REGULATION OF THE OXIDATION OF REDUCED NICOTINAMIDE-ADENINE DINUCLEOTIDE IN ISOLATED PLANT MITOCHONDRIA

by

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A thesis submitted for the degree of
Doctor of Philosophy of the University of London
and for the Diploma of Imperial College.

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February, 1977.

ABSTRACT

The results presented suggest that AMP may be involved in the control of electron transport through the complex NADH dehydrogenases in plant mitochondria.

The results of initial experiments indicated that carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was a potent uncoupler of oxidative phosphorylation in isolated Jerusalem artichoke (*Helianthus tuberosus*) mitochondria. FCCP markedly increased the respiration of mitochondria oxidizing NADH or succinate. When NAD^+-linked substrates were used, the response to FCCP was less than expected. Addition of ADP or AMP increased this low rate of respiration. The stimulation of respiration in the presence of FCCP by the adenine nucleotides was not inhibited by oligomycin and therefore could be distinguished from the increase in respiration due to oxidative phosphorylation. Spectrophotometric measurements showed that the addition of adenine nucleotides to these mitochondria resulted in simultaneous oxidation of endogenous NADH and reduction of cytochrome b, indicating enhanced electron flux between the two components.

ADP was only effective in bringing about the enhanced electron transfer in the presence of Mg^{2+}, whereas AMP did not require Mg^{2+}.

Bongkrekic acid, an inhibitor of the adenine nucleotide translocation, did not prevent the stimulation of respiration by AMP. It was concluded that AMP did not have to enter the mitochondria to bring about its effect. FCCP extensively increased the rate of respiration of the mitochondria oxidizing NAD^+-linked substrates in the presence of atractylate. In this case atractylate behaved

like AMP.

The AMP-stimulated electron transfer was inhibited by piericidin A, whereas the piericidin A-resistant pathway by-passing the phosphorylation site I was inhibited in the presence of AMP.

These findings indicate that the respiratory activity of plant mitochondria could be regulated directly by the concentration of AMP in the cytosol.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to Dr J. M. Palmer for his assistance and guidance throughout the course of this work.

I thank Mrs Jill Farmer for the care she has taken in typing this thesis and for her concern for my welfare during my stay in London.

I am indebted to my family, especially my mother and Sunthorn, my husband, for their encouragement and sacrifices throughout the period of my studies.

The Royal Thai Government has provided financial support, for which I am grateful.

CONTENTS

ABBREVIATIONS

AMP, ADP, ATP	adenosine 5'-mono-, di- and triphosphate
ATPase	adenosine triphosphatase
ANS	1-anilinonaphthalene-8-sulphonate
BSA	bovine serum albumin
m-Cl-CCP	carbonylcyanide-*m*-chlorophenylhydrazone
CoASH	coenzyme A
DEAE cellulose	diethylamino-ethyl cellulose
DNP	2,4-dinitrophenol
EDTA	ethylenediaminetetra-acetate
FCCP	carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone
GMP	guanosine 5'-monophosphate
IMP	inosine 5'-monophosphate
MOPS	3-(N-morpholino)propanesulphonic acid
NAD^+	nicotinamide-adenine dinucleotide
NADH	reduced nicotinamide-adenine dinucleotide
$NADP^+$	nicotinamide-adenine dinucleotide phosphate
NADPH	reduced nicotinamide-adenine dinucleotide phosphate
PEP	phosphoenolpyruvate
Pi	orthophosphate
S_{13}	5-chloro-3-*t*-butyl-2'-nitrosalicylanilide
TES	N-tris(hydroxymethyl)-methyl-2-aminoethanesulphonic acid
TMPD	NNN'N'-tetramethylphenylenediamine
TPP	thiamin pyrophosphate
tris	tris(hydroxymethyl)methylamine, or 2-amino-2-hydroxymethylpropane-1,3-diol

SYMBOLS

Å	Ångström unit = 10^{-10} m
ε	extinction coefficient
g	acceleration due to gravity (981 $cm.s^{-2}$)
K_i	dissociation constant of the enzyme-inhibitor complex (the inhibitor constant)
v	velocity (rate of reaction)

LIST OF ENZYMES

Trivial name	Systematic name	Enzyme Commission No.
Adenylate kinase	ATP:AMP phosphotransferase	2.7.4.3
ATPase	ATP phosphohydrolase	3.6.1.3
Catalase	Hydrogen-peroxide:hydrogen-peroxide oxidoreductase	1.11.1.6
Citrate synthase	Citrate oxaloacetate-lyase (*pro*-3S-$CH_2.COO^-$ → acetyl-CoA)	4.1.3.7
Glutamate-oxaloacetate transaminase (Aspartate aminotransferase)	L-Aspartate:2-oxoglutarate aminotransferase	2.6.1.1
Hexokinase	ATP:D-hexose 6-phosphotransferase	2.7.1.1
Isocitrate dehydrogenase (NAD^+)	threo-D_S-Isocitrate:NAD^+ oxidoreductase (decarboxylating)	1.1.1.41
Lactate dehydrogenase	L-Lactate:NAD^+ oxidoreductase	1.1.1.27
Malate dehydrogenase	L-Malate:NAD^+ oxidoreductase	1.1.1.37
Malic enzyme (NAD^+ requiring)	L-Malate:NAD^+ oxidoreductase (decarboxylating)	1.1.1.39
NADH dehydrogenase	NADH:(acceptor) oxidoreductase	1.6.99.3
α-Oxoglutarate dehydrogenase	2-Oxoglutarate:lipoate oxidoreductase (decarboxylating and acceptor-succinylating)	1.2.4.2
PEP carboxykinase (ATP requiring)	ATP:oxaloacetate carboxy-lyase (transphosphorylating)	4.1.1.49
Pyruvate dehydrogenase	Pyruvate:lipoate oxidoreductase (decarboxylating and acceptor-acetylating)	1.2.4.1
Pyruvate kinase	ATP:pyruvate 2-*o*-phospnotransferase	2.7.1.40
Succinate dehydrogenase	Succinate:(acceptor) oxidoreductase	1.3.99.1

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INTRODUCTION

The investigations presented in this thesis were undertaken in order to unravel the questions of whether weak acid uncoupling agents could uncouple the oxidative phosphorylation of plant mitochondria, and whether adenine nucleotides were required as modulators for the action of the uncoupling agents.

1. The nature of the problem

In contrast to the familiar observation that uncoupling agents (uncouplers) cause an increase in the rate of oxygen uptake by mitochondria without the formation of ATP from ADP and inorganic phosphate, plant mitochondria frequently show complicated and unreliable patterns of response to the addition of weak acid uncoupling agents. There have been many reports concerning the failure of weak acid uncouplers to stimulate respiration in plant mitochondria and the ability of adenine nucleotides to stimulate respiration of plant mitochondria in the presence of uncouplers. Wiskich & Bonner (1963) found that both ADP and ATP could stimulate the oxidation of succinate by sweet potato (*Ipomea batatus*) mitochondria in the presence of dinitrophenol. They suggested that the stimulation of succinate oxidation under these conditions was due to the removal by ATP of oxaloacetate, a known inhibitor of succinate dehydrogenase (Pardee & Potter, 1948; Avron & Biale, 1957); ADP would only become active after conversion to ATP by residual oxidative phosphorylation or by the action of adenylate kinase (Bonner, 1973). Drury, McCollum, Garrison & Dickinson (1968)

reported that, in tomato fruit mitochondria, the stimulation of succinate oxidation by the adenine nucleotides in the presence of dinitrophenol was not sensitive to oligomycin and concluded that it was not necessary to convert ADP into ATP in order to relieve the inhibition of succinate dehydrogenase. Singer, Oestreicher, Hogue, Contreiras & Brandao (1973b) have reported that, in mitochondria isolated from mung bean (*Phaseolus aureus*) and cauliflower (*Brassica oleracea*) more than half of the succinate dehydrogenase is in the deactivated state. In addition to succinate, both ADP and ATP can activate the enzyme in intact and sonicated plant mitochondria in the presence of oligomycin (Oestreicher, Hogue & Singer, 1973) whereas ADP does not activate the mammalian enzyme and ATP is only effective in intact mitochondria (Gutman, Kearney & Singer, 1971; Singer, Kearney & Kenney, 1973a).

Succinate is not the only substrate whose oxidation fails to respond predictably to the addition of uncouplers. The rates of oxidation of NAD^+-linked substrates have been found to be slow in the presence of uncouplers (Ikuma & Bonner, 1967b; Laties, 1953 & 1973). Laties (1973) showed that, in the presence of an uncoupler, the rate of oxidation of citrate, pyruvate-plus-malate or succinate-plus-pyruvate could be stimulated by ADP. The stimulation by ADP was found to be insensitive to oligomycin which inhibits the phosphorylation of ADP to ATP (Lardy, Johnson & Connelly, 1958; Lardy, Connelly & Johnson, 1964; Wiskich & Bonner, 1963). Laties (1973) therefore concluded that ADP was involved in implementing 'uncoupler effectiveness'. Laties (1973) could find no evidence of ATP or AMP having a similar effect in activating the rate of oxidation in his studies, thus his conclusions appear to

be in conflict with those of Bonner (1973) who stated 'the mitochondria must have had a small amount of ATP in order for the uncoupler to be effective'.

Observations have also been made which suggest that ADP could stimulate electron flow in plant mitochondria in the absence of uncouplers, other than by acting as a phosphate acceptor in oxidative phosphorylation. Raison, Lyons & Campbell (1973b) have described the phenomenon of 'conditioning' in mitochondria isolated from several species of plants. The main characteristic of this phenomenon, which has also been reported by Ikuma & Bonner (1967a), is that under certain conditions the rate of oxidation in the presence of ADP, obtained in response to the first limited addition of ADP, is markedly lower than that obtained in response to subsequent addition of ADP. Furthermore, it was noted that following the first addition of ADP the increase in the rate of oxidation was gradual whereas the subsequent addition of ADP resulted in an immediate increase in respiration. Raison, Laties & Crompton (1973a) also showed that, if mitochondria were incubated with substrate and ADP in the absence of phosphate, the rate of oxidation initiated by the addition of phosphate was rapid and considerably higher than that initiated by the addition of ADP to mitochondria incubated with substrate and phosphate. Thus they concluded that the incubation of mitochondria with ADP under non-phohphorylating conditions resulted in 'conditioning'. Incubation in the presence of ATP did not have a similar effect.

As 'conditioning' is observed with substrates other than succinate, it is not attributed to the activation of succinate dehydrogenase. There are reports concerning the role of ADP and other

adenine nucleotides as a modulator of other enzymes, in animals, fungi and bacteria, such as pyruvate dehydrogenase (Shen, Fall, Walton & Atkinson, 1968; Linn, Pettit & Reed, 1969) and NAD^+-linked isocitrate dehydrogenase (Chen & Plaut, 1963; Hathaway & Atkinson, 1963; Sanwal, Zink & Stachow, 1964; Sanwal & Stachow, 1965). The possibility of similar effects of adenine nucleotides on the enzymes in plant mitochondria, which has not been considered by Laties (1973) and Raison *et al.* (1973b), will be discussed in detail in the Results section.

Laties (1973) concluded that the ability of ADP to stimulate the oxidation in the presence of an uncoupler was not dependent on its role in bringing about partial conditioning because he observed that the stimulation in this case was immediate. Therefore he considered that ADP had two separate roles in stimulating non-phosphorylating electron flow in plant mitochondria.

In order to assess the problem, it is necessary to consider the current knowledge of the properties and functions of plant mitochondria with special reference to their response to ADP and uncoupling agents, namely the mechanism of oxidative phosphorylation. However, the complex nature of the branched electron transport of plant mitochondria deserves some mention, as it is likely that the regulation of electron transfer in plant mitochondria may be different from that in animal mitochondria and may result in the unusual observations discussed above.

2. The general properties of plant mitochondria

Although the general conclusion derived from various reviews on structure and functions of mitochondria (Hackett, 1959; Mercer, 1960; Crane, 1961; Bonner, 1965; Liebermann & Baker, 1965; Hanson & Hodges, 1967; Pullman & Schatz, 1967; Chance, Bonner & Storey, 1968; Lardy & Ferguson, 1969; Racker, 1970a; van Dam & Meyer, 1971; Ikuma, 1972; Palmer & Hall, 1972; Öpik, 1974; Palmer, 1976) is that mitochondria isolated from plants and animals are similar with respect to the principal metabolic processes, it is recognized that there are significant differences between plant and animal mitochondria. Some of these differences will be briefly mentioned here.

While it is apparent that the basic organization of the two membranes of plant mitochondria is like that of animal mitochondria (Parsons, Bonner & Verboon, 1965; Öpik, 1974), the amount of carbohydrate (galacturonic acid) in the outer membrane of plant mitochondria can be as much as 10% of the total membrane mass, which may account for their osmotic stability, in contrast to a negligible amount in rat liver outer membrane (Mannella & Bonner, 1975). Mannella & Bonner (1975) have reported that the molar ratio of cholesterol/phospholipid of the outer mitochondrial membrane of mung bean hypocotyl is high, in fact higher than that of rat liver (Graham & Green, 1970; Colbeau, Nachbauer & Vignais, 1971), but that of potato (*Solanum tuberosum*) tuber is low, like those of guinea pig liver (Parsons & Yano, 1967) and pig heart (Comte, Maïsterrena & Gautheron, 1976).

The distribution of the respiratory chain components, the

enzymes of the tricarboxylic acid cycle (Krebs cycle) and other enzymes in plant mitochondria has not been thoroughly studied, but is believed to resemble that in animal mitochondria (Klingenberg & Pfaff, 1966; Sottocasa, Kuylenstierna, Ernster & Bergstrand, 1967a & b; Brdiczka, Pette, Brunner & Miller, 1968; Ernster & Kuylenstierna, 1969; see also Ashwell & Work, 1970). From very few reports on enzyme distribution, it appears that the outer membranes of higher plants also have an antimycin A-insensitive NADH-cytochrome c reductase (Moreau & Lance, 1972; Douce, Mannella & Bonner, 1973), whose activity is inhibited by *p*-chloromercuribenzoate (Day & Wiskich, 1975), but lack monoamine oxidase and kynurenine hydroxylase (Okamoto, Yamamoto, Nozaki & Hayaishi, 1967; Schnaitman & Greenawalt, 1968). Kynurenine hydroxylase is present on the outer membrane of *Neurospora crassa* mitochondria (Cassady & Wagner, 1971).

The presence of transport systems for phosphate, dicarboxylic acids, α-oxoglutarate and citrate (Chappell, 1968) has been confirmed in plant mitochondria (Phillips & Williams, 1973a & b; Day & Wiskich, 1974; Wiskich, 1975), so has the translocator specific for adenine nucleotides (Klingenberg, 1970; Souverijn, Huisman, Rosing & Kemp, 1973; Passam, Souverijn & Kemp, 1973; Jung & Hanson, 1973; Earnshaw & Hughes, 1976; Wickes & Wiskich, 1976; Vignais, Douce, Lauquin & Vignais, 1976). It has been noted, however, that in buffered KCl solution plant mitochondria show spontaneous swelling, a process distinguishable from the rapid osmotic adjustment, at a rate about five times as rapid as in rat liver mitochondria (Lyons, Wheaton & Pratt, 1964; Lyons & Pratt, 1964; Stoner & Hanson, 1966). Spontaneous swelling in plant

mitochondria is retarded by the presence of Ca^{2+} (Lyons & Pratt, 1964; Hanson, Malhotra & Stoner, 1965; Truelove & Hanson, 1966) and reversed by the addition of substrate (Wilson, Hanson & Mollenhauer, 1969) or ATP and Mg^{2+} (Lyons & Pratt, 1964; Stoner & Hanson, 1966).

The respiratory activities of plant mitochondria (Hackett, 1959; Crane, 1961; Ikuma, 1972; Palmer, 1976) indicate that the organization of their electron transport chain (respiratory chain) is different from and more complicated than the prevalent system in animal mitochondria (Klingenberg, 1968; Palmer & Hall, 1972; Palmer & Coleman, 1974; Singer & Gutman, 1974). Nevertheless, there is no doubt about the existence in plant mitochondria of the pathway of electron transfer resembling that in their animal counterparts (Chance *et al.*, 1968; Ikuma, 1972). It is also regarded that there are three sites of phosphorylation associated with this pathway (Hanson & Hodges, 1967; Ikuma, 1972), site I between the NADH dehydrogenase and the ubiquinone, site II between b- and c-cytochromes, and site III between cytochromes a and a_3.

3. Branched electron transport chain in plant mitochondria

The employment of various respiratory inhibitors together with the kinetic analyses of the oxidation-reduction of various members of the respiratory chain has been useful in determining the sequence of reactions and the arrangement of respiratory chain components (Chance & Williams, 1956; Schatz & Racker, 1966; Chance, 1972; Storey & Bahr, 1972). Furthermore, mitochondria from different types of tissues or organisms can be distinguished

by their responses to the inhibitors, presumably because of the diversity of their functions in the respective tissues (Hackett, 1959; Crane, 1961; Ikuma, 1972; Palmer, 1976). In general animal mitochondria are very sensitive to respiratory inhibitors; amytal, rotenone and piericidin A block electron transport between the endogenous NADH dehydrogenase and ubiquinone (Ernster, Dallner & Azzone, 1963; Hall, Wu, Crane, Takahashi, Tamura & Folkers, 1966; Jeng, Hall, Crane, Takahashi, Tamura & Folkers, 1968); antimycin A and 2-heptyl-4-hydroxyquinoline N-oxide inhibit between the cytochromes b and c_1 (Chance & Williams, 1956; Estabrook, 1962; Jackson & Lightbrown, 1958); cyanide and azide are inhibitors of cytochrome oxidase (Keilin, 1936; Keilin & Hartree, 1939). On the contrary, the respiratory activities of plant mitochondria show varying degrees of sensitivity to these inhibitors. The most frequently quoted and often a subject of much attention is the cyanide-resistant respiration in mitochondria from certain plant tissues (Bendall, 1958; Bendall & Bonner, 1971; Passam & Palmer, 1972; Ikuma, 1972; Meeuse, 1975). It is apparent, with mounting information on the activities of plant mitochondria in the presence of respiratory inhibitors, that the organization of the electron transport chain in plant mitochondria is very complicated.

(a) The cyanide-resistant (alternative) oxidase

The presence of cyanide-resistant respiration (the alternative oxidase) has been detected in mitochondria from several plant tissues, although it is often present in varying levels; in the presence of ADP mung bean mitochondria are about 30% insensitive to

0.1 mM KCN (Ikuma & Bonner, 1967c; Bendall & Bonner, 1971), mitochondria from spadices of skunk cabbage (*Symplocarpus foetidus*) are about 70% resistant to KCN (Hackett & Haas, 1958; Ikuma & Bonner, 1967c; Storey & Bahr, 1969b; Bendall & Bonner, 1971), whereas total insensitivity can be observed in mitochondria from spadices of *Arum maculatum* (Passam & Palmer, 1972). Cyanide-resistant respiration is absent in potato tuber mitochondria but can be detected in mitochondria isolated from potato slices which have been repeatedly washed for some hours in distilled water (Bonner, Christensen & Bahr, 1972). In sweet potato mitochondria, cyanide only partially inhibits the oxidation of malate or succinate (Wiskich & Bonner, 1963; Tomlinson & Moreland, 1975), but it completely inhibits the oxidation of exogenous NADH (Tomlinson & Moreland, 1975). Hence it is apparent that electrons from the exogenous NADH dehydrogenase (Douce *et al.*, 1973) have no access to the cyanide-resistant pathway.

It has been noted that antimycin A inhibits the respiration of these cyanide-resistant mitochondria to the same extent as does cyanide (Bendall, 1958; Ikuma & Bonner, 1967c; Bendall & Bonner, 1971; Passam & Palmer, 1972; Ikuma, 1972). Therefore the cyanide-resistant pathway seems to branch from the normal electron transport chain before the site of antimycin A inhibition.

Due to the observation that a b-type cytochrome (named cytochrome b_7), which is found in high proportion in mitochondria from mature spadices of *Arum* (Bendall & Hill, 1956) and *Sauromatum* (Bonner *et al.*, 1972), remains oxidized in the presence of cyanide or antimycin A (Bendall & Hill, 1956; Bendall, 1958), it has been suggested that cytochrome b_7 is part of the respiratory chain in-

volving cyanide-resistant respiration. However, there is no direct correlation between the amount of cytochrome b_7 and the activity of cyanide-resistant respiration (Bendall, 1958). In skunk cabbage mitochondria, although the b-cytochromes are oxidized in the presence of cyanide or azide, they are largely reduced by the addition of antimycin A (Chance & Hackett, 1959; Bonner, 1961; Storey & Bahr, 1969a; Bendall & Bonner, 1971). Cytochrome b components therefore play no significant part in the antimycin A-resistant respiration in skunk cabbage mitochondria. The same conditions are also found in the mi-1 mutant of *N. crassa* ('poky') (Lambowitz, Slayman, Slayman & Bonner, 1972; von Jagow, Weiss & Klingenberg, 1973).

From the kinetics of oxidation of various components of the respiratory chain of skunk cabbage mitochondria in the presence of succinate, Storey & Bahr (1969a) and Erecinska & Storey (1970) have concluded that the high potential flavoprotein, Fp_{hf} is directly involved in the cyanide-resistant pathway. In a recent report on the mi-1 mutant of *N. crassa* mitochondria (von Jagow *et al.*, 1973), the branching point is considered to be at the level of ubiquinone (UQ) because the redox state of UQ responds to changes in the electron transfer activity in both pathways.

Bendall & Bonner (1971) proposed that the cyanide-resistant respiration was mediated through an alternative oxidase which was not a cytochrome. The alternative oxidase in plant mitochondria differs from the cyanide-resistant oxidase (cytochrome o) in intestinal parasites (Cheah, 1968 & 1975), trypanosomes (Hill & Cross, 1973; Hill, 1976) and some bacteria (Castor & Chance, 1959; Broberg & Smith, 1967; Sekuzu & Takemori, 1972) in that the plant

alternative oxidase does not react with carbon monoxide (Bendall & Hill, 1956; Yocum & Hackett, 1957; Meeuse, 1975). Following the discovery by Schonbaum, Bonner, Storey & Bahr (1971) that hydroxamic acids, especially *m*-chlorobenzhydroxamic acid (*m*-CLAM), are very effective inhibitors of the cyanide-resistant respiration, there is a suggestion that a non-haem iron protein may be associated with the alternative oxidase. However, Palmer (1972) has shown that the effect of the iron chelator potassium thiocyanate (KSCN), earlier reported to inhibit the cyanide-resistant oxidation of succinate (Bendall & Bonner, 1971), is primarily on the succinate dehydrogenase. At very high concentrations of KSCN, the oxidation of NADH or ascorbate/TMPD is also affected, probably because of the chaotropic activity of KSCN. The intense iron-sulphur signal seen in the electron paramagnetic resonance (EPR) spectrum of *Arum* submitochondrial particles (Cammack & Palmer, 1973) has been thought to be associated with the succinate dehydrogenase (Palmer, 1976).

Electron transfer via the alternative oxidase is not associated with phosphorylation of ADP to ATP. In skunk cabbage mitochondria, the presence of cyanide results in a loss of ATP synthesis at phosphorylation sites II and III, whereas that at site I is unimpaired (Hackett & Haas, 1958; Storey & Bahr, 1969b). In *Arum* mitochondria, the overall efficiency of oxidative phosphorylation is very low (Hackett & Simon, 1954; Passam & Palmer, 1972; Lance, 1974). The low level of oxidative phosphorylation has been suggested to be due to endogenous uncoupling or active ATPase (Lance, 1974), together with the increased participation of the alternative pathway (Passam & Palmer, 1972; Meeuse, 1975).

Wilson & Smith (1971) have suggested that free fatty acids might act as endogenous uncouplers in *Sauromatum* spadix during the odoriferous period (D-day), diverting the energy of oxidation into heat production. As the rapid respiration of *Sauromatum* spadix mitochondria is also cyanide-insensitive (Bonner, 1965; Wilson & Smith, 1971), it appears that uncoupling is not the only factor that causes the elevation of temperature.

Studies of the development of *Sauromatum* inflorescences have indicated that thermogenesis in arum lilies is controlled primarily by the light/dark regime which probably leads to production in the staminate flower primordia of a triggering hormone ('calorigen') (Buggeln & Meeuse, 1971; Meeuse, 1975). Metabolic changes in the spadix leading to the production of heat (and smell) in the spadix is believed to be induced by the hormone, which is a water-soluble non-proteinaceous substance (Buggeln & Meeuse, 1971). Buggeln & Meeuse (1971) also detected calorigen-like substances in flowers of *A. maculatum* and other members of the Araceae family.

The existence of branched electron transport pathways in plant mitochondria raises the question of the relative activity of each branch under natural conditions and in the absence of inhibitors. Investigation into the sensitivity to either cyanide or aromatic hydroxamic acids of the oxygen uptake by mitochondria from mung bean hypocotyls (Bahr & Bonner, 1973a) has indicated that during active phosphorylation of ADP (state 3 in the terminology of Chance & Williams, 1956) the entire electron flux is carried by the cytochrome (cyanide-sensitive) pathway, while the alternative pathway is fully active in state 4 (when all the ADP had been phosphorylated). In skunk cabbage mitochondria the cytochrome pathway is

the major pathway in the absence of cyanide or antimycin A (Storey & Bahr, 1969a; Bahr & Bonner, 1973a), as seen by the high efficiency of their oxidative phosphorylation (Hackett & Haas, 1958; Storey & Bahr, 1969b). Bahr & Bonner (1973a) have shown that the participation of the alternative oxidase in the total electron flux is more in state 4 than in state 3, therefore the contribution of the alternative oxidase is apparently regulated by the activity of the cyanide-sensitive pathway which is in turn controlled by the rate of oxidative phosphorylation. Bahr & Bonner (1973b) have proposed that the branching point consists of two separate components in equilibrium with each other (each pathway receives its reducing equivalents from one of the carriers) and it is the equilibrium between these two components that regulates the relative flux through the two pathways.

(b) The external NADH dehydrogenase

Unlike animal mitochondria, plant mitochondria readily oxidize exogenously added NADH coupled to oxidative phosphorylation with P/O ratio less than that of the oxidation of endogenous NADH (Wiskich & Bonner, 1963; Cunningham, 1964; Ikuma & Bonner, 1967a; Wilson & Hanson, 1969; Srivastava & Sarkissian, 1970). (Day, Rayner & Wiskich (1976) reported that mitochondria from fresh red beetroot (*Beta vulgaris*) did not oxidize added NADH. However, the ability to oxidize NADH was shown to develop in mitochondria from slices of beetroot incubated in aerated dilute $CaSO_4$ solution.) Subsequent studies have provided evidence to suggest that exogenous NADH is oxidized by a dehydrogenase located on the outer surface of the inner membrane (Palmer & Passam, 1971; Douce *et al.*,

1973; Palmer & Coleman, 1974; Palmer, 1976). Divalent metal ions, such as Ca^{2+} and Sr^{2+}, stimulate the oxidation of exogenous NADH but have no direct effect on the rate of oxidation of endogenous NADH (malate-plus-pyruvate) (Hackett, 1961; Miller, Dumford, Koeppe & Hanson, 1970; Miller & Koeppe, 1971; Coleman & Palmer, 1971). The oxidation of exogenous NADH is not inhibited by compounds like amytal and rotenone (Ikuma & Bonner, 1967c; Miller *et al.*, 1970; Coleman & Palmer, 1972), but is as sensitive to inhibition by antimycin A as the oxidation of endogenous NADH (Lieberman, 1960; Miller *et al.*, 1970; Coleman & Palmer, 1972). Thus it appears that electrons from both NADH dehydrogenases share a common pathway from the level of ubiquinone to oxygen (Palmer, 1976).

The presence of the external NADH dehydrogenase indicates that plant mitochondria can oxidize NADH in the cytosol, whereas in animal mitochondria cytosolic NADH has to be oxidized indirectly by a shuttle system involving malate dehydrogenase and aspartate-oxoglutarate aminotransferase (Chappell, 1968). There is evidence that, in Jerusalem artichoke mitochondria, the NAD^+-specific malic enzyme (Macrae & Moorhouse, 1970; Macrae, 1971b) is also present in the intermembrane space and can supply NADH to the external NADH dehydrogenase (Coleman & Palmer, 1972; Palmer & Arron, 1976; Palmer, 1976).

Since the oxidation of exogenous NADH by plant mitochondria appears to require the presence of Ca^{2+} (Coleman & Palmer, 1971), any alteration of the calcium concentration in the intermembrane space or the cytosol will affect the rate of oxidation of NADH (Palmer, 1976). The presence of organic acids has also been

observed to influence the oxidation of exogenous NADH. High concentrations of citrate can inhibit the oxidation of exogenous NADH, presumably by removing Ca^{2+} (R.C. Cowley & J.M. Palmer, unpublished data). Succinate also inhibits the oxidation of NADH but by a different mechanism, because the inhibitory effect of succinate can be reversed by malonate (Palmer, 1976). It is believed that this inhibition is the result of electron flux from succinate which alters the redox equilibrium of a component of the respiratory chain (possibly the ubiquinone) so that electrons cannot flow from the NADH dehydrogenase. A similar mechanism has been suggested to operate in the regulation of electron flow in animal mitochondria (Gutman *et al.*, 1971; Trumpower & Katki, 1975).

(c) The piericidin A-resistant pathway for the oxidation of endogenous NADH

There have been reports of incomplete inhibition by rotenone or piericidin A during the oxidation of NAD^+-linked substrates in plant mitochondria (Ikuma & Bonner, 1967c; Miller & Koeppe, 1971; Brunton & Palmer, 1973; Day & Wiskich, 1974; Brunton, 1975). The oxidation of NADH in submitochondrial particles prepared from mung bean mitochondria is partially inhibited by rotenone (Wilson & Bonner, 1970), whereas that in particles prepared from beef heart mitochondria is completely inhibited by piericidin A (Jeng *et al.*, 1968) which acts at the same site as rotenone (Ernster *et al.*, 1963). These reports indicate that there is an alternate pathway in plant mitochondria for the oxidation of endogenous NADH which by-passes the rotenone or piericidin A inhibition.

Ikuma & Bonner (1967c) found that the oxidation of malate in

mung bean mitochondria was 40-50% inhibited by rotenone and the ADP/O ratio was reduced from 2.3 to 1.3. Brunton & Palmer (1973) demonstrated that piericidin A caused partial inhibition of the oxidation of NAD^+-linked substrates in wheat mitochondria with the subsequent loss of phosphorylation at site I. Thus it appears that phosphorylation at site I is closely associated with the site of piericidin A inhibition.

Much information on piericidin A-insensitivity and phosphorylation at site I has been obtained from work on mitochondria isolated from fungi grown under different conditions. Mitochondria isolated from *Candida utilis* grown under iron- or sulphur-limited conditions were found to lack piericidin A sensitivity and the ability to phosphorylate at site I, and they did not show an EPR signal at g = 1.94 (Light, Ragan, Clegg & Garland, 1968; Garland, 1970). By varying the balance between iron and glycerol (carbon source), it was possible to observe phosphorylation at site I in the absence of both piericidin A sensitivity and an EPR signal at g = 1.94 (Clegg, Ragan, Haddock, Light & Garland, 1969; Clegg & Garland, 1971). Subsequently it has been discovered that, during the logarithmic growth phase, the oxidation of NAD^+ linked substrates by *C. utilis* mitochondria is insensitive to rotenone and is not linked to phosphorylation at site I (Katz, 1971; Katz, Kilpatrick & Chance, 1971; Ohnishi, 1972). Both rotenone/piericidin A sensitivity and site I phosphorylation are acquired, together with the increase in the EPR signal at g = 1.94 (at 77^oK), characteristic of iron-sulphur proteins of mammalian type NADH dehydrogenase (Orme-Johnson, Orme-Johnson, Hanson, Beinert & Hatefi, 1971; Gutman, Singer & Beinert, 1972), during the transition to

the stationary growth phase (Katz *et al.*, 1971; Grossman, Cobley, Singer & Beinert, 1974). Apparently, the acquisition of phosphorylation site I, rotenone sensitivity and the EPR signal at g = 1.94 involves active protein synthesis because their appearance can be prevented by cycloheximide (Grossman *et al.*, 1974, Singer & Gutman, 1974). It has been shown that the type of NADH dehydrogenase present in the logarithmic growth phase is different from that in the stationary phase; the former has relatively low ferricyanide reductase activity but high juglone (5-hydroxy-1,4-naphthoquinone) reductase activity (Grossman *et al.*, 1974; Singer & Gutman, 1974).

In *Saccharomyces* mitochondria, operation of phosphorylation at three sites is observed during the stationary growth phase but only two sites are operative during the logarithmic phase (Mackler & Haynes, 1973; Ohnishi, 1973). However, the EPR signal at g = 1.94 and rotenone/piericidin A sensitivity do not appear to be present under any conditions (Ohnishi, 1973). Therefore, the question of whether functional phosphorylation at site I and piericidin A sensitivity depend on the presence of the iron-sulphur protein with an EPR signal at g = 1.94 (Singer & Gutman, 1974) seems to be a matter of controversy. As it has not been possible to obtain the EPR signal at g = 1.94 and piericidin A sensitivity without phosphorylation at site I (Ohnishi, 1973), the current opinion is that there is a correlation between phosphorylation at site I, piericidin A sensitivity and an EPR signal at g = 1.94. In order to explain the additional phosphorylation site in *Saccharomyces* mitochondria, it has been suggested that the substrates are oxidized by $NADP^+$-linked dehydrogenases and that the trans-

hydrogenation of the NADPH-NAD^+ results in the synthesis of ATP (Van de Stadt, Nieuwenhuis & van Dam, 1971; Palmer & Coleman, 1974).

In *Arum* spadix mitochondria, an increase in piericidin A-resistant oxidation of malate and cyanide-resistant alternative oxidase are observed concomitantly (J.M. Palmer, personal communication). It is possible that different NADH dehydrogenases may operate depending on the stages of growth of the spadix, similar to the conditions in *C. utilis* (Grossman *et al.*, 1974). The data on oxidative phosphorylation in other plant mitochondria (Ikuma & Bonner, 1967c; Brunton & Palmer, 1973) indicate that a different control mechanism is involved. Brunton & Palmer (1973) have concluded that the piericidin A-resistant pathway is not operative until the inhibitor is present.

The presence of endogenous piericidin A-resistant NADH dehydrogenase and its activity in Jerusalem artichoke mitochondria will be investigated in this study.

4. Oxidative phosphorylation

The precise way in which the energy made available during the oxidoreduction (redox) reactions in the mitochondrion may be utilized for the synthesis of ATP from ADP and inorganic phosphate (Pi) - the so-called oxidative phosphorylation - has been intensively studied by many workers. For several years two contesting theories, the chemical theory and the chemiosmotic theory, have dominated the field of oxidative phosphorylation.

(a) The chemical theory of coupling

From the observation that in intact mitochondria the rate of respiration increases markedly if the mitochondria are supplied with ADP and Pi, it can be deduced that respiration is closely linked with phosphorylation (Lardy & Wellman, 1952). Slater (1953) proposed a mechanism, based on analogy with substrate-linked phosphorylation (Racker & Krimsky, 1952), whereby the synthesis of ATP might be coupled to the electron transfer reactions in mitochondria. The essential feature of this proposal is that the free-energy change during the oxidation-reaction of two adjacent components of the respiratory chain is conserved in the form of a high-energy intermediate which subsequently reacts with ADP and Pi to form ATP. Since it has been established that the oxidation of one molecule of NADH results in the synthesis of three molecules of ATP (Ochoa, 1943; Lehninger, 1951; Chance & Williams, 1956), the formation of the high-energy intermediate must occur at three places in the respiration chain. When the respiratory chain components are arranged in order of their redox potentials (Chance, 1972), there are three positions where the free-energy changes of electron transfer between the pairs of adjacent components appear to be sufficiently large to support the synthesis of ATP. These positions also correspond with the phosphorylation (coupling) sites observed by monitoring the changes in the level of reduction of respiratory components due to the addition of ADP (the 'cross-over technique') (Chance & Williams, 1955b & 1956; Schatz & Racker, 1966; Singer & Gutman, 1974).

The original hypothesis of Slater (1953) has been modified and expanded so as to account for other phenomena connected with

respiration, such as the uptake of metal cations (Harris, Judah & Ahmed, 1966; Lehninger, Carafoli & Rossi, 1967; Hanson & Hodges, 1967), nicotinamide adenine nucleotide transhydrogenation (Danielson & Ernster, 1963a & b) and reversed electron transport (Chance & Hollunger, 1957; Chance, 1961; Chance & Fugmann, 1961; Löw, Krueger & Ziegler, 1961; Klingenberg & Häfen, 1963; Ernster & Lee, 1967). These reactions can be driven by respiration or the hydrolysis of ATP, so they are said to be energy-linked. In addition, there is competition between oxidative phosphorylation on the one hand and cation accumulation (Rossi & Lehninger, 1963; Lehninger, 1966) and transhydrogenation (Lee & Ernster, 1968) on the other. These observations have led to the conclusions that there is a common non-phosphorylated high-energy intermediate (X~I, according to the terminology of Chance & Williams, 1956) linking the high-energy intermediates between the three coupling sites, and that cation accumulation, transhydrogenation and ATP synthesis are connected with respiration by this common factor (see schemes in Ernster, Lee & Janda (1967), Pullman & Schatz (1967) and Greville (1969)).

Several proposals as to the nature of the high-energy intermediates have been put forward and subsequently summarized in the reviews by Lardy & Ferguson (1969) and van Dam & Meyer (1971). The proposed intermediates include the phosphoimidazole (Boyer, 1963; Bieber & Boyer, 1966), the phosphorylated sulphonium intermediate (Lambeth & Lardy, 1969) and the chelate between ubiquinone and non-haem iron (Moore & Folkers, 1964). Following the observations by Wilson & Dutton (1970a & b) that ATP ('energization') can change the midpoint potentials (by about 275 mV) of cytochrome

a_3 and a species of cytochrome b (this component has been named cytochrome b_T), Chance, Wilson, Dutton & Erecinska (1970) concluded that these cytochromes are directly involved in energy conservation. There is considerable doubt concerning this interpretation. Wikström & Lambowitz (1974) demonstrated that the apparent change in the midpoint potential (E_m) of cytochrome b_T was due to reversed electron transport induced by ATP. Furthermore, it has not been possible to show that 'energization' alters the E_m of cytochrome b_{566} which is considered to be the plant counterpart of cytochrome b_T of animal mitochondria (Dutton & Storey, 1971; Lambowitz, Bonner & Wikström, 1974).

As for the enzyme responsible for the synthesis of ATP, Kielley & Kielley (1951 & 1953) suggested that the ATPase associated with the membrane fraction of mitochondria might represent the reversal of the synthesis of ATP which normally occurs in mitochondria. Kielley & Bronk (1957 & 1958) demonstrated that phosphorylating submitochondrial particles prepared by sonic disintegration of mitochondria contained active ATPase. The identity of the stalked spheres of 85 Å in diameter which are observed on the matrix side of negatively stained inner mitochondrial membranes under electron microscope (Fernandez-Moran, 1962; Stoeckenius, 1963 & 1970; Parsons *et al.*, 1965; Öpik, 1974) has been established as the ATPase. It has been possible to obtain the enzyme ATPase in the membrane-free forms from ox heart (Pullman, Penefsky, Datta & Racker, 1960; Horstman & Racker, 1970; Andreoli, Lam & Sanadi, 1965; Senior & Brooks, 1970), rat liver (Catterall & Pedersen, 1971; Lambeth & Lardy, 1971) and the yeast *S. cerevisiae* (Schatz, Penefsky & Racker, 1967; Tzagoloff & Meagher, 1971).

The membrane fraction devoid of the stalked spheres can carry out electron transport but not oxidative phosphorylation, except when it is recombined with the ATPase fraction (Penefsky, Pullman, Datta & Racker, 1960).

(b) The chemiosmotic theory

Denouncing the existence of the chemical high-energy intermediate, Mitchell (1961, 1966 & 1968) has proposed that electron transport and phosphorylation are coupled via the transmembrane electrochemical potential of hydrogen ions (termed the proton motive force, PMF) which is made up of the pH gradient across the membrane and the membrane potential (an electrical component). The chemiosmotic theory applies to chloroplasts and bacteria as well as mitochondria but only the mitochondrial system will be discussed here.

According to Mitchell's chemiosmotic theory, both the respiratory chain and the reversible ATPase function as translocators of protons across the inner mitochondrial membrane which has low permeability to protons, cations and anions. Mitchell (1961, 1966, 1968 & 1972) has proposed that the components of the respiratory chain are arranged in such a way that during respiration protons are generated on one side of the coupling membrane. The ATPase is also anisotropic and arranged so that it is accessible to protons only on one side and the movement of protons through the ATPase in the opposite direction to that created by the respiratory chain components, in the presence of ADP and Pi, results in the formation of ATP.

It has been shown that protons are translocated outwards

during the oxidation of substrate and the hydrolysis of ATP in intact mitochondria from rat liver (Mitchell & Moyle, 1965, 1967 & 1968) and certain bacteria (Mitchell, 1962), whereas protons are taken up by the grana membrane of spinach chloroplasts during electron transport (Jagendorf & Hind, 1963; Neumann & Jagendorf, 1964; Jagendorf & Neumann, 1965). The membrane potential developed across the coupling membrane during electron transport can be detected and measured by means of synthetic penetrating ions (Grinius, Jasaitis, Kadziauskas, Liberman, Skulachev, Topali, Tsofina & Vladimirova, 1970; Bakeeva, Grinius, Jasaitis, Kuliene, Levitsky, Liberman, Severina & Skulachev, 1970; Jasaitis, Kuliene & Skulachev, 1971; Skulachev, 1971 & 1975). It has been found that penetrating anions (e.g. tetraphenylboron) are extruded by mitochondria during electron transport, indicating a membrane potential with negative charges inside. Submitochondrial particles take up anions but extrude cations (e.g. tetraphenylphosphonium cation), indicating opposite polarity of membrane potential to that of the mitochondrion.

The central postulate of the chemiosmotic theory, that ATP synthesis may be driven by the PMF across the coupling membrane, has received experimental support. The synthesis of ATP from ADP and Pi as a result of an artificial pH gradient of about 4-5 pH units across the coupling membrane, in the presence of inhibitors of electron transport, has been demonstrated in spinach chloroplast grana (Jagendorf & Uribe, 1966) and in rat liver mitochondria (Reid, Moyle & Mitchell, 1966). Cockrell, Harris & Pressman (1967) have shown that an artificial membrane potential, generated by the unequal distribution of potassium ions, across

the membrane of submitochondrial particles can be used to synthesize ATP. Recently, Thayer and Hinkle (1975a & b) have demonstrated ATP generation in submitochondrial particles induced by a combination of a pH gradient and a membrane potential.

The credibility of Mitchell's chemiosmotic theory has been discussed in comparison with the chemical theory (Slater, 1967 & 1971; Greville, 1969; Racker, 1970a & b, 1976; Skulachev, 1971 & 1975). The main criticisms of the chemiosmotic theory are concerned with the adequacy of the PMF to drive the synthesis of ATP (Cockrell, Harris & Pressman, 1966; Slater, Rosing & Mol, 1973) and the mechanism whereby protons are translocated (Skulachev, 1971 & 1975; Williams, 1969; Papa, 1976).

Mitchell & Moyle (1969b) have estimated the PMF of rat liver mitochondria in state 4 to be about 230 mV. Thermodynamic data indicate that the synthesis of ATP in the mitochondria requires a PMF of 250-350 mV (Cockrell *et al.*, 1966; Slater *et al.*, 1973), which is more than the value of the PMF found by Mitchell & Moyle (1969b).

Mitchell & Moyle (1965, 1967 & 1968) have measured the number of protons translocated per atom of oxygen consumed (H^+/O ratio) by rat liver mitochondria and obtained H^+/O ratios of 6 and 4 with β-hydroxybutyrate and succinate, respectively, as substrate. Hence two protons would be translocated per two reducing equivalents ($2e^-$) for each coupling site (cf. Brand, Reynafarje & Lehninger, 1976). In order to explain the observed stoichiometry of proton translocation ($2H^+$ per coupling site), Mitchell has postulated that the components of the electron transport chain are arranged in a series of three loops traversing the inner mitochon-

drial membrane (see diagrams in Mitchell, 1966 & 1967; Greville, 1969; Palmer & Hall, 1972). At three points on the chain on the outer surface of the membrane, the species being transferred changes from H_2 to $2e^-$ and the resultant pair of protons (i.e. $2H^+$) is liberated into the incubating medium. To account for three oxidoreduction loops, at least three hydrogen carriers and three electron carriers would be required, and they would have to be arranged alternately down the respiratory chain. In the original scheme of Mitchell's loops, the ubiquinone was placed between cytochromes b and c_1 so as to act as the hydrogen carrier for the last (third) loop at the oxygen terminal, cytochromes c_1, c, a and a_3 being the electron carriers (Mitchell, 1967). This localization of the ubiquinone is inconsistent with the established position of ubiquinone between the dehydrogenase segment and cytochrome b (Kröger & Klingenberg, 1967; Klingenberg, 1968; Storey & Bahr, 1972).

Recently Mitchell (1975a & b) has proposed an ingenious scheme, the protonmotive Q cycle, describing a mechanism whereby the translocation of protons across the mitochondrial membrane, from the matrix to the cytosol side, may take place during the transfer of reducing equivalents through the cytochromes b-c_1 segment (Complex III - Hatefi, Haavik, Fowler & Griffith, 1962) of the electron transport chain. In this scheme, two species of cytochrome b, cytochrome c_1, the ubiquinone semiquinone, as well as the oxidized and reduced UQ are involved in the redox reactions; both the oxidized and reduced UQ act as mobile carriers and the transfer of one electron gives rise to the translocation of two protons. The possible existence of the protonmotive Q cycle has already been

discussed in a few reviews (Papa, 1976; Racker, 1976; Rieske, 1976). The proposition of the protonmotive Q cycle has also prompted further research (Trumpower, 1976; Rich & Moore, 1976).

Mitchell's proposal of oxidoreduction loops implies that the proton translocation in mitochondria is a primary process and has stimulated research into the existence and arrangement of both electron and hydrogen carriers in the respiratory chain associated with vesicular membrane structure (Greville, 1969; Racker, 1970a & b, 1976). Evidence for asymmetric organization of the inner mitochondrial membrane has been obtained by the use of specific antibodies against individual respiratory components (Racker, 1970 a & b), impermeant *p*-mercuriphenyl sulphonate and diazobenzene sulphonate which modify the surface components of the membrane (Schneider, Kagawa & Racker, 1972; Tinberg & Packer, 1976) and impermeant electron donor (NADH) or acceptor (ferricyanide) (Klingenberg & Buchholz, 1970; Tyler, 1970). Cytochromes c_1 and c are located on the cytosol side of the membrane facing the outer mitochondrial membrane. Cytochrome oxidase is transmembranous with cytochrome a on the cytosol side and cytochrome a_3 on the matrix side. The dehydrogenases are located on the matrix side. Nevertheless the respiratory chain segment between NADH dehydrogenase and the site of antimycin A inhibition is arranged along, rather than forming loops across, the membrane and is located close to its inner surface (Skulachev, 1975; Racker, 1976). Skulachev (1975), Ragan (1976) and Papa (1976) have suggested that the translocation of a proton by this segment is facilitated by a transmembranous proton pump (specific proton carrier protein) localized at each coupling site.

More recently the value of 2 for the stoichiometry of proton translocation per coupling site ($H^+/2e^-$) has been brought forward for reappraisal. Although Hinkle & Horstman (1971) and Hinkle, Kim & Racker (1972) have been able to obtain the $H^+/2e^-$ ratio close to 2 in both submitochondrial particles and reconstituted cytochrome oxidase vesicles, Junge, Rumberg & Schröder (1970) have reported ratios between 3 and 4 H^+ translocated per ATP in the case of photosynthetic electron transport. Measurement of Ca^{2+} transport coupled to respiration shows inward movement of $2Ca^{2+}$ per coupling site indicating that $4H^+$ are associated with each coupling site (Lehninger, Carafoli & Rossi, 1967; Brand, Chen & Lehninger, 1976). Brand, Reynafarje & Lehninger (1976) have also repeated the experiment first carried out by Mitchell & Moyle (1965, 1967) but found $H^+/2e^-$ ratio of 3. They have indicated that the $H^+/2e^-$ ratio of 2 found by Mitchell & Moyle (1965, 1967) was underestimated due to movements of endogenous ions, particularly phosphate. The controversy over the stoichiometry of proton translocation does not invalidate the theory that the PMF provides the driving force for oxidative phosphorylation and transport of ions, but it brings attention to the mechanism of proton translocation.

Another theory of the electron transport phosphorylation mechanism, that the energy coupling process may involve protein conformational changes in a manner similar to muscle contraction, was first suggested by Boyer (1965). In the original form, the change in protein conformation accompanying electron transport was thought to be the driving force for the formation of a covalent bond in oxidative phosphorylation. Studies on oxygen exchange reactions in mitochondria have led Boyer, Cross & Momsen (1973) to

conclude that very little energy may be required for the actual synthesis of ATP at the catalytic site of the ATPase but the release of ATP from the ATPase by conformational change requires energy from electron transport.

The morphological changes of the inner mitochondrial membrane observed concomitantly with the metabolic transitions of the mitochondria (Chance & Packer, 1958; Hackenbrock, 1968; Green, Asai, Harris & Penniston, 1968) may support the conformational coupling theory (Green, 1970; Baltscheffsky & Baltscheffsky, 1974). The changes in intensity of the fluorescence of 1-anilinonaphthalene-8-sulphonate (ANS), added to a mitochondrial suspension, have been considered as evidence for protein conformational changes in the mitochondrial membrane (Chance, Azzi, Mela, Radda & Vainio, 1969; Azzi, Chance, Radda & Lee, 1969; Brocklehurst, Freedman, Hancock & Radda, 1970). McCarty & Fagan (1973) have found some evidence to indicate that chloroplast ATPase undergoes a conformational change on illumination.

The conformational coupling theory has been considered as a variant of either the chemical theory or the chemiosmotic theory, depending on the origin of the energy source of protein conformational change (Racker, 1970a & b, 1976; Slater, 1971; Harold, 1972). More recently, Boyer (1975a & b) has conceded that changes in protein conformation may be due to movements of ions induced by proton gradient and/or the membrane potential. Ion-induced conformational change of enzyme protein is evident in the case of Ca^{2+}-ATPase of sarcoplasmic reticulum (Knowles & Racker, 1975; Racker, 1976).

5. Mechanism of action of uncoupling agents

The mechanism of action of uncoupling agents on electron transport and the synthesis of ATP has been mentioned in all reviews and papers concerning oxidative phosphorylation (Pullman & Schatz, 1967; Lardy & Ferguson, 1969; van Dam & Meyer, 1971; Greville, 1969; Palmer & Hall, 1972; Skulachev, 1971 & 1975; Racker, 1976) and recently discussed in detail by Hanstein (1976). At the present time several hundreds of agents are known to possess the ability to uncouple processes of oxidation and phosphorylation.

According to the chemical coupling theory, the proposed function of all uncoupling agents is to hydrolyze the high-energy intermediate X∿I, thus preventing phosphorylation. The large range of chemicals of divergent structures makes this explanation unattractive.

Mitchell (1966 & 1968) proposed that uncoupling agents induce the conductivity of the coupling membrane towards ions, thereby collapsing the PMF. The lowering of the PMF across the mitochondrial membrane would result in an increase in the rate of respiration (Mitchell, 1966). The uncoupling agents can be grouped, on the basis of the chemiosmotic theory, according to their modes of action (Palmer & Hall, 1972).

(a) Lipid-soluble weak acids, typified by dinitrophenol, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) and 5-chloro-3-*t*-butyl-2'-chloro-4'-nitrosalicylanilide (S_{13}), are supposed (Mitchell, 1966) to diffuse in, through the mitochondrial membrane, as the undissociated acids. Once inside the mitochon-

dria, they ionize and the resulting anions are then expelled from the organelle down the electrochemical gradient. Supporting evidence for such a mechanism has been obtained from the finding that these uncouplers can increase the proton conductivity of artificial phospholipid membranes (Skulachev, Jasaitis, Navickaite, Yaguzhinsky, Liberman, Topoli & Zofina, 1969; Skulachev, 1971). Mitchell & Moyle (1967) have shown that FCCP can cause a rapid collapse of the pH differential across rat liver mitochondrial membrane.

(b) Lipid-soluble cations (e.g. dimethyldibenzylamine) and anions (e.g. tetraphenylboron) have been shown to increase the electrical conductivity of artificial phospholipid membranes as a result of the net transfer of ions through the membrane (Skulachev *et al.*, 1969). The cation dimethylbenzylamine has been shown to uncouple whole mitochondria, which are negatively charged on the inside (Skulachev *et al.*, 1969).

(c) Ion conducting macrocyclic and polypeptide antibiotics (ionophores), which form lipid-soluble complexes with cations, can promote the unidirectional entry of cations into rat liver mitochondria (Chappell & Croft, 1965). The chemiosmotic theory can account for the uncoupling and osmotic actions of the ionophores (Mitchell, 1966). The activities of the ionophores have been summarized by Lardy, Graven & Estrada-o (1967), Palmer & Hall (1972) and Pressman (1976). Valinomycin is a neutral ionophore which brings about uncoupling by catalyzing the transport of K^+ (Rb^+ or Cs^+) along the electrochemical gradient. Thus valinomycin will produce complete uncoupling in mitochondria but not in submitochondrial particles (Mitchell, 1968; Palmer & Hall, 1972).

Gramidicin can transport K^+, Na^+, Ni^+, Rb^+, Cs^+ and H^+ across the membrane by forming an ion conducting channel (Urry, Goodall, Glickson & Mayers, 1971; Pressman, 1976).

(d) This last class of uncoupling agents, electrophilic alkylating agents, was reported by Skulachev *et al.* (1969) but not much is known about the mechanism (Hanstein, 1976). N,N-dichloroethyl-*p*-aminophenylacetic acid has been shown to release respiratory control in mitochondria and slightly increase electrical conductance of membranes (Skulachev *et al.*, 1969).

MATERIALS AND METHODS

1. Materials used in the studies

Jerusalem artichoke tubers were obtained from the University of London Botanical Supply Unit in batches of about 50 kg in autumn and early winter. The tubers were stored in plastic bags at 0-4°C (cold room) after the surface soil had been removed.

White potatoes were purchased from a local market. Rootstock of *Arum maculatum* was dug up (in April, 1976) from an area in North Kent and used the following day.

A young adult male Sprague-Dawley rat was kindly supplied by the Department of Zoology and Applied Entomology, Imperial College.

2. Chemicals

Enzymes, NADH, ATP, ADP, AMP were obtained from the Boehringer Corporation (London) Ltd. Bovine serum albumin, thiamin pyrophosphate chloride, GMP, IMP, oligomycin, potassium glutamate, malonic acid, oxaloacetic acid and most of the substrates for the mitochondrial respiration studies were obtained from the Sigma London Chemical Co., Kingston-upon-Thames, Surrey, U.K. Sucrose, succinic acid, potassium arsenite, EDTA, tris-(hydroxymethyl)-methylamine (tris), NNN'N'-tetramethylphenylenediamine (TMPD), magnesium chloride, potassium ferricyanide and sodium dithionite were from BDH Chemicals, Poole, Dorset, U.K. Potassium atractylate was obtained from Calbiochem Ltd., London, U.K. Other buffers, potassium phosphate (KH_2PO_4) and sodium metabisulphite ($Na_2S_2O_5$) were obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex,

U.K. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), piericidin A and bongkrekic acid were generous gifts from Dr P.G. Heytler, Professor T.P. Singer and Dr H.C. Passam respectively. 5-Chloro-3-*t*-butyl-2'-nitrosalicylanilide (S_{13}) was a generous gift from the Monsanto Company, St Louis, Missouri, U.S.A.

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. Uncouplers and piericidin A were dissolved in absolute ethanol.

3. Media used in the studies

Isolation media

(a) Isolation medium for mitochondria from Jerusalem artichoke, potato and *Arum* rootstock

0.5 M sucrose

5 mM EDTA

10 mM MOPS

0.1% (w/v) BSA

2 mM $Na_2S_2O_5$ (Stokes, Anderson & Rowan, 1967)

at pH 7.8

(b) Isolation/wash medium for rat liver mitochondria

0.25 M sucrose

0.1 mM EDTA

10 mM tris-HCl at pH 7.6

(c) Wash/suspending medium for Jerusalem artichoke, potato and *Arum* mitochondria

0.4 M sucrose

10 mM TES

0.1% (w/v) BSA at pH 7.2

Reaction media

(a) Standard reaction medium for Jerusalem artichoke mitochondria

0.3 M sucrose

5 mM KH_2PO_4

5 mM TES

2.5 mM $MgCl_2$ at pH 7.2

(b) Reaction medium for *Arum* rootstock mitochondria

0.3 M sucrose

5 mM KH_2PO_4

10 mM TES

5 mM $MgCl_2$ at pH 7.2

(c) Reaction medium for rat liver mitochondria

0.1 M KCl

25 mM sucrose

10 mM $MgCl_2$

1 mM EDTA

5 mM TES

10 mM KH_2PO_4 at pH 6.8

The pH's of the media were adjusted with KOH solutions.

4. Isolation of mitochondria

The temperature of centrifugation and of the solutions used during the isolation of mitochondria were maintained at 0-4°C.

(a) Jerusalem artichoke (*Helianthus tuberosus*) mitochondria

The method was essentially that of Palmer & Kirk (1974). Using a fine vegetable grater, peeled Jerusalem artichoke tubers (200 g) were grated in 300 ml isolation medium in an ice-cold dish. The suspension was filtered through two layers of muslin, and the filtrate was centrifuged at 20,000 rpm for 2 min in the Sorvall RC2-B refrigerated centrifuge using the SS-34 rotor (8 x 50 ml capacity, 48,000 *g* at 20,000 rpm). The pellets were suspended in the wash medium and gently homogenized using a 10-ml glass homogenizer with a teflon pestle, then diluted to 100 ml with the wash medium. Large particles were removed by accelerating the centrifuge rotor from 0 - 10,000 rpm (12,000 *g*) (20 seconds) and then bringing it to rest (3 minutes). The supernatant was 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 the protein concentration of about 20 mg/ml. The mitochondria obtained by this method were used throughout the studies except where otherwise indicated.

Large scale preparation of Jerusalem artichoke 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 isolation medium and disintegrated with a Moulinex Robot Marinette salad maker to a consistent slurry (about

30 - 45 seconds). The homogenate was filtered through 2 layers of muslin and centrifuged for 15 min at 8,000 rpm (10,500 *g*) using the Sorvall GSA rotor. Each pellet was resuspended in 10 ml wash medium, homogenized gently using a small glass homogenizer with teflon pestle and diluted to 50 ml with the wash medium. Cell debris and large particles were removed from the suspension by centrifugation in the SS-34 rotor, as described earlier. The supernatant was centrifuged at 20,000 rpm for 2 min and the washed mitochondria thus collected were resuspended in the wash medium to about 4 ml, giving the protein concentration of about 20 mg/ml.

(b) Potato (*Solanum tuberosum*) mitochondria

Potato mitochondria were prepared from 200 g peeled tubers, hand-grated and isolated in the same way as Jerusalem artichoke mitochondria, except that starch granules were first removed by centrifuging the filtrate, after grinding, at 2,000 rpm (500 *g* using the Sorvall SS-34 rotor) for 5 min. The mitochondria were washed once in 100 ml of the wash medium. The final pellets were suspended and made up to 1 ml with the wash medium. The protein concentration in the final suspension was about 20 mg/ml.

(c) *Arum maculatum* rootstock mitochondria

The *Arum* rootstock was scrubbed well and the roots were removed. The rootstock (470 g) was cut into small cubes and ground in 700 ml isolation medium with a Moulinex Robot Marinette salad maker for 30 seconds. The homogenate was filtered through 3 layers of muslin. Starch was removed from the filtrate by centrifugation at 2,000 rpm (650 *g* in the Sorvall GSA rotor, 6 x 250 ml

capacity) for 15 min. The mitochondria were obtained from the supernatant by centrifugation at 8,000 rpm (10,500 *g* in the Sorvall GSA rotor) for 15 min. The mitochondria were washed once in 4 x 50 ml wash medium as described for Jerusalem artichoke mitochondria. The final pellets were suspended and made up to 6 ml with the wash medium. The protein concentration in the mitochondrial suspension was about 25 mg/ml..

(d) Rat liver mitochondria

Rat liver mitochondria were isolated from a 200 g young adult male Sprague-Dawley rat using the method described by Weinbach (1961). The liver (about 10 g) was excised from the rat, killed by a blow in the cervical region, rinsed in ice-cold isolation medium and cut into small strips. The cut liver was placed in 50 ml isolation medium and homogenized (10-15 passes) by hand using a Wesley Coe glass homogenizer with a loosely fitting teflon pestle (0.4 mm smaller in diameter than the glass body). Cell debris and red blood cells were removed by centrifugation for 10 min at 2,300 rpm (600 *g*) using the Sorvall SS-34 rotor and the supernatant was further centrifuged at 10,000 rpm (12,000 *g*) for 10 min. The fluffy material on the surface of the sedimented mitochondria was removed by rinsing the pellet with 1-2 ml of isolation medium. The mitochondria were resuspended in 10 ml isolation medium by gentle aspiration with a Pasteur pipette. The mitochondrial suspension was diluted to 50 ml with the isolation medium and centrifuged at 12,000 *g* for 10 min. The final pellet was resuspended and made up to 5 ml with isolation medium giving the protein concentration of about 20 mg/ml.

5. Preparation of submitochondrial particles (EDTA particles)

Submitochondrial particles were prepared from Jerusalem artichoke mitochondria by sonic disintegration using a Dawe Soniprobe type 7530A (Dawe Instruments Ltd., London). The mitochondria from the large scale preparation were diluted to 8 ml with ice-cold sonicating medium (0.4 M sucrose, 5 mM TES, and 1 mM EDTA at pH 8.5) in a thick-wall vessel. Sonication was carried out at a current output of 4-5 amps for a total period of 30 s in three 10-second periods, allowing both the vessel and the sonicating probe to cool in ice between the sonicating periods. The sonicated suspension was centrifuged at 20,000 rpm (48,000 *g*) for 10 min using the Sorvall SS-34 rotor and the pellet was discarded. The supernatant was centrifuged for 1 hr at 45,000 rpm (100,000 *g*) using a Beckman Spinco preparative ultracentrifuge with type 50 rotor. The red-brown gelatinous pellet consisting of submitochondrial particles was taken up to 0.5 ml with the wash medium containing 0.4 M sucrose, 10 mM TES and 0.1% (w/v) BSA at pH 7.2. The protein content in the final suspension was about 40 mg/ml.

6. Preparation of partially purified citrate synthase from Jerusalem artichoke mitochondria

Citrate synthase (EC 4.1.3.7) was isolated from Jerusalem artichoke mitochondria according to the method of Bogin & Wallace (1969). Frozen mitochondria (6 ml suspension) from 1 kg peeled tubers were thawed at room temperature and diluted with 12 ml of cooled 50 mM tris-HCl buffer at pH 7.5. The mitochondrial suspension was divided into two portions and sonicated using a Dawe

Soniprobe type 7530A at the maximum output of 4-5 amps. The total sonication time was 60 s, divided into four sonicating periods of 15 s. Both the vessel containing mitochondria and the soniprobe were allowed to cool in ice between these sonicating periods. After sonication the mitochondrial suspension was centrifuged at 20,000 *g* for 10 min and the precipitate was discarded. The supernatant was frozen at -30°C (in the deep freezer) for 5 min and thawed at room temperature. The procedure was repeated four times. The suspension was then centrifuged at 20,000 *g* for 10 min. The supernatant was subjected to fractionation by ammonium sulphate precipitation.

Most of the citrate synthase activity was contained in the fraction precipitating between 40-65% saturation with ammonium sulphate (specially low in heavy metals). This precipitate was collected by centrifugation at 30,000 *g* for 20 min and dissolved in 10 ml of 10 mM tris-HCl buffer at pH 7.2. The suspension was dialysed for 15 hours against 4 lit of 1 mM tris-HCl solution at pH 7.0 containing 1 mM cysteine. The dialysing solution was changed once after 1 hour. The precipitate which remained after dialysis was removed by centrifugation at 10,000 *g* for 10 min. The supernatant (12 ml) contained the citrate synthase activity.

The method of Shepherd & Garland (1969) for purifying citrate synthase from rat liver mitochondria was adapted for use with the dialysed enzyme which was heavily contaminated with malate dehydrogenase. The dialysed enzyme was pumped onto a DEAE-cellulose column (0.9 x 26 cm previously equilibrated with 10 mM tris-HCl at pH 7.3. The column was washed with 50 ml of the tris-HCl buffer, then with 50 ml of 10 mM potassium phosphate in the tris-HCl

buffer. According to Shepherd & Garland (1969), citrate synthase from rat liver mitochondria could be eluted with 18 mM phosphate, but no citrate synthase activity was detected in the 5-ml fractions when the eluting solution contained 20 mM phosphate. Therefore the concentration of phosphate in the tris-HCl buffer was gradually raised, 10 mM at a time. Citrate synthase was eluted from the DEAE-cellulose column when the eluting solution contained 50 mM phosphate. Five 5-ml fractions which contained high activities of citrate synthase were combined and concentrated to 1.6 ml using a Sartorius membrane filter (Collodion Bag SM13200, Sartorius-Membranefilter GmbH, Göttingen, Germany). This concentrated material constituted the partially purified citrate synthase; some activity of malate dehydrogenase was still detected in this material.

7. Assays and analytical procedures

(a) Determination of concentration of protein

Mitochondrial protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) after first solubilizing 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 of protein) in a test tube, and the mixture was made up to 0.5 ml with 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% $CuSO_4.5H_2O$ 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. With vigorous mix-

ing, 0.5 ml of diluted Folin & Ciocalteu's phenol reagent (1 part of the phenol reagent + 2 parts of distilled water) was added to the test tube. The absorbance of the coloured solution was read at 600 nm after 30 min, in a Unicam SP 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 be determined from a calibration curve prepared by treating 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.

(b) Determination of the concentrations of ADP and AMP

The concentrations of ADP and AMP in solutions were determined by the method of Adam (1962) using the Boehringer ADP/AMP test kit. The determination of ADP is based on the following enzyme reactions catalyzed by pyruvate kinase (PK) and lactate dehydrogenase (LDH).

$$\text{ADP} + \text{phosphoenolpyruvate (PEP)} \overset{\text{PK}}{\rightleftharpoons} \text{ATP} + \text{pyruvate}$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \overset{\text{LDH}}{\rightleftharpoons} \text{lactate} + \text{NAD}^+$$

The concentration of ADP could be determined by the amount of NADH oxidized in the mixture.

Since AMP was found to be a contaminant in ADP solutions, it was necessary to establish the amount of AMP present in each preparation of ADP solution used in the ADP/O determination. The amount of AMP in the solution of ADP was determined by adding adenylate kinase, which catalyzes the reaction

$$\text{AMP} + \text{ATP} \overset{(\text{Mg}^{2+})}{\rightleftharpoons} 2\ \text{ADP}\ ,$$

to the reaction mixture after the concentration of ADP had been found.

The reaction was carried out in a cuvette with 1 cm light-path and the change in absorbance at 340 nm was monitored in an Aminco DW-2 dual wavelength spectrophotometer (American Instrument Co., Silver Spring, Maryland, U.S.A.) set in the split beam mode. The reaction mixture consisted of 1 ml of buffer at pH 7.5 (0.1 M triethanolamine-HCl and 25 mM Na_2CO_3), 0.1 ml PEP solution (10 mM PEP, 0.4 M $MgSO_4$ and 1.3 M KCl), 5 µl NADH solution (50 mM NADH), 5 µl LDH (5 mg/ml) and a known volume of ADP solution (3-5 µl of approximately 25 mM solution were normally used). The contents of the cuvette were thoroughly mixed and the absorbance at 340 nm was recorded. Pyruvate kinase (5 µl of 2 mg/ml solution) was added and the reactions were allowed to proceed until no further decrease in the absorbance was detected (about 5 min). Using the extinction coefficient of 6.22×10^3 litre.mol^{-1}.cm^{-1} for NADH at 340 nm, the concentration of ADP (mM) could be calculated from the formula

$$[\text{ADP}]\,(\text{mM}) = \frac{\Delta E_1 \times V_1}{6.22 \times v}$$

where ΔE_1 = the decrease in absorbance after adding PK

V_1 = the volume in the cuvette (ml)

v = the volume of ADP solution (µl).

For the determination of AMP, 5 µl adenylate kinase (2 mg/ml) were added to the cuvette and the decrease in absorbance was recorded after 15 min. The concentration of AMP could be calculated from

$$[\text{AMP}]\ (\text{mM}) = \frac{\Delta E_2 \times V_2}{6.22 \times 2 \times v}$$

where ΔE_2 = the decrease in absorbance after adding adenylate kinase

V_2 = the volume in the cuvette (ml) after adding adenylate kinase

The content of AMP in the ADP solution was normally found to be about 10%.

(c) Activation of succinate dehydrogenase

The method used was that elaborated by R. C. Cowley (personal communication). Mitochondria were incubated at 26°C for 4 min with 20 µl of 50 mM ATP, pH 7.2, 20 µl of 50 mM KH_2PO_4 (both amounts are per mg of mitochondrial protein) and $MgCl_2$ to a final concentration of 5 mM. After this activation procedure the mitochondria were kept in an ice bath and the succinate oxidase remained active through the course of each experiment.

(d) Oxygen consumption

The rate of oxygen uptake by the mitochondria was measured polarographically using a small Rank oxygen electrode (Rank Bros, Bottisham, Cambridge, U.K.) with an internal diameter of 9 mm, in conjunction with a Servoscribe RE 511.20 (single channel) or Servoscribe 2 RE 520.20 (two-channel) potentiometric recorder (Smiths Industries Ltd, London). A polarizing voltage of 0.7 V was applied across the oxygen electrode and the voltage input for the recorder was set at 10 mV.

Reactions were carried out in the reaction chamber surrounded

by a water jacket connected to a water bath, the temperature of which was maintained at 25°C by a Julabo Paratherm II thermostatic heater (Scienco Western, Trowbridge, Wiltshire, U.K.). Rapid mixing in the reaction chamber was achieved by a small magnetic stirrer (Rank Bros). Using an Eppendorf microlitre pipette with a disposable tip (Eppendorf, Hamburg, Germany), the mitochondrial suspension was added to the reaction medium to give a final volume of 1 ml in the reaction chamber. Reactants (substrates, etc.) were introduced into the closed reaction chamber through the narrow opening in the stopper by means of micro syringes.

The rates of oxygen consumption (in nmol O_2/min per mg of mitochondrial protein) were calculated from the oxygen electrode traces according to the method of Chance & Williams (1955a & 1956) using 240 μM as the concentration of oxygen in the reaction medium (Chance & Williams, 1955a; Brunton & Palmer, 1973).

At the beginning of each experiment, the oxygen electrode was calibrated at the polarizing voltage and input for the recorder as stated above. The recorder pen was set at the 100% level on the chart paper when the reaction chamber contained only the reaction medium. The zero-oxygen level was found by adding a few crystals of sodium 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.

(e) Determination of ADP/O and respiratory control ratios

The ADP/O and respiratory control ratios during the oxidation of substrates by the mitochondria were calculated from the oxygen electrode traces according to the methods of Chance & Williams

(1955a & 1956). A substrate was added to the mitochondria suspended in the standard reaction medium (i.e. with Mg^{2+} and Pi) to start respiration. An addition of ADP (0.1 - 0.2 µmol) brought about an increase in the rate of respiration (state 3) which declined to a slow rate (state 4) on depletion of ADP.

The ADP/O ratio was calculated from µmoles of ADP phosphorylated per µatom of oxygen consumed. The amount of oxygen consumed during the phosphorylation of ADP to ATP was calculated from the oxygen electrode trace during respiration in state 3 as described by Chance & Williams (1955a).

The respiratory control ratio was calculated from the ratio: the rate of oxygen uptake in state 3/the rate of oxygen uptake in state 4 (Chance & Williams, 1956).

The concentration of oxygen in the reaction medium was then taken to be 240 µM (Chance & Williams, 1955a; Brunton & Palmer, 1973).

(f) Reduction of potassium ferricyanide

The reduction of potassium ferricyanide was monitored in the Aminco DW-2 dual-wavelength spectrophotometer at 420 nm, either in the split-beam mode, or in the dual-beam mode using 490 nm as the reference wavelength (Palmer & Kirk, 1974). In a 1 ml cuvette with 1.0 cm light-path, the mitochondria were suspended in the standard reaction medium containing potassium ferricyanide (1 mM or 2 mM) and potassium cyanide (1.5 mM). In the experiment in which the phosphorylation of ADP per pair of electrons (ADP/$2e^-$) was measured, KCN was not included because it led to a decrease in the value of ADP/$2e^-$. (Ikuma & Bonner (1967c) also found that both

the respiratory control and the ADP/O ratios of malate oxidation in mung bean mitochondria decreased in the presence of KCN.)

(g) Determination of ADP/$2e^-$ ratio from the reduction of potassium ferricyanide

The ratio of ADP/pair of electrons (ADP/$2e^-$) using ferricyanide as the electron acceptor was estimated from the spectrophotometer traces in a manner analogous to the estimation of the ADP/O ratio (Lee, Sottocasa & Ernster, 1967; Douce, Christensen & Bonner, 1972). The reduction of $Fe(CN)_6^{3-}$ was monitored at 420 nm with 490 mn as the reference wavelength as described in the preceding section. KCN was not included in the reaction mixture for the reason given in the preceding section. Thus the measurement of the ADP/$2e^-$ ratio was carried out when the concentration of $K_3Fe(CN)_6$ was between 1-2 mM since it was found that, when $K_3Fe(CN)_6$ in this range of concentrations was added to the oxygen electrode, it could inhibit the oxygen uptake of the mitochondria.

The reaction was started by adding the substrate to the cuvette containing mitochondria, necessary cofactor(s) and 2 mM $K_3Fe(CN)_6$. An increase in the rate of reduction of $Fe(CN)_6^{3-}$ was detected upon adding ADP (100 nmol), i.e. respiration in state 3. Using the millimolar extinction coefficient $\varepsilon_{420} = 1\ mM^{-1}.cm^{-1}$ (Lee *et al.*, 1967) the number of electrons reaching $Fe(CN)_6^{3-}$ during the phosphorylation of ADP could be obtained. Hence the ADP/$2e^-$ ratio could be calculated.

(h) The fluorescence of 1-anilinonaphthalene-8-sulphonate (ANS)

The changes in the level of fluorescence of ANS were monitored in the Perkin-Elmer Fluorescence Spectrophotometer model MPF-3. ANS (250 nmoles in 0.1 ml ethanol) was added to the mitochondria, which had been washed in the wash medium without BSA and suspended in 2.5 ml of the standard reaction medium in a 3 ml cuvette. The fluorescence of ANS was excited at 366 ±3 nm and its emission was monitored at 470 ±5 nm (Azzi, 1969). The changes in the level of fluorescence at 470 nm were recorded with the Hitachi Recorder Model QPD-33 set at 3.0 sensitivity. The basal fluorescence of the system was suppressed electronically by the appropriate controls on the instrument.

(i) Detection of respiration-supported proton extrusion by Jerusalem artichoke mitochondria

In a large Rank oxygen electrode assembly (3 ml capacity) with a top opening to accommodate a small combined glass electrode (5 mm external diameter for use with a Pye Unicam pH meter model 290), nitrogen gas was bubbled into 2.8 ml of reaction medium consisting of 0.3 M sucrose and 5 mM potassium phosphate (pH 7.2) without other buffer until the oxygen content of the medium was almost zero. Jerusalem artichoke mitochondria were added to the medium to give the final volume of 3 ml and the pH electrode was assembled. Potassium succinate (10 mM, pH 7.2) was added and the reaction was allowed to become anaerobic. Small volumes of oxygen-saturated reaction medium were added to study the changes in the pH of the system. Simultaneous changes in oxygen levels and proton concentrations were recorded with the Servoscribe RE 511.20 potentiometric recorder set at 2 mV on both channels.

(j) Reduction of cytochrome b

The mitochondria (about 2 mg of protein) were suspended in a cuvette containing the standard reaction medium to the total volume of 1 or 3 ml. Thiamin pyrophosphate or glutamate and glutamate-oxaloacetate transaminase were included for the oxidation of pyruvate or malate as appropriate. The levels of reduction of cytochrome b were monitored in the Aminco DW-2 dual-wavelength spectrophotometer, at 432 nm with 410 nm as the reference wavelength (Chance & Williams, 1956), and 3.0 nm slit width, using a light-path of 1 cm.

(k) Reduction of endogenous nicotinamide adenine dinucleotide

The levels of reduction of nicotinamide-adenine nucleotide in the mitochondria were monitored in the Aminco DW-2 dual-wavelength spectrophotometer, at 340 nm with 374 nm as the reference wavelength (Chance & Williams, 1956), using 3.0 nm slit width, the UV filter, and a light-path of 1 cm. The mitochondria (about 2 mg of protein) were suspended in the standard reaction medium to the total volume of 1 ml in a cuvette.

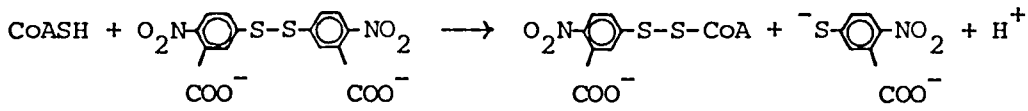
(l) Assay of citrate synthase

The method is that of Shepherd & Garland (1969).

Reactions were carried out in 0.1 M tris-HCl buffer pH 8.0 in 1 ml cuvettes with 1 cm light-path. The sample cuvette contained 0.1 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB, Ellman's reagent), 0.05 mM acetyl-S-CoA and 20 µl of enzyme; the reference cuvette contained the tris-HCl buffer, DTNB and acetyl-S-CoA. The reaction was started by adding 0.2 mM oxaloacetate (20 µl of 10 mM solution) to the sample cuvette, bringing the total volume in the

cuvette to 1.0 ml. Using an Aminco DW-2 dual-wavelength spectrophotometer in split-beam mode, the activity of citrate synthase was measured by monitoring the increase in absorption at 412 nm (with 2 nm slit width) due to the mercaptide ion produced from the reactions:-

$$\text{Acetyl-S-CoA} + \text{oxaloacetate}^{2-} + H_2O \rightleftharpoons \text{citrate}^{3-} + \text{CoASH} + H^+$$



The rate of release of CoASH could be calculated using 13,600 as the molar extinction coefficient of the mercaptide at 412 nm.

(m) Assay of malate dehydrogenase

The activity of malate dehydrogenase was assayed by the method described by Coleman & Palmer (1972). The oxidation of NADH, linked to the reduction of oxaloacetate, was followed at 340 nm in the Aminco DW-2 dual-wavelength spectrophotometer in the split beam mode. The reaction medium consisted of 1 mM oxaloacetate, 0.25 mM NADH and 20 mM TES at pH 7.5. The reaction was started by adding the enzyme protein, bringing the total volume in the cuvette to 1 ml.

RESULTS

1. Organization and complexity of the NADH dehydrogenase in isolated Jerusalem artichoke mitochondria

(a) Oxidation of various substrates and the ADP/O ratios

Jerusalem artichoke mitochondria isolated by the method described could oxidize Krebs-cycle intermediates and exogenously added NADH, and showed respiratory control (Chance *et al.*, 1968; Chance & Williams, 1956) in an oxygen electrode, indicating coupled oxidative phosphorylation. Addition of a limited amount of ADP to the mitochondria in the presence of a substrate caused an increase in the rate of oxygen uptake which reverted to the original rate when ADP was exhausted. The rates of oxidation of various substrates by the mitochondria and their corresponding ADP/O and respiratory control ratios, as measured in the oxygen electrode, are reported in Table 1. The terminology of the oxidation rates in the presence and absence of ADP, referred to as 'state 3' and 'state 4' respectively, and the method used to calculate the ADP/O ratios are those of Chance & Williams (1955a, 1956).

Although the ADP/O ratios were not the same in all preparations, they were always less than, and appeared to represent a similar percentage of, the theoretical values within one preparation. It seemed reasonable to attribute these lower-than-theoretical ADP/O ratios to the inefficiency of the ATP synthesizing enzyme, or to the integrity of the mitochondrial membranes providing the required potential for ATP synthesis. The presence of the pathway of electron transport by-passing the first coupling site (Brunton, 1975) in these mitochondria was unlikely to contribute to the low ADP/O ratios

TABLE 1 The respiratory activities of Jerusalem artichoke mitochondria

Substrate	ADP/O ratio		Oxidation rate nmol O_2/min/mg protein		Respiratory control ratio
	average value	% of theoretical value	State 3	State 4	
5 mM ascorbate + 0.5 mM TMPD	0.61	61	215	164	1.31
1 mM NADH	1.26	63	130	47	2.77
10 mM succinate	1.28	64	136	61	2.23
15 mM pyruvate + 1 mM malate + 170 µg TPP	2.07	69	58	24	2.42
25 mM malate + 10 mM glutamate + 20 µg glutamate-oxaloacetate transaminase	1.95	65	56	28	2.00
10 mM citrate + 1 mM malonate	1.68	56	27	20	1.35
10 mM α-oxoglutarate + 170 µg TPP + 1 mM malonate	2.29	57	29	17	1.71

The mitochondria were suspended in the reaction medium, containing the necessary cofactors or inhibitor as indicated, to the total volume of 1 ml in the oxygen electrode. The substrates at the specified final concentrations were added to start the reactions. Oxidation in state 3 was brought about by adding small amounts of ADP to the reaction mixture; 150 nmol ADP per addition except ascorbate/TMPD oxidation when 100 nmol ADP were used. The oxidation rates reported were those produced by the second addition of ADP. The ADP/O ratios were calculated by the method of Chance & Williams (1956). The amount of mitochondrial protein per assay was approximately 0.2 mg for ascorbate/TMPD oxidation, 0.5 mg for NADH or succinate and 1 mg for the NAD^+-linked substrates except α-oxoglutarate when 2 mg were employed. Each value quoted was the average of three experiments. For succinate oxidation the mitochondria were activated as described in Methods.

found for the NAD^+-linked substrates because the ADP/O ratio for the oxidation of ascorbate plus TMPD was also about sixty per cent of the expected value (i.e. 0.61 instead of 1 for one site of phosphorylation).

Another non-phosphorylating pathway of electron transport to oxygen, i.e. the cyanide-resistant alternate oxidase (Storey & Bahr, 1969b; Bendall & Bonner, 1971; Passam & Palmer, 1972), which has been shown to be induced in potato mitochondria (Bonner *et al.*, 1972) by washing potato slices for some hours in several changes of water before isolating the mitochondria, could perhaps account for the low efficiency of energy conservation observed. However, the mitochondria used in these experiments were not isolated from 'washed' Jerusalem artichoke tubers, and cyanide resistance has not been observed in freshly prepared Jerusalem artichoke mitochondria (Passam, 1971). Therefore it was unlikely that the low efficiency of energy conservation observed was the result of the non-phosphorylating alternate oxidase. No other non-phosphorylating pathway is known. It was unlikely that the ATPase activity, i.e. the hydrolysis of ATP, was responsible for the low efficiency of ATP synthesis (low ADP/O ratios) obtained because of the presence of an endogenous inhibitor (Pullman & Monroy, 1963; Passam & Palmer, 1973) which seems to prevent the hydrolysis but not the synthesis of ATP in intact Jerusalem artichoke mitochondria.

From Table 1, although the oxidation rates were reported in the order of the number of coupling sites (from 1 to 3, plus one substrate-level phosphorylation for α-oxoglutarate), the highest rate of oxidation (in state 3) was observed with ascorbate plus TMPD as the substrate. The rates of oxidation of succinate and NADH came

second on the list and citrate and α-oxoglutarate were oxidized at the slowest rates. It was apparent that the rate limiting step in the oxidation of substrates in the mitochondria was not at the level of cytochrome oxidase, but further up the electron transport chain and probably closer to the first site of phosphorylation or at the level of the NAD^+-linked dehydrogenases.

It was usual to find that the oxidation of succinate by isolated Jerusalem artichoke mitochondria was slow at first, and the rate of oxidation increased with time after addition of succinate. This was due to the activation by succinate of the succinate dehydrogenase, which was often found in isolated plant mitochondria to be in the deactivated state (Singer *et al.*, 1973b). The activation could also be accomplished by preincubating the mitochondria with ATP (Wiskich & Bonner, 1963; Oestreicher *et al.*, 1973). The procedure for the activation of Jerusalem artichoke mitochondria as described in Methods was elaborated by R. C. Cowley (personal communication) and was regularly employed in experiments involving succinate oxidation. After activation, the 'activated mitochondria' could oxidize succinate rapidly and at a constant rate. Unfortunately the 'activated mitochondria' could no longer oxidize pyruvate-plus-malate, which was generally used as the representative of the NAD^+-linked substrates. It is possible that the incubation of mitochondria with ATP and magnesium led to the inactivation of pyruvate dehydrogenase, a situation reported for mitochondria from beef heart (Schuster & Olson, 1974) and rabbit heart (Chiang & Sacktor, 1975).

Variations in the rate and pattern of oxidation of malate by the mitochondria were often found. After a long period of storage

of the tubers, the oxidation of malate by itself was slow and non-linear (decreasing), as reported by Passam (1971). An oxygen electrode trace in Fig. 1 demonstrates the oxidation of malate at a very slow rate under these conditions. The state-3 rate and the ADP/O ratio decreased as the oxidation proceeded. Inclusion of glutamate and glutamate-oxaloacetate transaminase in the reaction mixture to remove oxaloacetate facilitated a relatively rapid and constant rate of oxidation of malate (as reported in Table 1) with a constant ADP/O ratio. Hence, the decline in the rate of oxidation of malate and the decrease in the ADP/O ratio appeared to be the results of accumulation of oxaloacetate. Without the oxaloacetate trap the decline in the oxidation of malate may be easily explained by the property of the equilibrium of the reaction catalyzed by the malate dehydrogenase which favours the reduction of oxaloacetate rather than malate oxidation (Davies, 1969).

There is evidence that another enzyme, the NAD^+-specific malic enzyme, may also be responsible for the oxidation of malate in plant mitochondria (Macrae & Moorhouse, 1970; Macrae, 1971a, b & c; Coleman & Palmer, 1972). By analysing the products of the oxidation of malate in Jerusalem artichoke mitochondria, Coleman & Palmer (1972) found that pyruvate was another product and its level continued to rise although the accumulation of oxaloacetate stopped when the oxidation of malate by the malate dehydrogenase had reached equilibrium. The existence of the malic enzyme in Jerusalem artichoke mitochondria has been confirmed by Brunton (1975) and Palmer & Arron (1976). Macrae (1971c) showed that isolated malic enzyme was inhibited by oxaloacetate. However, addition of oxaloacetate to Jerusalem artichoke mitochondria oxidizing malate only slightly inhibited

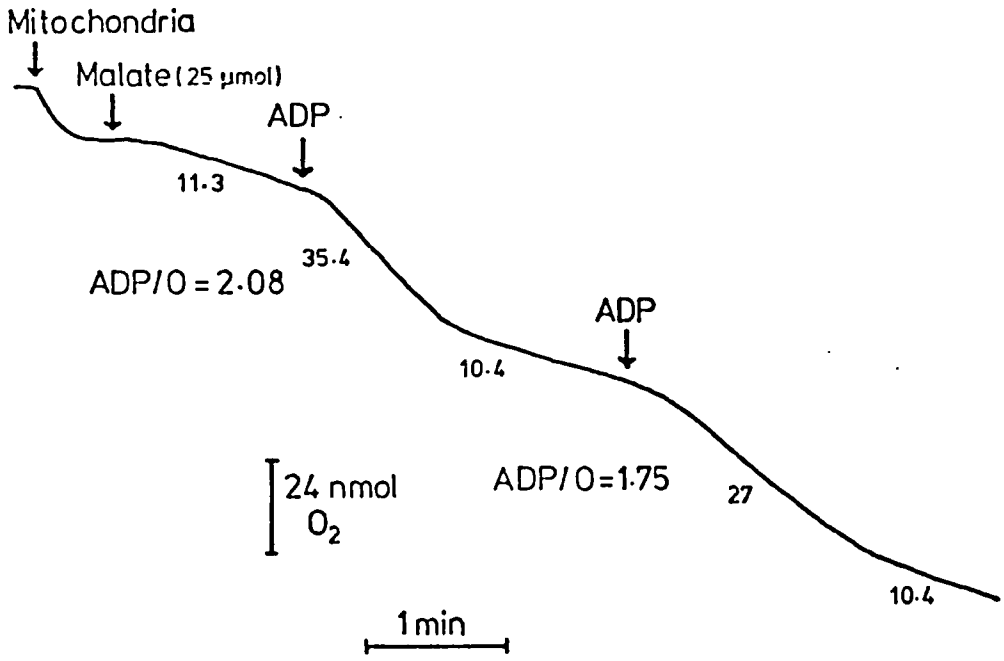


Fig. 1 Oxygen electrode trace showing malate oxidation by Jerusalem artichoke mitochondria

Malate was added to mitochondria (1.2 mg of protein) suspended in 1 ml reaction medium (as in Methods). ADP was added as indicated at 147.4 nmol per addition. ADP/O ratios were calculated according to Chance & Williams (1955a). Numbers underneath the trace indicate the rates of oxygen consumption in nmol/min per mg of mitochondrial protein.

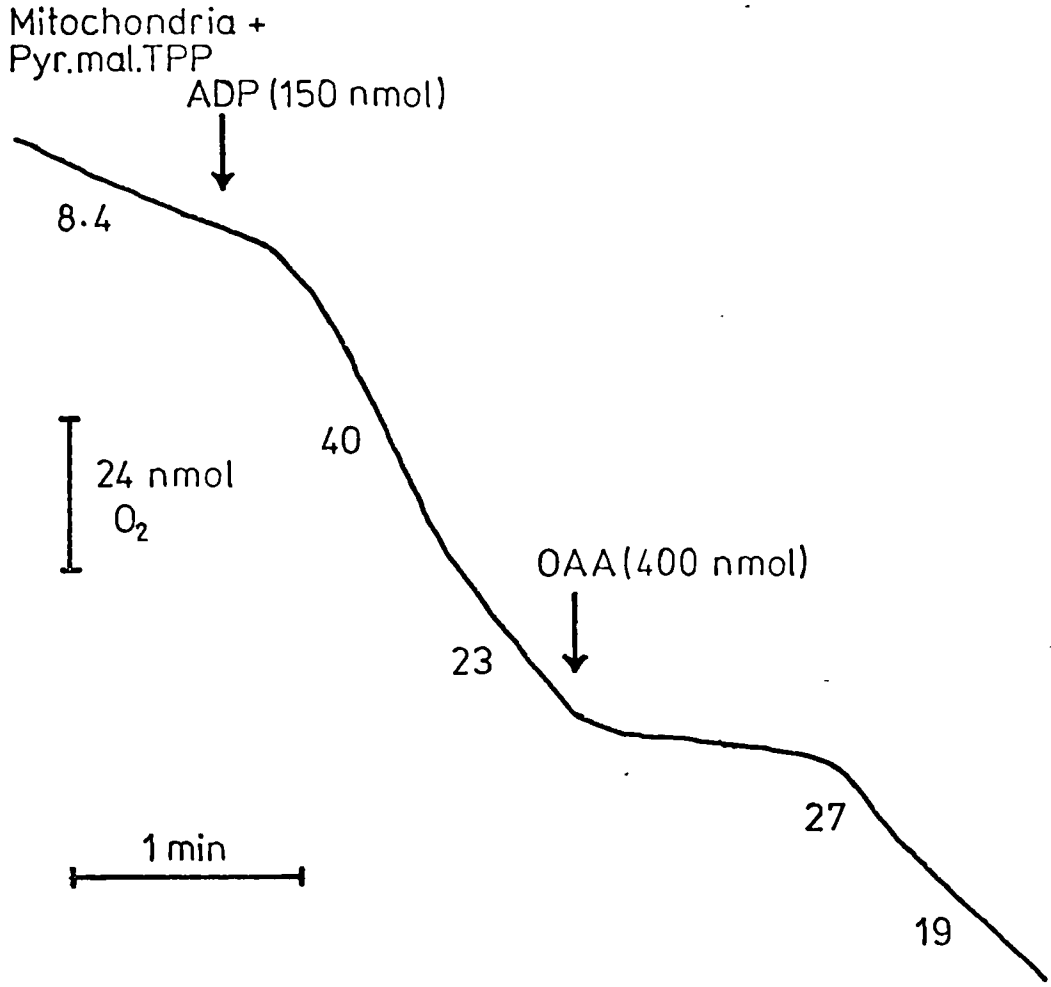


Fig. 2 The effect of oxaloacetate on the oxidation of pyruvate

Jerusalem artichoke mitochondria, 1.86 mg of protein in 1 ml reaction medium, were oxidizing pyruvate (15 mM) with a catalytic amount of malate (0.5 µmol) and 170 µg TPP in an oxygen electrode. Oxaloacetate (400 nmol) was added in state 4 as indicated. The numbers under the oxygen electrode trace indicate the rates of oxygen consumption in nmol/min per mg of protein.

the oxidation (Brunton, 1975). Therefore the oxidation of malate by the mitochondria must have been brought about by the balance between the activities of the malate dehydrogenase and the malic enzyme, not by either enzyme alone.

It is much more difficult to explain the decrease in the ADP/O ratio during the oxidation of malate when the oxaloacetate trap was absent. The first possibility might be that the NADH produced was also oxidized by the piericidin A-resistant pathway by-passing the phosphorylation site I (Brunton & Palmer, 1973; Palmer & Coleman, 1974). However, the evidence from Brunton & Palmer (1973) suggested that this pathway (in wheat mitochondria at least) only operated when the inhibitor piericidin A was present. The second and more plausible explanation is that in the presence of ATP, oxaloacetate could be removed by the action of PEPcarboxykinase (ATP requiring; EC 4.1.1.49), which had been found in plant mitochondria (Davies, 1956), thereby regenerating ADP. The evidence for this reaction in Jerusalem artichoke mitochondria is shown in Fig. 2; when oxaloacetate was added to the mitochondria oxidizing pyruvate in state 4, there was an inhibition of oxygen uptake followed by a short period of respiration faster than the state-4 rate, and then the respiration returned to state 4. This result indicated that the removal of oxaloacetate when ATP was present produced ADP which could become available for oxidative phosphorylation.

(b) Specificity of oxidative phosphorylation towards adenine nucleotides

Apart from the discovery that succinyl-CoA synthetase of Jerusalem artichoke mitochondria is specific for adenine nucleotides (Palmer & Wedding, 1966), it has been shown by Passam & Palmer (1973)

that the phosphorylation reaction of the reversible ATPase of Jerusalem artichoke submitochondrial particles is specific for adenine nucleotides. The implication of these results is that only adenine nucleotides (ADP and AMP) can act as the phosphate acceptors in these mitochondria. Indeed, addition of ADP or AMP brought about the stimulation of oxygen uptake by the mitochondria in the presence of α-oxoglutarate or NADH (Fig. 3). Addition of GMP and IMP did not cause any stimulation, therefore confirming that neither could act as the phosphate acceptor in these mitochondria.

A close examination of the oxygen electrode trace of the oxidation of NADH in Fig. 3 revealed that the addition of AMP did not bring about the increase of oxygen uptake immediately, unlike the trace of the oxidation of α-oxoglutarate, where the addition of AMP after a pulse of ADP caused an immediate stimulation. This observation can be explained by the following account.

It has been established that the translocation of adenine nucleotides into animal mitochondria for oxidative phosphorylation is achieved by a translocator, specific for ADP and ATP, in a 1:1 exchange process between exogenous and endogenous adenine nucleotides; exogenous ADP is the preferred species transported into the mitochondria (Pfaff & Klingenberg, 1968; Souverijn *et al.*, 1973). AMP itself cannot be transported by the translocator, but must first be converted into ADP by the action of adenylate kinase (Brierly & O'Brien, 1965; Souverijn, 1974) which is present in the intermembrane space of the mitochondrion (Siekevitz & Potter, 1955; Klingenberg & Pfaff, 1966; Sottocasa *et al.*, 1967 a & b). The existence of the translocator specific for ADP has been confirmed in Jerusalem artichoke mitochondria (Passam *et al.*, 1973; Passam & Coleman, 1975).

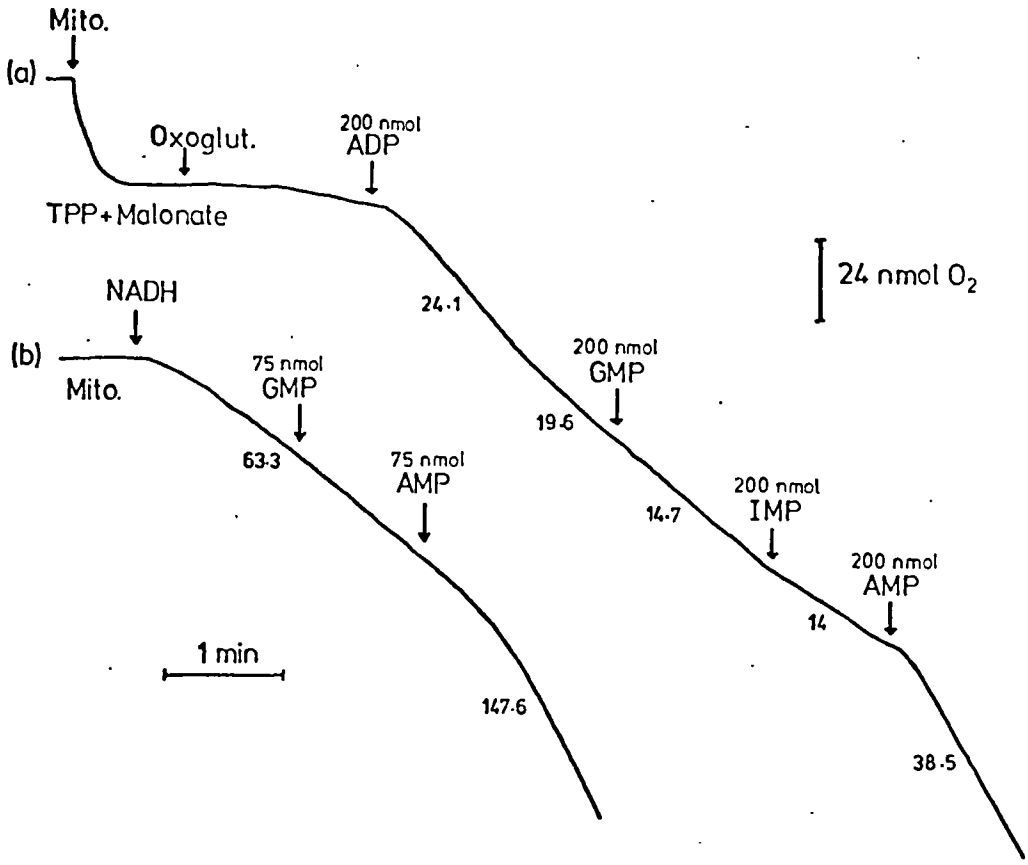


Fig. 3 Requirement of adenine nucleotides for the oxidation of α-oxoglutarate

(a) α-Oxoglutarate (10 mM) was added to a 1 ml oxygen electrode containing mitochondria (1.73 mg of protein) suspended in the standard reaction medium plus 170 µg TPP and malonate (1 mM). ADP, GMP, IMP and AMP were added in aliquots of 200 nmol as indicated.

(b) GMP and AMP (75 nmol) were added to the mitochondria (0.46 mg of protein) oxidizing 1.2 mM NADH in the standard reaction medium.

Numbers under the traces indicate rates of oxygen consumption in nmol O_2/min per mg of protein.

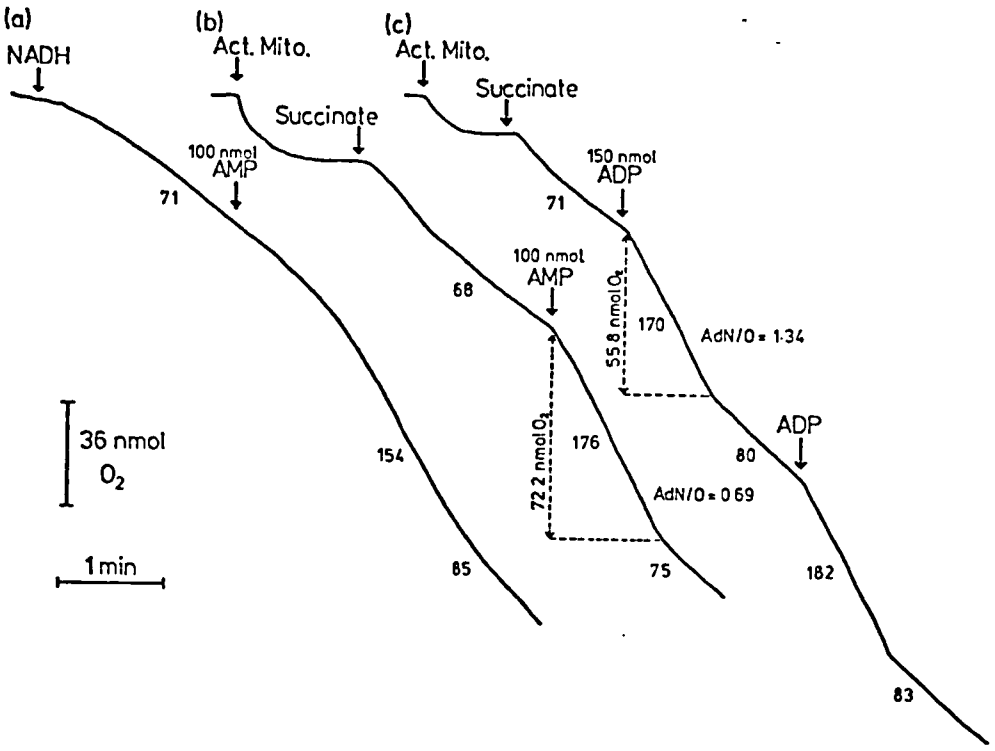


Fig. 4 The effect of AMP on respiration of Jerusalem artichoke mitochondria

Oxygen electrode traces showing the stimulation of respiration by 100 nmol AMP and 150 nmol ADP. The mitochondria (0.43 mg of protein) were suspended in the standard reaction medium (0.3 M sucrose, 5 mM KH_2PO_4, 2.5 mM $MgCl_2$ and 5 mM TES at pH 7.2) to the total volume of 1 ml in the oxygen electrode; activated mitochondria (by preincubation with ATP and Mg^{2+}, as in Methods) were used for succinate oxidation, traces b and c. The P/O ratios were calculated according to Chance & Williams (1955a,1956). Numerals under the traces indicate oxygen consumption in nmol O_2/min per mg of protein.

Passam & Coleman (1975) have also demonstrated that AMP stimulates oxygen uptake by the mitochondria only in the presence of ATP and Mg^{2+}, indicating involvement of adenylate kinase in the utilization of AMP.

An experiment was carried out to verify the finding of Passam & Coleman (1975), and the result is shown in Fig. 4. Again, the addition of AMP to Jerusalem artichoke mitochondria oxidizing NADH (in the presence of Mg^{2+}) brought about the stimulation of respiration after a lag period (Fig. 4a). If ATP was present initially, together with Mg^{2+}, as in the case of activated mitochondria oxidizing succinate, the stimulation was immediate (Fig. 4b). AMP also induced twice as much oxygen uptake per mole as did ADP (Fig. 4c), so that the resultant adenine/O (AdN/O) ratio was half of that from the addition of ADP.

The lag period before the stimulation of respiration by the addition of AMP (Fig. 3; Fig. 4a) was probably due to the presence of a very small amount of non-specifically bound ATP (Passam *et al.*, 1973) on the membrane, thereby limiting the production of ADP by adenylate kinase. After some time more ATP was available because of its efflux in exchange for the translocation of ADP and the production of ADP from AMP could be stimulated. Then the rise in respiration could be observed.

(c) Reduction of potassium ferricyanide in mitochondria and submitochondrial particles

The reduction of ferricyanide supplied as a non-penetrating electron acceptor (Mitchell & Moyle, 1969a; Klingenberg & Buchholz, 1970) in Jerusalem artichoke mitochondria is demonstrated in Fig. 5.

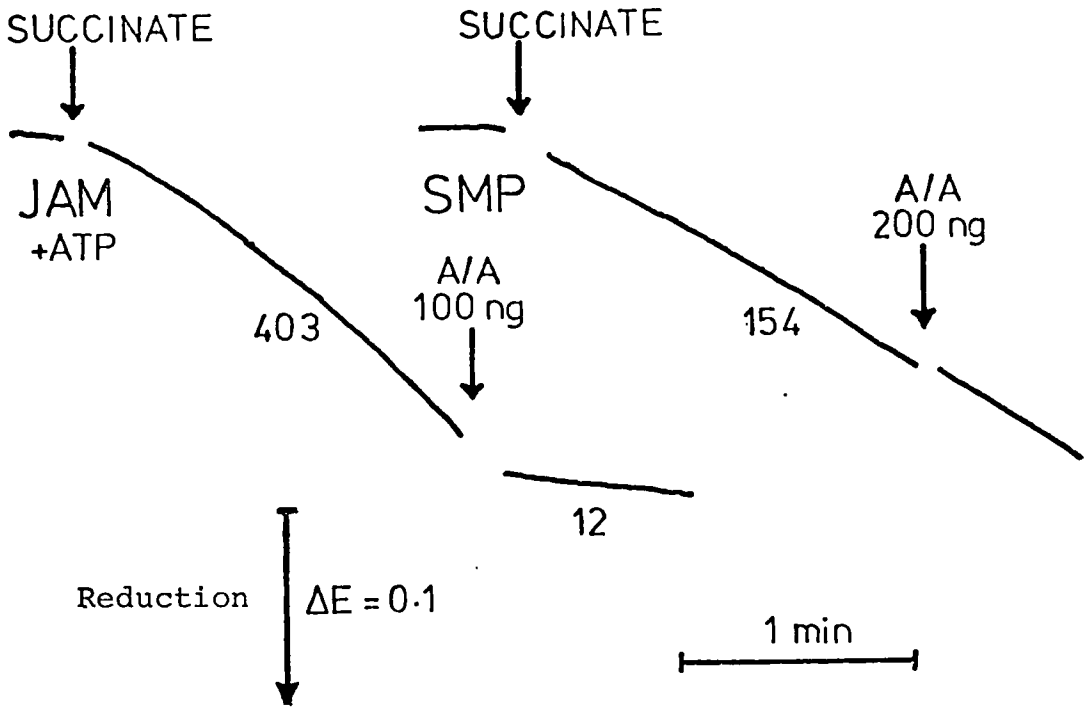


Fig. 5 The effect of antimycin A on ferricyanide reduction

The reduction of ferricyanide was monitored at 420 nm in the split beam mode. Jerusalem artichoke mitochondria (0.33 mg protein - JAM) and the submitochondrial particles (0.54 mg protein - SMPs) were incubated for 5 min in 1 ml reaction medium plus 0.5 µmol ATP, then 1 µmol $K_3Fe(CN)_6$, 1.5 µmol KCN and FCCP at the final concentration of 2×10^{-7} M were added. Succinate (20 mM) was added to start the reaction. Numbers under the traces indicate the rates of reduction of ferricyanide in nmol/min per mg of protein.
A/A = antimycin A.

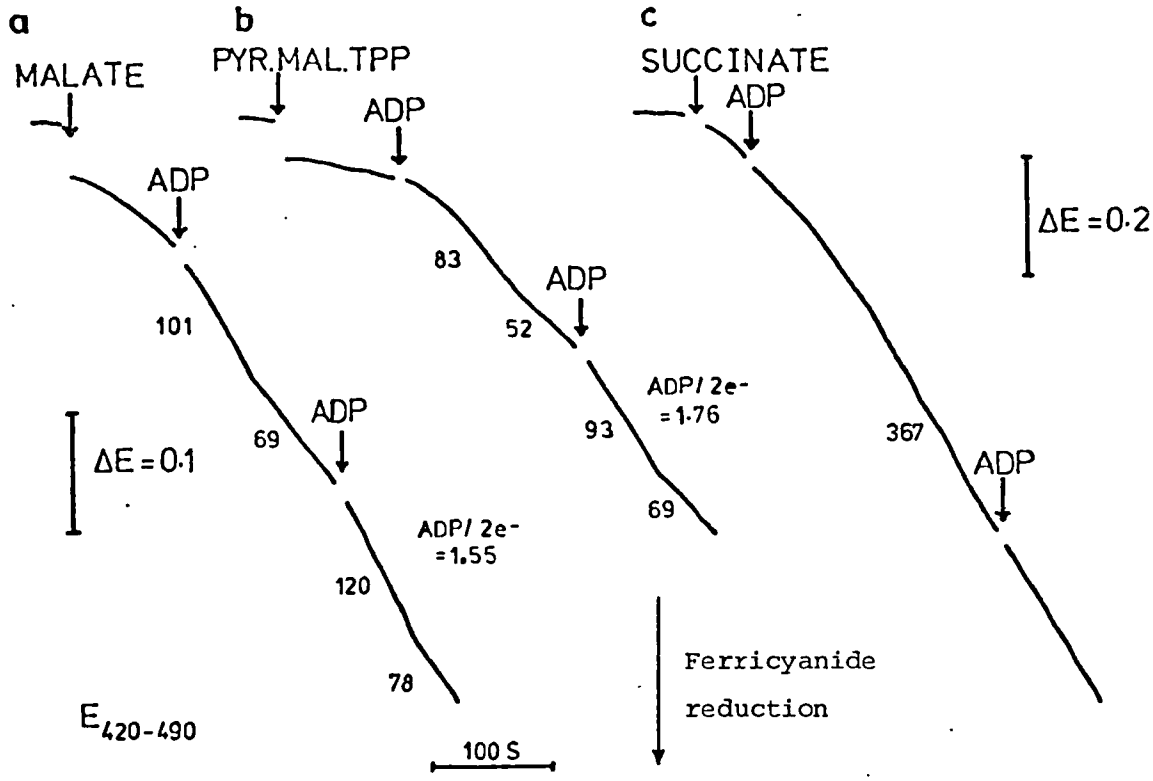


Fig. 6 Reduction of ferricyanide and oxidative phosphorylation of NAD^+-linked substrates

The reduction of ferricyanide was followed at 420 nm, using 490 nm as the reference wavelength (Palmer & Kirk, 1974) and the millimolar absorbance coefficient $\varepsilon_{420} = 1\ mM^{-1}cm^{-1}$ (Lee *et al.*, 1967) (at 490 nm there is no absorption by ferricyanide). Using 1 ml cuvettes with 1 cm light-path, Jerusalem artichoke mitochondria were suspended in the standard reaction medium plus 2 mM $K_3Fe(CN)_6$. For malate oxidation 10 mM glutamate and 20 µg glutamate-oxaloacetate transaminase were included in the reaction medium. The volume in the cuvette before addition of substrate was 1.02 ml; 97.6 nmol

Fig. 6 (cont)

ADP was added where indicated. The concentrations of substrates were 25 mM malate, 15 mM pyruvate + 1 mM malate + 170 µg TPP (Pyr. Mal. TPP), and 10 mM succinate. The mitochondrial protein per assay was 1.14 mg in a & b and 0.62 mg in c. For the oxidation of succinate, the mitochondria were activated as described in Methods. The numbers under the traces indicate the rates of reduction of ferricyanide in nmol/min per mg of protein.

Reduction of ferricyanide occurred when succinate was added to the intact mitochondria and, under this condition, was inhibited by antimycin A (trace on the left) because ferricyanide could only intercept electrons in the region of cytochrome c (Pressman, 1955; Estabrook, 1961). In submitochondrial particles prepared by sonicating the mitochondria, there was no permeability barrier because the membranes were turned inside-out (Lee *et al.*, 1967) and ferricyanide could accept electrons directly from succinate dehydrogenase. Therefore the succinate-ferricyanide reduction was insensitive to the addition of antimycin A (trace on the right). The complete insensitivity to antimycin A of the submitochondrial particles indicated that the matrix side of the inner mitochondrial membrane was uniformly exposed (see Harmon *et al.*, 1974).

The ADP-stimulated oxidation of NAD^+-linked substrates using ferricyanide as the terminal electron acceptor is shown in Fig. 6 (a & b). It was possible to estimate the ADP/pair of electrons (ADP/$2e^-$) ratio (Lee *et al.*, 1967; Douce *et al.*, 1972) in a manner analogous to the estimation of ADP/O ratio (Chance & Williams, 1956). In this experiment the ADP/$2e^-$ ratio was found to be approaching 2, i.e. one less than the ADP/O ratio because the third site of phosphorylation (site III) was not traversed. As it was not possible to see respiratory control in the oxidation of succinate by ferricyanide (Fig. 6c), the ADP/$2e^-$ ratio for succinate could not be estimated in this study.

(d) <u>The presence of a pieridicin A-resistant pathway of oxidation of endogenous NADH in Jerusalem artichoke mitochondria</u>

Piericidin A has been shown to be a potent inhibitor of electron transport in mammalian mitochondria (Jeng *et al.*, 1968); it specific-

ally reacts at very low concentrations at a site near the NADH dehydrogenase identical with the site of rotenone inhibition. Jeng *et al.* (1968) showed that 0.036 nmol (approx. 15 ng) piericidin A per mg of protein completely inhibited NADH oxidase in beef heart submitochondrial particles. In plant mitochondria the situation is not simple; intact mitochondria can oxidize added NADH (as shown in Table 1 and Fig. 4a) via the externally located NADH dehydrogenase and hence is insensitive to rotenone or piericidin A (Palmer & Passam, 1971; Coleman & Palmer, 1971). In addition, oxidation of NAD^+-linked substrates which is partially inhibited by piericidin A or rotenone has been reported (Ikuma & Bonner, 1967c; Brunton & Palmer, 1973; Day & Wiskich, 1974; Brunton, 1975). The findings indicate the presence of a piericidin A-resistant pathway of oxidation of endogenous NADH in plant mitochondria.

The sensitivity to piericidin A of the oxidation of endogenous NADH by Jerusalem artichoke mitochondria was investigated. It was found that the oxidation of NAD^+-linked substrates was partially sensitive to piericidin A (Fig. 7), and the level of piericidin A-resistance was about 30% of the oxidation rate in state 3.

The piericidin A-resistant respiration was coupled to oxidative phosphorylation, though with a low respiratory control ratio. The oxygen electrode trace in Fig. 8 shows residual phosphorylation after addition of piericidin A to mitochondria oxidizing malate (in the presence of TPP) in state 3. When ADP was exhausted, the respiration decreased until more ADP was added. The resultant ADP/O ratio was 1.10, whereas that obtained, in the same preparation, from malate oxidation (+ TPP) without piericidin A was 2.17. Brunton & Palmer (1973) found that addition of rotenone to barley-scutella mitochon-

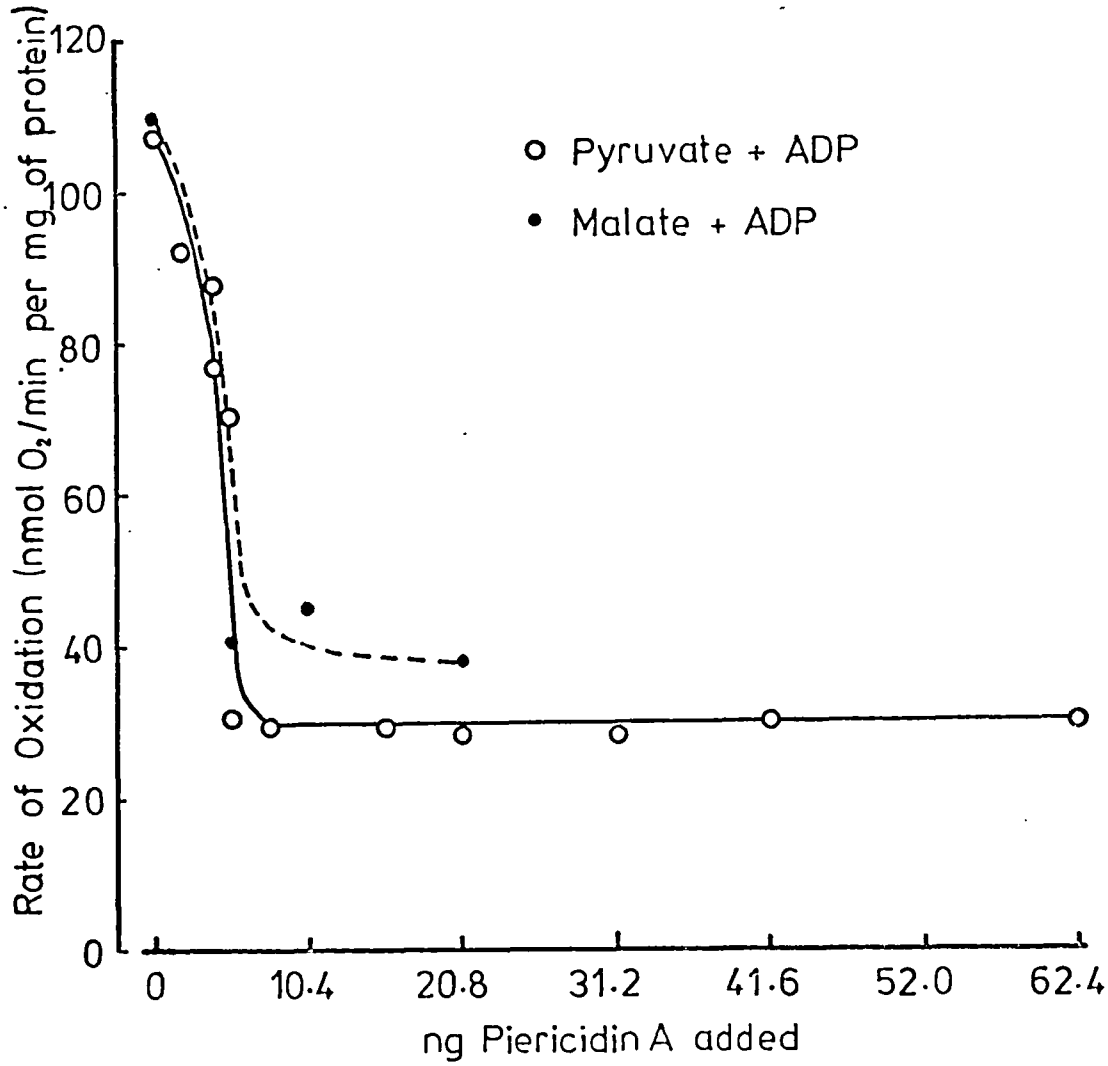


Fig. 7 Inhibition of the oxidation of NAD^+-linked substrates by piericidin A

Piericidin A dissolved in ethanol was added at the appropriate concentrations to the 1 ml-oxygen electrode containing Jerusalem artichoke mitochondria (0.77 mg of protein) oxidizing either 15 mM pyruvate (plus 1 mM malate and 170 µg TPP) or 25 mM malate (in the presence of 10 mM glutamate and 20 µg glutamate-oxaloacetate transaminase) in state 3 which was brought about by addition of 300 nmol ADP. The rates plotted were the final rates in the presence of piericidin A.

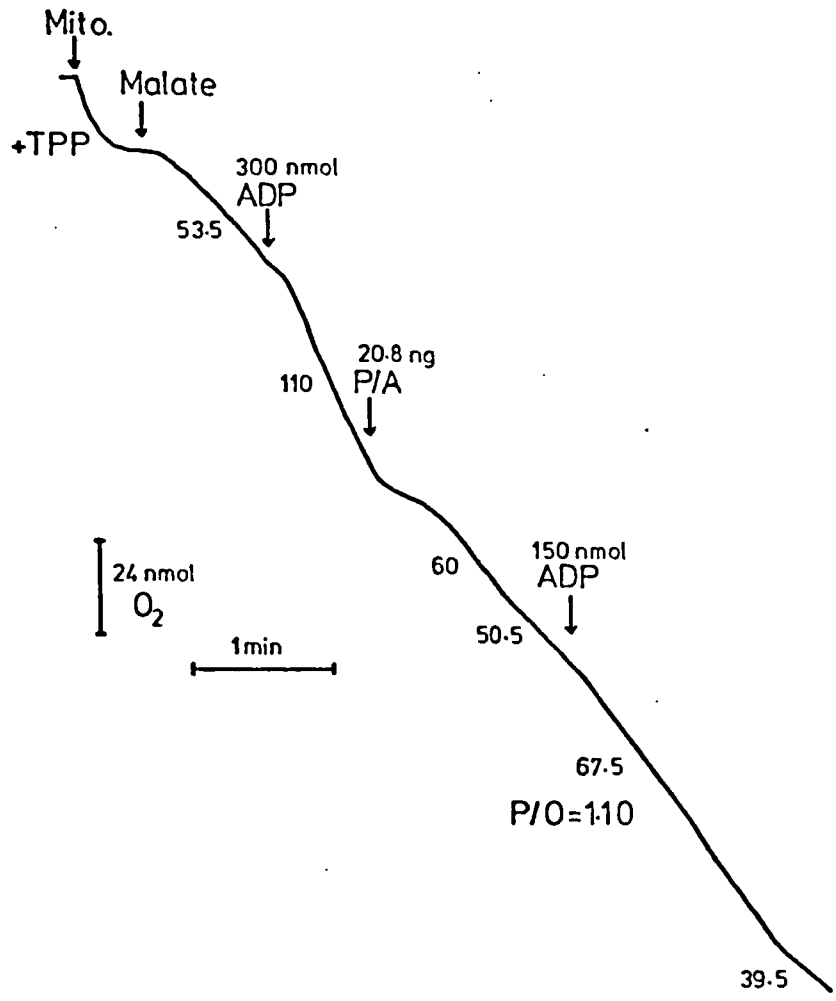


Fig. 8 Oxidative phosphorylation of the piericidin A-resistant pathway

The oxygen electrode trace shows the effect of 50 pmol (20.8 ng) piericidin A (which was enough to cause maximal inhibition as shown in Fig. 7) on the oxidation of malate by Jerusalem artichoke mitochondria. The mitochondria (0.77 mg of protein) were suspended in 1 ml standard reaction medium plus 170 µg TPP. The numbers under the trace indicate rates of oxygen consumption in nmol O_2/min per mg of protein.

dria oxidizing NAD^+-linked substrates caused the ADP/O ratio to fall by the equivalent of one phosphorylation site. Thus it was suggested that the resistant pathway was linked to two phosphorylation sites, by-passing the first site. In this experiment the decrease of ADP/O ratio was just over 1 (2.17 - 1.10) which must be considered to be more than an equivalent loss of one site of phosphorylation, considering that the oxidative phosphorylation was 70% effective.

One explanation for the resultant ADP/O ratio of 1.10 (Fig. 8) instead of 1.4, was that it was due to the uncoupling activity of piericidin A; Vallin & Löw (1969) found that piericidin A stimulated the hydrolysis of ATP by mammalian submitochondrial particles. However, the ADP/O ratio obtained from the oxidation of succinate, by the mitochondria from the same preparation, and in the presence of the same concentration of piericidin A was 1.41, with normal respiratory control ratio. Therefore, piericidin A seemed to be unable to uncouple in the presence of succinate. In fact, the data of Vallin & Löw (1969) indicated that the presence of succinate (plus NAD^+) prevented pieridicin A from stimulating the release of Pi by submitochondrial ATPase.

In the same paper, Vallin & Löw (1969) also studied the energy-linked reduction of NAD^+ (Ernster & Lee, 1967) by succinate (reversed electron transport). They noted that if piericidin A (in the range of concentrations which did not inhibit the NADH oxidase) was added after an anaerobic preincubation of the particles with ATP, or after succinate had been added in the aerobic state, the inhibition of the reduction of NAD^+ by piericidin A was decreased. They therefore concluded that the degree of inhibition of the energy-linked reversed electron transport by piericidin A depended upon the oxidation-

reduction (redox) state of a critical component, presumably the ubiquinone; the inhibition by piericidin A was less effective the more extensively the ubiquinone was reduced. This conclusion arose from the earlier suggestion by Moore & Folkers (1964) that a planar chelate between the ubiquinone and non-haem iron might be a prerequisite for the formation of the primary high-energy bond. Due to the structural similarity between piericidin A and ubiquinone, piericidin A could substitute the ubiquinone molecule, causing the inhibition of the formation of the high-energy bond and hence the reversed electron transport. For this reason, uncoupling (lowering of ADP/O ratio) by piericidin A was seen in the presence of malate (Fig. 8) but not succinate.

Alternatively, the apparent uncoupling in the presence of piericidin A and malate (not succinate) may perhaps be explained in the light of Mitchell's chemiosmotic theory. The synthesis of ATP in the mitochondrion depends on the electrochemical potential (the protonmotive force, PMF) arising from the electron transfer during the oxidation of substrate (Mitchell, 1966; Greville, 1969). Since the rate of piericidin A-resistant oxidation of malate (Fig. 8) was slow compared with the oxidation of succinate (see Table 1 and Fig. 4), it was likely that the net PMF of the piericidin A-resistant oxidation of malate might be smaller and less effective for the synthesis of ATP than the PMF generated from the oxidation of succinate. It is obvious that the slow rate of piericidin A-resistant oxidation would also affect the level of reduction of the ubiquinone. As a result it looks as though the level of reduction of ubiquinone controlled the coupling activity of the mitochondria (cf. Vallin & Löw, 1969).

2. The response of mitochondrial activities to the uncoupling agent carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP)

Laties (1973) reported that in the absence of added ADP the rate of consumption of oxygen by potato mitochondria was not increased on addition of the weak acid uncoupling agent carbonylcyanide-*m*-chlorophenylhydrazone (*m*-Cl-CCP), except when exogenous NADH was used as the substrate. Earlier work by Passam (1971) indicated, however, that *m*-Cl-CCP acted as an uncoupler of oxidative phosphorylation in plant mitochondria because it abolished ^{32}P-esterification during the oxidation of succinate in the presence of ADP by mitochondria from Jerusalem artichoke tubers and *Arum* spadices. Laties (1973) showed that in the presence of the uncoupler the rate of oxidation of citrate, pyruvate-plus-malate or succinate-plus-pyruvate could be stimulated by ADP in an oligomycin insensitive manner. Hence Laties concluded that under these conditions ADP was involved in bringing about "uncoupler effectiveness", a role distinct from the conventional phosphate acceptor in oxidative phosphorylation.

Although the failure of weak acid uncouplers to stimulate oxidation of NAD^+-linked substrates had been earlier reported (Laties, 1953; Ikuma & Bonner, 1967b), the publication by Laties (1973) prompted the investigation into the effects on Jerusalem artichoke mitochondrial activities of the weak acid uncoupler carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) which is closely related to *m*-Cl-CCP and has been shown to effectively uncouple oxidative phosphorylation from electron transport in animal mitochondria (Heytler & Prichard, 1962; Reid *et al.*, 1966).

(a) The permeability of the mitochondrial membrane to protons

According to the chemiosmotic theory proposed by Mitchell (1966, 1968), the oxidoreduction activity of electron transport in the mitochondrion is accompanied by proton translocation outwards across the coupling membrane which is relatively impermeable to ions. The protonmotive force (the pH gradient and the membrane potential), thus generated, brings about ATP synthesis in the presence of a phosphate acceptor and the proton-translocating reversible ATPase system. Any substance affecting the permeability of the membrane to ions can collapse this electrochemical potential, i.e. the protonmotive force, and therefore uncouples oxidative phosphorylation. The weak acid uncoupler FCCP has been shown to cause a rapid collapse of the pH differential across the coupling membrane of rat liver mitochondria created by respiration (Mitchell & Moyle, 1967).

(i) Respiration-supported proton extrusion by Jerusalem artichoke mitochondria

It was possible to demonstrate respiration-driven proton extrusion in Jerusalem artichoke mitochondria using succinate as the oxidizable substrate (Fig. 9). The decrease in pH of the suspending medium was detected as soon as oxygen was introduced into the medium (in this case oxygen was liberated from H_2O_2 by catalase which was present in the medium). When oxygen was exhausted, the expelled protons returned to the mitochondria. (Note: the oxygen electrode showed a delay of 10 sec in registering the exhaustion of oxygen.) After addition of FCCP at the concentration of 2×10^{-7} M, there was very little decrease in pH due to the addition of oxygen and an increase in pH was not detected when oxygen was exhausted.

As explained by Mitchell (1968), FCCP conducted a rapid trans-

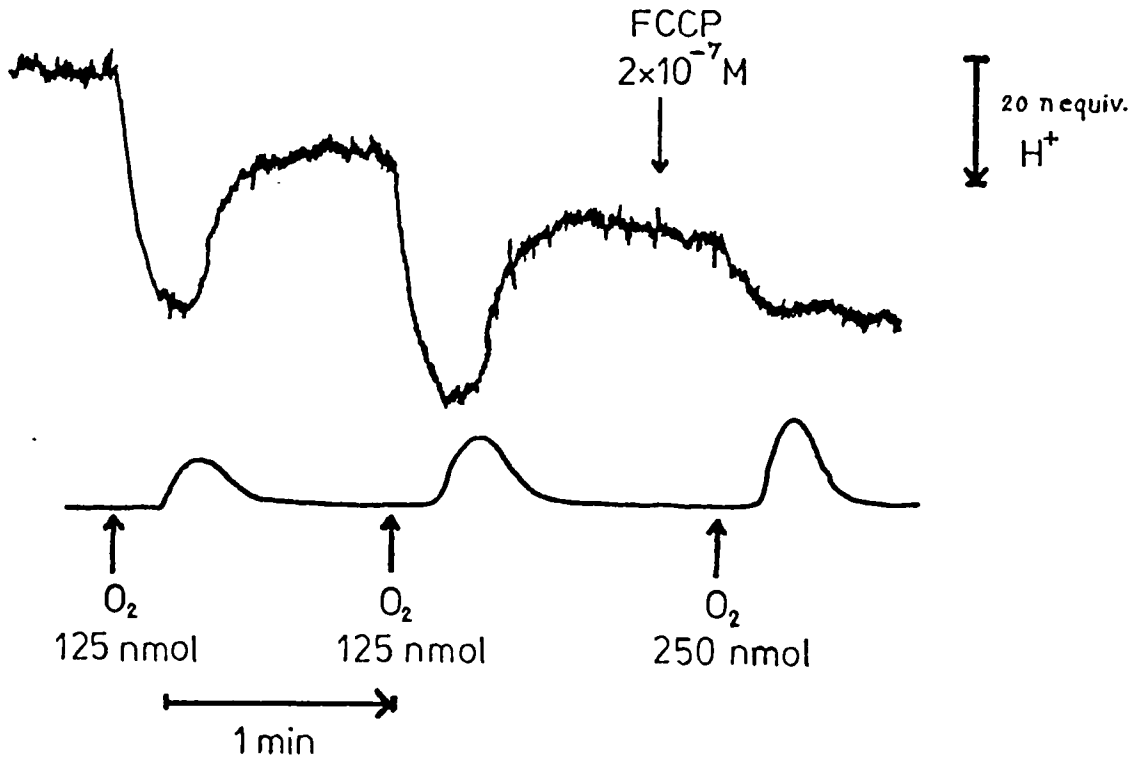


Fig. 9 Respiration-supported proton extrusion in Jerusalem artichoke mitochondria and the effect of FCCP

The mitochondria (4 mg of protein) were incubated for 10 min anaerobically with 0.3 M sucrose, 5 mM potassium phosphate pH 7.2, 10 mM succinate and 26 units of catalase in a 3 ml oxygen electrode fitted with a small pH electrode (as described in the Methods). Pulses of oxygen were introduced into the reaction mixture by adding appropriate amounts of hydrogen peroxide solution (50 mM). FCCP (in ethanol) was added to give the final concentration of 2×10^{-7} M. The upper trace indicates the change in concentration of H^+ in the medium. The lower line records the level of oxygen in the electrode, the linear part being the anaerobic state.

fer of protons into the mitochondria, therefore the pH differential was abolished. According to the chemical coupling theory, the proton pump is secondary to the high-energy intermediate (Chance *et al.*, 1967; Greville, 1969; see also Skulachev, 1971 & 1975) and by hydrolysing the high-energy intermediate (Slater, 1953; Chance & Williams, 1956; Ernster *et al.*, 1967) FCCP could prevent the formation of a pH gradient. Whether the pH gradient is a direct result of electron transport or not, it was evident that FCCP could abolish it (Fig. 9).

It was also apparent that prior to the addition of FCCP, the time taken by the mitochondria to consume oxygen was the same as that taken by the mitochondria, in the presence of FCCP, to consume twice the amount of oxygen. Thus FCCP seemed to stimulate respiration of the mitochondria.

(ii) The fluorescence changes of 1-anilinonaphthalene-8-sulphonate (ANS)

Another method of detecting the energy-dependent changes in mitochondria and submitochondrial particles is the use of the amphiphilic fluorescent probe 1-anilinonaphthalene-8-sulphonate (ANS). Azzi *et al.* (1969), Chance *et al.* (1969) and Brocklehurst *et al.* (1970) showed that energization by adding a substrate or ATP to submitochondrial particles resulted in an enhancement of the fluorescence of added ANS. In the mitochondria, energization decreased ANS fluorescence (Azzi, 1969). Although there is no conclusive interpretation, the change of ANS fluorescence has been employed as a convenient indicator for the energy coupling in these systems.

The transition of fluorescence of ANS associated with the res-

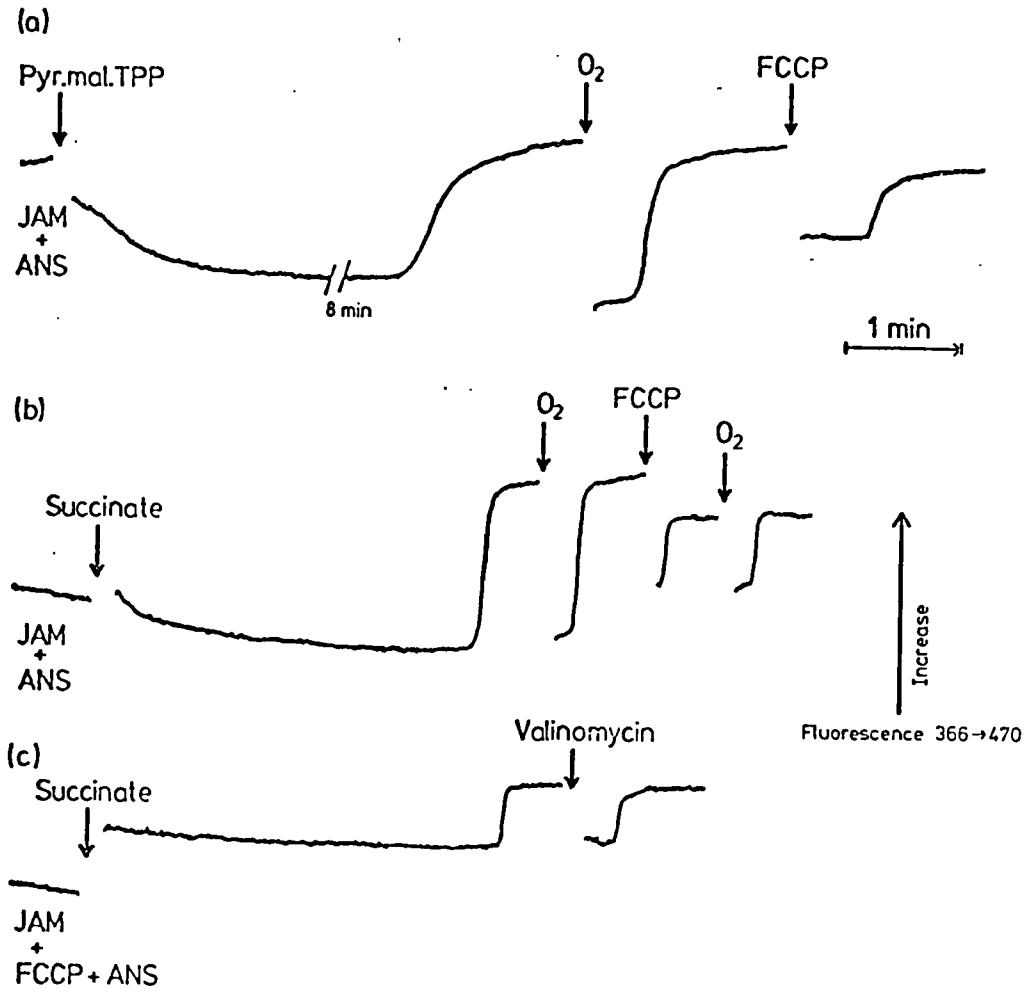


Fig. 10 The changes in the levels of fluorescence of ANS

Jerusalem artichoke mitochondria which had been washed in the wash medium without BSA (see Methods) were suspended in the standard reaction medium (4.9 mg of protein in 2.7 ml) in a cuvette in the Perkin-Elmer fluorescence spectrophotometer. The fluorescence of ANS was excited at 366 nm with slit width 6 nm and recorded at 470 nm with slit width 10 nm (Azzi, 1969) setting the sample sensitivity at 3. Where indicated, in (a) a mixture of pyruvate, malate and TPP (giving final concentrations of 15 mM pyruvate, 1 mM malate and 170 µg TPP), in (b) and (c) succinate (10 mM) were added. The concentrations of FCCP and valinomycin were 2×10^{-7} M and 2.5×10^{-7} M respectively.

piration of Jerusalem artichoke mitochondria has been observed. In Fig. 10, addition of pyruvate-plus-malate and TPP (a) or succinate (b) resulted in a decrease in the level of fluorescence of ANS until the mixture became anaerobic when the fluorescence increased. By stirring the cuvette a small amount of oxygen was re-introduced and a short period of decrease in fluorescence could be observed.

Skulachev and co-workers (Jasaitis *et al.*, 1971; Skulachev, 1971) have explained on the basis of the chemiosmotic theory that the changed levels of fluorescence of ANS are connected with the electrochemical potential across the membrane, brought about during the oxidation of substrate by the mitochondria, i.e. the changes are related to the movement of ANS across the mitochondrial membrane. The fluorescence of ANS in the mitochondrial suspension is due to the portion of ANS bound in the mitochondrial membrane (Radda, 1971; Skulachev, 1971). The concentration of ANS in the membrane is dependent upon its concentration in the incubation mixture and in the intramitochondrial matrix. During substrate oxidation, protons are extruded from the mitochondria, hence the mitochondrial interior becomes more negative. Being an anion, ANS leaves the mitochondria electrophoretically, the effect of which is a decrease in the concentration of ANS inside the mitochondrial matrix. Extruded ANS is diluted in the large volume of the incubating medium, and does not significantly increase the ANS concentration outside the mitochondria. Therefore, phenomena related to the movement of ANS across the membrane are determined almost entirely by the concentration of ANS in the matrix. Thus, the concentration of ANS in the mitochondrial membrane, and hence the intensity of ANS fluorescence shown in Fig. 10, decreased during the oxidation of pyruvate and succinate. When

respiration ceased, ANS could return to the mitochondria and the fluorescence therefore increased.

After FCCP had been stirred into the cuvettes, the cycles of changes in the level of fluorescence could still be observed, although they were smaller in magnitude (Fig. 10a & b). FCCP slightly quenched the fluorescence of ANS, so the total fluorescence was less than that before the addition of FCCP. Other workers have reported that FCCP completely abolishes the energy-linked transitions of the fluorescence of ANS (Azzi, 1969; Azzi *et al.*, 1969; Takeuchi, 1975). In this experiment, if FCCP was added to the mitochondrial suspension before the substrate, the decrease in fluorescence due to the addition of substrate was not seen (Fig. 10c). However, when O_2 was exhausted an increase in fluorescence similar to the residual changes in Fig. 10a and b was still observed, and addition of more FCCP or valinomycin did not affect it.

It is possible that the respiration of the mitochondria gives rise to two effects concerning the ANS in the membrane: a movement of ANS across the membrane and a change in the environment of the binding site of ANS in the membrane (Radda, 1971). The persistent changes in fluorescence of ANS in the presence of FCCP (Fig. 10) may be due to the change in environment of the mitochondrial membrane which always takes place together with electron transport. Since ANS is reputed to display a high fluorescence yield in non-polar environments (Stryer, 1965; Dodd & Radda, 1967; Radda, 1971), and in the mitochondrial membrane, it is bound at polar-non-polar regions (Rubalcava *et al.*, 1969; Radda & Vanderkooi, 1972). One conjecture is that the formation of water during the mitochondrial electron transport may occur near the binding site of ANS, resulting in a

decrease in its hydrophobicity which can lead to a decrease in the binding of ANS. Therefore, this portion of transition of the fluorescence of ANS in mitochondria must occur in the presence of oxygen irrespective of the presence of FCCP.

A conclusion can be drawn from the studies of mitochondrial proton expulsion and ANS fluorescence that FCCP can effectively abolish the electrochemical potential in Jerusalem artichoke mitochondria as well as in animal mitochondria.

(b) The stimulation of respiration by FCCP

By a definition based on simple observations, uncoupling agents are compounds which prevent the synthesis of ATP but allow electron transport to proceed in an uncontrolled manner (Chance & Williams, 1956; Palmer & Hall, 1972). The results in the preceding section have demonstrated that, with respect to the parameters tested (Fig. 9 & 10), FCCP is a potent uncoupling agent in mitochondria isolated from Jerusalem artichoke tubers. Its effect on the respiration of the mitochondria must now be studied.

(i) Oxidation of NADH and succinate

The effect of FCCP on the rate of respiration of the mitochondria oxidizing added NADH or succinate (activated mitochondria) is shown if Fig. 11. Addition of FCCP (at 2×10^{-7} M) stimulated respiration, and the stimulated rate of uptake of oxygen was equal to or even greater than that obtained in the presence of ADP. The response was of equal magnitude when the uncoupler was added either before ADP or after the exhaustion of a pulse of ADP (i.e. in state 4).

(ii) Oxidation of an NAD^+-linked substrate

In contrast to the oxidation of NADH and succinate, the oxidation

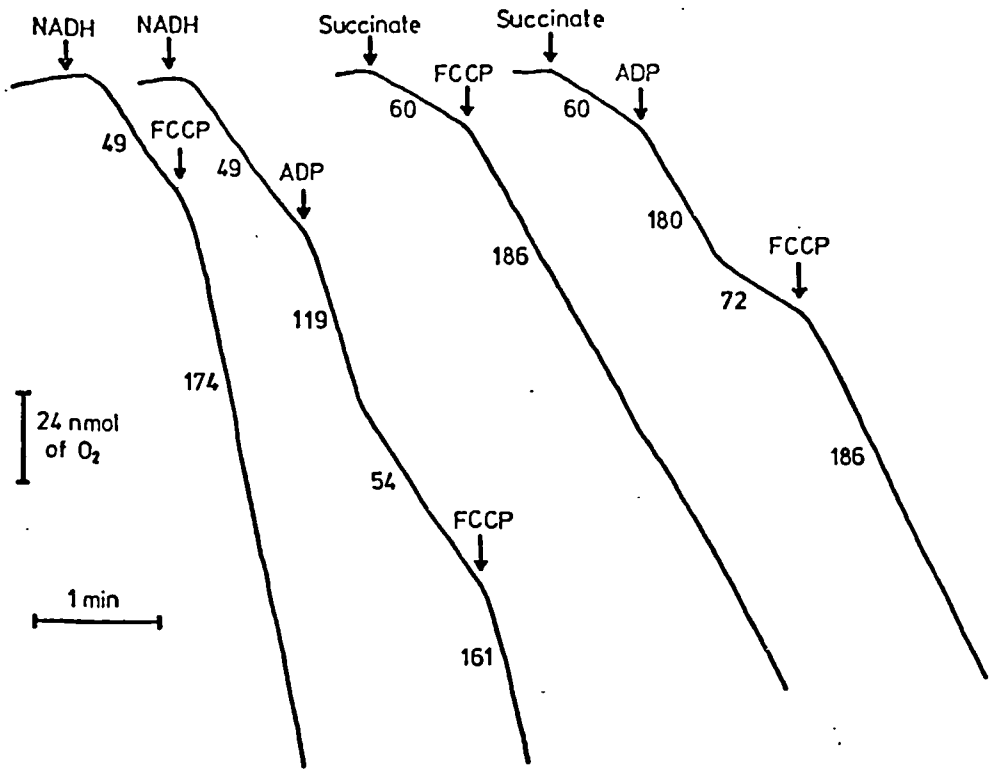


Fig. 11 Influence of FCCP on the rates of oxidation of NADH and succinate

Oxygen-electrode traces showing the effect of FCCP on the rate of oxidation of 1 mM NADH and 10 mM succinate. The mitochondria were suspended in a reaction volume of 1 ml. Respiration was stimulated by adding 100 nmol of ADP or the uncoupler (2×10^{-7} M). For the oxidation of NADH, 1 mg of mitochondrial protein was used per assay and 0.4 mg of activated mitochondria was used for succinate oxidation. Values under the traces are the rates of oxygen consumption in nmol of O_2/min per mg of protein.

of pyruvate-plus-malate was not fully stimulated by FCCP (Fig. 12); this was especially apparent if the uncoupler was added before ADP. The rate of oxidation of pyruvate-plus-malate in the presence of FCCP was characterized by an initial fairly rapid phase followed by a second, more prolonged, slower phase.

The failure of FCCP to stimulate the oxidation of pyruvate was in agreement with the result obtained by Laties (1973), though, in these mitochondria, FCCP was able to abolish the energy-linked transitions of the fluorescence of ANS during the oxidation of pyruvate as well as succinate (Fig. 10). In contrast to the findings of Laties (1973), it was found that FCCP stimulated the oxidation of succinate as effectively as it did NADH oxidation. It is unlikely that FCCP could only uncouple the two phosphorylation sites involved in the oxidation of NADH and succinate but not the first site, because it is thought that the non-phosphorylated high-energy state associated with each coupling site is in equilibrium with a common high-energy state (Ernster *et al.*, 1967). Therefore, efficient uncoupling by FCCP at one site, by hydrolysing the high-energy intermediate of oxidative phosphorylation (Slater, 1953; Chance & Williams, 1956), would result in uncoupling at all other sites. Resistance to uncoupling at any one site is most improbable when the electrochemical gradient across the membrane (Mitchell, 1966 & 1968) is abolished because of the equilibration of the acid-base gradient across the membrane by FCCP. The lack of stimulation of oxidation of the NAD^+-linked substrate by FCCP must have been the result of other factor(s).

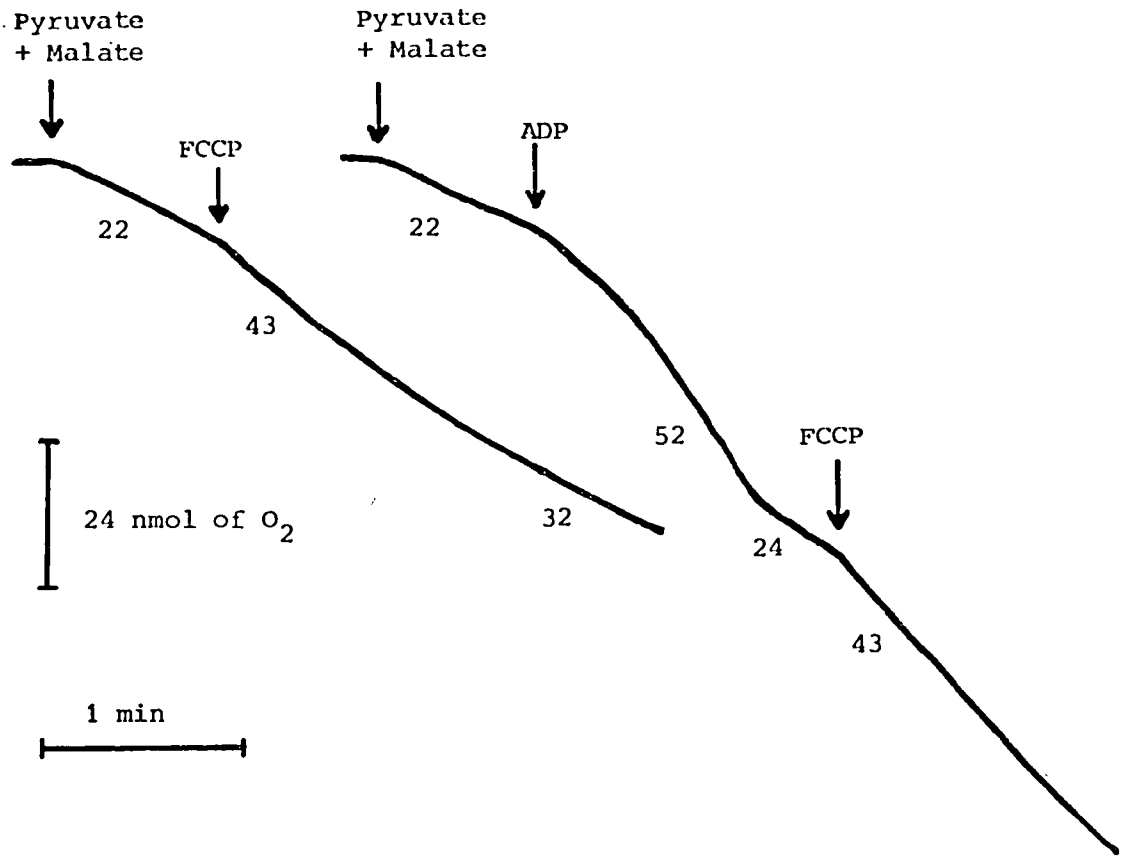


Fig. 12 Influence of FCCP on the oxidation of pyruvate-plus-malate

The conditions of assays were the same as in Fig. 11 except that 170 µg of thiamin pyrophosphate (TPP) was included in the reaction medium and pyruvate (15 mM) plus malate (1 mM) was used as the substrate. The oxygen-electrode traces were obtained by using 1 mg of mitochondrial protein per assay, and 100 nmol of ADP or FCCP at 2×10^{-7} M were added as indicated.

3. The response of uncoupled respiration to adenine nucleotides

(a) Stimulation of oxygen uptake in the presence of FCCP

Experiments were carried out in order to investigate the possibility that adenine nucleotides might stimulate the respiration of Jerusalem artichoke mitochondria in the presence of an uncoupling agent as already reported by Laties (1973) in potato mitochondria.

It was found that respiration of the unactivated mitochondria oxidizing succinate was slightly stimulated by FCCP. However, the uncoupled oxidation could be increased by ATP and to a lesser extent by ADP but not by AMP (Fig. 13). This result confirmed the findings of previous authors (Wiskich & Bonner, 1963; Drury *et al.*, 1968; Oestreicher *et al.*, 1973) that the adenine nucleotides (ATP and ADP) were involved in the activation of succinate dehydrogenase.

If pyruvate-plus-malate was used as the substrate, the addition of ADP or AMP 1 min after the uncoupler resulted in a marked stimulation of the uncoupled rate. In fact, AMP was apparently more efficient in stimulating the respiration, in the presence of FCCP, than ADP, as shown in Fig. 14. At about 50 μM, AMP produced maximum stimulation, whereas the concentration of ADP had to be raised to about 125 μM in order to get the same effect.

The addition of ATP at the same level was without effect. Neither GMP nor IMP could stimulate oxygen uptake by the mitochondria in the presence of the uncoupler.

The effect of AMP on the uncoupled rates of oxidation of several substrates is shown in Table 2. Inhibitors were added where necessary to prevent the further oxidation of the products of the principal reaction from complicating the result. Clearly the stimulation of oxygen uptake by AMP was only observed when substrates using the

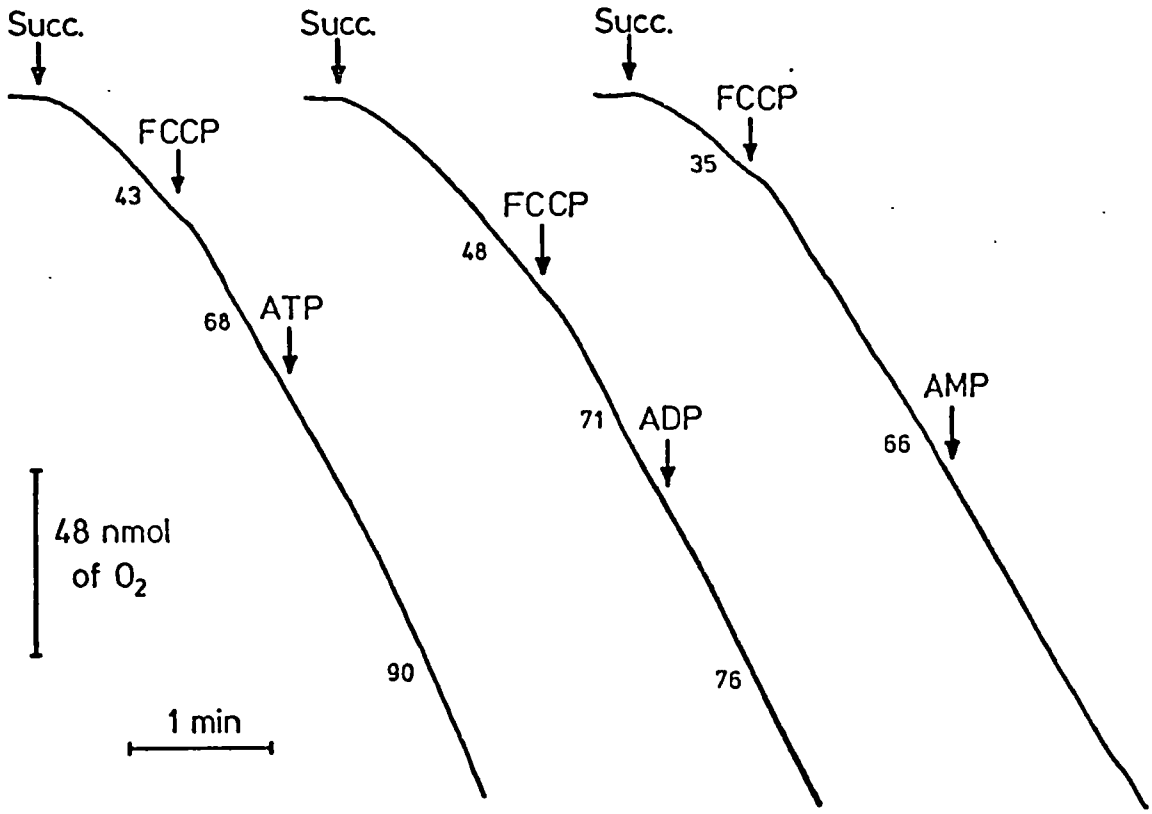


Fig. 13 The effect of adenine nucleotides on the oxidation of succinate in the presence of FCCP

Succinate (10 mM) was added to the unactivated mitochondria (0.96 mg of protein) in the oxygen electrode (total volume 1 ml). Additions of FCCP (2×10^{-7} M) and the adenine nucleotides (150 µM for ATP and ADP, 75 µM for AMP; see Fig. 14) were made where indicated. Values under the traces are the rates of oxygen uptake in nmol of O_2/min per mg of mitochondrial protein.

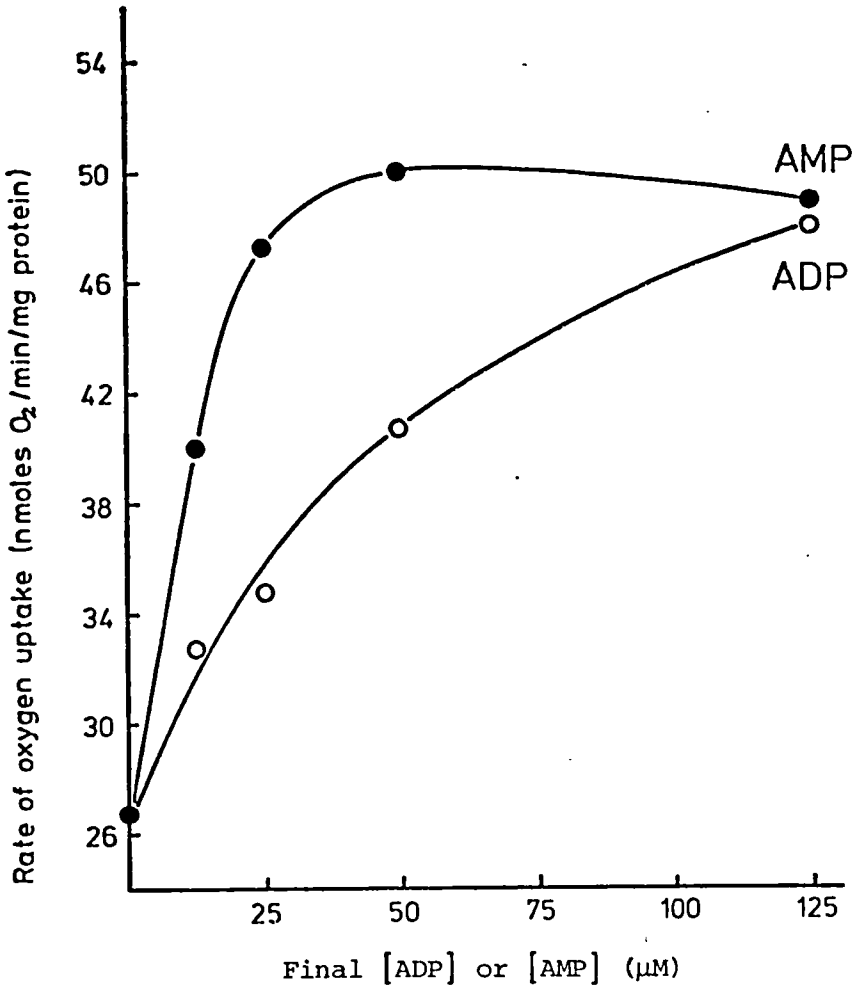


Fig. 14 Stimulation of the uncoupled rate of oxidation of pyruvate-plus-malate by different concentrations of adenine nucleotides (AMP or ADP)

The rates of oxygen uptake were calculated from oxygen electrode traces. Mitochondrial protein (1 mg) was suspended in 1 ml reaction volume; the substrate added was pyruvate (15 mM) plus malate (1 mM) and 170 μg TPP. The uncoupler FCCP (2×10^{-7} M) was added 1 min after the substrate, and appropriate amounts of ADP (o) or AMP (•), as solutions of their potassium salts, were added 1 min after the uncoupler. The rate in the presence of uncoupler and absence of adenine nucleotide was taken to be that immediately before adding the nucleotide.

TABLE 2 The influence of AMP on the uncoupled rate of oxidation of various substrates

Substrate	Rate of oxygen uptake nmoles O_2/min/mg protein		
	-FCCP	+FCCP	+FCCP + AMP
1 mM NADH	49.2	174.4	174.4
10 mM succinate	60	186	186
15 mM pyruvate + 1 mM malate + 170 µg TPP	22	26.2	50.3
10 mM 2-oxoglutarate + 170 µg TPP	7.7	11.6	57.9
10 mM 2-oxoglutarate + 170 µg TPP + 0.5 mM malonate	6.8	6.8	27
10 mM citrate	15	26	39
10 mM citrate + 1 mM arsenite	-	26	39
100 mM malate + 170 µg TPP	12	29	57
100 mM malate + 170 µg TPP + 1.5 mM arsenite	12	18	34.7

Substrates at the specified final concentrations were added to Jerusalem artichoke mitochondria suspended in 1.0 ml of reaction mixture; where indicated, thiamin pyrophosphate (TPP) or inhibitors were added before the reaction was started. In each assay 1 mg of mitochondrial protein was used, except when measuring the oxidation of succinate when 0.4 mg was used. FCCP (2×10^{-7} M) was added 1 min after the substrate and AMP (75 µM) was added 1 min after the FCCP. When NAD^+-linked substrate was used the rate of oxygen uptake in the absence of FCCP or in the presence of FCCP and AMP was approximately linear. The rate in the presence of FCCP alone often decreased with time and the rate quoted was that obtained immediately before the addition of AMP.

endogenous nicotinamide-nucleotide pool were supplied, and where the uncoupler failed to stimulate the oxygen uptake. With α-oxoglutarate, it was possible that the requirement for adenine nucleotide was directly concerned with substrate-level phosphorylation; this was not the case for other substrates.

(b) The effect of oligomycin

The investigator's first reaction to the results in Table 2 was to conclude that the uncoupler FCCP was not effective enough to completely uncouple oxidative phosphorylation in the presence of NAD^+-linked substrates, although the result in Fig. 10 proved that FCCP was equally effective in abolishing the electrochemical potential across the mitochondrial membrane in the presence of pyruvate-plus-malate or succinate, and therefore phosphorylation of adenine nucleotides resulted in the stimulation of oxygen uptake.

Whether the adenine nucleotide acted as the phosphate acceptor in the presence of FCCP could be resolved by determining its sensitivity to oligomycin (Lardy *et al.*, 1958 & 1964; Wiskich & Bonner, 1963; Stoner & Hanson, 1966). It was found that the stimulation by adenine nucleotides in the presence of the uncoupler was insensitive to oligomycin, whereas that without the uncoupler (i.e., state-3 respiration) was inhibited by oligomycin (Fig. 15). This result clearly indicated that the stimulation of respiration by adenine nucleotides in the presence of FCCP was not the result of oxidative phosphorylation, and that FCCP was effective in uncoupling oxidative phosphorylation in Jerusalem artichoke mitochondria. Therefore, the adenine nucleotides must have another role which results in the stimulation of electron transport; this role is the subject of further investigation.

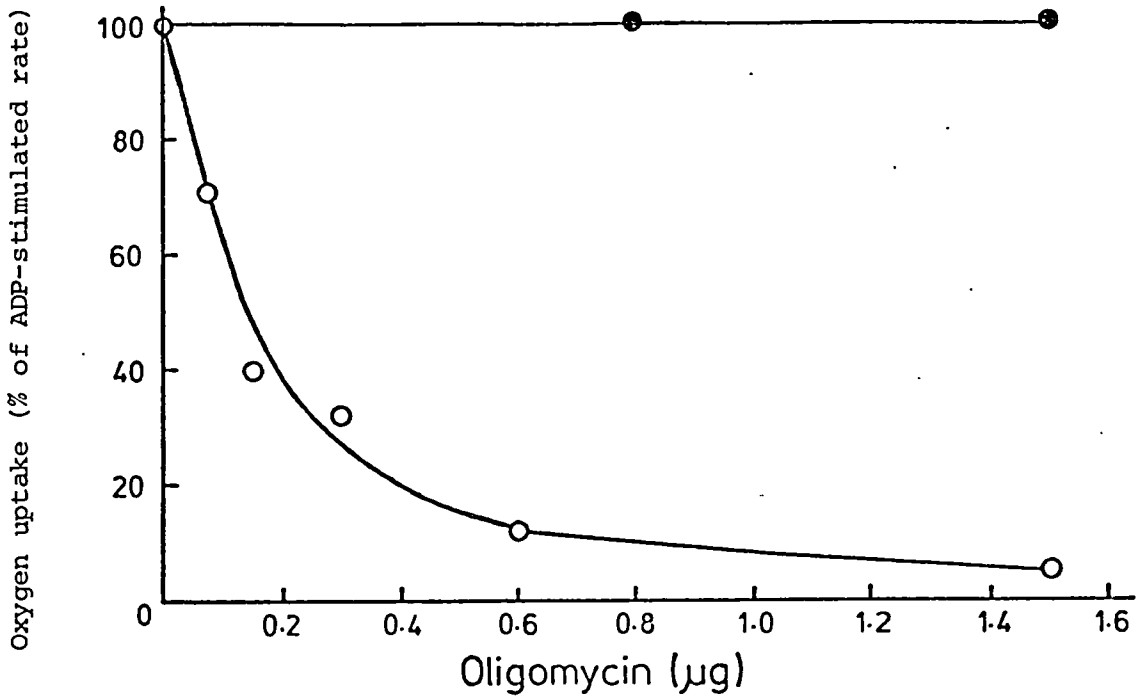


Fig. 15 Influence of oligomycin on the ADP-stimulated oxidation of pyruvate-plus-malate in the presence or absence of the uncoupler FCCP

Jerusalem artichoke mitochondria (1 mg of protein) were oxidizing pyruvate (15 mM) plus malate (1 mM) and 170 µg TPP in an oxygen electrode (1 ml reaction volume). When measuring the control treatment (o) the appropriate amount of oligomycin was added once a linear state-3 rate had been obtained from an addition of 125 nmol of ADP. When measuring the effect of oligomycin in the presence of the uncoupler (2×10^{-7} M) (•) the uncoupler was added 1 min after the substrate and ADP (125 nmol) was added 1 min after the uncoupler. The 100% values were 56 nmol of O_2/min per mg of protein for the control and 35 nmol of O_2/min per mg of protein in the presence of the uncoupler.

4. The problem of "conditioning" in plant mitochondria

So far, all the results show that the adenine nucleotides stimulate the oxidation of NAD^+-linked substrates in the presence of an uncoupling agent. There have also been observations which suggest that ADP could stimulate electron flow in the absence of uncoupling agents, other than by acting as a phosphate acceptor in oxidative phosphorylation. Raison *et al.* (1973a & b) have described the phenomenon of "conditioning" (as already described in the introduction) in plant mitochondria.

In some preparations of Jerusalem artichoke mitochondria, the phenomenon of "conditioning" was observed. When "conditioning" was apparent, the rate of oxygen uptake obtained in the presence of NAD^+-linked substrates and no added adenine nucleotide was very much lower than the subsequent rate in state 4. This is apparent in trace (a) of Fig. 16. The first addition of ADP slowly increased the oxygen uptake of mitochondria in the presence of pyruvate-plus-malate, and the stimulated rate (state 3) was less than that obtained after the second addition of ADP. If oligomycin was present (at the concentration which was shown to inhibit oxidative phosphorylation (Fig. 15)), the pre-ADP rate (hereafter referred to as the substrate rate) could be stimulated to a rate similar to that characteristic of state 4 either by AMP or ADP (Fig. 16b & c). Subsequently, an addition of FCCP (Fig. 16b) could bring about an immediate increase of respiration to the same level as that in state 3.

When succinate or exogenous NADH was supplied as the substrate, the substrate rate was not different from the rate in state 4, and neither AMP nor ADP had any effect when added after oligomycin.

It is apparent that "conditioning" may be the result of the

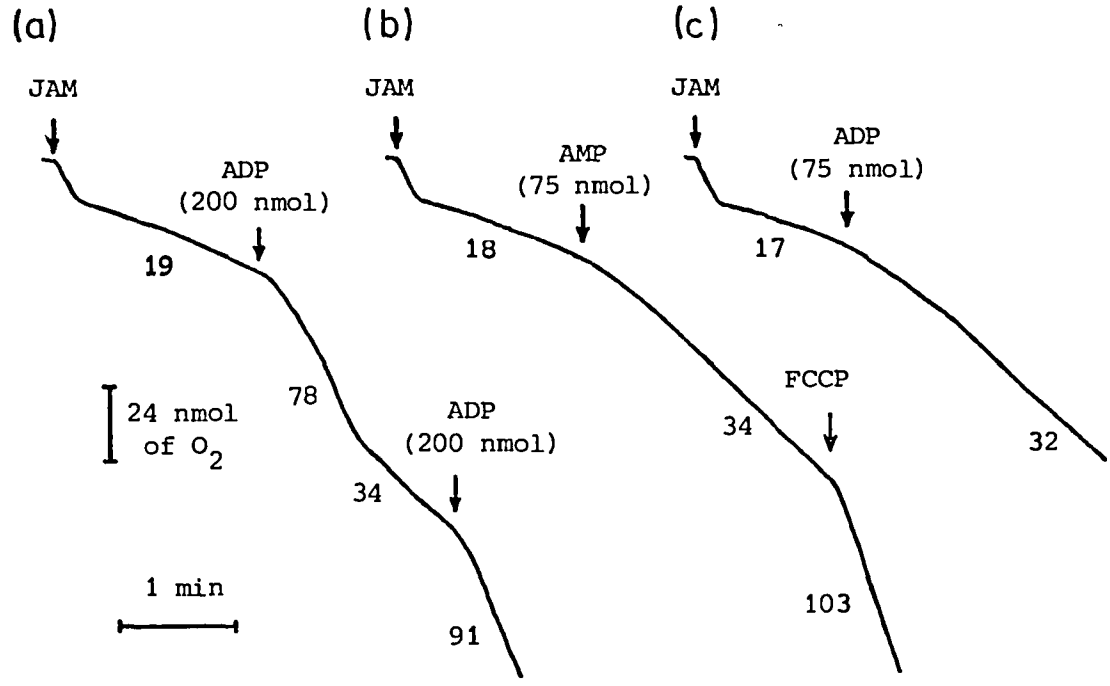


Fig. 16 Influence of adenine nucleotides on the rate of oxidation in the presence of oligomycin and absence of FCCP

Jerusalem artichoke mitochondria (JAM) (1 mg of protein) were added to 1.0 ml of reaction mixture containing 15 mM pyruvate plus 1 mM malate and 170 µg TPP in an oxygen electrode. Oligomycin was added in (b) and (c) at the rate of 1.2 µg per mg of protein; (a) was the control. Respiration was then stimulated by adding either ADP or AMP as indicated. The concentration of FCCP in trace (b) was 2×10^{-7} M. The values under the traces are the rates of oxygen consumption in nmol/min per mg of protein.

ability of adenine nucleotides to stimulate respiration, and this ability does not concern oxidative phosphorylation.

The next question is by what mechanism the adenine nucleotides bring about the stimulation of electron flow. There are two possibilities, namely, that the adenine nucleotides allosterically activate the NAD^+-linked dehydrogenase or that the effect is direct on a member of the electron transport chain.

Since the stimulation of respiration by the adenine nucleotides is observed when any NAD^+-linked substrate is used, allosteric activation of all the dehydrogenases is unlikely. However, control of NAD^+-linked dehydrogenases purified from animals and micro-organisms by adenine nucleotides does exist, and the possibility of similar control in Jerusalem artichoke mitochondria is discussed here.

In animals, although the activity of pyruvate dehydrogenase (EC 1.2.4.1) is inhibited by ATP, the reactivation of the enzyme is catalyzed by a Mg^{2+}-dependent phosphatase and not by ADP or AMP (Linn *et al.*, 1969). The pyruvate dehydrogenase complex from *Escherichia coli* is subject to control by several metabolic modulators, including nucleotides. Shen *et al.* (1968) reported that the *E. coli* enzyme was stimulated by AMP. Schwartz & Reed (1970) have shown that the enzyme responds to energy charge (Atkinson, 1968). Information on purified plant pyruvate dehydrogenase is lacking.

In Jerusalem artichoke mitochondria, it was necessary to include a small amount of malate and thiamin pyrophosphate (TPP) in order to observe oxygen uptake when pyruvate was used as the substrate. It was possible that the synthesis of citrate might be involved in regulating the rate of oxidation of pyruvate, therefore the activity of citrate synthase (EC 4.1.3.7) was examined. So far,

ATP has been shown to be a competitive inhibitor for acetyl-CoA in the synthesis of citrate by the citrate synthase from pig heart (Hathaway & Atkinson, 1965), rat liver (Shepherd & Garland, 1966) and lemon fruit (Bogin & Wallace, 1966); ADP and AMP have only small effects.

In this study the activity of citrate synthase of Jerusalem artichoke was found to be inhibited by ATP. At 1 mM ATP, citrate synthase activity of the dialyzed ammonium sulphate precipitate of the enzyme (prepared as described by Bogin & Wallace, 1969) was inhibited by 34%. For the partially purified enzyme 40% inhibition was observed at 1 mM ATP and 66% at 5 mM ATP (Fig. 17). ADP inhibited the activity of citrate synthase to a lesser extent (Fig. 17), whereas AMP had hardly any effect on the enzyme. In the presence of Mg^{2+} (10 mM) the inhibition of citrate synthase by ATP (5 mM) decreased (35% inhibition instead of 66%), although Mg^{2+} itself could inhibit the enzyme activity (at 5 mM Mg^{2+} the activity of the enzyme was 87.5% of control) (see also Kosicki & Lee, 1966; Parvin, 1969).

Allosteric activation of the NAD^{+}-specific isocitrate dehydrogenase (EC 1.1.1.41) by AMP is observed in mitochondria isolated from *Neurospora crassa* (Sanwal *et al.*, 1964; Sanwal & Stachow, 1965) and yeast (Hathaway & Atkinson, 1963). In beef heart, AMP has no effect on this enzyme (Plaut & Sung, 1954), although the enzyme exhibits activation and stabilization by ADP (Chen & Plaut, 1963). Cox & Davies (1967) have shown that activation of the enzyme activity by AMP or ADP is not observed in plant mitochondria. Although ATP and other nucleoside triphosphates inhibit isocitrate dehydrogenase in swede (*Brassica napus* L.) mitochondria, the inhibition appears to be

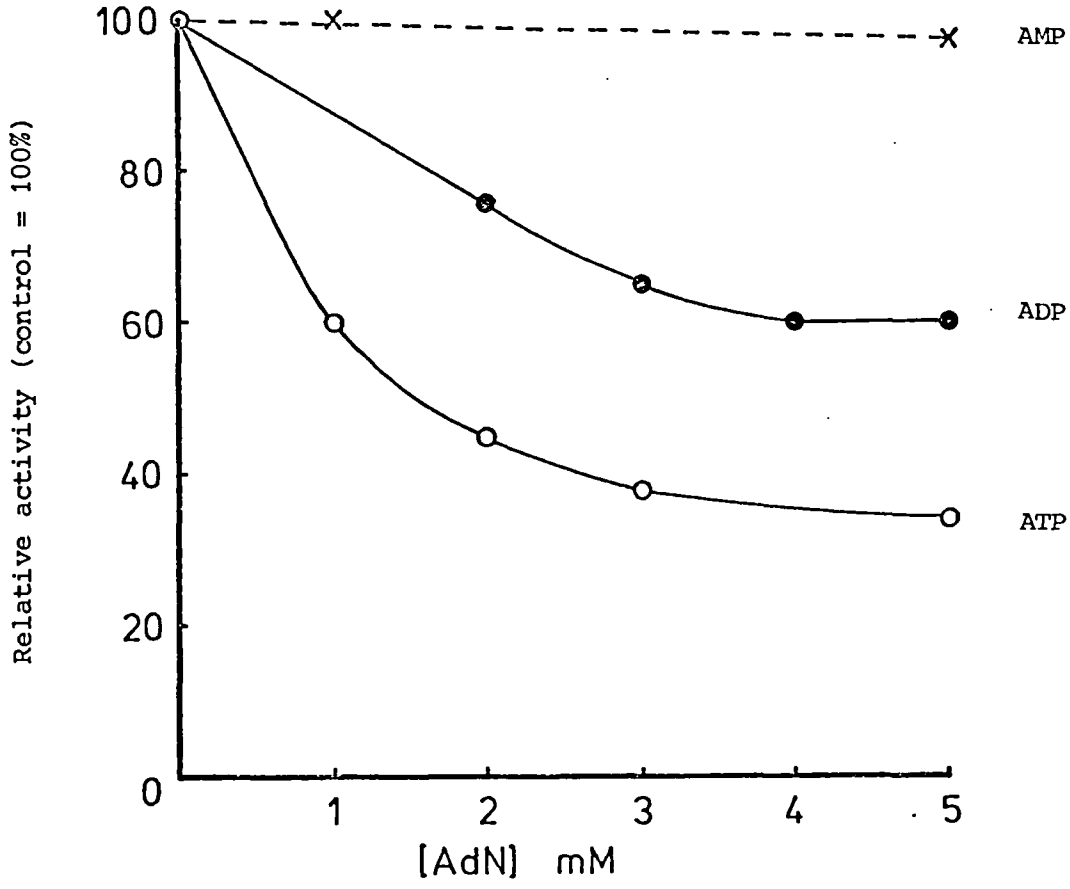


Fig. 17 Inhibition of citrate synthase by adenine nucleotides

The activity of the partially purified citrate synthase was measured spectrophotometrically at 412 nm (Methods). The reaction mixture (1.0 ml) contained 0.1 M tris-HCl pH 8.0, 0.05 mM acetyl-CoA, 0.1 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and 20 µl of the enzyme. The reaction was started by adding potassium oxaloacetate to give the final concentration of 0.2 mM. Using the molar extinction coefficient of DTNB (at 412 nm) of 13,600, the activity of the control was 2.94 nmol of CoASH released per min.

due to the formation of a complex with the activating cation such as Mn^{2+} or Mg^{2+} (Coultate & Dennis, 1969).

Adenine nucleotide activation of the α-oxoglutarate dehydrogenase (EC 1.2.4.2) from pea (Davies & Kenworthy, 1970) and cauliflower (Wedding & Black, 1971) has been reported. However, this effect and the involvement of adenine nucleotides in substrate level phosphorylation (Kaufman & Alivisatos, 1955; Palmer & Wedding, 1966) are not easily discernible.

Kuramitsu (1966) reported that AMP, ADP and ATP activated the reduction of NAD^+ by commercial pig heart malate dehydrogenase (EC 1.1.1.37) and all three adenine nucleotides inhibited the reaction catalyzed by the enzyme in the opposite direction. However, the account of this enzyme which appeared in the Worthington Enzyme Manual (1972) did not include the three adenine nucleotides in the list of effectors. No allosteric regulation by adenine nucleotides has been observed in malate dehydrogenases from other sources (Englard & Siegel, 1969; Kitto, 1969; Davies, 1969).

Direct effect of adenine nucleotides on the electron transport chain was, therefore, thought most likely and investigations into particular members of the electron transport chain were undertaken.

5. Effects of adenine nucleotides on the reduction of components of the electron transport chain

(a) The level of reduction of cytochrome b

Attempts were made to find the locus of the stimulation brought about by AMP or ADP in the electron transport chain. In plant mitochondria a single Soret (γ) peak of the b cytochromes is observed

although there are three absorption peaks in the α region at 77°K (Bonner, 1961; Baker & Lieberman, 1962; Lance & Bonner, 1968; Chance *et al.*, 1968; Storey, 1969; Passam, 1971). The b cytochrome has the usual ratio of intensities of the Soret to the α bands of approximately 10 and shows a great change in reduction levels during state 3-state 4 transition (Chance & Williams, 1956). Thus, to start with, the level of reduction of cytochrome b of Jerusalem artichoke mitochondria in the Soret band was investigated.

Measurements of the level of reduction of cytochrome b (Fig. 18) in the presence of pyruvate-plus-malate revealed an unexpected result. It was observed that the addition of pyruvate-plus-malate to Jerusalem artichoke mitochondria failed to reduce cytochrome b to any great extent (trace b), whereas in a similar experiment using rat liver mitochondria (trace a) the same substrate resulted in a marked reduction of cytochrome b. The addition of ADP to the two types of mitochondria had markedly different effects on cytochrome b (Fig. 18), whereas the rate of oxygen uptake in both types increased (result not shown here). It resulted in oxidation of cytochrome b in rat liver mitochondria, presumably because respiratory control was released, allowing electrons to flow out from cytochrome b (Chance & Williams, 1955b & 1956). In Jerusalem artichoke mitochondria the response was exactly opposite, suggesting that ADP allowed electrons to flow more readily into cytochrome b. Once the ADP had become converted into ATP by oxidative phosphorylation, respiratory control was imposed and cytochrome b became further reduced as expected. In these mitochondria, cytochrome b could not be kept reduced long after the exhaustion of ADP; the absorbance slowly decreased until the oxygen was all

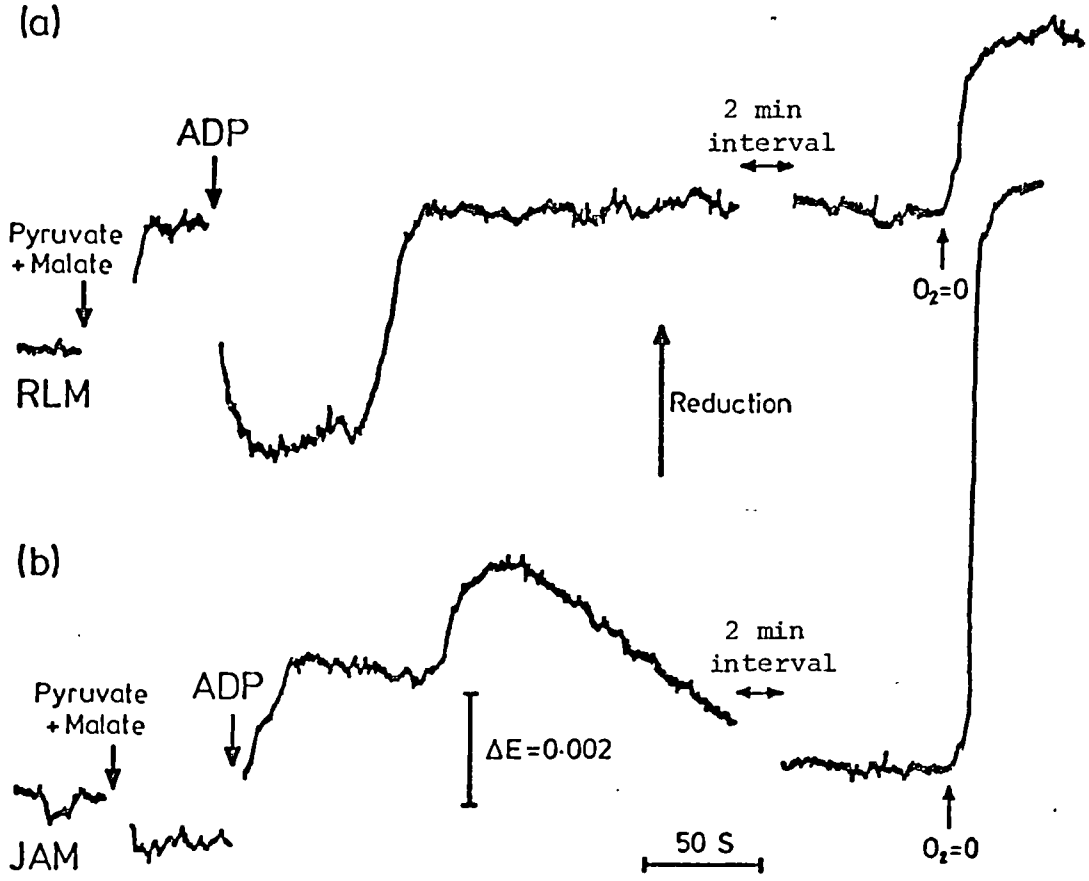


Fig. 18 Effects of ADP on the reduction of cytochrome b by pyruvate-plus-malate in rat liver and Jerusalem artichoke mitochondria

Trace (a), rat liver mitochondria (100 µl containing 3 mg of protein) were added to the cuvette containing 2.9 ml of reaction medium (0.1 M KCl, 25 mM sucrose, 10 mM $MgCl_2$, 1 mM EDTA, 510 µg of TPP, 5 mM TES and 10 mM KH_2PO_4 at pH 6.8). The additions during the experiment were pyruvate (15 mM) plus malate (1 mM) and ADP (0.5 µmol). Reduction of cytochrome b was measured in an Aminco DW-2 dual wavelength spectrophotometer at 432 nm with 410 nm as the reference wavelength (Methods).

Fig. 18 (cont)

Trace (b), Jerusalem artichoke mitochondria (100 µl containing 2.5 mg of protein) were added to 2.9 ml of the standard reaction medium (Methods) plus 510 µg of TPP. The additions and conditions of measurement were the same as for trace (a). Increased absorbance indicates reduction of cytochrome b.

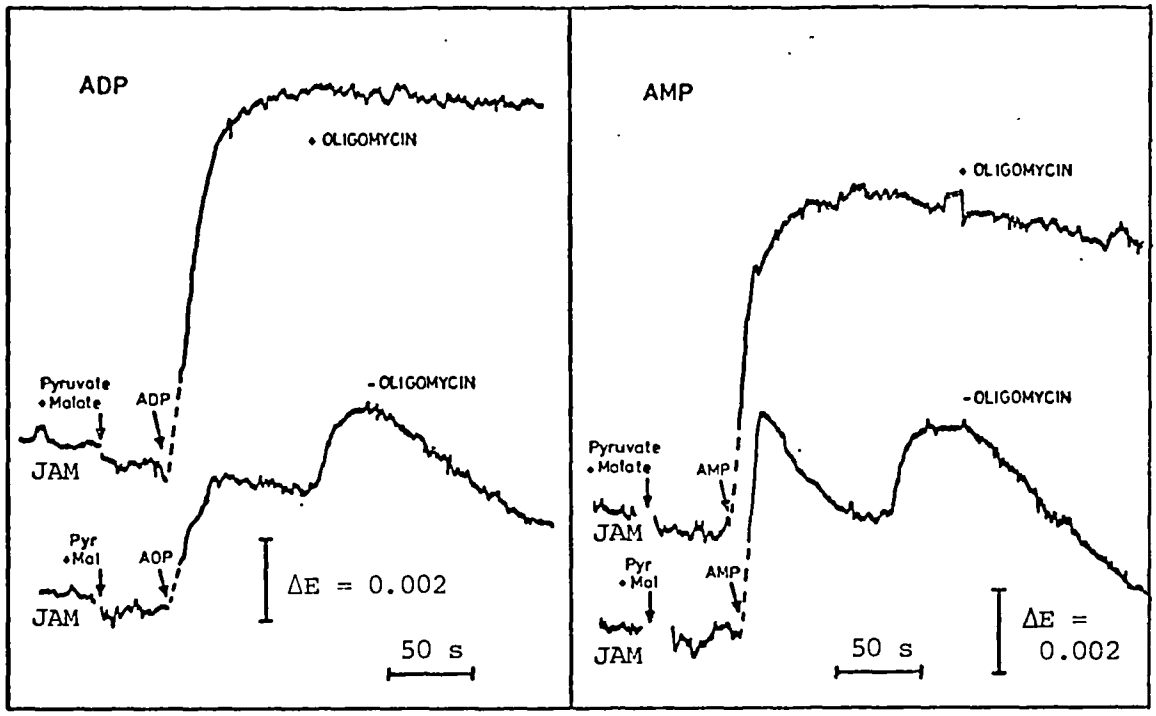


Fig. 19 Effect of oligomycin and adenine nucleotides on the reduction of cytochrome b

Jerusalem artichoke mitochondria (JAM) (100 µl containing 2 mg of protein) were added to the cuvette containing 2.9 ml of standard reaction medium plus 510 µg of TPP. The amount of oligomycin, when present, was 2.5 µg per cuvette. Additions made during the experiment were 15 mM pyruvate plus 1 mM malate and 0.5 µmol of ADP or AMP. Other conditions of assay were as described in Fig. 18 and Methods. Upward deflections indicate reduction of cytochrome b.

consumed and the cytochrome became fully reduced (Fig. 18b). In rat liver mitochondria the level of reduction of cytochrome b remained constant in state 4 until it became further reduced when all the oxygen was exhausted (Fig. 18a).

Fig. 19 shows the changes in the level of reduction of cytochrome b when either ADP or AMP was added in the absence or presence of oligomycin. The addition of both ADP and AMP resulted in the rapid reduction of cytochrome b (upper traces) when oligomycin was present in the reaction mixture, indicating that the enhanced flow of electrons into cytochrome b was independent of oxidative phosphorylation. In the absence of oligomycin the response to AMP was more complex than the response to ADP. AMP caused a more rapid and more extensive reduction of cytochrome b (lower trace on the right of Fig. 19) than did ADP (lower trace on the left). The slow oxidation to state-3 level of cytochrome b which followed this initial rapid phase of reduction could be due to the conversion of AMP into ADP by the action of the enzyme adenylate kinase. The ADP thus produced could then act as a phosphate acceptor in oxidative phosphorylation. When all ADP was phosphorylated, the level of reduction of cytochrome b again increased to state-4 level.

(b) Effect of Mg^{2+} on the reduction of cytochrome b by adenine nucleotides

When the study of the reduction of cytochrome b was repeated in the presence of oligomycin, but in the absence of Mg^{2+} (Fig. 20), only AMP was capable of promoting the rapid reduction of cytochrome b. Thus it seemed that AMP was primarily responsible for promoting electron flow into cytochrome b. The requirement of Mg^{2+}

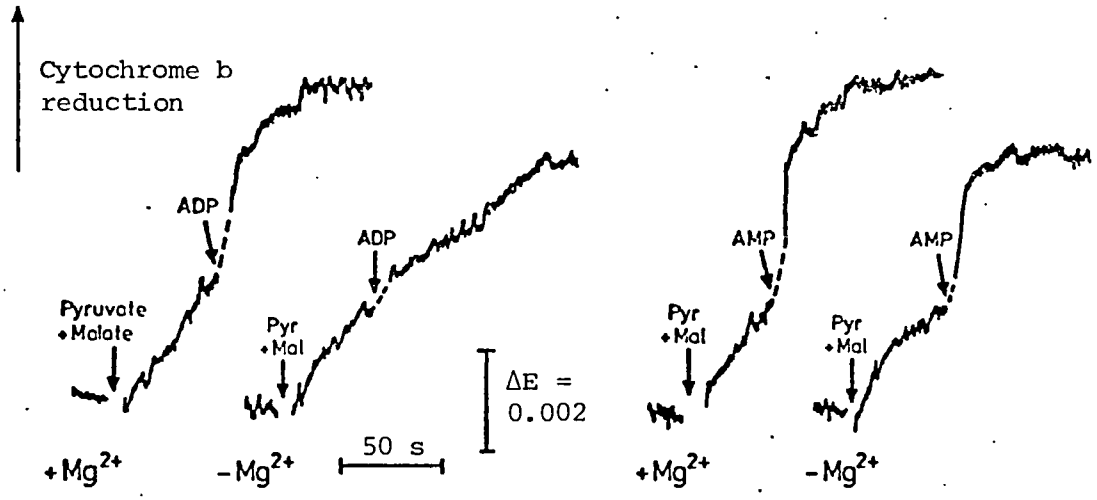


Fig. 20 Effect of Mg^{2+} and adenine nucleotides on the reduction of cytochrome b

Jerusalem artichoke mitochondria (100 µl containing 2 mg of protein) were added to the cuvette containing 2.9 ml of magnesium-free reaction medium with 510 µg of TPP and 2.5 µg of oligomycin. The concentration of Mg^{2+} when present was 2.5 mM. Additions made during the experiment were 15 mM pyruvate (Pyr) plus 1 mM malate (Mal) and ADP or AMP (0.5 µmol). Other conditions of assay were as described in Fig. 18 and Methods.

for ADP to cause the reduction of cytochrome b indicated that ADP was converted to AMP via the Mg^{2+}-requiring adenylate kinase (Kalckar, 1943) to bring about its effect.

(c) Effect of piericidin A on the reduction of cytochrome b by AMP

The observation that the addition of AMP brought about the reduction of cytochrome b in Jerusalem artichoke mitochondria but not in rat liver mitochondria led one to investigate whether the cytochrome b reduced was a normal component of the electron transport chain and by which pathway it was reduced. An experiment was set up to test whether the reduction of cytochrome b by pyruvate-plus-malate, which was enhanced by the addition of AMP, was sensitive to piericidin A. The result of this test is shown in Fig. 21; the addition of piericidin A to the cuvette, containing the mitochondria,substrate, oligomycin and AMP, led to the oxidation of cytochrome b. Hence, it was concluded that the cytochrome b reduced on the addition of AMP was a normal member of the electron transport chain and was reduced by the piericidin A-sensitive pathway.

(d) Effects of AMP on the reduction of endogenous nicotinamide-adenine dinucleotide and cytochrome b in the presence of FCCP

From the studies of the reduction of cytochrome b in Jerusalem artichoke mitochondria, it could be concluded that AMP was primarily responsible for the increased electron flow into cytochrome b, hence increased oxygen uptake, by having a direct effect on the respiratory chain in a manner which was independent of oxidative phosphoryl-

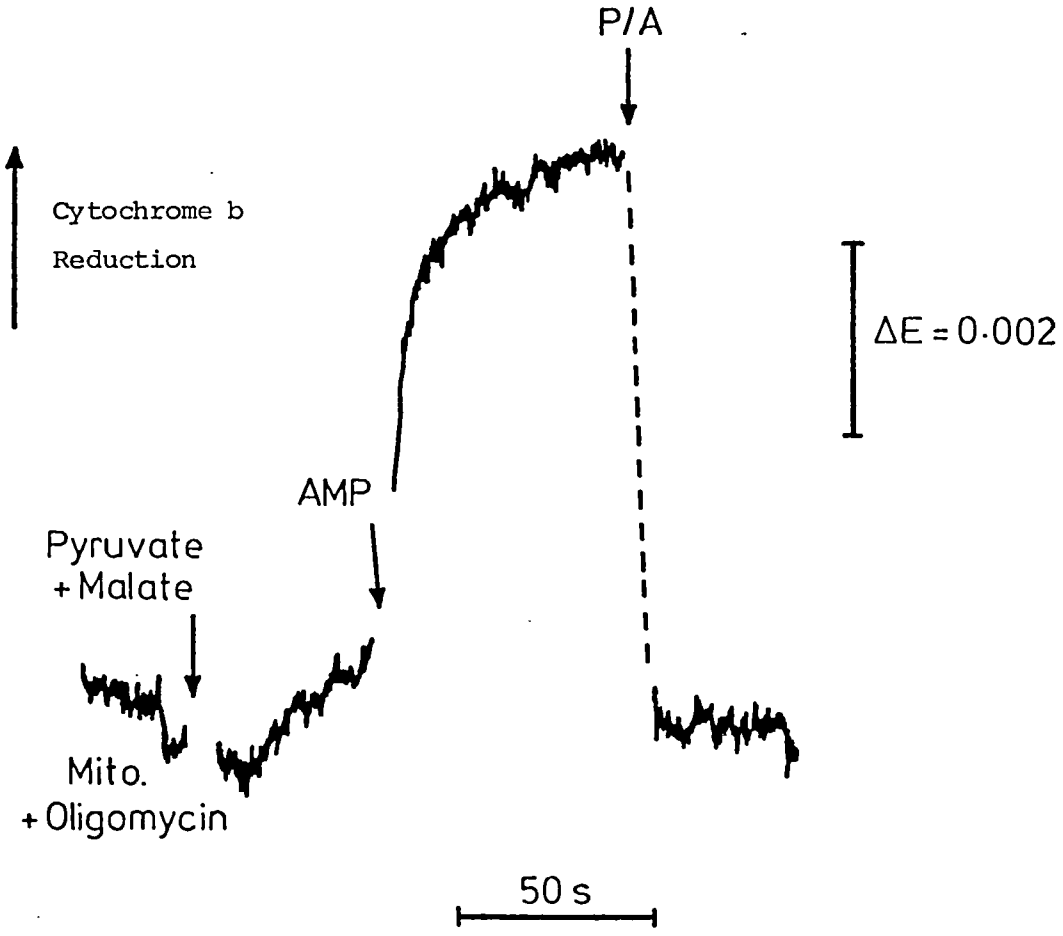


Fig. 21 Effect of piericidin A on the reduction of cytochrome b

Jerusalem artichoke mitochondria (2 mg of protein) were suspended in the standard reaction medium (total volume of 3 ml) containing 510 µg TPP and 2.5 µg oligomycin. Pyruvate (15 mM) plus malate (1 mM) was added to start the reaction; AMP (0.5 µmol) and 60 ng piericidin A (P/A) were added as indicated. Other conditions of assay were as described in Fig. 18.

ation. In order to establish the locus of AMP interaction more closely, attention was focussed on the oxidation-reduction of the mitochondrial nicotinamide-adenine dinucleotide (nicotinamide nucleotide).

Fig. 22 shows simultaneous measurements of the level of reduction of endogenous nicotinamide nucleotide and cytochrome b in the presence of FCCP and the effects of the addition of AMP. The spectrophotometric traces of both components were obtained consecutively using identical cuvettes. Since pyruvate interfered with the absorption of NADH at the wavelengths used, malate was used as the substrate and glutamate and glutamate-oxaloacetate transaminase were employed to remove oxaloacetate. This substrate system also ensured the efficient operation of malate dehydrogenase without involving the enzyme complex pyruvate dehydrogenase, so as to exclude the possibility of allosteric regulation by adenine nucleotides. The traces show that the addition of AMP resulted in the immediate reduction of cytochrome b and the oxidation of nicotinamide nucleotide indicating that AMP enhanced the electron flux from the nicotinamide nucleotide pool along the respiratory chain.

The finding that the endogenous nicotinamide nucleotide pool became oxidized on addition of AMP provided the evidence that the stimulation of respiration by AMP was not the result of an activation of any of the dehydrogenases of the NAD^+-linked substrates which would have caused a reduction of the nicotinamide nucleotide. It also ruled out the possibility that AMP activated the uptake of substrates into the mitochondria.

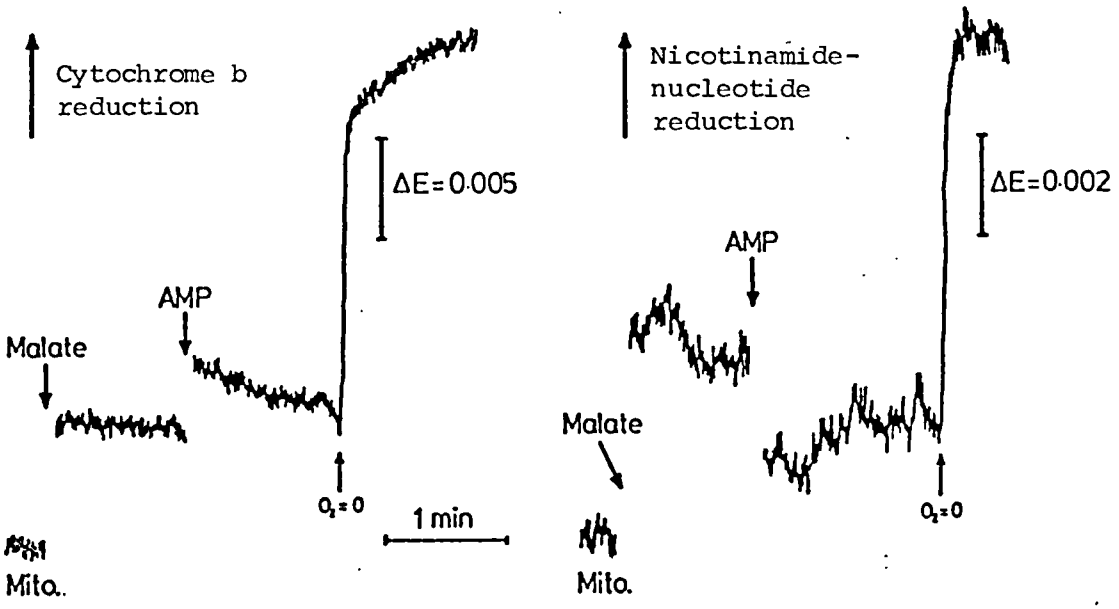


Fig. 22 Effects of AMP on the reduction of cytochrome b and endogenous nicotinamide nucleotide of Jerusalem artichoke mitochondria oxidizing malate

In 1 ml cuvettes with 1 cm light-path, mitochondria (Mito.) (2.2 mg of protein) were suspended in the standard reaction medium plus 10 mM glutamate, 20 µg glutamate-oxaloacetate transaminase, 3 µg oligomycin and 0.1 µM FCCP. Additions made during the experiment were 25 mM malate and 75 nmol AMP. The reduction of cytochrome b and endogenous nicotinamide nucleotide were followed at 432 and 340 nm, with 410 and 374 nm respectively as reference wavelengths, in an Aminco DW-2 dual-wavelength spectrophotometer (Methods). The assays were done consecutively using two identical cuvettes.

6. Location of the action of AMP

(a) Investigation of the effect of AMP in submitochondrial particles

So far, all results had indicated that AMP activates the flow of electrons between the endogenous nicotinamide nucleotide and cytochrome b, through the piericidin A-sensitive section of the electron transport chain. It was unlikely that the stimulation was brought about by a direct interaction between AMP and the endogenous NADH dehydrogenase because AMP is not actively transported into the mitochondria by the adenine nucleotide translocator (see section 1(b); Passam *et al.*, 1973; Passam & Coleman, 1975). To prove this it was necessary to ascertain whether AMP stimulated the oxidation of added NADH by submitochondrial particles, the polarity of whose membranes was the reverse of that in intact mitochondria (see section 1(c)) thus exposing the endogenous NADH dehydrogenase to the incubating medium.

Jerusalem artichoke submitochondrial particles, prepared as described in the Methods section, were able to oxidize NADH as depicted by the oxygen electrode trace in Fig. 23. In these submitochondrial particles there appeared to be a residual control of electron transport by the phosphorylating system as FCCP could stimulate their oxygen consumption. However, the addition of AMP did not affect the oxidation of NADH in the presence of FCCP.

The failure of AMP to stimulate the oxidation of NADH by the submitochondrial particles in the presence of FCCP indicated that there was no stimulatory site for AMP on the endogenous NADH dehydrogenase in this preparation. Since it is believed that AMP

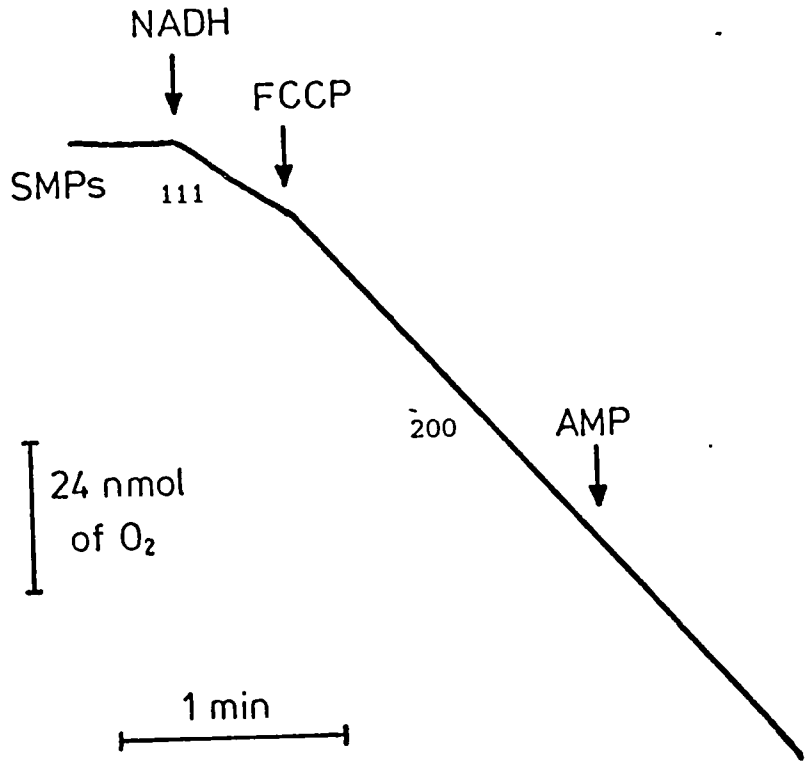


Fig. 23 Oxidation of NADH by submitochondrial particles

Jerusalem artichoke submitochondrial particles (SMPs), prepared as described in Methods, were suspended in the standard reaction medium (0.22 mg of protein in 1 ml) in an oxygen electrode. The concentrations of NADH, FCCP and AMP used were 1 mM, 0.2 µM and 75 µM respectively. The numbers under the trace are the rates of oxygen uptake in nmol/min per mg of protein.

can cross the outer mitochondrial membrane but not the inner membrane (Klingenberg & Pfaff, 1966; Pfaff, Klingenberg, Ritt & Vogell, 1968; Passam & Coleman, 1975), it was envisaged that the site of action of AMP was on the outer surface of the inner mitochondrial membrane. Therefore AMP was unable to stimulate respiration in submitochondrial particles because its site of action was shielded from the incubating medium by the change in membrane polarity caused by sonication.

Alternatively, one might argue that the stimulation of respiration by AMP occurred only in intact mitochondria (if AMP could somehow get across the inner mitochondrial membrane); sonication altered the natural and highly organized structure of the NADH dehydrogenase so that its interaction with AMP was prevented.

(b) Effect of bongkrekic acid on the stimulation of respiration by AMP

The failure of AMP to stimulate the respiration of submitochondrial particles in the presence of FCCP (described in the previous section) did not afford any conclusion concerning the site of action of AMP. In order to establish whether the site of action of AMP was on the outer surface of the inner mitochondrial membrane, it was necessary to use a different approach.

Bongkrekic acid, a product of the growth of the bacterium *Pseudomonas cocovenenans* on defatted press cake of coconut (bongkrek), has been shown to inhibit state-3 respiration of artichoke mitochondria (Passam *et al.*, 1973; Passam & Coleman, 1975) presumably by blocking the translocation of ADP into the mitochondria as in the case of animal mitochondria (Henderson & Lardy, 1970;

TABLE 3 Effect of bongkrekic acid on the stimulation of mitochondrial respiration by AMP

Substrate		O_2 uptake (nmol/min per mg of protein)						
		Expt. 1				Expt. 2		
		Substrate rate	State 3 (+AMP)	State 4	Uncoupled (+FCCP)	Substrate rate	Uncoupled rate	Uncoupled + AMP
1.2 mM NADH	-BA	75	156	85	188	75	206	206
	+BA	61	61	-	160	60	157	154
10 mM Succinate	-BA	73	177	75	179	73	171	171
	+BA	57	57	-	160	57	162	160
25 mM Malate + 10 mM glutamate + 20 µg glutamate-oxaloacetate transaminase	-BA	52	83	43	94	55	85	97
	+BA	37	43	-	81	36	40	62
15 mM Pyruvate + 1 mM malate + 170 µg TPP	-BA	18	57	26	54	18	22	57
	+BA	18	28	-	41	17	26	63

TABLE 3 (cont.)

Jerusalem artichoke mitochondria were suspended in reaction medium containing 0.3 M sucrose, 5 mM TES, 5 mM potassium phosphate and 2.5 mM $MgCl_2$ at pH 6.8 in a 1 ml oxygen electrode for 10 min with or without bongkrekic acid (BA) at 2.5 nmol/mg of protein. The substrates were added to start respiration. In Expt. 1, 100 nmol of ADP was added to bring about state-3 rates and the uncoupler FCCP (1×10^{-7} M) was added in state 4. In Expt. 2, AMP was added 1 min after the uncoupler, and the uncoupled rates reported are those immediately before additions of AMP. For NADH or succinate oxidation, 0.43 mg of mitochondrial protein was used per assay, 0.86 mg for malate oxidation and 1.72 mg for pyruvate oxidation. The substrate rate is that produced when substrate was added to the mitochondria before the addition of AMP.

Henderson, Lardy & Dorschner, 1970; Klingenberg, Grebe & Heldt, 1970). This inhibitor was therefore employed as a tool to examine whether AMP needed to cross the mitochondrial membrane before it could stimulate the flow of electrons from the endogenous NADH pool.

The data in Table 3 show that the incubation of the mitochondria with bongkrekic acid prevented the oxidation in state 3 of all substrates as the result of addition of AMP (Expt. 1). Since earlier results (Fig. 4; Passam & Coleman, 1975) have indicated that oxidative phosphorylation of AMP in Jerusalem artichoke mitochondria is achieved through its conversion to ADP by adenylate kinase in the intermembrane space and the translocation of ADP into the matrix, it was likely that bongkrekic acid inhibited the state-3 oxidation by blocking the translocation of ADP produced from AMP.

The stimulation of electron flow from the endogenous NADH pool by AMP could be differentiated from oxidative phosphorylation by the finding that it was insensitive to bongkrekic acid. When bongkrekic acid was present, AMP could still increase the slow substrate rates during the oxidation of malate and pyruvate to the rates characteristic of state 4 (Expt. 1). In addition, if FCCP was present as well as bongkrekic acid, AMP could only stimulate the respiration of NAD^+-linked substrates, but not NADH or succinate (Table 3, Expt. 2). The results favoured the suggestion that AMP did not have to enter the mitochondrial matrix to stimulate the flow of electrons between NADH and cytochrome b.

(c) Effects of atractylate on the respiration of Jerusalem artichoke mitochondria

Passam *et al.* (1973) reported that atractylate, which normally acts as an inhibitor of adenine nucleotide translocation in animal

mitochondria, was not effective in Jerusalem artichoke mitochondria. Subsequently, there have been other reports, concerning the inhibition by atractylate of plant mitochondrial adenine nucleotide translocators (Jung & Hanson, 1973; Earnshaw & Hughes, 1976; Wickes & Wiskich, 1976; Vignais *et al.*, 1976), which are in conflict with the results of Passam *et al.* (1973).

In pursuit of other evidence to support the observation obtained during the studies using bongkrekic acid, it was necessary to establish whether atractylate could affect the respiration of Jerusalem artichoke mitochondria. By varying the concentration of atractylate (0 - 200 μM) in the assay medium, and measuring the stimulation of oxygen uptake brought about by different amounts of ADP during the oxidation of NADH, it was found that atractylate inhibited the stimulation of oxygen uptake by ADP. The inhibition was competitive with respect to the concentration of ADP, as shown by the Dixon plot (Dixon, 1953; Dixon & Webb, 1964) in Fig. 24. From the Dixon plot the K_i fell in the range of 1 - 2 μM, which was 10 times as great as that found in rat liver mitochondria (0.1 μM) (Vignais, Duee, Vignais & Huet, 1966).

The next attempt was to show that the stimulation of oxygen uptake by AMP in the presence of FCCP still took place in the presence of atractylate. However, the most unexpected results were obtained. While FCCP hardly stimulated the oxygen uptake in the control (Fig. 25a), it resulted in an immediate increase in the oxidation of pyruvate by the mitochondria in the presence of atractylate (Fig. 25b & c). The FCCP-stimulated respiration in the presence of Mg^{2+} or EDTA (no Mg^{2+}) at various concentrations of atractylate are shown in Table 4. Apparently higher rates of un-

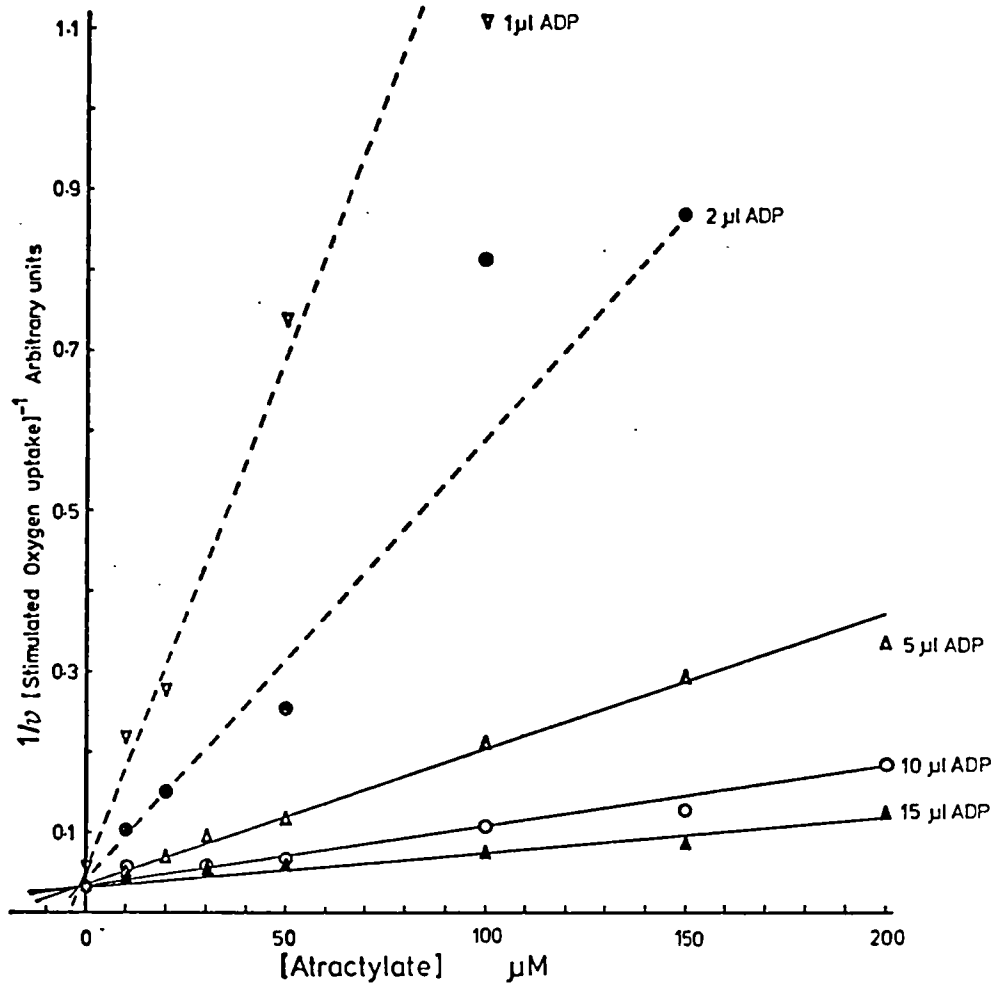


Fig. 24 The Dixon plot (Dixon, 1953) of ADP-stimulated oxygen uptake by Jerusalem artichoke mitochondria oxidizing NADH in the presence of atractylate

In an oxygen electrode, the mitochondria were oxidizing 1 mM NADH in the standard reaction medium (0.58 mg of protein in 1 ml) in the presence of 10 mM glucose, 0.3 mg hexokinase and the appropriate amount of potassium atractylate. Stimulation of oxygen uptake was brought about by adding the solution of ADP (containing 18.9 mM ADP and 2.35 mM AMP). The stimulated rate of oxygen uptake (v) was the rate (per min) of the decrease in oxygen concentration in the oxygen electrode (per cent) in state 3 minus the substrate rate.

coupled respiration were obtained at higher concentrations of atractylate. Subsequently the stimulation by AMP became less.

The results in Fig. 25 and Table 4 show two interesting facts. Firstly, the addition of atractylate enables a subsequent addition of FCCP to stimulate respiration to a very high rate. In this respect atractylate seems to be able to substitute for AMP (see Fig. 16b), probably by acting at the AMP-binding site which causes the stimulation of electron flow. At present there is no concrete evidence to disprove the alternative possibility that the site of action of atractylate is different from that of AMP, but somehow it results in the same effect - an increase in respiration on the addition of FCCP. In this case the reason that the further increase due to the addition of AMP is reduced by higher concentrations of atractylate (Table 4) could be that some other step in the dehydrogenation of endogenous NADH, which provides the electron flux, becomes the rate-limiting step. Therefore it is not possible to see the additive affect of AMP and atractylate. However, this second proposition seems unattractive because it unnecessarily complicates matters by bringing in the extra binding site for atractylate for which there is no definite evidence.

The second point of interest is that when EDTA was included in the reaction medium, together with atractylate, AMP seemed unable to increase the rather high respiration rate brought about by FCCP (Fig. 25c and Table 4). Although the oxygen electrode traces in Fig. 25 show that the absence of Mg^{2+} (+EDTA) did not reduce the overall rate in the presence of both atractylate and FCCP (before the addition of AMP), it was possible that the respiratory activities of the mitochondria became limited upon the extraction of Mg^{2+}.

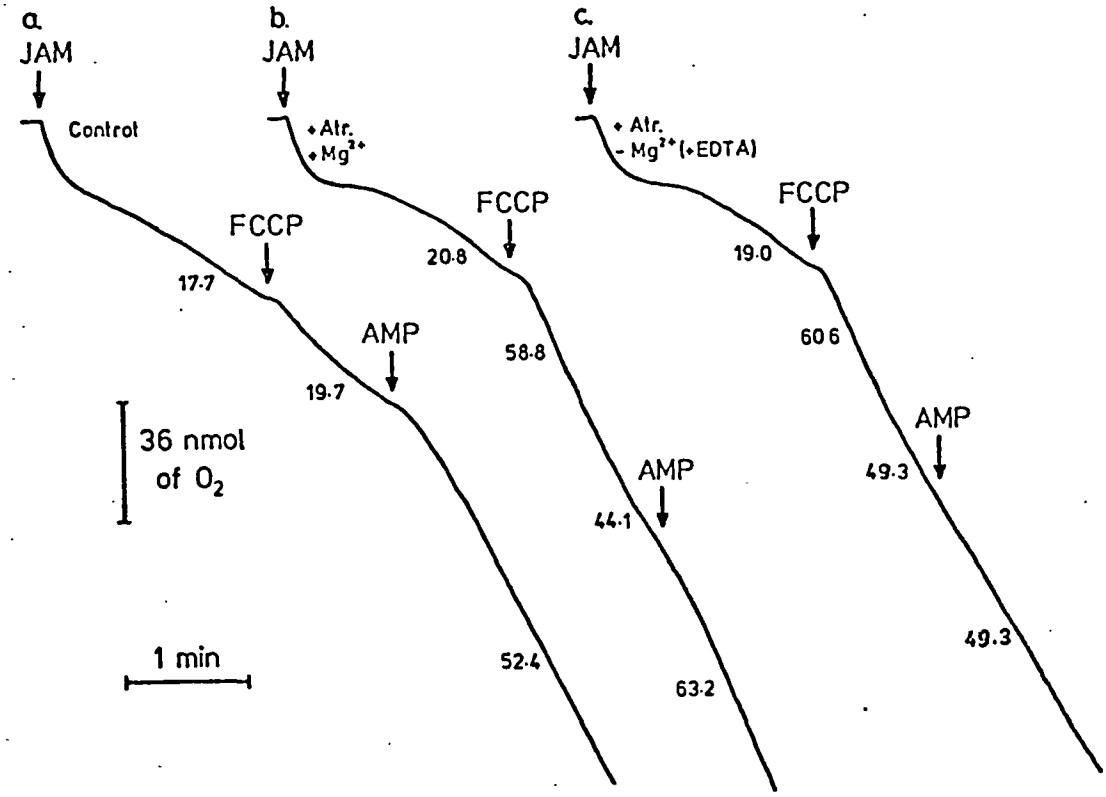


Fig. 25 Effects of atractylate on the response of mitochondria to additions of FCCP and AMP

Jerusalem artichoke mitochondria (JAM) (1.43 mg of protein) were added to 0.95 ml of the standard reaction medium (a & b) containing 15 mM pyruvate, 1 mM malate and 170 µg TPP. In b & c, atractylate (200 nmol) was also included; in c the reaction mixture contained 5 mM EDTA but no Mg^{2+}. FCCP (2×10^{-7} M) and AMP (75 nmol) were added where indicated. The numbers under the oxygen electrode traces are the rates of oxygen uptake in nmol/min per mg of protein.

TABLE 4 Effects of Mg^{2+} and atractylate on the stimulation of non-phosphorylating respiration by AMP

Atractylate present nmol	Rates of oxygen uptake in the presence of FCCP nmol O_2/min per mg of protein					
	+ Mg^{2+} - EDTA			- Mg^{2+} + EDTA		
	Before AMP	After AMP	Increase (caused by AMP)	Before AMP	After AMP	Increase (caused by AMP)
0	19.7	52.4	32.7	24.2	46.7	22.5
50	34.6	58.0	23.4	38.9	43.3	4.4
100	not measured			53.6	58.0	4.4
200	44.1	63.2	19.1	49.3	49.3	0

FCCP (2×10^{-7} M) was added to Jerusalem artichoke mitochondria (1.43 mg of protein) oxidizing pyruvate (plus malate) in the presence or absence of Mg^{2+} (2.5 mM) and various amounts of atractylate. The addition of AMP (75 nmol) was made 1 min after FCCP. The rates of oxygen uptake before and after AMP additions were calculated from the oxygen electrode traces. In the reaction medium without Mg^{2+}, 2.5 mM EDTA was present. Other conditions of assay were as described in Fig. 25.

Therefore, further stimulation of electron transfer by AMP would not be feasible after the electron flux had been enhanced by atractylate. At present it is not known in what way the presence of EDTA in the reaction medium may affect the respiratory activities of the mitochondria. The problem of the requirement of divalent cations, which could be removed by EDTA, for the activity of the externally located NADH dehydrogenase (Hackett, 1961; Hanson *et al.*, 1965; Miller *et al.*, 1970; Coleman & Palmer, 1971) was not involved in this experiment. Coleman & Palmer (1971) showed that the presence of a chelating agent in the reaction medium did not affect the oxidation of succinate or malate by Jerusalem artichoke mitochondria. In this study the synthesis of ATP, which requires the presence of Mg^{2+} (Kielley & Kielley, 1953; Bronk & Kielley, 1957; Linnane, 1958; Penefsky *et al.*, 1960; Racker, 1961), was not involved.

An alternative explanation for the failure of AMP to increase the rate of uncoupled respiration in the presence of high concentrations of atractylate is that atractylate may be able to bind more tightly at the AMP-binding site if all traces of Mg^{2+} are removed by EDTA, thus preventing the binding of AMP at that site.

In the presence of EDTA, although ADP is incapable of bringing about the stimulation of electron flux (see Fig. 20), there is evidence that ADP can interfere with the stimulation brought about by AMP. Fig 26. shows the inhibition of the AMP-stimulated non-phosphorylating respiration by ADP in the presence of EDTA but without Mg^{2+}. It was also found that under these conditions ATP could bring about a similar inhibition (result not shown). This added information may not directly support the idea that atractylate

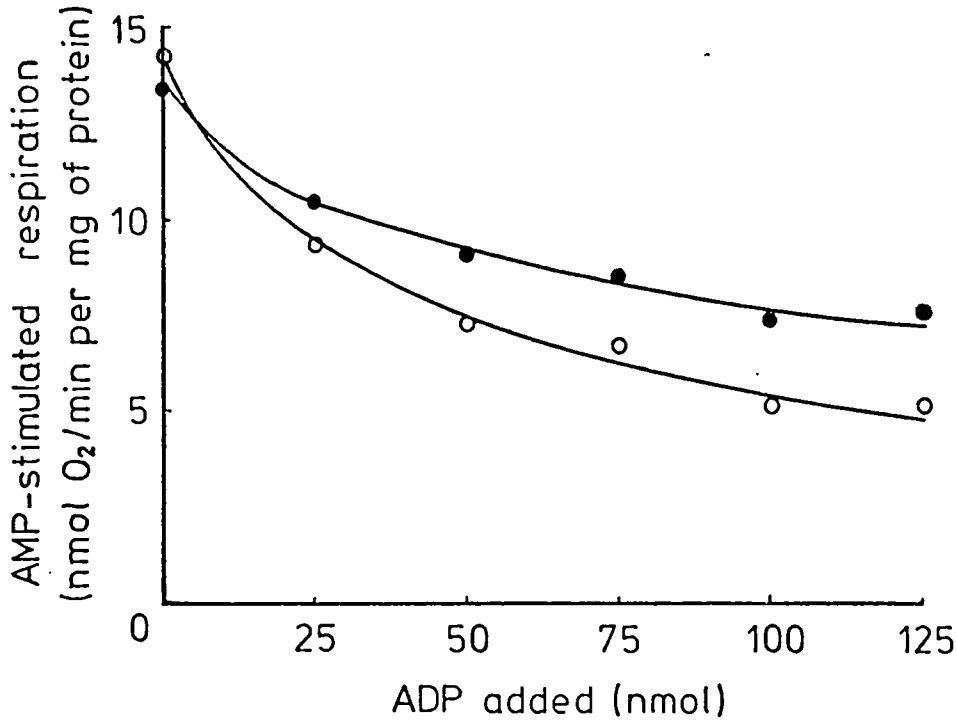


Fig. 26 The interference of the AMP-stimulated respiration by ADP in the presence of EDTA

The reaction mixture consisted of the standard reaction medium (minus Mg^{2+}), 15 mM pyruvate, 1 mM malate, 170 µg TPP, 5 µg oligomycin and 5 mM EDTA. To start the reaction, Jerusalem artichoke mitochondria (2.1 mg of protein) were added to the reaction mixture bringing the total volume in the electrode to 1 ml. Appropriate amounts of ADP were added to the electrode when a constant rate of respiration was established (about 3 min after the start of respiration). AMP was added immediately after ADP. The increased rates of oxygen uptake due to the addition of 75 nmol (o) and 125 nmol (●) of AMP are shown.

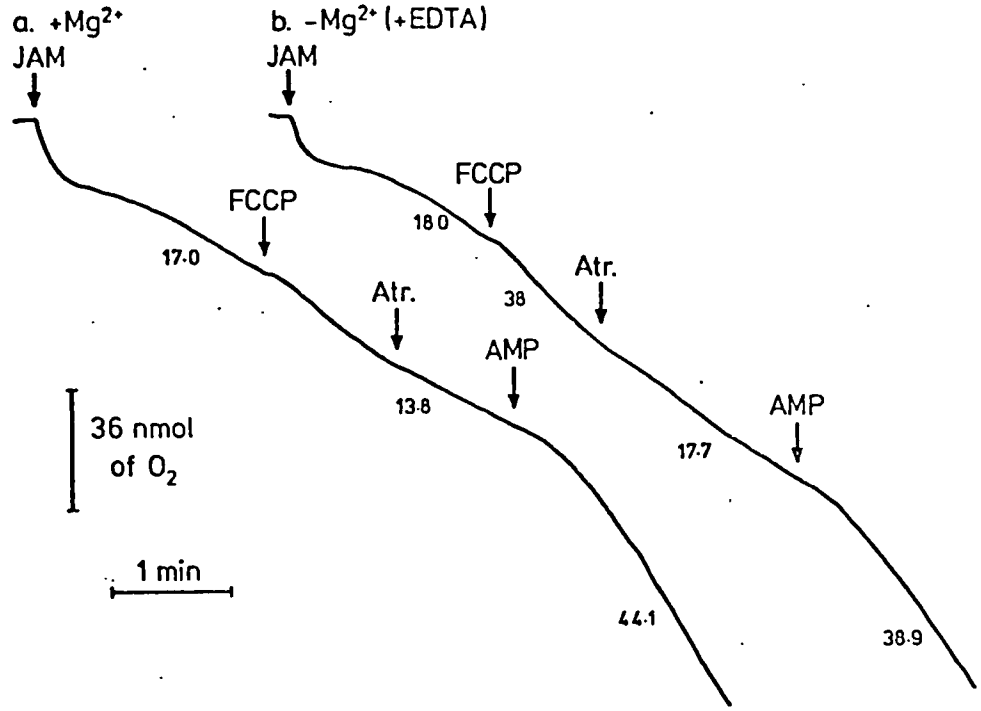


Fig. 27 Response of the uncoupled respiration to atractylate and AMP

Conditions of assay were as described in Fig. 25, except that FCCP (2×10^{-7} M) was added before atractylate (100 nmol) and AMP (75 nmol). Mg^{2+} (2.5 mM) was present in a, none in b. EDTA (5 mM) was included in b.

may also interfere with AMP in the presence of EDTA, but it demonstrates that inhibition of action of AMP by other structurally related substances is possible.

Fig. 27 shows that if atractylate was added to the mitochondria after FCCP, it did not stimulate the non-phosphorylating respiration regardless of the presence of Mg^{2+}. Here, AMP was capable of increasing the oxygen uptake. This result suggests that FCCP, if added before atractylate, probably alters the conformation of the AMP-binding site so as to prevent the action of atractylate but not AMP.

7. Rate of respiration in relation to the sequence of addition of AMP and FCCP

The discovery that the sequence of addition of atractylate and FCCP affected the final rate of respiration (Figs. 25 and 27) led to an investigation as to whether the AMP-stimulated non-phosphorylating respiration would be subject to a similar influence. Fig. 28 shows oxygen electrode traces of the oxidation of pyruvate by Jerusalem artichoke mitochondria and the result of adding FCCP. It is apparent that the final rate of respiration brought about by adding AMP 1 min after FCCP (trace a) was faster than that if AMP was added 5 min after FCCP (trace b). This result suggests that a delay in adding AMP after FCCP resulted in a low rate of respiration.

The rates of uncoupled respiration shown in Table 5 were obtained from an experiment in which the effect of the sequence of adding AMP and FCCP on the final rate of respiration was investigated.

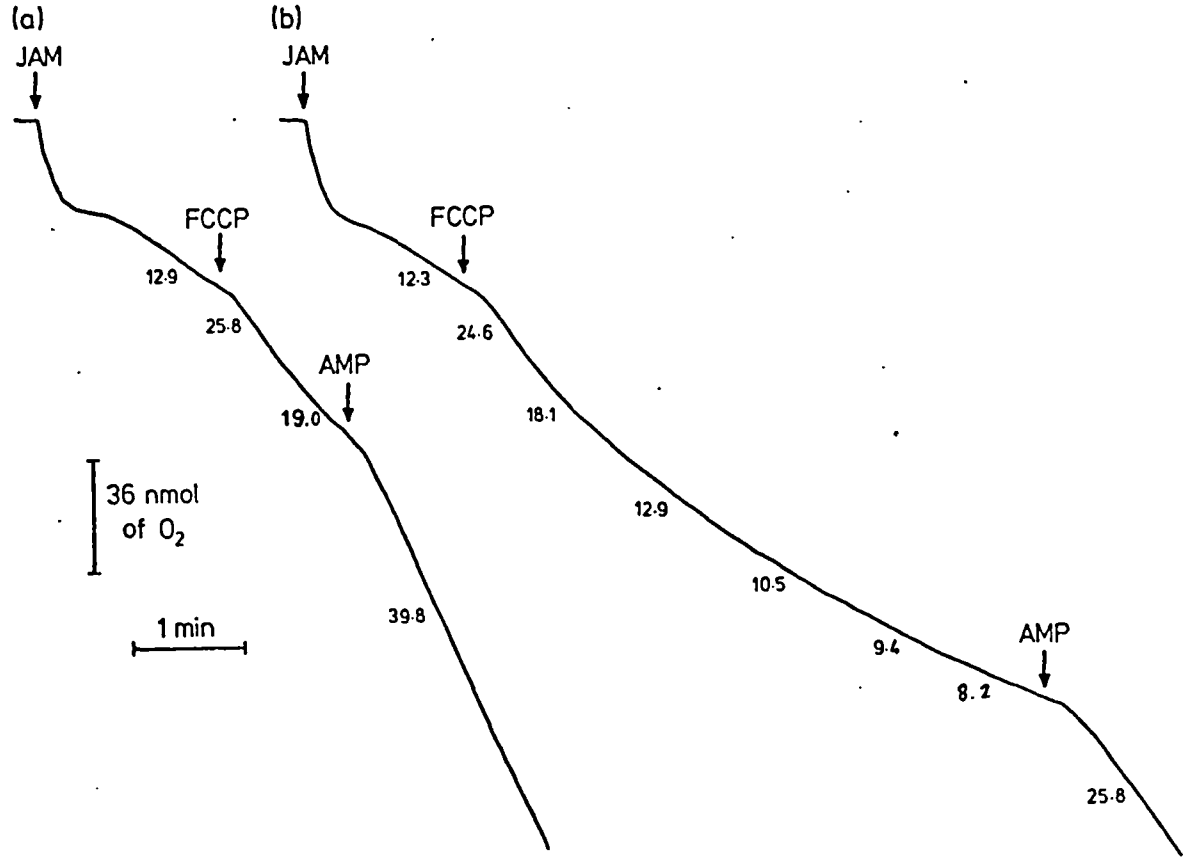


Fig. 28 The effect of the delay in adding AMP after FCCP on the final rate of respiration

FCCP (2×10^{-7} M) was added 100 s after the addition of Jerusalem artichoke mitochondria (0.1 ml containing 2.05 mg of protein) into 0.9 ml of the standard reaction medium plus 15 mM pyruvate, 1 mM malate and 170 µg of TPP, in an oxygen electrode. AMP (75 nmol) was added 1 min (a) and 5 min (b) after FCCP. The numbers under the traces are the rates of oxygen uptake in nmol/min per mg of protein.

TABLE 5 Final rates of oxygen uptake of mitochondria according to the sequence of additions of FCCP and AMP (Analysis of variance)

	Rate of O_2 consumption (% of total O_2/min)		
	Group A (AMP added 1 min after FCCP)	Group B (AMP added immediately after FCCP)	Group C (AMP added before FCCP)
	42.0	41.0	48.0
	37.5	41.0	51.0
	38.0	42.5	50.0
	39.0	41.0	48.0
	36.0	43.0	45.5
	-	42.0	44.5
Total	$T_1 = 192.5$	$T_2 = 250.5$	$T_3 = 287.0$
Mean	$\bar{X}_1 = 38.5$	$\bar{X}_2 = 41.75$	$\bar{X}_3 = 47.83$
	$n_1 = 5$	$n_2 = 6$	$n_3 = 6$

Grand Total $= T_1 + T_2 + T_3 = G = 730$

Total number of observations $N = 17$

Correction factor $C = \frac{G^2}{N} = \frac{(730)^2}{17} = 31347.06$

Total sum of squares about the mean $= SX^2 - C = 305.94$

Total sum of squares between groups $= \frac{(T_1)^2}{n_1} + \frac{(T_2)^2}{n_2} + \frac{(T_3)^2}{n_3} - C$

$= 250.74$

TABLE 5 (cont.)

Source of variation	Sum of Squares	Degree of Freedom	Mean Square
Total	305.94	16	
Between groups	250.74	2	125.37
Residual	55.20	14	3.94

Variance ratio. $F = \frac{125.37}{3.94} = 31.82$

The F ratio for the 1% point is 6.51, so the differences between the three groups were significant.

Oligomycin was included in each assay. AMP was added 1 min after FCCP in group A, immediately after FCCP in group B and 1 min before FCCP in group C. The rates of respiration reported are the changes in the concentration of oxygen (per cent) in the oxygen electrode per min. The analysis of variance using the F-ratio test (Snedecor, 1956) shows that the differences between the rates in each group are significant. Because the addition of AMP before FCCP results in the fastest rate of respiration, it can be concluded that FCCP interferes with the activity of AMP if it is added to the mitochondria before AMP.

Another feature of the oxygen electrode traces shown in Fig. 28 (and earlier in Fig. 12) is that the rate of respiration after the addition of FCCP decreased with time. At a glance it might have been concluded that FCCP brought about this effect. Only when the rate of respiration of the mitochondria, in the presence of pyruvate and enough oligomycin to inhibit the oxidation in state 3 but without FCCP (Fig. 29), was examined that it was also found to decrease with time. (The decrease in the rate of oxygen uptake is also clearly shown in Fig. 30.) Fig. 27 also shows that the addition of AMP, either 5 min (trace a) or 2 min (trace b) after the start of respiration, increased the rates of respiration to a constant level. Hence the decrease in the rate of respiration observed was not due to the decrease in the ability of the mitochondria to oxidize pyruvate because they were in the oxygen electrode too long, but it was probably the result of another factor which is the subject of the following discussion.

Fig. 30 shows that, when there was no other addition, the pattern of oxygen uptake of the mitochondria in the presence of

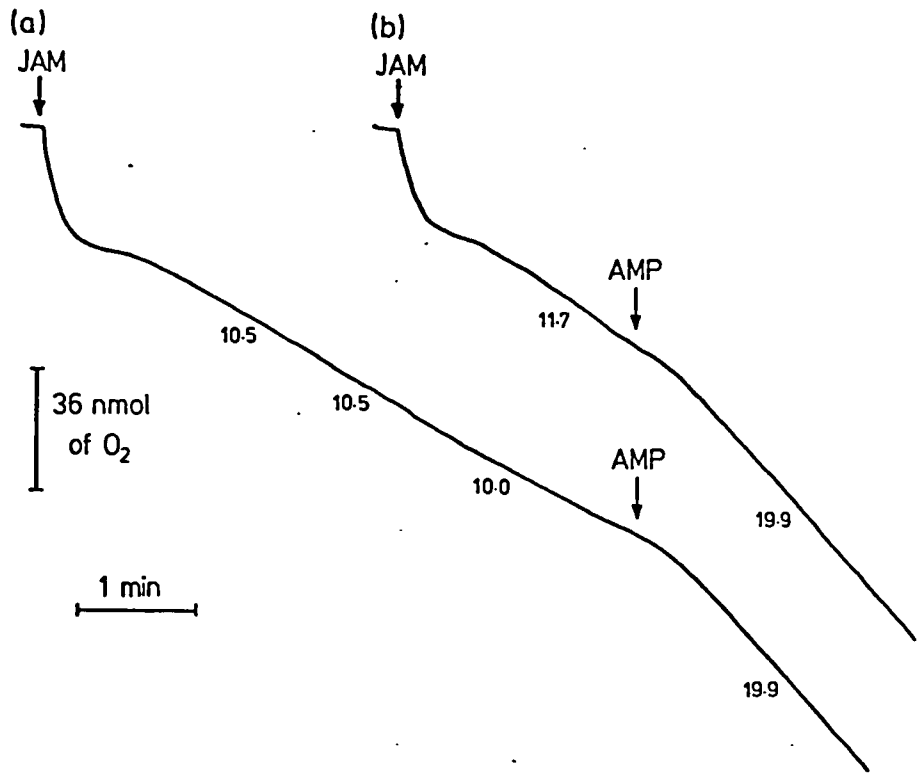


Fig. 29 The effect of time on the oxidation of pyruvate and its stimulation by AMP

Jerusalem artichoke mitochondria (2.05 mg of protein) were oxidizing 15 mM pyruvate in the standard reaction medium plus 1 mM malate, 170 µg of TPP and 2.5 µg of oligomycin in the total volume of 1 ml. AMP (75 nmol) was added 5 min (a) and 2 min (b) after the start of the reaction. The numbers under the oxygen electrode traces are the rates of oxygen uptake in nmol/min per mg of protein.

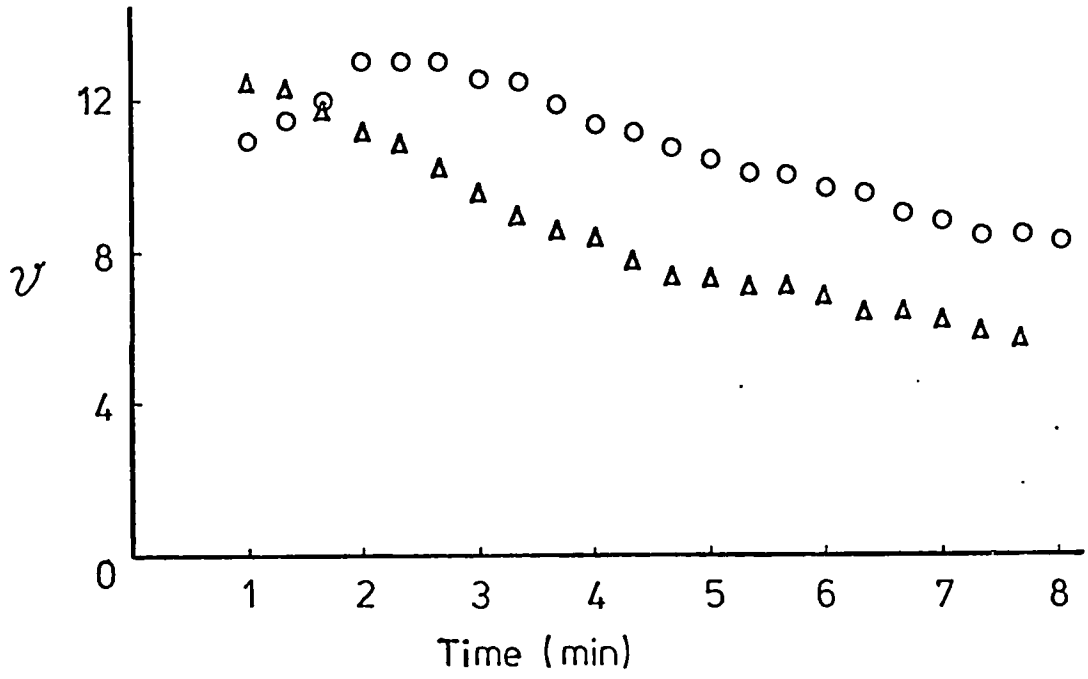


Fig. 30 The time course of oxygen uptake by mitochondria oxidizing citrate or pyruvate

At time zero, Jerusalem artichoke mitochondria (1.86 mg of protein) were added to the standard reaction medium containing 10 mM citrate (O) or 15 mM pyruvate plus 1 mM malate and 170 µg of TPP (Δ) in a 1 ml oxygen electrode. The rate of oxygen uptake (v) is expressed as the decrease in oxygen concentration in the electrode (% of total O_2) per min.

Since the rates of oxygen uptake were not constant, v was estimated from the tangent to the oxygen electrode trace at the specified time after the addition of mitochondria.

citrate is similar to that in the presence of pyruvate plus malate and TPP. Since it has been shown earlier that the addition of AMP stimulates the oxidation of endogenous NADH, it is likely that the oxidation of both substrate systems is subject to control by the $NADH/NAD^+$ ratio. When AMP is absent endogenous NADH cannot be oxidized fast enough, so the high $NADH/NAD^+$ ratio decreases the activity of the enzyme producing NADH and the overall effect is seen by the decrease in respiration.

It is possible that the activity of isocitrate dehydrogenase, which may be controlled by the $NADH/NAD^+$ ratio (Coultate & Dennis, 1969), may control the oxidation of pyruvate by the mitochondria similar to the situation in blowfly flight-muscle mitochondria (Johnson & Hansford, 1975) when AMP is absent. Alternatively, the activity of the pyruvate dehydrogenase complex may be regulated directly by the $NADH/NAD^+$ ratio similar to the case of the pyruvate dehydrogenase complex from *E. coli* (Hansen & Henning, 1966).

The pattern of oxygen uptake by the mitochondria in the presence of citrate was slightly different from that of pyruvate in that the rate of oxidation of citrate increased during the first two minutes. As citrate is known as a positive effector of isocitrate dehydrogenase in plant mitochondria (Coultate & Dennis, 1969; Cox & Davies, 1969), it was possible that citrate might at first increase the production of NADH until the activity of isocitrate dehydrogenase became inhibited by the high $NADH/NAD^+$ ratio when AMP was absent.

8. Occurrence of the stimulation of respiration by AMP under other conditions

(a) Stimulation of respiration of Jerusalem artichoke mitochondria in the presence of other uncoupling agents

An experiment was done to investigate the effect of AMP under other conditions, especially whether AMP was effective when uncoupling agents other than FCCP were added to the mitochondria. Fig. 31 shows that AMP was able to stimulate oxidation of pyruvate by Jerusalem artichoke mitochondria in the presence of dinitrophenol (DNP), S_{13} (5-chloro-3-*t*-butyl-2-chloro-4-nitrosalicylanilide) and valinomycin. Thus it was quite clear that the stimulation of oxidation of NAD^+-linked substrates by AMP lay in the nature of the mitochondria rather than the uncoupling agents.

(b) Stimulation of respiration by AMP in other plant mitochondria

Laties (1973) reported that only ADP was effective in stimulating the uncoupled respiration of potato mitochondria; AMP was ineffective. Since AMP has been shown to be responsible for the stimulation of non-phosphorylating respiration in Jerusalem artichoke mitochondria, its effect on potato mitochondria was tested.

The oxygen electrode traces of potato mitochondria oxidizing pyruvate plus malate and TPP are shown in Fig. 32. The uncouplers FCCP and dinitrophenol, at the concentrations which produced maximum stimulation of respiration of the mitochondria in the presence of NADH, partially stimulated the oxidation of pyruvate. Addition of AMP increased the uncoupled respiration of potato mitochondria in the presence of pyruvate (Fig. 32).

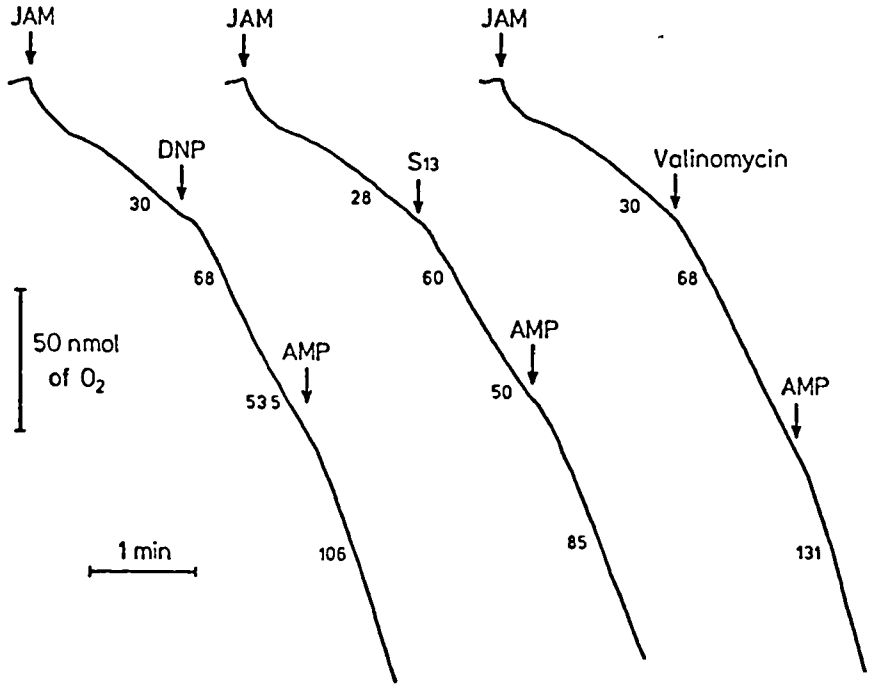


Fig. 31 Activation of non-phosphorylating respiration of Jerusalem artichoke mitochondria by AMP in the presence of various uncoupling agents

Jerusalem artichoke mitochondria (50 µl suspension containing 1.12 mg of protein) were added to the standard reaction medium containing 15 mM pyruvate, 1 mM malate and 170 µg TPP bringing the final volume in the oxygen electrode to 1 ml. The concentrations of the uncouplers added in the oxygen electrode were 5×10^{-5} M dinitrophenol (DNP), 1×10^{-6} M 5-chloro-3-*t*-butyl-2-chloro-4-nitro-salicylanilide (S_{13}) and 1.2×10^{-7} M valinomycin. The amount of AMP added was 75 nmol per assay.

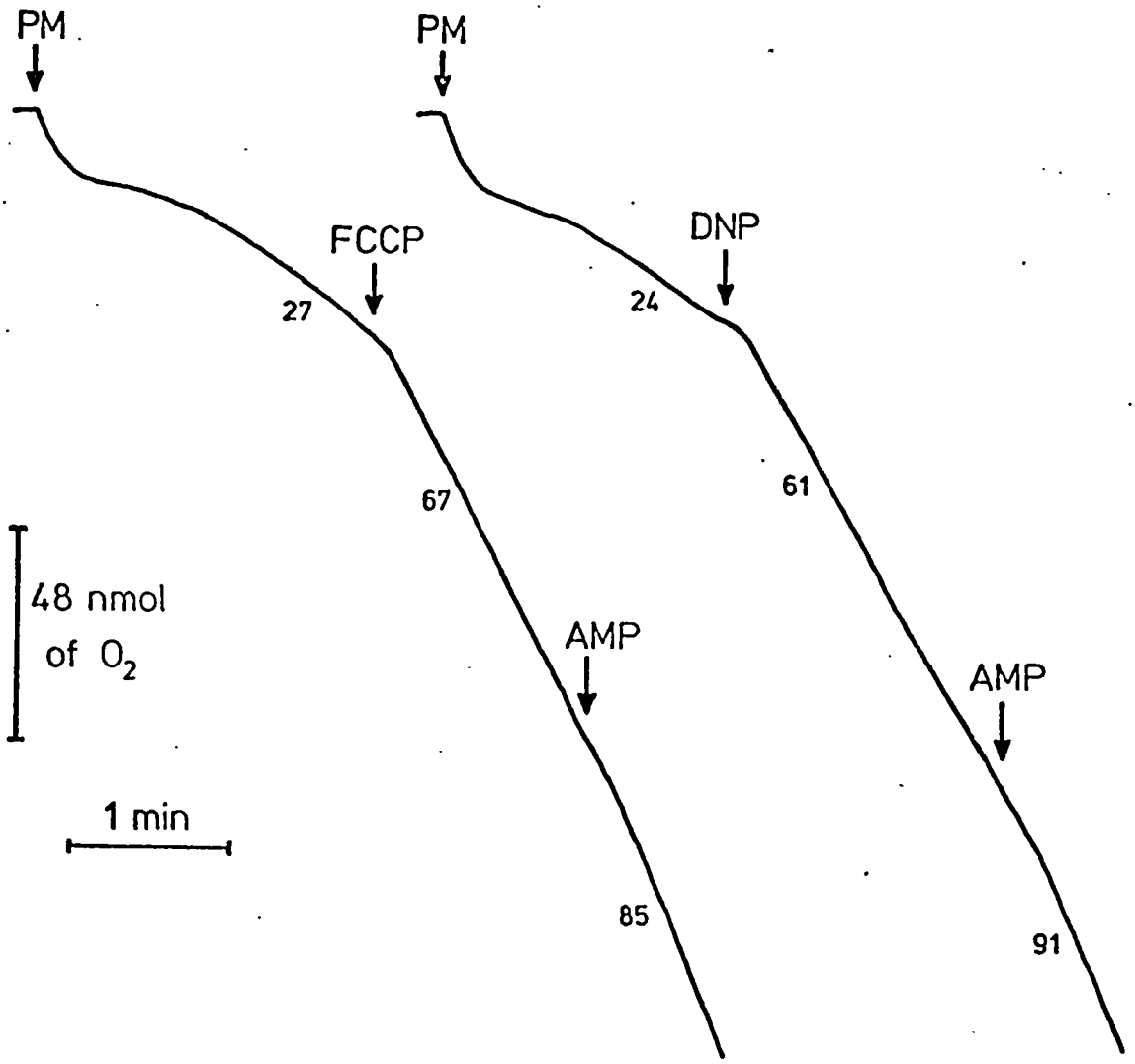


Fig. 32 The effect of AMP on the respiration of potato mitochondria in the presence of FCCP and DNP

The oxygen uptake was started by adding potato mitochondria (PM) (1.1 mg of protein) to the standard reaction medium containing 15 mM pyruvate, 1 mM malate and 170 µg of TPP, bringing the final volume in the oxygen electrode to 1 ml. Other additions were FCCP (2×10^{-7} M), DNP (4×10^{-5} M) and 75 nmol of AMP (75 µM). The numbers under the traces are the rates of oxygen uptake in nmol/min per mg of protein.

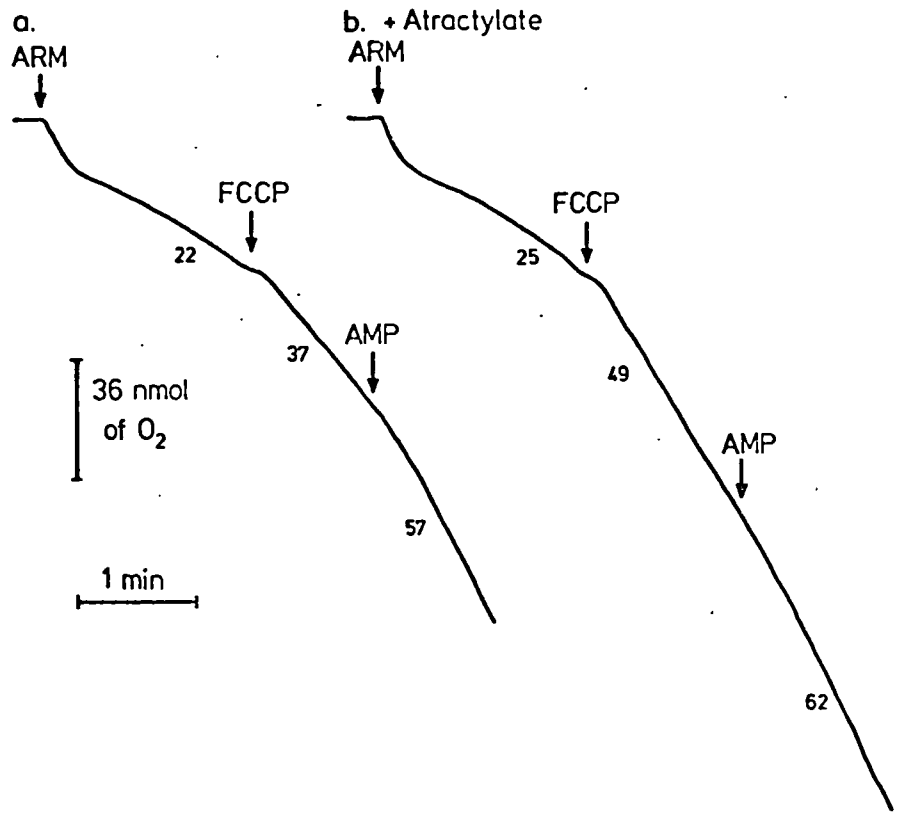


Fig. 33 Activation of non-phosphorylating respiration by AMP in *Arum* rootstock mitochondria

Arum rootstock mitochondria (ARM) (1.34 mg of protein per assay) were oxidizing 15 mM pyruvate and 1 mM malate (plus 170 µg TPP) in the reaction medium for *Arum* rootstock mitochondria (Methods). The total reaction volume in the oxygen electrode was 1 ml. In b, 200 nmol of potassium atractylate was included in the reaction medium. FCCP (2×10^{-7} M) and AMP (75 nmol) were added as indicated. The numbers under the traces are the rates of oxygen uptake in nmol/min per mg of protein.

AMP was also found to stimulate the uncoupled NAD^+-linked respiration in mitochondria prepared from *Arum* rootstock (Fig. 33a). Moreover, if atractylate was initially present in the medium (Fig. 33b) the same result as in Jerusalem artichoke mitochondria was obtained, that is, the addition of FCCP increased the rate of respiration more in the presence of atractylate than in its absence.

9. Regulation of the oxidation of endogenous NADH in Jerusalem artichoke mitochondria

In section 1(d) it has been shown that there exists a pathway of electron transfer from the endogenous pool of NADH in Jerusalem artichoke mitochondria which is resistant to piericidin A and coupled to two sites of phosphorylation (Figs. 7 and 8). By measuring the ADP/O ratios both in the presence and absence of piericidin A, Brunton & Palmer (1973) concluded that in cereal mitochondria the oxidation of NADH by the piericidin A-resistant pathway, which by-passes the phosphorylation site I, was not operative until the inhibitor was present. The ADP/O ratios obtained in section 1(a) also indicate that by-passing of phosphorylation at site I does not take place in the absence of piericidin A because the ADP/O ratios of NAD^+-linked substrates appear to be equivalent to three sites of phosphorylation, taking into account that in these mitochondria the respiratory chain-linked phosphorylation is about 60-70% efficient (Table 1). Thus, in the mitochondria, there seems to be a regulating system for determining the pathway whereby electrons are transferred between the endogenous NADH pool and

cytochrome b. The main aim of this section is to elucidate how this regulating system operates.

It is believed that piericidin A inhibits electron transport at the level of the iron-sulphur components of the electron transport chain (Jeng *et al.*, 1968; Brunton & Palmer, 1973; Singer & Gutman, 1974). As the addition of piericidin A to plant mitochondria in the presence of NAD^+-linked substrates leads to a loss of one phosphorylation site (Brunton & Palmer, 1973), the piericidin A-sensitive electron transport pathway, which AMP is capable of stimulating, appears to be closely linked to the phosphorylation site I. Therefore an investigation into the influence of AMP on the pathway not linked to phosphorylation at site I has been undertaken.

Fig. 34 shows oxygen electrode traces of Jerusalem artichoke mitochondria oxidizing citrate in the presence of oligomycin and piericidin A. (The reason for using citrate as the substrate here is that in the presence of FCCP and no AMP the rate of oxidation of citrate is constant.) As expected, the addition of AMP did not stimulate the rate of oxygen uptake because piericidin A inhibits the AMP-stimulated electron flow between NADH and cytochrome b (Fig. 21). In the presence of FCCP (Fig. 34b) AMP appears to cause a gradual decrease in the rate of piericidin A-resistant oxidation, which suggests that AMP is capable of inhibiting the piericidin A-resistant pathway.

This view is supported by data presented in Fig. 35; trace (a) shows that piericidin A slightly inhibits the uncoupled rate of oxidation of citrate in the absence of AMP and the piericidin A-resistant rate appears to be constant. It is shown in trace (b)

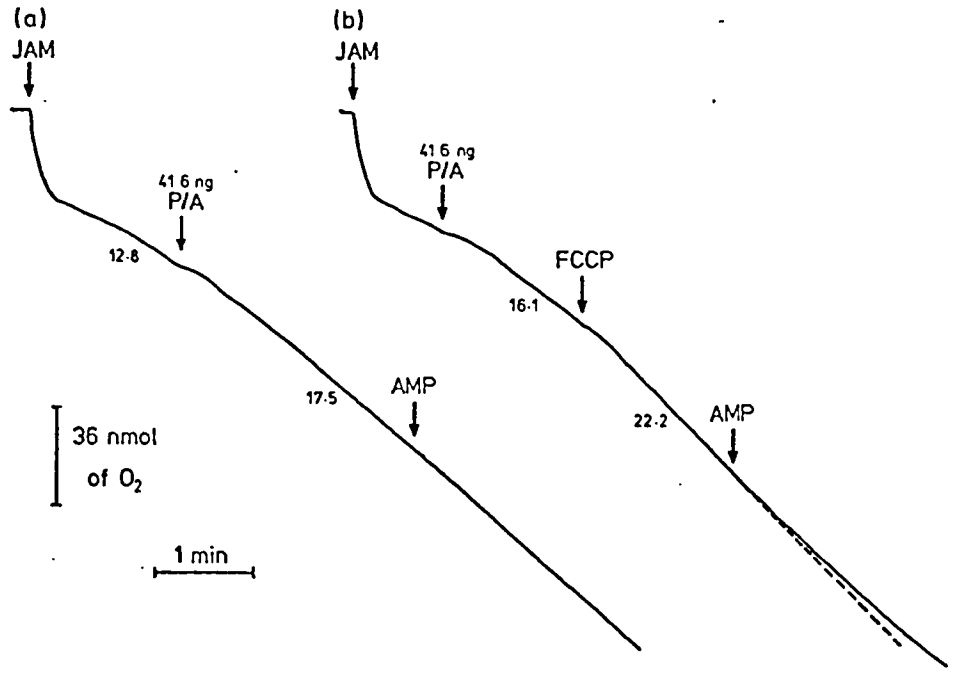


Fig. 34 Effect of AMP on the oxidation of citrate in the presence of oligomycin and piericidin A

Jerusalem artichoke mitochondria (0.1 ml containing 1.84 mg of mitochondrial protein) were added to the standard reaction medium containing 3 μg oligomycin and 10 mM citrate in a 1-ml oxygen electrode. The concentration of FCCP was 2×10^{-7} M and the amount of AMP added was 75 nmol per assay. The numbers under the traces are the rates of oxygen uptake/min per mg of protein. The dotted line in (b) indicates the respiration without AMP.

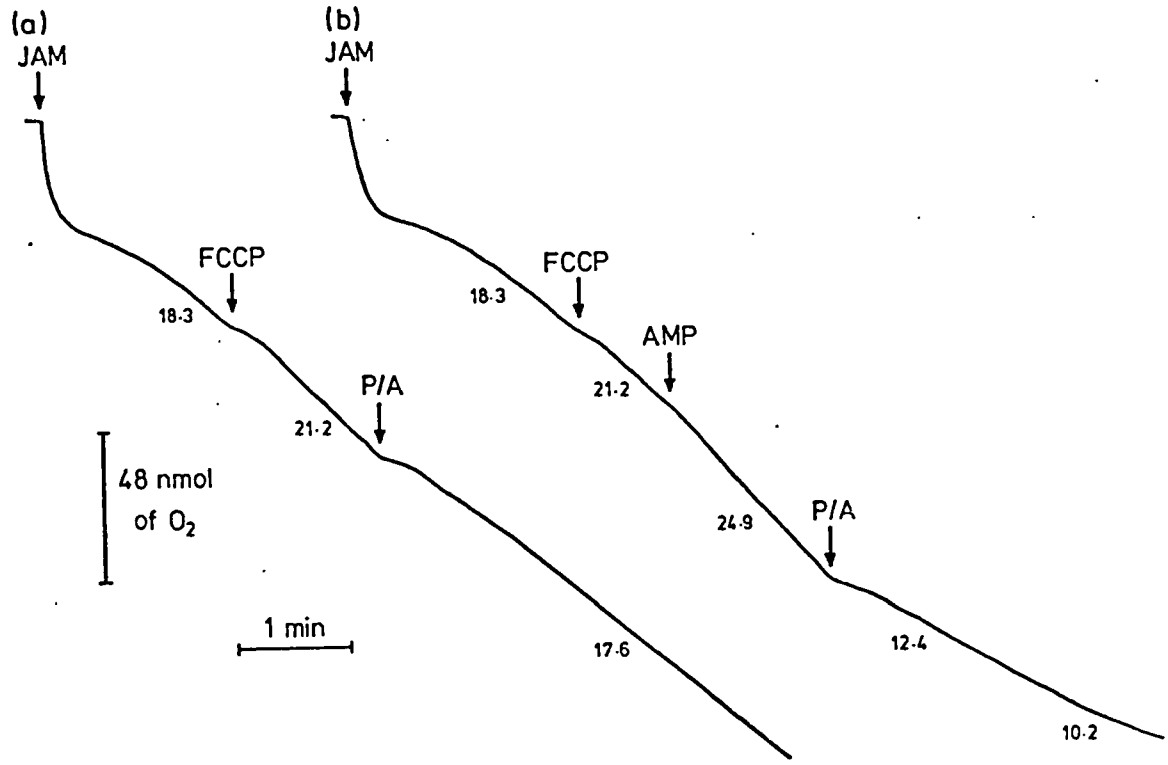


Fig. 35 The oxidation of citrate and its inhibition by piericidin A

The oxidation of citrate by Jerusalem artichoke mitochondria was started by adding the mitochondria (1.6 mg of protein) to the standard reaction medium containing 10 mM citrate (1 ml reaction volume) in the oxygen electrode. Other additions were FCCP (2×10^{-7} M), 41.6 ng piericidin A (P/A) and in (b) 75 nmol of AMP.

that AMP stimulates the uncoupled rate and the addition of piericidin A results in a more significant inhibition; the residual rate after the addition of piericidin A is much slower than that in trace (a), i.e. without AMP.

Using pyruvate (plus malate and TPP) as the substrate, it was also found that if piericidin A was added in state 4 after a pulse of ADP (Fig. 36a), the piericidin A-resistant rate was faster than that when the inhibitor was added after FCCP and AMP (Fig. 36b).

The results shown in Figs. 35 and 36 seem to indicate that when electrons are encouraged to flow along the piericidin A-sensitive pathway (in the presence of AMP) the passage of electrons through the other pathway is deterred.

At this point it is tempting to suggest that on the other hand the presence of ATP may favour the operation of a piericidin A-resistant pathway because of the small inhibitory effect of piericidin A on the oxidation in state 4 (Fig. 36a). The proposition was put to test by studying the effect of piericidin A on the state-3 oxidation of pyruvate by the mitochondria in the presence and absence of ATP. Yeast hexokinase (EC 2.7.1.1) and glucose were added to the suspension of mitochondria so as to remove ATP from the medium. Fig. 37 shows that in state 3 the piericidin A-resistant rates were practically the same whether some ATP was present in (trace a) or absent from (trace b) the reaction medium. In case (a), piericidin A was added to the reaction mixture when about half of the added ADP had been phosphorylated. Although the exact amount of ATP in the reaction medium was not known, the amount of ATP in (a) was likely to be higher than that in (b). Thus the operation of the piericidin A-resistant pathway

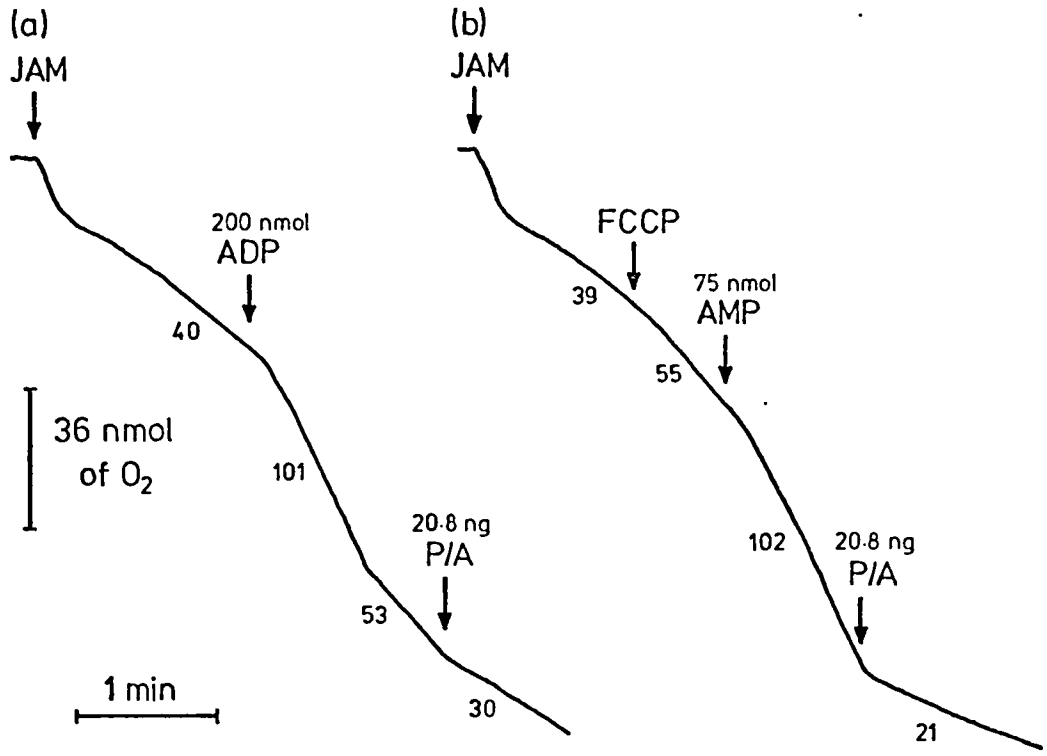


Fig. 36 Inhibition of oxidation of pyruvate by piericidin A

Jerusalem artichoke mitochondria (JAM) (0.77 mg of protein) were added to the reaction medium containing 15 mM pyruvate, 1 mM malate and 170 µg TPP in a 1-ml oxygen electrode. In trace (a), 20.8 ng piericidin A (P/A) was added in state 4 after an addition of ADP (200 nmol). In trace (b), the same amount of piericidin A was added after FCCP (2×10^{-7} M) and 75 nmol of AMP. The numbers under the oxygen electrode traces are the rates of oxygen consumption in nmol/min per mg of mitochondrial protein.

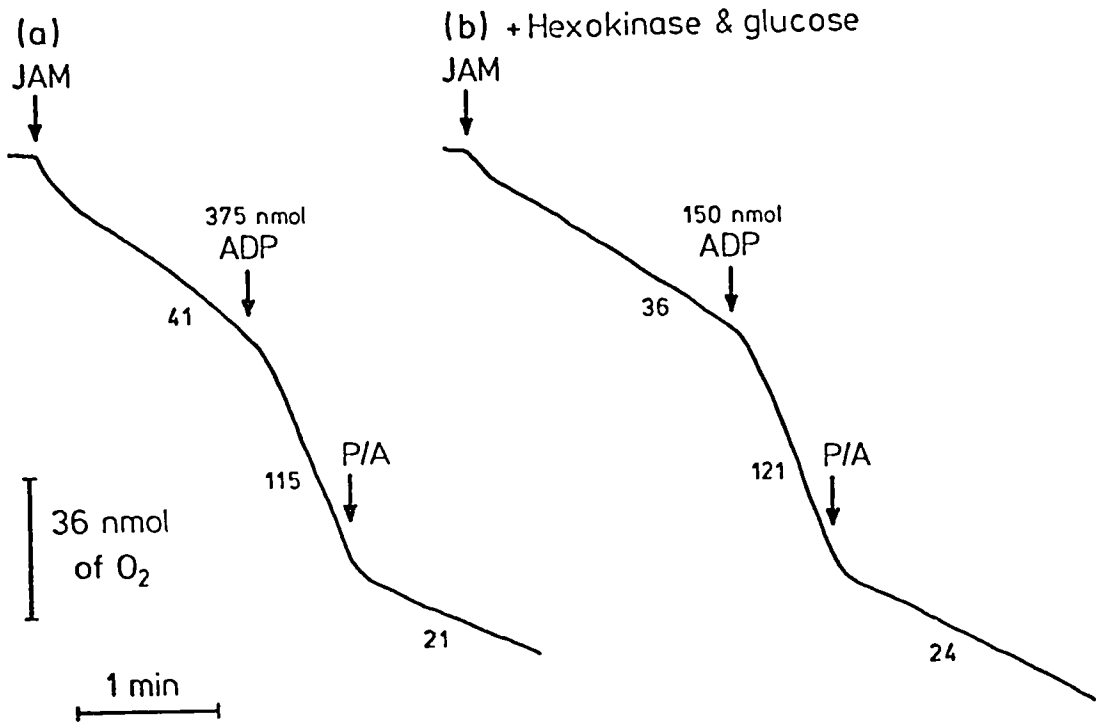


Fig. 37 Influence of ATP in the reaction medium on the inhibition of pyruvate oxidation by piericidin A

The basic reaction mixture contained the standard reaction medium and 15 mM pyruvate plus 1 mM malate and 170 µg TPP. In (b), hexokinase (200 µg) and glucose (10 mM) were included to remove ATP. Jerusalem artichoke mitochondria (0.79 mg of protein) were added to start the reaction, bringing the total volume to 1 ml in the oxygen electrode. In (a), 375 nmol ADP was added to start oxidation in state 3; in (b) 150 nmol ADP was added. Piericidin A (10.4 ng) was added in state 3. The numbers under the oxygen electrode traces are the rates of oxygen uptake in nmol/min per mg of protein.

seemed not to depend on the concentration of ATP outside the mitochondria.

It should be pointed out that if the operation of the piericidin A-resistant pathway depended upon the concentration of ATP in the mitochondrial matrix, varying the concentration of ATP outside the mitochondria would not affect the sensitivity of the mitochondria to piericidin A.

An alternative suggestion in explanation of the result in Fig. 37 is that the operation of the piericidin A-resistant pathway depends upon the state of oxidation of the mitochondria, but not upon the presence of ATP *per se*. If this was the case, adding piericidin A to the mitochondria in state 3 (Fig. 37) would naturally produce the same effect regardless of the amount of ATP present. The evidence that, in the presence of oligomycin, AMP did not inhibit the piericidin A-resistant respiration without the addition of FCCP (Fig. 34a) also indicates that the presence of AMP *per se* does not affect the piericidin A-resistant respiration. Because piericidin A appeared to produce a small inhibition if added in state 4 (Fig. 36a) but its maximum inhibitory effect was obtained when the piericidin A-sensitive, AMP-stimulated electron transport pathway was operative (Fig. 35b and 36b), it is only possible to conclude that the operation of the piericidin A-sensitive, AMP-stimulated electron transport may inhibit the piericidin A-resistant pathway of Jerusalem artichoke mitochondria.

DISCUSSION

The results obtained in the preliminary experiments indicated that Jerusalem artichoke mitochondria used in this study were in many ways similar to other plant mitochondria (Hanson & Hodges, 1967; Chance *et al.*, 1968; Ikuma, 1972; Palmer, 1976). The mitochondria were capable of phosphorylating ADP during the oxidation of exogenously added NADH and Krebs-cycle acids, as evident from their activities in the oxygen electrode, presumably by the same mechanism which operates in animal mitochondria (Chance & Williams, 1956; Palmer & Hall, 1972). The observation that the oxidation of NAD^+-linked substrates by the mitochondria was partially inhibited by piericidin A and the piericidin A-resistant respiration was coupled to phosphorylation at sites II and III, but not at site I, clearly demonstrated the complex organization of the respiratory chain of Jerusalem artichoke mitochondria. Some evidence of a mechanism by which plants regulate the flux of electrons along the branched respiratory chain has emerged from the investigation into the anomalies concerning the response of plant mitochondria to uncoupling agents. The significance of these findings will be discussed in relation to the role of mitochondria in the cell.

Uncoupling of oxidative phosphorylation and stimulation of respiration of mitochondria

It was shown in this study that FCCP could abolish the electrochemical gradient, as measured by the expulsion of H^+ (Fig. 9) and ANS fluorescence changes (Fig. 10), formed during the respiration

of Jerusalem artichoke mitochondria. With respect to these effects on the mitochondria, FCCP appeared to be a potent uncoupling agent (Mitchell, 1968; Greville, 1969: Skulachev, 1971) and therefore should enhance electron transport when it was limited by the lack of phosphate acceptor. The addition of FCCP to the mitochondria oxidizing succinate or exogenous NADH resulted in a high rate of respiration very often faster than that in state 3 (Fig. 11). On the contrary, the rate of respiration of the mitochondria in the presence of NAD^+-linked substrates was not fully stimulated by the uncoupler (Fig. 12), similar to the results previously obtained by other workers (Ikuma & Bonner, 1967*b*; Bonner, 1973; Laties, 1973). In conformity with either of the theories of oxidative phosphorylation, it was unlikely that uncoupling by FCCP was effective only at the two phosphorylation sites involved in the oxidation of succinate and exogenous NADH, leaving site I fully operational. It was therefore thought that incomplete uncoupling by FCCP at the first site of phosphorylation during the oxidation of NAD^+-linked substrates, as suggested by Bonner (1973) and Laties (1973), was not responsible for the lack of stimulation of respiration in the presence of the uncoupler. It should be noted that not only FCCP, but also other uncouplers tested, DNP, S_{13} and valinomycin, failed to increase to any extent the rate of respiration of Jerusalem artichoke, potato and *Arum* rootstock mitochondria in the presence of NAD^+-linked substrates.

It was confirmed in this study that adenine nucleotides could stimulate the respiration of plant mitochondria in the presence of uncouplers of oxidative phosphorylation. As the stimulation was also observed in the presence of oligomycin either with or without

an uncoupler, it was concluded that under these conditions the function of adenine nucleotides was not that of the phosphate acceptor so as to release the respiratory control. The stimulation of respiration in the presence of NAD^+-linked substrates could be brought about by ADP and AMP but not by ATP, and thus could be distinguished from the activation of succinate dehydrogenase by ADP and ATP (Drury *et al.*, 1973; Oestreicher *et al.*, 1973). Further investigation however indicated that AMP, and not ADP, was primarily responsible for the stimulation of the oxidation of NAD^+-linked substrates. AMP was effective at lower concentrations than ADP. During the investigation into the level of reduction of cytochrome b in the mitochondria (which will be discussed later on), the reduction of cytochrome b was found to be more rapid when it was induced by AMP than by ADP (Fig. 20). Finally the most compelling evidence was that when Mg^{2+} was omitted from the medium only AMP could promote the rapid reduction of cytochrome b (Fig. 20). These observations suggest that ADP had to be converted into AMP probably by the action of adenylate kinase, and the lack of Mg^{2+} prevented the enzyme from converting ADP into AMP. Laties (1973) reported that AMP would not increase the rate of respiration of potato mitochondria in the presence of an uncoupling agent. It was therefore surprising when the experiment was repeated to find that AMP was capable of stimulating the oxidation of an NAD^+-linked substrate by potato mitochondria in the presence of FCCP or 2,4-dinitrophenol (Fig. 32). In addition, the stimulation by AMP could be demonstrated in mitochondria isolated from the rootstock of *A. maculatum* (Fig. 33).

Attempts to find the locus of the stimulation of electron

flow induced by AMP revealed that in Jerusalem artichoke mitochondria without added adenine nucleotide the level of reduction of cytochrome b was very low in the presence of NAD^+-linked substrates. In a comparable experiment using rat liver mitochondria a higher level of reduction of cytochrome b was observed. The addition of AMP, or ADP in the presence of Mg^{2+}, to Jerusalem artichoke mitochondria induced a rapid flow of electrons from the endogenous pool of nicotinamide-adenine dinucleotide to cytochrome b, as clearly shown under non-phosphorylating conditions by simultaneous oxidation of endogenous NADH and reduction of cytochrome b (Fig. 22). The oxidation of endogenous NADH as a result of the addition of AMP ruled out the alternative theory that the stimulation of respiration originated from the allosteric activation of the NAD^+-linked dehydrogenase which would have resulted in the more rapid reduction of endogenous NAD^+. Because the rapid reduction of cytochrome b induced by AMP could be inhibited by piericidin A, it was concluded that the stimulation of the uncoupled rate of oxidation of NAD^+-linked substrates by AMP was the result of the activation of electron flow, which is independent of oxidative phosphorylation, through the piericidin A-sensitive NADH dehydrogenase.

The low level of reduction of cytochrome b of plant mitochondria in the presence of NAD^+-linked substrates has also been reported by Lambowitz & Bonner (1974) and has been attributed to some form of accessibility barrier between the endogenous NADH dehydrogenase and cytochrome b. The increased electron flux from the endogenous pool of NADH to cytochrome b observed when AMP was added indicates that the accessibility barrier between the two components can be lowered by AMP. The lack of a marked increase in

the rate of respiration on addition of uncoupling agents to plant mitochondria in the presence of NAD^+-linked substrates is consistent with the suggestion of the existence of an accessibility barrier. It can be concluded that the action of AMP is not to bring about uncoupling, but to increase the rate of electron flux along the respiratory chain. The finding that the addition of FCCP to the mitochondria pre-treated with AMP (and oligomycin) resulted in a maximum rate of respiration (Fig. 16b) is in accord with this conclusion. The phenomenon of 'conditioning' (Raison *et al.*, 1973 a & b) can be accounted for by this rationale.

Site of action of AMP

Since there was evidence to suggest that AMP could not traverse the inner mitochondrial membrane (Fig. 4; Klingenberg, 1970), the observation that AMP, not ADP, was primarily responsible for the activation of electron transport along the piericidin A-sensitive NADH dehydrogenase presented some problems in locating the site of action of AMP. All of the results however indicated that AMP acted on the outside of the inner membrane. This conclusion was derived from the following observations: (a) AMP did not increase the rate of oxidation of NADH by submitochondrial particles prepared by sonicating the mitochondria (Fig. 23); (b) the activation of electron transport to oxygen by AMP was observed in the presence of inhibitors of the adenine-nucleotide translocators, bongkrekic acid (Table 3) and atractylate (Fig. 27); (c) under certain conditions atractylate behaved like AMP (Fig. 25).

Because it has been shown that the polarity of the membranes of submitochondrial particles obtained by sonication is opposite to

that of mitochondria (Lee *et al.*, 1967), the oxidation of NADH by submitochondrial particles is effected by the NADH dehydrogenase normally located on the matrix side of the membrane. The failure of AMP to stimulate the oxidation of NADH by the submitochondrial particles indicated that there was no stimulatory site for AMP on the endogenous NADH dehydrogenase.

It has been established that AMP is not actively transported on the adenine nucleotide translocator into the mitochondria (in this study; Klingenberg, 1970; Passam & Coleman, 1975). The phosphorylation of AMP appears to take place via the action of adenylate kinase, in the intermembrane space, in which AMP is converted into ADP, and ADP is then translocated into the mitochondria for oxidative phosphorylation. This situation is confirmed by the data which show that the state-3 rate of oxidation of all substrates, resulting from the formation of ADP from AMP, was inhibited by bongkrekic acid (Table 3). The stimulation of the non-phosphorylating oxidation of NAD^+-linked substrates by AMP was unaffected by bongkrekic acid. This observation suggested that AMP did not enter the mitochondrial matrix to bring about the stimulation because bongkrekic acid has been shown to inhibit the translocation of ADP into plant mitochondria (Passam *et al.*, 1973; Vignais *et al.*, 1976). It was unlikely that the stimulation observed in the presence of bongkrekic acid was the result of AMP entering the mitochondrial matrix by other means because if AMP had entered the matrix it would have been transphosphorylated and respiration in state 3 would have been observed.

The result of the experiment using atractylate was in agreement with that obtained with bongkrekic acid. AMP could increase

the low rate of oxidation of NAD^+-linked substrates by the mitochondria in the presence of FCCP and atractylate which was also shown to inhibit the respiration in state 3, in a competitive manner with respect to the concentration of ADP. There was no doubt that atractylate could inhibit the translocation of ADP into plant mitochondria as the inhibition had clearly been demonstrated by Vignais *et al.* (1976). The inhibition however required considerably higher concentrations of atractylate in comparison with those reported for animal mitochondria (Vignais *et al.*, 1966; Klingenberg, 1970; Vignias *et al.*, 1976), which probably accounted for the negative result obtained by Passam *et al.* (1973).

It was noted in the experiment with atractylate that the addition of FCCP to Jerusalem artichoke or *Arum* rootstock mitochondria incubated with atractylate resulted in a rapid rate of respiration (Figs 25 & 33). Atractylate thus could substitute for AMP (see Fig. 16b), presumably by acting at the AMP-binding site which caused the stimulation of electron flow. This observation confirms the earlier suggestion that the site of action of AMP is on the outer surface of the inner mitochondrial membrane because it has been established from the studies with animal mitochondria that atractylate does not traverse the inner mitochondrial membrane (Vignais, Vignais & Defaye, 1973; Vignais, 1976) and only interacts with the membrane on the cytosolic side (Vignais, Vignais & Stanislas, 1962; Scherer & Klingenberg, 1974; Shertzer & Racker, 1974).

With respect to the present data, it is not known whether atractylate could bind at the AMP-binding site thus resulting in an increased electron flux or whether it interacts at a different

site which results in the same effect. It was also observed that when the mitochondria were incubated with atractylate at high concentrations in the presence of EDTA, which probably removed Mg^{2+}, the addition of FCCP resulted in a very high rate of respiration and AMP could not increase the respiration rate any further (Fig. 25c, Table 4). When Mg^{2+} was present in the medium as well as atractylate, AMP could increase the FCCP-stimulated respiration (Fig. 25b). If the ability of FCCP to increase the rate of respiration of the mitochondria in the presence of atractylate was the result of the binding of atractylate at the AMP-binding site, the affinity of the binding of atractylate seemed to increase when Mg^{2+} was absent (in the presence of EDTA) (Table 4) and might prevent the interaction of AMP. This apparent tight binding of atractylate to the AMP-binding site in the absence of Mg^{2+} is unlike the binding of atractylate to the adenine nucleotide translocator which is enhanced in the presence of Mg^{2+} (Klingenberg, Grebe & Scherer, 1975; Vignais, 1976). It should be emphasized here that atractylate could substitute for AMP only if it was added to the mitochondria before FCCP. It seems that FCCP can alter the conformation of the AMP-binding site to some extent so that it may interact with AMP, but not atractylate.

At present there is insufficient information on the binding of atractylate and adenine nucleotides in plant mitochondria (Vignais *et al.*, 1976). An extensive study on the binding of atractylate in beef heart mitochondria has led to the conclusion that atractylate only interacts with the adenine nucleotide translocator which does not bind AMP (Klingenberg, 1970; Klingenberg *et al.*, 1975). If this situation were also true in plant mitochondria, it would be

necessary to conclude that the binding of atractylate at the adenine nucleotide translocator of plant mitochondria might be different from that in animal mitochondria with respect to the role of Mg^{2+} and had some effects on the AMP-binding site. The implication of this conclusion would be that the AMP-binding site might be close physically and functionally to the adenine nucleotide translocator assuming that they are separate entities, which might be advantageous to plant mitochondria. Recent studies on the phosphorylation of extramitochondrial ADP in animal mitochondria have indicated a functionally and physically close relationship between the adenine nucleotide translocator and the ATPase (Vignais, Vignais & Doussiere, 1975; Out, Valeton & Kemp, 1976).

The exact mechanism by which AMP in the external compartment brings about the stimulation of the oxidation of endogenous NADH is not yet known. Palmer (1976) has suggested that AMP might interact with the outer extremity of the proton pumping loop of the NADH dehydrogenase because of the observation that AMP enhanced electron transport via the piericidin A-sensitive NADH dehydrogenase which is in close association with the phosphorylation site I. Further search for substances which can specifically substitute for AMP without interacting with other functions may be of benefit to the insight into the mechanism of action of AMP.

Regulation of the oxidation of endogenous NADH in plant mitochondria

Evidence presented in this study indicates the existence of an endogenous pathway of oxidation of NADH in Jerusalem artichoke mitochondria, which is resistant to the inhibition of piericidin A

and not coupled with phosphorylation at site I. This piericidin A-resistant NADH dehydrogenase appears to be active under state-4 conditions when the activity of the pathway linked with oxidative phosphorylation is restricted by the lack of phosphate acceptor. There was some indication that while the addition of AMP resulted in the stimulation of electron transfer through the piericidin A-sensitive NADH dehydrogenase, the oxidation of NADH via the piericidin A-resistant pathway was inhibited (Fig. 36). However it was found that the inhibition of electron transfer through the piericidin A-resistant pathway was not due to the presence of AMP *per se*. The operation of the piericidin A-resistant pathway seemed to depend upon the activity of the piericidin A-sensitive pathway which is in turn regulated by AMP and the activity of oxidative phosphorylation. This conclusion is in agreement with the finding that by-passing of phosphorylation at site I does not occur in state 3 in the absence of piericidin A (Brunton & Palmer, 1973). The regulation of electron transfer through this segment of the respiratory chain appears to be similar to that suggested by Bahr & Bonner (1973a & b) for the operation of the alternative oxidase in mitochondria from mung bean hypocotyl and skunk cabbage.

If it is considered that the function of the mitochondrion is not simply to supply ATP but to play a part in the metabolism of carbon compounds by producing carbon skeletons for biosynthetic purposes, the significance of the existence of the non-phosphorylating electron transport pathways in plant mitochondria becomes quite clear (see Palmer, 1976). It is generally believed, as Racker (1976) has stated, that "ATP is generated only when it is needed". So the advantage of having the non-phosphorylating

piericidin A-resistant NADH dehydrogenase in mitochondria of plant tissues is that the Krebs cycle may operate in the presence of a high level of ATP in order to supply carbon skeletons for anabolic syntheses such as proteins which should be favourable in the presence of ATP.

It is generally considered that the rate of electron flux through the respiratory chain is influenced by the availability of ADP for the synthesis of ATP and has been shown to be controlled by the phosphorylation potential ($[ATP]/[ADP] \times [Pi]$) which is an expression of the energy status of the cell (van Dam & Meyer, 1971; Lehninger, 1975; Williamson, 1976). AMP is usually thought to have an influence on the respiratory chain via its conversion into ADP by the action of adenylate kinase. Thus AMP produced in the cytosol as a result of synthetic processes is converted into ADP by adenylate kinase in the mitochondrial intermembrane space (Sottocasa *et al.*, 1967a & b; Klingenberg, 1970). The direct interaction between cytosolic AMP and the respiratory chain of plant mitochondria elucidated in this study may serve as an extra control mechanism to ensure maximum energy production by the respiratory chain which also consists of a non-phosphorylating pathway. The appearance of AMP in the cytosol due to syntheses of proteins, lipids and purines will activate the flow of electrons along the phosphorylating pathway, whereas the non-phosphorylating pathway is inhibited, and therefore all of the three phosphorylation sites will be operative so that ATP is produced with high efficiency.

Another possible reason for the necessity of the control mechanism by cytosolic AMP is that the activity of isocitrate

dehydrogenase in plant mitochondria is not controlled by the ATP/ADP ratio (Cox & Davies, 1967) as in animal mitochondria (Chen & Plaut, 1963) but seems to be regulated by the $NADH/NAD^+$ ratio (Coultate & Dennis, 1969). The stimulation of the oxidation of NADH by AMP could also release the inhibition of isocitrate dehydrogenase by a high $NADH/NAD^+$ ratio. Evidence for this mode of operation of AMP is apparent during the oxidation of citrate by Jerusalem artichoke mitochondria (Figs 30, 34 & 35), as already discussed in section 7 of Results.

In some plant mitochondria, the activity of the piericidin A-resistant pathway of oxidation of endogenous NADH appears to be parallel to the operation of the cyanide-resistant alternative oxidase (Wakiyama & Ogura, 1970: J.M. Palmer & R. Cammack, in preparation). Since the findings in this study indicate that AMP plays an important role in the regulation of energy (ATP) production in plant cells, it may be possible to speculate that the appearance of AMP in the cytosol favours the operation of electron transport linked with the production of ATP and thus it inhibits not only the piericidin A-resistant pathway but also the cyanide-resistant pathway, neither of which is coupled to the synthesis of ATP.

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on the addition of valinomycin. This more sensitive effect is similar to that reported by Hunter & Capaldi (1974) in oxidase vesicles prepared by using a phospholipid extract from mitochondria. The stimulation of respiration by valinomycin can also be seen to occur in our vesicles when the ionophore is added before carbonyl cyanide trifluoromethoxyphenylhydrazone. There is no evidence of any significant synergistic effect.

Partial inhibition of respiration with NaN_3 abolishes the differences in valinomycin effect between the two types of vesicles (Fig. 2) and induces an inhibiting effect of valinomycin on oxidase activity. In 166μM-NaN_3 (approx. 45% inhibition of respiration). Valinomycin added either before or after carbonyl cyanide trifluoromethoxyphenylhydrazone causes a rise in steady-state cytochrome *c* reduction values.

The present results confirm the reported differences between the valinomycin sensitivity of cytochrome *c* oxidase vesicles prepared by using purified natural phospholipids (in this case from egg yolk) and those prepared by using an extract of mitochondrial phospholipids. The latter appear to be particularly sensitive to valinomycin. The partial release of respiratory control by valinomycin indicates a controlling effect of K^+ on respiration. One way in which this could arise would be if the vesicles possessed a capability for proton–cation exchange, allowing a partial conversion of an electrogenic proton movement into an electrogenic K^+ translocation. Addition of valinomycin as well as carbonyl cyanide trifluoromethoxyphenylhydrazone would then be required for full release of respiratory control. On this interpretation, the difference in valinomycin sensitivity between the two types of vesicles could result from differences in K^+/H^+ exchange capacity. After azide inhibition, respiration is inhibited by valinomycin added either before or after carbonyl cyanide trifluoromethoxyphenylhydrazone in both types of vesicles. This could indicate the partial conversion of membrane potential into an osmotic gradient of a potassium salt, in this case KN_3 (Palmieri & Klingenberg, 1967). The discharge of the gradient by valinomycin would then induce a diffusion potential inhibitory to the cytochrome *c* oxidase reaction.

We thank Jack Pedersen and Freda Nicholls for expert technical assistance. J. M. W. acknowledges a travel grant from Odense University.

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Stimulation of Electron Transport and Activation of Reduced Nicotinamide–Adenine Dinucleotide Dehydrogenase in Jerusalem-Artichoke Mitochondria

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Isolated plant mitochondria respond in a very complex manner to the addition of adenine nucleotides and weak acid uncoupling agents. Raison *et al.* (1973) showed that ADP was capable of stimulating the oxidation of citrate in the presence of sufficient oligomycin to completely inhibit oxidative phosphorylation. Laties (1973) reported that ADP was necessary to obtain a maximal response to the uncoupling agent carbonyl cyanide *m*-chlorophenylhydrazone.

Results obtained in our laboratory confirm these observations and support the suggestion that adenine nucleotides have a role in enhancing electron transport in addition to

their roles as phosphate acceptors. We consistently observed that when Jerusalem-artichoke mitochondria were supplied with NAD^+-linked substrates such as pyruvate plus malate the rate of oxidation obtained after addition of the substrate and before addition of ADP was significantly slower than the rate obtained in the state 4 condition, after the exhaustion of a pulse of ADP. It was possible to stimulate the pre-ADP rate to the anticipated state 4 rate by the addition of AMP or ADP in the presence of sufficient oligomycin to completely inhibit oxidative phosphorylation as shown in trace (*a*) in Fig. 1. AMP was more effective in bringing about the stimulation; maximal stimulation was induced by 25 μM-AMP or 125 μM-ADP. ADP was ineffective in the absence of Mg^{2+}; this presumably prevented the conversion of the ADP into AMP via the Mg^{2+}-dependent adenylate kinase. Trace (*b*) in Fig. 1 showed that the addition of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone resulted in very little stimulation until AMP was added. The dependence of the uncoupled rate on the presence of adenine nucleotide was only observed when using NAD^+-linked substrates, AMP or ADP caused no stimulation of the uncoupled rate when succinate or exogenous NADH were used as the electron donor.

Measurement of the redox state of cytochrome *b* showed that the addition of pyruvate plus malate did not result in any appreciable reduction; however, the addition of AMP resulted in the immediate and extensive reduction of cytochrome *b*. Thus indicating that

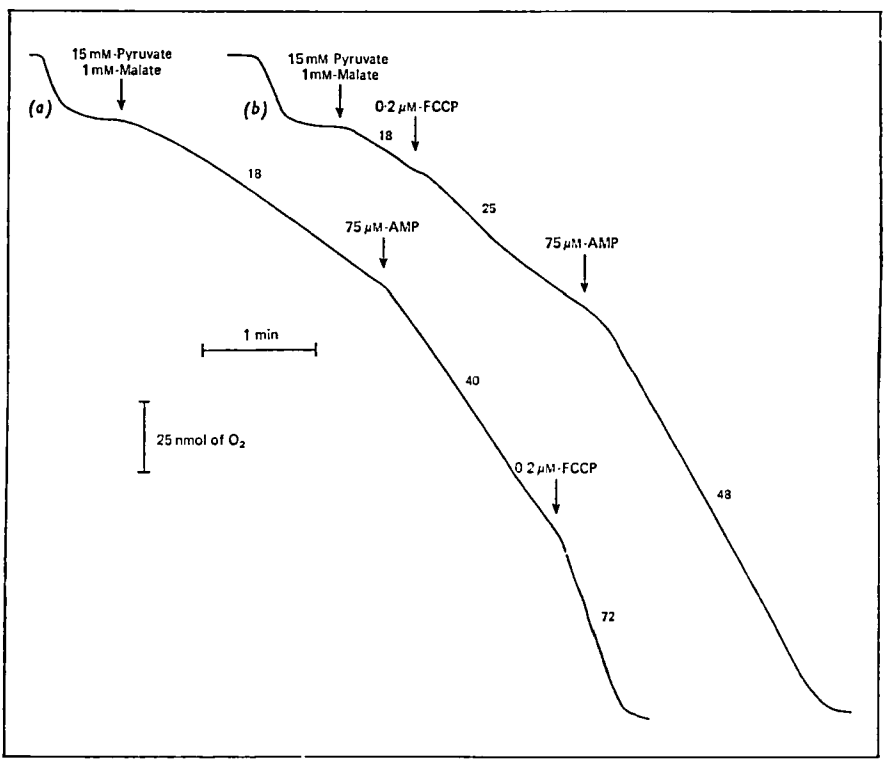

Fig. 1. *Rates of oxygen consumption by Jerusalem-artichoke mitochondria*

The mitochondria were isolated and assayed as described by Palmer & Kirk (1974). All assays were carried out in the presence of 2 μg of oligomycin/mg of mitochondrial protein. FCCP, carbonyl cyanide trifluoromethoxyphenylhydrazone.

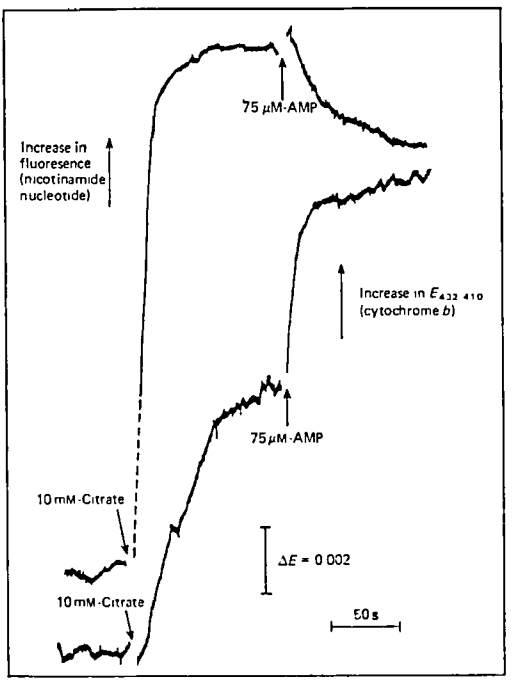

Fig. 2. *Changes in the redox state of endogenous nicotinamide nucleotides and cytochrome b*

All assays were carried out in the presence of 2 μg of oligomycin/mg of mitochondrial protein.

AMP stimulated respiration by increasing the flow of electrons to cytochrome *b*. Both succinate and exogenous NADH reduced cytochrome *b* in the absence of added adenine nucleotide, when using these substrates the addition of AMP or ADP resulted in the partial reoxidation of cytochrome *b* owing to the release of respiratory control.

The increased rate of electron flow to cytochrome *b* from NAD^+-linked substrates could have been due to the lack of endogenous NADH caused either by the lack of substrate entering the mitochondrion or by the allosteric repression of the NAD^+-linked dehydrogenase. Alternatively it could have been due to the inability of electrons to move freely between the endogenous NADH and cytochrome *b* owing to some restriction within the respiratory chain. Simultaneous measurements of the redox state of the endogenous nicotinamide nucleotides and cytochrome *b* in the presence of oligomycin are shown in Fig. 2. It was apparent that the reduction of cytochrome *b* was accompanied by the simultaneous oxidation of the endogenous nicotinamide nucleotides. Such an observation indicated that AMP appears to activate the NADH dehydrogenase and facilitate the flow of electrons from endogenous NADH to cytochrome *b*.

This work was supported by grants from the Science Research Council, The Royal Society and the Central Research Fund of the University of London.

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The Metabolism of Carcinogenic Polycyclic Hydrocarbons by Tissues of the Respiratory Tract

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Carcinogenic polycyclic hydrocarbons undoubtedly require metabolic activation, a process currently thought to involve epoxide formation. Previous studies have shown that the NADPH-dependent microsomal mono-oxygenase that catalyses the formation of epoxides is present in preparations of human and rat lung tissue (Grover *et al.*, 1973, 1974). These tissues are also capable of the further metabolism of hydrocarbon epoxides since, with both species, the conversion of benz[*a*]anthracene 5,6-oxide into the corresponding dihydrodiol, catalysed by microsomal epoxide hydrase, and into the corresponding glutathione conjugate, catalysed by glutathione *S*-epoxide transferase, was detected (Grover *et al.*, 1973; Grover, 1974).

The polycyclic hydrocarbons that are present in tobacco smoke (Wynder & Hoffman, 1959) are suspected of contributing to the increased incidence of respiratory cancer in man (Doll & Hill, 1950). The human respiratory tumours (Kreyberg type 1), whose occurrence appears to be most closely associated with tobacco smoking (Kreyberg, 1962), arise in the bronchial epithelium. In view of this, efforts are being made to investigate the metabolic activation of polycyclic hydrocarbons in bronchial mucosa, which is probably more relevant to the etiology of human lung cancer than the mainly alveolar tissue preparations that were used in the earlier work.

In the present studies, the metabolism of the ^{3}H-labelled polycyclic hydrocarbons benz[*a*]anthracene (sp. radioactivity 610mCi/mmol), 7-methylbenz[*a*]anthracene (sp. radioactivity 457mCi/mmol) and benzo[*a*]pyrene (sp. radioactivity 475mCi/mmol) by Wistar rat and Syrian hamster trachea and by human bronchial tissue maintained in short-term culture was examined. The freshly removed animal trachea were sliced into rings and portions weighing approx. 200mg were placed in plastic tissue-culture flasks (30ml) (Falcon Plastics, Oxnard, Calif., U.S.A.) that contained Dulbecco's MEM (10ml) (Biocult Ltd., Paisley, U.K.) supplemented with foetal calf serum (10%, w/v) and incubated at 30°C under 10% CO_2 in air. Segments of human bronchus that were macroscopically free of tumour were placed in culture in a similar way within 1h of removal at thoracotomy. After 24h in culture, the medium surrounding the respiratory tissue was replaced by medium (10ml) to which a ^{3}H-labelled hydrocarbon (4μg) (The Radiochemical Centre, Amersham, Bucks., U.K.) had been added as a solution in ethanol (2μl). After incubation for a further 24h, the media were removed, extracted with ethyl acetate (2×1vol.) and the pooled extracts were evaporated to dryness. The residue was redissolved in ether, small amounts of appropriate authentic reference compounds were added and the mixtures were examined on t.l.c. developed with benzene–ethanol (9:1, v/v) as described previously (Grover *et al.*, 1974). Bands that contained the reference compounds were marked off under u.v. light and these and intermediate bands were removed, transferred to glass vials, and the radioactivity present was determined by liquid-scintillation counting. Protein present in homogenates of tracheal and bronchial tissues was determined (Fincham, 1954) by using casein as a standard protein. Other specimens were examined histologically.

With benz[*a*]anthracene, radioactive products were formed that were inseparable on t.l.c. from 5,6-dihydro-5,6-dihydroxybenz[*a*]anthracene, 8,9-dihydro-8,9-dihydroxybenz[*a*]anthracene and 9-hydroxybenz[*a*]anthracene. With 7-methylbenz[*a*]anthracene radioactive products with the chromatographic characteristics of the related 5,6- and 8,9-dihydrodiols were formed together with 7-hydroxymethylbenz[*a*]anthracene. The metabolism of [^{3}H]benzo[*a*]pyrene by respiratory tissues yielded products with the chromatographic characteristics of 4,5-dihydro-4,5-dihydroxybenzo[*a*]pyrene, 7,8-dihydro-7,8-dihydroxybenzo[*a*]pyrene and 9,10-dihydro-9,10-dihydroxybenzo[*a*]pyrene; 3-hydroxybenzo[*a*]pyrene was also detected. The results obtained show that rat

Biochem. J. (1975) **152**, 637–645
Printed in Great Britain

The Activation of Non-Phosphorylating Electron Transport by Adenine Nucleotides in Jerusalem-Artichoke (*Helianthus tuberosus*) Mitochondria

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(*Received* 18 *August* 1975)

In isolated plant mitochondria the oxidation of both succinate and exogenous NADH responded in the expected manner to the addition of ADP or uncoupling agents, and the uncoupled rate of respiration was often in excess of the rate obtained in the presence of ADP. However, the oxidation of NAD^+-linked substrates responded in a much more complex manner to the addition of ADP or uncoupling agents such as carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone to mitochondria oxidizing pyruvate plus malate failed to result in a reliable stimulation; this uncoupled rate could be stimulated by adding AMP or ADP in the presense of oligomycin or bongkrekic acid. Spectrophotometric measurements showed that the addition of AMP or ADP resulted in the simultaneous oxidation of endogenous nicotinamide nucleotide and the reduction of cytochrome *b*. ADP was only effective in bringing about these changes in redox state in the presence of Mg^{2+} whereas AMP did not require Mg^{2+}. It was concluded that AMP activated the flow of electrons from endogenous nicotinamide nucleotide to cytochrome *b*, possibly at the level of the internal NADH dehydrogenase.

Plant mitochondria frequently respond in a complex and often unreliable manner to the addition of ADP or weak acid uncoupling agents. There have been many reports that the addition of adenine nucleotides to uncoupled mitochondria results in the stimulation of electron transport. Wiskich & Bonner (1963) found that both ADP and ATP would stimulate succinic oxidase in the presence of dinitrophenol. They suggested that the simulation of succinic oxidase under these conditions was due to the removal by ATP of oxaloacetate, a known inhibitor of succinate dehydrogenase; ADP would only become active after conversion to ATP by residual oxidative phosphorylation or by the action of adenylate kinase (Bonner, 1973). Drury *et al.* (1968) reported that the stimulation of electron transport by adenine nucleotides in the presence of dinitrophenol was not sensitive to oligomycin and concluded that it was not necessary to convert ADP into ATP in order to relieve the inhibition of the succinate dehydrogenase. Recent observations (Oestreicher *et al.*, 1973) have shown that the succinic dehydrogenase from plant mitochondria can be activated both by ATP and by ADP, thus explaining why it was unnecessary to convert ADP into ATP in order to bring about the stimulation.

Succinate is not the only substrate whose oxidation failed to respond predictably to the addition of uncoupling agents. The rate of oxidation of NAD^+-linked substrates has often been found to be slow in the presence of uncoupling agents (Ikuma & Bonner, 1967). Laties (1973) showed that the uncoupled rate of oxidation of citrate, pyruvate-plus-malate or succinate-plus-pyruvate could be stimulated by ADP; he found the stimulation by ADP was insensitive to oligomycin and concluded that ADP was directly involved in bringing about 'uncoupler effectiveness'. Laties (1973) could find no evidence that either ATP or AMP had a similar effect in activating the uncoupled rate of oxidation, thus his conclusions appear to be in conflict with those of Bonner (1973) who stated 'the mitochondria must have had a small amount of ATP in order for the uncoupler to be effective'.

The papers discussed so far suggest that ADP stimulates oxidation in the presence of uncoupling agents. Observations have also been made which suggest that ADP could stimulate electron flow in the absence of uncoupling agents, other than by acting as a phosphate acceptor in oxidative phosphorylation. Raison *et al.* (1973*b*) have described the phenomenon of 'conditioning' in plant mitochondria. The main characteristic of this phenomenon was that, under certain conditions, the rate of oxidation in the presence of ADP, obtained in response to the first limited addition of ADP, was markedly lower than that obtained in response to subsequent additions of ADP. Raison *et al.* (1973*a*) showed that if mitochondria were incubated with substrate and ADP in the absence of phosphate, the rapid rate of oxidation initiated by the addition of phosphate was consider-

ably higher than if the mitochondria were incubated with substrate and phosphate and the oxidation initiated by the addition of ADP. Thus they concluded that incubation of mitochondria with ADP under non-phosphorylating conditions resulted in 'conditioning'. Incubation in presence of ATP did not have a similar effect.

Laties (1973) concluded that the ability of ADP to stimulate the uncoupled rate of oxidation was not dependent on its role in bringing about partial conditioning and thus he considered that ADP had two separate roles in stimulating non-phosphorylating electron flow in plant mitochondria. The purpose of the experiments described in the present paper was to examine the influence of adenine nucleotides on the non-phosphorylating rate of electron transport in Jerusalem-artichoke mitochondria and to attempt to establish where in the metabolic sequence they had their effect.

Materials and Methods

Chemicals

NADH, ATP, ADP, AMP and glutamate–oxaloacetate transaminase (EC 2.6.1.1) were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; malate, pyruvate, glutamate, oxoglutarate, citrate, malonate, thiamin pyrophosphate, GMP, IMP and oligomycin were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.; sucrose, succinate, EDTA, $MgCl_2$ and potassium arsenite from BDH Chemicals, Poole, Dorset, U.K.; Mops [3-(*N*-morpholino)-propanesulphonic acid], Tes [*N*-tris(hydroxymethyl)-methyl-2-aminoethanesulphonic acid] and KH_2PO_4 from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and bongkrekic acid were generous gifts from Dr. P. G. Heytler and Dr. H. C. Passam respectively.

Isolation of mitochondria

Jerusalem-artichoke mitochondria were isolated from the tubers by using the method described by Palmer & Kirk (1974). Rat liver mitochondria were isolated from a 200g young adult male Sprague–Dawley rat by using the method described by Weinbach (1961).

Activation of succinate dehydrogenase

Mitochondria were incubated at 26°C for 4min with 20μl of 50mM-ATP, pH7.2, 20μl of 50mM-P_i (both amounts are per mg of mitochondrial protein) and $MgCl_2$ to a final concentration of 5mM. After this activation procedure the mitochondria were kept in an ice bath and the succinate oxidase remained active through the course of each experiment. This method was that of Cowley (R. C. Cowley, personal communication).

Oxygen consumption

Oxygen uptake was measured in a small Rank oxygen electrode (Rank Bros., Bottisham, Cambridge, U.K.) with an internal diameter of 9mm, in conjunction with a Servoscribe RE 511.20 potentiometric recorder. Reactions were carried out at 25°C. The standard reaction mixture consisted of 0.3M-sucrose, 5mM-potassium phosphate, 2.5mM-$MgCl_2$ and 5mM-Tes at pH7.2. Mitochondrial suspension was added to give a final volume of 1ml in the electrode.

Measurement of the levels of reduction of cytochrome b and nicotinamide nucleotide

Measurement of the levels of reduction of cytochrome *b* and nicotinamide nucleotide was carried out in an Aminco DW-2 dual-wavelength spectrophotometer, with a light-path of 1cm at 432 and 340nm, with 410 and 374nm respectively as reference wavelengths. The mitochondria were suspended in the same reaction medium as that used in the oxygen electrode so that the final volume in the cuvette was 1 or 3ml. Other additions were as indicated in the legends to the Figures.

Protein determination

Mitochondrial protein was determined by the method of Lowry *et al.* (1951) after first solubilizing the protein with deoxycholate; bovine serum albumin was used as standard.

Results

Investigation of succinate oxidase in uncoupled Jerusalem-artichoke mitochondria confirmed the findings of previous authors (Wiskich & Bonner, 1963; Oestreicher *et al.*, 1973) that it was activated by both ATP and ADP but not by AMP. This stimulation appeared to be different from that occurring with NAD^+-linked substrates reported in the present paper. All assays of succinate oxidase activity reported in the present study were carried out with activated mitochondria, as described in the Materials and Methods section; mitochondria used with other substrates were not activated. The oxygen-electrode traces in Fig. 1 show that when mitochondria were supplied with either added NADH or succinate as the electron donor, the rate of oxygen uptake in the presence of the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was equal to or

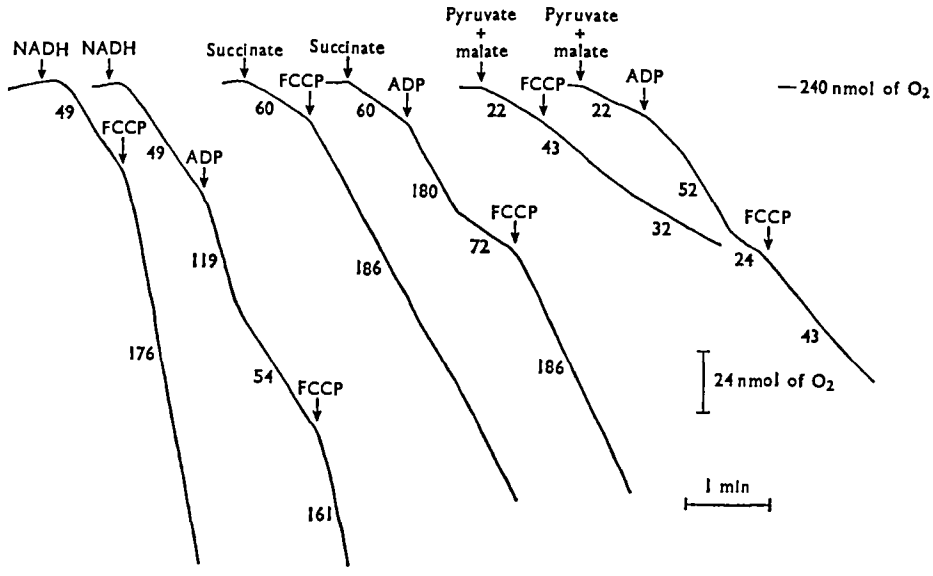

Fig. 1. *Influence of the uncoupler carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) on the rate of oxidation*

Oxygen-electrode traces showing the effect of the uncoupler (02/μM) on the rate of oxidation of NADH (1 mM), succinate (10mM) or pyruvate (15mM) plus malate (1 mM). When pyruvate-plus-malate were used as the substrate, 170μg of thiamin pyrophosphate was included in the reaction medium. The mitochondria were suspended in a total reaction volume of 1.0ml and the respiration was stimulated by adding 100nmol of ADP or the uncoupler (0.2/μM). For the oxidation of NADH or pyruvate-plus-malate, 1 mg of mitochondrial protein was used per assay, and 0.4mg was used for succinate oxidation. Values under the traces are the rates of oxygen consumption in nmol of O_2/min per mg of protein.

greater than that obtained in the presence of ADP. The response was of equal magnitude if the uncoupler was added before ADP or after the exhaustion of a pulse of ADP (i.e. state 4 as defined by Chance & Williams, 1956). In contrast the oxidation of pyruvate-plus-malate was not fully stimulated by the uncoupler; this was especially apparent if the uncoupler was added before ADP. The rate of oxidation of pyruvate-plus-malate in the presence of the uncoupler was characterized by an initial fairly rapid phase followed by a second, more prolonged, slower phase. The addition of ADP or AMP 1 min after the uncoupler, i.e. in the early stages of the slow phase, resulted in a marked stimulation of the uncoupled rate. The addition of ATP was without effect. Fig. 2 shows that AMP was apparently more efficient than ADP in bringing about the stimulation. Parallel studies showed that GMP or IMP did not have a similar effect. Studies using oligomycin (Fig. 3) and bongkrekic acid (Table 1) showed that whereas these compounds readily inhibited the rate of oxidation in the presence of ADP (i.e. state 3 as defined by Chance & Williams, 1956) they were completely without effect on the adenine nucleotide-enhanced rate in the presence of the uncoupler.

The effect of AMP on the uncoupled rate of oxidation of other substrates is given in Table 2. Inhibitors were added where necessary to prevent the further oxidation of the products of the principal reaction from complicating the result. Stimulation was only observed when substrates using the endogenous nicotinamide-nucleotide pool were supplied.

In some of our preparations we observed the phenomenon of 'conditioning' (already defined in the introduction) described by Raison *et al.* (1973*b*). When conditioning was apparent we noticed that the rate of oxygen uptake obtained in the presence of substrate and no added adenine nucleotide was very much lower than the subsequent rate in state 4. This is apparent in trace (*a*), Fig. 4. The slow pre-ADP rate could be stimulated to a rate similar to that in state 4, in the presence of oligomycin, either by ADP or by AMP (Fig. 4). When succinate or exogenous NADH were supplied as the substrate the pre-ADP rate (hereafter referred to as the 'substrate rate') was not significantly different from the rate in state 4, and neither AMP nor ADP had any effect when added after oligomycin.

Attempts were made to find the locus of the stimulation brought about by AMP or ADP in the

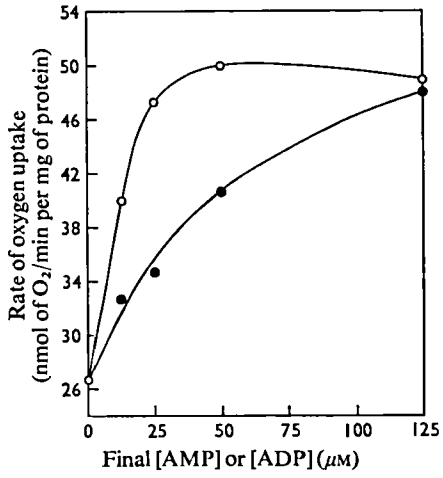

Fig. 2. *Stimulation of the uncoupled rate of oxidation of pyruvate-plus-malate by different concentrations of adenine nucleotides*

The rates of oxygen uptake were calculated from oxygen-electrode traces. Mitochondrial protein (1 mg) was suspended in 1.0 ml reaction volume; the substrate added was pyruvate (15 mM) plus malate (1 mM) and 170 μg of thiamin pyrophosphate. The uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (0.2/μM) was added 1 min after the substrate and appropriate amounts of ADP (●) or AMP (○) were added 1 min after the uncoupler. The rate in the presence of uncoupler and absence of adenine nucleotide was taken to be that immediately before adding the nucleotide.

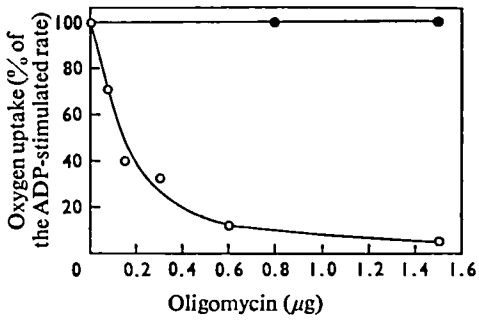

Fig. 3. *Influence of oligomycin on the ADP-stimulated oxidation of pyruvate-plus-malate in the presence or absence of the uncoupler carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP)*

Jerusalem-artichoke mitochondria (1 mg of protein) were suspended in 1.0 ml reaction volume containing pyruvate (15 mM) plus malate (1 mM) and 170 μg of thiamin pyrophosphate. When measuring the control treatment (○) 125 nmol of ADP was added 2 min after the substrate and the appropriate amount of oligomycin added once a linear state-3 rate had been obtained. When measuring the effect in the presence of the uncoupler (0.2/μM) (●) the uncoupler was added 1 min after the substrate and the ADP (125 nmol) was added 1 min after the uncoupler. The 100% values found in this experiment were 56 nmol of O_2/min per mg of protein for the control and 35 nmol of O_2/min per mg of protein in the presence of the uncoupler.

Table 1. *Effect of bongkrekic acid (BA) on the stimulation of mitochondrial protein by AMP*

The rates of oxygen uptake of Jerusalem-artichoke mitochondria were measured polarographically in a 1 ml-volume electrode. Mitochondria were suspended in reaction medium containing 0.3 M-sucrose, 5 mM-Tes, 5 mM-phosphate and 2.5 mM-$MgCl_2$ at pH 6.8 in the electrode for 10 min with or without bongkrekic acid at 2.5 nmol/mg of protein. The substrates were added to start respiration. In Expt. 1, 100 nmol of AMP was added to bring about state-3 rates and the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (0.1/μM) was added in state 4. In Expt. 2, AMP was added 1 min after the uncoupler, and the uncoupled rates reported are those immediately before additions of AMP. For NADH oxidation, 0.43 mg of mitochondrial protein was used per assay and 0.86 mg for malate oxidation. The 'substrate rate' is that produced when substrate was added to mitochondria before the nucleotide addition.

		O_2 uptake (nmol/min per mg of protein)						
		Expt. 1				Expt. 2		
Substrate		Substrate rate	State 3	State 4	Uncoupled (FCCP)	Substrate rate	Uncoupled rate	Uncoupled+ AMP
1.2 mM-NADH	−BA	75	156	85	188	75	206	206
	+BA	61	61	—	160	60	157	154
25 mM-Malate (potassium salt) +10 mM glutamate +20 μg glutamate–oxaloacetate transaminase	−BA	52	83	43	94	55	85	97
	+BA	37	43	—	81	36	40	62

Table 2. *Influence of AMP on the uncoupled rate of oxidation of various substrates*

Substrates at the specified final concentrations were added to Jerusalem-artichoke mitochondria suspended in 1.0ml of reaction mixture; where indicated, thiamin pyrophosphate (TPP) or inhibitors were added before the reaction was started. In each assay 1 mg of mitochondrial protein was used, except when measuring the oxidation of succinate when 0.4mg was used. The uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) (0.2/μM) was added 1min after the substrate and AMP (75μM) was added 1 min after the uncoupler. When NAD^+-linked substrate was used, the rate of oxygen uptake in the absence of uncoupler or in the presence of the uncoupler and AMP was approximately linear with time. The rate in the presence of the uncoupler alone often decreased with time and the rate quoted was that obtained immediately before the addition of AMP.

Substrate	Rate of oxygen uptake (nmol of O_2/min per mg of protein)		
	−FCCP	+FCCP	+FCCP+AMP
1mM-NADH	49.2	174.4	174.4
10mM-Succinate	60	186	186
15mM-Pyruvate+1mM-malate+170μg of TPP	22	26.2	50.3
10mM-2-Oxoglutarate+170μg of TPP	7.7	11.6	57.9
10mM-2-Oxoglutarate+170μg of TPP+0.5mM-malonate	6.8	6.8	27
10mM-Citrate	15	26	39
10mM-Citrate+1mM-arsenite	—	26	39
100mM-Malate+170μg of TPP	12	29	57
100mM-Malate+170μg of TPP+1.5mM-arsenite	12	18	34.7

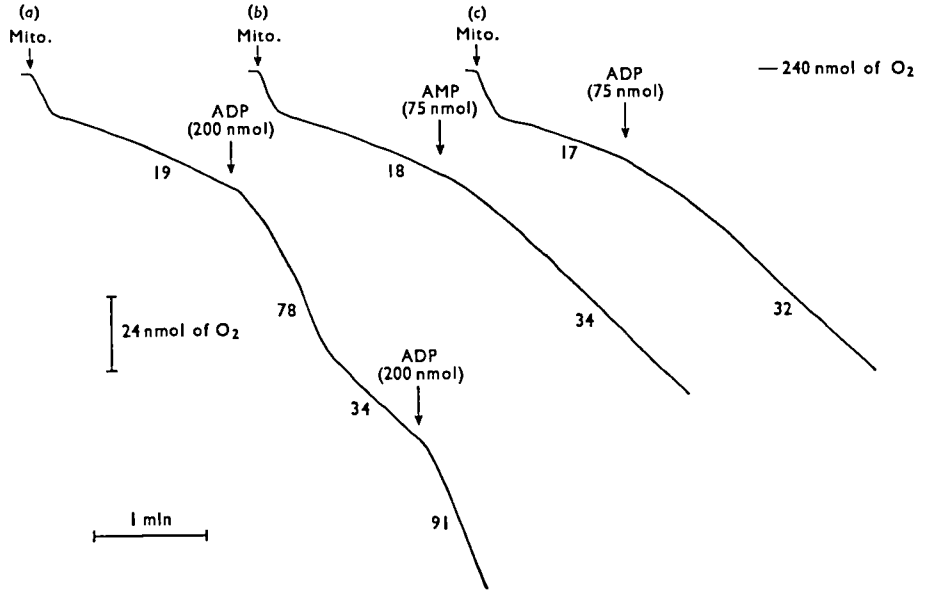

Fig. 4. *Influence of adenine nucleotides on the rate of oxidation in presence of oligomycin and absence of carbonyl cyanide p-trifluoromethoxyphenylhydrazone*

Jerusalem-artichoke mitochondria (Mito.) (1 mg of protein) were added to 1.0ml of reaction mixture containing pyruvate (15mM) plus malate (1mM) and 170μg of thiamin pyrophosphate. Oligomycin was added when necessary at the rate of 1.2μg per mg of protein. The respiration was then stimulated by adding either ADP or AMP as indicated. The values under the oxygen-electrode traces are the rates of oxygen consumption in nmol/min per mg of protein. (*a*) Control; (*b*) and (*c*), +oligomycin.

electron-transport chain. Measurements of the level of reduction of cytochrome *b* (Fig. 5) revealed the unexpected observation that the addition of pyruvate-plus-malate to Jerusalem-artichoke mitochondria failed to reduce cytochrome *b* to any great extent (trace *b*), whereas in a similar experiment using rat liver mitochondria (trace *a*) the same substrate resulted in a marked reduction of cytochrome *b*. The

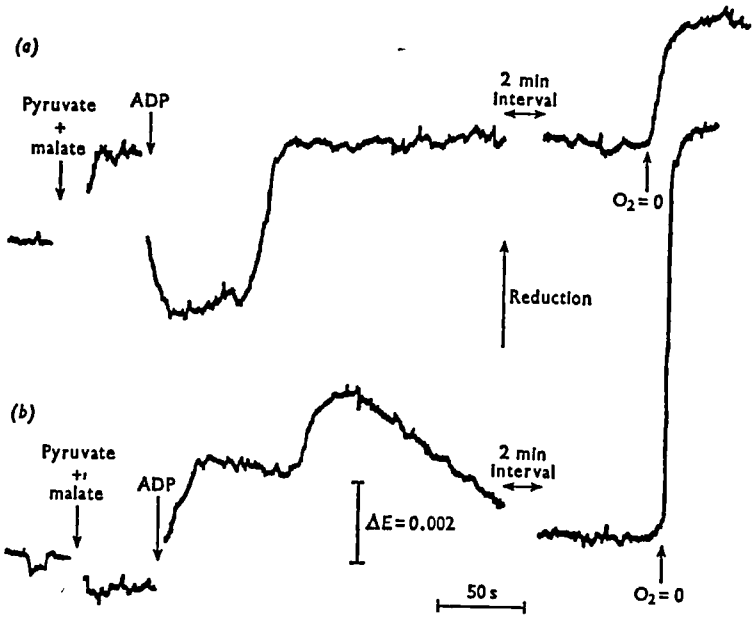

Fig. 5. *Reduction of cytochrome b by pyruvate-plus-malate in either rat liver (a) or Jerusalem-artichoke (b) mitochondria*

Trace (*a*), rat liver mitochondria (100μl containing 3mg of protein) were added to the cuvette containing 2.9ml of reaction medium (0.1M-KCl, 0.025M-sucrose, 0.01M-$MgCl_2$, 0.001M-EDTA, 510μg of thiamin pyrophosphate, 0.005M-Tes and 0.01M-KH_2PO_4 at pH6.8). The additions during the experiment were pyruvate (15mM) plus malate (1mM) and ADP (0.5μmol). Reduction of cytochrome *b* was measured in an Aminco DW-2 dual-wavelength spectrophotometer at 432nm (with 410nm as the reference wavelength) a band-pass of 1nm and light-path of 1cm. Trace (*b*), Jerusalem-artichoke mitochondria (100μl containing 2.5mg of protein) were added to 2.9ml of standard reaction mixture, as defined in the Materials and Methods section, plus 510μg of thiamin pyrophosphate. The additions and conditions of measurement were as defined for trace (*a*). Increase in absorbance indicates reduction of cytochrome *b*.

addition of ADP to the two types of mitochondria had markedly different effects on cytochrome *b*, whereas the rate of oxygen uptake in both types increased. It resulted in oxidation of cytochrome *b* in rat liver mitochondria, presumably because respiratory control was released, allowing electrons to flow out from cytochrome *b*. In Jerusalem-artichoke mitochondria the response was exactly opposite, suggesting that ADP allowed electrons to flow more readily into cytochrome *b*. Once the ADP had become converted into ATP by oxidative phosphorylation, respiratory control was imposed and cytochrome *b* became further reduced as expected. In these mitochondria, cytochrome *b* could not be kept reduced long after the exhaustion of ADP; the absorbance slowly decreased until oxygen was all consumed and the cytochrome became fully reduced (Fig. 5). In rat liver mitochondria the level of reduction of cytochrome *b* was constant in state 4 until it became further reduced when oxygen was exhausted. Fig. 6 shows the changes in the level of reduction of cytochrome *b* when either ADP or AMP was added in the absence or presence of oligomycin. The addition of both ADP and AMP resulted in the rapid reduction of cytochrome *b* in the presence of oligomycin, indicating that the enhanced flow of electrons into cytochrome *b* was independent of oxidative phosphorylation. In the absence of oligomycin the response to AMP was more complex than the response to ADP. AMP caused a more rapid and more extensive reduction of cytochrome *b* than did ADP. The slow oxidation to the state-3 level which followed this initial rapid phase of reduction could be due to the conversion of AMP into ADP via endogenous adenylate kinase and ATP. The ADP thus produced could then act as a phosphate acceptor in oxidative phosphorylation. If the experiment was repeated in the presence of oligomycin, but in the absence of Mg^{2+} (Fig. 7), only AMP was capable of promoting the reduction of cytochrome *b*, thus it seemed as if AMP was primarily responsible for promoting electron flow into cytochrome *b*, and ADP was only active if converted into AMP via the enzyme adenylate kinase.

To establish the mechanism by which AMP induced the reduction of cytochrome *b* and to

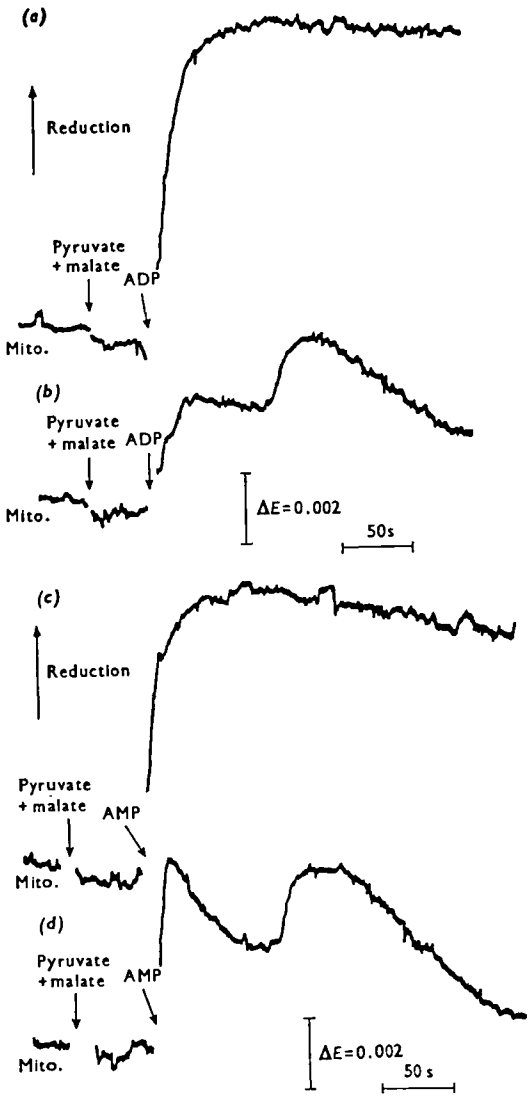

Fig. 6. *Effect of oligomycin and adenine nucleotides on the reduction of cytochrome b*

Jerusalem-artichoke mitochondria (Mito.) (100μl containing 2mg of protein) were added to the cuvette containing 2.9ml of standard reaction medium plus 510μg of thiamin pyrophosphate. The amount of oligomycin, when present (traces *a* and *c*), was 2.5μg per cuvette. Additions made during the experiments were pyruvate (15mM) plus malate (1mM) and 0.5μmol of ADP (traces *a* and *b*) or AMP (traces *c* and *d*). Other conditions of assay were as described in Fig. 5.

investigate whether this phenomenon was related to the stimulation of electron transport by AMP in the presence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, simultaneous measurements of the level of reduction of endogenous nicotinamide nucleotide and cytochrome *b* were made. The traces of both components shown in Fig. 8 were obtained during consecutive runs using malate in the presence of glutamate, and glutamate–oxaloacetate transaminase to remove oxaloacetate. This substrate system ensured the efficient operation of the malate dehydrogenase (EC 1.1.1.37) which is believed not to be allosterically regulated by adenine nucleotides. The experiment was also carried out in the presence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and oligomycin to prevent any interaction between the phosphorylating system and the redox components. The traces (Fig. 8) show that the addition of AMP resulted in the immediate reduction of cytochrome *b* and oxidation of the nicotinamide nucleotide.

Discussion

Results presented in the present paper suggest that the ability of adenine nucleotides to stimulate the oxidation of succinate or NAD^+-linked substrates in the presence of uncoupling agents is the result of different mechanisms. The oxidation of succinate could be stimulated by ATP and ADP or partially stimulated by using glutamate and glutamate–oxaloacetate transaminase. Thus it would appear that the stimulation resulted from the removal of an inhibition caused by oxaloacetate. The oxidation of NAD^+-linked substrates was stimulated by ADP or AMP whereas ATP was ineffective. All of our results point to AMP and not ADP being primarily responsible for activating the uncoupled rate of pyruvate oxidation. First, AMP was effective at lower concentrations than ADP; second, the rate of reduction of cytochrome *b* was more rapid when induced by adding AMP than by ADP. Finally the most compelling evidence was that ADP was less effective in stimulating respiration and reducing cytochrome *b* if Mg^{2+} was omitted from the medium. The lack of Mg^{2+} would prevent the activity of adenylate kinase from converting ADP into AMP. These observations suggest that ADP had to be converted into AMP before it was able to activate the uncoupled flow of electrons from NAD^+-linked substrates. We cannot explain why our observations differ from those of Laties (1973), who reported that AMP would not stimulate in the presence of uncoupler. We repeated the experiments by using potato (*Solanum tuberosum*) mitochondria, as used by Laties, but nevertheless found AMP active. However, it seems significant that Laties (1973) found that the activation by ADP was second-order with respect to the concentration of ADP; thus the adenylate kinase enzyme system may also be active in bringing about the stimulation under the conditions he used.

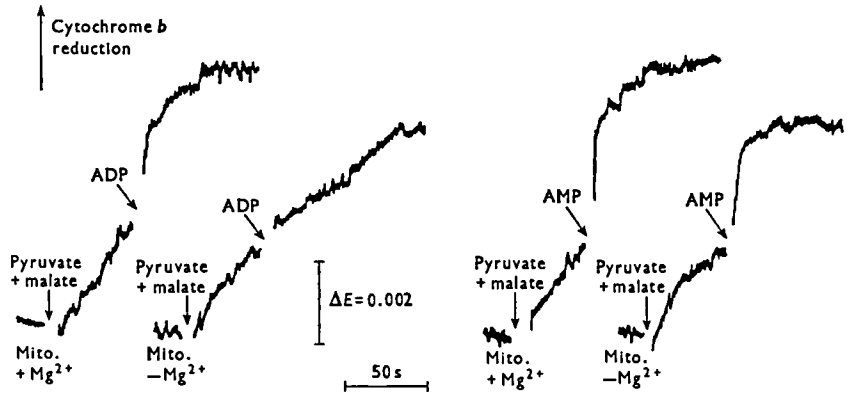

Fig. 7. *Effect of Mg^{2+} and adenine nucleotides on the reduction of cytochrome b*

Jerusalem-artichoke mitochondria (Mito.) (100 μl containing 2 mg of protein) were added to the cuvette containing 2.9 ml of magnesium-free reaction medium containing 510 μg of thiamin pyrophosphate and 2.5 μg of oligomycin. The concentration of Mg^{2+} added was 2.5 mM. Additions made during the experiment were pyruvate (15 mM) plus malate (1 mM) and ADP or AMP (0.5 μmol). Other conditions of assay were as described in Fig. 5.

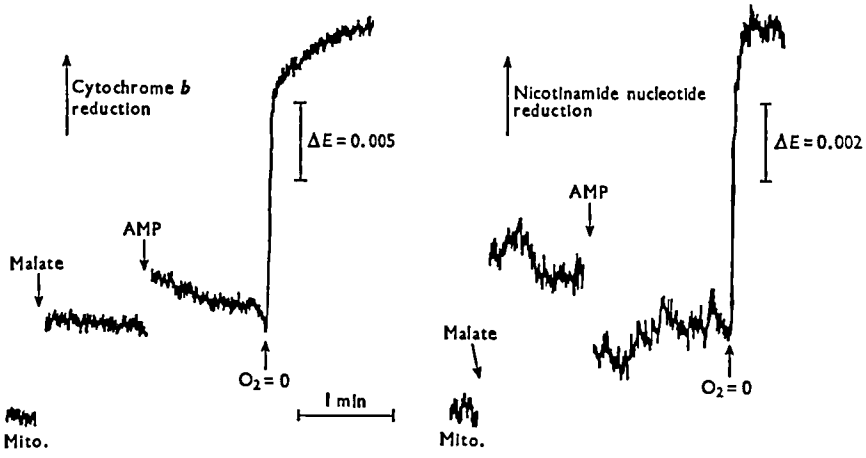

Fig. 8. *Effect of AMP on the reduction of cytochrome b and endogenous nicotinamide nucleotide of Jerusalem-artichoke mitochondria oxidizing malate*

In a 1 ml cuvette with 1 cm light-path, mitochondria (Mito.) (2.2 mg of protein) were suspended in standard reaction mixture plus 10 mM glutamate, 20 μg of glutamate–oxaloacetate transaminase, 3 μg of oligomycin and 0.1/μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. The reduction of cytochrome *b* was followed at 432 nm with 410 nm as the reference wavelength. The reduction of endogenous nicotinamide nucleotide was followed at 340 nm with 3.0 nm slit width and 374 nm as the reference wavelength in an Aminco DW-2 dual-wavelength spectrophotometer (Chance & Williams, 1956).

Our results showed that carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone uncouple oxidative phosphorylation from electron transport efficiently when either succinate or exogenous NADH was supplied as the electron donor. Since electrons from both substrates only traverse two terminal sites of oxidative phosphorylation (Coleman & Palmer, 1972) it seems that the uncoupler was capable of uncoupling both these sites of oxidative phosphorylation. If AMP is therefore to bring about the stimulation of oxidation of NAD^+-linked substrates by increasing uncoupler effectiveness, as suggested

by Laties (1973), its effect must be restricted to the first site of phosphorylation, associated with the NADH dehydrogenase. Such a possibility seems unlikely because it is thought that the non-phosphorylated high-energy state associated with each coupling site is ultimately in equilibrium with a common non-phosphorylated high-energy state and therefore efficient uncoupling at one site results in uncoupling at all other sites. It therefore seems more likely to us that AMP is necessary, not to bring about uncoupling, but to have the uncoupled state reflected in an enhanced flux of electrons along the respiratory chain. The observation that addition of AMP resulted in the simultaneous oxidation of the nicotinamide nucleotide pool and reduction of cytochrome *b* is consistent with this theory. It also rules out the alternative theory that the stimulation was the result of the allosteric activation of the NAD^+-linked dehydrogenase, thereby causing the stimulation by producing more endogenous NADH. The simultaneous oxidation of the nicotinamide nucleotide and reduction of cytochrome *b* by AMP indicate the existence of some form of accessibility barrier (Lee & Slater, 1972; Lambowitz & Bonner, 1974) between these components which is lowered by the addition of AMP. Studies using piericidin A [which inhibits electron transfer between NADH and cytochrome *b* (Jeng *et al.*, 1968)] and antimycin A [which inhibits between cytochrome *b* and *c* (Chance & Williams, 1956)] show that the cytochrome *b* reduced on the addition of AMP was reduced via a piericidin A-sensitive pathway and oxidized via an antimycin A-sensitive pathway, providing evidence that it was a normal member of the electron-transport chain. We thus conclude that the activation of the uncoupled rate of oxidation of NAD^+-linked substrates by AMP was the result of activation of electron flow through the piericidin A-sensitive NADH dehydrogenase.

We have attempted to demonstrate AMP activation directly by showing that the oxidation of exogenous NADH by submitochondrial particles, the polarity of whose membranes is reversed, was stimulated by AMP. This experiment failed because AMP appears to act on the outside of the inner membrane. It is thought that AMP cannot enter intact mitochondria using the normal adenine-nucleotide translocator (Klingenberg, 1970), thus any stimulation of electron flow caused by AMP, and not ADP, resulting from the action of adenylate kinase situated in the intermembrane space, has to result from AMP interacting on the outside of the inner membrane. This situation is confirmed by the data in Table 1, which show that the state-3 rate of oxidation, resulting from the formation of ADP from AMP, was inhibited by bongkrekic acid whereas the stimulation of the uncoupled rate of malate oxidation was unaffected by bongkrekic acid. Since bongkrekic acid has been shown to inhibit the adenine-nucleotide translocator in Jerusalem-artichoke mitochondria (Passam *et al.*, 1973) it seems as if AMP does not have to enter the mitochondria to bring about the enhancement of electron flow. This observation could provide the basis of an interesting physiological control mechanism whereby the AMP concentration in the cytosol could regulate the rate of oxidation of NADH inside the inner mitochondrial membrane.

We thank Mrs Jill Farmer for her skilled technical assistance. This research was supported by grants from the Science Research Council, The Royal Society and the Central Research Fund, University of London. R. S. acknowledges the receipt of a Royal Thai Government Scholarship.

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