STUDIES ON ELECTRON TRANSPORT AND ENERGY-LINKED REACTIONS IN BEEF HEART MITOCHONDRIA AND ESCHERISCHIA COLI

A thesis submitted to the University of Warwick in partial fulfilment for the degree of Doctor of Philosophy

by

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To my wife

The work described in this thesis was carried out by myself in the School of Molecular Sciences at the University of Warwick, between September 1966 and September 1968, under the supervision of Dr. D. E. Griffiths.

A. J. Sweetman

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ABBREVIATIONS

Abbreviations used are as described in The Biochemical Journal - Policy of the Journal and Instructions to Authors (Revised 1970). London: The Biochemical Society.

Exceptions are:-

piericidin	-	piericidin A
3H piericidin	-	3H piericidin A

ABSTRACT

The work described in this thesis was concerned primarily with the possible role of quinones in mitochondrial and bacterial reactions. In this respect electron transport and energy-linked reactions were both examined and the major sources of material used for this purpose were beef heart and Escherischia coli. Various approaches were utilized namely, studies with inhibitors, extraction-reactivation experiments, ultraviolet irradiation techniques and quinone deficient mutants.

An extensive study was made of the effect of piericidin A, which had been proposed as a ubiquinone analogue, on various bacterial and mitochondrial reactions. It was shown that piericidin was acting at the same site as rotenone in the NADH dehydrogenase region of the respiratory chain of beef heart mitochondria. The possible nature, concentration and specificity of this site was examined. At high concentrations piericidin also inhibited succinate oxidation, possibly due to damage of the mitochondrial membrane system. The effect of piericidin on various other mitochondrial and bacterial reactions was also described. Of particular interest was the inhibition by piericidin of the energy-dependant reduction of $NADP^+$ by NADH in both beef heart submitochondrial particles and small particles derived from E. coli.

The ATP-dependant reduction of $NADP^+$ by NADH and the ATP-dependant reduction of NAD^+ by succinate catalysed by E. coli small particles were both fully characterised.

Extraction techniques were used for studying electron transport and energy-linked reactions in beef heart submitochondrial particles. Reactivation of NADH oxidation, succinate oxidation and the energy-linked reduction of $NADP^+$ by NADH to pentane extracted particles was achieved by the addition of ubiquinone homologues at concentrations equal to those originally present in the particles. The reactions had the same sensitivity to inhibitors of electron transport and oxidative phosphorylation as the normal reactions in unextracted particles.

Extraction techniques, ultraviolet irradiation and ubiquinone deficient mutants were used to study the role of quinones in electron transport and energy-linked reactions in E. coli. The results obtained were discussed fully in Chapter VI.

CONTENTS

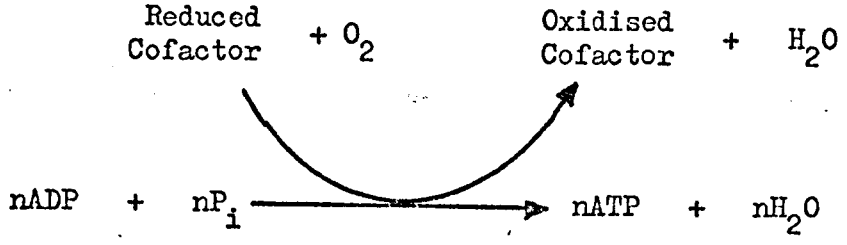
CHAPTER I.

THE RESPIRATORY CHAIN AND OXIDATIVE PHOSPHORYLATION.

INTRODUCTION.

Oxidative phosphorylation describes the process whereby energy derived from breakdown of intracellular substrates is conserved through ATP formation within the mitochondria. For example 90% of the energy made available in the complete oxidation of glucose is derived from oxidative phosphorylation. The process is of paramount importance in biological systems because ATP is the primary source of energy in a large number of processes such as muscle contraction, ion transport and various anabolic reactions.

The reduced cofactors NADH and $FADH_2$ produced by the oxidation of fatty acids and citric acid cycle intermediates are oxidised via a series of redox components called the respiratory chain to give oxidised cofactors and water. The significance of this is that there are large free energy changes associated with the oxidations, which are used to synthesise ATP from ADP and inorganic phosphate by the process of oxidative phosphorylation. According to the scheme, ATP formation is



said to be coupled to the oxidation - reduction reactions of the respiratory chain. More than one ATP molecule may be formed for each molecule of cofactor oxidised. Although this concept was established about thirty years ago the fundamental mechanism of energy coupling in the respiratory chain still remains unexplained. This is probably because the oxidative phosphorylation system is intimately associated with the mitochondrial membrane; it therefore defies the standard enzymological approaches that have been used to elucidate other metabolic pathways. Disruption of the

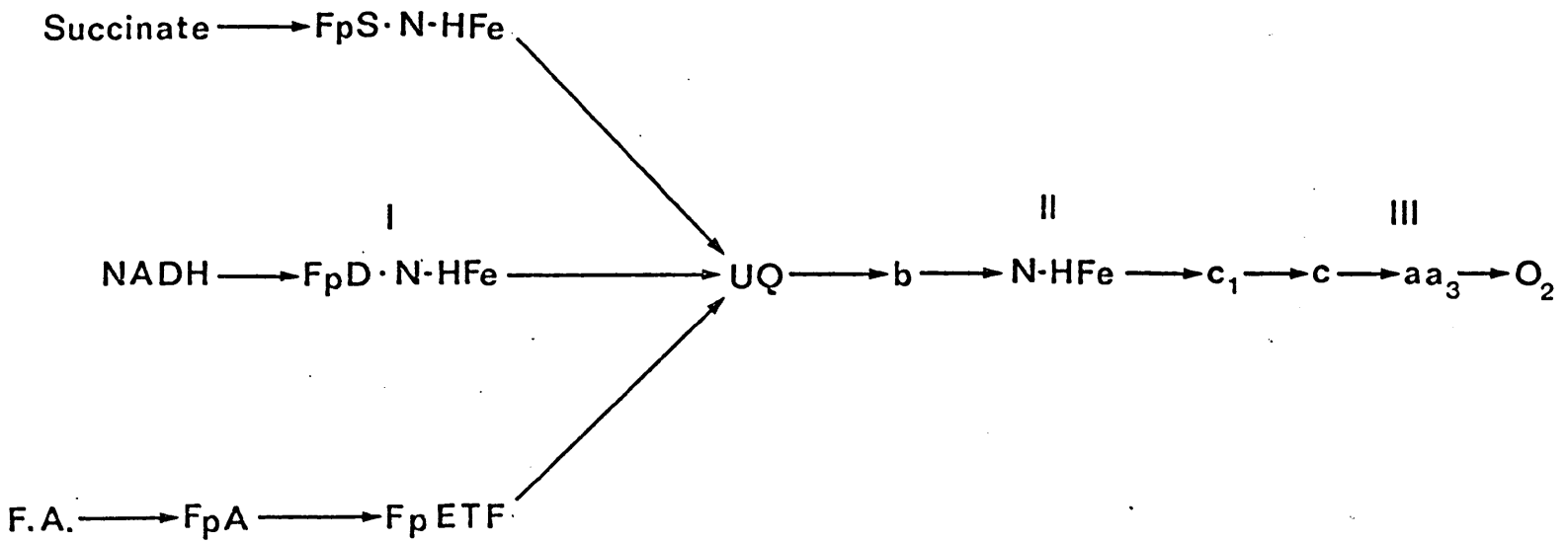


Fig. 1. THE RESPIRATORY CHAIN IN ANIMAL MITOCHONDRIA. Key: FpS - succinic dehydrogenase flavoprotein. FpD - NADH dehydrogenase flavoprotein. FpA - fatty acyl CoA dehydrogenase flavoprotein. FpETF - electron transfer flavoprotein. F.A. - fatty acyl CoA. N-HFe - nonhaem iron containing proteins. UQ - ubiquinone. b, c_1, c, aa_3 - cytochromes. I, II, III - proposed sites of ATP synthesis.

highly organised membrane structure usually results in a loss of phosporylative capacity, so indirect methods of study have to be used. In this connection sophisticated spectroscopic techniques, studies with inhibitors and extraction-reactivation experiments have all been employed. These techniques have resulted in the identification, and in some cases the isolation, of several new oxidation-reduction components, but the search for intermediates in the phosphorylation process has so far been unsuccessful. This has led us from a chemical intermediate hypothesis to two other attempts at explaining oxidative phosphorylation. These are the conformation change hypothesis and the chemiosmotic theory.

Various aspects of the respiratory chain and the three theories of oxidative phosphorylation will be discussed in the following chapter. More detailed consideration of other aspects of mitochondrial reactions are to be found in several recent reviews (1-16). The NADH dehydrogenase flavoprotein enzyme system, energy-linked reactions in mitochondria and bacteria, and the role of ubiquinone in mitochondrial and bacterial reactions are discussed in detail in the appropriate chapters of this thesis.

The Respiratory Chain.

Information with regard to the number and sequence of electron carriers is still incomplete and some of the components have not yet been identified. It is also not known which, if any, of the carriers participate directly in energy coupling. Fig. 1 shows current ideas as to the sequence and composition of the respiratory chain in animal tissues (15). The cytochromes form the backbone of the chain; they accept the reducing equivalents from a variety of substrates, each of which has its own specific dehydrogenase. The dehydrogenases are either flavoproteins, as in succinic dehydrogenase, or NAD-linked enzymes such as malic dehydrogenase. They are connected to the respiratory chain by two hydrogen carrying molecules, NAD and ubiquinone. Several techniques have been employed to determine the sequence shown in Fig. 1; these include measurements of the redox potentials of the individual components, kinetic determinations of the reaction sequence and studies on the donor and acceptor specificity of isolated fractions of the chain.

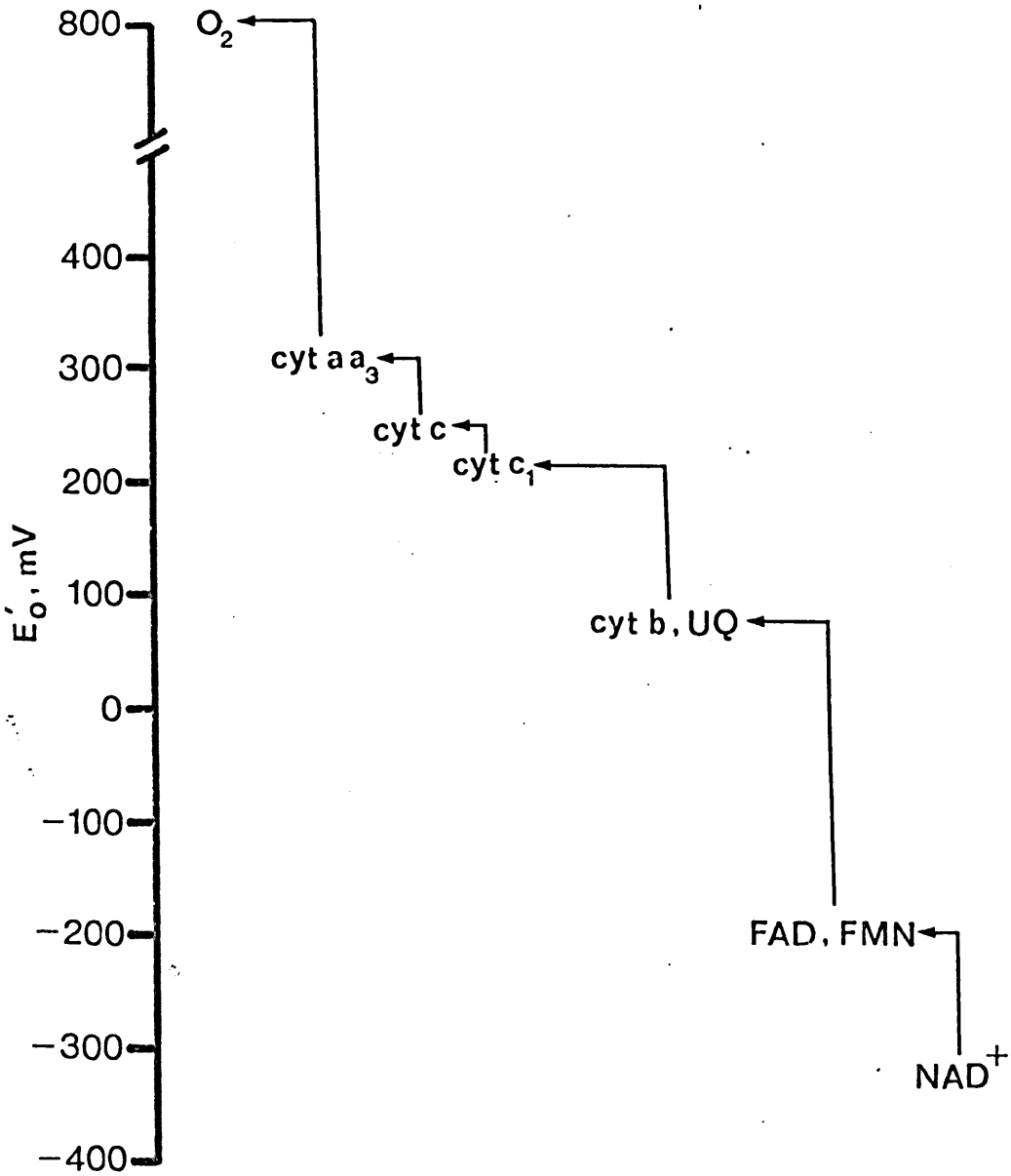


Fig. 2. REDOX POTENTIALS OF RESPIRATORY CARRIERS. Abbreviations as in Fig. 1.

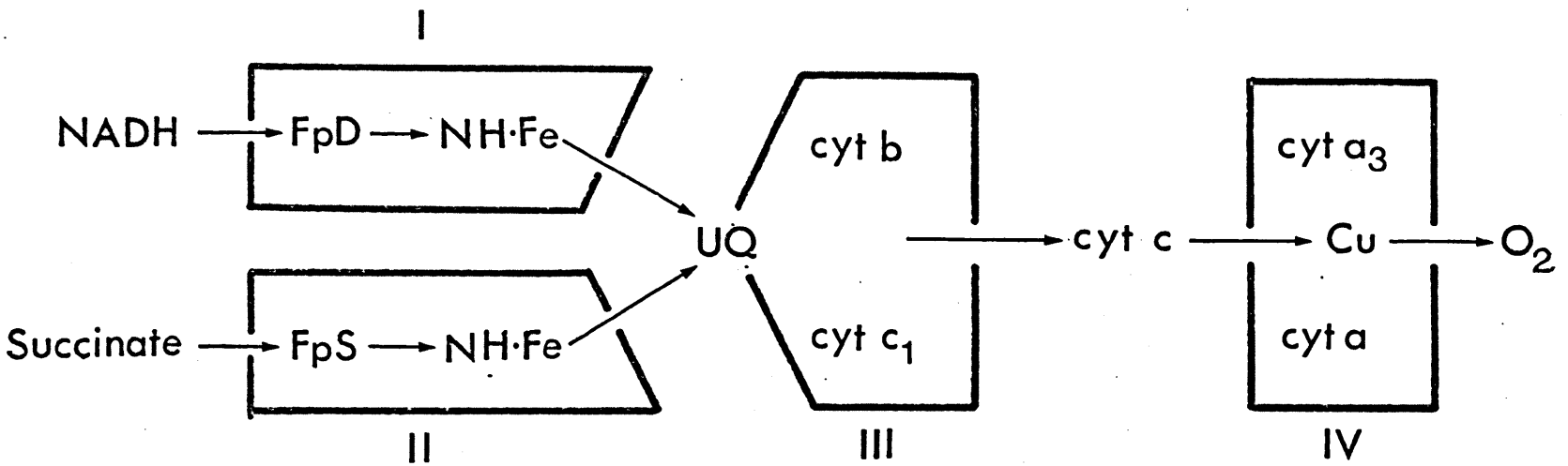


Fig. 3. STOICHIOMETRIC RECONSTRUCTION OF THE RESPIRATORY CHAIN. Adapted from Green and Oda (21). I, II, III, IV - represent the four complexes of the respiratory chain (see text).

The complete redox potential difference between NADH and oxygen is about 1100 mV, but it may be split up into small increments, because of the different redox potentials of the individual components (Fig. 2). When this was done then the sequence of carriers obtained followed very closely that shown in Fig. 1, except in the cytochrome b - ubiquinone region. This discrepancy may be because the redox potentials of the isolated molecules are markedly different to those occuring in the local environment of the respiratory chain.

The kinetics of oxidation of various components of the respiratory chain in rat liver mitochondria have been measured by improved stopped flow techniques (18). The results showed that all the cytochromes, except for cytochrome b, were rapidly oxidised by added oxygen. On the other hand flavoproteins and NADH were much slower to react. However, the reaction sequence obtained showed good agreement with sequences determined by other methods. The scheme outlined below shows the half times in msec for oxidation of respiratory carriers by 15μM oxygen at 25° in rat liver mitochondria (18).

$$O_2 \longrightarrow a_3 \longrightarrow a \longrightarrow c \longrightarrow c_1 \longrightarrow b \longrightarrow Fp \longrightarrow NAD$$

0.40 0.51 2.5 5.0 80 500 750

Further attempts at establishing the sequence of events have been carried out by fractionating the respiratory chain into individual enzyme complexes. Success in this direction has been achieved by Hatefi et al (19) and by King and Takemori (20). The four complexes of Hatefi, namely; NADH ubiquinone reductase, succinate ubiquinone reductase, ubiquinol cytochrome c reductase and cytochrome c oxidase were capable of recombining with a 1:1:1:1 stoichiometry (Fig. 3). The reconstituted respiratory chain was sensitive to the same compounds that specifically inhibited electron transfer in intact mitochondria (22,23). The interpretation placed on the findings was that there was a specific interaction between the individual complexes which resulted in a stoichiometric reorganisation of the respiratory chain. However, more accurate determinations of the molecular weights of the individual complexes showed

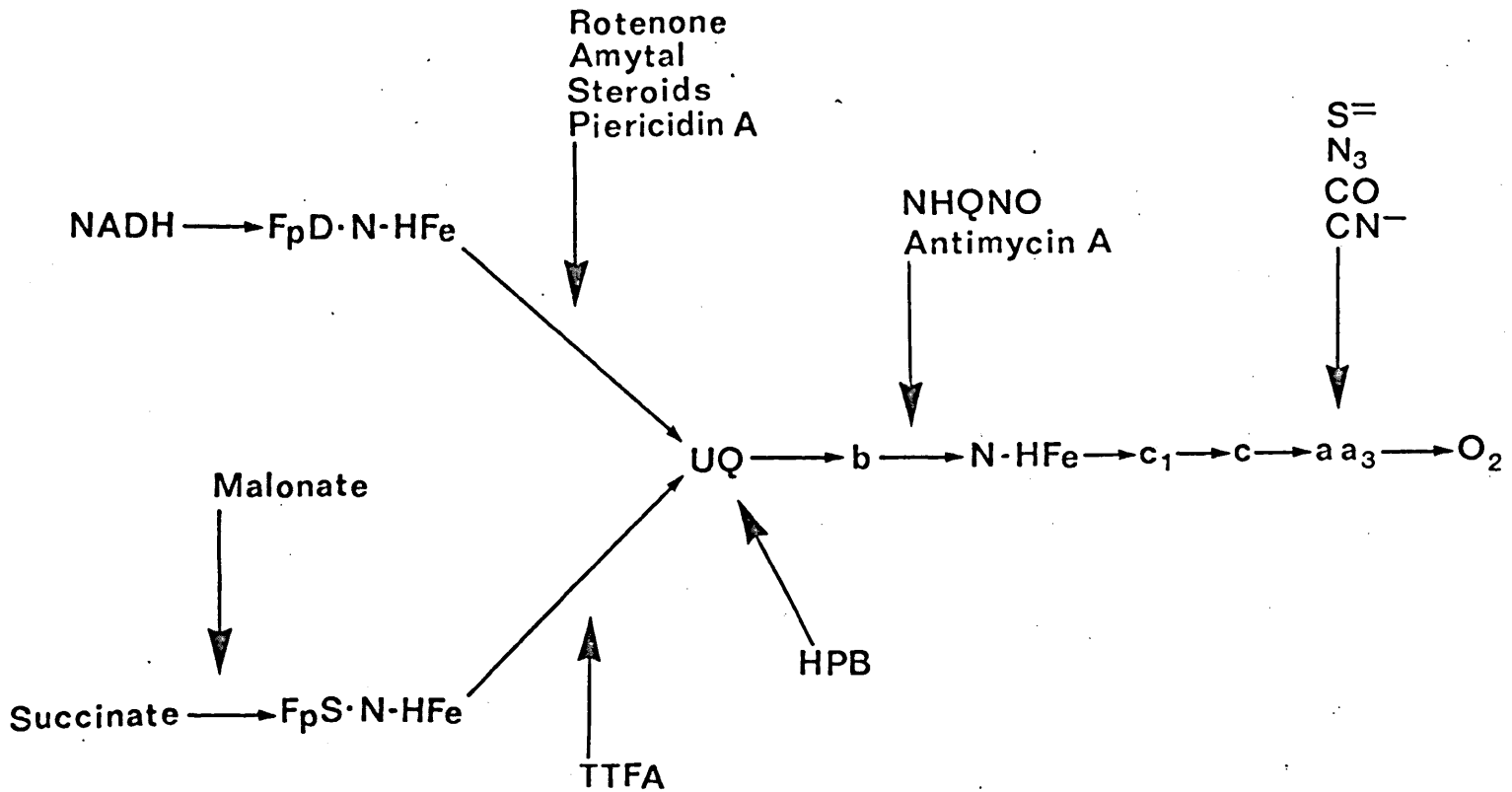


Fig. 4. SITE OF ACTION OF INHIBITORS IN THE RESPIRATORY CHAIN. HPB - 2,3-dimethoxy-5-hydroxy-6-phytyl-1,4-benzoquinone. TTFA - thenoyltrifluoroacetone. For other abbreviations see Fig. 1.

that the complexes were not combined in a 1:1:1:1 ratio, because there was three times as much Complex IV as of any of the other three complexes (24). Further experiments indicated that any proportions of two or more complexes could be combined, provided precautions were taken to avoid precipation (10). This evidence excluded stoichiometry as a factor in the reconstitution experiments. If there was no precise orientation of the complexes then how could they interact to give a reconstituted electron transfer chain? The answer to this question may lie in the existence of diffusible hydrogen and electron carriers, such as NAD^+, ubiquinone and cytochrome c. For example, ubiquinone could collect hydrogen from the various dehydrogenases and then diffuse to the cytochromes where reduction would take place. Thus diffusible links between the complexes could overcome the random occurence of the dehydrogenases and the cytochromes. Klingenberg and his associates (17, 25-27), in the course of their studies on the role of ubiquinone in mitochondria, have arrived at similar conclusions. The major difference between their work and that of Green is that they have assumed a stoichiometric arrangement of the cytochromes in intact mitochondria, because of the 1:1:1 ratio of cytochrome a:cytochrome c:cytochrome b. They therefore postulated that only NAD^+ and ubiquinone were diffusible hydrogen carriers (17).

Various inhibitors have been useful in the study of the respiratory chain, because a specific inhibitor will divide the chain into two segments; one before and one after the inhibition site. The partial reactions occuring on either side of the site can then be examined and important information about the electron transfer sequence obtained. The proposed sites of action of the most frequently used inhibitors are shown in Fig. 4.

Oxidative Phosphorylation.

It has been known for thirty years that when a pair of electrons traverses the respiratory chain from NADH to molecular oxygen three molecules of ATP are formed from ADP and inorganic phosphate (28,29). Thus there are three sites for ATP production in the chain; these sites

correspond to Complexes I, III and IV (see Fig.1). Complex II does not contribute to the coupling process, but it does allow the entry of succinate, which can then produce two molecules of ATP via Complexes III and IV.

Although it has been demonstrated conclusively that ATP synthesis is coupled to electron transport, the mechanism of the process has remained a mystery. Several theories have been proposed to account for the experimental findings. But in spite of this, no evidence is yet available which points to any particular mechanism as being the solution to the problem. All three theories under consideration at the present time propose an energy conservation step prior to the formation of ATP. The major point of conflict between the theories is the question of the nature of this step. The "chemical" hypothesis proposes that the energy of the oxidation-reduction reactions is conserved as an energy rich compound (30) which eventually transfers its energy to ADP and inorganic phosphate to give ATP. The "chemiosmotic" hypothesis states that the energy of oxido-reduction is utilized to produce a membrane potential, which can be coupled to the synthesis of ATP (31). The "conformation" hypothesis maintains that the energy is conserved as a conformational change in a mitochondrial protein, prior to ATP synthesis (32,33).

(i) The Chemical Hypothesis.

According to this hypothesis a series of chemical reactions leads from a high energy form of a respiratory carrier, via a phosphorylated high energy intermediate, to ATP. There are many variations of the reaction sequence (34-36), but all follow the same general pattern:

$$AH_2 + B + X \rightleftarrows A\sim X + BH_2$$

$$A\sim X + P_i \rightleftarrows A + X\sim P$$

$$X\sim P + ADP \rightleftarrows ATP + X$$

sum: $$AH_2 + B + P_i + ADP \rightleftarrows BH_2 + A + ATP$$

It is postulated that an intermediate X combines with the electron carrier A during the oxidation reduction reaction thus forming a high energy compound A~ X. A~ X then transfers its high energy bond to give a phosphorylated compound, which is capable of phosphorylating ADP to give ATP. The involvement of non-phosphorylated intermediates is supported by the findings that energy-linked reactions (1) and ion transport (9) can be supported by respiration, in the absence of inorganic phosphate and in the presence of oligomycin.

A considerable amount of information has been collected from studies with compounds that affect the phosphorylation process. The most important groups of compounds in this respect are uncouplers and energy transfer inhibitors. For example, the classical uncoupler DNP (63) interferes with phosphorylation in that it prevents ATP formation, but allows respiration to continue, often at an increased rate. The precise mode of action of DNP is unknown; possibilities are that it might prevent formation of the high energy intermediate or it might dissipate the intermediate once it has formed. The antibiotic oligomycin is an example of an energy transfer inhibitor (64). Its effect differs from that of DNP because respiration as well as ATP formation are prevented. Oligomycin has been particularly useful in the characterisation of mitochondrial energy-linked reactions (see Chapters III and IV of this thesis).

At the present time the chemical hypothesis suffers from a considerable disadvantage because there is no convincing evidence available as to which, if any, of the respiratory carriers is involved in the coupling process. Furthermore none of the phosphorylated intermediates so far suggested have been shown to be involved. For example, some indications for the participation of NAD (37-39), vitamin K (40,41) and ubiquinone (42) have not been followed by convincing experimental evidence. The isolation of protein bound phosphohistidine led to the proposal that this was an intermediate in oxidative phosphorylation (43). However, it was later shown that most of the phosphohistidine was involved in the succinyl thiokinase reaction (44,45), but a role for the remainder in oxidative phosphorylation is still a possibility (46). The search for non-phosphorylated intermediates has been even less successful, although

Chance (47-49) has observed a special form of cytochrome b, which appeared in the high energy state and which disappeared on addition of the uncoupler dicoumarol. The results of the experiments were disappointing in that no changes were observed in any of the other respiratory carriers, making it unlikely that they are directly involved in high energy intermediate formation.

Various model systems have prompted fresh experimental approaches to the problem, but so far none of the schemes have been validated. One of the most attractive models is based on the observation that chemical oxidation of quinol phosphates by bromine resulted in the phosphorylation of AMP to ADP (50). These experiments led to the formulation of cyclic schemes involving quinone methide intermediates (51-53). The hypotheses were open to biochemical testing because the methyl ⇄ methide transition, occuring during the operation of the cycle, would result in the incorporation of added tritium into the ring methyl group of the quinone. However, no one was able to detect any significant incorporation under a variety of conditions, in beef heart mitochondria (7), rat liver mitochondria (55,56), Mycobacterium phlei (56) and Rhodospirillum rubrum (7,55). The possibility that only a small fraction of the total ubiquinone pool is participating in the reaction must be considered, because small changes in ubiquinone would not have been detected in the experiments.

The meagre evidence for the existence of phosphorylated or non-phosphorylated high energy intermediates has resulted in the postulate that a high energy state, rather than the formation of a covalent compound, is responsible for ATP synthesis. According to this idea ATP would be the first phosphorylated compound formed from inorganic phosphate. The chemiosmotic and conformation hypothesis take account of this suggestion and also attempt to explain the requirement for an intact membrane, which is rather difficult to explain on the basis of a purely chemical system.

(ii) The Chemiosmotic Hypothesis.

Mitchell (31) has proposed that the primary event in oxidative phosphorylation is not the formation of a high energy intermediate, but

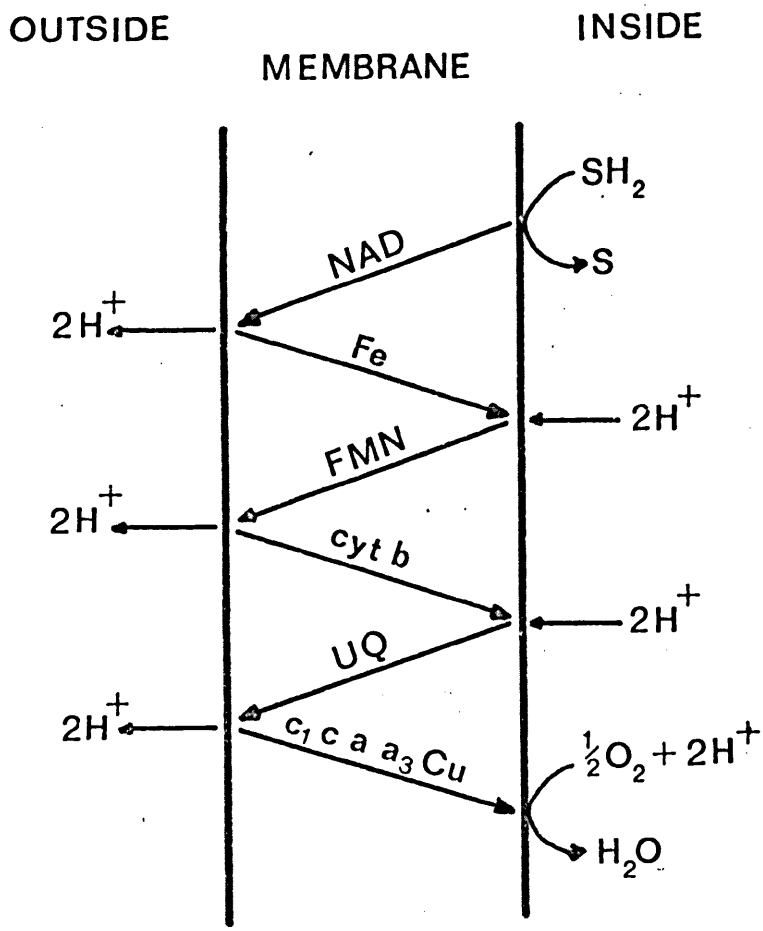


Fig. 5. PROTON TRANSLOCATION IN ELECTRON TRANSPORT. SH_2 - reduced substrate. S - oxidised substrate.

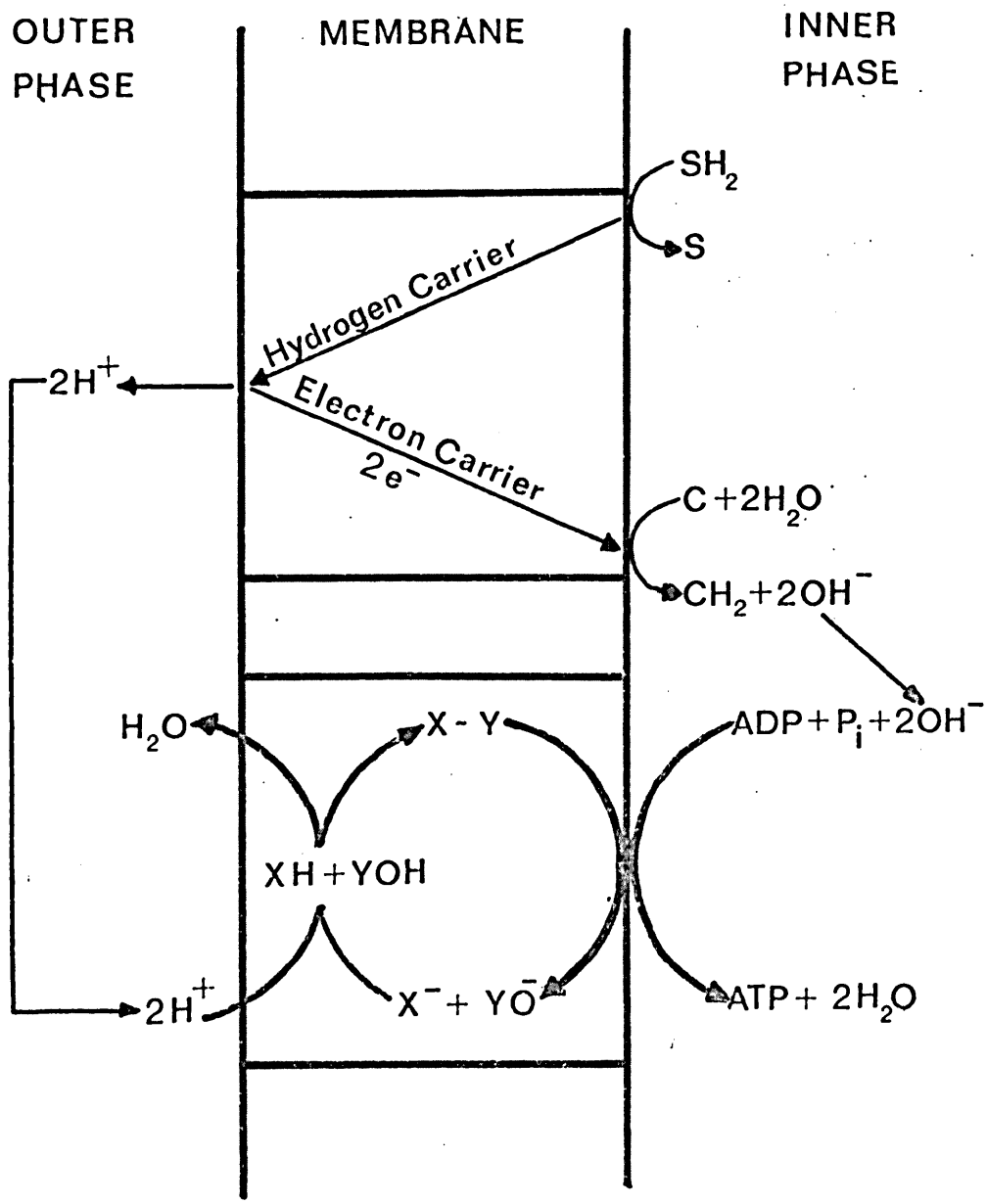


Fig. 6. RELATIONSHIP BETWEEN ELECTRON TRANSPORT AND OXIDATIVE PHOSPHORYLATION. (see text for details).

the generation of an electrochemical gradient. The gradient could be produced by a respiration-dependant translocation of protons through the mitochondrial membrane. In the presence of ADP and inorganic phosphate the free energy of the electrochemical potential could be used to synthesise ATP by reversal of the membrane-bound ATPase. Two points integral to Mitchell's hypothesis are that the phosphorylating membranes must be relatively impermeable to ions and that the mitochondrial ATPase must be anisotropic.

The way in which Mitchell envisages the coupling of electron transport to ATP synthesis is as follows: alternate hydrogen and electron carriers in the respiratory chain cause the transfer of protons across a membrane. A hydrogen carrier accepts protons on one side of the membrane and it is then oxidised by an electron carrier. This then releases protons on the other side of the membrane, and at the same time hydroxyl ions are moved in the opposite direction to the protons. This results in a separation of charge (Fig. 5). The sum of hydrogen and electron carriers resulting in the transport of one pair of protons is termed a "loop", and is equivalent to the coupling site in the chemical hypothesis. Thus for the complete oxidation of NADH by molecular oxygen there are three loops and three molecules of ATP are synthesised; this is in agreement with the observed P:O ratio of three for NADH oxidation. If ADP and inorganic phosphate are present at the active site of the vectorial ATPase, the ejected protons can be used to reverse the ATPase to give ATP (Fig. 6).

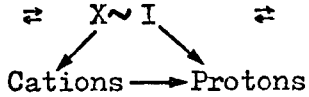
The electrochemical potential mentioned above could be due to a membrane potential or a pH gradient, or a combination of both. Mitchell (57) has calculated that to bring a solution containing ADP and inorganic phosphate to equilibrium, i.e. 50% ATP, requires either a pH gradient of 3.5 units or a membrane potential of 210 mV. Because the pH gradient needed is so large Mitchell currently believes that a membrane potential makes the major contribution to the electrochemical potential. Furthermore, to counteract the large pH changes, K^+ and Cl^- could be exchanged for H^+ and OH^-, thus maintaining the membrane potential.

It has proved difficult to design experiments that will distinguish unambiguously between the chemiosmotic and the chemical hypothesis.

Although many results of the experiments carried out are in agreement with a chemiosmotic mechanism they are also compatible with the chemical hypothesis. For example, proton transport has been observed on addition of oxygen and ATP to anaerobic mitochondrial suspensions (58); and it was found that two protons were ejected per molecule of ATP synthesised or hydrolysed. It is not yet clear whether proton transport is a primary process directly linked to electron transport (A), or whether it is a secondary process due to cation transport or hydrolysis of a high energy intermediate (B)

(A) Electron Transport $\rightleftarrows$ Protons $\rightleftarrows$ ATP

(B) Electron Transport $\rightleftarrows$ X~I $\rightleftarrows$ ATP



Of great importance was the finding that mitochondrial membranes were almost impermeable to protons (58) and that uncoupling agents such as DNP markedly increased the proton permeability. According to the chemiosmotic hypothesis, the mode of action of uncouplers is therefore thought to be due to their proton conducting properties, rather than to a dissipation of a high energy intermediate, as suggested in the chemical hypothesis. The chemical diversity of the compounds does favour a non-specific proton conducting effect, as opposed to a specific chemical interaction.

One of the most convincing experiments supporting the chemiosmotic hypothesis is probably the acid bath experiment carried out by Jagendorf and Uribe (59). Chloroplasts were incubated at pH 4.0 and then injected into a medium containing ADP and inorganic phosphate at pH 8.0 in the dark. Large amounts of ATP were synthesised under these conditions and uncouplers decreased the amount of ATP produced. Similar results were obtained with mitochondria when the incubation time was restricted to one second (60).

The failure of the antibiotics nigericin and valinomycin to uncouple oxidative phosphorylation was thought to be incompatible with the

chemiosmotic hypothesis. Nigericin causes K^+/H^+ equilibration; it therefore disrupts the pH gradient, but in spite of this it does not uncouple oxidative phosphorylation. Valinomycin promotes K^+ transport across the mitochondrial membrane; this results in the collapse of potassium membrane potentials, but there is still no uncoupling of oxidative phosphorylation. Mitchell has explained these anomalies by saying that nigericin will not affect the membrane potential, since the $K^+ : H^+$ exchange is electrically neutral (61). Thus even though nigericin changes the pH gradient it will not alter the membrane potential. In the case of valinomycin Mitchell maintains that although the membrane potential will be affected, the pH gradient will be unaltered. This serves to emphasise the point that either a pH gradient or a membrane potential can be used for ATP synthesis. These conclusions have been supported by Jackson and Crofts (62) who have shown that nigericin and valinomycin together uncouple photophosphorylation, although neither compound is effective on its own.

Until oxidative phosphorylation is demonstrated in a membrane free system, or when a high energy derivative of a respiratory chain carrier is conclusively shown to be involved in phosphorylation, the problem of differentiation between the two hypotheses will remain. Perhaps a technique permitting direct measurement of mitochondrial membrane potentials would resolve a few of the difficulties.

(iii) The Conformation Hypothesis.

The conformation hypothesis, like the chemiosmotic hypothesis proposes that high energy intermediates are not necessary for ATP synthesis; but suggests that there is a high energy state which leads to the synthesis of ATP. This proposal was first suggested by Boyer (65) and has also been championed by Green (33). According to the hypothesis the free energy of oxido-reduction is conserved as a conformation change, either in a respiratory carrier or in an associated protein; this change is then coupled to the synthesis of ATP from ADP and inorganic phosphate. In the absence of electron flow the mitochondrial membranes are in a non-energised state; as a consequence of electron flow the membrane attains the

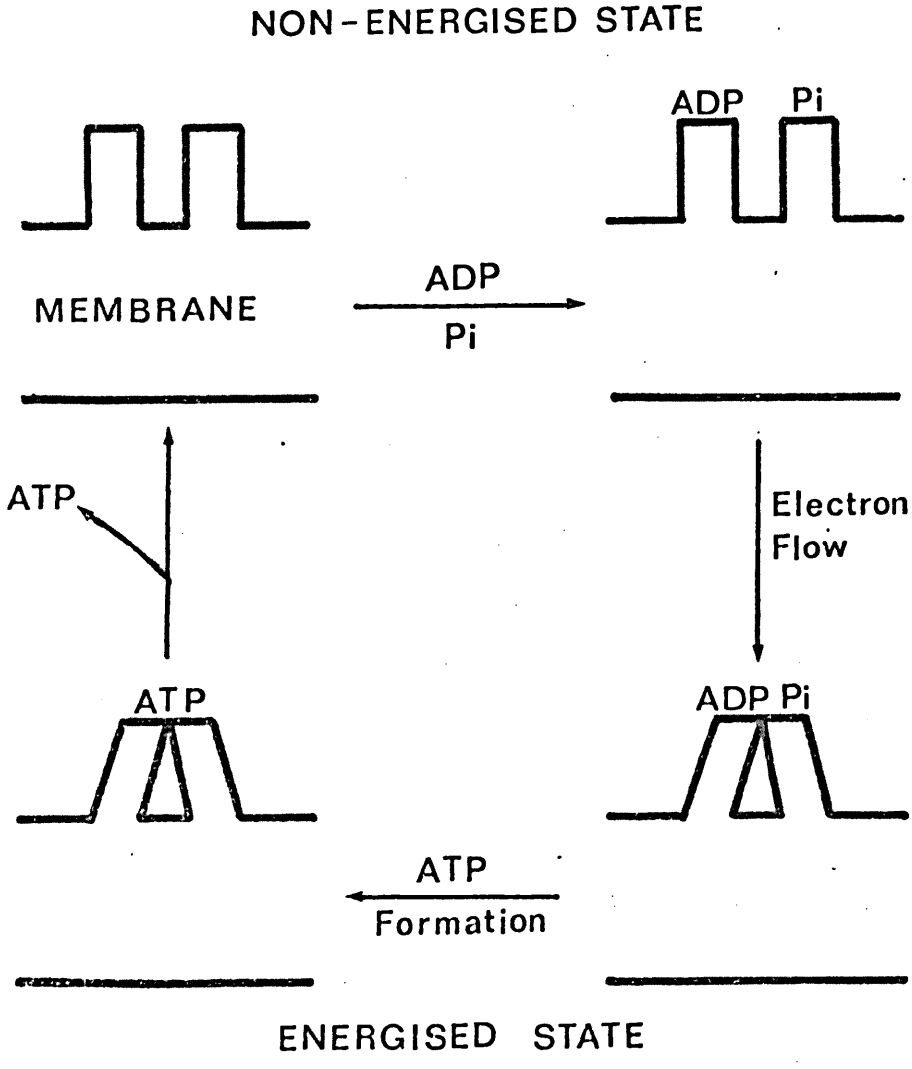


Fig. 7. SYNTHESIS OF ATP BY A MEMBRANE CONFORMATION CHANGE.

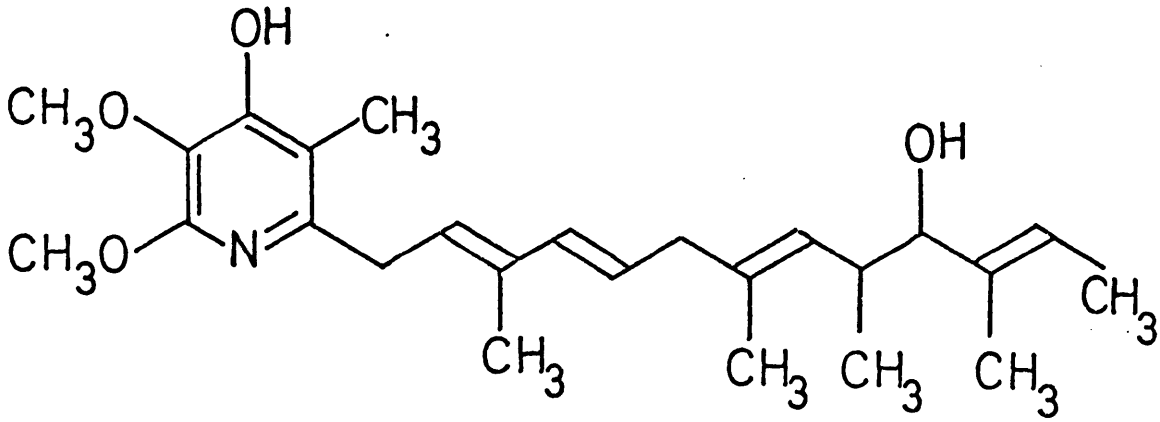
energised state and ATP formation can then take place (Fig. 7). The bulk of the evidence supporting this hypothesis is based on electron microscopic observations of structural changes in mitochondria in different metabolic states (54). However time dependant studies on these changes have not been carried out and Chance (12) has pointed out that the various structures have been found in different regions of the same mitochondrion. This would appear to be a rather unimportant objection because there is no reason why the various reactions should not be occuring at the same time. Of greater significance are the drastic procedures required to prepare the mitochondria for examination with the electron microscope. The possibility of artefacts must be ruled out before the conformation hypothesis can be brought into line with the other two contenders for the mechanism of oxidative phosphorylation.

Summary.

After thirty years investigation the mechanism of oxidative phosphorylation still remains obscure. The chemical hypothesis has held sway for some time now, but the failure to find any high energy chemical intermediates has led to other suggestions. The new hypotheses involve the generation of a high energy state, which may be either a membrane potential or a conformation change. Which of these theories is correct is still open to question. Indeed the answer may lay in another, as yet unproposed, theory.

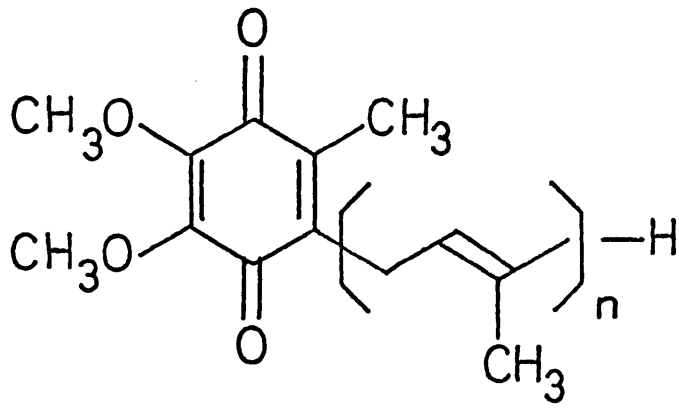
STRUCTURE OF PIERICIDIN A

I



STRUCTURE OF UBIQUINONE

II



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CHAPTER II

INTERACTIONS OF PIERICIDIN A WITH MITOCHONDRIAL AND BACTERIAL ENZYMES.

INTRODUCTION.

The isolation and some of the physiological properties of piericidin A was first described by Tamura et al (66). The compound was obtained from the mycelia of Streptomyces mobaraenis and was shown to be a potent insecticide, probably with limited practical use since it was found to be highly toxic towards mammals and fish. Structurally piericidin A (I) resembles ubiquinone (II) (see ref. 67): both compounds possess an aromatic ring with adjacent methoxy groups, ring methyl and oxygen functions, and a lipophilic side chain, which is isoprenoid in ubiquinone and derived from acetate and propionate in piericidin A (68).

Because of its apparent structural relationship to ubiquinone Hall et al (69) examined the effects of piericidin on mitochondrial electron transport reactions. Reactions such as the oxidation of NADH and succinate have been shown to involve ubiquinone (70). At high concentrations piericidin was found to act as a competitive inhibitor towards ubiquinone in the succinic oxidase system (71). This was demonstrated as a release of piericidin inhibition of succinic oxidase on addition of ubiquinone homologues. It became clear that piericidin had a differential effect on mitochondrial oxidations when it was shown that the oxidation of NAD-linked substrates was much more sensitive to piericidin than the oxidation of succinate (7, 71). It was suggested that piericidin was acting in the NADH dehydrogenase region of the respiratory chain at a site which may be identical to that of rotenone, and at concentrations far lower than the concentration of any of the known respiratory components (71). There was, however, little agreement as to the site of action of rotenone and other inhibitors such as barbiturates and steroids which were suspected to act in the NADH dehydrogenase region. The inhibition site of rotenone has been variously placed on the substrate side of the flavoprotein (1, 72) and on the oxygen side of the flavoprotein (73, 74).

The availability of the new inhibitor, piericidin, opened up possibilities for a fresh approach to the study of the role of ubiquinone in mitochondria. Initial studies (7) showed that ubiquinone-dependant restoration of NADH oxidase to lyophilised, pentane extracted mitochondria was competitively inhibited by piericidin. The specific interaction of piericidin with the NADH dehydrogenase region of the respiratory chain also provides a tool for a study of that region.

The studies described in this chapter were designed to attempt to answer some of the following questions.

(i) What are the effects of piericidin on mitochondrial reactions? Is piericidin a specific inhibitor of the respiratory chain? Is ubiquinone involved in piericidin inhibition?

(ii) What is the site of action of piericidin and is this the same as other known inhibitors, such as rotenone, barbiturates and steroid hormones?

(iii) Is piericidin interacting with an unknown component of the NADH dehydrogenase region of the respiratory chain, as has been suggested by Hall et al (71), or is it stoichiometric with one of the known components?

(iv) What are the structural requirements of piericidin as an inhibitor of NADH oxidase?

(v) Is there any chemical rationale between the different groups of inhibitors?

(vi) It has been suggested that ubiquinone may play a role in oxidative phosphorylation (75). If piericidin acts by competing with ubiquinone does it interfere with reactions involving oxidative phosphorylation such as the energy-linked transhydrogenase and the energy-linked reversal of electron transport?

(vii) Can piericidin be used as a tool in the study of bacterial reactions?

(viii) What is the nature of the component that binds piericidin to mitochondria?

MATERIALS AND METHODS.

Chemicals.

Analytical grade laboratory chemicals were purchased from Hopkin and Williams Ltd., Chadwell Heath, Essex. Enzymes were obtained from Boehringer und Soehne, G.m.b.H., Mannheim, Germany. Standard biochemicals were from Sigma Chemical Co., St. Louis, Mo., U.S.A. Rotenone was supplied by the Aldrich Chemical Co., Milwaukee, Wisconsin, U.S.A. Rotenone analogues were a gift from Prof. N.Takahashi, University of Tokyo, and was supplied as a solution in chloroform. Before use the crude piericidin solution was purified by thin layer chromatography. 10mg amounts were spotted onto alumina G plates (0.75mm thick) and eluted with chloroform; the piericidin separated into four components at R.f. 0.1, 0.3, 0.6 and 0.9. Only spot III (R.f. 0.6) exhibited the characteristic ultraviolet spectrum of piericidin (λmax at 233nm, 238nm and 267nm) and represented about 60% of the crude piericidin (89). All the fractions were tested for their inhibitory activity towards NADH oxidase; none of the spots showed any activity except the one corresponding to piericidin A. Piericidin A purified in this manner was used in all the inhibitory studies described in this chapter. Piericidin and pyridine analogues were synthesised by C.J.Coles at the University of Warwick. 3H piericidin was prepared from piericidin A by catalytic exchange labelling with tritium gas in a solution of cyclohexane, using platinum oxide as a catalyst. No reduction of the olefinic side chain of piericidin A was detected. The specific activity of the radioactive compound was 56.5mC/mmole. Tritiated reduced piericidin A was prepared by catalytic hydrogenation of 3H piericidin A in methanol (89). The specific activity remained unchanged.

Preparation of Mitochondria.

Beef hearts were used as the source for all mitochondrial preparations. The method used was a modification of that described by Sanadi and Fluharty (86). The fibrous tissue, blood vessels and heart valves were quickly removed from six beef hearts. The heart muscle was cut into cubes and minced; 125g lots of the mince were then homogenised in 250ml ice cold 0.25 M-sucrose containing 10 mM-tris base, in a Waring

Blendor for 30 sec. Six homogenates were combined and mixed with 1 litre 0.25 M-sucrose and the pH was adjusted to 7.6 with 1.0 M-tris base, using bromothymol blue as an external indicator. The homogenates were then centrifuged at 1,100 x g for 10 min at 2°. The resulting supernatants were combined and centrifuged at 14,000 x g for 10 min. The mitochondrial pellets, at the bottom of the tubes, were combined and suspended in 0.25 M-sucrose and the pH was adjusted to 7.6 with tris base. The suspension was centrifuged at 27,000 x g and the supernatant and the loosely packed portion of the pellet (light mitochondria) were discarded. The remaining tightly packed layer (heavy mitochondria) was suspended in a medium containing 4 mM-magnesium chloride, 1 mM-ATP, 1 mM-sodium succinate and 0.25 M-sucrose at pH 7.6. The suspension was stored at -15°. When required the mitochondria were thawed and collected at 27,000 x g and resuspended in 0.25 M-sucrose at pH 7.6.

Preparation of Submitochondrial Particles.

Submitochondrial particles were prepared by sonic disintegration according to the method of Hansen and Smith (87). Heavy beef heart mitochondria, prepared as decribed above, were suspended at a protein concentration of 20mg/ml in 0.25 M-sucrose containing 50 mM-tris HCl buffer (pH 7.6), 4 mM-magnesium chloride, 1 mM-ATP and 1 mM-sodium succinate. The suspension was sonicated in 11ml batches at 0-2° for 40sec at maximum output from the large probe of an MSE 60 watt cell disintegrator. Any unbroken mitochondria were removed from the combined suspensions by centrifugation at 27,000 x g. The supernatants were carefully removed with a pasteur pipette and centrifuged for 30min at 100,000 x g. The sediment was resuspended in 0.25 M-sucrose using a loose fitting glass homogeniser and recentrifuged at 100,000 x g, to remove any loosely bound enzymes. The pellet was finally resuspended in a medium containing 0.25 M-sucrose, 4 mM-magnesium chloride and 25mM-tris HCl buffer (pH 7.6) and used immediately.

Preparation of Bacterial Particles.

Wild type E.Coli K 12 was grown on a minimal medium with succinate as the sole energy source. The cells were harvested in the log phase of

growth and the small particles were prepared by sonication and isolated by differential centrifugation (see methods section of Chapter III).

Measurement of Enzyme Activities.

All enzyme activities were measured at 30°. Water insoluble inhibitors, such as piericidin and rotenone, were added to the incubation media as their ethanolic solutions. Suitable controls were carried out with equivalent amounts of ethanol.

(i) NADH Oxidase.

The oxidation of NADH by mitochondrial and bacterial preparations was measured by determining the decrease in absorption at 340nm in a Beckman DK 2 recording spectrophotometer. Experimental and blank cuvettes contained 670μmole sucrose, 27μmole tris-HCl (pH 7.5) and 0.1 - 0.2mg mitochondrial protein. 0.32μmole NADH was added to the experimental cuvette to initiate the reaction and the final volume was 3ml. 1-2mg bacterial protein were used in measuring the reaction in E. coli.

(ii) Succinate Oxidation.

Oxygen uptake by mitochondria (2 - 3mg protein), or by bacterial particles (3 - 4mg protein), in the presence of succinate, was measured polarographically, using an oxygen electrode (Rank Brothers, Bottisham, Cambs.). The reaction medium comprised, 10μmole sodium succinate, 18μmole magnesium chloride, 750μmole sucrose and 150μmole tris HCl (pH 7.5). The final volume was 3ml.

(iii) Pyruvate and Malate Oxidation.

Pyruvate plus malate oxidation was measured polarographically in the same way as succinate oxidation, except that 10μmole pyruvate plus 10μmole malate were used as substrate.

(iv) Energy-Linked Reduction of NAD^+ by Succinate.

This reaction was measured by the method of Griffiths and Robertson (85). Blank and experimental cuvettes of 1cm light path contained 675μmole sucrose, 135μmole tris HCl buffer (pH 8.0), 16μmole magnesium chloride,

3μmole potassium cyanide, 3μmole NAD^+ and 15μmole sodium succinate. 0.3 - 1.0mg submitochondrial particles were added to both cuvettes and allowed to equilibrate for 3min. The reaction was initiated by the addition of 6μmole ATP to the experimental cuvette, to give a final volume of 3ml. The resulting increase in absorbance at 340nm was measured in a Beckman DK 2 recording spectrophotometer.

A similar method was developed for measuring the reaction in bacterial preparations. This is described in detail in Chapter III.

(v) Energy-linked Transhydrogenase.

(a) ATP-driven reaction: blank and experimental cuvettes of 1cm light path contained 675μmole sucrose, 16μmole magnesium chloride, 3μmole potassium cyanide, 135μmole tris HCl buffer (pH 8.0) and 0.3 - 1.0mg beef heart submitochondrial particles. The following additions were then made to the experimental cuvette; 10μl yeast alcohol dehydrogenase (290μg) and 10μl ethanol. The absorption was determined until a steady trace was obtained, when 40nmole NAD^+ was added to the experimental cuvette. The non-energy-linked transhydrogenase was initiated by the addition of 0.1μmole $NADP^+$; this reaction was followed for 2 - 3min, then the energy-linked reaction was started by the addition of 6μmole ATP.

Measurement of the reaction in bacterial particles is described in detail in Chapter IV.

(b) Succinate-driven reaction: blank and experimental cuvettes of 1cm light path contained 675μmole sucrose, 16μmole magnesium chloride, 0.6nmole rotenone, 135μmole tris HCl buffer (pH 8.0), 3μg oligomycin and 0.3 - 1.0mg beef heart submitochondrial particles 10μl yeast alcohol dehydrogenase (290μg), 10μl ethanol and 40nmole NAD^+ were added to the experimental cuvette, followed by 0.6μmole $NADP^+$. The energy-linked transhydrogenase driven by high energy intermediates, generated by the aerobic oxidation of succinate, was initiated by the addition of 10μmole sodium succinate.

It was not possible to measure this reaction in E. coli small particles, because NADH oxidation in this bacterium is not inhibited by rotenone.

(vi) Cytochrome b Reduction.

Cytochrome b reduction was measured using a double beam spectrophotometer (Aminco-Chance) set at the wavelength pair 430 - (410)nm. The incubation medium was 875μmole sucrose, 21μmole magnesium chloride, 4μmole potassium cyanide and 7.2mg mitochondrial protein. The suspension was incubated for 5min in a cuvette of 1cm light path before addition of substrate (5μmole pyruvate plus 5μmole malate) to give a final volume of 4 ml.

(vii) Flavin Reduction.

Measurements of flavin reduction were made as described above for cytochrome b reduction, except that the wavelength pair was 465 - (510)nm and 9.2 mg mitochondrial protein were used. Substrate additions were 5μmole sodium pyruvate plus 5μmole sodium malate, or 5μmole sodium succinate.

(viii) Yeast Alcohol Dehydrogenase.

Enzyme activity was measured at 340nm in a Beckman DK 2 spectrophotometer with NAD^+ and ethanol as substrates. The additions made to a cuvette of 1 cm light path were 50μmole tetrasodium pyrophosphate buffer (pH 9.0), 3μmole NAD^+ and 0.1ml ethanol. After temperature equilibration 50μg yeast alcohol dehydrogenase was added to start the reaction. The final volume was 3 ml. Ethanolic solutions of inhibitors were added before the enzyme such that the total amount of ethanol present was always 0.1ml.

(ix) Beef Liver Glutamic Dehydrogenase.

The incubation medium contained 125μmole tetrasodium pyrophosphate buffer (pH 9.0), 3μmole NAD^+ and 50μg beef liver glutamic dehydrogenase. After equilibration the reaction was started by the addition of 50μmole sodium glutamate. The final volume was 3ml and the increase in absorbance at 340nm over the first 30sec was taken as the measure of enzyme activity.

(x) Pig Heart Malic Dehydrogenase.

The incubation medium contained in a volume of 3ml. 125μmole sodium phosphate buffer (pH 7.6), 0.6μmole oxaloacetate and 0.32μmole NADH. 0.25μg pig heart malic dehydrogenase was added to the experimental cuvette to start the reaction, which was measured by the decrease in absorbance at 340nm

in a Beckman DK 2 recording spectrophotometer.

Beef heart mitochondrial malic dehydrogenase was measured in the same way except that 16µg mitochondrial protein was added, instead of the crystalline enzyme.

Soluble beef heart malic dehydrogenase was prepared by centrifuging beef heart mitochondria at 100,000 x g for 2hr and collecting the supernatant. The activity was measured as described above, except that 2.5µg supernatant protein was used.

Protein.

Protein was determined by the method of Gornall et al (88), after solubilisation of the mitochondrial or bacterial pellet with deoxycholate (0.33% w/v) and bovine serum albumen was used as a standard.

Measurement of 3H piericidin Binding.

18 - 25mg mitochondrial protein were incubated with 1,000µmole sucrose and 200µmole tris HCl buffer (pH 7.6) containing 2% (w/v) bovine serum albumen (BSA) in a final volume of 5ml. Various amounts of 3H piericidin or 3H reduced piericidin were added to the reaction tubes and the mitochondria were incubated with the radioactive inhibitor for 8min at 30°. The tubes were then cooled on ice for 5min and the mitochondria were collected by centrifugation at 27,000 x g for 10min. The resulting pellets were washed in the sucrose-tris-BSA medium and finally suspended in 4ml sucrose-tris. 0.1ml aliquots were added to 10ml methanol:toluene (30:70) containing 0.8% (w/v) 2,5-diphenyloxazole (PPO) and 0.05% (w/v) 1,4-[2-(5-phenyloxazoyl)] benzene (POPOP) and counted in a Packard Tri-Carb Scintillation Counter. At the concentrations employed there was no quenching by piericidin.

Effect of Unlabelled Inhibitors on 3H Piericidin Binding.

The incubations were carried out as described above, except that varying amounts of unlabelled inhibitors were incubated with the mitochondria for 8min at 30°. After this time 3H piericidin was added to each tube and incubated for a further 8min. The procedure for washing and scintillation counting was the same as that described above.

Lyophilisation and Acetone Extraction of Mitochondria.

25mg mitochondria were incubated with varying amounts of 3H piericidin in sucrose-tris-BSA medium as described above. The mitochondria were washed and an aliquot was removed for scintillation counting. The remaining mitochondria were collected by centrifugation at 27,000 x g and suspended in 0.15 M-potassium chloride to produce a protein concentration of 20mg/ml. The mitochondrial suspensions were then lyophilised on an oil pump, using a liquid nitrogen trap. The lyophilised mitochondria were homogenised with two individual 10ml portions of acetone, with centrifugation used to separate the soluble and insoluble fractions after each homogenisation. The extracted mitochondria were dried on a rotary evaporator, to remove any residual acetone, and finally suspended in 2ml sucrose-tris prior to scintillation counting. The acetone extracts were combined, dried over anhydrous sodium sulphate, filtered, and evaporated under a stream of nitrogen in a small conical flask. The dried extract was dissolved in 2 ml ethanol and evaporated to a small volume under a stream of nitrogen and then spotted on a thin layer chromatography plate, to test for 3H piericidin binding to mitochondrial lipids (see Results section).

In some experiments mitochondria were lyophilised, extracted with acetone and suspended in sucrose-tris-BSA, before addition of 3H piericidin.

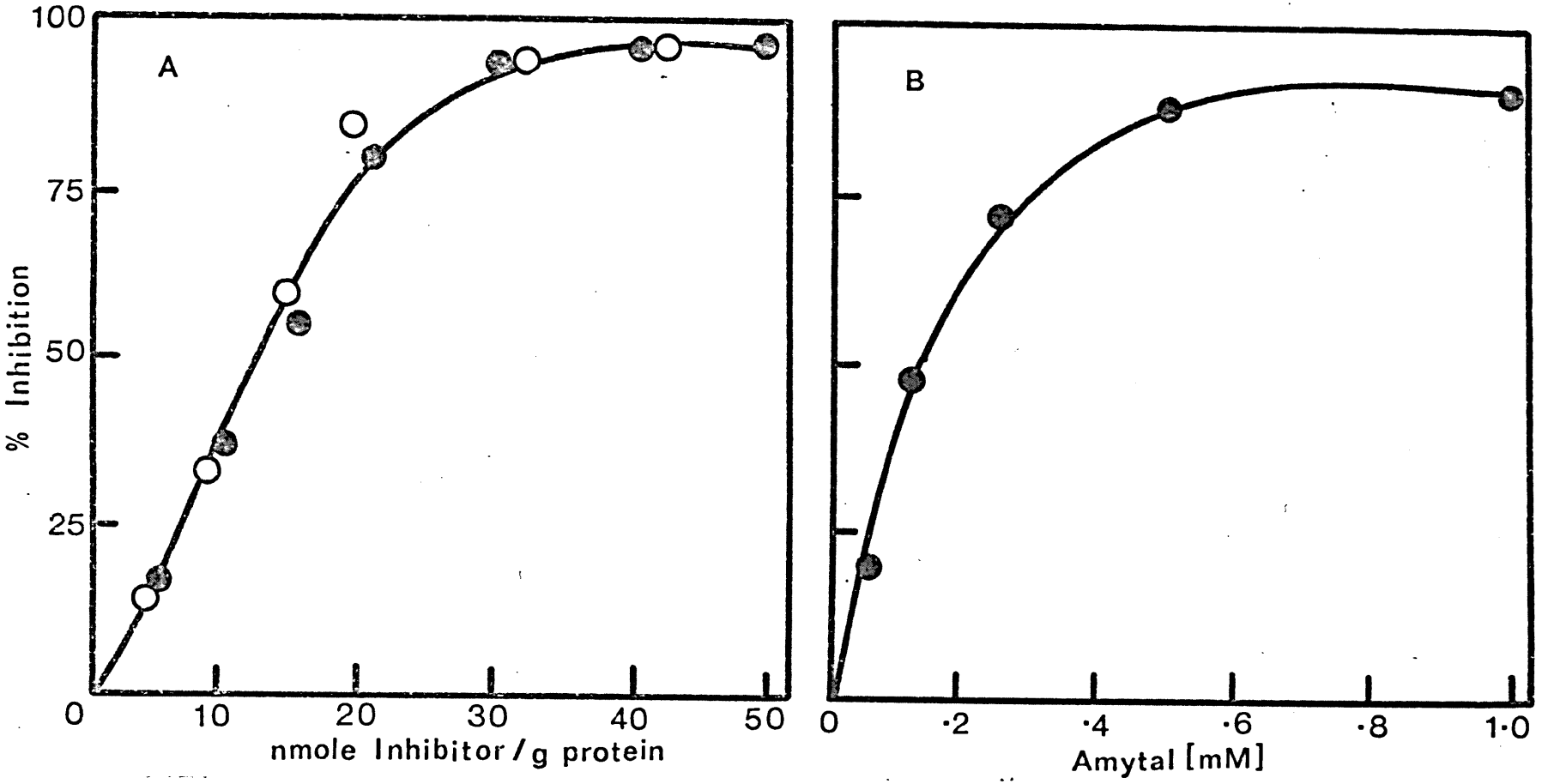


Fig. 1. THE EFFECT OF PIERICIDIN A, ROTENONE AND AMYTAL ON NADH OXIDATION IN BEEF HEART MITOCHONDRIA. The incubation medium contained 670µmole sucrose, 27µmole tris-HCl (pH7.5) and 0.1-0.2mg mitochondrial protein. 0.32µmole NADH was added to the experimental cuvette to give a final volume of 3.0ml. The decrease in absorbance at 340nm was taken as a measure of the enzyme reaction. The temperature of the incubation was 30°. A: ● rotenone, O piericidin A B: amytal.

Table 1

EFFECT OF PIERICIDIN ROTENONE AND AMYTAL ON NAD-LINKED OXIDATIONS IN BEEF HEART MITOCHONDRIA

INHIBITOR	CONCENTRATION (nmole/g protein)	%INHIBITION
Rotenone	41	38
	70	55
	138	88
Piericidin A	52	41
	79	60
	160	91
Amytal	0.33 (mM)	42
	0.9	66
	2.4	78

Oxygen uptake was measured polarographically using 10μmole pyruvate plus 10μmole malate as substrate. The reaction medium was 750μmole sucrose, 150μmole tris-HCl buffer (pH 7.5), 18μmole magnesium chloride and beef heart mitochondria (3mg protein). The final volume was 3.0ml and the temperature of the incubation was 30°. Piericidin, rotenone and amytal were added to the incubation medium to give the concentrations shown.

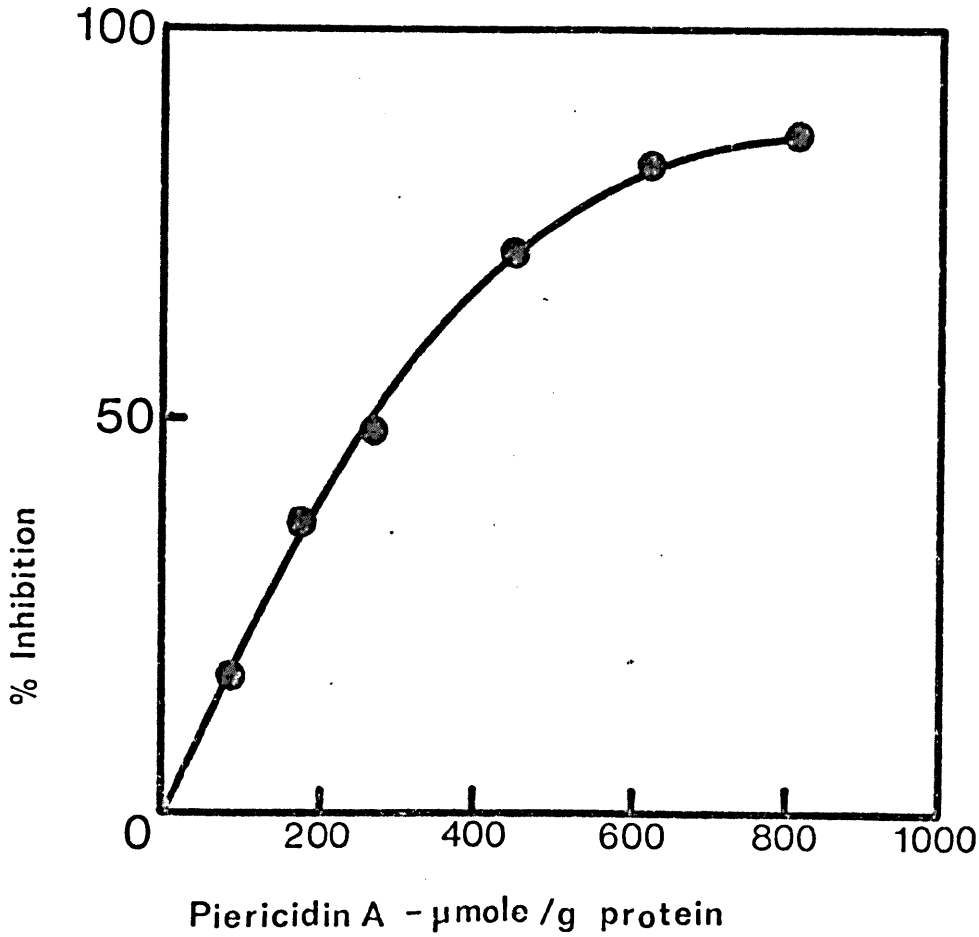


Fig. 2. THE EFFECT OF PIERICIDIN A ON SUCCINATE OXIDATION IN BEEF HEART MITOCHONDRIA. Oxygen uptake by mitochondria (3mg protein) was measured using an oxygen electrode. The reaction medium was 750µmole sucrose, 150µmole tris-HCl (pH 7.5), 18µmole magnesium chloride and 10µmole sodium succinate in a final volume of 3.0ml. The temperature of the incubation was 30°.

RESULTS.

(i) Electron Transport Reactions.

Piericidin was found to be a potent inhibitor of NADH oxidation in beef heart mitochondria; complete inhibition was achieved at a concentration of about 30 nmole/g mitochondrial protein. The amount of pieridiidin required for maximum inhibition was identical to the concentration of rotenone required for a similar effect (74). Amytal, a known inhibitor of this region (76), was also shown to inhibit NADH oxidase, but at much higher concentrations than either piericidin or rotenone (Fig. 1).

The oxidation of NAD-linked substrates, such as pyruvate plus malate and glutamate plus malate, were also inhibited by piericidin, rotenone and amytal (Table 1). The concentrations required for inhibition were slightly higher than those determined for NADH oxidation, but this may be due to the methods employed for measuring the activities. NADH oxidation was measured spectrophotometrically and NAD-linked substrate oxidations were measured polarographically.

The effects of piericidin, rotenone and amytal on succinate oxidation were tested. At the concentrations employed neither rotenone nor amytal had any effect on succinate oxidation, but piericidin was found to be inhibitory. The degree of inhibition, however, was not significant until high concentrations were employed. The maximum inhibition attainable was 80% at a concentration of 1,000μmole/g mitochondrial protein (Fig. 2). Piericidin is therefore an inhibitor of both NAD-linked and succinate oxidations in beef heart mitochondria, but is about 10,000 times more potent towards NAD-linked oxidations. The results suggest that low concentrations of piericidin, rotenone and amytal exhibit specific effects towards the NADH dehydrogenase region of the respiratory chain. Furthermore, the concentrations of piericidin and rotenone required for inhibition are of the same order as the NADH dehydrogenase content of beef heart mitochondria (77). It is possible that these two compounds are titrating stoichiometrically with a component of the NADH dehydrogenase enzyme system.

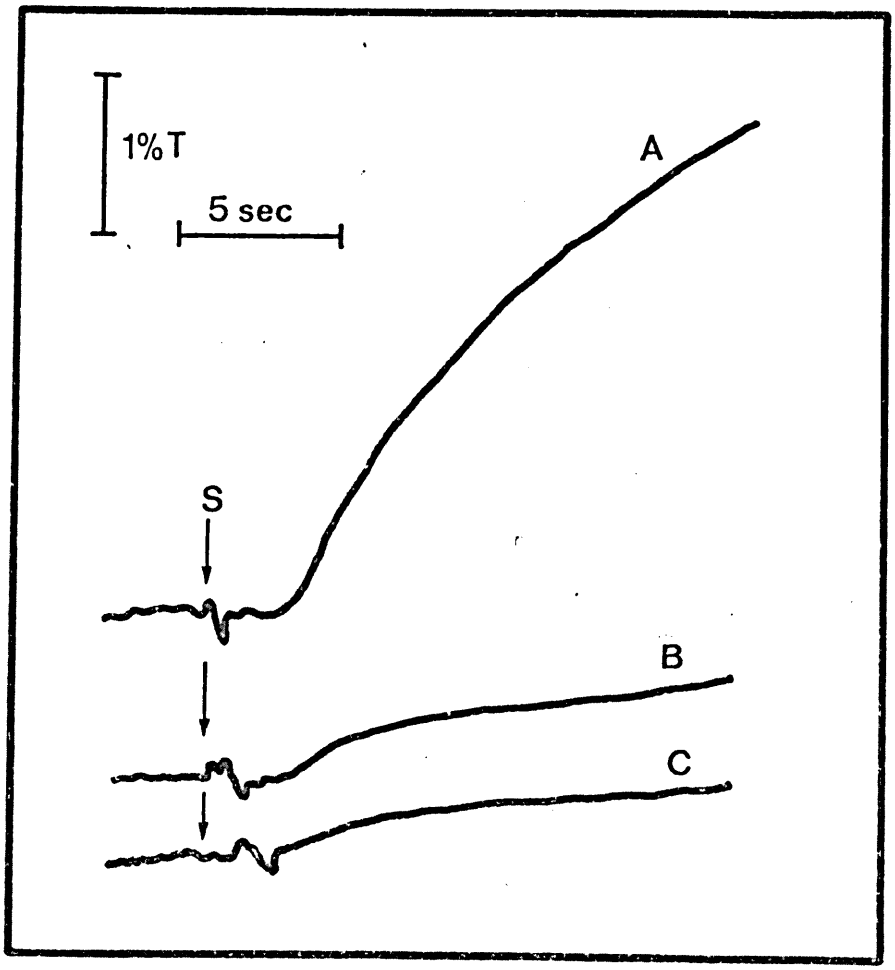


Fig. 3. THE EFFECT OF PIERICIDIN A AND ROTENONE ON CYTOCHROME b REDUCTION IN BEEF HEART MITOCHONDRIA. Cytochrome b reduction was measured at the wavelength pair 430-(410)nm. The incubation medium was 875µmole sucrose, 21µmole magnesium chloride, 4µmole KCN and 7.2mg mitochondrial protein in a volume of 4.0ml. The system was allowed to equilibrate for 5min at 30° before addition of substrate (5µmole pyruvate + 5µmole malate). A:control with no inhibitor. B:rotenone. C:piericidin A. Substrate was added at the point marked S on the tracing.

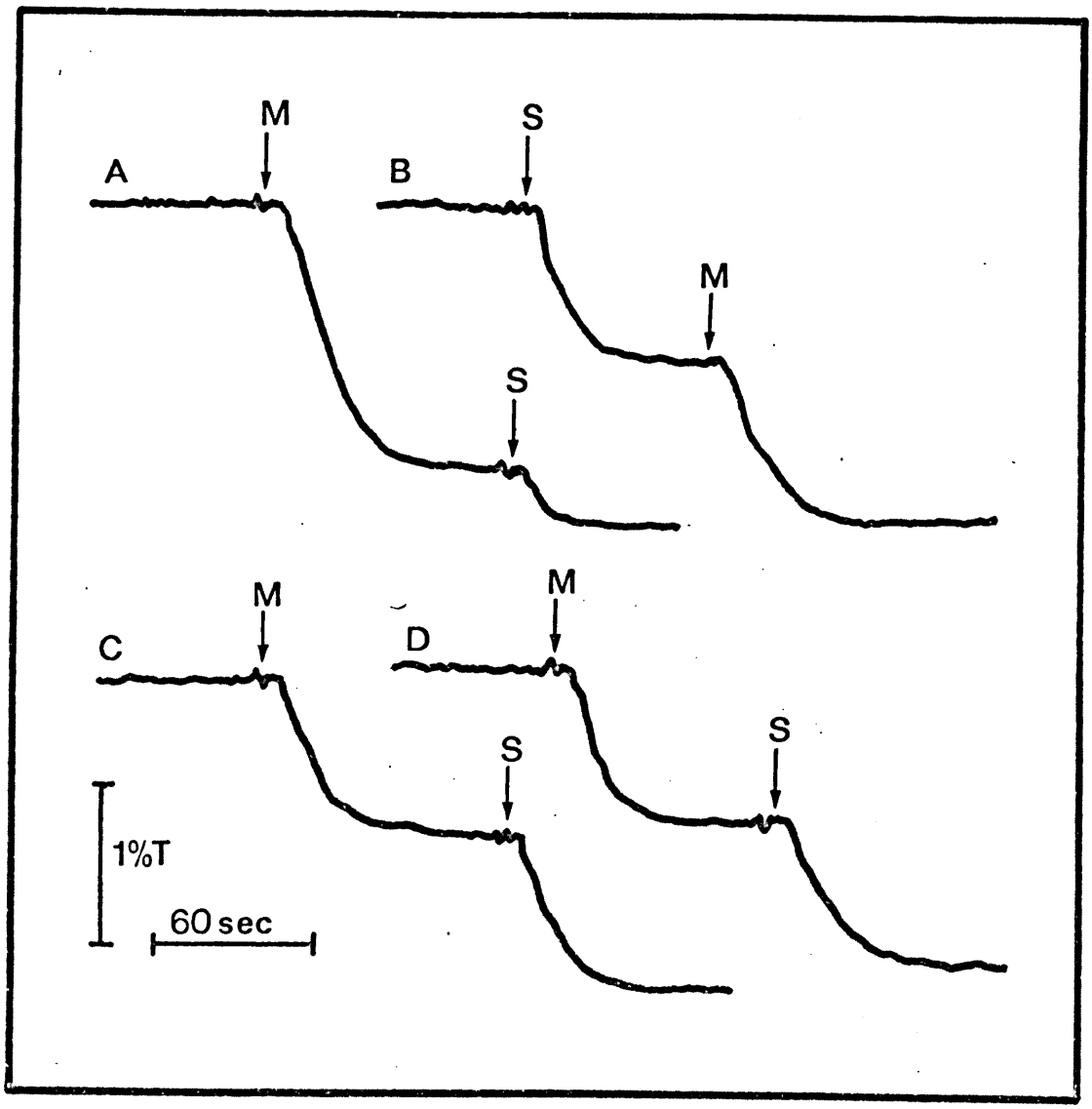


Fig. 4. THE EFFECT OF PIERICIDIN A AND ROTENONE ON "FLAVIN" REDUCTION IN BEEF HEART MITOCHONDRIA. Measurements were carried out using the wavelength pair 465-(510)nm. Conditions were as described in Fig. 3 except that 9.2mg mitochondrial protein were used. At point M 5µmole pyruvate + 5µmole malate were added and at point S 5µmole succinate were added. A and B: controls with no inhibitor. C: rotenone. D: piericidin. The temperature of the incubation was 30°.

(ii) Site of Action of Piericidin and Rotenone.

Double-beam spectrophotometry allows the site of action of inhibitors in the respiratory chain to be determined more precisely, because the rates of oxidation and reduction of individual components of the respiratory chain, such as the flavoproteins and cytochromes, can be measured.

Measurements of cytochrome b reduction using the wavelength pair 430-(410)nm showed that the cytochrome was rapidly reduced on addition of pyruvate and malate to cyanide inhibited mitochondria. In the presence of amounts of piericidin and rotenone sufficient to give 100% inhibition of NADH oxidation, cytochrome b reduction was reduced by about 80%.(Fig. 3). It is concluded that the point of action of the two inhibitors was before the cytochrome b - ubiquinone region of the respiratory chain. Flavin reduction was measured at 465-(510)nm in the presence of cyanide with pyruvate and malate as substrate. Reduction of the remainder of the 465nm absorbing material could be brought about by the addition of succinate. When pyruvate plus malate were added first to the mitochondria 85% of the 465nm absorbing material was reduced; the remainder was reduced by succinate to give a value of 100%. When succinate was added first, 50% of the material was reduced and 100% reduction was not observed until the addition of pyruvate plus malate (Fig. 4). These results showed that NAD-linked substrates were capable of reducing a portion of the 465nm material which was also reducible by succinate, whereas succinate only reduced the succinic dehydrogenase flavoprotein. In the presence of inhibitory amounts of piericidin or rotenone 50% of the total reducible material was reduced by pyruvate plus malate; the remainder was reduced by succinate to give a value of 100% (Fig. 4). This showed that succinic dehydrogenase was not reduced by pyruvate plus malate in the presence of piericidin or rotenone, but the reduction of NADH dehydrogenase was unaffected (see discussion).

(iii) Binding of Piericidin.

Piericidin labelled with tritium was synthesised and utilised for binding studies so that the nature of the interaction of the inhibitor with

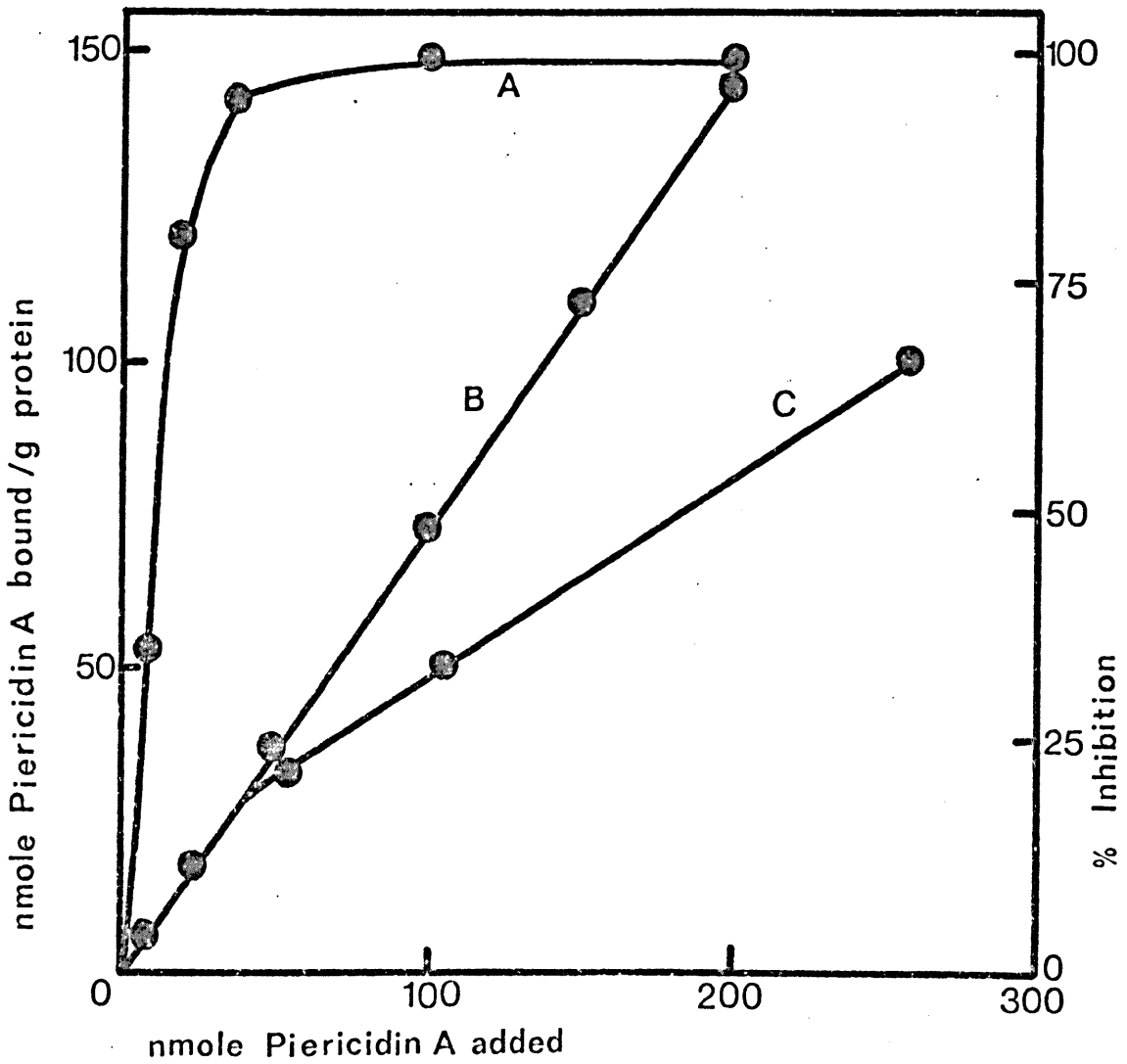


Fig. 5. COMPARISON OF INHIBITION OF NADH OXIDASE BY PIERICIDIN A AND 3-H PIERICIDIN BOUND TO BEEF HEART MITOCHONDRIA. NADH oxidase was measured as described in the legend to Fig. 1. The experimental details of 3-H piericidin binding are described in the Methods section. A-inhibition of NADH oxidase. B-binding of 3-H piericidin in sucrose-tris medium. C-binding of 3-Hpiericidin in sucrose-tris-BSA medium.

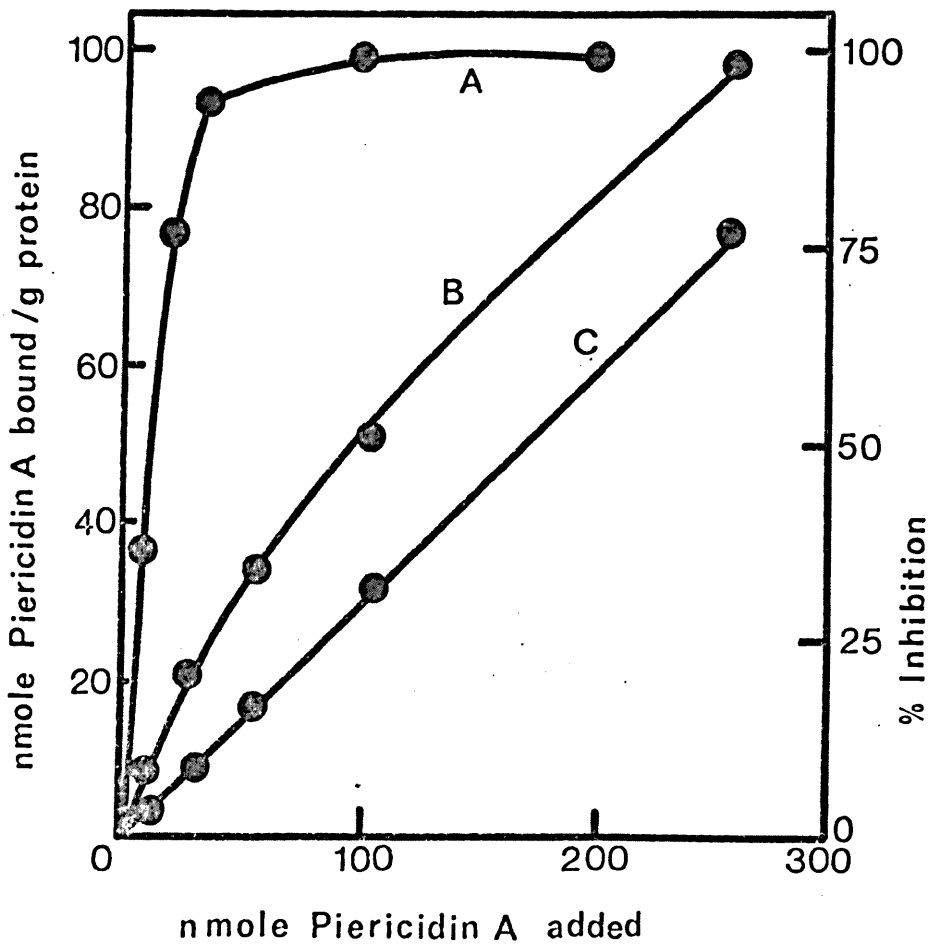


Fig. 6. EFFECT OF PRETREATMENT WITH UNLABELLED PIERICIDIN A ON THE SUBSEQUENT BINDING OF 3-H PIERICIDIN A. Experimental details are described in the Methods section. A-inhibition of NADH oxidase. B-binding of 3-H piericidin A in sucrose-tris-BSA medium. C-binding of 3-H piericidin A in sucrose-tris-BSA medium after preincubation with 35.8nmole unlabelled piericidin A.

Table 2

EFFECT OF REPEATED WASHING ON BINDING OF 3H-PIERICIDIN TO MITOCHONDRIA

PIERICIDIN A ADDED (nmole/g protein)	PIERICIDIN A BOUND (nmole/g protein)			
	1 Wash	3 Washes	5 Washes	7 Washes
10.4	8.16	7.44	6.92	6.80
26.0	17.72	15.76	14.80	13.44
52.0	27.76	22.76	21.24	19.16
104.0	31.36	24.12	20.80	19.12

Binding of 3H-piericidin A was measured as described in the Methods section. After incubation with 3-H piericidin 25mg amounts of beef heart mitochondria were repeatedly washed by centrifugation in sucrose/tris/BSA medium. 0.1ml aliquots were removed for scintillation counting at the stages shown above.

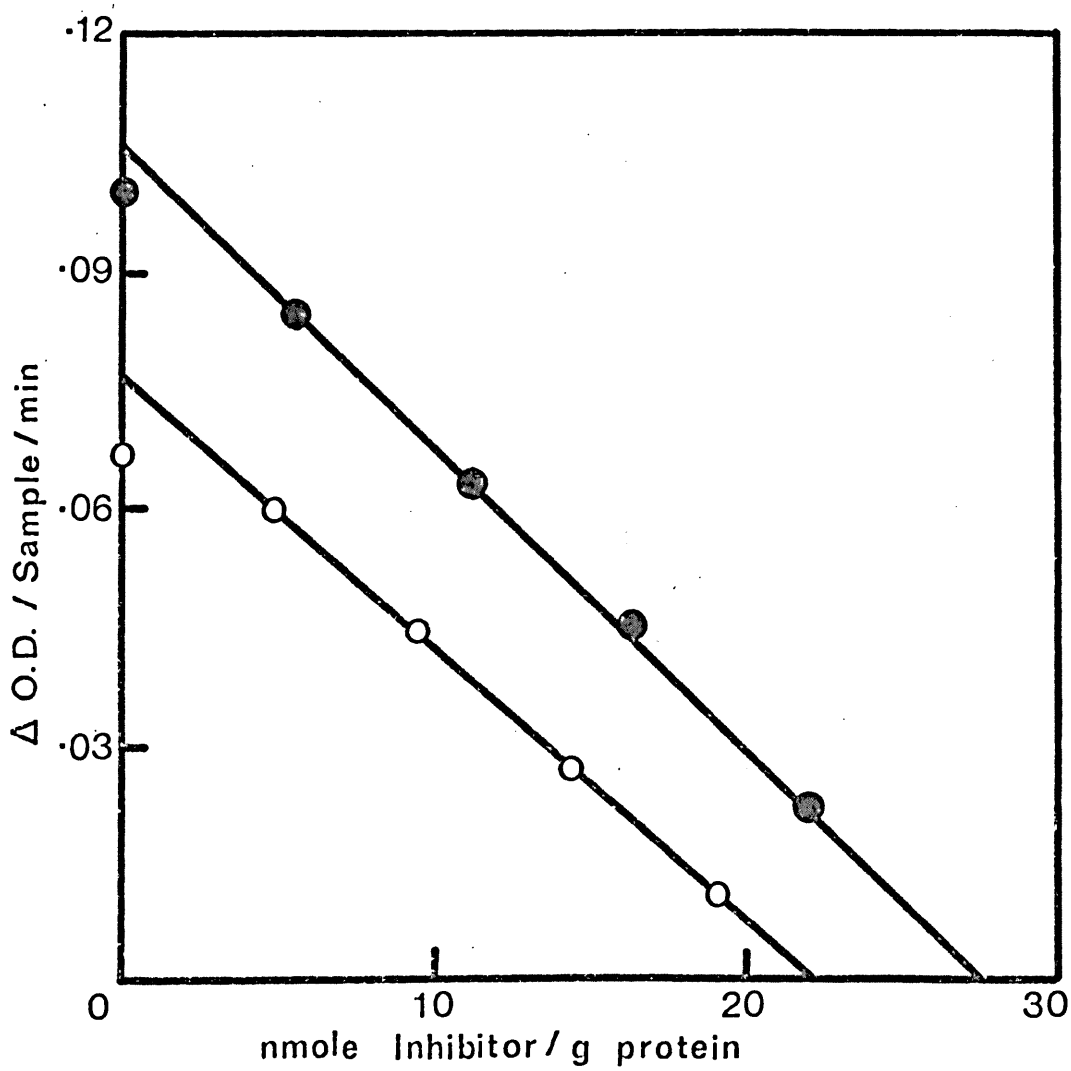


Fig. 7. TITRATION OF THE RESPIRATORY CHAIN OF BEEF HEART MITOCHONDRIA WITH PIERICIDIN A AND ROTENONE. NADH oxidation was measured as described in the legend to Fig. 1. ● rotenone. O piericidin A.

the respiratory chain, could be studied in more detail.

The curve for titration of beef heart mitochondria with 3H piericidin in a sucrose-tris medium is shown in Fig. 5. The effect on NADH oxidation was the same as that for non-radioactive piericidin. Curve B (Fig. 5) shows that binding of 3H piericidin proceeds in a linear manner even at concentrations several times that required to inhibit NADH oxidation maximally. This result indicates that piericidin binding is not restricted to the specific site in the NADH dehydrogenase region of the respiratory chain. The extent of non-specific binding was decreased considerably by the addition of BSA to the titration medium (Curve C). The presence of BSA causes the curve to become biphasic in nature, with an initial rapidly rising portion giving rise to a more gentle slope than was obtained in the absence of BSA. Fig. 6 shows that the rapidly rising portion of the curve could be abolished by titrating the mitochondria to maximal inhibition with unlabelled piericidin before addition of radioactive piericidin to the sucrose-tris-BSA medium. It is concluded that the initial part of the curve is due to binding of piericidin at the specific site.

Repeated washing of beef heart mitochondria with sucrose-tris-BSA after titration of the particles with 3H piericidin, resulted in complete removal of the non-specific binding and complete retention of the specifically bound material (Table 2). There was no change in the extent of inhibition observed, even after several washes, and there was good coincidence between the binding curve and the curve for inhibition of NADH oxidation. 3H reduced piericidin had the same binding characteristics as 3H piericidin.

(iv) Concentration of the Piericidin-Sensitive Factor.

The data presented above provide us with three methods for calculating the amount of piericidin bound to the specific site. Measurements of the rate of NADH oxidation in the presence of varying amounts of piericidin showed a linear decrease over the range of piericidin used. The minimum amount of piericidin required for maximum inhibition can be derived from the intersection of the curve with the abscissa (Fig. 7). This value corresponds to the concentration of the piericidin sensitive factor and was found to be 21.9nmole/g mitochondrial protein. Similar experiments

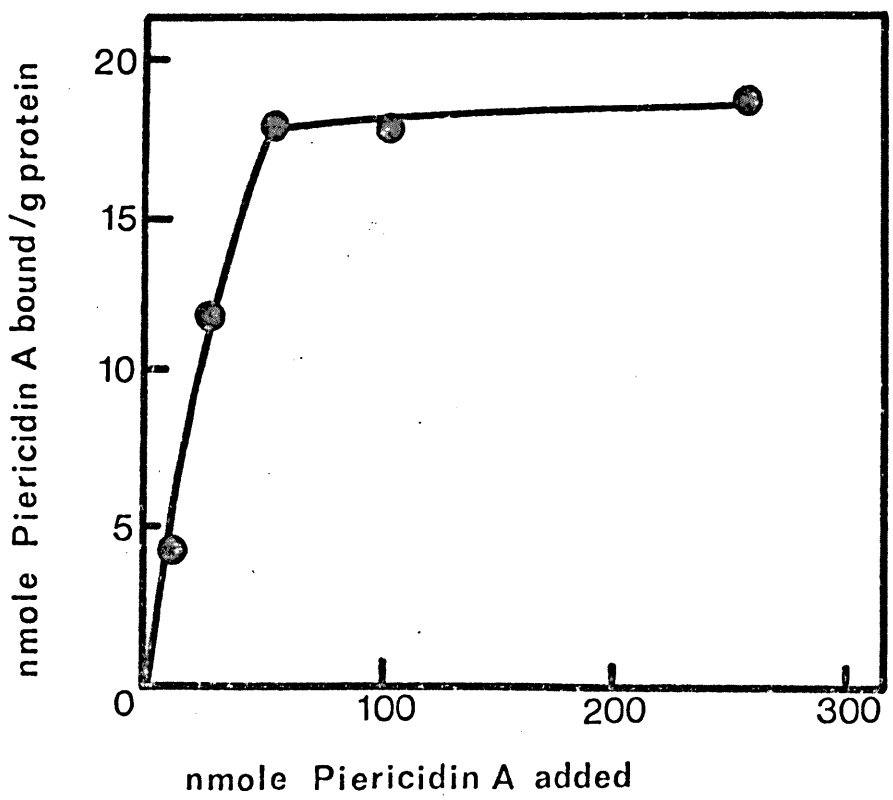


Fig. 8. 3-H PIERICIDIN A BOUND AT THE SPECIFIC SITE. This curve was obtained by subtracting curve C from curve B (see Fig. 6).

Table 3a

CONCENTRATION OF PIERICIDIN SENSITIVE FACTOR IN BEEF HEART MITOCHONDRIA

METHOD OF ESTIMATION	CONCENTRATION OF FACTOR (nmole/g protein)	
	PIERICIDIN	ROTENONE
Titration: NADH oxidase (Fig. 7)	21.9	27.5
Curve B - Curve C (Fig. 6)	18.6	-
Repeated washing (Table 2)	19.2	-
ATP-dependant reduction of NAD+ by succinate (Table 11)	19.6	21.4
NADH dehydrogenase content of beef heart mitochondria (see ref. 77)	18.7	

All methods used were as described in the legends to the figures and tables mentioned above.

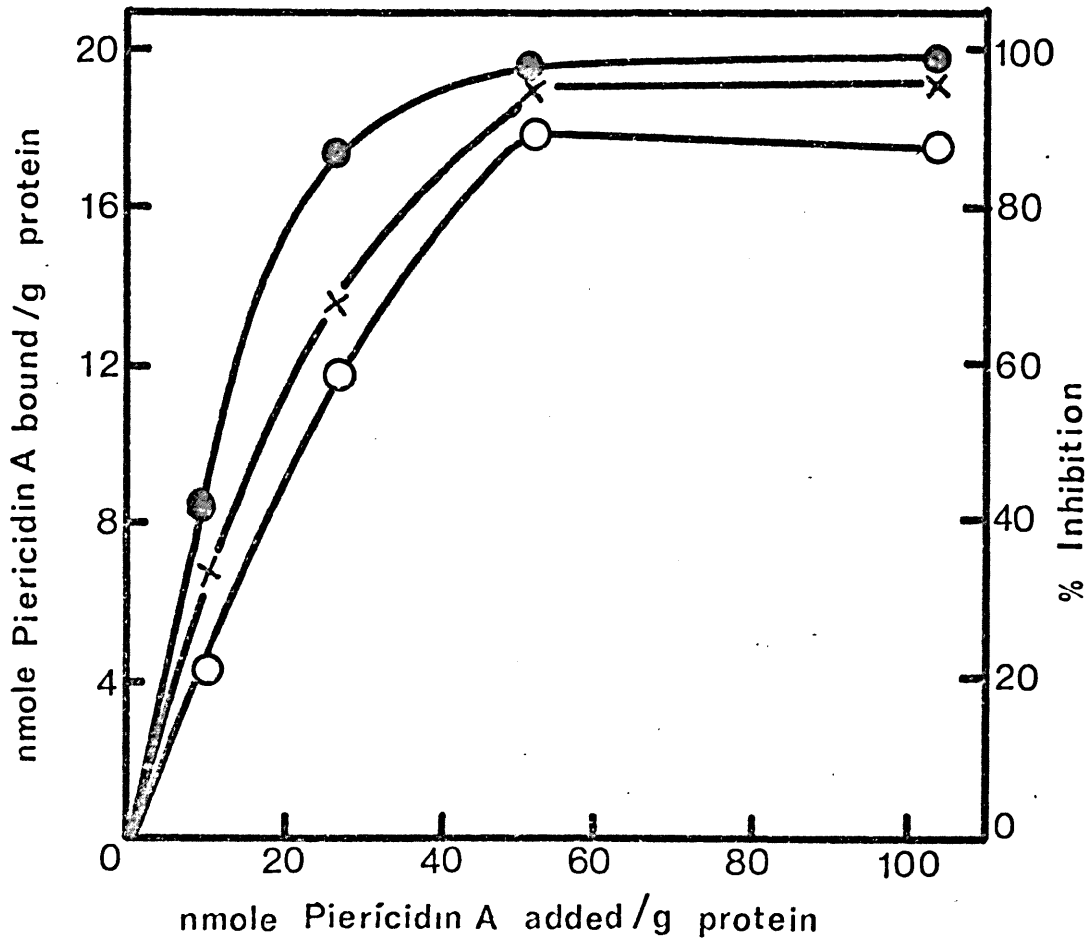


Fig. 9. COMPARISON OF BINDING AND INHIBITION OF NADH OXIDASE. Experimental details are described in the Methods section. ● NADH oxidase inhibition. X effect of repeated washing on 3-H piericidin binding (see Table 2). O effect of preincubation with unlabelled piericidin A (see Fig. 8).

were carried out with rotenone: the rotenone sensitive factor was estimated to be equivalent to 27.5nmole/g mitochondrial protein.

In the experiment shown in Fig. 6 the specific binding of 3H piericidin was abolished by the prior addition of unlabelled piericidin, leaving only non-specific binding. Subtraction of curve C (non-specific binding) from curve B (non-specific + specific binding) will give a measure of the piericidin specifically bound. The curve obtained is shown in Fig. 8 and the maximum 3H piericidin bound to mitochondrial protein was 18.6nmole/g.

Repeated washing of the 3H piericidin treated mitochondria with sucrose-tris-BSA resulted in removal of all the non-specifically bound 3H piericidin (Table 2). After 7 washes there was no further decrease in 3H piericidin binding but NADH oxidation was still maximally inhibited. The amount of 3H piericidin remaining bound after 7 washes is a further measure of the concentration of the piericidin sensitive site. The value obtained was 19.2nmole/g mitochondrial protein.

The different values for the piericidin sensitive factor together with the NADH dehydrogenase content of beef heart mitochondria are included in Table 3a(77). The results obtained were in good agreement with each other and with the NADH dehydrogenase content of the mitochondria. This agreement could be fortuitous, but is consistent with the site of action of piericidin proposed above i.e. piericidin does not prevent reduction of the NADH dehydrogenase flavin moiety, but prevents its reoxidation. Finally, there is good correlation between the amount of piericidin bound and the degree of inhibition observed (Fig. 9).

(v) Nature of Piericidin Sensitive Factor.

Burgos and Redfearn (74) proposed that lipid constituents might be involved in the NADH dehydrogenase system on the basis of its sensitivity towards organic solvents, surface active agents, and lipophilic inhibitors such as rotenone. Piericidin is also a lipophilic molecule, so the question of lipid involvement in 3H piericidin binding was examined in a number of ways.

Mitochondria were incubated with 3H piericidin at the concentrations

Table 3

EFFECT OF ACETONE EXTRACTION ON 3H PIERICIDIN BINDING IN BEEF HEART MITOCHONDRIA

	3H PIERICIDIN ADDED (nmole)	
	Experiment I	Experiment II
	0.65	1.3
cpm added	84,500	169,000
cpm bound	66,066	140,956
cpm bound (One acetone wash)	7,240	17,020
cpm bound (two acetone washes)	2,600	7,780

3H piericidin binding was measured as described in the Methods section. 25mg amounts of beef heart mitochondria were incubated with 3H piericidin at the concentrations shown and 0.1ml aliquots were removed for scintillation counting. The remaining mitochondria were lyophilised and extracted with 10ml acetone. The mitochondria were dried on a rotary evaporator and a further sample was removed for scintillation counting. The portion remaining was re-extracted with 5ml acetone, dried and counted.

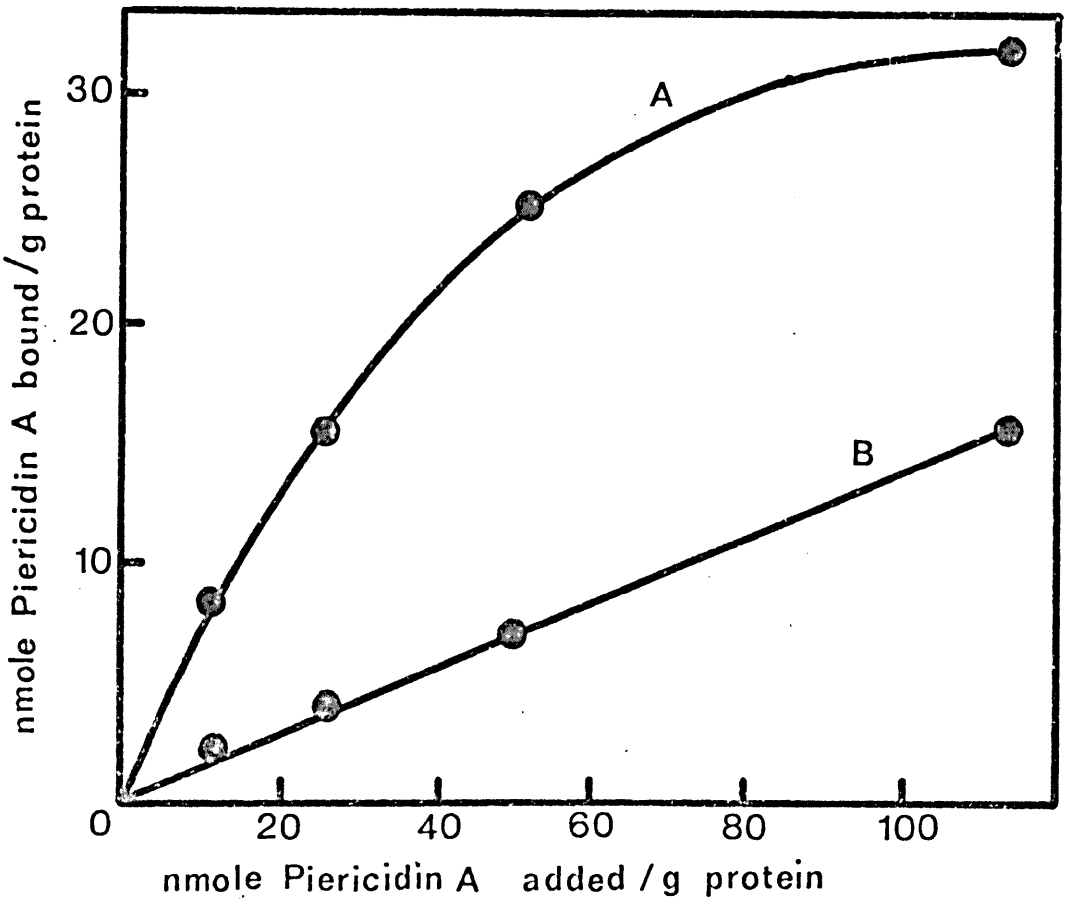
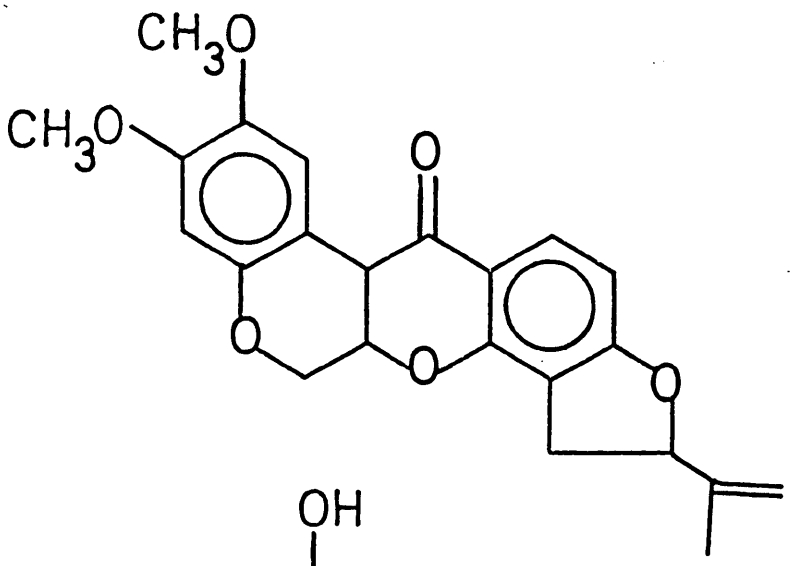


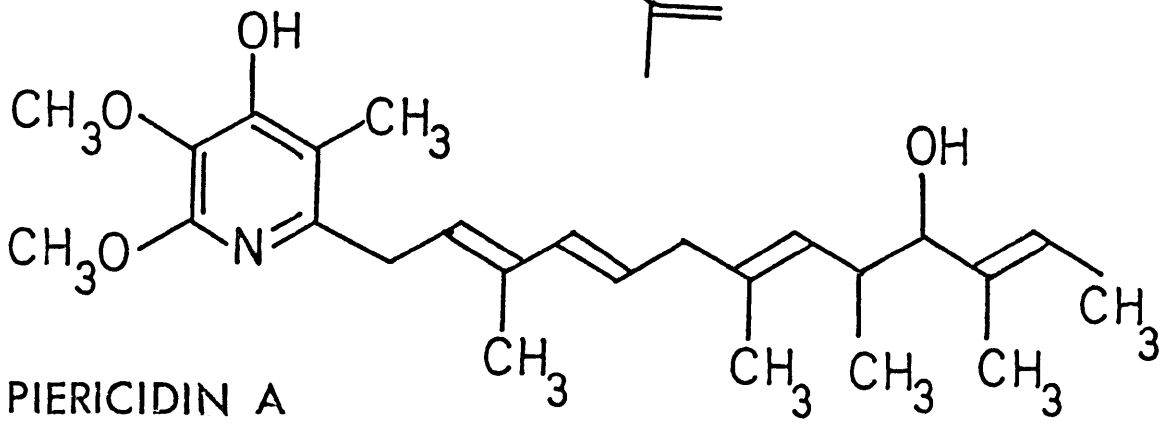
Fig. 10. BINDING OF 3-H PIERICIDIN TO ACETONE-EXTRACTED BEEF HEART MITOCHONDRIA. Mitochondria were lyophilised and extracted with acetone as described in the Methods section. The extracted mitochondria were incubated with varying amounts of 3-H piericidin, washed once with sucrose-tris-BSA and the amount of 3-H piericidin remaining bound was determined as described previously. A-3-Hpiericidin A bound to normal mitochondria. B-3-H piericidin A bound to acetone extracted mitochondria.

shown in Table 3 and the quantity of 3H piericidin remaining bound to the mitochondria was determined. The concentrations of piericidin employed were such that the majority was bound at the specific site. The mitochondria were then lyophilised and extracted twice with dry acetone. This treatment resulted in the removal of 95% of the 3H piericidin together with the mitochondrial lipids. Fleischer et al (78) have investigated the lipid composition of beef heart mitochondria by chromatography, using various solvent systems, after acetone extraction. The acetone extracts obtained in the present experiments were chromatographed on Silica Gel N, using chloroform: methanol: water (65: 25: 4) to separate the mitochondrial phospholipids. 95% of the radioactivity on the plate was found in association with the neutral lipid fraction, which moved with the solvent front, and no radioactivity was detected in the phospholipid spots. The neutral lipid fraction was chromatographed on two systems: wet benzene: acetone (25: 1) and chloroform: acetone (7: 3). 85% of the radioactivity was located at the Rf values peculiar to piericidin, and no radioactivity was found associated with any of the neutral lipid spots.

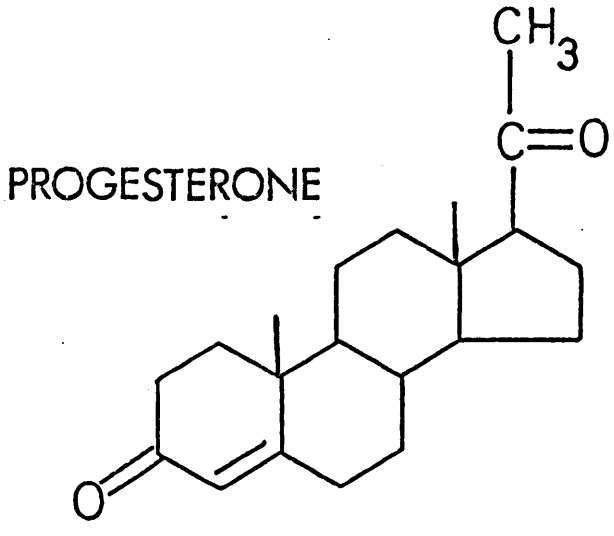
Piericidin appears to be removed unchanged from its specific binding site by acetone extraction, this indicates that it does not undergo any metabolic change before exerting its action. Although piericidin is not linked to any of the mitochondrial lipids, the removal of the compound with the lipid fraction does indicate that lipids play an essential role in the binding of piericidin at the specific site. To test this hypothesis the extent of piericidin binding to acetone extracted mitochondria was determined. Mitochondria were lyophilised and extracted with acetone to remove the lipid constituents (78). The extracted mitochondria were incubated with various amounts of 3H piericidin and the extent of piericidin binding was compared with that occuring in normal mitochondria. Fig. 10 shows that binding of 3H piericidin was decreased by 70 - 80% in acetone extracted mitochondria; this bound piericidin was easily removed by washing with sucrose-tris-BSA. These results show that binding of 3H piericidin at the specific site is completely dependant on the presence of mitochondrial lipids and that some non-specific binding can occur under these conditions.



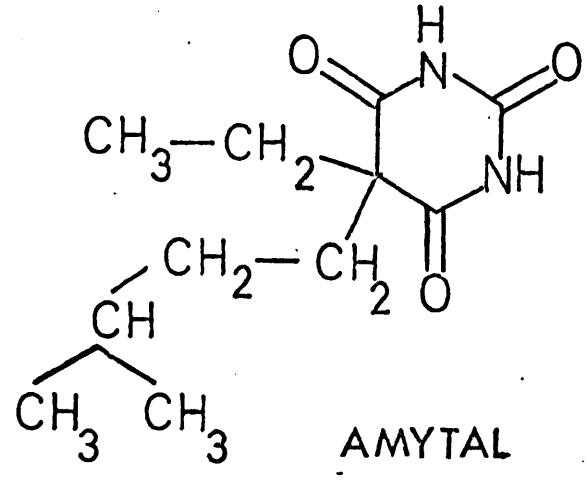
ROTENONE



PIERICIDIN A



PROGESTERONE



AMYTAL

Fig. 11. STRUCTURE OF INHIBITORS.

Table 4

EFFECT OF VARIOUS COMPOUNDS ON 3H PIERICIDIN BINDING

INHIBITOR	UNLABELLED INHIBITOR (conc.)	DECREASE 3H PIERICIDIN BINDING (nmole/g protein)	% INHIBITION NADH OXIDASE
Piericidin A (nmole/g protein)	0	0	0
	7.15	1.59	26
	14.30	2.97	62
	35.80	6.10	89
	71.50	7.97	98
Rotenone (nmole/g protein)	0	0	0
	5.5	2.09	21
	13.75	2.75	59
	27.50	4.73	85
	55.00	7.87	95
Amytal (mM)	0	0	0
	1	1.32	71
	3	5.66	85
	5	7.75	92
Progesterone (μM)	0	0	0
	25	1.75	45
	100	2.48	87
	500	5.52	100
Diethyl Stilboestrol (μM)	0	0	0
	25	0.50	70
	100	1.64	92
	500	4.96	100
Antimycin A (mg/g protein)	0	0	0
	2.75	0	100
	5.50	0.11	100

Unlabelled inhibitors were added to 25mg amounts of mitochondria at the concentrations shown. After incubation for 8min at 30° 26nmole 3H piericidin was added and incubated for a further 8min. 3H piericidin binding was then determined as described in the Methods section.

(vi) Specificity of Piericidin Binding Site.

Many compounds are known to inhibit the oxidation of NAD-linked substrates in mitochondria by exerting their effect on the NADH dehydrogenase region of the respiratory chain. It is not known if these compounds act at the same locus or whether they react with different parts of the same region of the chain. An examination of the types of compound possessing inhibitory action shows them to be of widely differing chemical structure (Fig. 11). The availability of labelled piericidin and its ability to bind at a specific site in the NADH dehydrogenase system provided a method for testing the other groups of unlabelled inhibitors, since it was shown that unlabelled piericidin was capable of preventing binding of 3H piericidin at the specific site (Fig. 6). It was reasoned that any inhibitor binding at the same site as piericidin would prevent binding of 3H piericidin.

Table 4 shows that piericidin, rotenone, amytal, progesterone and diethylstilboestrol were all capable of decreasing 3H piericidin binding when present in the medium in concentrations that produced inhibition. The more potent inhibitors piericidin and rotenone showed good correlation between the degree of inhibition observed and the extent of the decrease in binding of 3H piericidin obtained. The less active compounds amytal, progesterone and diethylstilboestrol appeared to bind less strongly to the inhibitory site. This was indicated by the observation that binding did not follow inhibition over the whole concentration range studied, particularly at low concentrations. The low binding capacity of these three inhibitors may account for the higher concentrations required for inhibition as compared to piericidin and rotenone. In support of this hypothesis, the steroid hormone oestrogen, which inhibits NADH oxidase to a maximum of 75% only at high concentrations, did not prevent 3H piericidin binding to any great extent (a maximum of 1.56nmole/g mitochondrial protein at 1mM-oestrogen). It is concluded that piericidin, rotenone, amytal and the steroid hormones are acting on the same component in the respiratory chain. Antimycin A, an inhibitor which does not act in the NADH dehydrogenase region of the respiratory chain, did not prevent 3H piericidin binding, showing that reversal of binding is specific to inhibitors that act in the same place as piericidin.

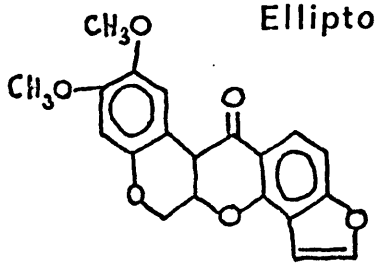
Table 5

EFFECT OF VARIOUS ROTENOIDS ON NADH OXIDATION

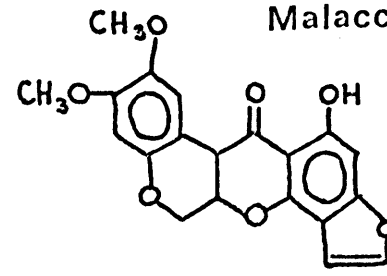
ROTENOID	CONCENTRATION FOR 50% INHIBITION (nmole/g protein)	RATIO
Rotenone	11	1
Elliptone	50	4.5
Malaccol	82	7.5
Rotenoid I	15×10^6	1.3×10^6
Rotenoid II	7.5×10^6	6.8×10^5

NADH oxidase was measured as described in the Methods section.

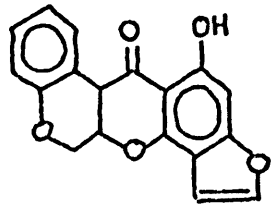
Elliptone



Malaccol



Rotenoid I



Rotenoid II

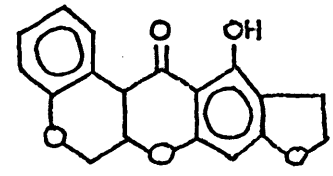
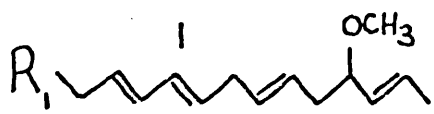


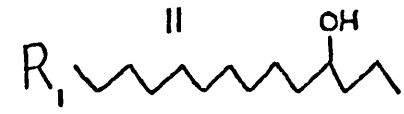
Table 6

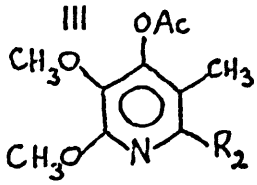
EFFECT OF PIERICIDIN ANALOGUES ON NADH OXIDATION IN BEEF HEART MITOCHONDRIA

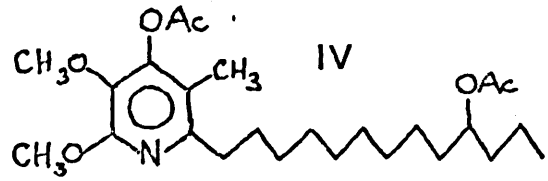
	COMPOUND	CONCENTRATION FOR 50% INHIBITION (nmole/g protein)	RATIO
	Piericidin A	11	1
I	Piericidin B	25	2.3
II	Octahydro piericidin A	25	2.3
III	Piericidin A monoacetate	12,000	1,100
IV	Octahydro Piericidin A diacetate	13,000	1,200
V	Compound I	2.5×10^7	2.3×10^6

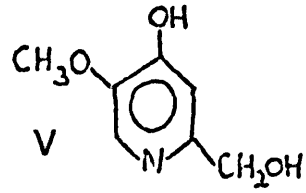
NADH oxidase was measured as described in the Methods section.











R_1 = Piericidin A nucleus

R_2 = Piericidin A side-chain

(vii) Structural Requirements for Piericidin and Rotenone as Inhibitors of NADH Oxidase.

The effects of various rotenoids on NADH oxidation and their structural requirements as inhibitors, have been discussed in some detail by Burgos and Redfearn (74). Table 5 shows the effect of five rotenoids on NADH oxidation by beef heart mitochondria. Rotenone was the most potent of the compounds tested. Elliptone, which differs from rotenone in that the C-5' side-chain is missing, was only slightly less potent than rotenone. The activity was reduced further by the introduction of a hydroxyl group at position 11, as in malaccol. The most dramatic decrease in activity was observed when the methoxyl groups were absent, as in 2,3-desmethoxy malaccol, which was 100,000 times less potent than malaccol. To summarize, for a rotenoid to be a potent inhibitor it must possess ring methoxyl groups and to a lesser extent the lipophilic side-chain at C-5'. Further decrease in lipophilicity, achieved by the introduction of a hydroxyl group at position 11, also results in a slight loss of activity.

Similar studies were carried out using piericidin analogues (Table 6). Again, the parent compound, piericidin A, was the most active and reduction of the unsaturated hydrocarbon side-chain gave only a slight loss of potency. Methylation of the side-chain hydroxyl group to produce piericidin B had little effect on the activity, indicating that this group was not involved in the inhibitory process. Acetylation of the ring hydroxyl group brought about a 1,000 fold decrease in potency. Cleavage of the side-chain from piericidin A was not achieved, but the synthetic compound 2-hydroxymethyl, 4-hydroxy, 5-methoxy-pyridine served to demonstrate that the lipophilic side-chain is probably essential for activity. These studies show that at least two factors are important if a piericidin is to be a potent inhibitor (i) a long lipophilic side-chain (ii) a ring hydroxyl group.

(viii) Binding Characteristics of Piericidin and Rotenone Analogues.

Various analogues were tested for their ability to decrease the binding of 3H piericidin. It was found that elliptone, malaccol, piericidin B and reduced piericidin A all abolished binding of radioactive

Table 7

EFFECT OF PIERICIDIN AND ROTENONE ANALOGUES ON 3H PIERICIDIN BINDING IN BEEF HEART MITOCHONDRIA

COMPOUND	nmole added/g protein	% INHIBITION	3H PIERICIDIN BOUND (nmole/g protein)
Piericidin A	14.3	58	13.20
Rotenone	14.0	57	12.90
Elliptone	66.5	60	12.97
Piericidin B	35.0	65	12.32
Octahydro piericidin A	40.0	62	12.80
Malaccol	105.0	67	12.53
None	0	0	16.23

Unlabelled analogues were added to 25mg amounts of beef heart mitochondria at the concentrations shown. After incubation for 8min at 30° 26nmole 3H piericidin A were added and incubated for a further 8min. 3H piericidin binding was then determined as described in the Methods section. All the analogues tested decreased 3H piericidin binding to the same extent indicating that they were all acting at the same site.

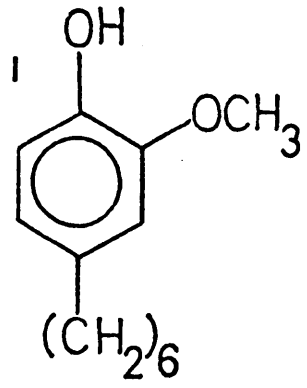
Table 8

EFFECT OF PYRIDINE ANALOGUES ON NADH OXIDATION IN BEEF HEART MITOCHONDRIA

COMPOUND	CONCENTRATION FOR 50% INHIBITION (μM)	RATIO
I	45	1.0
II	100	2.2
III	110	2.4
IV	120	2.7
V	290	6.4
VI	350	7.8
VII	600	13.3
VIII	800	17.8
IX	900	20.0
X	1200	26.7
XI	3000	65.6
XII	5000	111.0
XIII	5500	122.0

NADH oxidation was measured as described in the Methods section. Pyridine analogues were added to the incubation medium to give the concentrations shown. The formulae for the pyridine analogues are given on the following page.

I



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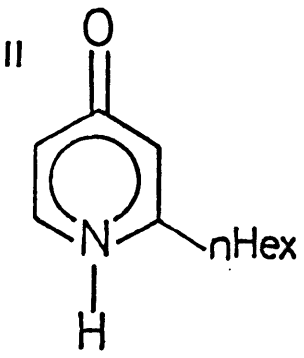

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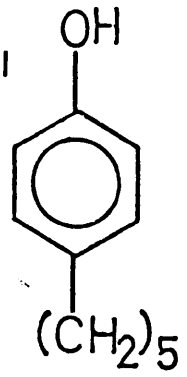

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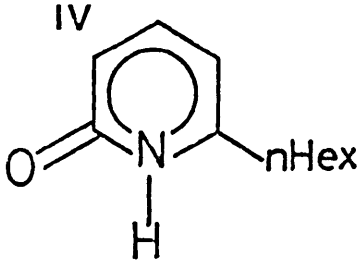

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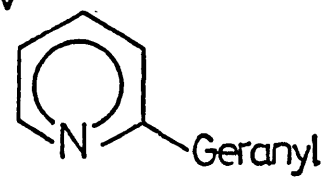

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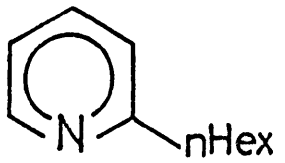

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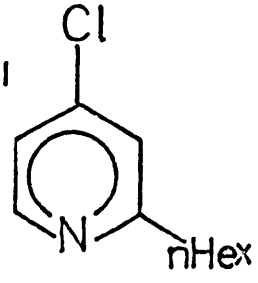

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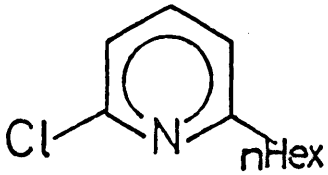

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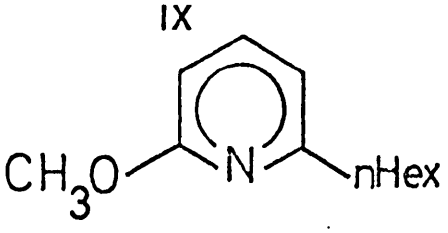

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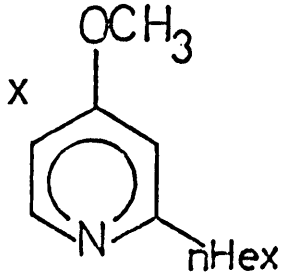

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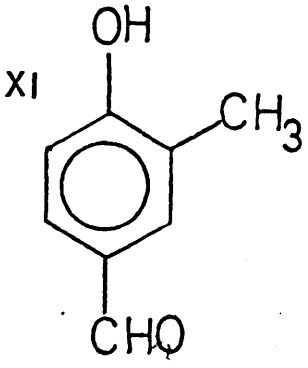

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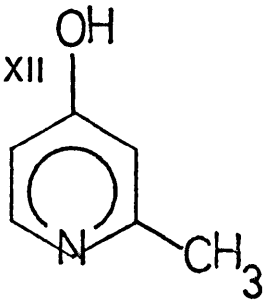

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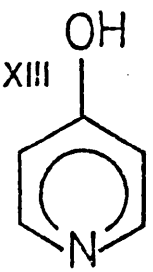


nHex = nHexyl

piericidin at the specific side. There is a good correlation between the ability of a compound to inhibit NADH oxidase and to prevent subsequent binding of 3H piericidin (Table 7), although the compounds differ in their inhibitory potency. It is concluded that these compounds are all acting at the same site and that this site is responsible for inhibition of NADH oxidase. No binding was detected with the less potent analogues, desmethoxy malaccol, piericidin A monoacetate and reduced piericidin A diacetate at the inhibitory concentrations tested. It seems likely, therefore that these weak inhibitors do not bind very strongly to the respiratory chain (cf oestrogen).

(ix) The Effect of Pyridine Analogues on NADH Oxidation.

Piericidin is a pyridine substituted at all five carbons in the ring. Attempts were made to simplify the piericidin structure by synthesising a series of pyridine analogues; their inhibitory action towards NADH oxidase was then tested (Table 8).

Compounds 1 - 4 were the most potent tested, but none of the derivatives approached piericidin in its effect. They were, however, comparable in activity to the steroids and barbiturates and may represent a new group of compounds inhibiting NADH oxidase by acting on the NADH dehydrogenase system.

Compounds 5 - 10, which have no ring oxygen function but still possess a lipophilic side chain, were less potent than those analogues (1-4), which had both a ring oxygen and a lipophilic side chain. The last group (11-13), which had a ring oxygen but no side chain were the least potent of the analogues tested. These results indicate that the requirements for a good inhibitor are (i) a ring oxygen group (ii) a lipophilic side chain and (iii) a ring methoxy group. The latter is thought to be necessary because the most potent inhibitor (compound 1) possesses this group; as does compound 11, which is more active than compounds 12 and 13.

(x) The Effect of Piericidin on Other Mitochondrial Dehydrogenases.

Piericidin binds and inhibits at a specific site in the NADH dehydrogenase region of the respiratory chain; also it appears to bind to

Table 9

EFFECT OF PIERICIDIN A ROTENONE AND AMYTAL ON VARIOUS MITOCHONDRIAL DEHYDROGENASES

ENZYME	INHIBITOR	CONCENTRATION (μM)	% INHIBITION
Yeast ADH	Rotenone	0.33	18
		3.3	30
		33.3	60(max)
	Piericidin	320	6
	Amytal	330	3
Beef Liver GDH	Rotenone	1.6	48
		2.5	60
		6.6	85
	Piericidin	1.6	43
		3.2	65
		7.9	83
	Amytal	3300	0
Pig Heart MDH	Rotenone	33	2
	Piericidin	3.2	40
		7.9	65
		16.0	94
	Amytal	3300	5

The enzymes were purchased from Boehringer und Soehne, G.m.b.H., Mannheim, Germany. Enzyme activities were measured as described in the Methods section. Abbreviations: ADH - yeast alcohol dehydrogenase; GDH - beef liver glutamic dehydrogenase; MDH - pig heart malic dehydrogenase.

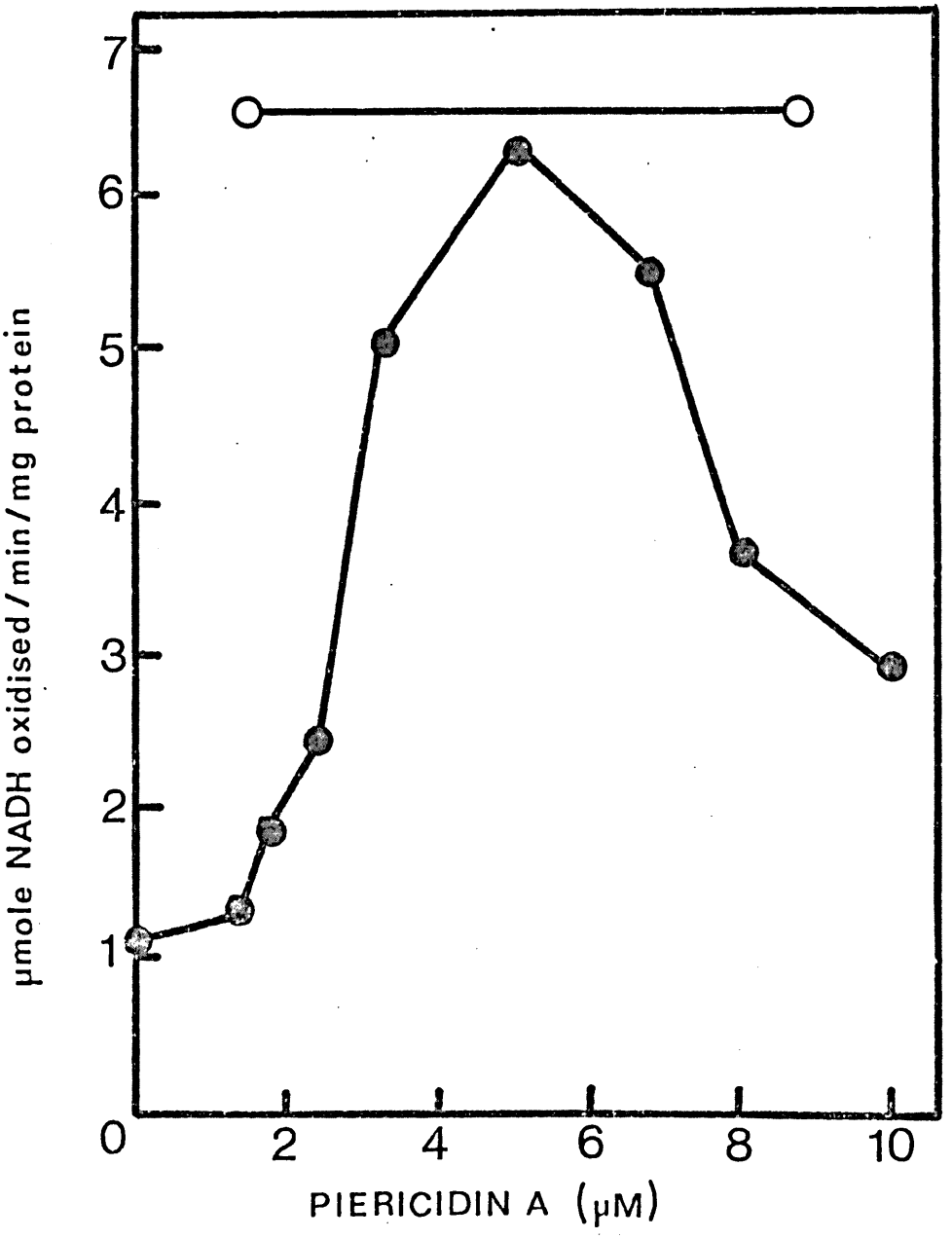


Fig. 12. EFFECT OF PIERICIDIN A ON BEEF HEART MITOCHONDRIAL MALIC DEHYDROGENASE. Conditions were identical to those described in the legend to Table 9, except that 16µg mitochondrial protein were added instead of the crystalline enzyme. 1.0mM KCN was added to prevent oxidation of NADH via the respiratory chain. ● piericidin A. O level of activity produced by incubation of the mitochondria with 5ug triton X-100.

non-specific sites in beef heart mitochondria. Earlier reports in the literature have shown that rotenone is an inhibitor of other mitochondrial dehydrogenases such as alcohol dehydrogenase (79) and glutamic dehydrogenase (80) and that certain steroid hormones inhibit glutamic dehydrogenase (81, 82). It is possible, therefore, that non-specific binding of piericidin is due to interaction with other mitochondrial dehydrogenases, and that piericidin, like rotenone, might inhibit these enzymes. A study was made of the effects of piericidin, rotenone and amytal on certain mitochondrial dehydrogenases and of the binding of piericidin to these enzymes.

Table 9 shows that alcohol dehydrogenase was inhibited by rotenone but not by piericidin or amytal at the concentrations tested. On the other hand, glutamic dehydrogenase was inhibited by both piericidin and rotenone. The inhibitory pattern was identical for both compounds, suggesting that they were acting in the same way on this enzyme. Amytal had no effect on the enzyme at a concentration of 3.3mM. Piericidin alone inhibited malic dehydrogenase; 100% inhibition occuring in the region of 10-20μM. Kinetic studies of the interaction of piericidin with pig heart malic dehydrogenase showed that inhibition was of the mixed competitive - non-competitive type (cf ref.79), whether NADH or oxaloacetate was the substrate that was varied. These results show that piericidin is capable of inhibiting certain purified mitochondrial dehydrogenases and this might account for the non-specific binding observed in earlier experiments. Malic dehydrogenase was selected for a study of the effect of piericidin on a mitochondrial enzyme in situ. The malic dehydrogenase activity of intact beef heart mitochondria was found to be relatively low (1.2μmole NADH oxidised/min/mg mitochondrial protein). Surprisingly the activity was increased 5-6 fold, (6.5μmole NADH oxidised/min/mg mitochondrial protein) by low (1-5μM) concentrations of piericidin (see Fig. 12). The same degree of stimulation was obtained with triton X-100, a non-ionic detergent which is known to lyse mitochondria (83). When the concentration of piericidin was increased beyond 5μM then inhibition of the stimulated malic dehydrogenase occured, with maximum inhibition at 20μM as before. Neither piericidin nor triton X-100 produced any stimulation of the soluble malic dehydrogenase located in the 100,000 x g supernatant, ruling out any direct activation of the enzyme by these compounds.

Table 10

BINDING OF 3H PIERICIDIN TO VARIOUS PROTEINS

	BEEF LIVER GDH	PIG HEART MDH	BSA
Mg protein present	1.0	1.0	1.0
Mg protein recovered	0.8	0.78	0.82
cpm bound/mg protein	2.22×10^6	1.91×10^6	2.49×10^6
% added 3H piericidin bound	71	61	79

1.0mg amounts of the proteins shown above were incubated with 3H piericidin A (2.54×10^6cpm) for 30min in 4.0ml 0.25M-sucrose containing 10mM-tris-HCl (pH 7.5) at 30°. At 30min 0.1ml 10% (w/v) TCA was added and the precipitated proteins were collected by centrifugation and the supernatant was discarded. The proteins were resuspended in 1.0ml buffer containing 0.05ml 10% (w/v) DOCA and 0.1ml aliquots were removed for scintillation counting. The total protein recovered was determined by the biuret reaction. Abbreviations: GDH - glutamic dehydrogenase; MDH - malic dehydrogenase; BSA - bovine serum albumin; TCA - trichloracetic acid; DOCA - deoxycholate.

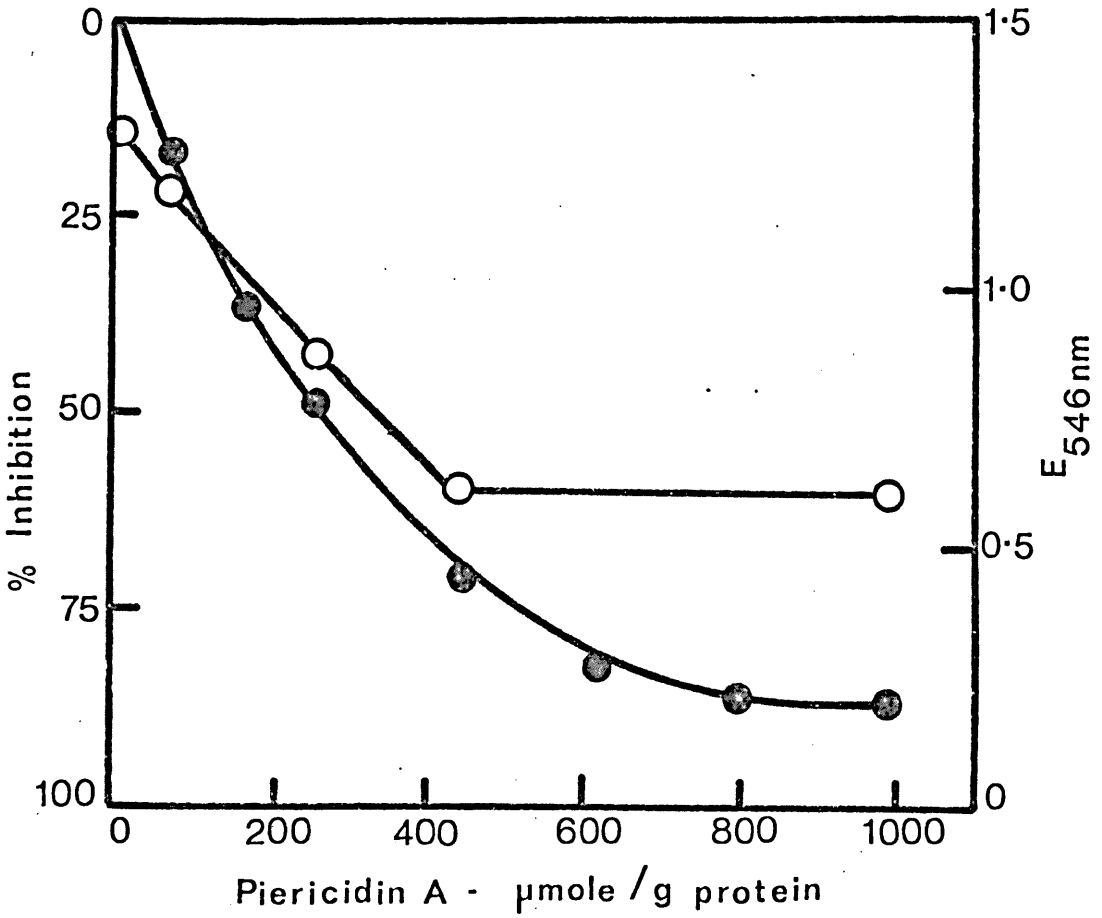


Fig. 13. THE EFFECT OF PIERICIDIN A ON SUCCINATE OXIDATION AND LYSIS OF BEEF HEART MITOCHONDRIA. Succinate oxidation was measured polarographically as described in Fig. 2. Using the same medium, the extent of mitochondrial lysis was measured by determining the decrease in absorbance at 546nm. Beef heart mitochondria were suspended in 3.0ml sucrose-tris-magnesium and the absorbance was measured. Piericidin A was added at the concentrations shown, the contents of the cuvettes were mixed and the absorbance was again determined after 10min. 3.0mg mitochondrial protein were used in all the determinations and the temperature of the incubations was 30°. ○ Lysis. ● Succinate oxidation.

Table 11

EFFECT OF PIERICIDIN A ON ENERGY-LINKED REDUCTION OF NAD^+ BY SUCCINATE IN BEEF HEART MITOCHONDRIAL PARTICLES

INHIBITOR	CONCENTRATION (nmole/g protein)	% INHIBITION
Piericidin A	6	9
	12	49
	18	91
Rotenone	5	10
	12.5	54
	20	90
Amytal	170 (μM)	33
	340	66
	850	96

Blank and experimental cuvettes contained 675μmole sucrose, 135μmole tris-HCl buffer (pH 8.0), 16μmole magnesium chloride, 3μmole KCN, 3μmole NAD^+ and 15μmole succinate. 0.8mg beef heart submitochondrial particles were added to both cuvettes and allowed to equilibrate for 3min. The reaction was started by the addition of 6μmole ATP to the experimental cuvette, to give a final volume of 3ml. The resulting increase in absorbance was measured at 340nm in a Beckman DK 2 recording spectrophotometer at 30°. Inhibitors were added 30sec before the ATP.

Since malic and glutamic dehydrogenases are inhibited by piericidin, then it is possible that these enzymes are candidates for the non-specific binding of the inhibitor. In this connection Table 10 shows that both enzymes, as well as BSA, are able to bind 3H piericidin. It was not possible to show release of bound 3H piericidin by washing with BSA because this protein could not be separated from the enzymes after TCA precipitation. It is concluded that piericidin is not a specific inhibitor of NADH dehydrogenase because piericidin is able to combine with other mitochondrial dehydrogenases.

(xi) Mechanism of Piericidin Inhibition of Succinate Oxidation.

The indication that piericidin could bring about lysis of mitochondria at high concentrations (see effect of piericidin on mitochondrial malic dehydrogenase in the previous section) suggested a possible mechanism for the inhibition of succinate oxidation observed at high piericidin concentrations. Maximum lysis occured at a concentration of 0.5μmole/mg mitochondrial protein and maximum inhibition of succinate oxidation was in the region of 0.8-1.0μmole/mg protein. There was a good correlation between the extent of lysis and the degree of inhibition of succinate oxidation over the complete concentration range (Fig.13). These results are in agreement with those obtained for the effect of the non-ionic detergent triton X-100 on succinate oxidation (83).

(xii) The Effect of Piericidin on Energy-Linked Reactions.

Quinones were first implicated as intermediates in energy-linked reactions by Chance et al (84) and it has also been suggested that piericidin is a quinone analogue (69, 71). Because of this relationship energy-linked reactions were tested for their sensitivity towards piericidin. The ATP-dependant reduction of NAD^+ by succinate, catalysed by beef heart submitochondrial particles, was inhibited by the same low levels of piericidin required for inhibition of NADH dehydrogenase (Table 11). This was consistent with the findings of Griffiths and Roberton (85) who showed that rotenone and amytal inhibited the energy-linked reversal of electron transport at the same concentrations as were required for inhibition of NADH oxidation. The present findings lend support to the proposal

Table 12

EFFECT PIERICIDIN A ROTENONE AND AMYTAL ON ENERGY-LINKED REDUCTION OF $NADP^+$ BY NADH IN BEEF HEART SUBMITOCHONDRIAL PARTICLES

REACTION	INHIBITOR	CONCENTRATION (μM)	% INHIBITION
ATP driven	Piericidin	22	46
		34	73
		45	90
	Rotenone	33	0
	Amytal	3300	0
Succinate driven	Piericidin	13	37
		32	84
		42	93
	Rotenone	33	0
	Amytal	3300	0

The reactions were measured as described in the Methods section. Inhibitors were added 30sec before the addition of ATP or succinate and corrections were made for the non-energy-linked reaction.

Table 13

EFFECT PIERICIDIN A ON ELECTRON TRANSPORT REACTIONS IN E. COLI

SUBSTRATE	PIERICIDIN A (nmole/ mg protein)	% INHIBITION
NADH	25	57
	50	68
	100	82
	150	87
Succinate	25	35
	50	60
	100	80
	150	87
NADPH	25	41
	50	63
	100	88

Oxidase activities were measured polarographically using an oxygen electrode. The reaction medium contained 750µmole sucrose, 150µmole tris-HCl buffer (pH 7.5), 18µmole magnesium chloride and 3.5mg bacterial particles. The amounts of substrates added were 10µmole sodium succinate or 5µmole NADH or 5µmole NADPH. Inhibitors were added approximately 3min after the substrate. The temperature of the incubation was 30°.

that the NADH dehydrogenase enzyme system is involved in both reactions (85). The concentration of the piericidin sensitive factor in the reaction was found to be 19.6nmole/g mitochondrial protein and this was in good agreement with the result obtained for NADH oxidation (21.9nmole/g protein). Similar studies with rotenone gave a value for the rotenone sensitive factor of 21.4nmole/g protein.

The energy-linked transhydrogenase of beef heart submitochondrial particles does not involve NADH dehydrogenase and consequently neither rotenone nor amytal inhibit the reaction. Piericidin also did not affect the energy-linked transhydrogenase at concentrations which inhibited NADH dehydrogenase, but at higher concentrations the energy-dependant reaction was inhibited. Maximum inhibition occured in the region of 40-50μM, and the sensitivity of the reaction to piericidin was independant of the energy source: either ATP or high energy intermediates generated by the aerobic oxidation of succinate could be used (Table 12). Attempts to reverse the inhibition by the addition of ubiquinone homologues were unsuccessful and these experiments were complicated by the finding that ubiquinone was an inhibitor of the reaction.

(xiii) The Effect of Piericidin on Bacterial Electron Transport.

The inhibition of the respiratory particles prepared from E.coli was quite different from that of beef heart mitochondria. The levels of piericidin needed to inhibit NADH oxidation were much higher than in the mammalian system; 100nmole/mg bacterial protein as compared to 0.02nmole/mg mitochondrial protein. The amount of piericidin required for inhibition of NADH oxidation was the same as that required for inhibition of both succinate and NADPH oxidation (Table 13); there was no differentiation, as in the beef heart preparation. It is concluded that piericidin inhibits the oxidation of all three substrates at the same site, and this site is in the common terminal portion of the respiratory chain.

(xiv) The Effect of Piericidin on Bacterial Energy-Linked Reactions.

In Chapters III and IV of this thesis the ATP-dependant reduction of NAD^+ by succinate (energy-linked reversal of electron transport) and the ATP-dependant reduction of $NADP^+$ by NADH (energy-linked transhydrogenase),

Table 14

EFFECT PIERICIDIN A ON ENERGY-LINKED REACTIONS IN E. COLI

REACTION	PIERICIDIN A (nmole/mg protein)	% INHIBITION
ATP-driven reduction of NAD^+ by succinate	80	+35
	120	+24
	160	+5
	200	17
ATP-driven reduction of $NADP^+$ by NADH	80	28
	120	44
	160	83
	200	100

The incubation medium for the ATP-driven reduction of NAD^+ by succinate contained 375µmole sucrose, 75µmole tris- HCl, (pH 8.0), 10µmole magnesium chloride, 3µmole NAD^+, 5µmole sodium sulphide, 20µmole succinate and 2.5mg bacterial protein (small particles). The energy-dependant reaction was started by the addition of 6µmole ATP to the experimental cuvette to give a final volume of 2.0ml. The temperature of the incubation was 30°. Inhibitors were added 30sec before ATP.

The incubation medium for the ATP-driven reduction of $NADP^+$ by NADH contained 375µmole sucrose, 75µmole tris-HCl buffer (pH 8.0), 10µmole magnesium chloride, 0.05µmole NAD^+, 200µg yeast alcohol dehydrogenase, 180µmole ethanol, 5µmole sodium sulphide and 2.5mg bacterial protein (small particles). $NADP^+$ was added to the experimental cuvette, followed by 6µmole ATP to give a final volume of 2.0ml. The temperature was 30° and inhibitors were added 30sec before the ATP. Both reactions were measured at 340nm.

catalysed by E. coli small particles, are described. The present section deals with the effect of piericidin on these reactions.

Unlike the beef heart enzyme system, the ATP-dependant reduction of NAD^+ by succinate, catalysed by the bacteria was not inhibited by piericidin. At concentrations up to 500nmole/mg bacterial protein (five times greater than was required for inhibition of NADH and succinate oxidation) no inhibitory effect was observed. In many experiments the reaction was increased in the presence of piericidin (Table 14). This might be due to incomplete block of respiration by the sulphide in the reaction medium. This means that leakage of electrons from succinate to oxygen could have been prevented; this would have allowed the electrons to travel back more efficiently to NAD^+, thus producing an increase in the reaction rate. A similar effect was obtained with NHQNO (see Chapter III).

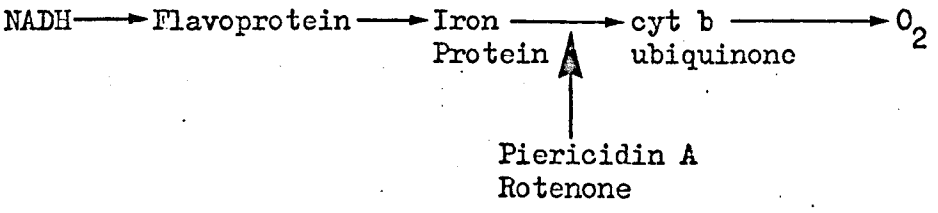
The energy-linked transhydrogenase is inhibited 100% by piericidin at a concentration of 200nmole/mg bacterial protein (Table 14). A similar effect was observed with beef heart submitochondrial particles. Again attempts to reverse the inhibition with ubiquinone homologues were complicated by the inhibitory effect of these compounds on the reaction.

DISCUSSION.

The pattern of inhibition of NADH oxidation by piericidin A was strikingly similar to that obtained with rotenone, amytal and certain steroid hormones. Piericidin A was unusual, however, in that it also inhibited the oxidation of succinate by beef heart mitochondria, but at much higher concentrations than those required for NADH oxidase. One of the most active areas, and one of the most controversial, in the study of electron transport has been the sequence of electron carriers in the NADH dehydrogenase region of the respiratory chain. Purified NADH dehydrogenases (90-92) containing nonhaem iron, FMN and labile sulphide have different molecular weights and different specificities with regard to electron acceptors. There seems to be little agreement as to which one of the enzymes, if any, represents the normal dehydrogenase. Furthermore, little is known about the concentration of the enzyme in mitochondria although estimates have been made (77). The redox state of the flavoprotein in mitochondria is unknown, but Chance et al (93) using a combination of double-beam spectrophotometry and fluorimetry have proposed that there are two flavoproteins (FpD1 and FpD2) in the NADH dehydrogenase. Hatefi (94) found that the absorption changes observed by Chance were greater than could be accounted for in terms of flavin content alone. He assumed that at least half the colour change was due to oxidation-reduction of protein-bound iron and maintained that there was one flavoprotein and one iron protein. It is not surprising to find that against this confusing background there was considerable dispute about the site of action of inhibitors, such as rotenone and amytal.

The site of action of rotenone has been variously placed on the substrate side of the flavin (1, 72), between FpD1 and FpD2 (93), between a flavoprotein and an iron protein (94), at the nonhaem iron protein (95) and on the oxygen side of the nonhaem iron (96). In the present work measurements of cytochrome b reduction, in the presence and absence of piericidin and rotenone, confirm that the site of action of these compounds is on the substrate side of cytochrome b. Measurements of the components absorbing in the 465nm region (i.e. flavin plus nonhaem iron)

showed that in cyanide inhibited mitochondria pyruvate and malate were capable of reducing both NADH and succinate dehydrogenase components; reduction of the remainder was achieved by the addition of succinate. Succinate, on the other hand only reduced the succinic dehydrogenase component. The presence of piericidin or rotenone had no effect on the reduction of the 465nm material in the NADH dehydrogenase segment of the chain. These results indicate that piericidin and rotenone act at the same site in the NADH dehydrogenase region of the respiratory chain. It is concluded that this site is on the oxygen side of the 465nm absorbing material; i.e. on the oxygen side of both the NADH dehydrogenase flavin and the NADH dehydrogenase nonhaem iron, at the point indicated in the following scheme.



These conclusions are in agreement with those of Palmer et al (96) who demonstrated by EPR spectroscopy that the nonhaem iron signal at $g = 1.94$ appeared unchanged in the presence of piericidin or rotenone. The site of action of piericidin and rotenone is therefore identical in intact mitochondria and submitochondrial particles.

It has been reported in the literature that rotenone (76) and piericidin (71) were titrating stoichiometrically with a component present in mitochondria in lower concentrations than any of the known redox agents. These conclusions may be based on an overestimate of the NADH dehydrogenase content of mitochondria, since the values have been based on the total flavin reducible by NADH (98), whereas Cremona and Kearney (77) have shown that the FMN content of NADH dehydrogenase is some twenty times less than the total flavin: they arrived at a value of 18.7nmole NADH dehydrogenase/g mitochondrial protein. If 18.7nmole is a realistic figure then the concentration of piericidin (21.9nmole/g) and rotenone (27.5nmole/g) required for maximum inhibition of NADH oxidase is identical to the NADH dehydrogenase content.

Radioactive piericidin was synthesised to determinewhether piericidin and rotenone were acting at the same site and to resolve the question of stoichiometry. The binding curves obtained with 3H piericidin showed that piericidin was bound to mitochondria even at concentrations beyond those required for maximum inhibition of NADH oxidase (110). Thus, binding of 3H piericidin is by no means specific for the site which is responsible for inhibition of NADH oxidase. Titrating with piericidin in the presence of BSA results in a decrease in the binding of 3H piericidin. The curve resolves itself into an initial rapidly rising portion and a second linear portion with a more gentle slope. This latter portion could be abolished by repeatedly washing the mitochondria with BSA, leaving the initial portion unchanged. Of special interest in this respect was that the inhibition of NADH oxidase was unaffected, showing that this part of the bound 3H piericidin was responsible for inhibition of NADH oxidase. BSA therefore has little effect on the specifically bound piericidin, but it causes marked dissociation of piericidin from the non-specific sites.

Pretreatment of mitochondria to maximum inhibition with unlabelled piericidin and subsequent titration with 3H piericidin causes abolition of the initial rapidly rising portion of the curve, but has no effect on the non-specific sites; thus 3-H piericidin does not displace the tightly bound unlabelled compound from the specific site. The amount of piericidin binding to the specific site (18.6 - 19.2nmole/g) shows good agreement with the NADH dehydrogenase content of the mitochondria (18.7nmole/g) and is in agreement with the values determined for rotenone and piericidin by direct titration of NADH oxidase with unlabelled inhibitor. These values are two to three times lower than those determined by Horgan et al (99) for titration of electron transport particles with 14C piericidin A and 14C rotenone, but this may be due to increased purification of the particle over the intact mitochondria. The excellent coincidence between the binding curves, as determined in the washing experiments, and the experiments involving pretreatment with unlabelled inhibitor, and the inhibition curve of NADH oxidase, shows that tightly bound piericidin is responsible for inhibition of NADH oxidase.

Other inhibitors of NADH oxidase are capable of preventing 3H piericidin from binding to the specific site when added to the mitochondria before 3H piericidin; thus rotenone abolished the binding of subsequently added 3H piericidin in the same manner as unlabelled piericidin. The less potent compounds amytal, progesterone and diethylstilboestrol were also effective, but the decrease in 3H piericidin binding was less dramatic than with piericidin or rotenone. It is concluded that because of the good correlation between inhibitory capacity and ability to prevent piericidin binding that piericidin, rotenone, amytal, progesterone and diethylstilboestrol all act at the same site.

The involvment of lipids in the inhibition of NADH oxidase was first suggested by Burgos and Redfearn because of the lipophilic nature of the molecule (74). Extraction of 3H piericidin treated mitochondria with dry acetone resulted in removal of the mitochondrial lipids, together with 95% of the tightly bound 3H piericidin. The 3H piericidin was removed unchanged; it was not covalently bound to any of the phospho-lipids or neutral lipids. It appears that lipids are essential for binding of piericidin to the specific site. In support of this hypothesis it is found that the amount of piericidin bound to acetone extracted mitochondria is 80% less than that binding to normal mitochondria.

An examination of the effect of various piericidin and rotenone analogues on NADH oxidase and 3H piericidin binding shows that certain structural features are essential for both. Small decreases in the lipophilicity of the rotenone molecule caused a slight decrease in inhibitory potency, and removal of the 2- and 3- methoxyl groups brought about a large decrease. Also of interest was the finding that methyl rotenone (a compound in which the C-12 oxygen group has undergone a reaction) was a poor inhibitor of NADH oxidase (74); a similar relationship was found with 3H piericidin binding. Rotenone, elliptone and malaccol all decrease the binding subsequently added 3H piericidin at the concentrations which result in inhibition of NADH oxidase. The inhibitors with low activity towards NADH oxidase did not decrease 3H piericidin binding, even at high concentrations. Thus in all cases the ability of a particular rotenoid to act as a potent inhibitor was closely related to its ability to bind at the specific site in the NADH dehydrogenase.

Studies with piericidin analogues show that methylation of the side chain hydroxyl group (piericidin B) or reduction of the side chain (reduced piericidin A) has little effect on inhibitory or binding capacity. Acetylation of the ring hydroxyl group (piericidin A monoacetate, reduced piericidin A diacetate) causes a thousand-fold decrease in activity towards NADH Oxidase and gives no decrease in 3H piericidin binding. Cleavage of the side chain of piericidin A probably results in considerable loss of activity as shown by the failure of the model compound 2-hydroxymethyl, 4-hydroxy, 5-methoxy pyridine to inhibit NADH oxidase, except at high concentrations. A requirement for the ring oxygen group and the side chain are established; unfortunately a desmethoxy piericidin was not synthesised. In a series of synthetic pyridines, however, the most potent compound was one containing a ring hydroxyl, ring methoxyl and a six carbon side chain; compounds without the ring methoxyl group were less potent inhibitors. To summarize, the essential structural requirements for both piericidin and rotenone are (i) lipophilic nature of the molecule (ii) ring oxygen function (iii) methoxyl groups in rotenone and possibly in piericidin.

The binding experiments showed that the NADH dehydrogenase system was not the only component in beef heart mitochondria which interacted with piericidin. For example it was found that high concentrations of piericidin were capable of inhibiting succinate oxidation (see also refs. 71,100). Furthermore rotenone is a non-specific inhibitor which affects alcohol dehydrogenase (79) and glutamic dehydrogenase (80) and which also binds non-specifically to mitochondria (101-103). Because of the non-specific binding sites a survey was made of the effects of piericidin, rotenone and amytal on various mitochondrial reactions. None of the three dehydrogenases tested were sensitive to amytal. Piericidin and rotenone both inhibited glutamic dehydrogenase; rotenone alone inhibited alcohol dehydrogenase and piericidin alone inhibited malic dehydrogenase. The pattern of inhibition obtained does not suggest a common mode of action for the inhibitors on these enzymes. The closest agreement with the NADH dehydrogenase system is found with glutamic dehydrogenase; other inhibitors of NADH dehydrogenase also inhibit both

enzymes (cf steroids 81, 82 and o-phenanthroline 104, 105). Studies on the mode of action of rotenone and piericidin on glutamic dehydrogenase might be useful in elucidating the reactive groups involved in electron transport. Although there is no suggestion that lipids are involved in glutamic dehydrogenase, these compounds do bring about conformation changes in the enzyme, which might be related to the mechanism of action of NADH dehydrogenase. Interaction of piericidin with beef heart mitochondrial malic dehydrogenase was complicated by the finding that piericidin caused a release of the enzyme from mitochondria at concentrations below those required for inhibition. The amount of malic dehydrogenase released was comparable to that released on treatment of the mitochondria with triton X-100. It is possible that micromolar amounts of piericidin lead to disorganisation of the mitochondrial membranes which causes either a release of the enzyme or increased penetration of substrates. There is a close correlation between the extent of lysis of mitochondria by triton X-100 and the degree of inhibition of succinic dehydrogenase (83), and a similar relationship is found with piericidin (Fig. 13). Thus inhibition of succinate oxidation by piericidin may be due to damage of mitochondria and not to competion with ubiquinone as suggested by Crane and his colleagues (71,100). They found that piericidin inhibition of succinate oxidation was reversed by ubiquinone homologues but it was also reversed by α-tocopherol, indicating a non-specific requirement for the fatty part of the molecule. Hatefi (94) also concluded that ubiquinone was not involved in piericidin inhibition because he could find no site of inhibition of piericidin in complex III of the respiratory chain, in combination with either complex I or complex II.

A differential effect of piericidin on mitochondrial energy-linked reactions was observed (97). The energy-linked reduction of NAD^+ by succinate was inhibited by piericidin at the same low concentrations that inhibited NADH oxidase. This substantiates previous conclusions that the NADH dehydrogenase is involved in both reactions (85). However, it does not support the finding of Vallin and Low (108) that the energy-linked reduction of NAD^+ by succinate is ten times more sensitive to piericidin than NADH oxidation. This finding remains unexplained, especially since

Table 15

COMPARISON OF EFFECT PIERICIDIN A ON ENZYME REACTIONS IN BEEF HEART MITOCHONDRIA AND E. COLI

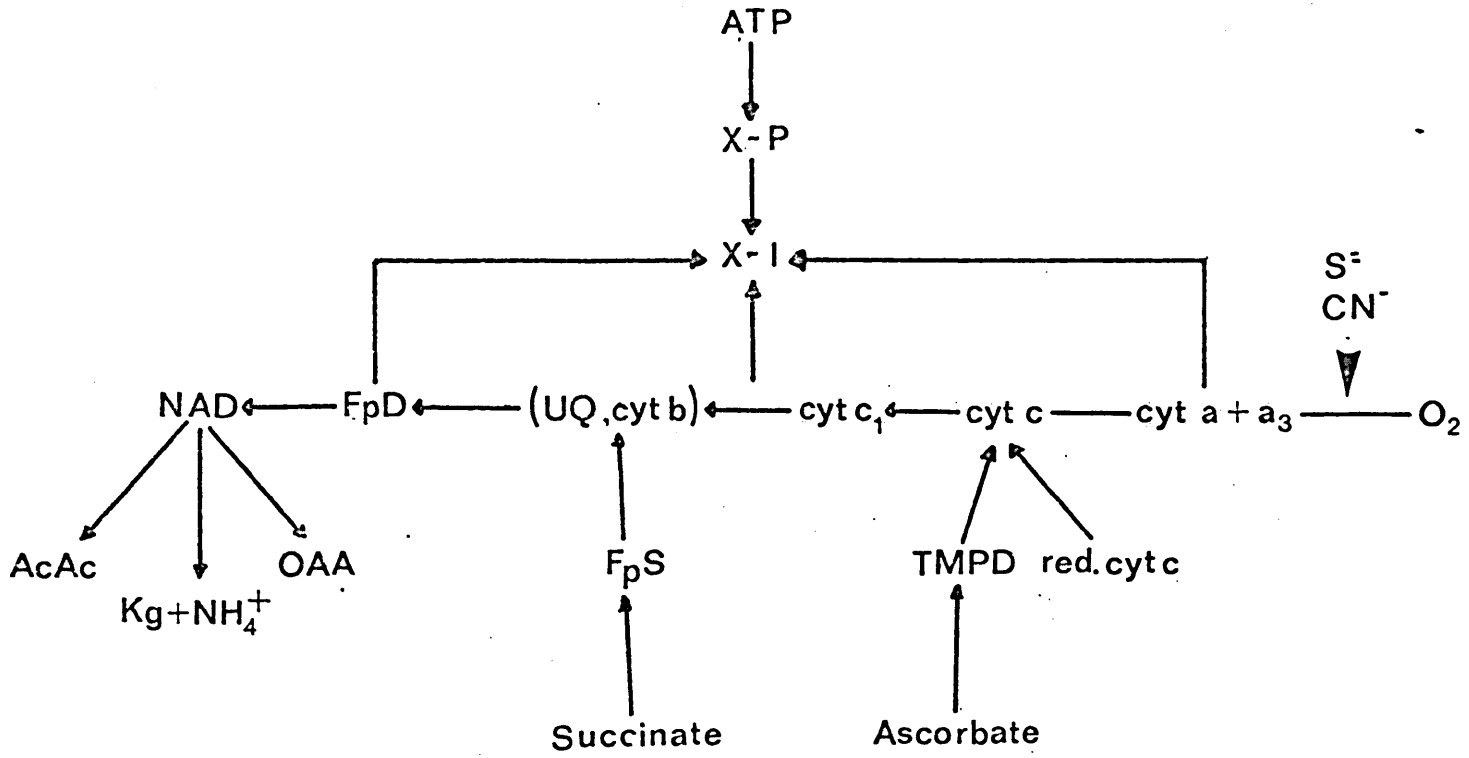
REACTION	CONCENTRATION FOR 50% INHIBITION	
	BEEF HEART	E. COLI
NADH oxidation	12.5nmole/g	23μmole/g
Succinate oxidation	600μmole/g	42μmole/g
ATP-driven reduction NAD^+ by succinate	12.2nmole/g	none
ATP-driven reduction $NADP^+$ by NADH	24μM	130μmole/g

All enzyme activities were measured as described in the Methods section.

the value for the piericidin sensitive factor in the energy-linked reaction (19.6nmole/g) is in good agreement with the value obtained by direct titration of NADH oxidase (21.9nmole/g).

The NADH dehydrogenase system is not involved in the energy-linked transhydrogenase reaction, since higher concentrations of piericidin are required to inhibit the latter. Rotenone and amytal do not share the ability of piericidin to inhibit this reaction; it is possible that piericidin is interacting with a component, possibly a quinone, that is not available to either rotenone or amytal. However, attempts to reverse piericidin inhibition with ubiquinone-(50) were unsuccessful, possibly because the quinone was not getting to the specific site. Another possibility is that piericidin is interacting with the energy transfer system of the submitochondrial particles (106).

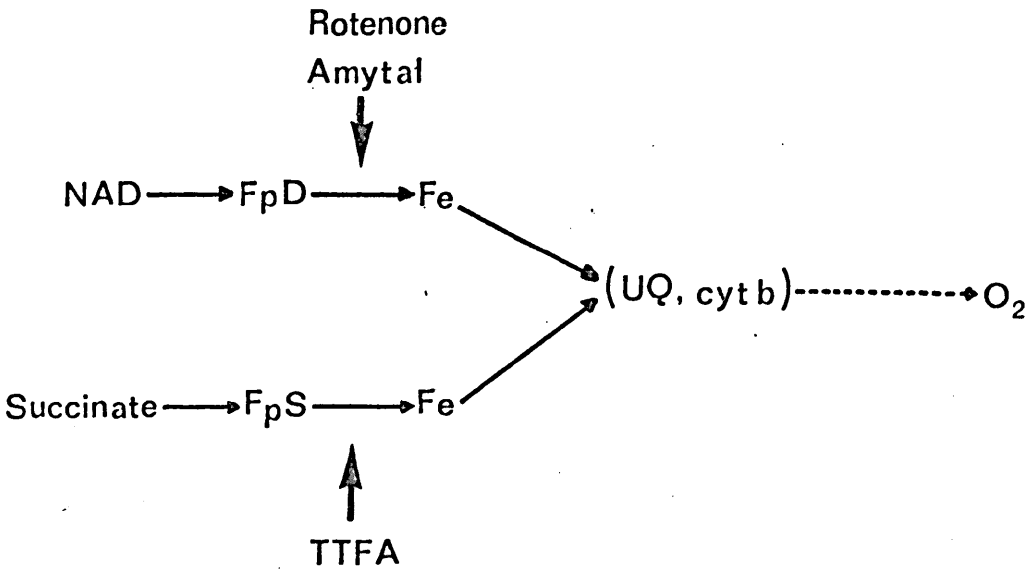
Unlike the mammalian reactions, both NADH and succinate oxidation in E.coli were inhibited by piericidin at the same concentrations (cf Azotobacter vinelandii, ref. 109). The point of action is on the terminal portion of the respiratory chain since ATP-dependant reduction of NAD^+ by succinate is not inhibited by piericidin. It is concluded that the piericidin sensitive site present in the NADH dehydrogenase region of the respiratory chainof beef heart mitochondria is not present in E. coli. This is supported by the finding that amytal and rotenone do not affect the oxidation of NADH by E.coli small particles (107). Table 15 summarizes the differences between the action of piericidin on beef heart enzymes and its effect on E. coli enzymes. The important points to note are: (i) there is a differential effect of piericidin on the NADH and succinate oxidation in beef heart mitochondria, whereas the two reactions in E.coli are inhibited by the same concentrations of piericidin. (ii) NADH oxidation and energy-linked reduction of NAD^+ by succinate are inhibited by the same concentrations of piericidin in beef heart mitochondria, but in E.coli the energy-linked reduction of NAD^+ by succinate is not affected by piericidin. (iii) The energy-linked transhydrogenase is inhibited by piericidin in both species. Points (i) and (ii) can be explained in part if E.coli has no piericidin sensitive site equivalent to the site present in the NADH dehydrogenase region of the mammalian respiratory chain. Point (iii) tentatively suggests a common mechanism for the energy-linked transhydrogenase in E.coli and beef heart (see Chapters V and VI of this thesis).



SCHEME 1. POSSIBLE PATHWAYS OF ENERGY-LINKED REVERSAL OF OXIDATIVE PHOSPHORYLATION
AcAc - acetoacetate, OAA - oxaloacetate, α-kg - α-ketoglutarate, TMPD - tetramethyl phenylenediamine, X~I and X~P are proposed high-energy intermediates of oxidative phosphorylation.

SCHEME II

SITE OF ACTION OF INHIBITORS OF ELECTRON TRANSPORT



Key: TTFA; Thenoyltrifluoroacetone

CHAPTER III.

ENERGY LINKED REDUCTION OF NAD^+ BY SUCCINATE IN ESCHERICHIA COLI

INTRODUCTION.

The first experimental evidence for the reversal of oxidative phosphorylation was presented by Chance and Hollunger (111), who showed that reduction of intramitochondrially bound NAD^+ by succinate was dependant upon the aerobic generation of high energy intermediates by the oxidation of succinate. Later investigators (85, 112) using submitochondrial particles, found that NAD^+ could be reduced by succinate in the presence of cyanide and ATP. They concluded that the energy requirement for the reaction could be satisfied either by the aerobic generation of high-energy intermediates, or by added ATP under anaerobic conditions.

Various electron donors have been employed for reduction of the respiratory carriers. The most popular has been succinate (85,113), but ascorbate plus TMPD (114) and reduced cytochrome c (115) have been used. The electron acceptor was normally NAD^+, especially when submitochondrial particles were employed as the enzyme source (85), but oxaloacetate, α-ketoglutarate + NH_4^+ and acetoacetate could replace NAD^+ in studies with intact mitochondria (116, 117).

Some of the possible pathways involved in the reaction are shown in Scheme I.

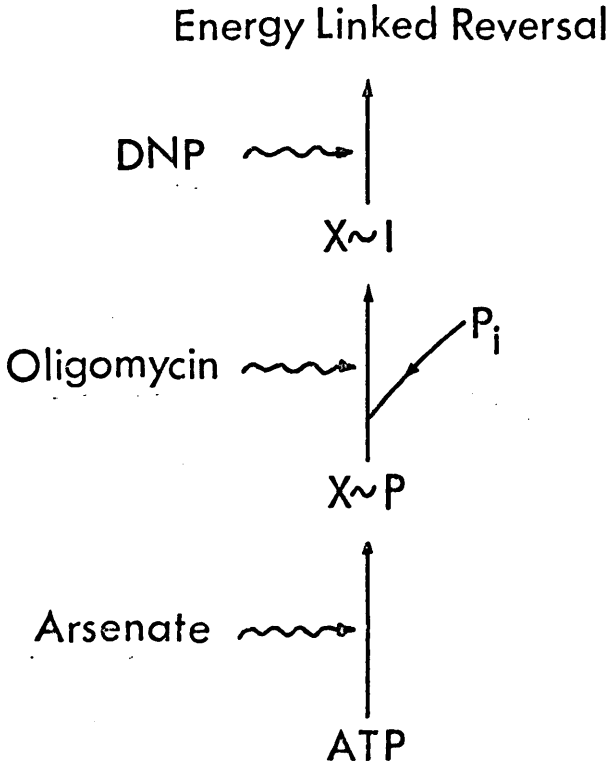
Energy-Linked Reduction of NAD^+ by Succinate.

The energy linked reduction of NAD^+ by succinate has been the most studied of the reaction pathways. The specific action of certain inhibitors suggests that both the succinic and the NADH dehydrogenase flavoprotein systems are involved. Inhibitors such as rotenone, amytal and thenoyltrifluoroacetone act on electron transport at the points shown in Scheme II. These compounds are also inhibitors of the energy-linked reduction of NAD^+ by succinate (85, 112), indicating that the same components are involved in both the forward and the reverse reactions.

The case for involvment of the NADH dehydrogenase system is supported by studies with tritiated NAD^+ (85). NADH dehydrogenase and

SCHEME III

ENERGY TRANSFER PATHWAY IN ENERGY-LINKED REVERSAL OF ELECTRON TRANSPORT



the final hydrogen transfer step in the energy-linked reduction of NAD^+ by succinate both require an enzyme of 'B' specificity. Krebs (118) has suggested that malic dehydrogenase, and not NADH dehydrogenase, was responsible for bringing about the reduction of the NAD^+. This was shown to be unlikely when it was found that malic dehydrogenase was 'A' specific towards NAD^+. That the reaction did not involve enzymes of the citric acid cycle was substantiated by the sensitivity of the reaction to energy-transfer inhibitors and uncouplers of oxidative phosphorylation. Dicoumarol, 2,4-dinitrophenol, carbonyl cyanide and oligomycin all inhibited the reduction of NAD^+ when employed in concentrations that were shown to inhibit oxidative phosphorylation (85).

One problem outstanding at the present time is the question of the link between the two flavoprotein systems. Is there a direct hydrogen transfer between the two enzymes? The participation of non-haem iron which is inferred from inhibition studies with the chelating agent thenoyltrifluoroacetone, probably excludes a direct transfer of electrons from succinic dehydrogenase to NADH dehydrogenase. In Scheme I the electrons are shown passing through the ubiquinone - cytochrome b region of the respiratory chain. It was Chance, Lee and Estabrook (84) who first proposed the involvment of ubiquinone in the reversal of electron transfer but no evidence was presented to support the conclusion.

The high-energy intermediate responsible for driving the reaction is thought to be a non-phosphorylated intermediate of the X~I type. The evidence for this is summarized below.

(i) Oligomycin did not inhibit the energy-dependant reduction of acetoacetate by succinate when driven by the aerobic oxidation of succinate (119).

(ii) Oligomycin inhibited the $^{18}OP_i - H_2O$ exchange reaction (126).

(iii) Arsenate did not relieve oligomycin inhibition of tightly coupled respiration (127).

(iv) Oligomycin inhibition of tightly coupled respiration was relieved by 2,4-dinitrophenol (127). It was concluded from these findings that oligomycin blocked a reaction between a non-phosphorylated intermediate and inorganic phosphate, indicating that phosphorylated intermediates were not involved in the reversal of electron transfer (Scheme III).

The conclusion was supported by the finding that energy-linked reversal proceeds at unchanged rates in the absence of inorganic phosphate (119).

Attempts to determine the stoichiometry of the reaction have lead to the conclusion that one high-energy bond is expended per molecule of NAD^+ reduced by succinate (119). Thus the following general equation can be written to describe the reaction.

$$\text{succinate} + NAD^+ + X\sim I \rightleftharpoons \text{fumarate} + NADH + H^+ + X + I$$

Reversal of electron transport in bacteria.

Aleem has demonstrated the energy-linked reduction of pyridine nucleotides in Nitrosomonas europea (120) and Thiobacillus novellus (121) by cytochrome c. The reaction may be of particular importance for the generation of reducing power in these chemoautotropic organisms, since the inorganic substrates they utilize have high redox potentials. A study of bacterial enzymes has certain advantages over an investigation with the corresponding mammalian enzymes. The effect of different growth conditions can be examined and it is possible to envisage the development of mutants. With this in mind, and with the encouragement of a brief report of the energy-linked reduction of NAD^+ by succinate in E. coli (122), we set out to examine the reaction in this bacterium in more detail.

The findings of Kashket and Brodie (122) can best be described by quoting all that they had to say on the subject. "Exogenous NAD^+ was reduced at a low rate (8mμmole/min/mg protein) when incubated with the small particles from E. coli and succinate under anaerobic conditions; in the presence of added ATP, however, the rate of NADH formation was increased 4-fold (23mμmole/min/mg protein). Further studies with inhibitors is necessary to confirm the succinate linked reduction of NAD^+ by this system".

The present work extends the studies of Kashket and Brodie to a demonstration of the energy requirement for the reduction of NAD^+ by succinate, together with the specificity of the energy source, the specificity of the electron acceptor, the effects of pH, magnesium and phosphate, the effects of temperature and of electron transfer inhibitors and uncouplers of oxidative phosphorylation and also the stoichiometry

of the reaction. A detailed method for the preparation of E. coli small particles is also described.

MATERIALS AND METHODS.

Growth of Bacteria.

Wild Type E. coli K12 were grown on a minimal medium using succinate as the sole energy source.

Composition of Minimal medium.

Compound	g/l
disodium hydrogen phosphate	18.9
potassium dihydrogen phosphate	6.3
magnesium sulphate: $7H_2O$	0.2
ammonium sulphate	2.0
sodium succinate	5.0

The pH of the medium was adjusted to 7.6 with 10% (w/v) KOH. The bacteria were then grown aerobically in 5 litre flasks containing 2 litres of medium, on a rotary shaker at 37°. The amount of growth was assessed with an EEL photoelectric colourimeter using a neutral density filter.

Preparation of bacterial particles.

Cells were harvested in the log phase of growth and collected at 4,000 x g. 4 litres of bacterial suspension were normally processed at any one time. The cells were then washed with cold water at 4,000 x g at 0° and the packed cells were suspended in water at a concentration of 0.5g wet weight/ml and the pH was adjusted to 7.4 with 40mM-tris-HCl (pH 8.2). 11ml aliquots of this suspension were sonicated at 2° for 3.5 min using maximum output from the large probe of the MSE 60-watt cell disintegrator. To counteract the heat generated by the sonicator the probe was precooled in ice and the bacterial suspension was kept on ice during the sonication. After removal of cell debris and large bacterial particles at 30,000 x g the small particle fraction was sedimented at

140,000 x g for 3hr. The pellet was finally suspended in 0.15M-KCl containing 10mM-magnesium chloride and the pH was adjusted to 7.4 with tris-HCl buffer (pH 8.2). Particles were normally used immediately after preparation but storage overnight did not affect the activity adversly. The protein concentration of the suspension was adjusted to fall in the range 30-50 mg/ml.

Estimation of protein content.

After solubilisation of the pellet with deoxycholate (0.33% w/v) protein was determined by the method of Gornall et al (88) using bovine serum albumen as the standard.

Estimation of NAD^+ and NADH.

Both compounds were estimated with alcohol dehydrogenase assay (123) using ethanol as substrate for NAD^+ and acetaldehyde as substrate for NADH estimation.

Energy-linked reduction of NAD^+ by succinate.

A Beckmann DK 2A recording spectrophotometer was used to determine the increase in absorbance at 340nm. Blank and experimental cuvettes of 1cm light path contained 375µmole sucrose, 75µmole tris-HCl buffer (pH 8.0), 10µmole $MgCl_2$, 3µmole NAD^+, 5µmole Na_2S, 20µmole succinate and bacterial particles (2-5 mg bacterial protein). The absorption was recorded until a steady trace was obtained, when the reaction was started by the addition of 6µmole ATP to the experimental cuvette to give a final volume of 2ml. The reaction was measured at 30° and water insoluble inhibitors were added as their methanolic solutions. Suitable controls with equivalent amounts of methanol alone were carried out.

Estimation of Nucleotide Triphosphatases.

Nucleotide triphosphatase activity was measured by determining the inorganic phosphate released (124) from the nucleotide triphosphate in 0.1ml aliquots removed from the reaction medium at appropriate time intervals. The reaction medium contained 650µmole nucleotide triphosphate (ATP, GTP, ITP) and bacterial particles (6mg bacterial protein) in a final volume of 3ml at 30°.

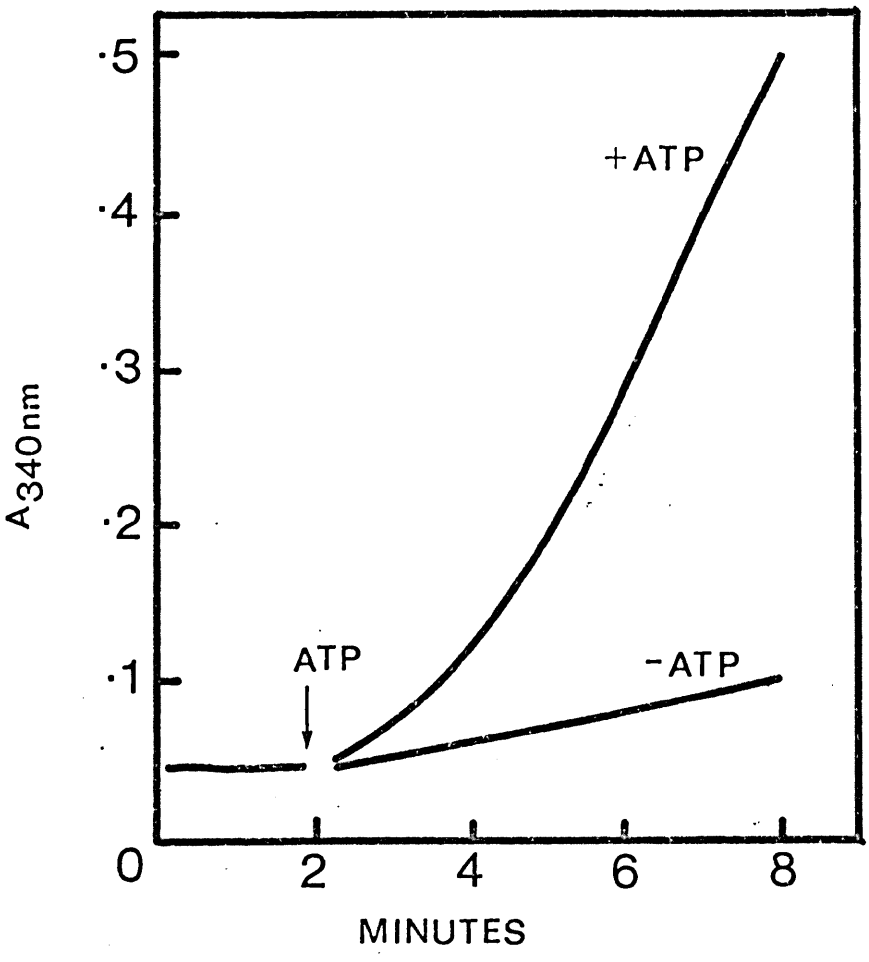


Fig. 1. TIME COURSE OF NAD^+ REDUCTION BY SUCCINATE. The incubation medium contained 375µmole sucrose, 75µmole tris-HCl (pH 8.0), 10µmole magnesium chloride, 3µmole NAD, 5µmole sodium sulphide, 20µmole succinate and 3.5mg bacterial protein (small particles). The energy-dependant reaction was started by the addition of 6µmole ATP to the experimental cuvette, to give a final volume of 2.0ml. The temperature of the incubation was 30°.

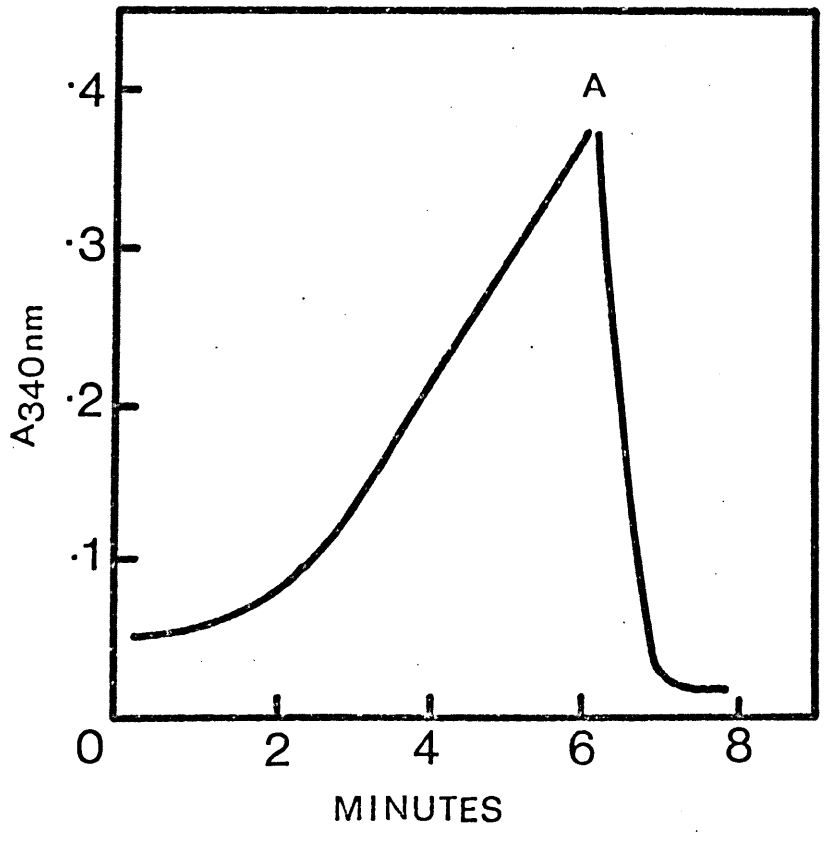


Fig. 2. DEMONSTRATION OF NADH FORMATION IN THE ENERGY-LINKED REDUCTION OF NAD BY SUCCINATE. Experimental conditions were the same as in Fig. 1. ATP was added at zero time and at 6min (point A on graph) 10μmole acetaldehyde and 20μg alcohol dehydrogenase (yeast) were added to the incubation medium. The drop below the control level was due to endogenous NADH present in the particles.

Table 1

REQUIREMENTS FOR ENERGY-LINKED NAD^+ REDUCTION BY SUCCINATE

CONDITIONS	NADH formed (nmole/min/mg protein)
Complete	10.41
$-Mg^{2+}$	1.86
-succinate	0
+fumarate	0
-ATP, +fumarate	0
$-NAD^+$	0
-ATP	0.73
-ATP, +ADP	0.5

Experimental conditions were as described in Fig. 1. Additions and ommisions were made as indicated above. 3µmole ADP and 10µmole fumarate were added where shown. 2.2mg small particle protein were present in all the assays.

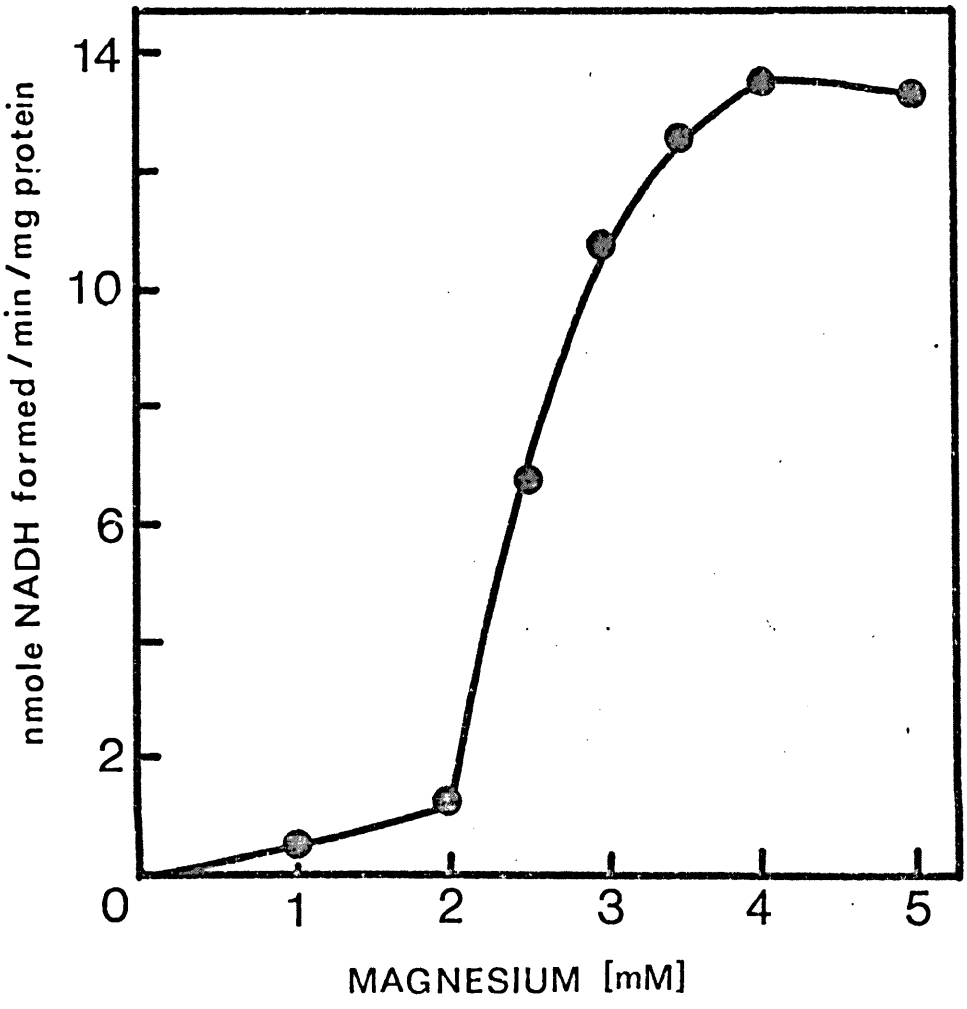


Fig. 3. EFFECT OF MAGNESIUM ON ENERGY-LINKED REDUCTION OF NAD BY SUCCINATE. Conditions as in Fig. 1, except that 2.5mg bacterial protein (small particles) were added. Magnesium was added to give the concentrations shown on the graph.

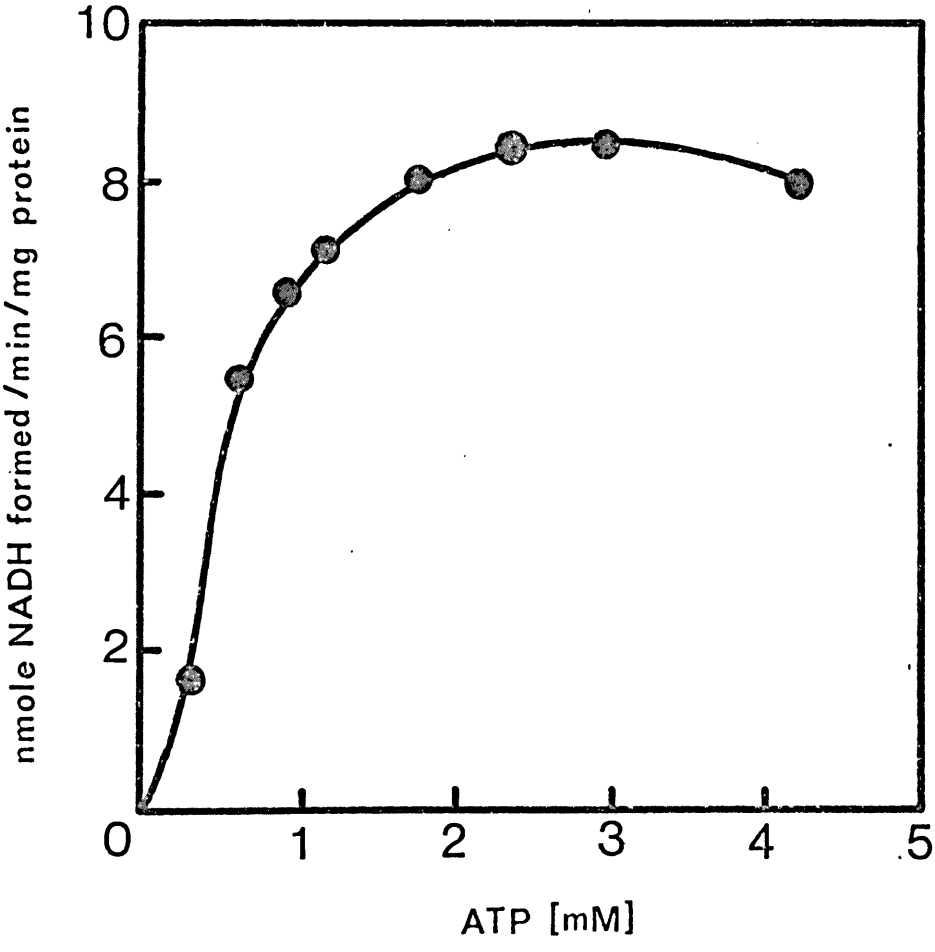


Fig. 4. EFFECT OF ATP ON THE ENERGY-LINKED REDUCTION OF NAD BY SUCCINATE. Conditions as in Fig. 1, except that 2.0mg bacterial protein (small particles) were added. ATP was added to give the concentrations shown on the graph.

Table 2

THE EFFECT OF VARIOUS NUCLEOTIDES ON THE REDUCTION OF NAD^+ BY SUCCINATE

NUCLEOTIDE ADDED	CONCENTRATION (mM)	NADH formed (nmole/min/mg protein)
ATP	1.5	4.67
ATP	3.0	5.42
ADP	3.0	0.30
AMP	3.0	0.34
ITP	3.0	1.02
GTP	3.0	0.41
CTP	1.5	0.17
UTP	3.0	0.28

Experimental conditions were as described in Fig. 1. Nucleotides were added to the incubation medium at the concentrations shown. 1.8mg small particle protein were present in all the assays.

RESULTS.

(i) Demonstration of the ATP-dependant reduction of NAD^+ by succinate.

A simple procedure for isolating a sub-cellular fraction from E.coli which contained the enzyme system catalysing the ATP dependant reduction of NAD^+ by succinate was developed (see Methods). The experiment illustrated in Fig. 1 shows the relative rates of the reaction in the presence and absence of ATP. The bacterial particles were incubated with succinate and NAD^+ in the presence of sulphide. Addition of ATP to the incubation medium caused an increase in the rate of reduction of NAD^+, after a short lag time. The reaction product was shown to be NADH both by its specific absorption at 340nm and by the rapid decrease in absorbance brought about by the addition of acetaldehyde plus alcohol dehydrogenase. Fig. 2 shows that 100% of the increase in absorption at 340nm could be accounted for by the formation of NADH.

The reaction was dependant on the presence of Mg^{2+} and succinate. Fumarate, added in the same concentrations as succinate, did not result in any reduction of NAD^+, in the presence or absence of ATP. (Table 1). The activity sometimes observed in the absence of magnesium was probably due to the small amount of magnesium in the suspension medium, corresponding to a final concentration of 0.25 - 0.5mM. When the particles were thoroughly washed and suspended in a medium deficient in magnesium, then no reaction was observed and an absolute requirement for magnesium was demonstrated (Fig. 3), with maximum activity at a concentration of 4mM-magnesium.

(ii) Specificity for ATP.

As replacement of ATP with ADP resulted in a loss of the reaction it seemed unlikely that regeneration of ATP via the adenylate kinase reaction was playing any part in this particular system. The reaction rate was found to be dependant on the amount of ATP added, indicating that ATP was acting as a substrate in the reaction. The Km for ATP was 470μM and maximum activity was observed in the region of 2 - 3mM-ATP (Fig 4).

Other nucleotides were tested for their ability to drive the energy-linked reduction of NAD^+ by succinate. Table 2 shows that only ITP produced any significant reduction of NAD^+. It was found that the ability of the various nucleotides to drive the reaction was related to the presence

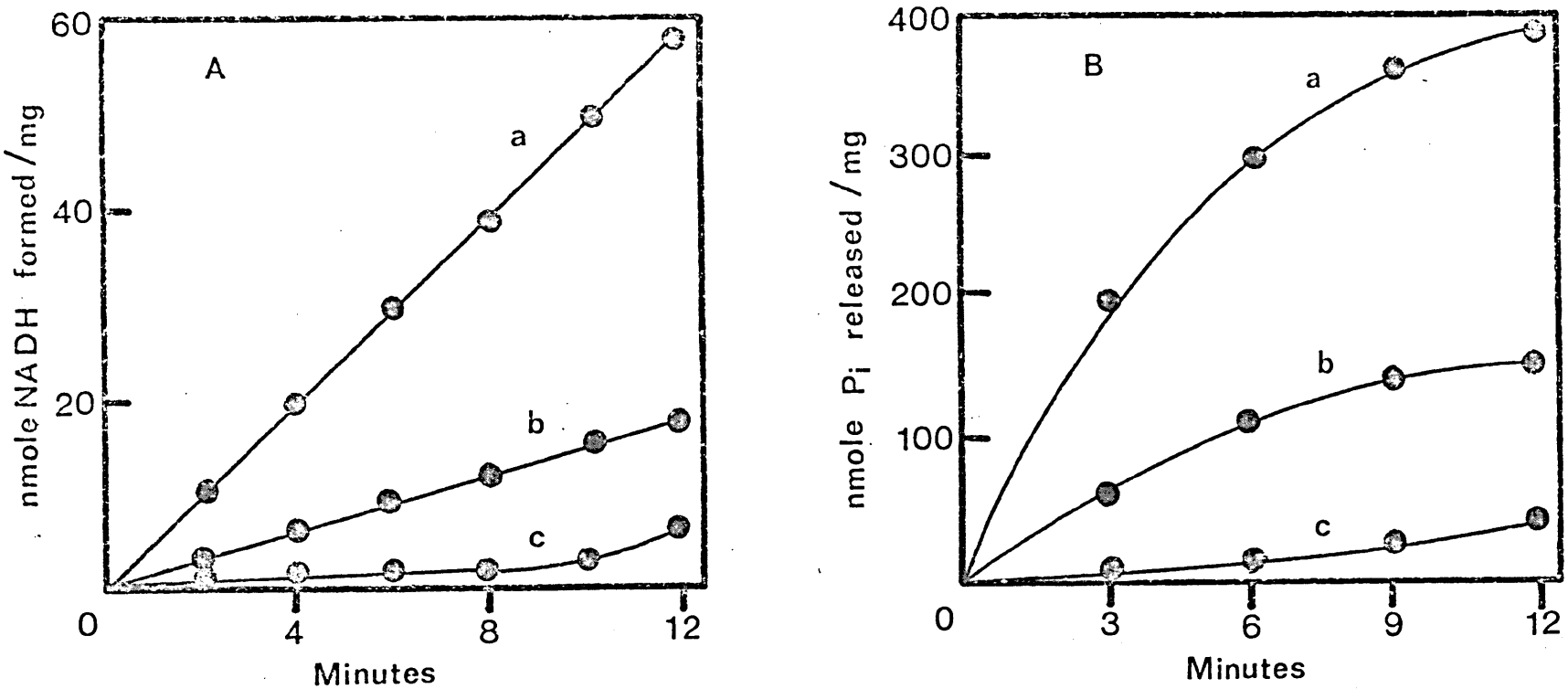


Fig. 5. ENERGY-LINKED REDUCTION OF NAD BY SUCCINATE AND NTPase ACTIVITY. The energy-linked reduction of NAD by succinate was measured as described in Fig. 1, except that 3µmole ATP, ITP or GTP were added as indicated. The protein concentration was 2mg (small particles). Phosphatase activity was determined by measuring the inorganic phosphate released from 6µmole ATP, ITP or GTP in a medium containing 650µmole sucrose, 130µmole tris-HCl buffer (pH 8.0) and 15µmole magnesium chloride in a final volume of 3.0ml. Graph A: Energy-linked reduction NAD by succinate; a - ATP dependant reaction; b - ITP dependant reaction; c - GTP dependant reaction. Graph B: Nucleotide triphosphatase activity; a - ATPase; b - ITPase; c - GTPase.

Table 3

REDUCTION OF NAD^+ AND NAD^+ ANALOGUES BY SUCCINATE

COMPOUND	λmax (nm)	E_{mM}	LAG TIME (min)	NADH formed
NAD^+	340	6.2	3	11.35
Nicotinamide hypoxanthine dinucleotide	338	6.2	4	10.02
3-Acetylpyridine -NAD	363	9.1	8	8.00
3-Pyridinealdehyde -NAD	358	9.3	13	7.35
3-Acetylpyridine hypoxanthine dinucleotide	361	9.0	8	5.34
3-Pyridinealdehyde hypoxanthine dinucleotide	356	9.4	20	2.20

Experimental conditions were as described in Fig. 1. Various analogues were substituted for NAD in the reaction as shown above. The analogues were obtained from Pabst Labs., Milwaukee, Wisconsin. Spectral properties are quoted from their circular OR-18. 2.5mg small particle protein were present in all the assays. NADH formed is given as nmole/min/mg protein.

of the corresponding nucleotide triphosphatases in the preparation: ATPase was the most active triphosphatase and ATP was the most efficient at driving the reaction. GTP, on the other hand, was only split to a small extent by the preparation and the resulting stimulation of NADH formation was minimal. An ITPase, operating at about 25 - 35% of the ATPase, resulted in energy-linked NADH formation at 20% of the ATP stimulated rate. These results are summarised in Fig. 5.

ADP added to the incubation medium, at concentrations equal to the amount of ATP present, caused about 50% inhibition of the reaction. This inhibition could be increased to 90% by the addition of inorganic phosphate plus ADP, even though phosphate alone had no effect on the reaction.

(iii) Specificity of the electron acceptor.

Several NAD^+ analogues were tested for their ability to act as electron acceptors in the system. The specificity for NAD^+ was relatively low (Table 3), although the lag period of the reaction was generally lengthened by the analogues. Substitution at the 6-position of the adenine ring with a hydroxyl group to give nicotinamide hypoxanthine dinucleotide did not result in any significant change in activity. Alterations of the 3-position of the pyridine ring to give 3-acetylpyridine-NAD^+ and 3-pyridinealdehyde-NAD^+ caused a 30 - 40% decrease in the rate of the reaction and a 2 - 3 fold increase in the lag time. Interference with both the 6-position of the adenine ring and the 3-position of the pyridine ring to give 3-acetylpyridine hypoxanthine dinucleotide and 3-pyridinealdehyde hypoxanthine dinucleotide resulted in further decreases in activity, and for the latter compound a considerable increase in the lag time. The Km for NAD^+ in the reaction was found to be very high - 4.3mM.

(iv) Relationship between NADH formed and ATP added.

ATPase activity, using relatively high concentrations of ATP (3mM) was five times greater than the rate of NAD^+ reduction by succinate. No significant difference between ATPase activity in the presence and absence of NAD^+ reduction could be detected. In fact, the ATPase activity decreased after 3 - 5min, but NADH formation still proceeded at a linear rate

ATP (uM)	NADH formed (uM)	ATP/NADH
1500	122.7	12.23
300	61.8	4.85
150	45.1	3.33
60	30.8	1.94

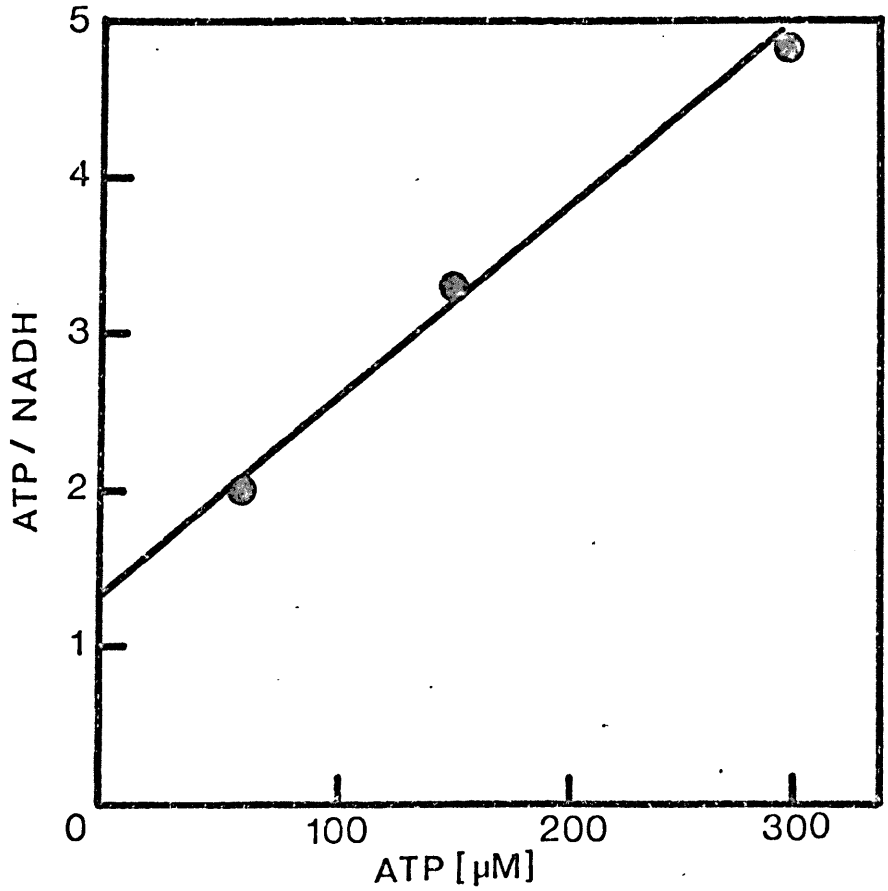


Fig. 6. RELATIONSHIP BETWEEN ATP ADDED AND NADH FORMED. Conditions were as described in Fig. 1. 2.5mg bacterial protein (small particles) were present. ATP was added at the concentrations shown and the reaction was allowed to go to completion, when the total amount of NADH formed was estimated from the increase in absorbance at 340nm.

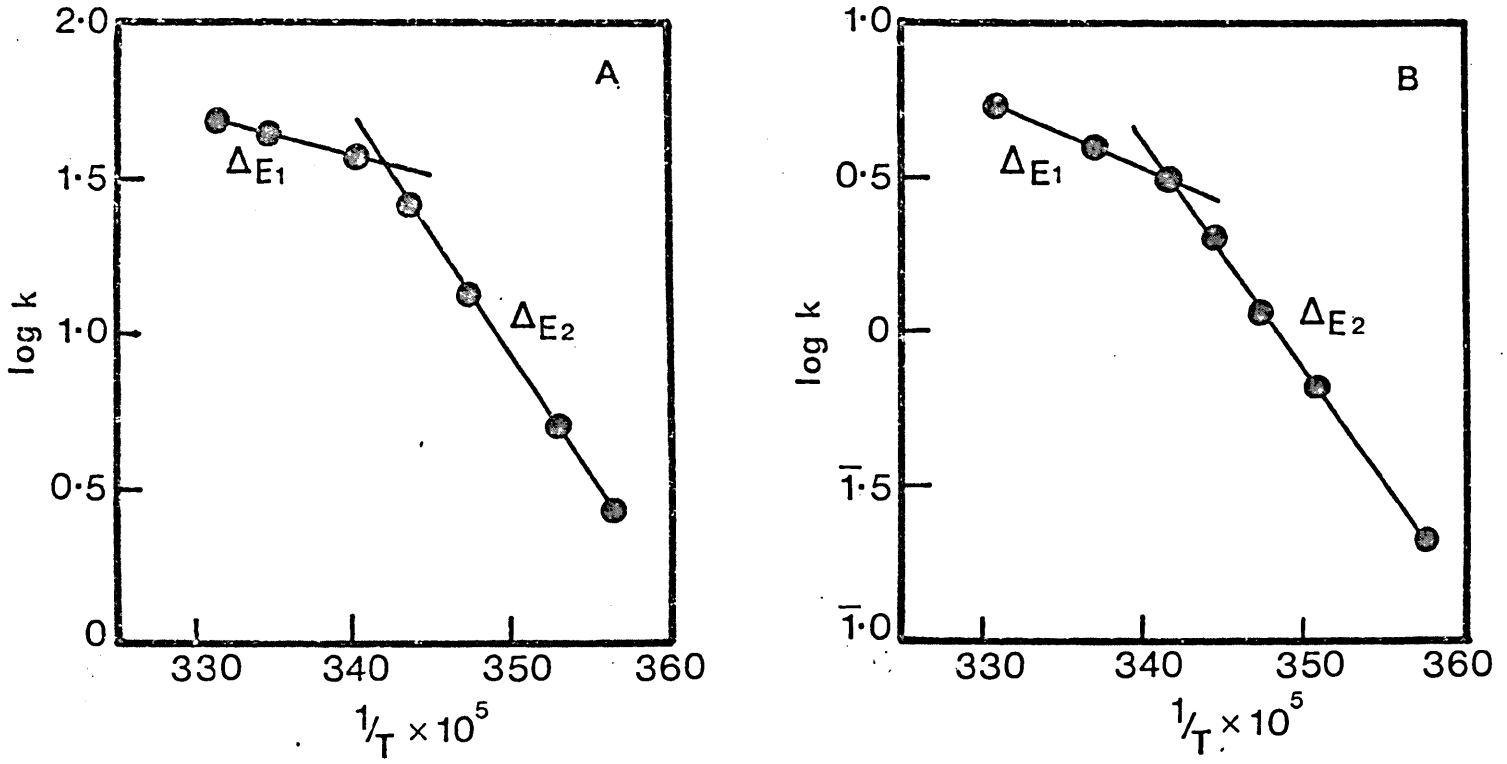


Fig. 7. ARRHENIUS PLOTS SHOWING THE EFFECT OF TEMPERATURE ON THE BACTERIAL ATPase AND ENERGY-LINKED REDUCTION OF NAD BY SUCCINATE. Graph A - ATPase, measured as described in Fig. 5 with 8.7mg. bacterial protein and 12µmole ATP. Graph B - Energy-linked reduction of NAD by succinate, measured as described in Fig. 1 with 2.5mg bacterial protein (small particles).
ATPase: ΔE1 = 9.7Kcal, ΔE2 = 33.3Kcal, transition temperature = 18.8°.
Reduction NAD: Δ E1 = 9.3Kcal, ΔE2 = 31.8Kcal, transition temperature = 19.5°.

for 15 - 20min. It was not possible under these conditions, to determine any relationship between ATP hydrolysed and NADH formed. To overcome these difficulties lower concentrations of ATP were employed in the hope that all the ATP which was hydrolysed might be used to drive the energy-linked reduction of NAD^+ by succinate. The reactions were allowed to go to completion and the amount of NADH formed was calculated. The ATP/NADH ratio decreased by decreasing ATP concentration (Fig. 6). When equilibrium was established, 0.062μmole NADH were formed with 0.12μmole ATP, giving an ATP/NADH ratio of 1.94/1. This was the lowest practicable value obtainable since further decreases in ATP concentration gave reaction rates that were difficult to measure. However, extrapolation of the data obtained to negligible ATP concentration gave a value of 1.3 and indicated that 1 - 2 ATP molecules were consumed per molecule of NADH formed.

(v) Temperature dependance of the energy-linked reduction of NAD^+ by succinate.

Haslam (125) has shown that energy-dependant reactions in sub-mitochondrial particles have characteristic Arrhenius plots, which have a sharp break in them. Reactions not dependant on oxidative phosphorylation such as the non-energy linked transhydrogenase, did not exhibit these sharp breaks. These studies have been extended here to include the energy-linked reduction of NAD^+ by succinate in bacterial particles. The results obtained (Fig. 7) were in agreement those of the mammalian system. There was a sharp break in the line at 19.5°; for temperatures above this level the activation energy was calculated to be 9.3Kcal, and below 19.5° a higher value (31.8 Kcal) was obtained. It was postulated (125) that at the transition temperature there was a conformational change in one of the enzymes involved in the reaction sequence. Haslam (125) suggested that it was the ATPase of the sub-mitochondrial particles which exhibited this temperature effect. The effect of temperature on the ATPase of the bacterial particles was determined (Fig. 7) and a transition temperature of 18.8° was calculated, with activation energies of 9.7Kcal and 33.3Kcal above and below this temperature respectively. The figures for the ATPase reaction are in good agreement with those for the energy-dependant reduction of NAD^+ by succinate, indicating that the ATPase reaction is involved in the reduction of NAD^+ by succinate.

Table 4

EFFECT OF INHIBITORS AND UNCOUPLERS ON NAD^+ REDUCTION

COMPOUND	CONCENTRATION (μM)	% INHIBITION
Malonate	2500 6250	49 66
TTFA	100 150	60 93
NHQNO	50 80	+45 17
Piericidin A	100 250	+35 17
Rotenone	50	0
Thyroxine	100	23
Dicoumarol	50 67	31 47
Pentabromophenol	84 168	47 90
2,4-DNP	180	0
Oligomycin	12.5μg	0

Experimental conditions were as described in Fig. 1. 2.5mg small particle protein were present in all the assays. Inhibitors that were insoluble in water were added as their methanolic solutions and suitable controls were carried out with equivalent amounts of methanol alone.

(vi) Effect of inhibitors of electron transport and uncouplers of oxidative phosphorylation.

(a) Electron transport inhibitors.

A variety of electron transport inhibitors were found to inhibit the energy-linked reduction of NAD^+ by succinate (Table 4). Malonate, an inhibitor of the succinate-cytochrome b segment of the respiratory chain, inhibited NADH formation in a competitive manner; 50% inhibition occuring at about 3mM. This characterises the reaction as one involving the succinic dehydrogenase flavoprotein system of the particles. A similar result was obtained with thenoyltrifluoroacetone, which is believed to interact with the nonhaem iron component of the succinic dehydrogenase. At low concentrations both 2-n-heptyl-4-hydroxy-quinoline-N-oxide (NHQNO) and piericidin stimulated the reduction of NAD^+ by about 50%. These compounds inhibit NADH and succinate oxidation in E. coli, at a point where their electron transfer pathways are common. Stimulation of the reaction is probably therefore due to incomplete inhibition of the oxidation of NADH by sulphide, completed by the presence of piericidin or NHQNO. At high concentrations NHQNO caused inhibition of NADH formation because of its interaction with the NADH dehydrogenase flavoprotein system of the particles. Rotenone, a specific inhibitor of the NADH dehydrogenase flavoprotein system in mammalian mitochondria (76), was without effect on the bacterial reaction.

(b) Uncouplers of oxidative phosphorylation.

The involvement of high-energy intermediates of oxidative phosphorylation in the reaction is supported by the action of dicoumarol and pentabromophenol (Table 4). These compounds inhibited the reduction of NAD^+ by about 50% at concentrations of 70μM and 80μM respecively. Oligomycin and 2,4-dinitrophenol had no effect on the reaction. This was in agreement with Kashket and Brodie, (107) who could find no effect with these compounds on oxidative phosphorylation by E. coli small particles.

DISCUSSION.

Oxidative phosphorylation in *E. coli* extracts has been described by a number of workers (see e.g. refs. 107, 122, 108). These studies showed that the mechanism of coupling of phosphorylation to oxidation, as well as the sequence of respiratory carriers, was markedly similar to that found in mitochondrial systems. The bacteria lack a definite organelle comparable to the mitochondrion in which energy production is isolated from the many other reactions of the cell and for this reason particulate fractions, presumably membrane fragments, prepared from bacteria frequently require the addition of a soluble component for coupled activity. It was of special interest when Kashket and Brodie (107) described the isolation of a small particle fraction from *E. coli* which was capable of catalysing phosphorylation of ADP coupled to the oxidation of NAD-linked substrates and succinate.

It has been known for some time that particles prepared from mitochondria were capable of catalysing oxidative phosphorylation. Later it was shown that these particles could drive certain energy-dependant reactions such as the energy-dependant reversal of electron transport and the energy-linked transhydrogenase (1). These reactions were shown to be closely integrated with the oxidative phosphorylation machinery of the particles and have proved to be important tools in the study of the mechanism of oxidative phosphorylation. In particular they have lead to the concept that the mitochondrion was able to utilize high-energy intermediates of oxidative phosphorylation as a source of energy for driving endergonic reactions (1). Knowledge of the occurence and mechanism of these reactions in bacteria (120 - 122) was very limited when the present studies were started.

The results obtained here show that a small particle fraction, prepared from *E. coli*, catalyses an energy-dependant reversal of electron transport and oxidative phosphorylation. The energy for the reaction was supplied by ATP. The bacterial particles were incubated under non-respiring conditions, in the presence of sulphide, with succinate as the electron donor and NAD^+ as the electron acceptor. The reaction observed was the ATP-dependant reduction of NAD^+ by succinate.

The effect of electron transport inhibitors supports the conclusion that the succinic dehydrogenase flavoprotein system and the NADH dehydrogenase flavoprotein system are involved. The electron transfer pathway between the two flavoprotein dehydrogenases is not elucidated by the present studies, but the involvment of nonhaem iron is inferred by the action of the chelating agent thenoyltrifluoroacetone. However, the possible involvment of ubiquinone, vitamin K and cytochrome b are not established.

The action of uncouplers indicates that high-energy intermediates of oxidative phosphorylation provide the necessary energy supply. In this connection the inhibitory effect of ADP + P_i shows that the "phosphate potential" i.e. $[ATP]/[ADP][P_i]$ is an important factor. These reagents may be acting by competing for a non-phosphorylated high-energy intermediate required for NAD^+ reduction. This conclusion is supported by the finding that inorganic phosphate, added alone, has no effect on the reaction rate. Unfortunately the mitochondrial energy transfer inhibitor oligomycin, which has been used to show that a non-phosphorylated high-energy intermediate is utilized for driving the energy-dependant reversal of electron transport in submitochondrial particles, has no effect on oxidative phosphorylation in E. coli.

The similar effects of temperature on both the ATP-driven reduction of NAD^+ by succinate and the particle ATPase provide further evidence for the participation of oxidative phosphorylation in the reaction. Both reactions give an Arrehnius plot with a sharp break at 19°, possibly due to a conformation change in the ATPase, which is probably involved in both reactions. Furthermore addition of fumarate to the reaction medium, in place of succinate, did not cause reduction of NAD^+ under any of the experimental conditions employed showing that NAD^+ reduction was not occuring via citric acid cycle oxidations, such as malic dehydrogenase, as originally suggested by Krebs (118).

It appeared that the energy-dependant reaction was associated with the phosphorylation process, since treatment of the particles with reagents and conditions which destroyed the capacity to couple phosphorylation to oxidation also resulted in loss of ability to carry out the ATP-dependant reduction of NAD^+ by succinate.

The reaction was completely dependant on the presence of magnesium. This is in agreement with previous results obtained with beef heart submitochondrial particles (112). The particles possessed nucleotide triphosphatase activity towards ATP and ITP and to a lesser extent GTP. No reaction was found with CTP and UTP. These activities correlated with the ability of the various nucleotide triphosphates to supply energy for NAD^+ reduction. CTP and UTP gave no significant reduction of NAD^+ but the other nucleotides were active in the order ATP>ITP>GTP. Similar results were obtained with mitochondria (133).

The energy requirement of the reaction was measured by comparing the amount of inorganic phosphate released from ATP with the amount of NADH formed. The ATP/NADH ratio varied from 12 to 1.9, the values decreasing with the concentration of ATP employed. By extrapolation to negligible ATP concentration a value of 1.3 was obtained. This indicates that 1 - 2 molecules of ATP are being consumed per molecule of NADH formed. It seems unlikely that all the ATP hydrolysed would be utilized for NAD^+ reduction; if this is the case then the results obtained give an over-estimate of the ATP/NADH ratio, so the ratio is more likely to be 1 than 2. Thus the following equation could be used to describe the reaction in E.coli particles.

$$\text{Succinate} + NAD^+ + ATP \rightleftarrows \text{Fumarate} + NADH + H^+ + ADP + P_i$$

Ratios of 1 have also been calculated for mammalian reactions (see review by Ernster and Lee, ref.1).

The ATP-dependant reduction of NAD^+ by succinate has been reported recently in certain other bacterial preparations. These bacteria may be divided into two main types (129). (a) Chemotropic: these organisms utilize either inorganic electron donors (lithtrophic) or organic compounds as electron donors (organotrophic). The reaction has been demonstrated in both types of organism. Lithotrophic bacteria such as Thiobacillus novellus (121) and Nitrosomonas europea (120) support the ATP-dependant reduction of NAD^+ by succinate, and so do organotrophic bacteria such as E.coli (122 and this thesis) and Micrococcus denitrificans (130). (b) Phototrophic: chromatophores prepared from Rhodospirillum rubrum catalysed an ATP or

light dependant reduction of NAD^+ by succinate (131, 132).

Further experiments on the ATP-driven reduction of NAD^+ by succinate in E. coli small particles and beef heart submitochondrial particles are described elsewhere in this thesis.

CHAPTER IV

ENERGY-LINKED REDUCTION OF $NADP^+$ BY NADH IN ESCHERICHIA COLI.

INTRODUCTION

The energy-dependant reduction of $NADP^+$ to NADPH by NADH (energy-linked transhydrogenase) was first described in submitochondrial particles by Danielson and Ernster (134). It was shown that energy for the reaction could be supplied either by added ATP, or by the aerobic oxidation of respiratory substrates. The sensitivity of the reaction to uncouplers and inhibitors of oxidative phosphorylation implies the participation of high-energy intermediates of oxidative phosphorylation.

Estimates of the energy requirement of the reaction demonstrated that one ATP molecule was utilized per NADPH molecule formed (133, 134). The submitochondrial particles contained a non-energy-dependant transhydrogenase, a reaction that could proceed in the absence of added ATP. It was shown (135) that the equilibrium constant ($K = [NAD^+][NADPH] / [NADH][NADP^+]$) was 0.79 for the non-energy-linked reaction. In the energy-dependant reaction there was a strong displacement of the equilibrium towards the formation of NAD^+ and NADPH with an equilibrium constant of 480. The following equation was proposed to describe the energy-linked reaction (1).

$$NADH + NADP^+ + \sim X \rightleftarrows NAD^+ + NADPH + X$$

$\sim X$ is a high-energy intermediate of oxidative phosphorylation, which can be generated either from ATP (A) or from the aerobic oxidation of respiratory substrates such as succinate (B).

$$(A) \quad ATP + X \rightleftarrows ADP + P_i + \sim X$$

$$(B) \quad AH_2 + B + X \rightleftarrows A + BH_2 + \sim X$$

n.b. A and B, in equation (B) represent components of the respiratory chain.

There were three possible pathways by which NAD^+ and NADPH could

be produced in the transhydrogenase reactions. (i) Transfer of hydrogen from NADH to $NADP^+$; (ii) transfer of phosphate from $NADP^+$ to NADH; (iii) exchange of adenine nucleotide or nicotinamide moieties of the two pyridine nucleotides. Griffiths and Roberton (136) carried out experiments using two types of ^{14}C-labelled NADH; the first labelled in the 7-position of the nicotinamide ring and the second in the 8-position of the adenine ring. Results showed that less than 0.3% of the available counts were transferred from ^{14}C-NADH to $NADP^+$, ruling out the group transfer mechanism. Experiments with NAD^+ and $NADP^+$, labelled with tritium in the 4-position of the nicotinamide ring, showed that both the energy-linked and the non-energy-linked reactions proceeded via a direct hydrogen transfer from the A-locus of NADH to the B-locus of $NADP^+$. These results were in agreement with those obtained by Ernster *et al* (137).

The energy-linked transhydrogenase has been demonstrated in certain bacterial preparations (120, 138-140), but studies have been limited because of technical difficulties. The present work describes the characterisation of the reaction in small particles derived from *E. coli* so that further experiments could be carried out on the mechanism of the reaction.

METHODS

Measurement of ATP-dependant reduction of $NADP^+$ by NADH.

NADPH production was determined, using a Beckman DK 2A recording spectrophotometer to measure the increase in absorbance at 340nm. Blank and experimental cuvettes of 1cm light path contained 375µmole sucrose, 75µmole tris-HCl buffer (pH 8.0), 10µmole magnesium chloride, 0.05µmole NAD^+, 200µg Sigma yeast alcohol dehydrogenase, 180µmole ethanol, 5µmole sodium sulphide and bacterial particles (2 - 5mg bacterial protein).

The non-energy-linked reaction was initiated by the addition of 1µmole $NADP^+$. After 2-3min the energy-linked reaction was started by the addition of 3-6µmole ATP to give a final volume of 2ml. All incubations were carried out at 30°.

Ethanol and alcohol dehydrogenase were employed as an NADH regenerating system in order to keep the level of NADH constant.

Other methods.

All other methods were described in the previous chapter.

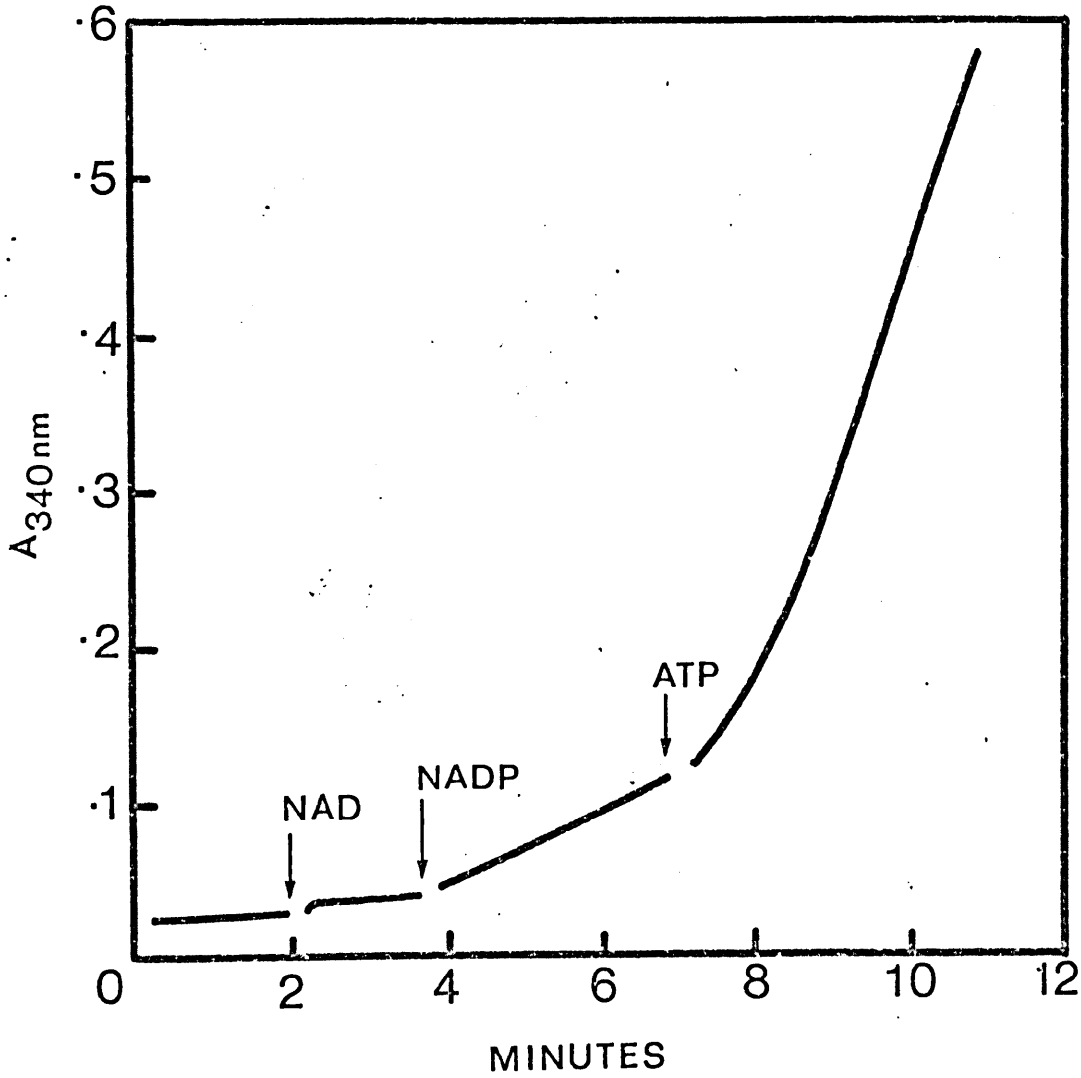


Fig. 1. TIME COURSE OF ATP-DEPENDANT AND ATP-INDEPENDANT REDUCTION OF NADP BY NADH. Blank and experimental cuvettes contained 375µmole sucrose, 75µmole tris-HCl buffer (pH 8.0), 10µmole magnesium chloride, 0.05µmole NAD, 200µg yeast alcohol dehydrogenase, 180µmole ethanol, 5µmole sodium sulphide and bacterial particles (3.5mg protein). The non-energy-linked reaction was initiated by the addition of 1µmole NADP at the point indicated. After 3min the energy-linked reaction was started by the addition of 6µmole ATP, to give a final volume of 2ml. The temperature of the incubation was 30°.

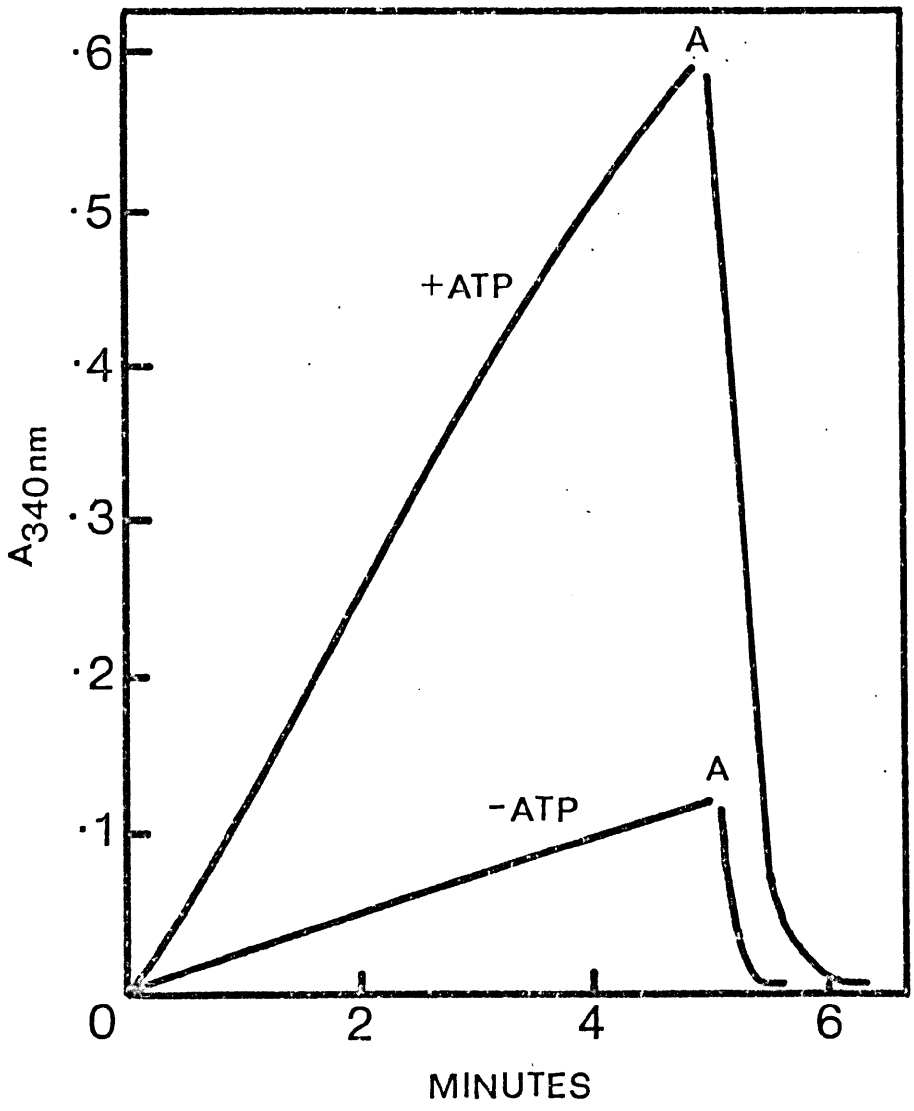


Fig. 2. DEMONSTRATION OF NADPH FORMATION IN ATP-DEPENDANT AND ATP-INDEPENDANT REDUCTION OF NADP BY NADH. The reactions were measured as described in Fig. 1, except that 3mg protein (small particles) were present. At the points marked A on the tracing 5umole glutathione and 5ug yeast glutathione reductase were added.

Table 1

REQUIREMENTS FOR REDUCTION OF $NADP^+$ BY NADH

CONDITIONS	NADPH formed (nmole/min/mg protein)
Complete	14.5
-NADH	0
$-NADP^+$	0
-ATP	3.2
-ATP, +ADP	5.3
-ATP, +AMP	3.2
-ATP, $-Mg^{2+}$	7.2
$-Mg^{2+}$	8.1

Experimental conditions were as described in Fig. 1. Additions and omissions were made as indicated above. 6μmole AMP and 6μmole ADP were added where shown. 2.5mg small particle protein were used in all the assays.

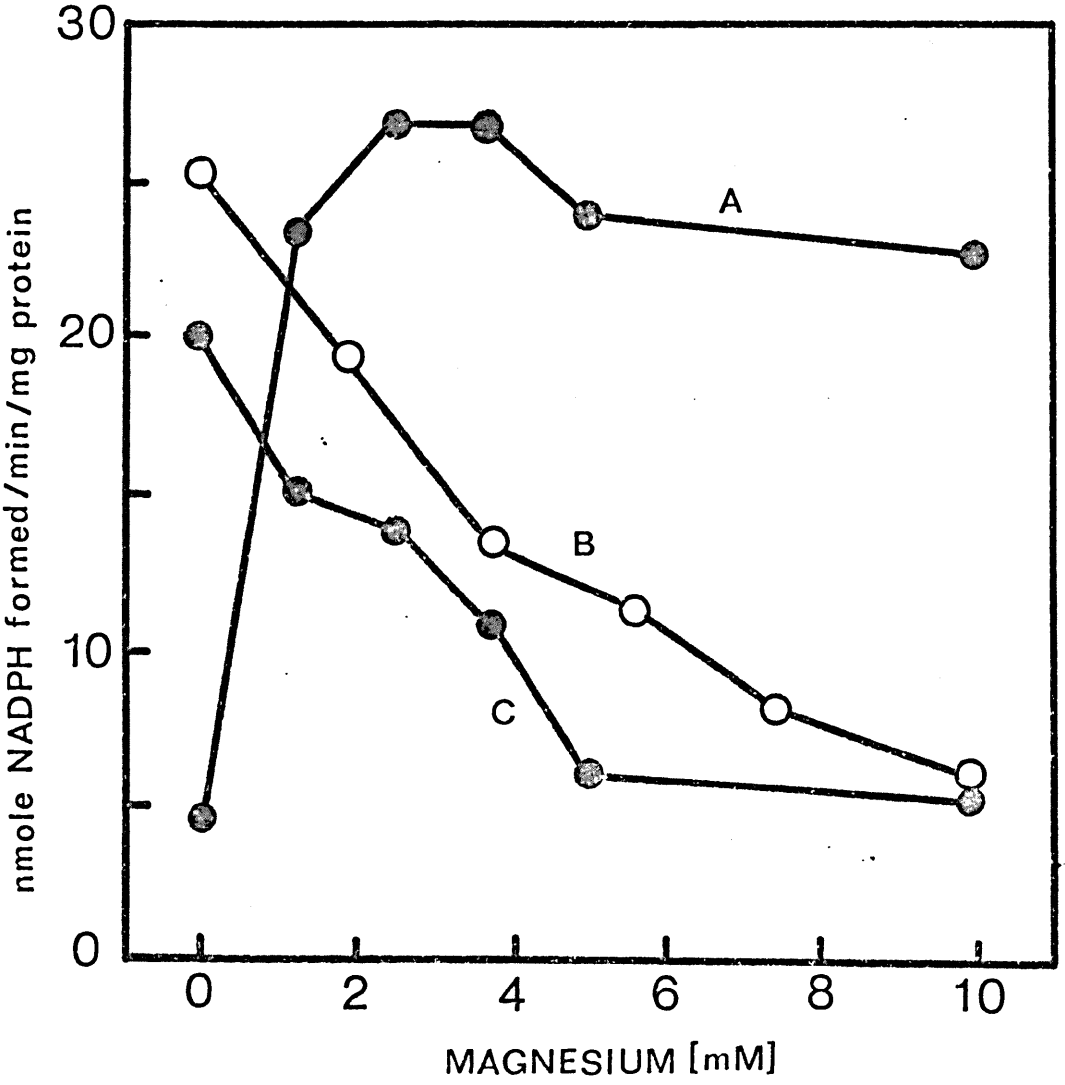


Fig. 3. EFFECT OF MAGNESIUM ON TRANSHYDROGENASE REACTIONS. The reactions were measured as described in Fig. 1, except that 2mg bacterial protein (small particles) were used. A - ATP-dependant reduction of NADP by NADH. B - ATP-independant reduction of NAD by NADPH. C - ATP-independant reduction of NADP by NADH.

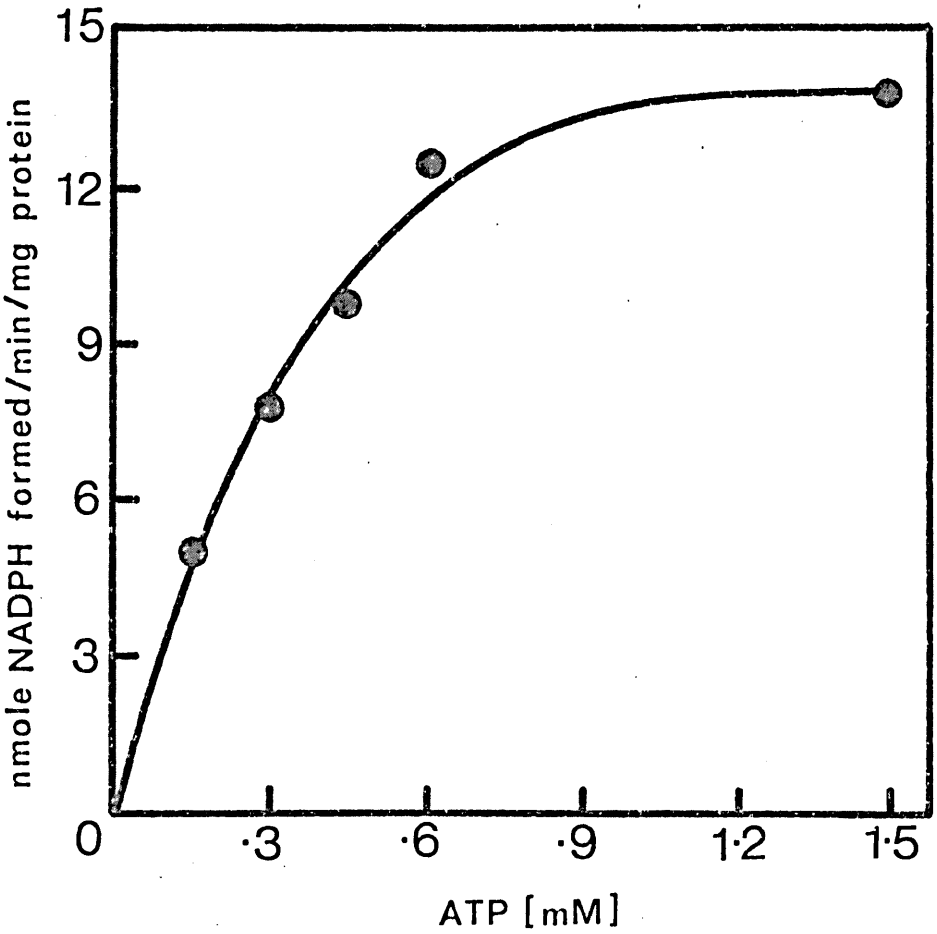


Fig. 4. EFFECT OF ATP ON ENERGY-DEPENDANT REDUCTION OF NADP BY NADH. Conditions as in Fig. 1, except that 3.0mg protein (small particles) were present. ATP was added to give the concentrations shown on the graph.

Table 2

THE EFFECT OF VARIOUS NUCLEOTIDES ON $NADP^+$ REDUCTION BY NADH

NUCLEOTIDE ADDED	NADPH formed (nmole/min/mg protein)
ATP	9.68
ADP	2.0
AMP	0
ITP	3.67
GTP	1.66
CTP	0
UTP	0

Experimental conditions were as described in Fig. 1. 2.0mg small particle protein were present in all the assays. The table shows the rate of NADPH formation observed after the addition of 3.0μmole of the various nucleotides indicated. Corrections have been made for the non-energy-linked reaction.

RESULTS.

(i) Demonstration of the ATP dependant reduction of $NADP^+$ by NADH.

In the presence of a powerful NADH regenerating system *E. coli* small particles catalysed the reduction of $NADP^+$ (non-energy-linked transhydrogenase) as shown by an increase in absorbance at 340nm. The reduction of $NADP^+$ was increased 4 - 5 times by the addition of ATP, after a lag time of about 1min. (Fig.1).

The formation of NADPH under these condition was demonstrated by the oxidation of the product by glutathione and glutathione reductase, as shown by a rapid drop in absorbance at 340nm, on addition of oxidised glutathione and the NADPH specific glutathione reductase (Fig. 2).

An absolute requirement for both NADH and $NADP^+$ was demonstrated for the energy-linked reaction (table 1). In the absence of ATP a slower rate of NADPH formation was observed; this rate was unaffected by AMP, but ADP produced a slight stimulation, indicating that ADP can act as an energy donor in this system. In the absence of ATP and magnesium and in the absence of magnesium alone the reaction was decreased by about 50%.

(ii) The effect of magnesium.

Magnesium had different effects on the energy-dependant and the energy-independant reactions. Magnesium was found to inhibit the non-energy-linked reaction going from NADH to NADPHand from NADPH to NADH (Fig. 3). Maximum inhibition of both reactions was achieved by 5mM magnesium. The energy-linked reaction, on the other hand, was stimulated by magnesium; optimum activity occuring at about 2.5mM magnesium. The activity was not completely abolished in the absence of magnesium, probably due to the presence of some endogenous magnesium in the bacterial particles.

(iii) Specificity for ATP.

The extent of the energy-linked transhydrogenase was found to be dependant on the amount of ATP added (Fig. 4) and the apparent Km for the reaction was 277μM. Apart from ATP the only nucleotide triphosphates capable of driving the reaction were ITP and to a lesser extent GTP (Table 2). The pattern was similar to that observed for the energy-linked

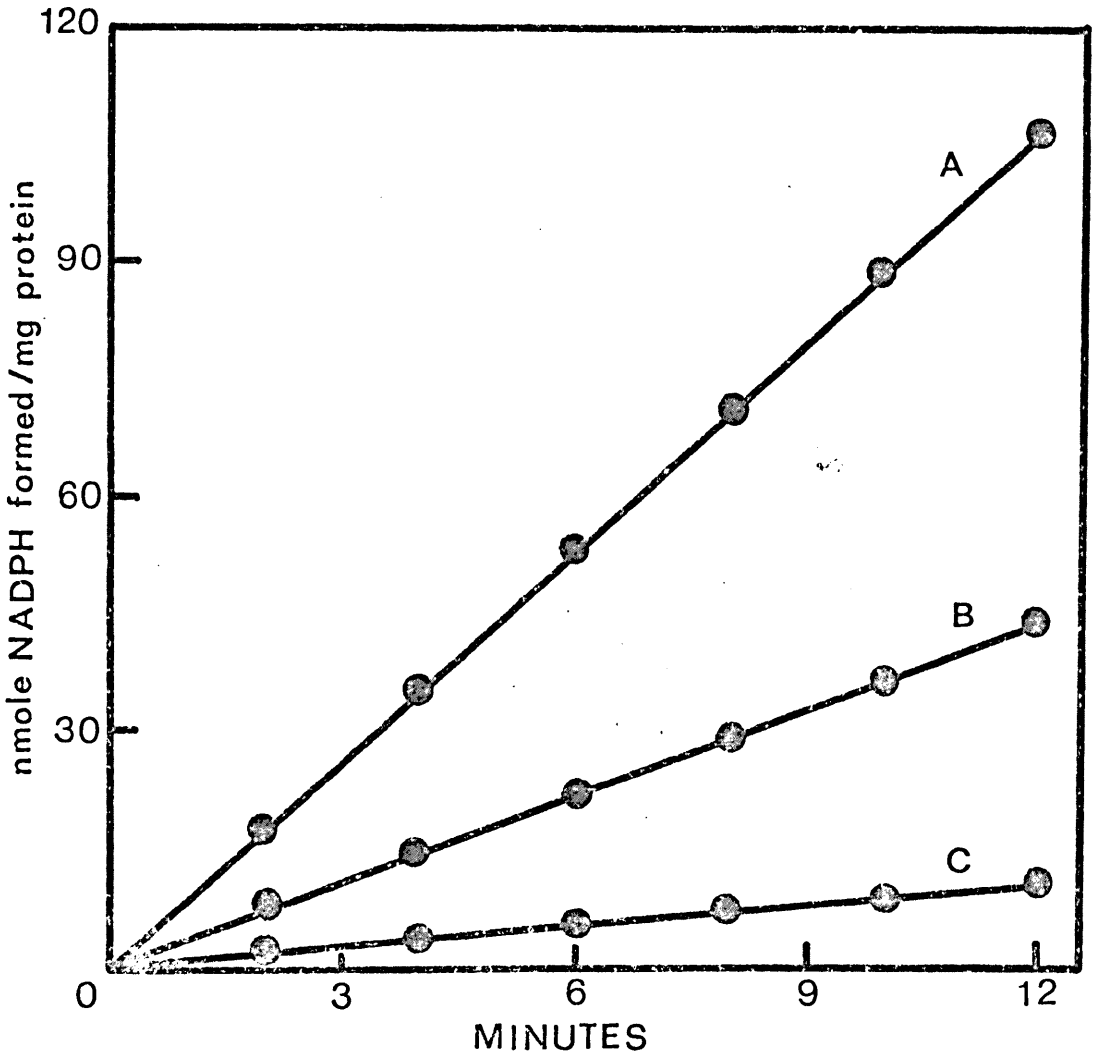


Fig. 5. ENERGY-LINKED REDUCTION OF NADP BY NADH AND NTPase ACTIVITY. The reaction was measured as described in Fig. 1, except that 2mg protein (small particles) were present. A - ATP-dependant reaction. B - ITP-dependant reaction. C - GTP-dependant reaction. The NTPase activities are shown in Chapter III (Fig. 5).

Table 3

ABILITY OF NAD^+ AND NAD^+ ANALOGUES TO ACT AS DONORS FOR ENERGY-LINKED $NADP^+$ REDUCTION

COMPOUND	λmax (nm)	E_{mM}	NADPH formed (nmole/min/mg protein)	
			non-energy linked	energy-linked
NAD^+	340	6.2	4.1	12.42
Nicotinamide hypoxanthine dinucleotide	338	6.2	1.95	0
3-acetylpyridine -NAD	363	9.1	1.72	1.18
3-pyridinealdehyde -NAD	358	9.3	2.25	0
3-acetylpyridine hypoxanthine dinucleotide	361	9.0	2.4	0
3-pyridinealdehyde hypoxanthine dinucleotide	356	9.4	1.9	0

Experimental conditions were as described in Fig. 1. Various analogues were substituted for NAD as shown above. The analogues were obtained from Pabst Labs., Milwaukee, Wisconsin. Spectral properties are quoted from their circular OR-18. 2.5mg small particle protein were present in all the assays.

ATP (uM)	NADH formed (uM)	ATP/NADPH
600	51.4	11.67
300	33.5	8.95
150	28.7	5.23
60	18.8	3.19

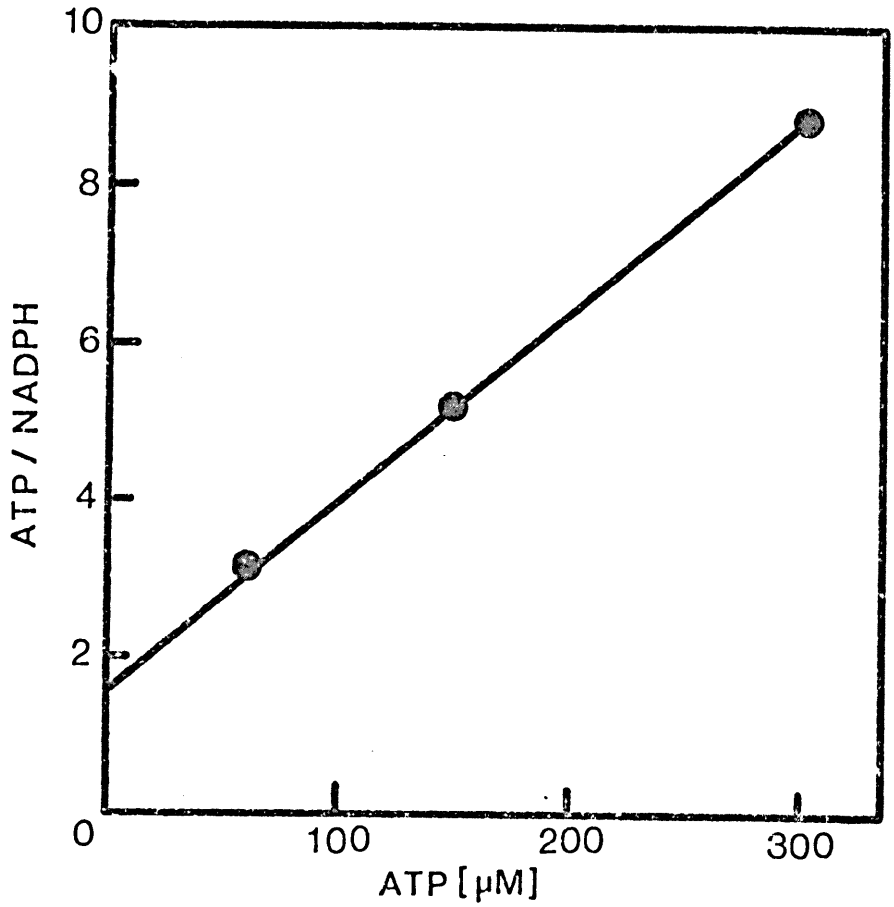


Fig. 6. RELATIONSHIP BETWEEN ATP ADDED AND NADPH FORMED. Conditions were as described in Fig. 1. 2.7mg protein (small particles) were present. ATP was added at the concentrations shown and the reaction was allowed to go to completion, when the total amount of NADPH formed was estimated from the increase in absorbance at 340nm.

reduction of NAD^+ by succinate (see previous chapter) i.e. ability to drive the reaction was related to the corresponding nucleotriphosphatase activity (Fig. 5). A slight stimulation of the reaction was observed in the presence of phosphate.

(iv) Specificity of the electron donor.

The specificity for NAD^+ analogues in the non-energy-linked transhydrogenase was relatively small (Table 3); all the analogues tested were capable of supporting at least 50% of the control rate. However, the energy-linked reaction was specific for NAD^+; the only analogue tested which produced any measurable response was 3-acetylpyridine-NAD^+.

(v) Relationship between NADPH formed and ATP added.

Direct phosphorylation of NADH by ATP to give NADPH is a possible pathway for the reaction. This was excluded because the amount of NADPH formed exceeded the total amount of NADH in the sytem e.g. in one experiment 0.096μmole NADPH were formed in the ATP-dependant reaction in a system containing 0.05μmole NADH.

Another possibility was that ATP was acting as a catalyst in the reaction. Although the particle ATPase was not stimulated in the presence of the ATP-dependant transhydrogenase, it was possible to show a relationship between the ATP split and the NADPH formed in the presence of low concentrations of ATP (Fig.6). These results suggested that it is unlikely that ATP is catalytic in function. The ATP/NADPH ratio decreased with decreasing ATP concentration to a minimum value of 3.19. By extrapolation to negligible ATP concentration a ratio of ATP/NADPH of 1.55 was obtained. Therefore 1-2 ATP molecules were consumed per NADPH molecule formed (cf. energy-linked reduction of NAD^+ by succinate).

(vi) Effect of temperature.

In the previous chapter it was shown that the energy-linked reduction of NAD^+ by succinate and the ATPase of the bacterial particles were characterised by Arrehnius plots which had a sharp break in them. It was concluded that the break was due to a conformational change in the ATPase, and that the ATPase was taking part in both reactions. The effect

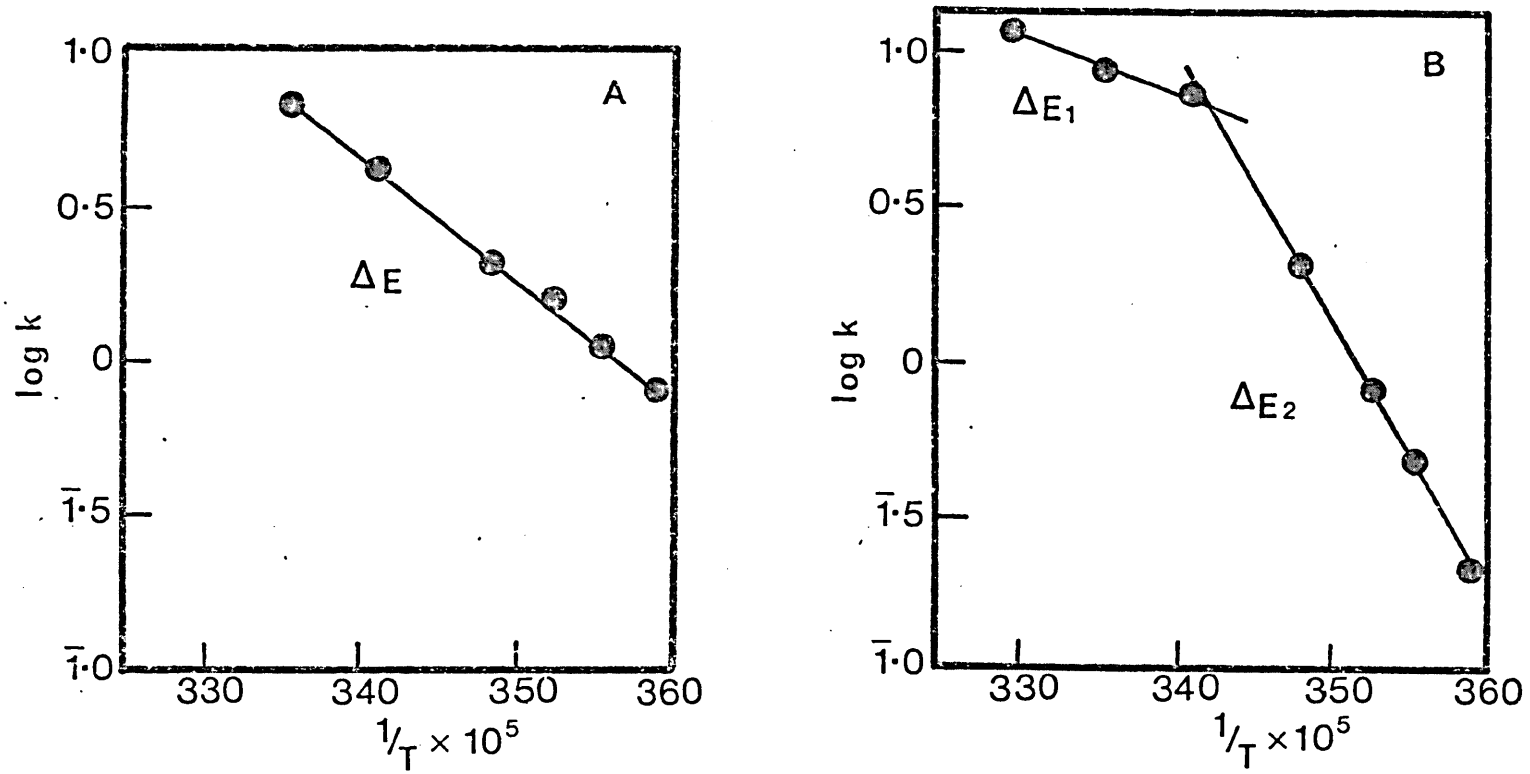


Fig. 7. ARRHENIUS PLOTS SHOWING THE EFFECT OF TEMPERATURE ON THE BACTERIAL ATP-DEPENDANT AND ATP-INDEPENDANT REDUCTION OF NADP BY NADH. Graph A - ATP-independant reaction. Graph B - ATP-dependant reaction. Reactions were measured as described in Fig. 1, except that 2.5mg bacterial protein (small particles) were added. A-ATP-independant reaction: Δ E = 16.5Kcal. B-ATP-dependant reaction: Δ E1 = 8.4Kcal, ΔE2 = 38.5Kcal, transition temp. = 19°. See Chapter III (Fig. 7) for temperature effects on ATPase and ATP-dependant reduction of NAD by succinate.

Table 4

EFFECT OF INHIBITORS AND UNCOUPLERS ON $NADP^+$ REDUCTION

COMPOUND	CONCENTRATION (μM)	%INHIBITION
Dicoumarol	33 66	70 97
Pentabromo-phenol	25 50	70 95
TTFB	1 2	42 73
2,4-DNP	90 180	50 66
Piericidin A	100 200	28 83
NHQNO	125 250	10 92
Thyroxine	50 100	56 77
Oligomycin	12.5μg	0

Experimental conditions were as described in Fig. 1. 2.5mg small particle protein were present in all the assays. Water insoluble inhibitors were added as their ethanolic solutions and suitable controls were carried out with equivalent amounts of ethanol alone.
TTFB - tetrachlorotrifluorobenzimidazole.

of temperature on the energy-linked and non-energy-linked transhydrogenases was measured (Fig. 7). The results for the energy-linked reaction showed a sharp break at 19°, with activation energies of 8.4 and 38.5Kcal above and below that temperature respectively. These figures are in good agreement with those obtained for the ATPase and ATP-dependant reduction of NAD^+ by succinate, indicating that the ATPase is involved in all three reactions. The non-energy-linked reaction had an activation of energy of 16.5Kcal and did not exhibit a break in the line, which showed that the reaction was not dependant on the particle ATPase.

(vii) Effect of inhibitors of electron transport and uncouplers of oxidative phosphorylation.

A variety of compounds were shown to inhibit the energy-dependant reduction of $NADP^+$ by NADH (Table 4). The mitochondrial uncouplers dicoumarol, pentabromophenol and tetrachlorotrifluorobenzimidazole inhibited the reaction by 50% at concentrations of 30μM, 18μM and 1μM respectively. The figures for dicoumarol and pentabromophenol are lower than the corresponding figures for the energy-linked reduction of NAD^+ by succinate. The reason for this is that these compounds have an inhibitory effect on the NADH regenerating system, resulting in an overestimate of their inhibitory effect towards the transhydrogenase.

Piericidin A (50% at 150μM) and NHQNO (50% at 190μM) both inhibited the energy-linked transhydrogenase, which contrasted with their effect on the energy-linked reduction of NAD^+ by succinate. Thyroxine also showed a differential effect; the tranhydrogenase was inhibited 50% at 44μM whilst the reduction of NAD^+ by succinate was inhibited 11% at the same concentration. Oligomycin, a mitochondrial energy-transfer inhibitor, had no effect on the reaction at the concentrations tested. The effect of uncouplers on the reaction characterise it as one involving high-energy intermediates of oxidative phosphorylation. None of the uncouplers affected the non-energy-linked reduction of $NADP^+$ by NADH, confirming that this reaction does not involve the participation of high-energy intermediates.

DISCUSSION.

Many of the anabolic reactions in a cell require NADPH as a source of reducing equivalents. This requirement could possibly be satisfied by $NADP^+$-linked Oxidations, but most of the cellular dehydrogenases are NAD^+-linked; thus other mechanisms must be utilized for NADPH production. $NADP^+$ is reduced in green plants and some algae by a process dependant on light energy (141), providing a source of NADPH for synthetic reactions. In non-photosynthetic tissues NADPH can be generated by an energy-linked transhydrogenase reaction, which is dependant on oxidative phosphorylation as the source of energy (1).

The results presented here show that E. coli small particles are capable of catalysing two kinds of transhydrogenase reaction. The reactions observed were the reduction of $NADP^+$ by NADH and the ATP-dependant reduction of $NADP^+$ by NADH. Both reactions resulted in the generation of reducing power in the form of NADPH.

The energy requirement for the energy-dependant reaction could be supplied by added ATP and to a lesser extent ITP and GTP. There was a relationship between the amount of ATP hydrolysed and the amount of NADPH formed. The minimum value determined for the ATP/NADPH ratio was 3.19, but extrapolation to negligible ATP concentration gave a value of 1.55. It is concluded that for the reaction in E. coli one molecule of ATP is hydrolysed per molecule of NADPH formed (see discussion in Chapter III). Equations for the two reactions might be written as follows:

(i) Non-energy-linked transhydrogenase.

$$NADH + NADP^+ \rightleftarrows NAD^+ + NADPH$$

(ii) Energy-linked transhydrogenase.

$$NADH + NADP^+ + ATP \rightleftarrows NAD^+ + NADPH + ADP + P_i$$

It has been proposed that the two reactions in submitochondrial particles involve the same hydrogen transfer enzyme (136), because both reactions show similar sensitivities towards inhibitors such as

triiodothyronine(142) and to specific antibodies prepared against the purified non-energy-linked transhydrogenase (143). Both reactions involve a direct hydrogen transfer from the A-locus of NADH to the B-locus of $NADP^+$, providing circumstantial evidence in support of the concept that both reactions utilize the same enzyme. Evidence against this concept is that the two reactions have different sensitivities to magnesium, different equilibria, and energy is required in one case and not in the other.

In bacterial preparation described here there are several differences between the two reactions that suggest they are catalysed by different enzymes or different forms of the same enzyme. ATP shifts the equilibrium towards NADPH formation with the result that the ATP-dependant reaction is 4-5 times faster than the non-energy-linked transhydrogenase. The activation energy of the non-energy-linked transhydrogenase is twice that of the ATP-dependant reaction between 20° and 30°. At 19° the energy-dependant reaction shows a sharp break in the Arrhenius plot and below this temperature the activation energy is increased to twice that of the non-energy-linked reaction, which has no break in the Arrhenius plot. The temperature effects on the energy-linked transhydrogenase are identical to those of the particle ATPase and the energy-linked reduction of NAD^+ by succinate. It is concluded that these three reactions have some reaction pathway in common, probably the ATPase: this pathway is not involved in the non-energy-dependant transhydrogenase.

Further differences are seen with the effect of magnesium on the two transhydrogenase reactions. The non-energy-dependant reaction is inhibited by magnesium in both directions, whereas the energy-linked reaction is completely dependant on magnesium for activity. The selectivity towards magnesium argues against the identity of the two enzymes as does the specificity towards adenine nucleotides.

Six analogues of NADH were tested for their ability to act as electron donors in the two reactions. All the analogues were able to form the corresponding NADPH analogue with the non-energy-linked enzyme at about 50% of the control rate. The energy-linked transhydrogenase, on the other hand, was active only towards 3-acetylpyridine-NADH and then at only 10% of the control rate, indicating that this enzyme was specific for NADH.

The action of uncouplers of oxidative phosphorylation characterises the energy-linked reaction as one involving the participation of high energy compounds derived from ATP hydrolysis. These reagents were without effect on the non-energy-linked reaction. The weight of the evidence is in favour of the hypothesis that in E. coli there is more than one hydrogen transfer enzyme responsible for the formation of NADPH from NADH. It is suggested that there are two enzymes; one of which requires ATP whilst the other does not.

The energy transfer pathway for the ATP-dependant reaction appears to be identical with that of the ATP-dependant reduction of NAD^+ by succinate. However, the hydrogen transfer pathway of the two reactions is quite different. The reduction of NAD^+ involves the NADH and succinate dehydrogenase flavoprotein systems, but there is no evidence to suggest that these enzymes also participate in the transhydrogenase. Furthermore, the transhydrogenase is selectively inhibited by piericidin and NHQNO compounds, which stimulate the energy-linked reduction of NAD^+ by succinate. If the electron transport chain is not involved in the transhydrogenase then there are obviously other sites of action of piericidin in the bacterial particles. These results are in agreement with studies on submitochondrial particles (see this thesis) which showed that piericidin inhibited the mitochondrial transhydrogenase. It seems likely that piericidin could be a useful tool in the study of transhydrogenase reactions.

The energy-linked transhydrogenase was first described in E.coli by Murthy and Brodie (138), although they could not find the reaction in Mycobacterium phlei. After completion of the present investigation a report describing the effect of inhibitors and uncouplers on oxidative phosphorylation and the energy-dependant transhydrogenase in E. coli was published (128). The investigators concluded that non-phosphorylated high-energy intermediates were involved in both reactions. Asano et al (144) concluded that the transhydrogenase in Micrococcus denitrificans was similar to that found in mammalian preparations. An energy-linked transhydrogenase has also been reported in a photosynthetic bacterium; Rhodospirillum rubrum (140,145).

Further experiments on the energy-linked transhydrogenase from E.coli small particles and beef heart submitochondrial particles are described elsewhere in this thesis.

CHAPTER V.

THE ROLE OF UBIQUINONE IN MITOCHONDRIAL REACTIONS.

INTRODUCTION.

Not long after its discovery by Morton (146) ubiquinone was shown to undergo oxidation-reduction reactions in mitochondria in the presence of respiratory substrates (147). Since that time a considerable amount of effort has been spent on examining the role of ubiquinone in electron transport and oxidative phosphorylation. The lipid solubility of the molecule and its characteristic absorption spectra in the oxidised and reduced forms have facilitated the experimental approaches employed. The first has allowed the extraction of the quinone from mitochondria with organic solvents and the second has afforded a method of measuring its concentration and determining its oxidation-reduction state. All studies carried out to date have clearly shown a function for ubiquinone in the electron transport system of mitochondria; but the precise nature of this function has caused some dispute.

Acetone extraction of beef heart mitochondria resulted in almost complete removal of ubiquinone and complete loss of ability to oxidise NADH or succinate (148). Restoration of succinate oxidation was achieved by addition of ubiquinone to the extracted mitochondria, but NADH oxidation was not restored. It was concluded that ubiquinone was on the main pathway of electron transport between succinate and oxygen and that restoration of NADH oxidation was not demonstrable because solvent extraction caused inactivation of the NADH dehydrogenase enzyme system. These results were criticised on the grounds that the rate of ubiquinone reduction was slower than that of the other respiratory components. It was suggested that ubiquinone occupied a position in a side pathway (149, 150). This conclusion was supported by later studies when it was shown that electron transport could occur in acetone extracted mitochondria from which all the ubiquinone had been removed (74). However, the possibility that some ubiquinone remained strongly bound or that acetone extraction caused structural reorganisation of the mitochondria cannot be excluded.

The question of ubiquinone's involvment in NADH oxidation was

resolved by Szarkowska (70). In her experiments extraction of lyophilised beef heart mitochondria with pentane resulted in complete loss of succinate and NADH oxidase; both activities were specifically restored by the addition of ubiquinone in the presence of mitochondrial phospholipids. The restored reactions were sensitive to inhibitors of electron transport in the same way as the normal reactions. One of the problems with these findings was that the concentration of ubiquinone required for complete restoration was considerably in excess of that originally present in the mitochondria. However, the results suggest that at least a part of the ubiquinone present in the mitochondria is necessary for electron transport. Support was given to this conclusion when it was shown that the redox behaviour of ubiquinone under various conditions closely resembled those of the other respiratory carriers (26). It was also shown that ubiquinone occupied a position between the flavoproteins and cytochrome c, but the relative positions of cytochrome b and ubiquinone were not determined.

Improvements (151) in the spectrophotometric technique (150) for measuring ubiquinone oxidation and reduction have now shown that the rate of oxidation of reduced ubiquinone is commensurate with a central role in the oxidation of succinate. However, some of the electrons were capable of bypassing ubiquinone and reducing cytochrome b instead. A similar type of split pathway has also been demonstrated by Jeng and Crane (152). They made use of the selectivity of piericidin and antimycin A to show that cytochrome b and ubiquinone could be independantly reduced by both NADH and succinate. The bulk of the evidence suggests that ubiquinone is involved in the main pathway of electron transfer between the flavoproteins and cytochrome c, but the possibility that some of the ubiquinone might be involved in other mitochondrial reactions must also be considered (74).

Various schemes have been proposed implicating quinones as possible high-energy intermediates in oxidative phosphorylation (51-53). The only experimental evidence for the operation of these schemes was obtained when it was shown that ubiquinol monophosphate was slowly oxidised by mitochondria with concomitant phosphorylation of ADP (42). However the rates of oxidation appeared to be too slow to have any physiological significance. Most of the evidence is against the participation of high-energy derivatives of quinones in oxidative phosphorylation in mitochondria (see Chapter I).

The present work was carried out to investigate the suggestion (84) that quinones were involved in mitochondrial energy-linked reactions, and constituted a new approach to the various suggestions that quinones were participating in oxidative phosphorylation (51-53). A role for quinones, other than that in electron transport, was considered to be possible because of the stoichiometric excess of ubiquinone relative to the cytochromes (17). Some of the results have been presented in preliminary form elsewhere (153).

METHODS.

Preparation of Ubiquinone Depleted Submitochondrial Particles.

Two methods were employed for preparing submitochondrial particles. (A) The first involved the preparation of submitochondrial particles from beef heart mitochondria by the method described in Chapter II. The particles were suspended at a protein concentration of 20mg/ml in 0.15M-KCl and were lyophilised for 3hr. The lyophilised particles were suspended in pentane and homogenised in a glass homogeniser with a teflon pestle and then shaken for 5min. The extraction procedure was repeated 4-5 times and the particles were collected by centrifugation and finally they were suspended in a solution containing 0.25M-sucrose and 0.01M-tris-HCl (pH 7.6). The transhydrogenase activity of the particles was measured spectroscopically and restoration was achieved by adding a solution of ubiquinone in ethanol directly to a suspension of the particles in a cuvette. (70).

(B) The second method involved the reincorporation of ubiquinone into lyophilised-extracted beef heart mitochondria with subsequent preparation of submitochondrial particles. Heavy beef heart mitochondria (20-30mg/ml) were suspended in 0.15M-KCl and lyophilised for 3hr. The lyophilised mitochondria (250-300mg protein) were suspended in 50ml pentane in a 250ml conical flask and stirred on ice for 30min. The extracted mitochondria were collected by centrifugation at 35,000 x g and homogenised in 20ml pentane and re-extracted for a further 10min. The twice extracted particles were sedimented at 35,000 x g and dried under vacuum to remove any residual pentane. Ubiquinone was incorporated into the mitochondria by suspending them in a solution of ubiquinone in pentane (2ml) and drying the resulting suspension under vacuum. A similar procedure was adopted for adding back the pentane extract (154). This technique gave five different mitochondrial preparations; (i) normal mitochondria, (ii) lyophilised mitochondria (iii) lyophilised-extracted mitochondria (iv) lyophilised-extracted mitochondria plus ubiquinone and (v) lyophilised-extracted mitochondria plus pentane extract. Submitbchondrial particles were prepared from all three batches by sonication as described in Chapter II.

Energy-Linked Reduction $NADP^+$ by NADH.

Blank and experimental cuvettes of 1cm light path contained 675µmole

sucrose, 16μmole magnesium chloride, 3μmole potassium cyanide, 135μmole tris-HCl buffer (pH 8.0) and 0.5-2.0mg beef heart submitochondrial particles. The following additions were made to the experimental cuvette; 10μl yeast alcohol dehydrogenase (290μg) and 10μl ethanol. The absorption was determined at 340nm using a Beckman DK 2 recording spectrophotometer until a steady trace was obtained, when 40nmole NAD^+ were added to the experimental cuvette. The non-energy-linked reaction was initiated by the addition of 0.6μmole $NADP^+$ to the experimental cuvette. After 2-3min 6μmole ATP were added to start the energy-linked reaction. When required ubiquinone was added as an ethanolic solution (50μl) to both the blank and experimental cuvettes. If added to the experimental cuvette alone, ubiquinone produced a large absorbance change which interfered with the measurement of the reaction. Immediately after addition of the quinone, the contents of the cuvettes were mixed thoroughly by stirring with a plastic rod for 15 sec. The final volume of the reaction mixture was 3.05ml and the incubation temperature was 30°. The problems associated with the addition of ubiquinone to the reaction medium were eliminated in the experiments in which ubiquinone was reincorporated into the submitochondrial particles prior to the measurement of the reaction.

Energy-Linked Reduction of NAD^+ by succinate.

NAD^+ reduction was measured by the method of Griffiths and Roberton (85). Blank and experimental cuvettes of 1cm light path contained 675μmole sucrose, 135μmole tris-HCl buffer (pH 8.0), 16μmole magnesium chloride, 3μmole potassium cyanide, 3μmole NAD^+ and 15μmole succinate. 0.5-2.0mg submitochondrial particle protein were added to both cuvettes and the system was allowed to equilibrate for 3min. The reaction was started by the addition of 6μmole ATP to the experimental cuvette, to give a final volume of 3.0ml. In experiments involving the addition of ubiquinone, the quinone was added to each cuvette and the contents of the cuvettes were mixed thoroughly by stirring the suspension for 15sec with a plastic rod. The reaction was measured at 340nm in a Beckman DK 2 recording spectrophotometer at 30°.

Other Methods.

NADH and succinate oxidation were determined polarographically by

the methods described in Chapter II. Ethanolic solutions of ubiquinone were added directly to the reaction chamber of the oxygen electrode and the contents were mixed by means of a magnetic stirrer.

All other methods used were as described in Chapter II of this thesis.

Table 1

RESTORATION OF ATP-DEPENDANT REDUCTION OF NADP+ BY NADH BY ADDITION OF UBIQUINONE IN BEEF HEART SUBMITOCHONDRIAL PARTICLES

CONDITIONS	REACTION RATES (nmole/min/mg protein)	
	EXPERIMENT I (Cyanide)	EXPERIMENT II (Rotenone)
Normal	69 (1.39)	75 (1.10)
Lyophilised	23 (0.90)	28.4 (0.78)
Lyophilised-extracted	2.4 (1.00)	2.0 (1.18)
Lyophilised-extracted plus ubiquinone	16.1 (1.00)	28 (1.18)

Pentane extracted submitochondrial particles were prepared by method A (see Methods section). ATP-dependant reduction of NADP+ by NADH was measured as described in the Methods section. Ubiquinone-(45) (130nmole) was added directly to both blank and experimental cuvettes and the reaction was measured after thorough mixing of the contents of the cuvettes. Cyanide (1.0μmole/ml) was used to block electron transport in experiment I and rotenone was used for the same purpose in experiment II. The figures in brackets refer to the amounts (mg) of submitochondrial particle protein employed. The time course of the reaction is shown graphically in Fig. 1. The concentration of rotenone employed was 0.5nmole/ml.

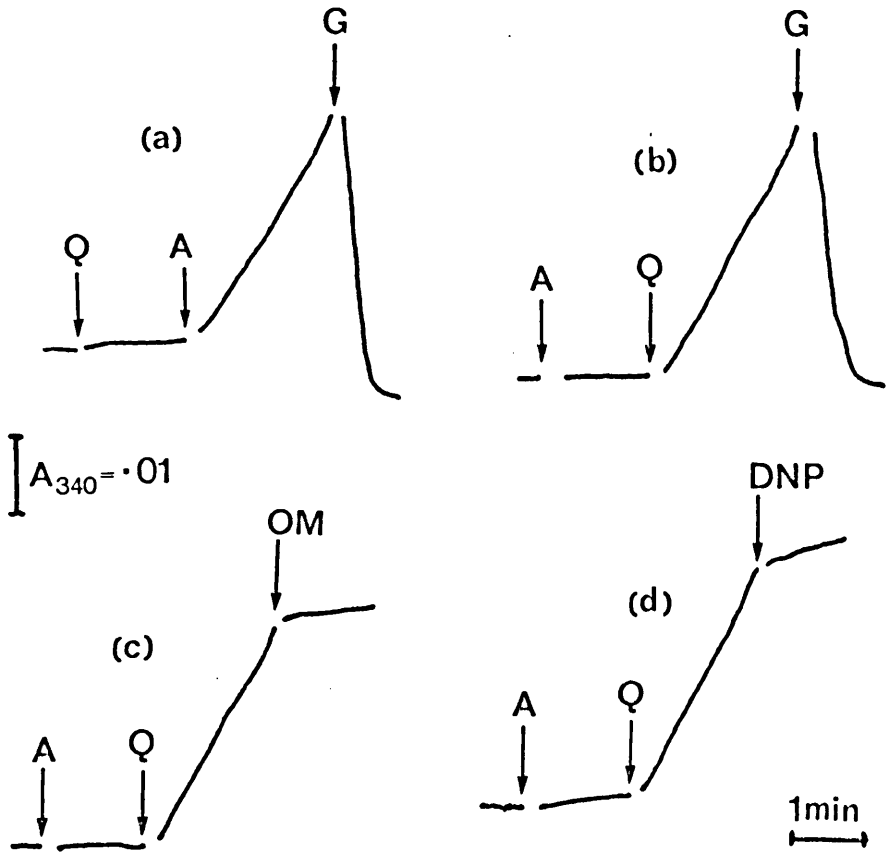


Fig. 1. RESTORATION OF ENERGY-LINKED REDUCTION OF NADP+ BY NADH BY UBIQUINONE-(45): EFFECT OF INHIBITORS AND FORMATION OF NADPH. Pentane extracted particles were prepared by Method A (see Methods section). Ubiquinone was added to the lyophilised extracted particles as described in Table 1. Cyanide (1.0μmole/ml) was used to block electron transport. 1.0mg submitochondrial particle protein was used in all the assays. The increase in absorbance was measured at 340nm in a Beckman DK 2 recording spectrophotometer at 30°. Key: Q - 130 nmole ubiquinone-(45) were added with thorough mixing; A - 6μmole ATP added; G - 5μmole glutathione plus 5μg yeast glutathione reductase; OM - 2.5μg oligomycin added; DNP - 2,4-dinitrophenol to give a final concentration of 500μM.

RESULTS AND DISCUSSION.

Table 1 shows the energy-linked transhydrogenase activities of normal, lyophilised and lyophilised extracted beef heart submitochondrial particles. The transhydrogenase activity was decreased to about 30% of the normal level by the lyophilisation procedure and to about 3% by subsequent extraction with pentane. Addition of a solution of ubiquinone - (45) to the reaction medium, which contained lyopilised extracted particles caused resoration of the transhydrogenase activity. In experiment I cyanide was used to prevent oxidation of the reduced adenine nucleotides: the degree of restoration of the energy-linked transhydrogenase was 70% of the lyophilised control. In experiment II rotenone, which inhibited NADH oxidation more effectively than cyanide, increased the degree of restoration to 100%. The energy-linked reduction of NAD^+ by succinate was also lost on extraction of the particles with pentane, but restoration of the reaction with ubiquinone, was not observed.

The effect on the transhydrogenase was not due to a non-specific absorbance increase produced by ubiquinone, because when ubiquinone was added before ATP, no restoration was obtained (Fig.1a and 1b). Furthermore in both cases addition of the NADPH specific enzyme glutathione reductase plus glutathione at the end of the experiment, caused a decrease in absorbance to the original level.

The restored reaction was inhibited by the uncoupler, 2,4-DNP and the energy transfer inhibitor oligomycin at the same concentrations as the normal reaction (Fig. 1c and 1d). This ruled out the possibility that ubiquinone was stimulating the non-energy-linked transhydrogenase, because this reaction was not sensitive to 2,4-DNP and oligomycin. One problem with the experiments was that the concentration of ubiquinone required for complete restoration was in excess of that originally present in the mitochondria. For example in experiment II (Table 1) the amount of ubiquinone added was equivalent to 110μmole/g particle protein, whereas the amount extracted was only 5.9μmole/g protein. This obviously casts some doubts on the relevance of the restoration obtained to the reaction occuring in normal particles. For this reason a second extraction technique was employed which allowed the reincorporation of ubiquinone into the particles at the concentration originally present.

Table 2

RESTORATION OF OXIDASE ACTIVITIES IN PENTANE EXTRACTED BEEF HEART SUBMITOCHONDRIAL PARTICLES

CONDITIONS	REACTION RATES (nmole/min/mg protein)		PROTEIN PRESENT (mg)
	SUCCINATE	NADH	
Normal	140	188	0.75
Lyophilised	125	142	0.70
Lyophilised-extracted	12	14	0.81
Lyophilised-extracted plus ubiquinone (reincorporated)	108	86	0.95
Lyophilised-extracted plus pentane extract	89	98	0.82
Lyophilised-extracted plus ubiquinone-(30) (direct addition)	25	24	0.81

Pentane extracted submitochondrial particles were prepared by Method B (see Methods section). Oxidase activities were measured polarographically. Submitochondrial particles (at the concentration shown) were incubated at 30° in a medium containing 750µmole sucrose, 150µmole tris-HCl buffer (pH 7.5) and 18µmole magnesium chloride (final volume 3.0ml). Either 10µmole succinate or 5µmole NADH were added as substrate. Ubiquinone (15nmole/mg protein) and pentane extract (containing an amount of ubiquinone equal to twice that originally present in the particles) were incorporated into the particles. The effect of a direct addition of ubiquinone is also shown.

Table 3

EFFECT OF UBIQUINONE ON ENERGY-LINKED ACTIVITIES IN PENTANE EXTRACTED BEEF HEART SUBMITOCHONDRIAL PARTICLES

CONDITIONS	REACTION RATES (nmole/min/mg protein)		PROTEIN PRESENT (mg)
	ATP-driven reduction of $NADP^+$ by NADH	ATP-driven reduction of NAD^+ by succinate	
Normal	72	48	1.5
Lyophilised	17.4	5.5	1.4
Lyophilised-extracted	0.9	0	1.6
Lyophilised-extracted plus ubiquinone (reincorporated)	9.0	0	1.9
Lyophilised-extracted plus pentane extract	14.1	4.5	1.6

Pentane extracted submitochondrial particles were prepared by Method B (see Methods section). Energy-linked activities were measured as described in the Methods section using the protein concentrations shown above. Ubiquinone (15nmole /mg protein) and pentane extract (containing an amount of ubiquinone equal to twice that originally present in the particles) were incorporated into the particles as described in the Methods section.

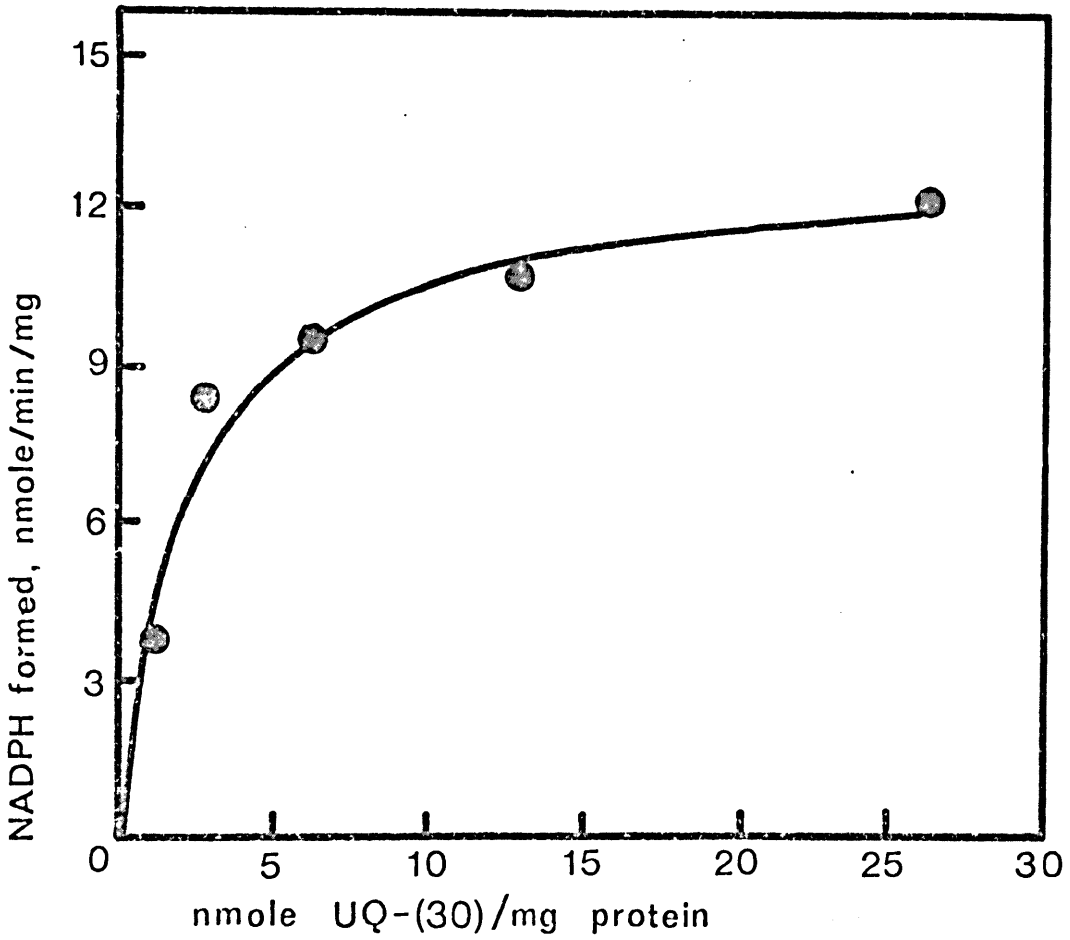


Fig. 2. RELATIONSHIP BETWEEN UBIQUINONE-(30) CONCENTRATION AND RESTORATION OF THE ENERGY-LINKED REDUCTION OF NADP+ BY NADH. Pentane extracted submitochondrial particles were prepared by Method B (see Methods section). Energy-linked activities were measured as described in the Methods section using 1.5mg particle protein. Ubiquinone-(30) was reincorporated into the particles at the concentrations shown.

Previous experiments have shown that both NADH and succinate oxidase enzyme systems were specifically restored by ubiquinone in beef heart mitochondria (7, 70). In order to evaluate the reincorporation technique the experiments were repeated with submitochondrial particles. Table 2 shows the results obtained. The extracted particles had virtually no oxidase activities; but reincorporation of ubiquinone resulted in restoration to 70-80% of the control (lyophilised) level. A similar degree of restoration was obtained when the pentane extract was reincorporated into the particles. On the other hand, when ubiquinone was added directly to the particles in the assay cuvette at the same concentrations as in the reincorporation experiments, only 20% reactivation was achieved.

The restored activities were the same as the original as they had the same inhibitor sensitivity to both antimycin A and cyanide. Thus when lyophilised-extracted particles were suspended in a solution of ubiquinone in pentane, and vacuum dried to remove the pentane, it was possible to achieve restoration by using concentrations of ubiquinone normally present in particles; and the restored reaction was still sensitive to electron transport inhibitors. These results were in contrast to those obtained previously with the direct method of addition (7, 70), when a considerable excess of ubiquinone was required for restoration: but they were in agreement with the recent findings of Ernster et al (155), who also employed the reincorporation technique. It is concluded that ubiquinone is an essential component of both the NADH oxidase and succinate oxidase systems of beef heart submitochondrial particles.

The pentane extraction procedure caused a complete loss of energy-linked activities in beef heart submitochondrial particles (Table 3). The energy-linked transhydrogenase could be restored to 80% of the control (lyophilised) activity by addition of the pentane extract of ubiquinone-(30) to the lyophilised-extracted preparation by the reincorporation technique. The extent of restoration of the transhydrogenase was dependant on the amount of ubiquinone-(30) added; and a near maximum effect was found at the concentration of ubiquinone normally present in the particles (Fig. 2). This was in contrast with the direct addition of ubiquinone, which required a twenty-fold excess of the quinone for maximum restoration.

The restored transhydrogenase reaction was sensitive to the same

Table 4

EFFECT OF INHIBITORS ON ATP-DEPENDANT REDUCTION OF $NADP^+$ BY NADH IN PENTANE EXTRACTED BEEF HEART SUBMITOCHONDRIAL PARTICLES

CONDITIONS	PROTEIN PRESENT (mg)	OLIGOMYCIN (2.5μg)		2,4-DNP (240μM)	
		+OM	-OM	+DNP	-DNP
Normal	1.5	9	70	13	65
Lyophilised	1.4	4.2	19	4.5	17.4
Lyophilised-extracted plus ubiquinone (reincorporated)	1.9	1.0	8.5	2.3	8.7

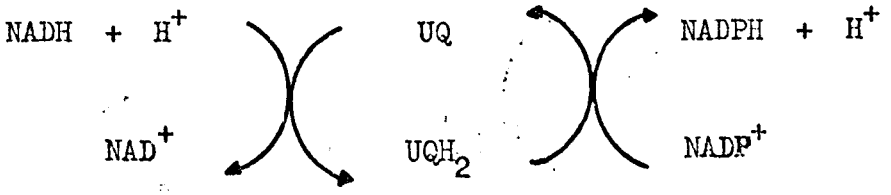
Pentane extracted submitochondrial particles were prepared by Method B (see Methods section). Energy-linked activities were measured as described in the Methods section using the protein concentrations shown above. Ubiquinone (15nmole /mg protein) was reincorporated into the particles as described in the Methods section. Inhibitors were added to give the concentrations shown. The figures in the table represent nmole NADPH formed/min/mg particle protein.

concentrations of 2,4-DNP and oligomycin that affected the control reactions (Table 4). The reactivation was a specific effect of ubiquinone since it could not be duplicated by vitamin K and phospholipid additions. The energy-linked reduction of NAD^+ by succinate was not reactivated by the addition of ubiquinone-(30), but was restored to 80% of the control level by the pentane extract.

These results suggest a specific role for ubiquinone in the energy-linked transhydrogenase reaction of beef heart submitochondrial particles. There are several possibilities for the mode of action of ubiquinone in this system: (i) ubiquinone participates in the phosphorylation process as high-energy intermediate; (ii) ubiquinone serves as a redox carrier in the hydrogen transfer sequence; (iii) ubiquinone causes a conformation change in one of the proteins involved in the reaction.

Both the energy-linked transhydrogenase and the energy-linked reduction of NAD^+ by succinate are thought to be driven at the expense of the same high-energy intermediate of oxidative phosphorylation (1). The failure of ubiquinone to restore the energy-linked reduction of NAD^+ by succinate constitutes evidence against the participation of a common quinone intermediate in both reactions. Ubiquinone involvment in reversed electron transfer has been demonstrated in rat heart mitochondria (26). However, the reaction studied in this case was not the ATP-dependant reduction of NAD^+ by succinate, but reversed electron transfer along the complete length of the respiratory chain, from cytochrome a to NAD^+. This reaction would be expected to involve ubiquinone, whereas the energy-linked reduction of NAD^+ by succinate could proceed via a direct electron transfer between the succinic dehydrogenase system and the NADH dehydrogenase system, thus eliminating the necessity for ubiquinone participation. However, loss of the energy-linked reduction of NAD^+ by succinate on extraction with pentane and the subsequent reactivation by reincorporation of the pentane extract, points to a role for a lipid component other than ubiquinone, in this reaction.

The way in which ubiquinone could act as a redox carrier in the energy-linked reduction of NADPH by NADH reaction is envisaged in the scheme depicted below. Reducing equivalents are



accepted from NADH by oxidised ubiquinone and transferred via the reduced form of the quinone to $NADP^+$, to give NADPH. However, ubiquinone could have a conformation change function in the reaction; and at the present time it is not possible to distinguish between the two theories. In this connection measurements of the oxidation-reduction state of ubiquinone in the reaction might be helpful in deciding its function. A problem with this approach would be interference from redox changes of ubiquinone in the respiratory chain. Green has recently reported that the transhydrogenase enzyme system represents a fifth respiratory chain complex (33). If this complex was isolated in a pure form, interference from other reactions would be minimal, and the role of ubiquinone could be studied more easily.

Piericidin does not act as an uncoupler or energy transfer inhibitor in beef heart mitochondria, yet it inhibits both the energy-linked transhydrogenase and energy-linked reduction of NAD^+ by succinate in beef heart submitochondrial particles (see Chapter II). This means that piericidin affects the hydrogen transfer processes in these reactions. Since it has been suggested that piericidin is a ubiquinone analogue (71,100) its effect on energy-linked reactions could be due to competition with a quinone component which participates in the reactions. This is not the case for the energy-linked reduction of NAD^+ by succinate because piericidin inhibition of this reaction is due to interaction with the NADH dehydrogenase enzyme system, in a rotenone-like manner (see Chapter II). However, competition with ubiquinone might explain the action of piericidin on the energy-linked transhydrogenase.

To summarize, it is concluded that ubiquinone is involved in the energy-linked transhydrogenase of beef heart submitochondrial particles, either as a hydrogen transfer component or by inducing a conformation change in one of the proteins involved in the reaction.

CHAPTER VI

THE ROLE OF UBIQUINONE IN ELECTRON TRANSPORT AND ENERGY-LINKED REACTIONS IN ESCHERICHIA COLI.

INTRODUCTION.

The role of quinones in mitochondrial metabolism has been thoroughly examined, but their function in bacterial systems has received relatively little attention. Mammalian mitochondria contain substantial amounts of ubiquinone and only have a low vitamin K content, whereas bacteria may have either ubiquinone or vitamin K derivatives, or both, in large amounts. The quinones found in bacteria appear to have an electron transport function, similar to that ascribed to mammalian quinones (173).

Ubiquinone-(40), which is the only quinone component found in Azotobacter vinelandii, is reduced by succinate, malate or NADH under anaerobic conditions or in the presence of cyanide (156, 157). Micrococcus denitrificans is the only bacterium so far investigated found to contain ubiquinone-(50) (this is the ubiquinone homologue normally found in mammalian cells): again the quinone is reduced on the addition of succinate or NADH under non-respiring conditions (158). Asano and Brodie (159) in their studies on Mycobacterium phlei showed that the endogenous naphthoquinone (vitamin $K_{2(45)}{}^{9H}$) was reduced by malate, but not by succinate. Escherichia coli W contains two natural quinones, vitamin K_2-(45) and ubiquinone-(40). It was concluded that the naphthoquinone was involved in NADH oxidation and the benzoquinone in succinate oxidation (122).

On the basis of inactivation of oxidative phosphorylation in M.phlei by long-wave ultraviolet light, and its subsequent reactivation by vitamin K, a role for vitamin K in the phosphorylation system in this organism has been proposed (160, 161). A phosphorylated derivative of the vitamin has been isolated and this has been proposed as a possible high-energy intermediate, which leads to the formation of ATP (162). Formation of the derivative was shown to be accompanied by a phosphate-dependant incorporation of tritium into a vitamin (163), but the location of tritium in the molecule was not consistent with the theoretical proposals (51-53) for the involvment of quinones in oxidative phosphorylation. There are obviously differences between this bacterial system and beef heart mitochondria, which do not

exhibit a phosphate-dependant incorporation of tritium into ubiquinone (7). Although the role of the phosphorylated derivative of vitamin K in M. phlei is unknown it may shed some light on the mechanism of energy coupling in this organism. Whatever the possible functions of quinones in oxidative phosphorylation in bacteria it is clear that either naphthoquinones or benzoquinones, or both, are implicated in electron transport in a wide variety of bacterial species.

Various techniques have been applied to the study of the role of quinones in bacterial reactions. Irradiation of bacterial particles with ultraviolet light has been used to destroy endogenous quinones, particularly vitamin K (122, 128, 160, 161). Activity can then be restored by adding back the quinone, but in some cases the effect is non-specific and a large variety of quinones can be used to effect restoration (164). Furthermore it has been shown that ultraviolet irradiation is not selective because another substance, in addition to the naphthoquinone, is destroyed by the procedure (159). Thus the results obtained are not very clear-cut because the technique is not specific and in some cases bypass reactions are caused which are not affected by electron transfer inhibitors (164).

Extraction of ubiquinone and subsequent determination of its redox state by a spectrophotometric method (165) has been used to study quinone function in bacteria. The results obtained show that ubiquinone is reduced by respiratory substrates under non-respiring conditions (107, 157, 158, 166). However, direct spectrophotometric evidence for the oxidation and reduction of quinones in intact bacterial particles has not been obtained.

More recently quinone deficient mutants have been employed to investigate the possible role of ubiquinone in electron transport in E. coli (167). A multiple aromatic mutant, E. coli 156, was found to lack ubiquinone when grown in the absence of 4-hydroxybenzoic acid; in the presence of 4-hydroxybenzoate normal levels of ubiquinone were synthesised. The lack of ubiquinone was correlated with low NADH and succinate oxidase activities, which could be restored by the addition of ubiquinone homologues. The development of these mutants presents a new approach to the study of quinone function in bacteria which is not available to mammalian preparations.

The studies described in this chapter are concerned with the possible involvment of quinones in bacterial membranes. The reactions

investigated were the oxidation of malate, NADH and succinate together with two energy-linked reactions, the ATP-dependant reduction of NAD^+ by succinate, the ATP-dependant reduction of $NADP^+$ by NADH, and the particle-bound ATPase reaction. E. coli K12 was chosen for the studies because this organism possesses energy-linked activities (see Chapters III and IV of this thesis) and because ubiquinone deficient mutants of E. coli were available.

METHODS.

Growth of Organisms.

E. coli K 12 was grown in a minimal medium with succinate as the sole energy-source by the method described in Chapter III. E. coli 156 was grown in the same medium supplemented with DL-phenylalanine, DL-tyrosine, DL-tryptophan (all 400μM), 4-aminobenzoic acid (1.0μM) and 4-hydroxybenzoic acid (0.1μM). This medium was used to grow an organism which contained ubiquinone. A quinone deficient organism was grown by omitting 4-hydroxybenzoic acid from the above medium (169). Other conditions were described for the wild type organism in Chapter III.

Irradiation of Bacterial Suspensions.

Cell suspensions were irradiated with ultraviolet light by the method of Brodie and Ballantine (160). A petri dish, standing on ice, was filled to a depth of 3mm with a suspension of bacterial particles (1.5-3.0mg protein/ml). The suspension was then irradiated at 360nm with an ultraviolet lamp at a distance of 10cm for 1hr. The enzyme activities of the particles were measured immediately after the irradiation procedure.

Pentane Extraction.

Pentane extraction was carried out by the method used for beef heart mitochondria and described in Chapter V. E. coli K 12 or E. coli 156 whole cells were suspended in 0.15M-KCl, to give a protein concentration of 20mg/ml, and lyophilised for 3hr. The lyophilised cells (200-300mg protein) were suspended in 50ml pentane and extracted for 30min. The extracted cells were collected by centrifugation and re-extracted with 20ml pentane for 10min. The twice extracted cells were re-centrifuged and dried under vacuum, to remove any residual pentane. Ubiquinone, vitamin K or the pentane extract were added to the extracted cells as described previously (Chapter V). Experiments were carried out using the six fractions so prepared viz: (i) normal cells (ii) lyophilised cells (iii) lyophilised-extracted cells (iv) lyophilised-extracted cells plus ubiquinone (v) lyophilised-extracted cells plus vitamin K (vi) lyophilised-extracted cells plus pentane extract. In experiments on energy-linked reactions, bacterial particles were prepared from the various fractions.

Estimation of Quinone Content.

The organisms were suspended in 0.15M-KCl, to give a protein concentration of 20mg/ml, and lyophilised for 3 hr. The dried cells were extracted three times with 40ml acetone. The combined extracts were evaporated and the residue was extracted twice with 5ml acetone, evaporated and applied in ethanol to activated plates of Kieselgel G. The plates were developed in benzene-chloroform (4:1 v/v). The quinone spots were eluted with ethanol and evaporated, to remove any residual benzene. The residue was extracted three times with ethanol and the quantity of each quinone was estimated using a Beckman DK 2 recording spectrophotometer. The absorbance at 248nm was used to estimate vitamin K and that at 275nm to estimate ubiquinone, after reduction of the quinone with borohydride (165).

Other Methods.

Bacterial particles were prepared and enzyme activities and protein concentrations were measured by the methods described in Chapters II, III and IV of this thesis.

Table 1

EFFECT OF ULTRAVIOLET IRRADIATION ON E. COLI ENZYME ACTIVITIES

ENZYME AND CONDITIONS	REACTION RATE (nmole/min/mg protein)	PROTEIN IN ASSAY (mg)
NADH oxidase		
non-irradiated	252	0.31
irradiated	16	0.31
irradiated + normal	280	0.62
Succinic oxidase		
non-irradiated	205	1.55
irradiated	0	1.55
irradiated + normal	232	3.1
ATP-dependant reduction of $NADP^+$ by NADH		
non-irradiated	18.9	3.1
irradiated	14.5	3.1
ATP-dependant reduction of NAD^+ by succinate		
non-irradiated	10.2	3.1
irradiated	9.8	3.1

Irradiated particles were prepared as described in the Methods section. NADH oxidation was measured at 340nm using a medium containing 670µmole sucrose, 27µmole tris-HCl (pH 7.5) and 0.32µmole NADH. Succinate oxidation was measured polarographically with a medium containing 750µmole sucrose, 150µmole tris-HCl (pH 7.5), 18µmole $MgCl_2$, and 10µmole succinate. ATP-dependant reduction of NAD^+ by succinate was measured at 340nm. The medium contained 375 µmole sucrose, 75 µmole tris-HCl (pH 8.0), 10µmole $MgCl_2$, 3µmole NAD^+, 5µmole Na_2S and 20µmole succinate. 6µmole ATP were added to start the reaction. $NADP^+$ reduction is described in detail in Table 4 of this Chapter.

RESULTS AND DISCUSSION.

Irradiation of bacterial particles with ultraviolet light at 360nm for one hour resulted in complete loss of both succinate and NADH oxidase activities (Table 1). The loss of oxidase activity was not due to the release of an inhibitor, caused by the irradiation process, since addition of non-irradiated particles to the assay medium, containing irradiated particles, resulted in normal activity in the added protein. The results were in agreement with those obtained previously (107, 122, 168). Addition of ubiquinone-(50) or vitamin K_2(45) had no effect on the irradiated activities; this was in agreement with previous findings (107, 168), but in contrast to the results of Kashket and Brodie (122) who showed that NADH oxidase was restored by vitamin K_2 and succinate oxidase by ubiquinone-(50). This discrepancy may be because E. coli W was used in Brodie's experiments and E. coli K 12 in the present experiments.

The effect of irradiation on bacterial energy-linked reactions is shown in Table 1. Both the energy-linked reduction of $NADP^+$ by NADH and the energy-linked reduction of NAD^+ by succinate were relatively stable to the same degree of irradiation that abolished the oxidase activities. The energy-linked reduction of $NADP^+$ by NADH was reduced by 24% and the energy-linked reduction of NAD^+ by succinate by only 2%. Thus if a light sensitive factor is involved in the energy-linked activities it does not appear to be susceptible to the treatment employed here. Certainly the light sensitive components necessary for normal oxidase activities are not participating in the energy-linked reactions. The energy-linked reduction of NAD^+ by succinate has not been studied previously, but other workers have shown that the energy-linked reduction of $NADP^+$ by NADH was stable to ultraviolet irradiation (128, 138).

The results obtained from the irradiation experiments are inconclusive and obviously do not give any information on the possible role of quinones in the enzyme systems studied. It is concluded that care should be taken in interpreting the results from such experiments because the effects observed may be non-specific, as shown by the failure of quinones to restore activity to the irradiated particles.

The pentane extraction technique (154), which was successful in

Table 2

EFFECT OF QUINONES ON OXIDASE ACTIVITIES IN PENTANE EXTRACTED E. COLI PARTICLES

CONDITIONS	SUCCINATE OXIDATION	PYRUVATE PLUS MALATE OXIDATION	PROTEIN IN ASSAY (mg)
Normal	184	188	1.9
Lyophilised	77	19	1.5
Lyophilised-extracted	11	6.6	1.8
Lyophilised-extracted plus pentane extract	47	8.2	2.2
Lyophilised-extracted plus ubiquinone-(30)	41	6.3	1.9
Lyophilised-extracted plus vitamin K2-(35)	17	13.2	1.4

Pentane extracted E. coli small particles were prepared as described in the Methods section. Oxidase activities were measured polarographically at 30°. Ubiquinone-(30) added was equivalent to 3.0nmole/mg protein, Vitamin K2-(35) added was equivalent to 15nmole/mg protein and the pentane extract added was equivalent to twice the amount of quinones originally present in the particles. The oxidase activities are given in nmole substrate oxidised /min/mg small particle protein.

Table 3

EFFECT OF INHIBITORS ON SUCCINATE OXIDATION IN E. COLI SMALL PARTICLES

INHIBITOR	% INHIBITION	
	NORMAL PARTICLES	LYOPHILISED-EXTRACTED PARTICLES PLUS UBIQUINONE-(30)
KCN 1.25mM	72	81
2.50mM	95	93
NHQNO 50μM	79	66
100μM	93	81

Pentane extracted E. coli small particles were prepared as described in the Methods section. Succinate oxidation was measured polarographically at 30°. Ubiquinone-(30) added was equivalent to 3.0nmole/mg bacterial protein. The normal rate of succinate oxidation was 180-200nmole succinate oxidised/min/mg protein. The rate for lyophilised-extracted particles plus ubiquinone was 40-50nmole succinate oxidised/min/mg protein. The inhibitors were added to the reaction chamber after the control rate of oxidation had been established.

Table 4

EFFECT OF QUINONES ON ENERGY-LINKED ACTIVITIES IN PENTANE EXTRACTED E. COLI PARTICLES

CONDITIONS	ENERGY-LINKED REDUCTION OF $NADP^+$ BY NADH	ENERGY-LINKED REDUCTION OF NAD^+ BY SUCCINATE	ATPase
Normal	15.0 (2.1)	10.2 (2.1)	48 (14.5)
Lyophilised	5.0 (2.0)	0 (2.0)	35 (12.3)
Lyophilised-extracted	0.5 (2.0)	0 (2.0)	21 (11.5)
Lyophilised-extracted plus pentane extract	3.0 (1.8)	0 (1.8)	24 (10.0)
Lyophilised-extracted plus ubiquinone-(30)	3.4 (1.8)	0 (1.8)	23 (10.0)

Pentane extracted particles were prepared as described in the Methods section. The energy-linked reduction of NAD^+ by succinate was measured as described in the legend to Fig. 1 and the particle ATPase reaction was measured as described in the Methods section of ChapterIII. Energy-linked $NADP^+$ reduction was measured at 340nm. Blank and experimental cuvettes of 1cm light path contained 375µmole sucrose, 75µmole tris-HCl,buffer (pH 8.0), 10µmole $MgCl_2$, 0.05µmole NAD^+, 200µg yeast alcohol dehydrogenase, 180 µmole ethanol, 5µmole Na_2S and bacterial particles. 1µmole $NADP^+$ was added to the experimental cuvette, and after 2-3min 6µmole ATP was added to give a final volume of 2.0ml. The reaction was measured at 30°. The rates of the enzyme reactions are given as nmole/min/mg protein. The figures in brackets relate to the amount of bacterial protein (mg) used in the assay.

demonstrating the involvment of quinones in mitochondrial reactions (see Chapter V), was used to study the bacterial reactions. Table 2 shows that lyophilisation caused a decrease in oxidase activities: succinate oxidase was decreased to 40% of the normal activity and pyruvate plus malate oxidation to 10% of the normal activity. In the case of succinate oxidation, extraction of the bacterial particles resulted in a decrease in activity to 14% of the control (lyophilised) level. This activity was restored to 50-60% of the control level by addition of the pentane extract or ubiquinone-(30) at the concentration originally present in the particles. Vitamin K did not effect any restoration, even at ten times the concentration originally present.

The results obtained for pyruvate plus malate oxidation were difficult to interpret because lyophilisation resulted in almost complete loss of this enzyme activity. The pentane extract produced a slight stimulation of activity and ubiquinone-(30) did not have a marked effect on the rate of the reaction. A ten-fold excess of vitamin K gave only a two-fold increase in activity. Isooctane extraction of E. coli has also been unsuccessful in attempts to restore malate or NADH oxidase activities by the addition of quinones (107, 168).

Despite the difficulty in demonstrating a quinone requirement for malate oxidation, it was possible to show thatsuccinate oxidation could be restored to the pentane extracted particles by the addition of ubiquinone-(30). This was the first demonstration of restoration of activity to a solvent extracted system in E. coli. The major significance of the results is that restoration was achieved at concentrations of ubiquinone equivalent to those normally present in the particles; thus the concentration before extraction was 1.5-2.0nmole/mg bacterial protein, and the concentration added in the experiments was 3.0nmole/mg protein. Furthermore the restored reaction was sensitive to the same concentrations of inhibitors that affected the normal reaction (Table 3). It was therefore unlikely that non-specific bypass reactions were set up by the quinone additions.

The effect of pentane extraction and quinone additions on E. coli energy-linked reactions is shown in Table 4. Lyophilisation caused a decrease in both the energy-linked reduction of $NADP^+$ by NADH and the ATPase, and a complete loss of the energy-linked reduction of NAD^+ by succinate.

Table 5

THE QUINONE CONTENT OF E. COLI WHOLE CELLS

ORGANISM	UBIQUINONE	VITAMIN K
E. coli K12	1.61	0.62
E. coli 156	0.07	0.02
E. coli 156 plus 4-OHB.	1.04	0.08

Bacteria were grown as described in the Methods section. E. coli 156 refers to E. coli 156 grown in the absence of 4-hydroxybenzoic acid. E. coli 156 plus 4-OHB refers to E. coli 156 grown in the presence of 4-hydroxybenzoic acid (0.1μM). The quinones were determined, after acetone extraction, by the technique described in the Methods section. Quinone concentrations are expressed as nmole ubiquinone or vitamin K present/mg bacterial protein.

Table 6

OXIDATION RATES IN E. COLI PARTICLES AND THE EFFECT OF UBIQUINONE-(10)

ORGANISM	NADH OXIDATION		MALATE OXIDATION		SUCCINATE OXIDATION	
	Control	+UQ	Control	+UQ	Control	+UQ
E. coli K12	230	252	194	205	180	185
E. coli 156	39	108	45	104	76	78
E. coli 156 plus 4-OHB	223	311	182	231	146	152

Bacteria were grown as described in the Methods section. E. coli 156 refers to E. coli 156 grown in the absence of 4-hydroxybenzoic acid. E. coli 156 plus 4-OHB refers to E. coli 156 grown in the presence of 4-hydroxybenzoic acid (0.1μM). Oxidase activities were measured using an oxygen electrode at 30°. 2.0mg bacterial protein were used in all the assays and the rates of the enzyme reactions are given as nmole substrate oxidised/min/mg bacterial protein. The columns marked "Control" refers to the reaction rates in the absence of added quinone. The columns marked "+UQ" refers to the reaction rates in the presence of added ubiquinone-(10) (0.19μmole/mg bacterial protein). Ubiquinone was added directly to the reaction chamber of the oxygen electrode, which contained the bacterial particles, 3min before the addition of substrate.

It was not possible to restore the latter reaction under any of the experimental conditions tested. This may have been because of destruction of the enzyme system by the lyophilisation procedure.

The energy-linked reduction of $NADP^+$ by NADH was decreased to 10% of the lyophilised control by pentane extraction, and this activity was restored to 60-70% of the control (lyophilised) level by the addition of ubiquinone-(30) or the pentane extract. This was a similar result to that obtained with the reaction from beef heart mitochondria (see Chapter V), and like the mammalian reaction, restoration was achieved at concentrations of ubiquinone equivalent to those normally present in the organism.

Whether the role of ubiquinone in the bacterial reaction is in electron transfer or in the energy transfer system is not decided by the present studies. However, it was shown that ubiquinone was not required for the ATPase activity located in the bacterial particles, because although pentane extraction resulted in some loss of ATPase, it was not restored by the addition of ubiquinone or the pentane extract. This was in contrast to the findings of Horio et al (154), who showed that quinones reactivated the ATPase system in pentane extracted Rhodospirillum rubrum chromatophores. It is concluded that in E. coli the ubiquinone requirement for the energy-linked reduction of $NADP^+$ by NADH is not due to quinone involvment in the bacterial ATPase.

Further information on the role of quinones in bacterial reactions was sought by examining the reactions in a mutant of E. coli. The mutant, E. coli 156, is a multiple aromatic mutant which requires tyrosine, phenylalanine, tryptophan, 4-aminobenzoic acid and 4-hydroxybenzoic acid for rapid aerobic growth (169). The mutant differs from E. coli K 12 in that it has only one major quinone (ubiquinone), whereas the wild-type has both ubiquinone, and vitamin K_2. When the mutant was grown in the absence of 4-hydroxybenzoate, the level of ubiquinone was found to be greatly reduced (Table 5). The organism therefore offered a method for studying the role of ubiquinone in the organism without resort to solvent extraction or ultraviolet irradiation.

The oxidase activities of E. coli K 12 and E. coli 156, the latter grown with and without 4-hydroxybenzoate, are shown in Table 6. Only very low rates of NADH, malate and succinate oxidation were found in the organisms

Table 7

THE EFFECT OF INHIBITORS ON OXIDATION RATES IN VARIOUS TYPES OF E. COLI

INHIBITOR	% INHIBITION					
	NADH OXIDATION			SUCCINATE OXIDATION		
	K12	156	156+4OHB	K12	156	156+4-OHB
Dicoumarol						
100μM	70	61	72	-	-	-
200μM	90	67	84	-	-	-
KCN						
1.25mM	70	51	65	72	76	71
2.50mM	91	68	85	95	82	90
Piericidin A						
100nmole/mg	82	87	82	80	77	75

Bacteria were grown as described in the Methods section. See Table 6 for conditions and control rates of oxidation. The inhibitors were added to the reaction chamber after the control rate of oxidation had been established.

Table 8

EFFECT OF UBIQUINONE-(10) ON DICOUMAROL INHIBITION OF NADH OXIDATION IN E. COLI 156

DICOUMAROL CONCENTRATION (μM)	% INHIBITION		
	E. COLI 156	E. COLI 156 + UQ	E. COLI 156 + 4OHB
50	48	59	57
100	61	75	72
150	65	82	79
200	67	88	84

Bacteria were grown as described in the Methods section. See Table 6 for conditions and control rates of oxidation. Dicoumarol was added to the reaction chamber after the control rate of respiration had been established. E. coli 156 + UQ refers to E. coli 156 incubated in the reaction chamber with ubiquinone-(10) (44nmole/mg bacterial protein) for 3min before the addition of substrate.

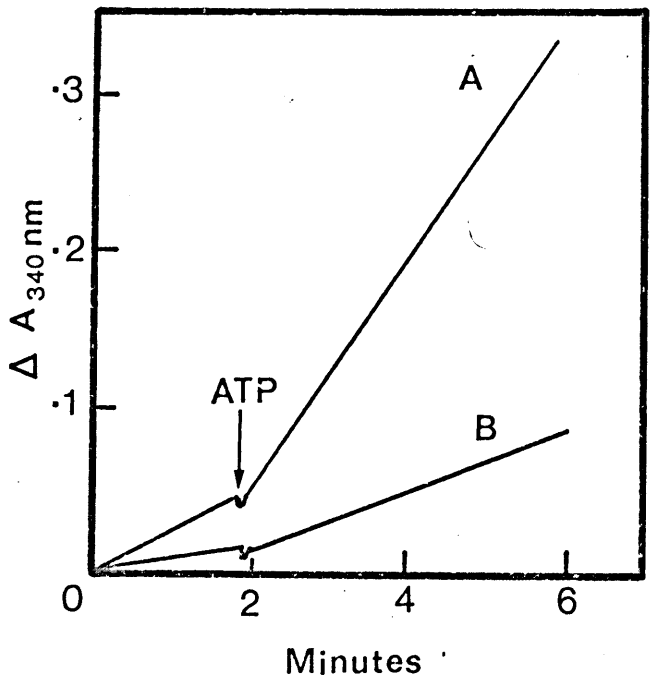
lacking ubiquinone, but in the mutants containing ubiquinone normal oxidation rates were observed. Thus the low rates of oxidation can be correlated with the lack of ubiquinone. Addition of ubiquinone-(10) to particles from the mutant, grown in the absence of 4-hydroxybenzoate, increased the rates of malate and NADH oxidation, but did not affect the rate of succinate oxidation (Table 6). The increase in oxidation rate was far greater than that observed with the mutant grown in the presence of 4-hydroxybenzoate or the wild-type organism. A non-specific bypass reaction was not involved because the reaction was sensitive to electron transport inhibitors (Table 7). The susceptibility of succinate oxidation to KCN and piericidin was identical in all the organisms; but although the sensitivity of NADH oxidation to piericidin was unaffected, the effects of KCN and dicoumarol were less marked in the quinone deficient mutant than in the other organisms.

It was found that addition of ubiquinone-(10) to the incubation medium containing ubiquinone deficient particles, resulted in an increase in dicoumarol sensitivity to the level of that found in the mutant which contained ubiquinone (Table 8). These results were similar to those recently described by Cox et al (170), who concluded that dicoumarol was not acting as a vitamin K antagonist, since their mutants did not contain any vitamin K. Similar conclusions can be drawn from the present experiments. Piericidin inhibition of NADH oxidase in the mutant containing ubiquinone was reversed by the addition of ubiquinone-(10) and ubiquinone-(30). For example in one experiment 169nmole piericidin/mg bacterial protein gave 88% inhibition of NADH oxidase, which was reduced to 21% inhibition by the addition of 225nmole ubiquinone-(10)/mg protein to the bacterial particles (see also ref 171).

The experimental evidence presented above clearly indicates that ubiquinone is involved in the oxidation of malate and NADH in E. coli 156. The lack of these oxidase activities in the ubiquinone deficient mutant, restoration of the activities by ubiquinone and the sensitivity of the reactions to inhibitors supports this conclusion. Similar conclusions have been reached by other workers (167, 170, 171), but they are at variance with the findings of Kashket and Brodie (122), who concluded that vitamin K was involved in NADH oxidation in E. coli W. The difference in results may

Table 9

ENERGY-LINKED REACTIONS IN E. COLI SMALL PARTICLES



ORGANISM	ENERGY-LINKED REDUCTION OF NAD+ BY SUCCINATE	ENERGY-LINKED REDUCTION OF NADP+ BY NADH
K12	10.4 (1.9)	15.0 (1.9)
156	0.48 (2.1)	2.9 (2.1)
156+4-OHB	0.4 (3.0)	12.5 (3.0)

Energy-linked activities were measured as described in the legends to Tables 1 and 4. The experiment was performed three times and the Table shows the mean of the three experiments. The tracing shows the time course of the ATP-dependant reduction of $NADP^+$ by NADH. A = E. coli 156 + 4-OHB; B = E. coli 156 - 4-OHB. Figures in brackets are amounts of protein (mg) present in the assay medium.

Table 10

SUMMARY OF RESULTS

EXPERIMENT	% REACTION			
	NADH OXIDATION	SUCCINATE OXIDATION	ENERGY-LINKED $NADP^+$ REDUCTION	ENERGY-LINKED NAD^+ REDUCTION
IRRADIATION				
Non-irradiated	100	100	100	100
irradiated	6	0	76	97
irradiated + ubiquinone	6	0	-	-
EXTRACTION				
lyophilised	100	100	100	0
lyophilised-extracted	33	14	10	0
lyophilised-extracted + ubiquinone	30	53	68	0
MUTANTS				
156 + 4-OHB	100	100	100	0
156 - 4-OHB	17	31	23	0
156 + UQ	52	33	-	-

Enzyme assays and conditions etc. are described in the legends to the previous Tables. The control activities i.e. non-irradiated, lyophilised and E. coli 156 grown in the presence of 4-hydroxybenzoic acid are taken as 100% and all other activities were compared to this control value. Those conditions which were not examined are indicated by a dash.

be due to the fact that different strains of E. coli could have different electron transfer pathways. Several studies have indicated substantial variations in quinone content of closely related strains of bacteria (157, 172).

The question of quinone participation in succinate oxidation is implied by the failure of the ubiquinone deficient mutant to oxidise succinate at normal rates. However lack of restoration of the reaction by added ubiquinone suggests that another component is also required. No other studies have been carried out on restoration of succinate oxidation in quinone deficient mutants of E. coli.

Preliminary studies showed that the level of the energy-linked reduction of $NADP^+$ by NADH was between two to four times higher in the ubiquinone containing organism than in the quinone deficient mutant (Table 9). This indicates that ubiquinone is required for normal activity, and supports the conclusions derived from the extraction experiments described above. The situation with the energy-linked reduction of NAD^+ by succinate was less satisfactory because significant levels of this reaction were not detected in either mutant.

The experimental findings described in this Chapter are summarized in Table 10. The irradiation and extraction experiments show that ubiquinone is involved in both the energy-linked reduction of $NADP^+$ by succinate and succinate oxidation in E. coli K 12. Although the oxidation of NAD-linked substrates was lost in both the above treatments, it was not possible, under the conditions employed, to restore activity. In the case of malate oxidation and pentane extraction this might have been due to the low levels of activity obtained after lyophilisation. Studies on E. coli 156 confirmed ubiquinone involvment in the energy-linked reduction of $NADP^+$ by NADH. The oxidation of respiratory substrates by the mutants was lower in the quinone deficient organism, but only NADH oxidation was restored by the addition of ubiquinone. The mutant appears to possess different electron transfer pathways to those found in the wild type, so the value of using mutants to investigate the pathway of wild type reactions must be questioned.

The most consistent results were obtained with the energy-linked reduction of $NADP^+$ by NADH. Both E. coli K 12 and E. coli 156 appear to

require ubiquinone for this reaction; this is also in agreement with the results obtained in beef heart mitochondria. Apart from quinone involvment, these reactions also exhibit certain other common features such as magnesium dependance, energy requirement, pH optima, susceptibility to uncouplers of oxidative phosphorylation, sensitivity to inhibitors (e.g. piericidin) and stoichiometry. Since the mechanism of the reaction appears to be similar in the two organisms, quinone deficient mutants could be useful in the study of this reaction.

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