are the considerations that excess magnesium does not reverse EGTA inhibition of NADH oxidation (section 3.1.3.1) (in spite of the relatively low affinity of EGTA for magnesium compared with other divalent cations, Sillen and Martell, 1964, 1971) although magnesium stimulates NADH oxidation in the absence of EGTA (this section). Also significant is the lanthanum inhibition of NADH oxidation, which will be discussed later.

### 3.3.3 Data obtained using the fluorescent probe 9AA

#### 3.3.3.1 Use of 9AA to investigate fixed charges associated with the mitochondrial membranes

There has recently been much interest in the nature of membrane-associated fixed charges, for example, on the surface of

thylakoid membranes (Davis and Gross, 1975; Searle *et al.*, 1977; Barber *et al.*, 1977; Barber, 1977) which have been determined to carry a net negative charge, using the monovalent fluorescent probe, 9-amino-acridine (9AA; experimental details, Methods, section 2.6.6.3).

Because of the apparent lack of chemical specificity and the determination of effective concentration by cation valency (section 3.3.1), a tentative suggestion was made that like thylakoid membranes, Jerusalem artichoke mitochondrial membranes might carry a net negative charge (Methods, section 2.6.6.2). In support of this view is the similarity of thylakoid membranes to Jerusalem artichoke mitochondrial membranes in the respect of their response to treatment with the chelators EDTA and EGTA (Møller, 1981). It was found that addition of mitochondria to a solution of 9AA in a low cation medium caused a decrease in fluorescence; subsequent addition of chelator (EDTA, EGTA) caused a further decrease in fluorescence (Møller, 1981). These observations were interpreted as representing initially the movement of 9AA into the diffuse layer (caused by unscreened negative charges on the mitochondrial membranes when suspended in low salt medium) thereby increasing the local concentration of 9AA such that a proportion of 9AA fluorescence was quenched. The subsequent addition of chelator caused a further decrease in fluorescence, which was interpreted to represent the removal of divalent cations from the membranes by chelator, thereby permitting the entry of more 9AA into the diffuse layer, such that further concentration quenching of 9AA fluorescence occurred. A similar account for quenching of 9AA fluorescence in the case of thylakoid membranes has been offered by Searle *et al.* (1977) and by Barber (1977).

The possibility that mitochondrial membranes carry a net negative charge is relevant to the problem of how calcium and other

cations may interact with mitochondria in the oxidation of NADH in the presence of reaction media of different composition. Fixed negative charges attract cations into the diffuse layer adjacent to the membrane surface (Barber *et al.*, 1977). The cation composition of this diffuse layer will depend upon the electrolyte composition of the medium in which the membranes are suspended. The double layer theory developed by Gouy (1910) and Chapman (1913) predicts that the effectiveness of the cation charge shielding is determined by its charge with the following order of effectiveness: $C^{3+} > C^{2+} > C^{+}$.

3.3.3.2 The effect of salts on the oxidation of exogenous NADH and the release of quenching of 9AA fluorescence

The effect of salts upon the rate of NADH oxidation and the associated release of quenching of 9AA fluorescence (section 2.6.6) were investigated in "low salt" mitochondria suspended in low salt reaction medium (section 2.3.4).

The fluorescent probe 9AA behaves in solution as a monovalent cation (section 2.6.6.1; Schuldiner *et al.*, 1972; Haraux and de Kouchkovsky, 1980). As stated in section 3.3.3.1, the addition of mitochondria to a solution of 9AA in a low salt medium causes a partial concentration quenching of 9AA fluorescence (Searle *et al.*, 1977; Barber, 1977). A typical monovalent cation (sodium) and divalent cation (magnesium) stimulate the rate of NADH oxidation and release the quenched fluorescence of 9AA in mitochondria from the same preparation, same protein concentration, suspended in low salt medium (figure 27). It is notable that for each cation, both stimulation of NADH oxidation and release of quenched fluorescence occurred over a similar concentration range. When expressed as percentage stimulation of NADH oxidation and percentage of the maximal release which can be achieved

Legend to figure 27

Rate of NADH oxidation in absence of salt, 125 nmol $O_2$/min/mg protein.

Isolation of mitochondria, pH 7.2, low salt procedure (section 2.4.4).

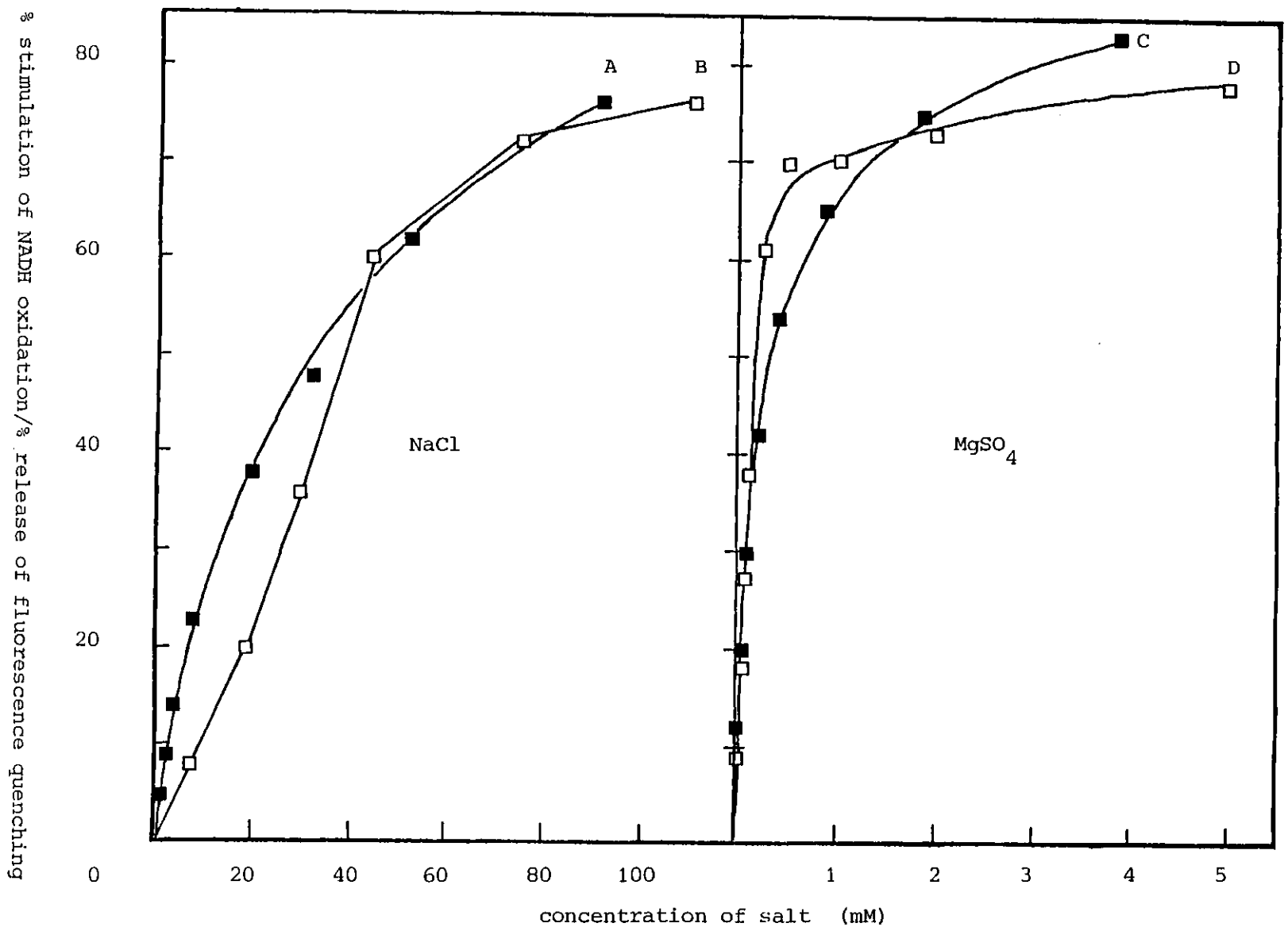
Additions: low salt reaction medium, pH 7.2; mitochondria; FCCP; salt; 9-AA if appropriate; NADH.

Protein concentration, 0.37 mg/ml.

The stimulation of oxidation of NADH (B & D) is expressed as the percent increase over the rate obtained without added salt.

The release of quenching of 9-AA fluorescence (A & C) is expressed as the percentage of the maximal release obtainable with excess salt.

Figure 27. The effect of salts on the oxidation of exogenous NADH and the release of quenching of 9-AA fluorescence



by very high concentrations of the respective ion, there was considerable overlap of the curves (figure 27). This similarity in response supports the view that in Jerusalem artichoke mitochondria the stimulation of oxidation of exogenous NADH may be due in part at least to the degree of screening of the fixed charges on the surface of the membrane by the cations in the diffuse layer.

Additional data confirming the similarity between the concentrations of various cations required to stimulate both the rate of NADH oxidation and the release of 9AA fluorescence are presented in table 14. Since mitochondria cause the fluorescence of 9AA to be quenched and because this can be released by a variety of cations of different valency, organic and inorganic, it is reasonable to suggest that, like thylakoid membranes (Davis and Gross, 1975; Barber, 1977), Jerusalem artichoke mitochondrial membranes are negatively charged. In table 14, values presented are the concentrations of various cations required to cause 50% of either maximum stimulation of the rate of NADH oxidation, or maximum release of fluorescence. There is a close correlation between the values presented for mono- and trivalent cations. However, when considering the divalent cations, it can be seen that a significantly lower concentration of the inorganic cations is required to produce half-maximal stimulation of the rate of exogenous NADH oxidation than is required for half-maximal release of fluorescence quenching using 9AA. Such a discrepancy could occur if the inorganic cations used were able to exert chemical selectivity and bind preferentially to those negative charges closely associated with bringing about the stimulation of NADH oxidation. It is significant to note that in the case of the organic divalent cation, $(DM)^{2+}$, which may be expected to lack the required chemical specificity to bind to the required groups, there

Table 14 Comparison between the effect of salts upon exogenous NADH oxidation and upon release of quenching of 9AA fluorescence

| Salt | Protein concentration in the assay<br>mg/ml | Concentration of salt at which fluorescence quenching is half maximally released<br>mM | Concentration of salt at which NADH oxidation is half maximally stimulated<br>mM |
|---|---|---|---|
| NaCl | 0.37 | 35 | 32 |
| KCl | 0.42 | N.D. | 21 |
| $MgSO_4$ | 0.37 | 0.32 | 0.10 |
| $CaCl_2$ | 0.47 | 0.37 | 0.12 |
| $(DM)Br_2$ | 0.37 | 0.34 | 0.22 |
| $(TEC)Cl_3$ | 0.37 | 0.031 | 0.023 |

Isolation, low salt procedure (section 2.4.4).

N.D., not done.

is less discrepancy between the concentrations necessary to stimulate the oxidation of NADH and release the quenching of fluorescence of 9AA.

### 3.3.4 The rate of NADH oxidation in mitochondria treated with EGTA and magnesium

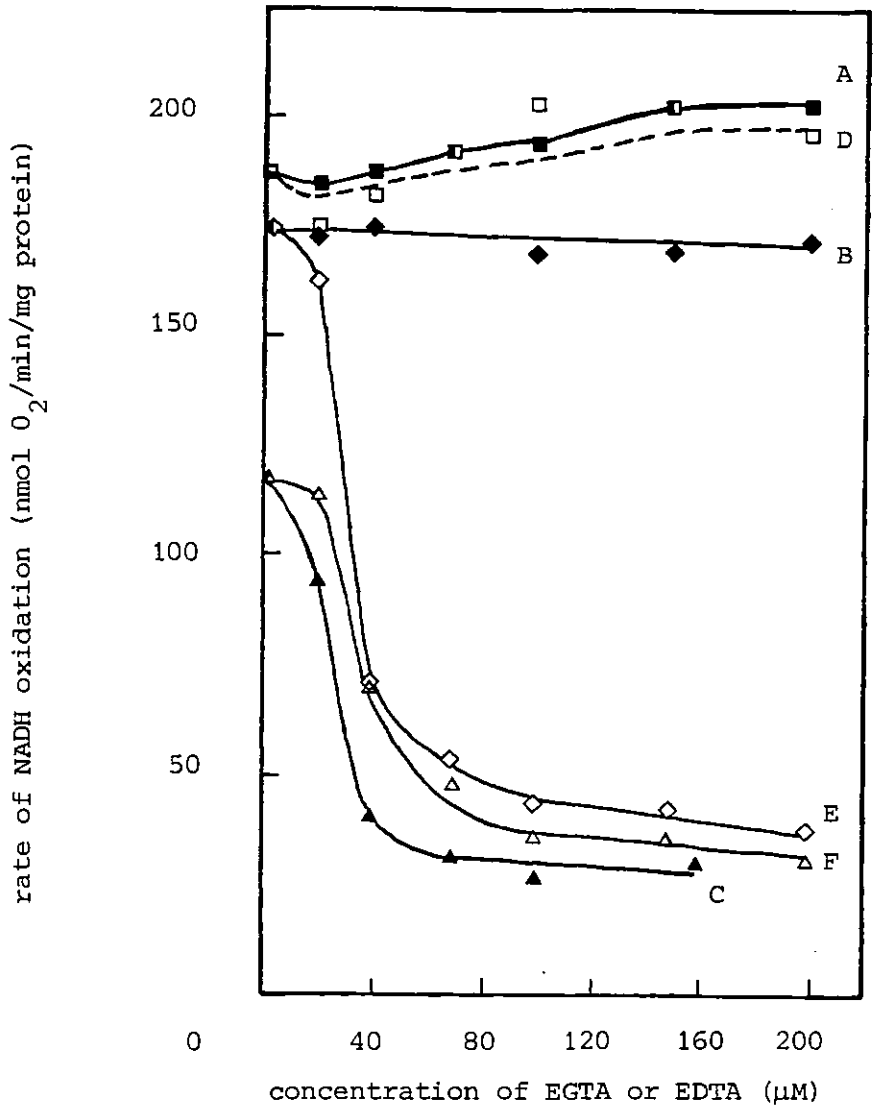
In earlier experiments, EGTA, a chelator with a preference for calcium over magnesium (section 3.1.2) was normally used in preference to EDTA, which has a much less clearly defined affinity for calcium than magnesium.

Results presented showing that magnesium stimulated NADH oxidation in low salt reaction medium in the absence of EGTA (section 3.3.1) seemed to be incompatible with earlier results which showed that excess magnesium fails to reverse EGTA-inhibited NADH oxidation (section 3.1.3.1). An experiment was devised in an attempt to resolve these observations.

The rate of exogenous NADH oxidation in low cation reaction medium, pH 7.2 was studied in the presence of a fixed concentration of divalent cation (1.5 mM calcium or magnesium) whilst the concentration of chelator (EDTA or EGTA up to 0.2 mM) was increased. Additions were as follows. Mitochondria were suspended in low salt reaction medium, followed by FCCP and then 1.5 mM salt. Respiration was initiated by the addition of NADH; chelator was added 1 min later. The inhibited rate of NADH oxidation was measured after about 3 min when the rate became linear.

Results are presented in figure 28. In the presence of magnesium, EGTA gives an S-shaped inhibition curve. The other three combinations of chelator and cation (EDTA-$Mg^{2+}$, EGTA-$Ca^{2+}$, EDTA-$Ca^{2+}$) give rates as expected in mitochondria assayed in standard medium containing magnesium. In agreement with data presented in section 3.1.3.1,

Figure 28. Effect of combinations of EGTA or EDTA and either magnesium or calcium on NADH oxidation




Isolation of mitochondria, pH 7.2, low salt procedure, section 2.4.4.

Additions, low salt reaction medium, pH 7.2, mitochondria, FCCP, salt and chelator as appropriate, NADH.

Protein concentration, 0.32mg/ml.

Key:

| | |
|---|---|
| A | calcium + EDTA |
| B | magnesium + EDTA |
| C | EDTA |
| D | calcium + EGTA |
| E | magnesium + EGTA |
| F | EGTA |

excess magnesium compared to EGTA does not permit NADH oxidation to proceed uninhibited. Indeed, massive inhibition of exogenous NADH oxidation occurred in mitochondria (0.32 mg/ml) at 40 μM EGTA in the presence of 1.5 mM magnesium, a 37-fold excess of magnesium. In the presence of EGTA, excess magnesium is insufficient to allow NADH oxidation to take place. It has been shown elsewhere (Møller, Johnston and Palmer, 1981) that magnesium gave "uninhibited" rates in the presence of EDTA not simply because of maximal screening conditions because maximal screening by $(DM)^{2+}$ could not remove inhibition by EGTA or EDTA. The most likely explanation for the data in figure 28 is that EDTA has removed $Ca^{2+}$ (and $Mg^{2+}$) from the membranes and that the addition of excess $Mg^{2+}$ displaces $Ca^{2+}$ from its EDTA complex, allowing it to return to specific sites on the membrane essential for NADH oxidation. The inability of $Mg^{2+}$ to give uninhibited rates in the presence of EGTA could be due to the lower stability constant of $Mg^{2+}$ with EGTA (5.3 cf. 8.7 with EDTA).

### 3.3.5 Effect of various combinations of cations upon the rate of NADH oxidation in EGTA-treated mitochondria

The influence of the EGTA stability constant on the ability of cations with different or unknown complexing abilities to reverse the EGTA inhibition of NADH oxidation was further studied using various combinations of these cations.

Additions were made as follows. Mitochondria were suspended in low salt reaction medium, followed by FCCP then 0.5 mM EGTA. NADH was added 30 sec later. Cations were added as indicated 2 min and 4 min later respectively. Results are presented in table 15.

The inhibited rate of NADH oxidation in the presence of EGTA and excess magnesium, reported in section 3.3.4 (figure 28) is seen to be stimulated back to "uninhibited" values by calcium (table 15).

Table 15 Effect of various combinations of cations on EGTA-inhibition of NADH oxidation

| Experiment | Sequence of additions | | | | | |
|---|---|---|---|---|---|---|
| | 1st addition | | 2nd addition | | 3rd addition | |
| | Chelator | Rate | Salt | Rate | Salt | Rate |
| 1. EGTA-$Ca^{2+}$ | EGTA, 0.50 mM | 56 | $Ca^{2+}$, 1.00 mM | 176 | - | - |
| 2. EGTA-$K^{+}$-$Ca^{2+}$ | EGTA, 0.50 mM | 51 | $K^{+}$, 60 mM | 26 | $Ca^{2+}$, 0.50 mM | 150 |
| 3. EGTA-$Mg^{2+}$-$Ca^{2+}$ | EGTA, 0.50 mM | 56 | $Mg^{2+}$, 6.00 mM | 41 | $Ca^{2+}$, 0.50 mM | 161 |
| 4. EGTA-$Mg^{2+}$-$Ca^{2+}$ | EGTA, 0.50 mM | 51 | $Mg^{2+}$, 1.00 mM | 41 | $Ca^{2+}$, 5.00 mM | 165 |
| 5. EGTA-$Mg^{2+}$-$Mn^{2+}$ | EGTA, 0.50 mM | 57 | $Mg^{2+}$, 5.00 mM | 39 | $Mn^{2+}$, 2.00 mM | 132 |
| 6. EGTA-$(lys.lys)^{2+}$-$Ca^{2+}$ | EGTA, 0.50 mM | 58 | $(lys.lys)^{2+}$, 1.00 mM | 70 | $Ca^{2+}$, 0.50 mM | 165 |
| 7. EGTA-$(TEC)^{3+}$-$Ca^{2+}$ | EGTA, 0.50 mM | 58 | $(TEC)^{3+}$, 0.05 mM | 73 | $Ca^{2+}$, 0.50 mM | 171 |

Isolation, low salt procedure (section 2.4.4). Rates, nmol $O_2$/min/mg protein.

Rates: in absence of salt, +60 mM $K^{+}$, +0.5 mM $Ca^{2+}$ and +0.5 mM $Mg^{2+}$; 126, 193, 187 and 185 nmol $O_2$/min/mg protein, respectively.

Protein concentration, 0.28 mg/ml.

When mitochondria are treated with EGTA, subsequent treatment with $Mg^{2+}$ or $K^{+}$ enhances the inhibition of NADH oxidation. Subsequent treatment with $(lys\text{-}lys)^{2+}$ or $(TEC)^{3+}$ (organic cations) led to a slight stimulation in the rate of NADH oxidation. In each of the above cases final treatment with $Ca^{2+}$ leads to a stimulation in the rate of NADH oxidation back to a typical maximum value. In the case of EGTA-$Mg^{2+}$ treatment, subsequent treatment with $Mn^{2+}$ is successful in stimulating the rate of NADH oxidation although the final rate is somewhat smaller than that achieved using $Ca^{2+}$ (table 15).

These data are interpreted as illustrating two types of stimulation of exogenous NADH oxidation. Non-specific stimulation of NADH oxidation is shown by $Ca^{2+}$, $K^{+}$ and $Mg^{2+}$. On the other hand, specific stimulation of NADH oxidation by calcium is shown indirectly with combinations of EGTA and certain divalent cations (table 15).

The addition of $K^{+}$ to EGTA-treated mitochondria significantly enhanced the level of inhibition of NADH oxidation (51→26 nmol $O_2$/min/mg protein; table 15). This observation confirms a similar finding by Earnshaw (1975) with corn mitochondria, who found that KCl caused a decrease in the binding of $Ca^{2+}$ to mitochondrial membranes and suggested that the stimulation by KCl was due to an increase in free $Ca^{2+}$ levels in the vicinity of the NADH dehydrogenase. Addition of $Ca^{2+}$ to EGTA-$K^{+}$ mitochondria reverses fully the inhibition of NADH oxidation.

It is possible that EGTA and high (2-5 mM) concentrations of magnesium could lead to a similar decrease in the binding of calcium to membranes rendering further calcium available for chelation with EGTA, thus explaining a slight enhancement of EGTA inhibition of NADH oxidation caused by the addition of magnesium, data not presented.

When the organic cations $(lys\text{-}lys)^{2+}$ and $(TEC)^{3+}$ are added to

mitochondria treated with EGTA, there is a slight stimulation in the rate of NADH oxidation (58→70 and 58→73 nmol $O_2$/min/mg protein respectively; table 15). The slight stimulation in rate is consistent with the interpretation that the organic cations are causing a non-specific stimulation of NADH oxidation by replacing inorganic cations removed from the diffuse layer by EGTA, calcium being required under these conditions for a full stimulation of the rate.

In the case of EGTA-$Mg^{2+}$ treated mitochondria, it is found that in addition to $Ca^{2+}$, $Mn^{2+}$ also stimulated the rate of NADH oxidation (39-132 nmol $O_2$/min/mg protein; table 15). This is interesting because, without chelator, low concentrations (<1.0 mM) of $Mn^{2+}$ were found to stimulate NADH oxidation but high concentrations (>10.0 mM) inhibited (section 3.3.1). The stability constant of EGTA for $Mn^{2+}$ (12.1-12.3, Sillén and Martell, 1964; 1971) is one order of magnitude higher than that for $Ca^{2+}$ (11.0) and very much higher than that for $Mg^{2+}$ (5.4). It is therefore likely that with these concentrations of cations and chelator (0.5 mM EGTA, 5 mM $Mg^{2+}$, 2 mM $Mn^{2+}$) most of the EGTA is present as the $Mn^{2+}$-complex and the concentration of $Mn^{2+}$ is too low to cause an inhibition of NADH oxidation as seen in section 3.3.2. Sufficient divalent cation ($Mn^{2+}$, $Mg^{2+}$) is probably present for full screening of electrostatic charges and sufficient calcium is available for partial activation of the NADH dehydrogenase (cf. 132 using $Mg^{2+}$ and $Mn^{2+}$ with e.g., 161 nmol $O_2$/min/mg protein using $Mg^{2+}$ and $Ca^{2+}$).

Data from a more extensive range of salts of various valency are presented elsewhere (Møller, Johnston and Palmer, 1981).

### 3.3.6 Effect of washing mitochondria in EGTA-magnesium medium

A washing programme was devised in which one portion of a mitochondrial preparation was washed twice with a medium containing

0.4 M sucrose, 2 mM TES, 1.5 mM magnesium and 0.2 mM EGTA with a final wash using standard wash medium. The remaining portion was treated as the control.

The rate of NADH oxidation was measured in both mitochondrial treatments, using low salt reaction medium and the effect of several salts upon the rate of NADH oxidation determined. It was found in both mitochondrial treatments that there was a non-specific response to salt additions, consistent with the stimulation of the rate of NADH oxidation being due to the screening of membrane-associated negative charges, but no evidence for a significant specific requirement of calcium was obtained in the test treatment (table 16). Once again it was not possible to obtain a mitochondrial preparation which specifically required calcium for the oxidation of NADH.

### 3.3.7 The effect of increasing concentrations of EGTA and EDTA on the rate of NADH oxidation in low salt media

Data presented by Coleman and Palmer (1971) indicated that EDTA was less efficient than EGTA at inhibiting NADH oxidation (i.e., a greater concentration of EDTA was required to produce a given level of inhibition). However, these data had been obtained using reaction media which contained magnesium. It was therefore decided to examine the effect of increasing concentrations of either EGTA or EDTA upon the rate of NADH oxidation in mitochondria isolated in low salt media and assayed in low salt reaction medium. The sequence of additions was as follows. Mitochondria were suspended in low salt reaction medium, FCCP was added, respiration was initiated by the addition of NADH. Chelator was added 1 min later. The rate was measured about 3 min later when linear.

The data in figure 29 show that as the concentration of

Table 16 Effect of EGTA-magnesium wash medium on rate of exogenous NADH oxidation

| Salt | Rate of NADH oxidation in mitochondria washed with EGTA-magnesium | Rate of NADH oxidation in control mitochondria |
|---|---|---|
| NaCl, 80.0 mM | 180 | 185 |
| $CaCl_2$, 1.0 mM | 191 | 185 |
| (DM)$Br_2$, 1.0 mM | 172 | 168 |
| (TEC)$Cl_3$, 0.09 mM | 169 | 162 |

Isolation of mitochondria, low salt procedure (section 2.4.4); mitochondria were then washed with standard wash medium to which 0.2 mM EGTA and 2.5 mM $MgCl_2$ had been added. A final wash with standard wash medium was given.

Additions, low salt reaction medium, mitochondria, FCCP, salt, NADH.

Rates, nmol $O_2$/min/mg protein.

Protein concentration, 0.28 mg/ml.

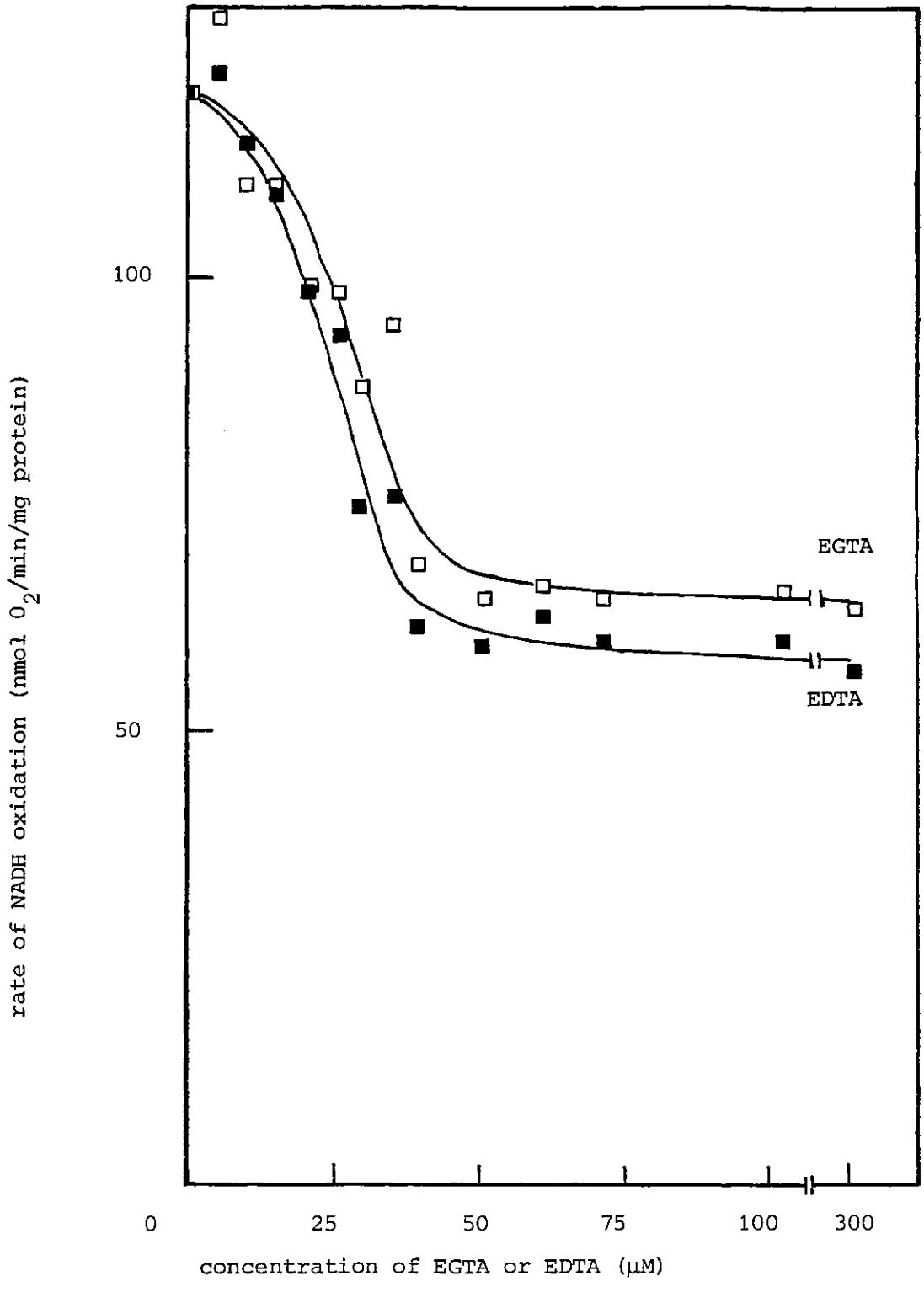



Figure 29. Chelator-inhibition of NADH oxidation in low salt reaction medium

Isolation of mitochondria, pH 7.2, low salt procedure, section 2.4.4.
Additions, low salt reaction medium, pH 7.2, mitochondria, FCCP, chelator, 0.5 min later, NADH. Rate measured 3 min after addition of NADH.

chelator is raised, the rate of NADH oxidation in mitochondria suspended in low salt medium is increasingly inhibited. It is noteworthy that the extent of the inhibition levels off at a rate of about 60-70 nmol $O_2$/min/mg protein (representing a maximum inhibition of approximately 50%) which is similar to the rate of oxidation found in mitochondria inhibited with EGTA but assayed in the presence of magnesium.

In contrast to the marked difference in concentration of chelator causing 50% inhibition of exogenous NADH oxidation in mitochondria assayed in the presence of magnesium (when EGTA was found to be much more effective at causing inhibition than EDTA, Coleman and Palmer, 1971) the results presented in figure 29 suggest that EDTA in the absence of magnesium is slightly more efficient than EGTA at inhibiting NADH oxidation (cf. 50% with 47%). This observation could be explained in terms of EDTA removing slightly more divalent cations from screening membrane-associated negative charges than EGTA because of high stability constants for both calcium and magnesium, thus making the local membrane environment more negative, therefore NADH would be more strongly repelled than would be the case in mitochondria treated with EGTA.

Finally mitochondria isolated in low salt media and assayed in low salt reaction medium appeared to have more or less the same sensitivity to treatment with chelator compared with mitochondria assayed in the presence of magnesium, as shown in figure 10. Although the relationship between the concentration of EGTA causing 50% inhibition of NADH oxidation and the concentration of mitochondrial protein was not investigated closely in mitochondria assayed in low salt reaction media, the data in figure 29 show that at 0.28 mg/ml 50% inhibition of exogenous NADH oxidation is observed at 40 μM EGTA or EDTA (equiva-

lent to 143 nmol chelator/mg protein). These values agree with the relationship between the concentration of EGTA causing 50% inhibition of NADH oxidation and the concentration of mitochondrial protein determined for mitochondria isolated by the EDTA-free procedure but assayed in the presence of magnesium as shown in figure 10.

# 4. DISCUSSION

## 4.1 Effect of a range of chelators on NADH oxidation

Jerusalem artichoke mitochondria oxidise exogenously added NADH, succinate and malate, both in the presence of FCCP and under State 3 conditions (Chance and Williams, 1956) at acceptable rates of $O_2$ consumption (tables 3 & 4) using a reaction medium containing magnesium (i.e., standard, table 2). The succinate-cytochrome c oxidoreductase activity is consistent with a high level of membrane integrity (section 3.1.1); this is essential for the study of an enzyme thought to be located in one mitochondrial compartment and influencing another.

If a chelator induces the inhibition of a biochemical process it is reasonable to infer that the inhibition may arise from the chelation of cations involved in that process. An example of chelator-induced enzyme inhibition can be found in kidney brush border neutral proteinase (E.C. 3.2.24.11). The action of this enzyme is inhibited by EDTA and reactivated by $Zn^{2+}$ (Kerr and Kenny, 1974). Because EGTA inhibits exogenous NADH oxidation and calcium reverses the inhibition (Coleman and Palmer, 1971) (figures 4 & 5) it seems plausible that calcium is involved in the oxidation. In considering how calcium may play a role in the oxidation of exogenous NADH, there are several possibilities. For example, perhaps the true substrate of the dehydrogenase is a NADH-$Ca^{2+}$ complex. Alternatively calcium may form part of the active centre for NADH, or perhaps calcium alters the conformation of the membrane-bound enzyme at a site removed from the active site, thus rendering the active site more accessible to NADH (section 3.1.5.1).

Furthermore if each of a range of chelators with various chemical structures is found to be effective at inducing an inhibition of

exogenous NADH oxidation, this is consistent with the inhibition being due to the removal of divalent cations.

When comparing a range of chelators only murexide did not inhibit exogenous NADH oxidation (table 6). It is thought that this may be because murexide binds $Ca^{2+}$ more weakly than the calcium-binding group in the membrane. The concentration of chelator found to inhibit exogenous NADH oxidation had no effect upon the rate of malate or succinate oxidation (table 5). Therefore the effect of chelators must be limited to that section of the respiratory chain between the dehydrogenase on the outer face of the inner membrane and ubiquinone. Because polar chelators do not gain access to the matrix space it is not possible to determine whether the NADH dehydrogenase located on the inner face of the inner membrane is calcium-dependent.

## 4.2 Identity of the divalent cation(s) involved in exogenous NADH oxidation

Calcium is strongly implicated as the principal divalent cation involved in exogenous NADH oxidation on the basis of the following observations. Calcium is more efficient than strontium and manganese at reversing the EGTA inhibition of NADH oxidation (table 7). Furthermore calcium is more effective than manganese at seasonally stimulating exogenous NADH oxidation in the presence of excess magnesium. However calcium and magnesium are by far the most common divalent cations in biological tissues (section 1.7.3; Epstein, 1972); strontium and manganese are relatively scarce. Data using the monovalent fluorescent probe 9AA (section 3.3.3) show that mitochondria are isolated with calcium and magnesium on the membranes in the ratio of approximately 1:1 (Møller, Johnston and Palmer, 1981). Finally calcium is further implicated because magnesium is totally ineffective at

preventing the EGTA inhibition of NADH oxidation (table 7) and because EGTA, which chelates calcium in much greater preference to magnesium (section 3.1.2) is very efficient at inhibiting exogenous NADH oxidation (table 5; Coleman and Palmer, 1971). It was reasonable to assume that the removal of calcium caused the inhibition in EGTA-treated mitochondria because this inhibition could be reversed by the addition of excess calcium (table 6; figures 3-5). Consequently it was expected that the addition of calcium to the chelator-treated mitochondria would lead to a stimulation of the rate of NADH oxidation back to the 'uninhibited' rates.

However, when using some chelators (namely DHPE, HSN, fluorescein complexone, calcichrome; table 6) the addition of excess calcium did not restore the rate of NADH oxidation. One interpretation could be that the inhibition for each of these chelators does not involve the chelation of calcium but is due to the specific binding of the chelator to a component of the electron transport chain at the outer surface of the inner membrane so that soluble calcium is denied access to the dehydrogenase. It was interesting to note that these four chelators were more readily soluble in ethanol (table 6) suggesting that their action may not have been at the outer face of the inner membrane but within the hydrophobic regions of the membrane. Thus data obtained by adding calcium to mitochondria treated with these four chelators (DHPE, HSN, fluorescein complexone and calcichrome) cannot be accounted for simply by the removal of calcium essential for exogenous NADH oxidation and were therefore equivocal with respect to the hypothesis that calcium was involved in the oxidation of exogenous NADH. On the other hand however, data obtained by adding calcium to mitochondria treated with EGTA, EDTA, HEDTA, DPTA, citrate and solochrome respectively

(when the rate of oxidation was stimulated) strongly supported the view that calcium was involved in the regulation of the exogenous NADH dehydrogenase.

The influence of calcium in stimulating the oxidation of NADH could not be accounted for by the uncoupling of mitochondria (Carafoli and Lehninger, 1971) since it was observed in the presence of FCCP (figure 3). This also suggests energy-dependent uptake of calcium into the matrix plays no role in the stimulation which seems likely to occur on the outer face of the inner membrane (Palmer and Coleman, 1974).

### 4.3 Stimulation of exogenous NADH oxidation by a wide range of cations

In this study the experimental programme was initiated using a reaction medium containing magnesium and phosphate (standard; table 2) which induced acceptable rates of exogenous NADH oxidation in the presence of FCCP. This reaction medium was used at the outset of this study for traditional reasons, having been developed for use in assays in which ADP is used to promote State 3 rates. These is also a feeling that divalent cations protect the integrity of mitochondrial membranes (e.g., Mannella and Bonner, 1975 and section 1.7.4). Moreover it has recently been suggested that divalent cations decrease membrane fluidity (Earnshaw and Cooke, 1981); this may be related to the maintenance of membrane integrity.

However when the reaction medium was simplified to exclude magnesium and phosphate, support for the stimulation of exogenous NADH oxidation by a wide range of cations was discovered (table 12; figure 25) in addition to that already presented for inorganic divalent cations (figure 2; table 7). Thus inorganic and organic cations, mono-, di- and trivalent, not just inorganic divalent cations are all

found to be equally stimulatory.

On the basis that maximum stimulation of exogenous NADH oxidation occurred over different concentration ranges, e.g. 100 μM for $(TEC)^{3+}$ (an organic trivalent), 1 mM for $(DM)^{2+}$ (an organic divalent) and 10 mM for $K^{+}$, the salts may be arranged by the valency of the cation (figure 25). The chemical nature of the anion and its valency seemed unimportant in the stimulation of NADH oxidation (e.g. $MgSO_4$ and $MgCl_2$ are equally stimulatory; table 12). These results are consistent with published data (Hackett, 1961; Miller *et al.*, 1970; Earnshaw, 1975) and provide evidence that the ability of a wide range of cations to stimulate NADH oxidation is due to an electrostatic screening of fixed negative charges associated with the mitochondrial membranes. This proposal is further supported because organic cations, which are unlikely to bind to membrane groups, are effective as well as inorganic cations. As outlined in section 3.3.2 the stimulation by a range of cations of different valency could be explained by a reduction in the membrane surface potential induced by altering the cation composition of the diffuse layer, thus decreasing repulsion of negatively charged NADH.

Additional strong support for this theory is obtained using the monovalent fluorescent probe 9AA (section 3.3.3). The addition of mitochondrial membranes to low salt reaction medium containing 9AA causes a partial quenching of fluorescence. In thylakoid membranes this partial quenching is considered to be due to the concentration quenching which follows the accumulation of 9AA into the diffuse layer associated with the membranes (Searle *et al.*, 1977; Barber, 1977). It is assumed that this explanation also accounts for the observations made in this study using mitochondrial membranes (section 3.3.4).

The quenched fluorescence can be released by increasing the concentration of cations thus displacing 9AA from the diffuse layer. The similarity in response to increase in concentration of both monovalent ($Na^+$) and divalent ($Mg^{2+}$) cations, both in the increase of fluorescence of 9AA and in the stimulation of rate of NADH oxidation (figure 27), is good evidence to support the view that the rate of oxidation of exogenous NADH is related to the degree of screening of the fixed charges on the surface of the membranes afforded by the cations in the diffuse layer. From these results it is assumed that mitochondrial membranes carry a net negative charge (like thylakoid membranes, Davis and Gross, 1975; Searle *et al.*, 1977; Barber, 1977; Chow and Barber 1980a,b). This has been confirmed by particle electrophoresis (S.P.J., I.M.M. and H.Y.N. unpublished data). These negative, fixed, surface charges will attract cations into the diffuse layer adjacent to the membrane surface (Barber *et al.*, 1977). The cation composition of this diffuse layer depends upon the electrolyte composition of the medium in which the membranes are suspended. The double layer theory (Gouy, 1910; Chapman, 1913) predicts that the effectiveness of the cation in charge shielding is determined by its charge with the following order of effectiveness: $C^{3+} > C^{2+} > C^{+}$ and not by the chemical nature of the cations. Data in figures 25 and 27 are consistent with the predictions of the classical double layer theory (Barber and Searle, 1978). The data in table 14 underline the similarity between the concentrations of various cations required to stimulate both the rate of NADH oxidation and the release of fluorescence of 9AA (cf. figure 27). There is a close correlation between the values presented for monovalent and for trivalent cations. However when considering the divalent cations it can be seen that compared with

the bulky organic cation $(DM)^{2+}$ significantly lower concentrations of the inorganic divalent cations produce half maximal stimulation of the rate of exogenous NADH oxidation than is required for half maximal release of fluorescence quenching using 9AA. This is not consistent with a purely non-specific stimulation related to a reduction in membrane surface potential. Indeed such a discrepancy could occur if the inorganic cations used were able to exert a measure of chemical selectivity and bind preferentially to those negative charges closely associated with bringing about the stimulation of NADH oxidation.

4.4 A specific requirement for calcium

It has been shown that a wide range of cations including magnesium stimulate the rate of NADH oxidation (section 4.3, figure 26, table 12). This is considered to be due to the production of maximal screening conditions (as assessed by 9AA fluorescence levels using $(DM)^{2+}$, calcium and magnesium, Møller, Johnston and Palmer, 1981). However in the presence of excess magnesium EGTA (which has a relatively low affinity for magnesium, section 3.1.2.1) is effective at producing an inhibition of NADH oxidation (figure 28). So under conditions of maximal screening there must be another reason for the inhibition of NADH oxidation observed in the presence of EGTA and magnesium (figure 28). Mitochondria are usually isolated with associated calcium, sufficient to promote maximal rates of NADH oxidation (section 3.2.3.3). Furthermore as discussed earlier (section 4.2 ) mitochondria are isolated with calcium and magnesium in the ratio of 1:1 in the EGTA-accessible compartment. As EGTA chelates calcium in far greater preference to magnesium it is proposed that even under conditions of maximal screening EGTA is able to chelate essential mitochondrial

calcium and thereby inhibit exogenous NADH oxidation. Having indirectly demonstrated a requirement for calcium in exogenous NADH oxidation, various aspects of its inhibition by EGTA are now considered.

## 4.5 Lack of calcium-dependent NADH oxidation in the absence of chelator

Attempts to demonstrate calcium-dependent exogenous NADH oxidation in the absence of chelators were always unsuccessful no matter whether the isolation procedure was standard or 'low salt', nor whether the mitochondria were washed once or several times in the presence and absence of EDTA or EGTA, nor whether the reaction medium was 'low cation' or excess magnesium (table 9, figures 11 & 13, section 3.2.3). However it was possible to demonstrate a requirement for a wide range of cations either mono-, di- or trivalent, inorganic or organic, evidently for screening the fixed negative charges on the membrane surfaces. A specific requirement for calcium could only be demonstrated directly in the presence of EGTA (figure 28). However following washing treatments involving chelators, the rate of NADH oxidation became much more sensitive to subsequent treatment with EGTA (table 9) when measured in a reaction medium containing magnesium. The initial exposure to chelator, whether in the isolation- or wash-medium, made a significant (i.e., ca. 1/20th; figures 9-11, 13; table 9) reduction in the concentration of EGTA causing 50% inhibition of exogenous NADH oxidation (section 3.2.1.4), hereafter referred to as the $I_{50\%}$ (EGTA). However subsequent exposure to chelators (up to 3 washes with EGTA wash medium, figure 11) had no significant additional effect compared with the sensitivity arising from the initial exposure (data not presented). The massive reduction in the $I_{50\%}$ (EGTA) after

a single exposure to chelator during isolation must represent the loss of a significant amount of mitochondrial calcium. As EGTA and EDTA lack access to the matrix (Reed and Bygrave, 1974; section 3.1.2.1) the loss of calcium must have been from the EGTA-accessible compartment which is probably similar to the extramatrix compartment (outer face of the inner membrane, both faces of the outer membrane and the intermembrane space). More specifically some calcium must have been lost both from the polyuronide component of the outer membrane (Mannella and Bonner, 1975) and from the exogenous NADH dehydrogenase on the outer face of the inner membrane. Because mitochondria could be washed free of EGTA following various washing regimes (i.e., maximal rates of NADH oxidation were obtained when assayed in standard reaction medium) this was consistent with the notion that EGTA-induced inhibition of NADH oxidation was due to the chelation of membrane-bound calcium rather than to an actual binding of EGTA to the membranes.

### 4.5.1 Proposed mobilisation of matrix calcium reserves

Increased sensitivity to EGTA treatment (and lack of calcium-dependent NADH oxidation in the absence of chelators) could result, it is proposed, if the amount of calcium involved in the oxidation of exogenous NADH is likely to be small, relative to the total mitochondrial calcium (table 10) and represents only a fraction of the substantial loss of calcium induced by the initial exposure.

It is interesting to speculate that calcium thus mobilised may be transported from the matrix to the outer surface of the inner membrane including the exogenous NADH dehydrogenase, because when assayed in a reaction medium containing magnesium, the dehydrogenase is always maximally activated (in the absence of EGTA) even though there are other sites for binding divalent cations (e.g., the outer membrane

polyuronides, Mannella and Bonner, 1975). This proposed transport of calcium to the dehydrogenase must be regulated in some way because NADH oxidation rates both initially and at minute intervals thereafter are identical for an EGTA-incubation period of 0.5 - 10.0 min (table 8). If transport of calcium from an EGTA-inaccessible space to an EGTA-accessible one were unchecked, mitochondria incubated the longest period would be expected to have the fastest rates of oxidation due to the mobilisation of the greatest amount of calcium; however this is not so.

Further support for the hypothesis that transport of calcium from the matrix may be involved in the activation of the exogenous NADH dehydrogenase in EGTA-treated mitochondria is found in the experiment described in figure 12. The data show that the oxidation of NADH in the presence of EGTA (i.e., inhibited rate) is stimulated by the addition of the divalent cation ionophore A23187 (Lardy *et al.*, 1967; Gómez-Puyou and Gómez-Lójero, 1977; section 3.2.3.2). As A23187 is considered to bind and transport calcium in preference to magnesium (Reed and Lardy, 1972a, b; Truter, 1976) and because there are calcium reserves in EGTA-treated mitochondria (table 10) stimulation of EGTA-inhibited NADH oxidation may be interpreted as representing the movement of calcium down a concentration gradient across the inner mitochondrial membrane thus activating the dehydrogenase.

4.5.2 Mitochondrial calcium content

Reference to table 10 shows that the calcium content of mitochondria may be reduced by between 25 - 50% using various washing media containing EDTA or EGTA. Thus there is a considerable portion of mitochondrial calcium which is resistant to EGTA treatment, consistent with the location of these reserves within the matrix, inaccessible

to EGTA. However, further calcium is lost when divalent cations (i.e., magnesium or $(DM)^{2+}$) are added to the chelator wash medium which indicates that some of the calcium, resistant to EGTA-only wash treatment is actually sited on the outer face of the inner membrane. One interpretation of these data is that chelator-only treatment effectively increases the surface potential by removing divalent cations from the double layer causing them to be replaced with monovalent ions ($H^+$, $K^+$). Thus the chelator has only limited access to the membrane-bound calcium, as discussed earlier (section 4.3). The addition of divalent cations would tend to lower the surface potential and permit the removal of additional calcium as observed (table 10). However magnesium- or $(DM)^{2+}$-chelator wash medium was ineffective at inducing calcium-dependent NADH oxidation in the absence of chelator (table 16). Therefore failure to demonstrate calcium-dependent NADH oxidation using EGTA-divalent cation wash medium (section 4.4) did not seem to be attributed to an increase in the surface potential (although this could be tested using higher concentrations of magnesium). Even though 'recovery' to EGTA treatment depends on the addition of NADH (table 8) and could be further stimulated by the addition of A23187 (figure 12), attempts to deplete the mitochondria of these calcium reserves by incorporating NADH and A23187 in the EGTA-wash medium were also unsuccessful in demonstrating a calcium-dependent exogenous NADH oxidation (figure 13). Perhaps under these conditions, the rate of solubilisation of matrix calcium salts (Earnshaw, 1975) mostly as hydroxylapatite (Weinbach and von Brand, 1965) becomes a limiting factor in the depletion of mitochondrial calcium reserves, for sufficient calcium remains to give maximum rates of NADH oxidation in mitochondria washed up to three times with EGTA media. It seems therefore that a direct

demonstration of calcium-dependent NADH oxidation is beyond the scope of the present study.

## 4.6 Conclusions on cations and the oxidation of exogenous NADH

Having discussed the experimental details, two important points have emerged concerning cations and the oxidation of exogenous NADH.

Firstly members of a wide range of cations of various valencies, inorganic and organic, stimulate the rate of NADH oxidation (figure 25, table 12; Johnston *et al.*, 1981; Møller and Palmer, 1981). This general response to cations (trivalent, most effective; monovalent, least effective) may be interpreted as being due to the screening of fixed negative charges associated with the lipid and protein components of the membrane. Screening of the charges decreases the electrostatic repulsion of the negatively charged substrate, NADH, resulting in an apparent increase in affinity between the enzyme and substrate. Screening also leads to an increase in the $V_m$ which may be attributed to an increased lateral mobility of membrane complexes resulting in an increased collision frequency and higher rates of electron transport (Møller and Palmer, 1981; Schneider *et al.*, 1980). This screening effect is of great importance in understanding the factors which determine the activity of membrane-bound enzymes. However, screening as a regulatory phenomenon is probably relatively unimportant *in vivo* because estimates of the cytosol concentration of cations suggest that under most physiological conditions *in vivo* there will be sufficient cations present to screen the various electronegatively charged membrane-bound organelles (Bowling, 1976).

The second major point resulting from this study is that there is a specific calcium requirement for exogenous NADH oxidation

independent of the general screening effect which may be demonstrated indirectly using EGTA. This specific requirement for calcium is a promising candidate for a physiological regulator of the exogenous NADH dehydrogenase. Examples of calcium activation of various enzymes are given in section 1.6.5. Regulation of the activity of certain key enzymes such as pyruvate dehydrogenase, isocitrate dehydrogenase and α-ketoglutarate dehydrogenase may be achieved experimentally using calcium in mammalian mitochondria (McCormack and Denton, 1980). Therefore it can be suggested tentatively that the concentration of free calcium provides the means for an *in vivo* regulation of various enzymes (Williamson and Cooper, 1980; Cooper, 1980). Free calcium concentrations could in turn be regulated by fluxes across the inner mitochondrial membrane as in mammalian mitochondria (Fiskum and Lehninger, 1980; Nicholls and Crompton, 1980; Racker, 1980). There is a different route for flux in each direction, so that free calcium concentration could be used to regulate cytosolic and mitochondrial concentrations of free calcium (Fiskum and Lehninger, 1980; Nicholls and Crompton, 1980).

It is now profitable to consider more closely the complex nature of the EGTA inhibition of NADH oxidation both when EGTA is added before oxidation is initiated and during NADH oxidation.

### 4.6.1 Exogenous NADH oxidation in mitochondria incubated with EGTA

When EGTA is added before NADH, the addition of NADH reveals an initial inhibition in the rate of oxidation followed by a time-dependent increase in $O_2$ consumption up to a partially 'recovered' rate (figures 7D-G, 8A, pH 7.2). Further illustrations of NADH oxidation for this sequence of additions are also provided at other

pH values (pH 6.0 - 9.0, figure 15B; pH 7.2, figures 14E and 16B; pH 8.2, figures 14D, 16C and 20B).

At pH 8.2 the rate of NADH oxidation in the presence of EGTA is less curvilinear than at pH 7.2 and for a given EGTA concentration the inhibition is greater at pH 8.2 (figure 14D, E). This is presumably so because its dissociation constant favours the formation of the EGTA-$Ca^{2+}$ complex.

4.6.2 Addition of EGTA to mitochondria in which NADH oxidation has been initiated

When EGTA is added to mitochondria oxidising NADH at pH 7.2, initially no inhibition is seen (figures 4A, 6B). However, an inhibition gradually develops, the magnitude of which is dependent on both EGTA concentration and the period of time from when EGTA is added (figure 7A-C). As the change in rate becomes negligible (figure 8B) the relationship between EGTA concentration and % inhibition of NADH may be represented as an 'S'-shaped curve (figure 7C) similar to that obtained for EGTA when added in the sequence EGTA-NADH (figure 7F-G).

4.6.3 Speculations on the role which calcium plays in the regulation of the exogenous NADH dehydrogenase

It is helpful to consider theoretical models when attempting to explain observations on the EGTA inhibition of NADH oxidation, both when EGTA is added before oxidation is initiated and during NADH oxidation. Speculation thereby provides a valuable platform for discussion. Each of the following models gives some insight into the experimental results.

4.6.3.1 Proposal for NADH-induced conformational changes in the dehydrogenase

The variations in the rate of NADH oxidation, depending on whether NADH oxidation is initiated before or after EGTA is added, must reflect the complex role that calcium plays in the regulation of the activity of the dehydrogenase. For example, observations for both sequences of addition suggest that the oxidation of NADH somehow renders the involved calcium less available to chelators. One interpretation of the data is that NADH renders the calcium less available to EGTA by the production of a conformational change in the dehydrogenase. Alternatively NADH could alter the affinity of the dehydrogenase for calcium so that the dehydrogenase competes successfully with EGTA for calcium essential for exogenous NADH oxidation. Indeed such a change in affinity could result from a conformational change induced by NADH. In the prolonged absence of NADH, calcium essential for exogenous NADH oxidation would be freely available for chelation with EGTA. Upon the subsequent addition of NADH, as calcium is not present at the dehydrogenase, no conformational change occurs and the dehydrogenase remains inactive so that the initial rate of oxidation in inhibited. This model therefore accounts for the observed initial rates of NADH oxidation and is further supported by data presented elsewhere (Møller, Johnston and Palmer, 1981).

However the initial rates of NADH oxidation are not maintained but tend to increase or decrease respectively depending upon sequence of addition of NADH and EGTA. This is most marked at pH 7.2 under fully screened conditions. When EGTA is added prior to NADH, the rate of oxidation gradually increases at pH 7.2 in a manner which suggests that calcium is being mobilised from an EGTA-inaccessible

compartment as discussed earlier; stimulation of this rate by A23187 supports this view.

4.6.3.2 Proposal that calcium binds NADH to the dehydrogenase

An alternative model for how calcium may regulate the exogenous NADH dehydrogenase proposes that calcium binds NADH to the dehydrogenase. It is then possible to account for the initial rates observed in the following manner. As NADH oxidation is initiated, $NAD^+$ is released from the membrane. NADH and EGTA then freely compete for the exposed membrane calcium. As EGTA gradually gains access to the essential calcium, the rate of NADH oxidation steadily declines until an equilibrium is reached between the calcium binding groups on the membrane associated with the dehydrogenase, the rate of calcium efflux from the matrix and the concentration of both EGTA and NADH.

4.6.3.3 Calcium and membrane integrity

As discussed earlier (section 4.6), general cation screening leads to higher rates of electron transport (Møller and Palmer, 1981) which may be attributed to increased lateral plane mobility of membrane complexes (Schneider *et al.*, 1980). In contrast data using the positively charged amine local anaesthetic prilocaine can be used to construct a model for a calcium-specific regulation of exogenous NADH oxidation on the basis that asymmetrically distributed negatively charged species and the calcium preferentially associated with them (Hope and Cullis, 1979) can increase the rigidity of membranes (e.g. plasma membranes, Gordon *et al.*, 1978) and therefore create local regions of laterally segregated lipids of different chemical and physical properties (Papahadjopoulos *et al.*, 1975;

Marinetti and Crain, 1978). Hence the removal of membrane-bound calcium as displaced by cationic drugs in plasma membranes (e.g. Hauser and Dawson, 1968; Papahadjopoulos, 1972; Low *et al.*, 1979) is likely to have profound effects on lateral lipid distribution. This is also likely to be the case when membrane-bound calcium is removed by chelation with EGTA from the outer face of the inner mitochondrial membranes from Jerusalem artichoke tubers. Thus by binding acidic phospholipids into clusters calcium locally increases the concentration of the protein components and stimulates the rate of exogenous NADH oxidation.

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# THE STIMULATION OF EXOGENOUS NADH OXIDATION IN JERUSALEM ARTICHOKE MITOCHONDRIA BY SCREENING OF CHARGES ON THE MEMBRANES

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## 1. Introduction

The ability of plant mitochondria to oxidize exogenous NADH is of great significance in the energy economy of the cell and it follows that the means of regulating such an oxidation must attract interest. The stimulation of exogenous NADH oxidation by various inorganic monovalent and divalent salts has been reported extensively [1], where divalent cations were noted to be effective at much smaller concentrations than monovalent cations, whereas the nature of the anion appeared to be unimportant. More recently, evidence presented in [2,3] was interpreted to indicate that the stimulation of exogenous NADH oxidation may be specifically dependent on $Ca^{2+}$ (with $Mn^{2+}$ and $Sr^{2+}$ being much less effective). In the present study, using a range of salts which include inorganic and organic cations, the oxidation of exogenous NADH has been re-examined in conjunction with an investigation into the behaviour of cations in the diffuse layer associated with the mitochondrial membrane using the fluorescent monovalent cation 9-aminoacridine (9-AA). The results presented here are consistent with the view that electrical charges at the membrane surface influence the ease with which the substrate NADH may approach the membrane-bound dehydrogenase.

*Abbreviations:* 9-AA, 9-aminoacridine; $(DM)Br_2$, decamethylene-bis-trimethylammonium bromide; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; $(TEC)Cl_3$, tris(ethylenediamine)cobalt (III) chloride; TES, *N*-tris (hydroxymethyl)methyl-2-aminoethane sulphonic acid

## 2. Materials and methods

### 2.1. *Materials*

NADH was obtained from C. F. Boehringer and Soehne GmbH, Mannheim. FCCP was a generous gift from Dr P. G. Heytler, Du Pont Chemicals. KCl, NaCl, $MgSO_4$, $MnCl_2$, $CaCl_2$ and $LaCl_3$ were obtained from BDH Chemicals Ltd, Poole, Dorset. $(DM)Br_2$ was from Sigma Chemical Co., $(TEC)Cl_3$ and 9-AA · HCl from Koch-Light Labs., Colnbrook, Bucks. and TES from Hopkin and Williams, Chadwell Heath, Essex.

### 2.2. *Isolation of mitochondria*

Mitochondria were isolated essentially as in [4]. They were then suspended in a medium containing 0.4 M sucrose, 2 mM TES (pH 7.2) (low salt medium) and removed by centrifugation at 48 000 × *g* for 2 min. The resulting pellet was finally suspended in a small volume of low salt medium to give a final preparation containing ~10 mg protein/ml.

### 2.3. *Oxygen consumption*

$O_2$ uptake by mitochondrial suspensions was measured in a Rank oxygen electrode assembly (Rank Bros., Bottisham, Cambridge) at 25°C in a low salt medium (0.3 M sucrose, 2 mM TES, pH 7.2). The concentration of oxygen was assumed to be 240 nmol $O_2$/ml. FCCP ($2 \times 10^{-7}$ M) and, where appropriate, the required amount of salt solutions were added to the oxygen electrode reaction vessel before $O_2$ consumption was initiated by the addition of 1 mM NADH.


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2.4. *Protein determination*

The protein content of suspensions of mitochondria was measured by the Lowry method [5] using crystalline bovine serum albumin (fraction V) as the standard.

2.5. *Measurement of 9-AA fluorescence*

The fluorescence of 9-AA was measured at room temperature in a Perkin-Elmer MPF-3 fluorescence spectrophotometer as in [6]. The excitation wavelength was 398 nm (slitwidth 2 nm) and the emission was measured at 456 nm (slitwidth 5 nm). At a sensitivity setting of 1.0 this gave a reading of 72–74% full-scale deflection on the recorder with 20 μM 9-AA. The medium (1.5 ml) was the same low salt medium as used in the oxygen electrode. 9-AA (20 μM), mitochondria and aliquots of stock salt solutions were added consecutively and readings taken after each addition.

## 3. Results and discussion

Data presented in fig.1 demonstrate that in a low salt medium (0.3 M sucrose, 2 mM TES, pH 7.2) the rate of exogenous NADH oxidation may be enhanced by the addition of KCl, $(DM)Br_2$ and $(TEC)Cl_3$, illustrating that inorganic and organic cations of various valencies may be equally stimulatory. However, it can be seen that maximum stimulation of the rate of NADH oxidation caused by these cations occurred over different concentration ranges, i.e., 100 μM for $(TEC)^{3+}$, 1 mM for $(DM)^{2+}$ and 80 mM for $K^+$. These results are consistent with observations made [1,3] using monovalent and divalent inorganic cations and provide good evidence that the stimulation of NADH oxidation lacks chemical specificity towards the stimulating cation. It therefore seems likely that the ability of cations to stimulate is due to an electrostatic screening of fixed charges associated with the

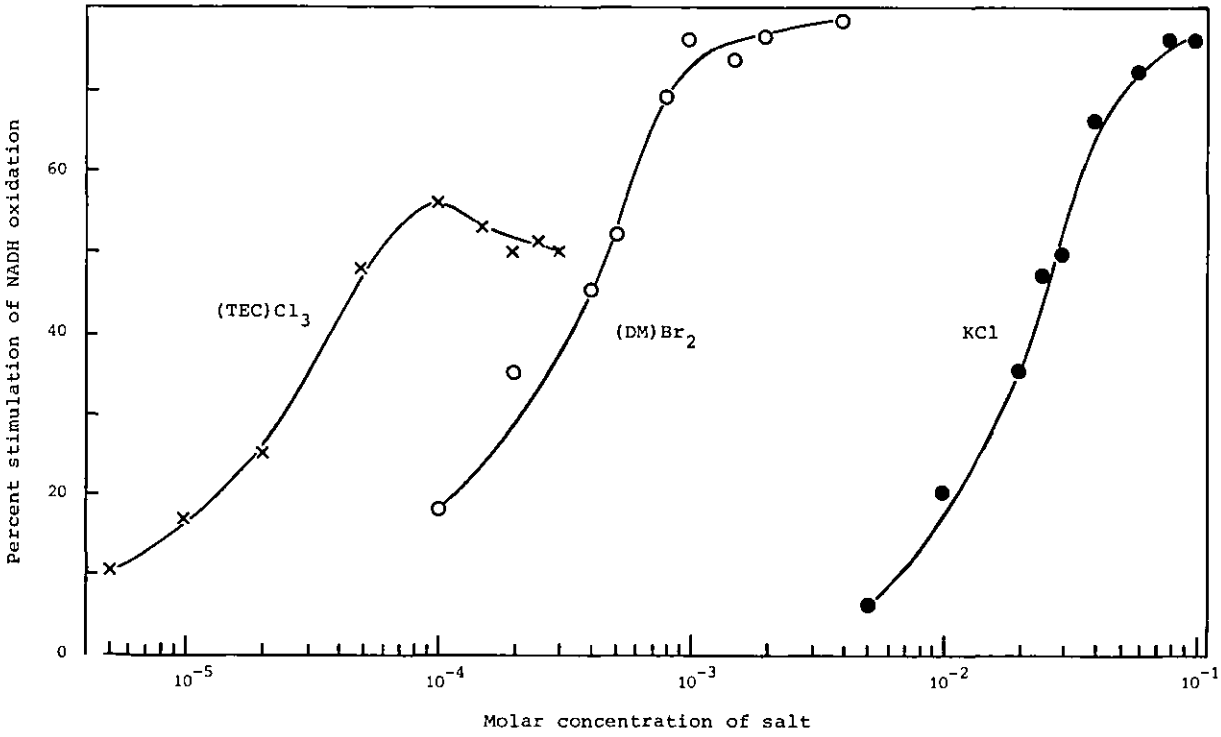


Fig.1. The stimulation, by salts, of the oxidation of exogenous NADH by Jerusalem artichoke mitochondria. The stimulation caused by $(TEC)Cl_3$ (×), $(DM)Br_2$ (○) and KCl (●) is expressed as the percent stimulation over the control containing no added salt. Each point represents a separate determination. The final protein concentration used in the assays was 0.4 mg/ml and the rate of oxygen consumption in the absence of salt was 134 nmol . $min^{-1}$ . (mg protein)$^{-1}$.

membrane surface. This theory was investigated further using the fluorescent cation 9-AA. The addition of mitochondrial membranes to a low-salt reaction medium containing 20 $\mu$M 9-AA causes a partial quenching of fluorescence which has been assumed, as in the case with thylakoid membranes [6,7], to be due to the concentration quenching following the accumulation of 9-AA into the diffuse layer associated with the membranes. The quenched fluorescence can be released by increasing the concentration of other cations thus displacing 9-AA from the diffuse layer. Data presented in fig.2a,b compare the ability of $Na^+$ and $Mg^{2+}$ to release the quenched fluorescence of 9-AA and stimulate the oxidation of exogenous NADH. The ability of the cations to release the quenching of fluorescence has been expressed as a percentage of the maximal release that can be achieved by very high concentrations of the ion in question. Both assays were carried out on the same preparation using equal concentrations of protein in each assay. It can be seen that the increase in fluorescence of 9-AA and the increase in NADH oxidation respond in a very similar manner to the increase in the concentration of both monovalent and divalent cations. This similarity in response suggests that in Jerusalem artichoke mitochondria the stimula-

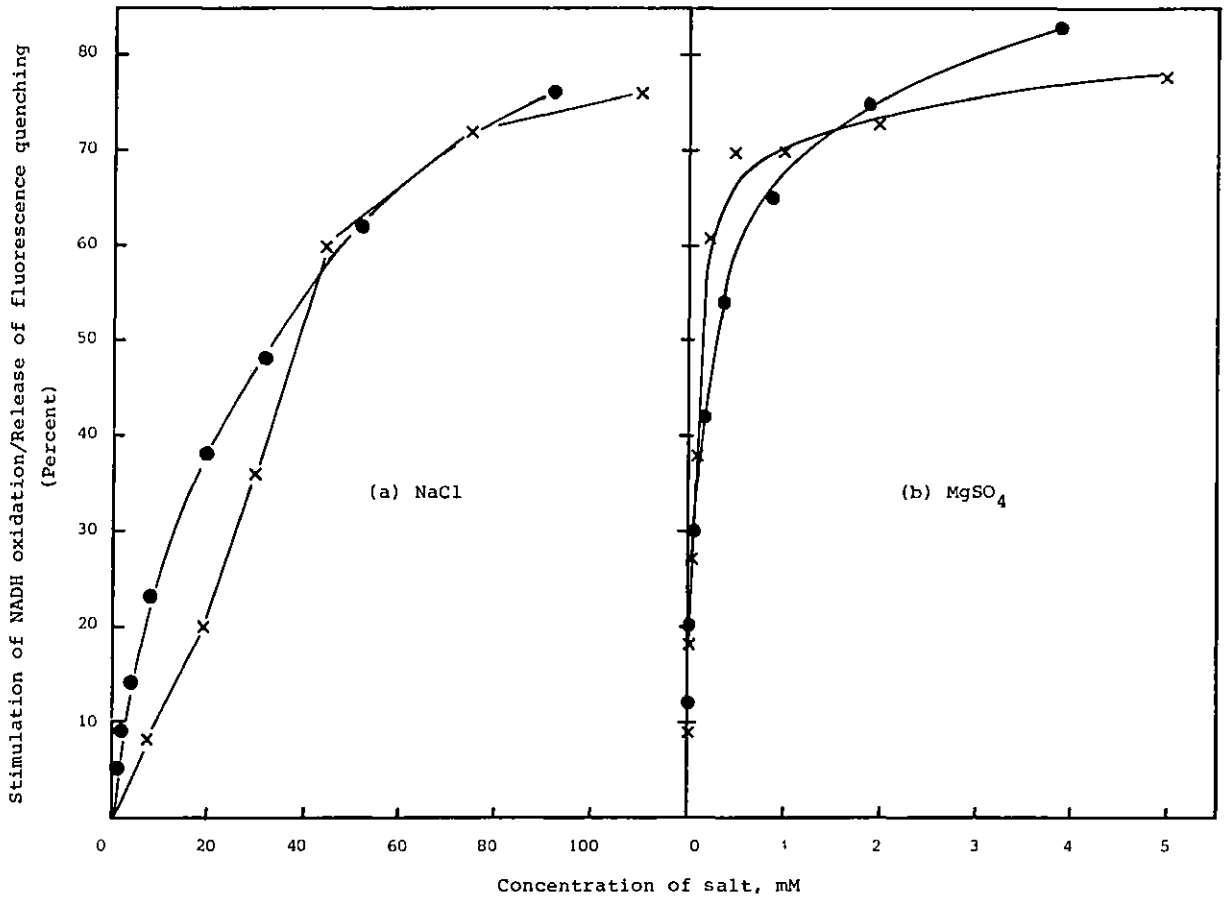


Fig.2. The effect of salts on the oxidation of exogenous NADH and the release of quenching of 9-AA fluorescence. The stimulation of oxidation of NADH (X) is expressed as the percent increase over the rate obtained without added salt. The release of quenching of 9-AA fluorescence (•) is expressed as the percentage of the maximal release obtainable with excess salt. The final protein level employed in all assays was 0.37 mg/ml and the rate of oxygen consumption in the absence of salt was 125 nmol . $min^{-1}$ . $(mg\ protein)^{-1}$ .

tion of oxidation of exogenous NADH may be due, in part at least, to the degree of screening of the fixed charges on the surface of the membrane by the cations in the diffuse layer. Since mitochondria cause the fluorescence of 9-AA to be quenched and because this can be released by a variety of cations it is suggested that, like thylakoid membranes [7,8], the membranes of the mitochondria are negatively charged. This has been confirmed by particle electrophoresis (I.M.M., H. Y. Nakatani, unpublished data). These fixed negative surface charges will attract cations into the diffuse layer adjacent to the membrane surface [9]. The cation composition of this diffuse layer will depend upon the electrolyte composition of the medium in which the mitochondrial membranes are suspended. The double layer theory as developed [10,11] predicts that the effectiveness of the cation in charge shielding is determined by its charge, with the following order of effectiveness:

$$C^{3+} > C^{2+} > C^{+}$$

and not by the chemical nature of the cation. It is clear that the data in fig.1,2 are consistent with the prediction of the classical double layer theory [12]. The data in table 1 underline the similarity between the concentrations of various cations required to stimulate both the rate of NADH oxidation and the release of 9-AA fluorescence. The values presented are the concentrations required to cause 50% of either maximum stimulation of the rate of NADH oxidation or maximum release of fluorescence. There is a close correlation between the values presented for monovalent and for trivalent cations. When considering the divalent cations, however, it can be seen that significantly lower concentrations of the inorganic divalents produce half-maximal stimulation of the rate of exogenous NADH oxidation than is required for half-maximal release of fluorescence quenching using 9-AA. Such a discrepancy could occur if the inorganic cations used were able to exert chemical selectivity and bind preferentially to those negative charges closely associated with bringing about the stimulation of NADH oxidation. It is significant to note that in the case of the organic divalent cation $(DM)^{2+}$, which may be expected to lack the required chemical specificity to bind to the required groups, there is less discrepancy between the concentrations necessary to stimulate the oxidation of NADH and release the quenching of fluorescence of 9-AA (table 1).

The data in fig.3 illustrate that certain cations may actually inhibit NADH oxidation, although release of 9-AA fluorescence caused by $Mn^{2+}$ and $La^{3+}$ is similar to that caused by cations which are stimulatory in NADH oxidation (I.M.M., unpublished data). In the case of $Mn^{2+}$ very low concentrations stimulate the oxidation of NADH although inhibition soon becomes apparent as the concentration is increased. In the case of $La^{3+}$ inhibition was observed at the lowest concentration tested. All the assays carried out in this study were conducted in the presence of FCCP and in the absence of $P_i$. If phosphate was present $La^{3+}$ had little influence on the rate of NADH oxidation and a precipitate of lanthanum phosphate was obtained. It is assumed that both $Mn^{2+}$ and $La^{3+}$ are capable of

Table 1
Comparison between the effect of salts upon exogenous NADH oxidation and upon release of quenching of 9-AA fluorescence

| Salt | Assay protein (mg/ml) | Concentration of salt at which fluorescence quenching is half-maximally released (mM) | Concentration of salt at which NADH oxidation is half-maximally stimulated (mM) |
|---|---|---|---|
| NaCl | 0.37 | 35 | 32 |
| KCl | 0.42 | – | 21 |
| $MgSO_4$ | 0.37 | 0.32 | 0.10 |
| $CaCl_2$ | 0.47 | 0.37 | 0.12 |
| $(DM)Br_2$ | 0.37 | 0.34 | 0.22 |
| $(TEC)Cl_3$ | 0.37 | 0.031 | 0.023 |

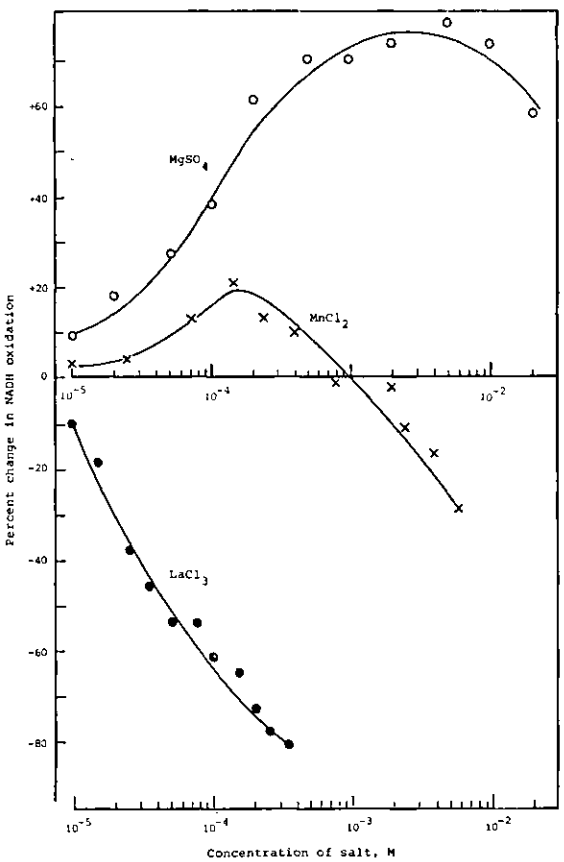


Fig.3. The effect of $Mg^{2+}$, $Mn^{2+}$ and $La^{3+}$ upon the oxidation of exogenous NADH. The change in rate of oxidation of NADH caused by $MgSO_4$ (○), $MnCl_2$ (×) and $LaCl_3$ (●) is expressed as a percent of the rate obtained in the absence of added salt. The protein concentration employed in the assays was ~0.4 mg/ml and the rate of oxygen consumption obtained in the absence of salt was 125 nmol . $min^{-1}$ . (mg protein)$^{-1}$ .

binding to sites on the mitochondrial membranes which influence adversely the oxidation of exogenous NADH.

The work in [13] has demonstrated that several enzymes located in various biological membranes (including outer and inner mitochondrial membranes) may be activated or inhibited by treatments which alter the density of the surface charge. At physiological pH, NADH is negatively charged and it is reasonable to assume that membranes with an overall negative charge will tend to repel NADH and other anionic substrates. The data here suggest that oxidation of exogenous NADH may be enhanced by treatments which alter the cation composition of the diffuse layer in such a way as to reduce the surface potential and allow this negatively charged substrate to approach the membrane surface.

## Acknowledgements

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# A specific role for $Ca^{2+}$ in the oxidation of exogenous NADH by Jerusalem-artichoke (*Helianthus tuberosus*) mitochondria

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1. The addition of chelators to a suspension of mitochondria in a low-cation medium containing 9-aminoacridine caused a decrease in 9-aminoacridine fluorescence. The chelators removed bivalent cations from the membranes and allowed more 9-aminoacridine to move into the diffuse layer. The relative effect of EGTA and EDTA on the fluorescence suggested that the mitochondria are isolated with about equal amounts of $Ca^{2+}$ and $Mg^{2+}$ on the membranes. 2. The removal of the bivalent ions by chelators resulted in the inhibition of NADH oxidation. The inhibition could not be removed by adding sufficient decamethylenebistrimethylammonium ion ($DM^{2+}$) to screen the fixed charges on the membranes and restore the fluorescence of 9-aminoacridine. This observation suggests that bivalent metal ions have a specific role in the oxidation of NADH. 3. $Ca^{2+}$ and not $Mg^{2+}$ reversed the inhibition of NADH oxidation caused by EGTA, whereas both reversed the inhibition caused by EDTA. This suggests that $Ca^{2+}$ plays a specific role and that $Mg^{2+}$ reverses the inhibition caused by EDTA by displacing the bound calcium from the chelator. 4. The results are interpreted as showing that $Ca^{2+}$ plays a specific role in the oxidation of external NADH in addition to its ability to screen electrostatically or bind to the fixed charges associated with the surface of the membrane.

In a previous paper (Johnston *et al.*, 1979) it was shown that cations enhanced, unspecifically, the oxidation of exogenous NADH by Jerusalem-artichoke (*Helianthus tuberosus*) mitochondria. This enhancement was closely correlated with the ability of the cations to release the quenched fluorescence of 9-aminoacridine. The non-specificity of the cations and the relative effectiveness of cations of different valencies ($C^{3+} > C^{2+} > C^{+}$) was consistent with the view that they acted by screening the fixed charges associated with the surface of the membranes. The screening would decrease the repulsion of the negatively charged substrate, NADH, and thus cause an increase in the effective substrate concentration near the active site of the membrane-bound NADH dehydrogenase (Johnston *et al.*, 1979).

Abbreviations used: $(DM)Br_2$, decamethylene-1,10-bistrimethylammonium bromide; $DM^{2+}$, the cation formed by the dissociation of $(DM)Br_2$; $(TEC)Cl_3$, tris-(ethylenediamine)cobalt(III) chloride; $TEC^{3+}$, the cation formed by the dissociation of $(TEC)Cl_3$; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulphonic acid.

In previous publications it has been proposed that $Ca^{2+}$ is specifically involved in the oxidation of exogenous NADH by both Jerusalem artichoke (Coleman & Palmer, 1971; Cowley & Palmer, 1978) and maize (*Zea mays*) (Miller *et al.*, 1970; Earnshaw, 1975) mitochondria. In Jerusalem artichoke the apparent requirement for $Ca^{2+}$ has only been observed in the presence of chelators like EGTA (Coleman & Palmer, 1971) and citrate (Cowley & Palmer, 1978). In maize, $Ca^{2+}$, $Ba^{2+}$ and $Sr^{2+}$ were the only bivalent cations causing a stimulation of NADH oxidation when the mitochondria were suspended in a 0.2 M-KCl medium in the absence of chelators (Miller *et al.*, 1970). However, no attempt was made in these investigations to distinguish between general screening of negative charges on the membrane surface (Johnston *et al.*, 1979) and more specific effects of the cations which stimulate NADH oxidation.

In the present investigation it is found that Jerusalem-artichoke mitochondria are isolated with a complement of both $Ca^{2+}$ and $Mg^{2+}$ on the membranes. When chelators are used to remove these bivalent cations an inhibition is observed even



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under conditions where the 9-aminoacridine fluorescence indicates that screening is maximal. This points to a specific effect of either $Ca^{2+}$ or $Mg^{2+}$, and it is shown that NADH oxidation specifically requires $Ca^{2+}$ for activity. However, the $Mg^{2+}$ on the membranes does seem to have a small and yet, unexplained, effect on NADH oxidation. Under certain conditions non-linear rates are observed, and this phenomenon will be discussed with respect to a possible reaction mechanism.

## Materials and methods

### *Preparation of mitochondria*

Mitochondria were prepared from Jerusalem-artichoke (*Helianthus tuberosus*) tubers bought in the market and stored at 4°C until used. The procedure was essentially that of Palmer & Kirk (1974) with the following modifications. The first centrifugation was at 48000*g* for 2min. The pellets were resuspended in 0.4M-sucrose/5mM-Tes (pH7.2)/0.1% (w/v) bovine serum albumin and, after a short centrifugation (accelerating to 12000*g* followed by immediate deceleration in a Sorvall RC-2B centrifuge with an SS-34 rotor) after which the pellets were discarded, the mitochondria were recovered from the supernatant by centrifugation at 48000*g* for 2min. These mitochondria were washed a second time in 0.4M-sucrose/2mM-Tes (potassium salt) (pH7.2)/0.1% (w/v) bovine serum albumin to minimize the number of cations present in the final preparation. The specific activity of the final preparation, with respect to oxygen uptake, could not be significantly increased by further purification on a density gradient, showing that the preparation was not extensively contaminated with other organelles.

### *Measurement of NADH oxidation*

The oxidation of exogenous NADH was measured as $O_2$ consumption in a Rank Brothers oxygen electrode (total volume 1.0ml) at 25°C in a low-cation medium consisting of 0.3M-sucrose/5mM-Tes (potassium salt) and 200nM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone at pH7.2. After the addition of mitochondria, a small amount of NADH (10–40$\mu$M) was added and this was allowed to be completely oxidized before the main addition of NADH (1mM) was made. The reason for the use of this initial 'sparker' NADH is discussed in the first part of the Results section.

When chelators were used, these were added after the sparker NADH had been completely oxidized and 30s before the main addition of NADH. $(DM)Br_2$ was added concomitant with the main addition of NADH.

### *Fluorescence of 9-aminoacridine*

The fluorescence of 9-aminoacridine was measured as described by Johnston *et al.* (1979).

### *Determination of protein concentration*

Protein concentration was determined by the method of Lowry *et al.* (1951) after solubilizing the mitochondria with 0.5% (w/v) deoxycholate. Bovine serum albumin (Sigma no. A-8022) was used as the standard.

### *Chemicals*

9-Aminoacridine was from Koch–Light Laboratories (Colnbrook, Bucks., U.K.). $(TEC)Cl_3$ was obtained from Alfa Products, U.S.A. (distributed by Lancaster Synthesis, Lancaster, U.K.). All other chemicals were of analytical grade and were supplied by BDH or Sigma, both of Poole, Dorset, U.K.

## Results

### *Use of 'sparker' NADH*

When the oxidation of exogenous NADH by Jerusalem-artichoke mitochondria was measured as described by Johnston *et al.* (1979), it was observed that the lag before a maximal linear oxidation rate was reached was longer in the presence of low concentrations of cations than it was in the presence of higher concentrations of cations. This is shown in Figs. 1(*b*) and 1(*d*). All cations tested were equally effective in shortening the time necessary to reach the maximal linear rate of NADH oxidation. In an attempt to decrease the duration of the lag in NADH oxidation, a low concentration of NADH was allowed to be completely oxidized in order to achieve a priming effect before the main addition of NADH was made. This small initial addition will hereafter be referred to as a 'sparker'. Results presented in Figs. 1(*a*) and 1(*b*) show that the lag in oxidation was decreased from 90 to 55s when a sparker addition of NADH was used in the low-cation medium and from 60 to 30s in the presence of 2mM-$(DM)Br_2$ (Figs. 1*c* and 1*d*). The duration of the lag and the effect of the sparker NADH appeared to vary with the age of the tubers (results not shown). To decrease such variability in the assays, to have consistent assay conditions and to shorten the lag in obtaining maximal linear rates of NADH oxidation, sparker NADH was used in all experiments.

### *Sequence of addition of chelator and main NADH*

Both Earnshaw (1975) and Cowley & Palmer (1978) have reported that chelators added after NADH cause less inhibition than an equal amount added before NADH. Since EGTA and EDTA were to be used extensively in the present investigation the effect of sequence of addition was characterized in a series of experiments. The results are shown in Table 1 and Fig. 2.

In a low-cation medium (total cation concen-

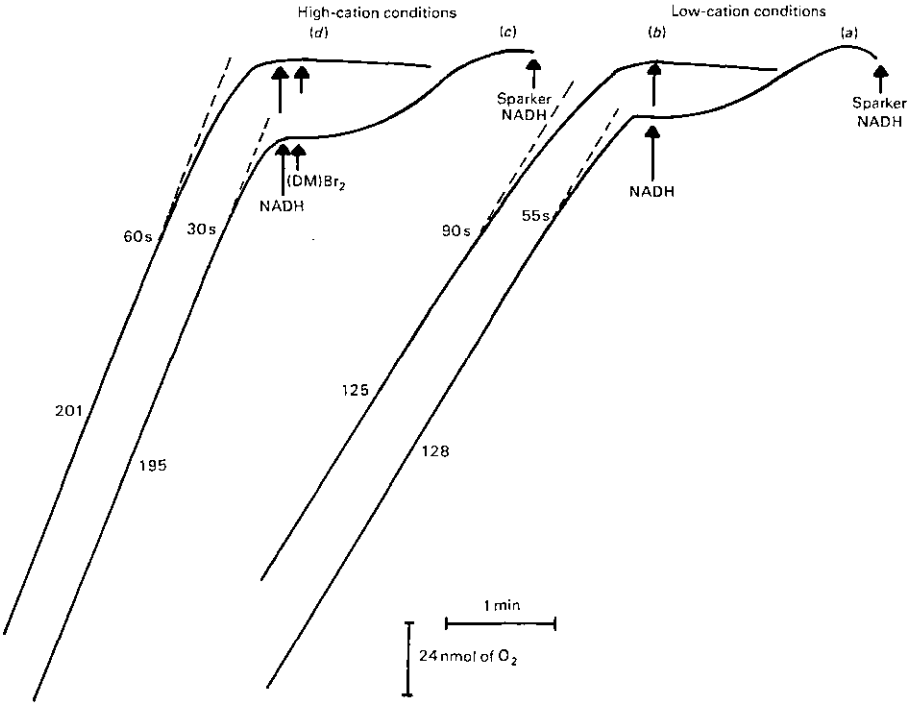


Fig. 1. *Effect of sparker NADH on the lag in attaining maximal linear rates of NADH oxidation*
NADH oxidation was measured as described in the Materials and methods section. Additions were made as indicated on the traces, and final concentrations were: 40 $\mu$M-sparker NADH (*a* and *c*), 2 mM-$(DM)Br_2$ (*c* and *d*), 1 mM-NADH and 0.48 mg of mitochondrial protein · $ml^{-1}$. The numbers on the traces are rates of NADH oxidation in nmol of $O_2 \cdot min^{-1} \cdot mg^{-1}$. The broken lines indicate where linearity was attained, and the duration of the lag is given.

Table 1. *Effect of sequence of addition of chelator and main NADH on the oxidation of NADH*
NADH oxidation was measured as described in the Materials and methods section (0.48 mg of protein · $ml^{-1}$) and chelator was added either 30 s before the main NADH addition [$\pm(DM)Br_2$] or after the main NADH addition at 65% $O_2$ when the rate of oxidation was linear. Rates (nmol of $O_2 \cdot min^{-1} \cdot mg^{-1}$) are given as means $\pm$ S.D. ($n$ = number of measurements on one preparation of mitochondria).

| Additions | Rate in low-cation medium | Inhibition (%) | Rate in low-cation medium + 2 mM-$(DM)Br_2$ | Inhibition (%) |
|---|---|---|---|---|
| Controls | 122 ± 8 (7) | — | 192 ± 8 (4) | — |
| 200 $\mu$M-EGTA | | | | |
| (*a*) Before main NADH | 62 ± 2 (2) | 49 | 60 ± 11 (2) | 68 |
| (*b*) After main NADH | 105 ± 7 (2)* | 16 | Curvilinear (see Fig. 2) | — |
| 200 $\mu$M-EDTA | | | | |
| (*a*) Before main NADH | 49 ± 6 (2) | 60 | 28 ± 6 (2) | 85 |
| (*b*) After main NADH | 94 ± 2 (2)* | 18 | Curvilinear (see Fig. 2) | — |

* Rates measured 1 min after addition of chelator.

tration about 3 mM-$K^+$), both chelators caused a much greater inhibition if added before the NADH (Table 1). When chelators were added before NADH, the rate steadily increased over a period of minutes (the duration was apparently dependent on the final rate) to give ultimately a maximal linear rate. This linear rate is the rate used to quantify the effect of chelators. Chelators added after NADH in

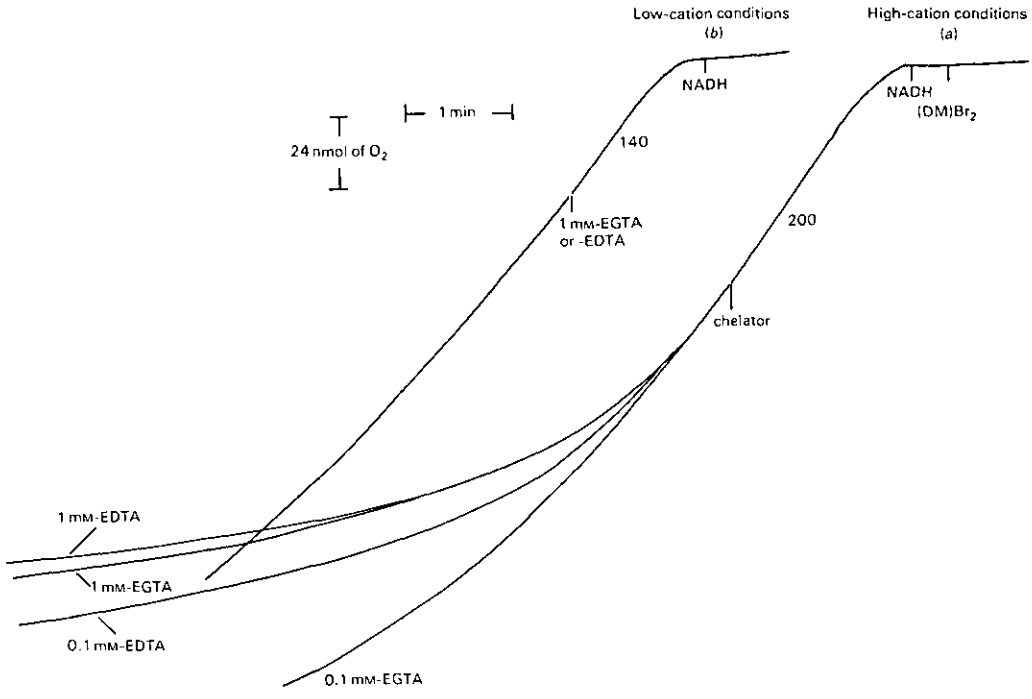


Fig. 2. *Effect of chelators on NADH oxidation when added during oxidation*
NADH oxidation was measured as described in the Materials and methods section and additions were made as indicated on the traces. Numbers on the traces are NADH oxidation in nmol of $O_2 \cdot min^{-1} \cdot mg^{-1}$. Protein concentrations were $0.28\,mg \cdot ml^{-1}$ in (*a*) and $0.46\,mg \cdot ml^{-1}$ in (*b*).

the low-cation medium caused an instant small inhibition that seemed to increase slowly with time (Fig. 2*b*).

When $DM^{2+}$ was added to the low-cation medium, the rate of NADH oxidation increased by 57% (Table 1), confirming previous observations (Johnston *et al.*, 1979). $DM^{2+}$ was used to obtain maximal screening conditions without interfering with the chelators by binding them (Møller *et al.*, 1980). In the presence of $DM^{2+}$ the chelators were more effective in inhibiting the oxidation of NADH (Table 1 and Fig. 2), but again an effect of sequence of addition was observed. Linear rates were reached if chelators were added before NADH. When added after NADH, chelators caused an inhibition that increased rapidly with time. The extent of this inhibition and the rate at which it became effective was dependent on the concentration of the chelator (Fig. 2*a*).

To avoid the problems of curvilinear rates, chelators were subsequently added 30 s before the addition of the main NADH.

*The effect of chelators on NADH oxidation and on the fluorescence of 9-aminoacridine*

In Table 1 and Fig. 2, EDTA was shown to cause

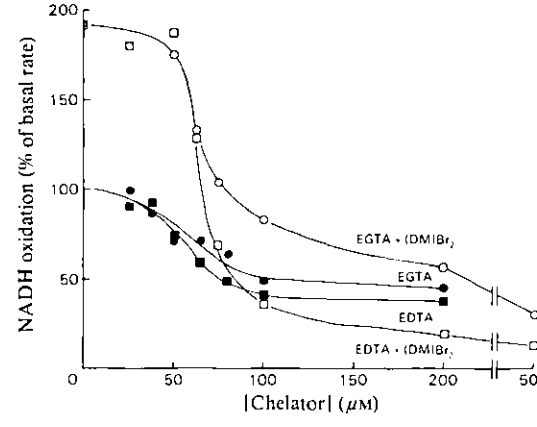


Fig. 3. *Effect of concentration of chelators on the oxidation of NADH under low-cation and high-cation conditions*
NADH oxidation was measured on two preparations of mitochondria as described in the Materials and methods section, with either 0.52 mg (low-cation medium) or 0.44 mg of mitochondrial protein/ml [+$(DM)Br_2$]. The rates are expressed as percentages of the rate in the low-cation medium in the absence of chelators (122 and 148 nmol of $O_2 \cdot min^{-1} \cdot mg^{-1}$ for the two preparations).

a significantly greater inhibition than EGTA. This is confirmed in Fig. 3, in which the effect of increasing concentrations of chelators on NADH oxidation is shown. Both chelators gave rise to S-shaped inhibition curves under conditions where screening would be expected to be near maximum [+2mM-$(DM)Br_2$] as well as in the low-cation medium alone. Both EGTA and EDTA cause equal inhibition of NADH oxidation below 50$\mu$M, whereas above this concentration EDTA proved more effective (Fig. 3). In the low-cation medium, inhibition by 100$\mu$M-EDTA was 57%, which was $14 \pm 5\%$ (mean $\pm$ S.D., $n =$ six preparations) more than that by 100$\mu$M-EGTA. Similarly, EDTA inhibited $23 \pm 5\%$ ($n = 5$) more than EGTA in the presence of $(DM)^{2+}$ (81 as against 57%).

$DM^{2+}$ has until now been assumed to give near-maximal screening conditions, even in the presence of chelators. That this is the case is shown in Fig. 4, where the fluorescence of 9-aminoacridine is measured at various concentrations of chelators either in a low-cation medium [2mM-Tes (potassium salt)] or in the presence of 2mM-$DM^{2+}$, -$Mg^{2+}$ or -$Ca^{2+}$. The addition of mitochondria to 9-aminoacridine in the low-cation medium caused a decrease in fluorescence from approx. 17 to 10 in arbitrary units (results not shown). This indicates that 9-aminoacridine, which is a univalent cation, was being concentrated in the diffuse layer adjacent to the negatively charged membranes of the mitochondria. The addition of EGTA caused a further decrease in fluorescence, and EDTA had an additional effect when added after EGTA (Fig. 4). EDTA alone had the same effect on fluorescence as EGTA followed by EDTA (results not shown). This difference in effect on 9-aminoacridine fluorescence between the two chelators will be discussed below.

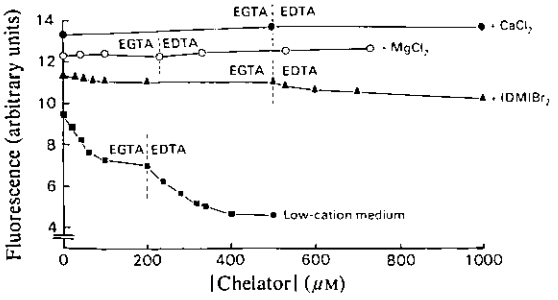


Fig. 4. *Sensitivity of the fluorescence of 9-aminoacridine to chelators in the presence of bivalent cations*

The fluorescence of 9-aminoacridine was measured as described in the Materials and methods section, either in the absence of any added cations (cation concentration about 1mM-$K^+$) or in the presence of 2mM-$(DM)Br_2$, -$MgCl_2$ or -$CaCl_2$ as indicated on the Figure. The results are from two preparations of mitochondria and the results in the low-cation medium were nearly identical. Protein concentrations were 0.52mg·ml$^{-1}$ [$(DM)Br_2$ and $CaCl_2$] and 0.46mg·ml$^{-1}$ ($MgCl_2$). Maximum release of fluorescence quenching was achieved by adding 33mM-$MgCl_2$ and the amount of fluorescence was just above that reached with 2mM-$CaCl_2$.

The addition of bivalent cations to a mitochondrial suspension containing 9-aminoacridine caused a marked increase in fluorescence (Fig. 4), $DM^{2+}$ being less effective than the bivalent-metal cations. The increase in fluorescence shows that the screening of the charges on the membranes was made more efficient by the bivalent cations causing a release of 9-aminoacridine into solution. Chelators had no effect on the fluorescence of 9-aminoacridine in the presence of $Ca^{2+}$ or $Mg^{2+}$ and only a small decrease was observed in the presence of $DM^{2+}$, indicating that a high degree of screening of fixed charges was maintained in all cases (Fig. 4).

The results presented so far show that chelators inhibit NADH oxidation (Table 1, Figs. 2 and 3), even under conditions where the fixed charges were fully screened (Figs. 3 and 4). Thus chelators have a role in causing the inhibition of oxidation of NADH in addition to the de-screening of fixed membrane charges.

*The role of $Ca^{2+}$ and $Mg^{2+}$ bound to the membranes*

The inhibitory effect of the chelators may be due to the removal of bivalent cations, specifically required for NADH oxidation, from the membranes of the mitochondria. Since EDTA binds $Mg^{2+}$ better than EGTA does, although they complex $Ca^{2+}$ equally well (Sillén & Martell, 1964, 1971), the relative efficiencies of the two chelators in causing a decrease in the fluorescence of 9-aminoacridine in the low-cation medium (Fig. 4) would seem to indicate that the mitochondria are isolated with a complement of about equal amounts of $Ca^{2+}$ and $Mg^{2+}$ on the membranes.

In Fig. 5 the effect of chelators on the fluorescence of 9-aminoacridine in a normal preparation of mitochondria is compared with that in mitochondria washed with EGTA + $Mg^{2+}$ as described in the legend. This treatment would be expected to remove $Ca^{2+}$ from the membranes and substitute it with $Mg^{2+}$, thus leaving the membranes relatively depleted of $Ca^{2+}$ and enriched with $Mg^{2+}$. The fluorescence of 9-aminoacridine in the presence of the control mitochondria shows a response to chelators very similar to that found in Fig. 4 [(fluorescence decrease with EGTA)/(fluorescence decrease with EDTA) = 0.60]. The treated mitochondria, on the other hand, show a much decreased response to EGTA and an increased response to

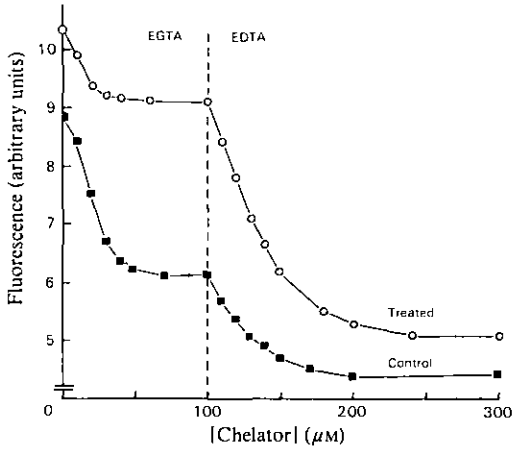


Fig. 5. *Effect of chelators on the fluorescence of 9-aminoacridine in a suspension of control mitochondria and mitochondria washed with EGTA + $Mg^{2+}$*

The pellets from the first centrifugation of an ordinary preparation (see the Materials and methods section) were divided into halves. One half was treated as described in the Materials and methods section. The other half was resuspended in 0.3M-sucrose/5mM-Tes/2.5mM-$MgCl_2$/1mM-EGTA, pH 7.2, and, after being pelleted, these mitochondria were washed in 0.3M-sucrose/2mM-Tes, pH 7.2, to remove the $Mg^{2+}$ and EGTA before the measurement of 9-aminoacridine fluorescence. Fluorescence of 9-aminoacridine was measured as described in the Materials and methods section by using protein concentrations of 0.46 (control) and 0.39 (treated) $mg \cdot ml^{-1}$.

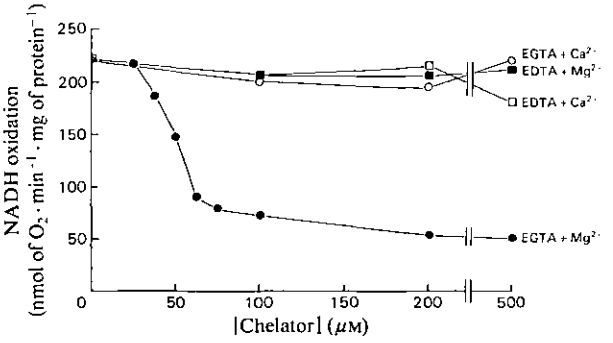


Fig. 6. *Effect of chelators on NADH oxidation in the presence of $Ca^{2+}$ or $Mg^{2+}$*

NADH oxidation was measured as described in the Materials and methods section (0.44mg of protein $\cdot ml^{-1}$), except that 2mM-$CaCl_2$ (open symbols) or 2mM-$MgCl_2$ (closed symbols) was added together with the main addition of NADH. EGTA (circles) or EDTA (squares) were added to the final concentrations indicated.

EDTA [(EGTA effect)/(EDTA effect) = 0.24; Fig. 5].

It would thus appear as if the relative efficiency of the two chelators in causing a decrease in the fluorescence of 9-aminoacridine in the presence of mitochondria is a good measure of the proportion of $Mg^{2+}$ and $Ca^{2+}$ present on the membranes.

In order to see whether NADH oxidation was dependent on $Mg^{2+}$ or $Ca^{2+}$, NADH oxidation was measured in the presence of 2mM of either of these cations and increasing concentrations of chelators. The results are shown in Fig. 6. EGTA gives an S-shaped inhibition curve in the presence of $Mg^{2+}$, which is very similar to the one measured in the presence of $DM^{2+}$ (Fig. 3). The other three combinations of chelators and cations show 'uninhibited' rates even at 500$\mu$M-chelator (Fig. 6). In the presence of EGTA, excess $Mg^{2+}$ is clearly not sufficient to allow NADH oxidation to take place. The fact that $Mg^{2+}$ gave 'uninhibited' rates in the presence of EDTA could not be solely due to the creation of maximal screening conditions (Fig. 4), since it has already been shown that maximal screening by $DM^{2+}$ could not remove inhibition by EGTA or EDTA (Figs. 3 and 4). Therefore it was thought that EDTA would remove $Ca^{2+}$ (and $Mg^{2+}$) from the membranes and that the addition of excess $Mg^{2+}$ would displace the $Ca^{2+}$ from its EDTA complex, allowing it to return to specific sites on the membrane essential for NADH oxidation. The inability of $Mg^{2+}$ to give 'uninhibited' rates in the presence of EGTA could be due to the lower stability constant of $Mg^{2+}$ with EGTA (5.3, as opposed to 8.7 with EDTA; Sillén & Martell, 1964, 1971). This point was pursued further by looking at the ability of a variety of cations with widely varying complexing abilities to reverse the inhibition of NADH oxidation caused by adding a chelator. The results obtained with EGTA are shown in Table 2. In all assays 2mM-$DM^{2+}$ was included to ensure maximal charge screening so that observed effects would not be due to differences in screening.

In the absence of EGTA, most of the cations had little or no effect on NADH oxidation, only $La^{3+}$ and $Cu^{2+}$ inhibiting strongly. The result with $La^{3+}$ is a confirmation of previous results (Johnston *et al.*, 1979). EGTA (200$\mu$M) inhibited 55% in the presence of $DM^{2+}$, which is similar to the amount of inhibition found in Table 1 and in Fig. 3. With the exception of $TEC^{3+}$, the abilities of cations to reverse this EGTA inhibition closely match the stability constants of their EGTA complexes. It is particularly noteworthy that 150$\mu$M-$La^{3+}$ or -$Cu^{2+}$, which are shown to be strongly inhibitory alone, reversed the inhibition by 200$\mu$M-EDTA almost completely. The addition of excess $La^{3+}$ or $Cu^{2+}$ (2mM) still inhibited strongly. The partial reversal

Table 2. *Reversal of EGTA inhibition of NADH oxidation by a range of cations*

The rate of NADH oxidation was measured as described in the Materials and methods section. EGTA was added after the sparker NADH and 30s before the main addition of NADH. The salt whose reversal of EGTA inhibition was to be investigated was added 1 min after the main NADH addition. The results are from two different preparations and the rates and S.D. values are given in the top of the Table. Protein concentrations were 0.43 and 0.48 mg·ml$^{-1}$. Stability constants are from Sillén & Martell (1964, 1971).

| Cation | NADH oxidation (nmol of $O_2$/min per mg of protein) | | Stability constant of cation–EGTA complex |
|---|---|---|---|
| | No chelator | 200 $\mu$M-EGTA | |
| None | 127 ± 2 (2) | 68 ± 6 (2) | |
| 2 mM-$CaCl_2$ | 204 ± 13 (2) | — | |
| 2 mM-$(DM)Br_2$ | 186 ± 14 (2) | 81 ± 3 (2) | |
| 2 mM-$(DM)Br_2$ + the following | | | |
| 100 mM-LiCl | 163 | 69 | 1.2 |
| 100 mM-NaCl | 199 | 36 | 1.4 |
| 150 $\mu$M-$MgCl_2$ | 202 | 54 | 5.3 |
| 2 mM-$MgCl_2$ | — | 41 | 5.3 |
| 150 $\mu$M-$AgNO_3$ | 139 | 71 | 7.0 |
| 2 mM-$AgNO_3$ | — | 60 | 7.0 |
| 150 $\mu$M-$SrCl_2$ | 199 | 207 | 8.1 |
| 2 mM-$SrCl_2$ | — | 230 | 8.1 |
| 150 $\mu$M-$CaCl_2$ | 207 | — | 11.0 |
| 2 mM-$CaCl_2$ | 207 | 230 | 11.0 |
| 150 $\mu$M-$FeSO_4$ | 151 | 159 | 11.9 |
| 2 mM-$FeSO_4$ | — | 202 → 71 | 11.9 |
| 150 $\mu$M-$MnCl_2$ | 189 | 176 | 12.2 |
| 2 mM-$MnCl_2$ | — | 89 | 12.2 |
| 150 $\mu$M-$LaCl_3$ | 26 | 182 | 15.7 |
| 2 mM-$LaCl_3$ | — | 17 | 15.7 |
| 150 $\mu$M-$CuCl_2$ | 9 | 142 | 17.7 |
| 2 mM-$CuCl_2$ | — | 0 | 17.7 |
| 100 $\mu$M-$(TEC)Cl_3$ | — | 136 | See the text |

caused by $TEC^{3+}$ is difficult to understand, since $TEC^{3+}$ contains a $Co^{3+}$ ion, where all co-ordination sites are occupied by ethylenediamine, and $TEC^{3+}$, therefore, should not complex EGTA. It could be explained, however, by assuming an exchange between the ethylenediamine and the EGTA at the $Co^{3+}$-co-ordination sites. The released ethylenediamine complexes $Ca^{2+}$ weakly and the net result would be an increase in free $Ca^{2+}$. A small amount of free $Co^{3+}$ in the $(TEC)Cl_3$ solution is also possible.

The reversal of EDTA-inhibited rates of NADH oxidation by cations (results not shown) is comparable with that obtained with EGTA, including a partial reversal by $TEC^{3+}$. The only exception is that $Mg^{2+}$ can reverse EDTA inhibition, as shown in Fig. 6. Again this is in perfect agreement with the known stability constants of the complexes between the cations and EDTA (Sillén & Martell, 1964, 1971).

Finally, it should be noted that an effect by chelators on the fluorescence of 9-aminoacridine (Figs. 4 and 5) is observed at low concentrations (10 $\mu$M), whereas no effect is observed on NADH oxidation below 30 $\mu$M (Figs. 3 and 6). In Table 3, data on NADH oxidation and the fluorescence of 9-aminoacridine for the same preparations are compared. The effect of a chelator is expressed as the concentration needed to give half-maximal inhibition of NADH oxidation (from Figs. 3 and 6) or half-maximal quenching of the fluorescence of 9-aminoacridine. It is clear that the first bivalent

Table 3. *Concentration of chelator at which half-maximal effect is observed on NADH oxidation and 9-aminoacridine (9-AA) fluorescence*

The data for the effect on NADH oxidation are from Figs. 3 and 6. The fluorescence of 9-aminoacridine was measured on the same three preparations.

| | [EGTA] ($\mu$M) | [EDTA] ($\mu$M) |
|---|---|---|
| Inhibition of NADH oxidation | | |
| (*a*) No cation added | 78 | 69 |
| (*b*) +2 mM-$(DM)Br_2$ | 70 | 66 |
| (*c*) +2 mM-$MgCl_2$ | 52 | — |
| Quenching of 9-AA fluorescence | 35 ± 8 (3) | 29 ± 6 (3) |

cations that are removed from the membranes by low concentrations of chelators influence the fluorescence of 9-aminoacridine, but have no involvement in NADH oxidation (Table 3).

## Discussion

In a previous paper the oxidation of exogenous NADH by Jerusalem-artichoke mitochondria isolated in a low-cation medium was shown to be stimulated in an unspecific manner by cations, the efficiency being determined only by the valency of the cation (Johnston *et al.*, 1979). The evidence given in the present paper shows that Jerusalem-artichoke mitochondria are isolated with about equal amounts of $Ca^{2+}$ and $Mg^{2+}$ associated with the membranes (Figs. 4 and 5) and that an inhibition of NADH oxidation is observed when these bivalent cations are removed by chelators, even in the presence of 2 mM-$DM^{2+}$ to give maximal screening of fixed membrane charges (Figs. 3, 4 and 5). This inhibition can be shown to be specifically due to the removal of $Ca^{2+}$, since an inhibition caused by EGTA is observed in the presence of excess $Mg^{2+}$ (Fig. 6). The fact that maximal activity is observed in the presence of EDTA and excess $Mg^{2+}$ (Fig. 6) is due to the displacement of $Ca^{2+}$ from its EDTA complex by $Mg^{2+}$. This effect is demonstrated with a variety of cations of different complexing ability (Table 2). Thus the oxidation of exogenous NADH by Jerusalem-artichoke mitochondria appears to have a specific requirement for $Ca^{2+}$ ($Sr^{2+}$ can possibly substitute).

It is possible that removal of the $Mg^{2+}$ from the membranes has some effect on NADH oxidation. In fact Fig. 3 quite clearly shows that EDTA inhibits significantly better than EGTA both in the low-cation medium and in the presence of $DM^{2+}$. This must be due to $Mg^{2+}$ being removed from binding sites on which $DM^{2+}$ cannot substitute. From Fig. 5 we know that such sites apparently exist, since chelators have a small but significant effect on the fluorescence of 9-aminoacridine even in the presence of $DM^{2+}$.

Not all the $Ca^{2+}$ on the membranes is equally important for the activity of the NADH dehydrogenase. The results presented in Table 3 and Figs. 3 and 6 show that some $Ca^{2+}$ can be removed by chelators without any effect on the NADH oxidation. Since the outer membrane is permeable to all molecules employed in the present study (Pfaff *et al.*, 1968) and since the mitochondrial inner membrane is not thought to be permeable to EDTA or EGTA (Imedidze *et al.*, 1978; Reed & Bygrave, 1974; Wehrle *et al.*, 1976), addition of chelators in the presence of 9-aminoacridine will yield information on the two surfaces of the outer membrane as well as on the outer surface of the inner membrane, where the NADH dehydrogenase appears to be located (Palmer & Passam, 1971). It is very likely that a portion of the bivalent cations that can be seen by the 9-aminoacridine technique (Figs. 4 and 5) are located on the outer membrane. The removal of these would be expected to have rather little effect on NADH oxidation, and this could account for the observed lack of inhibition by low concentrations of chelators (Figs. 3 and 6).

Having shown that $Ca^{2+}$ is required for the oxidation of exogenous NADH, the time-dependencies observed in Figs. 1 and 2 and the effect of sequence of addition of chelator and main NADH (Table 1) take on special significance, since they provide indirect evidence on the more detailed mechanism of the dehydrogenase.

The fact that chelators inhibited more in the low-cation medium when added before the NADH than after (Table 1 and Fig. 2) indicates that the oxidation of NADH somehow makes $Ca^{2+}$ less accessible to the chelators. This may be due to a conformational change of the dehydrogenase that locks $Ca^{2+}$ into the active site. To remove the $Ca^{2+}$ after it has been locked into place requires the close approach of the chelator. This is apparent from Fig. 2, which shows that EGTA and EDTA (both negatively charged at pH 7.2) when added after NADH inhibit more rapidly under screening conditions (+$DM^{2+}$, Fig. 2*a*) than in the low-cation medium (Fig. 2*b*), where the chelators would be repulsed by the relatively unscreened charges.

The lag before maximal linear rates of NADH oxidation were reached, observed in Fig. 1, could represent the 'locking-into-place' phase. As a result of the oxidation of NADH, $Ca^{2+}$ is mobilized from adjacent sites on the outer surface of the inner membrane or from the matrix by a translocation process and locked into the active site of the dehydrogenase in a time- and respiration-dependent manner. The function of the sparker addition of NADH in decreasing the duration of the lag phase could then be to induce this conformational change in the dehydrogenase enzyme.

In connection with the above speculation, it is noteworthy that the mitochondria that were relatively depleted of $Ca^{2+}$ after an EGTA + $Mg^{2+}$ wash (Fig. 5) showed significantly longer lags before linear rates were attained than did the controls (I. M. Møller, unpublished work). This could be due to the mitochondria having to mobilize $Ca^{2+}$ from a more distant or more respiration-demanding pool.

Lehninger *et al.* (1978) suggested that $Ca^{2+}$ movement across the inner membrane of mammalian mitochondria is regulated by the reduction level of the pyridine-nucleotide pool inside the mitochondria. $Ca^{2+}$ is taken up when the pyridine nucleotides are relatively reduced and a $Ca^{2+}$ efflux is observed under conditions where the pyridine

nucleotide pool is relatively oxidized. Future work will show whether the involvement of $Ca^{2+}$ in the oxidation of exogenous NADH in plant mitochondria fits a similar model.

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# THE EFFECT OF CATIONS ON EXOGENOUS NADH OXIDATION - GENERAL CHARGE SCREENING AND A SPECIFIC REQUIREMENT FOR $Ca^{2+}$

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Plant mitochondria can oxidize exogenously added NADH, presumably via a NADH dehydrogenase on the outer surface of the inner membrane (1). Both mono- and divalent cations have been reported to stimulate this NADH oxidation (2) and a specific role for $Ca^{2+}$ has been suggested (3, 4). Previous work did not, however, distinguish between specific effects and the general ability of cations to screen negative charges thereby allowing the negatively charged substrate to approach the active site (5).

Jerusalem artichoke mitochondria isolated in a low cation medium show a general unspecific stimulation of NADH oxidation (fig.1, ref.5). The efficiency of cations is $C^{3+} > C^{2+} > C^{+}$ consistent with the Gouy-Chapman theory of the diffuse layer (6). Under these conditions $CaCl_2$ has the same effect as $(DM)Br_2$ (5) and there is no indication of any specific effects of $Ca^{2+}$.

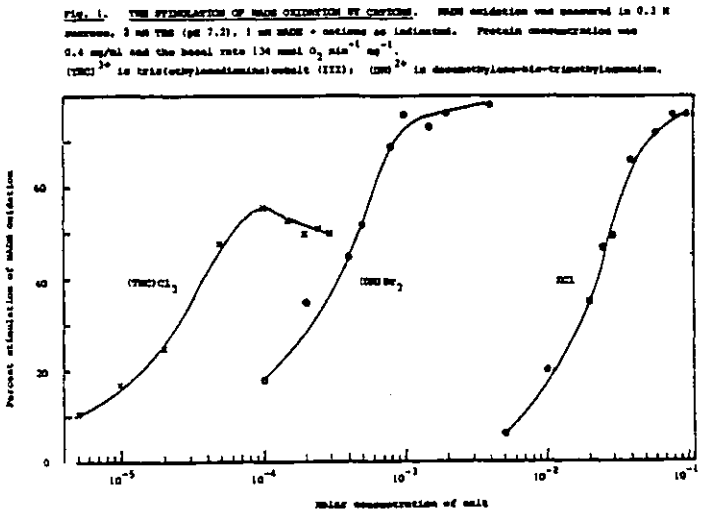


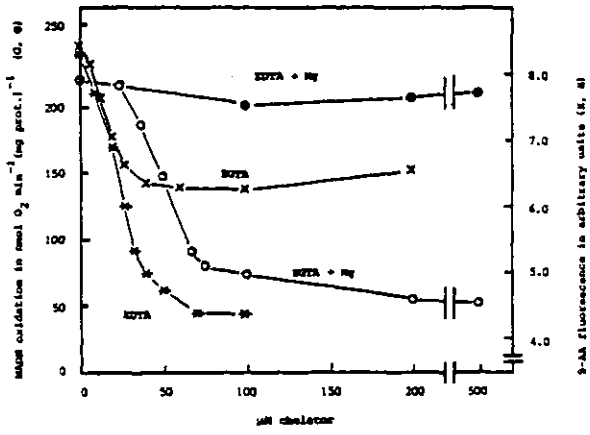

Fig. 1. [illegible]

In the absence of added cations EGTA and EDTA inhibit NADH oxidation (not shown) by removing divalent cations already present on the membranes. These consist of about equal amounts of $Ca^{2+}$ and $Mg^{2+}$ as shown by the 9-AA fluorescent curves in fig.2. 9-Aminoacridine (9-AA) is a fluorescent, monovalent cation. When divalent cations are removed by chelators from their association with the membranes the

concentration of 9-AA increases in the diffuse layer leading to a quenching of fluorescence.

$Ca^{2+}$ can reverse the inhibition of both chelators (not shown) while $Mg^{2+}$ cannot reverse EGTA inhibition even when present in great excess (fig.2). This and other evidence to be presented seems to point at a specific requirement for $Ca^{2+}$ in the oxidation of exogenous NADH.

Fig. 2. THE EFFECT OF CHELATORS ON 9-AA FLUORESCENCE AND NADH OXIDATION.
NADH oxidation and 9-AA fluorescence were measured in 0.3 M sucrose, 3 mM TES (pH 7.2).
The concentration of $Mg^{2+}$ was 2.5 mM and the protein concentration was 0.4 mg/ml.




## Conclusions

1) Cations stimulate NADH oxidation in Jerusalem artichoke mitochondria by screening the negative charges on the membranes thereby increasing the effective concentration of the negatively charged NADH at the active site.

2) When chelators are used to remove divalent cations from the membranes a specific requirement for $Ca^{2+}$ becomes apparent.

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