STUDIES ON THE INTERACTION OF THE COMPONENTS OF THE MITOCHONDRIAL ELECTRON TRANSFER SYSTEM.

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FOREWORD.

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GLOSSARY OF ABBREVIATIONS.

ADP ........................ Adenosine diphosphate.
AHQO ........................ 4-heptyl-2-hydroxyquinoline-N-oxide.
AMP ........................ Adenosine monophosphate.
Ant. A ..................... Antimycin A.
ATP ........................ Adenosine triphosphate.
Cyt. ........................ Cytochrome.
DGIP ........................ 2,6-dichlorophenolindophenol.
DNP ........................ 2,4-dinitrophenol.
ETP ........................ Electron transport particle.
ETP_H ........................ Phosphorylating ETP.
FAD ........................ Flavin adenine dinucleotide.
FMN ........................ Flavin mononucleotide.
FP ........................ Flavoprotein.
ICIO ........................ 7-iodo-5-chloro-8-hydroxyquinoline.
M.B. ........................ Methylene blue.
NAD ........................ Nicotinamide adenine dinucleotide.
NADP ........................ Nicotinamide adenine dinucleotide phosphate.
NHI or FePr ................ Non-haem iron.
PMS ........................ Phenazine methosulphate.
Succ. ........................ Succinate.
TFAA ................... Trifluoroacetylacetone.
TTA ................... 2-thenoyl trifluoroacetone.
UQ or Q .............. Ubiquinone (Coenzyme Q).
CHAPTER I

GENERAL INTRODUCTION

The Electron Transfer System and the Mitochondrion.

The living organism obtains energy for its vital processes by the oxidation of metabolites derived from foodstuffs. In order to achieve a high efficiency of energy conservation, the oxidations occur through a series of hydrogen and electron carriers (the electron transfer system), and at various stages the energy released is used to esterify inorganic phosphate to adenosine diphosphate (ADP) producing adenosine triphosphate (ATP) which is the primary energy source of the organism. This esterification process is called oxidative phosphorylation.

The established components of the electron transfer system are the substrate dehydrogenases, nicotinamide nucleotides, flavoproteins and cytochromes. Other components which have been the subjects of more recent study are quinones, non-haem iron (iron not associated with a haem residue) and a copper protein, but their precise modes of action are not, at the present, understood.

Dehydrogenation of each oxidisable substrate is brought about by a specific dehydrogenase which activates the substrate and effects the transfer of electrons and hydrogens to nicotinamide adenine dinucleotide (NAD or DPN). However the specific dehydrogenase for succinate does not
effect electron and hydrogen transfer to NAD, but to a
flavin. In fact both succinate and reduced NAD (NADH₂)
are oxidised by specific metalloflavoproteins. Electrons
are carried from the flavoproteins by the cytochrome sequence
to the enzyme, cytochrome oxidase, which catalyses the
reduction of oxygen to water.

The hydrogen and electron carriers of electron transfer
and the enzymes of oxidative phosphorylation are located
in the mitochondria. The mitochondria are small (3-5μ in
length, 0.2-0.5μ in diameter) subcellular particles with
an outside double membrane which is continuous with deep
inward invaginations or cristae. It is on these solid
structures that the electron transfer components and oxida­
tive phosphorylation enzymes are located. The inner fluid
matrix contains the soluble enzymes of the tricarboxylic
acid cycle.

The relationship between structure and function in
mitochondria has been investigated by their disruption
into submitochondrial fragments. Normal procedures for
production of such fragments result in particles which have
lost their capacity for phosphorylation but have retained
their electron transfer activity. They also generally
lose NAD and soluble NAD linked dehydrogenases. Submito­
chondrial particles which still have the capacity for
phosphorylation can be prepared by sonication or digitonin treatment of mitochondria in the presence of Mg\(^{++}\). In the absence of Mg\(^{++}\), a soluble, heat labile factor, essential for phosphorylation, is released from the particles. (Linnane and Ziegler, 1958; Fullman, Beneisky and Racker, 1958; Devlin and Lehninger, 1958). According to Green (1959) these phosphorylating particles (\(\Delta F_H\)) retain the distinctive three layer appearance of the membrane whilst nonphosphorylating particles possess only a single protein layer.

Recently controversy has arisen regarding the more precise location and orientation of the electron transfer and oxidative phosphorylation systems within the solid phase of the mitochondrion. Two schools of thought have arisen following the demonstration by Fernandez-Noran (1962) by electron micrograph examination of negatively stained preparations, that the external envelopes of the mitochondrion and the cristae are built up of paired arrays of particles about 75-100 Å in diameter and attached to the phospholipid - protein membrane by stalks about 40 Å long and 35 Å in diameter. Blair et al. (1963) are of the opinion that these particles contain a unit electron transfer system containing a stoichiometric amount of each electron transfer component. Such a particle, termed an
elementary particle (Blair et al. 1965) should have a molecular weight of $1.4 \times 10^6$. By freezing and thawing beef sarcosomes, treatment with surface active agents, ammonium sulphate fractionation and differential centrifugation, they have isolated a floating fraction which they claim contains elementary particles. These particles can be purified, with a concomitant increase in specific activity, down to an apparent molecular weight of $2.1 \times 10^6$. They can in fact be purified without much change in composition to the suggested particle molecular weight of $1.4 \times 10^6$, but a considerable loss of activity is encountered. They claim (Green and Wharton, 1965), that the particle is indistinguishable from that formed by recombination of the four segments of the respiratory chain. (These will be discussed later.) More recently Green (1962) has suggested that the elementary particles are of two kinds, those on the inner membranes and cristae, containing the enzymes of the respiratory chain, and those on the outer membrane containing the primary dehydrogenases. Sjöstrand (1964) however, thinks that these particles are artefacts and considers that they are formed during the staining procedures.

Chance et al. (1965) have attacked Green's postulates on the grounds that mechanical disruption of the cristae
may cause rearrangements of the stoichiometry and sequence of electron transfer. They have produced evidence (Chance and Parsons, 1963) that when the inner membrane is stripped by ultrasonic vibration, the released sub-units do not contain cytochromes $a$, $a$ or $c$ and that cytochromes $b$ and $c_1$ are only partially located in these structures. This treatment produces very little change in the composition of the actual membrane. They are of the opinion that the oxysome (functional unit of electron transfer) is distributed along the cristal membrane and is intimately associated with its structure. They conclude that the sub-units participate in the oxysome function and possibly contain the primary dehydrogenases. Whatever the function of these sub-units it is generally agreed that the lipid-protein structure of the membrane is essential to the integrity of electron transfer and phosphorylation processes. 60% of the total mitochondrial protein and 75% of the total mitochondrial lipid is, according to Green and Fleischer (1963), the structural material of what they call the mesolayer - that part of the membrane on which the elementary particles or sub-units are attached. The great majority of the mitochondrial lipid is phospholipid (Spiro and McKibbon, 1956). The mitochondrial structural protein
and lipid appear to play very closely linked functions. Criddle et al. (1962) have analysed the amino acid content of the mitochondrial structural protein. Several significant features were observed. There was a preponderance of amino acids with positive charges over those with negative charges, a high proportion with a hydrocarbon side chain and in general a high proportion of charged amino acids. The hydrophobic proteins require lipid as a bridge to the aqueous phase and the phospholipid appears to be strongly linked to them (Fleischer et al., 1963). The presence of lipids as a molecularly dispersed phase facilitates rapid equilibration of solutes between the hydrophobic and aqueous phases of the mitochondrion (Fleischer and Brierly, 1961). Green and Lester (1959) have also suggested that the lipid core of the mitochondrion might act as a nonaqueous stabilising medium for the highly unstable intermediates of oxidative phosphorylation. Another important aspect of the lipid-protein function is its possible role as a permeability barrier to an unrestricted flow of solutes (Green and Fleischer, 1963). They consider that the protein-lipid network has both contractile elements, which may be expressed in terms of contraction and swelling, and translocase properties expressed as energy dependent ion transport. They consider that lipid plays an essential function in both these activities.
The Components of the Electron Transfer System.

Although, in the living organism, it is paramount that the energy released during electron transfer is efficiently trapped by the oxidative phosphorylation mechanism, for the purposes of clarity, electron transfer and phosphorylation mechanisms are often considered separately. In fact a large percentage of the knowledge of the energy conserving mechanisms has been gleaned from the investigation of non-phosphorylating heart muscle preparations of the type originally prepared by Keilin and Hartree (1938). Fig. I.1 shows the probable sequence of components in such a preparation.

\[
\text{FF} \xrightarrow{\text{succ}} UQ \xrightarrow{\text{cyt. b}} \text{cyt. } c_1 \xrightarrow{\text{cyt. c}} \text{cyt. } a \xrightarrow{\text{cyt. } a_3} O_2
\]

**Fig. I.1**

Probable sequence of components in a heart muscle preparation.

The following section deals with the occurrence and properties of the different components.

**Nicotinamide adenine dinucleotide:** Most of the substrates providing reducing power for electron transfer processes are linked to the respiratory chain by NAD. This compound has been shown to undergo enzymic oxidation-reduction in
Fig. 1.2 The oxidation and reduction of nicotinamide adenine dinucleotide.
the nicotinamide residue (Fig. 1.2). This was demonstrated by Pullmann et al. (1954). NAD was first discovered in yeast juice by Harden and Young (1905). Although yeast is probably the richest source of NAD, it has been found in nearly all living organisms examined. Malate, α-ketoglutarate, β-hydroxybutyrate and pyruvate are the most important metabolites whose oxidation is coupled to the electron transfer system by NAD.

Nicotinamide adenine dinucleotide phosphate (NADP) is implicated in the tricarboxylic acid cycle as an essential cofactor in the oxidation of isocitrate to oxaloacetate. Although it was thought that reduced NADP might be reoxidised by a specific mitochondrial flavoprotein (Navazio et al. 1957), it is more likely that a mitochondrial NAD/NADP transhydrogenase serves this function (Furvis, 1958; Stein et al., 1959; Ernster, 1961).

Stoichiometrically NAD is the most abundant electron carrier in the mitochondrion, often present in a forty times excess over the individual cytochromes (Chance, 1956). To explain this great excess, Chance (1958) has suggested that the NAD is compartmented within the mitochondrion in such a manner that a particular compartment is available only to a particular substrate.
Fig. I.3  The oxidation and reduction of flavin mononucleotide or flavin adenine dinucleotide.
Metalloflavoproteins: NADH₂ is oxidised by the NADH₂ dehydrogenase flavoprotein, succinate by the succinate dehydrogenase flavoprotein. These have a flavin prosthetic group which is flavin adenine dinucleotide (FAD) in the case of succinate dehydrogenase, (Singer et al. 1956; Wang et al. 1956) and flavin mononucleotide (FMN) in the case of NADH₂ dehydrogenase. (Cremona and Singer, 1964.) The part of the prosthetic group which undergoes oxidation-reduction is a riboflavin residue (Fig. I.3) and is identical in both flavins. Both of these flavins have a characteristic 450 mμ absorption band which disappear on reduction. This has been used to study the oxidation-reduction of these components.

The more widely investigated of these enzymes is succinate dehydrogenase. This has been prepared in a soluble, highly purified form (Singer et al. 1956; Wang et al. 1956) and has a molecular weight of 200,000. The preparation and properties of this enzyme will be discussed in greater detail in Chapter IV. There are about 0.20 mμ moles of succinate dehydrogenase per mg. of protein in beef heart mitochondria, 0.28 in ETP_H and 0.43 in Green's elementary particle (Green and Wharton, 1963).

The relative instability of the NADH₂ dehydrogenase to solubilisation had rendered this enzyme less amenable
to the methods of study employed for succinate dehydrogenase. However, many attempts have been made to solubilise the enzyme (Ziegler et al. 1959; Savage, 1957; Singer, 1961) and preparations have been obtained with a molecular weight of 80,000. It has not been possible to reconstitute the NADH oxidase activity of an alkali inactivated mitochondrial preparation with any of these. According to Green and Wharton (1965) this dehydrogenase is twice as abundant in beef heart mitochondria as is succinate dehydrogenase, but on breaking down to the elementary particle the ratio becomes 1:1. The figures given for mitochondria, FTRH and elementary particle are respectively, 0.46, 0.38 and 0.43 mmoles/mg protein. Comparison with the individual cytochromes suggests a roughly 1:1 ratio in the elementary particle.

Cytochromes: The cytochromes are haemoproteins discovered by Keilin (1925) which act as electron carriers by virtue of reversible oxidation-reduction of the iron atom of their haem prosthetic group. They are characterised by the possession of sharp, selective absorption bands in the visible region. Three groups of cytochromes, a, b, and c, are components of the electron transfer system of mammalian mitochondria. Each has a different haem prosthetic group.
### Table I.1 Molar ratios of cytochromes in mitochondrial preparations.

In each case cytochromes $a + a_3$ were taken as 1.0.
Cytochrome c₁ was first observed by Yakushiji and Okunuki, (1940), and Keilin and Hartree (1939) suggested that "cytochrome a" was in fact two spectroscopically distinguishable cytochromes, a and a₂, the latter being the final, highly autoxidisable member of the respiratory chain. Cytochrome a₂ is thus synonymous with cytochrome oxidase. Other workers (Okunuki et al. 1958; Green, 1959) still maintain that cytochrome a is, in fact, a single entity.

There is relatively good agreement between the results of Chance (1952) and Green and Wharton (1963) regarding the stoichiometry of the various cytochromes in mitochondria. (Table I.1).

Cytochrome c₁ has been isolated in a highly purified form by Green (1959). It is now quite firmly established as a component of the direct sequence of electron transfer (Chance and Williams, 1956). Cytochrome c was first isolated by Keilin (1925) and he suggested that it might play a role in electron transfer. It has now been crystallised (Bodo, 1955; Hagihara et al. 1956) although it has been claimed that the water soluble form is an isolation artefact and that the actual functional form in the mitochondrion is lipid soluble (Widmer and Crane, 1958). There is little doubt that this
cytochrome functions in the direct sequence of electron transfer (Slater, 1949; Chance and Williams, 1956). Cytochrome c can easily be removed from a heart muscle mince (Tsou, 1952) or from rat liver mitochondria (Schneider et al. 1948, Jacobs and Sanadi, 1960). It is not possible to remove it as easily from submitochondrial particles. A considerable amount of cytochrome c is lost, in fact, from mitochondria during the normal procedures of heart muscle preparation.

Cytochromes a and a_3 have been purified by Okunuki et al. (1958) and Smith and Stotz (1954). Keilin and Hartree (1939) found that the addition of CO or HCN to a heart muscle preparation causes a split in the $\alpha$ and $\gamma$ absorption bands of what was previously called cytochrome a. The name 'cytochrome a' was retained for the factor which did not react with cyanide, the name 'cytochrome a_3' was introduced for the highly autoxidisable factor which did. It is possible that the prosthetic groups of cytochromes a and a_3 are attached to the same protein. The presence of a high concentration of copper in the cytochrome oxidase region has prompted some workers (Eichel et al. 1950) to suggest that the metal component of cytochrome a_3 is copper, but they later demonstrated quite clearly that the haemochromogen was in fact iron (Persson et al. 1953). Both cytochromes a and
are probably on the direct sequence of electron transfer. (Chance and Williams, 1956).

Cytochrome b has been studied since 1925 (Keilin, 1925), but its mode of participation in the respiratory chain is still uncertain. Green (1959) and Feldman and Wainio (1959) claim to have purified cytochrome b. The kinetics of cytochrome b reactions are anomalous to say the least, and have resulted in much discussion. Slater (1950) placed cytochrome b in the succinate branch of the respiratory chain as it was reduced much more slowly by NADH₂ than by succinate. Chance (1952) relegated cytochrome b to a side pathway in the succinate oxidase system, but later on the basis of studies on the actively phosphorylating mitochondria, concluded that when phosphorylation was occurring cytochrome b was on the direct pathway. This viewpoint has been criticised by Slater (1952) who finds it difficult to accept a fundamental change in the pathway without loss of efficiency when phosphorylation is uncoupled. A third stand was taken by Green and Lester (1959) who consider that cytochrome b lies off the succinate or NADH₂ oxidising chains under all circumstances.

Other Components of the Electron Transfer System: Although the presence of metals other than iron in mitochondria, has been known for some time, it is only recently that investigation of their role in electron transfer has been intensified
Studies on the composition of the segments of the respiratory chain (vide infra) have shown that in the segment which catalyses ferrocytochrome $c$ oxidation, copper is abundant. The segments which catalyse ubiquinone reduction by succinate or NADH$_2$ and ferricytochrome $c$ reduction by ubiquinol, contain a large concentration of non-haem iron. Electron paramagnetic resonance (EPR) studies (see Chapter IV and Beinert and Lee, 1961) and the study of the inhibitory effects of metal chelating agents (Tappel, 1960; Ziegler, 1961; Whittaker and Redfearn, 1963; Redfearn et al. 1964) leave little room for doubt that at least part of the non-haem iron is implicated in electron transfer processes. This will be discussed at greater length in Chapter IV. It is more difficult to define the role of copper in cytochrome oxidase, as no chelating agent so far examined has been found to affect cytochrome oxidase activity. (Green, 1961a). However there is evidence from chemical studies (Griffiths and Wharton, 1961a; Takemori et al. 1960) and EPR studies (Sands and Beinert, 1959) that the copper undergoes oxidation and reduction in a manner similar to that of the established components of electron transfer. The highly sensitive spectrophotometric methods developed to study the other components of the respiratory chain cannot be used to study the reactions of these metalloproteins, and there is consequently no data available concerning the kinetics of these
reactions. It is impossible, at present, to claim emphatically that these components are on the direct sequence of electron transfer.

A further component which cannot as yet be assigned a specific function in electron transfer processes is the lipid, ubiquinone (Coenzyme Q). This compound undergoes enzymic reduction to ubiquinol by succinate or $\text{NADH}_2$, which in turn can be oxidised by the cytochrome system of the respiratory chain. This compound will be discussed in a later section of this chapter (Lipid Cofactors in Electron Transfer).

The Electron Transfer System as an Entity

Molecular Composition: Although the components of the electron transfer system have been considered separately, it is important to bear in mind that they do not act merely as a juxtaposed, but unorganised collection of enzymes and coenzymes. All the available evidence points to the chain being an entity with a constant molecular composition and with precise molecular proportions among the oxidation-reduction carriers. Some idea of the stoichiometry has been included in the review of the components. Green and Wharton (1963) have shown that although there might not be close agreement in component stoichiometry of different mitochondrial preparations, on progressive breakdown through the electron transfer particle to the elementary particle, the precise
molecular proportions begin to appear. The elementary particle of Blair et al. (1963) suggests a basic component ratio of $f_{p_S}:f_{p_N}:c_{yt.c_1}:1:1:1$.

Segmentation of the Respiratory Chain: It is interesting to note that investigation of the respiratory chain from a direction opposite that of the breakdown of the mitochondria into elementary particles has also provided evidence that the electron transfer chain is an entity. Green and Wharton (1963) have described work in their laboratory in which the electron transfer system has been fragmented into four enzymic complexes by bile salt treatment and ammonium sulphate fractionation of beef heart mitochondria. These four complexes are formed by splitting the chain at what Green calls the mobile components of the respiratory chain, ubiquinone and cytochrome $c$, which, he considers, shunt electrons between the four complexes of the intact system. The four complexes produced are:

Complex II : Succinate - CoQ. reductase.
               (Ziegler and Doeg, 1962).
Complex III: Reduced CoQ. - cytochrome $c$ reductase.
               (Hatefi et al. 1962a; Rieske and Zaugg, 1962).
Complex IV : Cytochrome $c$ oxidase. (Griffiths and Wharton, 1961a).
The complexes will recombine in a 1:1:1:1 molecular ratio to form a particle indistinguishable in composition and properties from the elementary particle. (Hatefi et al. 1962b; Fowler and Richardson, 1963). The calculated total molecular weight of these complexes is in good agreement with the experimental figure for the elementary particle (Yang, 1963). Fig. 1.4 shows the relationship between and composition of the complexes (after Green and Wharton, 1963).

Besides reconstituting the complete respiratory chain the complexes will also recombine to give sub-elementary particles with the expected enzymic activity.

i.e. $I + III + IV \rightarrow NADH_2$ oxidase.

$II + III + IV \rightarrow$ Succinate oxidase

$I + III \rightarrow NADH_2 - \text{cytochrome } c$ reductase.

$II + III \rightarrow$ Succinate - cytochrome $c$ reductase.

$I + II + III \rightarrow$ Succinate or $NADH_2 - \text{cytochrome } c$ reductase.

$III + IV \rightarrow$ Ubiquinol oxidase.
On the other hand, Complexes I and IV or II and IV do not recombine and produce no new enzymic activity.

**Mechanisms of Electron Transfer.**

Although many theories have been propounded for the mechanism of electron transfer from one component to the next, they are all based on indirect evidence. The most plausible suggestion so far is the thermal collision theory. This postulates that electron transfer occurs by rotational or vibrational motion of the electron carriers about their points of attachment to the structure in which they are embedded. This view is supported by the finding (Chance, 1959) that reasonable agreement is obtainable between the measured reaction velocity in the 'soluble system' and that calculated for a closely packed molecular array in the particles. Furthermore, Chance and Spencer (1959) have shown that at liquid nitrogen temperatures the oxidation-reduction carriers maintain the same steady state oxidation-reduction level as in the system at normal temperatures, as if the electron transfer had been immobilised by a great reduction in the number of collisions. They also demonstrated that a similar effect, resulting in inhibition of electron transfer, could be brought about by increasing the viscosity of the supporting medium by adding glycerol. Once again steady state oxidation-reduction levels of the
carriers were maintained. A similar transfer mechanism has been postulated by Hagins and Jennings (1959) who proposed that energy transfer in the visual receptor is brought about by Brownian rotation.

A second possibility is that transfer occurs through conduction bands in the protein portion of the cytochromes. (Cardew and Eley, 1959). The main objections to this are that immobilization of the carriers by low temperatures or increased viscosity should not, if this theory is correct, impair the ability of the system to transfer electrons. Secondly it is difficult to reconcile this mechanism with the profound effect of the haem residue on the electron transfer properties of its protein. Chance (1959a) suggested that if this mechanism is implicated in electron transfer, it is certainly not the only one.

A third possibility is that electron and energy transfer occurs by a process of resonance energy transfer. The fact that splitting of the myoglobin-carbon monoxide molecule has a quantum requirement of unity is evidence that resonance energy transfer can occur from protein to haem of haemoproteins (Bücher and Caspers, 1946, 1947). Also the haem of the native protein will quench the fluorescence of the free protein. (Weber and Teale, 1959.) This has lead Keilin and Hartree (1953) and Dixon and Webb, (1958) to suggest that
electron and energy transfer in the cytochrome region can occur by a similar mechanism and that the photochemical relief of carbon monoxide poisoning of the respiratory chain is by a mechanism of resonance energy transfer from cytochromes other than the oxidase to the haem-CO compound. Chance (1959a) suggests that this mechanism is unlikely since the haem of the protein absorbing the light would quench the fluorescence and prevent transfer to the next component. The thermal collision theory is the only one which comes near to satisfying all the observed data.

Investigation of the Sequence of the Electron Transfer Components.

Oxidation Reduction Potentials: The order of magnitudes of the oxidation-reduction potentials of the components of the respiratory chain provides the only logical sequence for the order of reduction of each of the components. Oxidation-reduction potential is a measure of the potentiality of an oxidation-reduction system to accept electrons from, and become reduced by another oxidation-reduction system. For the purpose of defining a standard redox potential for a system, the potential of the system is measured against a normal hydrogen electron at reference conditions. A system with a negative potential will be oxidised by a standard hydrogen electrode.
On a purely logical basis if all the components were to act in a sequence of oxidation-reduction reactions, for the total reaction to be maximally efficient the electron transfer should occur from component to component in an increasing order of potentials. Fig. 1.5 shows the components of the electron transfer system set down in the order suggested by their redox potentials. This order agrees in many respects with that which has been deduced experimentally.

\[
\begin{align*}
&\text{NAD} \rightarrow \text{FP} \rightarrow \text{cyt. b} \rightarrow \text{UQ} \rightarrow \text{cyt c}_1 \rightarrow \text{cyt c} \rightarrow \text{cyt a} \\
&\quad -0.32 \quad -1.12 \quad -0.04 \quad 0.098 \quad 0.24 \quad 0.26 \quad 0.29 \\
&\quad \delta \rightarrow 0_2 \\
&\quad 0.82
\end{align*}
\]

**Fig. 1.5** Redox potentials of electron transfer components.

**Fragmentation of the Respiratory Chain:** The work carried out in Green's department in which the electron transfer chain has been segmented into what are considered to be four physiologically existing complexes which can recombine to give a complete respiratory chain, has provided important evidence as to the sequence of components. It has been suggested that non-haem iron might be active as an electron carrier in Complexes I, II and III. It also suggests that
different forms of cytochrome \( b \) might act at points in complexes II and III and defines the participation of cytochromes \( b \) and \( c_1 \) before cytochrome \( a \) in the oxidation-reduction sequence. Although this work has in some ways been extremely valuable, the possibility exists that purification and fragmentation of the particles disturbs the order of electron transfer through the components. Even at the level of the ET particle or heart muscle particle changes appear to have occurred from intact mitochondria in the kinetics of cytochrome \( b \), (Chance, 1952a; Chance and Williams, 1956) and ubiquinone (Chance, 1961) oxidation and reduction. It is quite conceivable and even probable that further breakdown of the particles will result in further disruption of the component sequence. Three of the components of the electron transfer system (NAD, ubiquinone, and cytochrome \( c \)) can easily be removed from mitochondria without causing any obvious alteration to them. It is then possible to study the oxidation-reduction reactions of these components when they are added to a heart muscle preparation. NADH\(_2\) will reduce added ubiquinone and cytochrome \( c \), ubiquinol will reduce cytochrome \( c \). This suggests that the order of these components is:

\[
\text{NAD} \rightarrow \text{UQ} \rightarrow \text{cyt.} c \rightarrow O_2
\]

The flavoprotein dehydrogenases are components of the NADH\(_2\)
and succinate-ubiquinone reductase segments of the chain and therefore probably act before ubiquinone (Hatefi et al. 1962, 1962a).

**Spectrophotometry:** The other components of the electron transfer chain - the cytochromes and metalloproteins have required the introduction of more refined techniques to determine their sequence of action in respiratory processes. In a brilliant series of spectrophotometric determinations of the rate and order of onset of oxidation of each component in the endogenous state, on exposure to oxygen, Chance and Williams (1955c) established the sequence of action of the cytochromes. The order of onset of oxidation was: cytochrome a₃, cytochrome a, cytochrome c, cytochrome b followed by the flavoproteins. It is probable that their sequence of action in the oxidation of metabolites is the opposite of this. On the basis of the results of these determinations Chance concluded that in phosphorylating mitochondria there was no reason for not including cytochrome b in the direct sequence. Apart from the suggested positions of non-haem iron the picture so far presented is similar to that detailed in Fig. I.1. There is no evidence as yet as to where in the cytochrome oxidase complex the copper might be acting. Non-haem iron, (Green and Wharton, 1963) appears to be implicated in each of the other complexes - probably after the flavin
moieties of Complexes I and II (Ziegler, 1961) and between cytochromes b and c\textsubscript{1} in Complex III (Rieske et al., 1964). The location of non-haem iron will be discussed in detail in a later chapter.

**Artificial Electron Acceptors and Electron Transfer Inhibitors**

The use of inhibitors which react with components of the respiratory chain has been of some value in determining the location of these components in electron transfer. The classic example is the discovery and location in the electron transfer system of cytochrome a\textsubscript{3} by its reaction with cyanide or carbon monoxide (Keilin and Hartree, 1939).

A more refined technique for the use of inhibitors was devised by Chance and Williams (1955, 1955a). This is the production of a crossover point on the addition of an inhibitor to mitochondria whose components are being maintained in the reduced state by the presence of substrate. During the passage of electrons from substrate to oxygen components attain a steady state oxidation-reduction level, when the rate of oxidation of a component equals its rate of reduction. (Chance, 1952, 1952a). Addition of an inhibitor will result in the oxidation of components acting on the oxygen side of its site of action and reduction of those on the substrate side. The redox state of the endogenous components was determined by direct spectrophotometry. Fig. I.6 shows the result of Antimycin A inhibition.
Artificial electron acceptors may be used to split up the electron transfer chain without the concomitant structural disruption occurring on physical segmentation of the system. The main disadvantages of this technique are doubt as to the site of action of the added electron acceptor, (Singer and Kearney, 1957) and the possibility that it may in fact have more than one site of action (Singer, 1961; Whittaker and Redfearn, 1964). The possibility of the involvement of a particular component in a particular segment may be studied by the action of an inhibitor, which reacts with the component, on the enzyme activity. The application of such a method to the study of non-haem iron participation in electron transfer is discussed in Chapter IV.

Oxidative Phosphorylation.

The energy released during electron transfer is used to phosphorylate ADP - this process is called oxidative phosphorylation. It is generally considered that the transfer of two electrons from NADH to oxygen is accompanied by the formation of three molecules of ATP, and from succinate to oxygen, two molecules of ATP. The sites of phosphoryla-
tion have been localised to some extent by the use of added cytochrome c as an artificial electron acceptor and donor. Borgström et al. (1955) have shown that two phosphorylations occur between NAD and cytochrome c, and Nielsen and Lehninger (1955) have shown one between cytochrome c and oxygen. Chance and Williams (1956) have demonstrated the precise position of the first two phosphorylation sites by the use of the crossover technique. In this case the difference spectrum is determined of mitochondria oxidising a substrate before and after ADP addition. The absence of ADP acts as a controlling mechanism at each phosphorylation site and consequently produces a similar effect to the addition of an inhibitor. Three crossover points were detected. The first two, which were definitely located, are between NADH₂ and its flavoprotein and between cytochromes b and c₁. The third is probably between cytochromes c and a. Thus it is considered that in the NADH₂ oxidase system, transfer of two electrons and reduction of one atom of oxygen results in the esterification of three phosphates. This is usually written in a shorthand form as a P:2e or P:O ratio of 3.

Recently, Smith and Hansen (1964) have suggested that P:O ratios of twice this value are obtainable, either because a single electron transfer could produce three phosphorylations and two electrons are necessary for the reduction of
one oxygen atom, or possibly because of the existence of more than three phosphorylation sites.

As has previously been observed the energy released is trapped in the ATP molecule. ATP is one of a class of compounds called 'energy rich' or 'high energy' phosphates. This group of phosphates is characterised by the unusually large value of the free energy change associated with the hydrolysis of the high energy phosphate of its members, about 7 kcals./mole compared with 2-4 kcals./mole for normal 'energy poor' phosphates. The term 'energy rich' is also often applied to the specific bond which will break to release the 7 kcals./mole of chemical energy. Such a bond is usually denoted by the symbol '−'. In this way the structure of ATP may be represented by the formula: Ad − O − P − P − P. The mechanism of formation of this compound from ADP and inorganic phosphate is one of the major unsolved problems of biochemistry. A working hypothesis for oxidative phosphorylation, which is general to all acceptable schemes so far propounded is that a carrier combines with some molecule to form a high energy intermediate, which in the presence of ADP and inorganic phosphate forms ATP. Chance and Williams (1955a, 1956) on the basis of work done on the difference spectrum of a mitochondrial preparation with ADP present (active state), when a high
oxidation rate exists, and a mitochondrial preparation with ADP absent (resting state) with a low respiratory rate, have suggested that a natural inhibitor, I, inhibits electron transfer in the resting state, and have suggested the following scheme:

\[
\begin{align*}
C_{\text{ox}} + I & \rightleftharpoons C_{\text{ox}} I \\
C_{\text{ox}} I + 2e & \rightleftharpoons \text{Cred} \sim I \\
\text{Cred} \sim I + x & \rightleftharpoons \text{Cred} + x \sim I. \\
x \sim I + \text{Pi} & \rightleftharpoons x \sim P + I \\
x \sim P + \text{ADP} & \rightleftharpoons x + \text{ATP}
\end{align*}
\]

(C is an electron carrier, I is a naturally occurring inhibitor. X is a compound forming a high energy intermediate.)

In the absence of ADP or Pi, \( C_{\text{ox}}I \) builds up and electron transfer slows down and stops. This is the phenomenon known as respiratory control.

Lehninger et al. (1954, 1958) has approached the problem from a rather different angle and has suggested the following scheme:

\[
\begin{align*}
i) \quad & C + X \xrightarrow{\text{electron transfer}} C \sim X \\
ii) \quad & C \sim X + \text{Pi} \rightleftharpoons C + X \sim P. \\
iii) & X \sim P + \text{ADP} \rightleftharpoons X + \text{ATP}
\end{align*}
\]

He approached the problem from what he describes as the 'back-door' by investigating the terminal reactions leading to the formation of ATP through a transphosphorylation to ADP. He examined the three characteristic transformations of ATP. These are:
1) An ATPase reaction, activated by dinitrophenol. (Boyer et al. 1956; Cooper and Lehninger, 1957). This is a sum of reactions iii) and ii) followed by hydrolytic breakdown of $\text{C} \sim \text{X}$.

2) An ATP - $P_i$ exchange reaction, inhibited by dinitrophenol and azide, (Cohn and Drysdale, 1955; Chan et al. 1960). This is the sum of reactions ii) and iii).

3) An ATP - ADP exchange reaction, inhibited by dinitrophenol but not azide, (Wadkins and Lehninger, 1958; Bronk and Kielle 1958). This is the same as reaction iii).

He is also of the opinion that the primary high energy intermediate is formed with the electron carrier in the oxidised state.

Slater, (1953, 1958) considers that a mechanism similar to that proposed by Chance is operating, but that the first high energy intermediate is formed with the reduced form of the carrier.

More recently the emphasis of phosphorylation studies has turned towards the isolation and characterisation of intermediates of oxidative phosphorylation. Peter et al. (1963, 1963a) have shown that one of the reactions of oxidative phosphorylation results in the formation of protein bound phosphohistidine. This has been demonstrated in intact rat liver mitochondria, and in mitochondrial
extracts. They suggest that a single activated imidazole structure is formed at each phosphorylation site which serves as a common intermediate in various energy linked reactions. The active imidazole structure could be a logical participant in a soluble preparation, necessary for coupling NAD reduction to ATP cleavage. Addition compounds with NAD (Pinchot and Horwanski, 1962) ubiquinone or cytochrome c (Smith and Hansen, 1962; Webster, 1962), might serve as precursors to the active imidazole. All these workers are of the opinion that the primary high energy intermediate at the first phosphorylation site of NADH₂ oxidase is formed with the nicotinamide nucleotide. Ernster (1961a), based on his studies on energy dependent electron transfer reversal, has proposed that the primary high energy intermediate is formed with the flavoprotein.

Another technique which has been valuable in the investigation of phosphorylation, has been the reconstitution of phosphorylation in a submitochondrial system which has been uncoupled by the preparative or other procedures. Smith and Hansen (1962) have described three discrete coupling proteins, released during preparation of submitochondrial beef heart particles each of which is a coupling factor for one of the energy trapping sites of the electron transfer system. Racker (1962, 1964) has reconstituted a
phosphorylating system by adding factors essential for the partial reactions of phosphorylation. One such factor \( F_1 \), released from urea treated submitochondrial particles, is essential for the ATP - \( F_1 \) exchange reaction in these particles. A further factor \( (F_2) \), essential for the same reaction is released from these particles on treatment with trypsin. This factor has been found to contain pyrophosphatase activity.

It does not appear that an absolutely standard mechanism for phosphorylation exists from organism to organism. The work of Brodie and his department has shown that reduced derivatives of vitamin \( K_1 \) are implicated in phosphorylation in *Mycobacterium phlei*. Vitamin \( K_1 \) (a naphthoquinone) apart from becoming reduced during electron transfer to a naphthohydroquinone, appears to cyclise to a naphthochromenol which is reduced to a naphthotocopherol. It is probable that this latter compound forms the primary phosphorylated intermediate.

**Lipid Cofactors in Electron Transfer.**

**Ubiquinone:** The presence of lipid in high concentration in mitochondria has been discussed already. The close association observed between mitochondrial lipid and structural protein led to the idea that the lipid was playing a purely structural role in the mitochondrion (Nygaard, 1953; Ball and Cooper, 1949; Edwards and Ball, 1954). Nason and
Lehnian (1955, 1956) introduced a technique which has since proved extremely valuable in the study of lipid cofactors of mitochondria. The mitochondrial preparation was shaken with an organic solvent, such as iso-octane and the solvent and aqueous layers separated by centrifugation. The enzyme activities of the solvent treated mitochondria were investigated. This technique was adopted by Green's group at Madison, who were able to prepare mitochondria on a very large scale. The extracts were investigated spectrophotometrically in the hope of identifying any lipid soluble electron transfer components. Crane et al. (1957) found that the iso-octane extracts contained a compound with an ultra-violet absorption maximum at 275\(\mu\) in ethanol. This compound was found to be a quinone which undergoes enzymic oxidation-reduction in a similar manner to the known components of the respiratory chain.

At this stage it became obvious that the compound they were dealing with was the same as a compound which had been investigated at Liverpool by Morton and his colleagues since 1953. This compound had been named 'SA', but on discovery of its quinonoid nature (Wilson, 1956) and its thus far universal distribution it was renamed 'ubiquinone' (Morton et al. 1957). The Madison group named the compound 'Coenzyme Q' because it appeared to play a coenzymic function in electron transfer.
The structure of the compound (Fig. 1.7) was found to be a fully substituted benzoquinone with a polyisoprenoid side chain on position six of the quinone nucleus. (Wolf et al. 1958; Morton et al. 1958). The number of isoprenoid units constituting the side chain varied with source of the quinone, from 6-10. The trivial name recommended for the quinone is 'ubiquinone' – abbreviated to $Q_6$ or $Q_{10}$, the subscript indicating the number of isoprenoid units in the side chain. $Q_{10}$ was the homologue isolated from mammalian tissues and spermatophytic plants. Ubiquinones with the shorter side chains ($Q_6$-$Q_9$) have been found in lower plants or animals and micro-organisms. Ramasarma (1961) presented a comprehensive review of the distribution of ubiquinone in various species and tissues. The most significant fact is the concentration of ubiquinone in tissues with a high respiratory rate. Crane (1962) was unable to find ubiquinone in some animal species, (lobster, Portuguese man-of-war, oyster and snail) although this may be because of interference with other lipids in the spectrophotometric assay.
The spectrum of ubiquinone was measured in ethanol. The concentration of ubiquinone was 46 μg./ml. A small crystal of sodium borohydride was added to the solution to reduce the ubiquinone to ubiquinol.
Ubiquinone has an absorption peak at 275 nm in ethanol (272 nm in cyclohexane) which disappears on reduction (Fig. I.3). This is used in the assay or determination of oxidation-reduction level of the quinone (Redfearn and Humphrey, 1960).

The discovery that ubiquinone is largely located in the mitochondria (Crane et al., 1957; Hemming, 1958) and the demonstration of enzymic oxidation–reduction of the quinone directed studies on this newly discovered lipid towards the investigation of a possible role for it, in electron transfer. Initial experiments supported this idea. Crane et al. (1957) demonstrated that ubiquinone was reduced by succinate or NADH₂ and that in the presence of air all the quinone becomes oxidised. They suggested that the site of action of the quinone was after the Antimycin A sensitive region as this inhibitor appeared to block ubiquinone reduction and have no effect on ubiquinol oxidation. These results were largely confirmed by Humphrey et al. (1958) although these workers showed quite conclusively that, in contrast to Crane's suggestion, Antimycin A completely inhibited ubiquinol oxidation but had no effect on the reduction of ubiquinone by either succinate or NADH₂. (Humphrey and Redfearn, 1959; Redfearn, 1959). This was eventually conceded by the Madison group (Green et al., 1959). Effects
of other inhibitors suggested that ubiquinone acted after the flavoproteins of both branches of the respiratory chain. Oxaloacetate and malonate inhibited reduction by succinate and amytal inhibited reduction by \( \text{NADH}_2 \). The oxidation of ubiquinol was inhibited by 2,3-dimercaptopropan-1-ol (BAL) and cyanide. A position between the flavoproteins and the Antimycin A sensitive region is consistent with the oxidation-reduction potential of the quinone.

To prove conclusively that ubiquinone was acting in the direct sequence of electron transfer it was also essential to show that its removal from the mitochondria inactivates electron transfer and that replacement reactivates it, and also that the rate of reduction of the quinone is consistent with the rate of oxidation of the substrate. Crane et al. (1957) showed that extraction of the mitochondria with heptane or iso-octane caused a loss of succinate oxidase activity. This could be regained by adding ubiquinone to the extracted preparation. Redfearn et al. (1960) demonstrated that this was almost certainly caused by inactivation of the preparation with residual small traces of iso-octane, and that the reactivation was a non-specific reversal of this inactivation, as observed by Deul et al. (1958) by \( \alpha \)-tocopherol or vitamin \( K_1 \).
Lester and Fleischer (1959) used a polar solvent—acetone—to extract the mitochondria, and found that this removes ubiquinone rapidly and almost completely. They showed unequivocally that this treatment caused a loss in succinate oxidase activity which could be specifically replaced by added ubiquinone. Redfearn (1961a) has shown that removal of 75% of the total ubiquinone causes a loss of only 10% of succinate oxidase activity. This and the great excess of ubiquinone (in mitochondria about 20x on an electron carrying basis) over the other cytochromes, raises the possibility of an ancillary function for ubiquinone. Recent work suggests that the reactivation might not be as specific to ubiquinones as first imagined. Ozawa et al. (1964) have demonstrated that reactivation can be brought about by a number of naturally occurring quinones, some of which might be expected to be inhibitory to succinate oxidase activity. (Redfearn and Whittaker, 1962). It is notable however that the reactivation they produce is to a much lower specific activity than that obtained by other workers.

The last, and possibly most important criterion for ubiquinone to be on the direct sequence of electron transfer, is that the kinetics of ubiquinone reduction should agree with those of succinate or NADH2 oxidation. Redfearn and Pumphrey (1960) assayed the rates of enzymic
The three possible schemes suggested by Redfearn (1961, 1961a) for the involvement of ubiquinone in the respiratory chain.
interconversions of ubiquinone by spectrophotometric determination of the ubiquinone extracted from mitochondrial aliquots denatured with \(-20^\circ\text{C}\) methanol containing pyrogallol at varying times after the onset of reduction or oxidation. They showed that the rate of reduction of the quinone by either succinate or NADH\(_2\) was considerably slower than the rate of oxidation of either of these substrates. What is more the rate of ubiquinol oxidation was also only a fraction of that of succinate oxidase activity. This suggests that electron transfer through ubiquinone does not account for the total electron flux through the system. Although Green et al. (1959) using a rapid flow technique concluded that the rates of ubiquinone reduction and succinate oxidation were equal, the results of Redfearn and Pumphrey (1960) were largely confirmed by Chance and Redfearn (1961) who measured the oxidation-reduction reactions of ubiquinone by direct spectrophotometry. This has led Redfearn (1961, 1961a) to suggest three possible schemes for the involvement of ubiquinone in the electron transfer system (Fig. 1.9). In the first scheme ubiquinone is in the direct sequence and links the flavoproteins with the cytochrome system. This scheme however does not agree with the kinetic evidence. The second possibility has ubiquinone as an 'interflavoprotein' link in a 'blind-alley' system.
It is difficult to reconcile this scheme with the extraction reactivation experiments, as complete inactivation by removal of the quinone should never be possible, although it would be consistent with ubiquinone as a source of reducing equivalents for some other system (Cornforth, 1959). The third scheme, which is consistent with most of the experimental data is that ubiquinone is acting on a branch pathway in parallel with a direct link between the flavoproteins and the Antimycin A sensitive site. An interesting possibility, discussed by Redfearn (1961a) is that ubiquinone and cytochrome b are alternative branch pathways functional in the resting and active states respectively. This was based on observations of the rates of reduction of each component in non-phosphorylating and intact phosphorylating systems. A similar scheme has been suggested by Green (1961).

Another technique useful in the study of respiratory chain components and particularly adaptable to the study of ubiquinone function, is the investigation of the steady state oxidation-reduction levels of the components in phosphorylating mitochondria. The concept of such a state was introduced by Chance (1952, 1952a). It is based on the fact that in a particular metabolic condition an electron transfer component will strike a balance between its
reduced and oxidised forms, when the rate of reduction by substrate equals the rate of oxidation by oxygen through the respiratory chain. A change in the metabolic state of the mitochondria might result in a significant change in steady state. Hatefi (1959) was the first person to investigate ubiquinone function using this technique. He used a method involving heat denaturation of the mitochondria and extraction of the quinone in its endogenous oxidation-reduction state. On the basis of the results, he suggested that ubiquinone was involved in the phosphorylating electron transfer system and that it might possibly form a high energy intermediate with the naturally occurring inhibitor 'I', in one of the phosphorylation steps.

Redfearn and Pumphrey (1960a) refined and improved the technique used by Hatefi and found that, contrary to his results, omission of phosphate from the reaction mixture, unlike ADF, did not result in increased oxidation of the quinone. The results of Redfearn and Pumphrey were generally in line with changes occurring in other respiratory components, and were supported by direct spectrophotometric evidence (Chance, 1961; Chance and Hagihara, 1961). More recently some doubt has been cast on the validity of the results obtained using the chemical determination devised by Pumphrey and Redfearn (1960), by Szarkowska and
Klingenberg (1962, 1963) using simultaneous spectrophotometric and chemical assays. The results, their validity and criticisms will be discussed in more detail in a later chapter.

Although these results conclusively implicate ubiquinone in electron transfer processes, it is not possible at present to define the role of ubiquinone with any degree of certainty.

Naturally Occurring Compounds Related to Ubiquinone: Morton's group in Liverpool discovered the cyclic isomeride of ubiquinone, which they called ubichromenol (Fig. I.10) (Laidman et al. 1959). There has been much controversy as to whether ubichromenol may or may not be an artifact of isolation. Links (1960) suggested ubichromenol was not a natural product but was formed by the action of alumina on ubiquinone. Morton's group (Hemming et al. 1961) repeated Links' work and claimed that only 14-15% of the ubichromenol
isolated was not of natural origin. There is evidence suggesting that most of the ubichromenol from commercially dried *Torula* yeast is formed from ubiquinone during factory drying and storage (Stevenson *et al*. 1962). There is little evidence for a biological role for this compound although some workers (Russell and Brodie, 1960; Clark and Todd, 1961) have suggested that chromanols are precursors of high energy phosphorylation intermediates.

A quinone, closely related to ubiquinone, was isolated by Kofler (1946) from plant tissues and named Kofler's quinone. Its structure (Fig. I.11) was elucidated by Kofler and Isler's group in Basle (Kofler *et al*. 1959, 1959a). This compound is more usually called plastoquinone because Crane (1957) demonstrated that it could only be found in photosynthesising tissues and was, in fact, concentrated in the chloroplasts. Bishop (1958) found that when lyophilised chloroplasts were extracted with organic solvents they lost their Hill reaction activity, and this could be
restored by adding plastoquinone. Crane et al. (1960) showed that illumination of isolated chloroplasts caused reduction of the endogenous quinone to plastoquinol. Investigation of the reduction of plastoquinone and its sensitivity to inhibitors (Redfearn and Friend, 1962; Friend and Redfearn 1963) and further extraction-reactivation experiments (Arnon and Horton, 1963; Whatley and Horton, 1963) have conclusively demonstrated a role for plastoquinone as an electron carrier in the photochemical reactions of photosynthesis. Among the interesting parallels between ubiquinone and plastoquinone is the existence of a cyclic isomeride of plastoquinone-solanochromene. This was isolated from flue-cured tobacco (Rowland, 1953). As with ubichromenol it is not certain whether or not this is an artefact of isolation.

The vitamins K are a third group of quinones actively involved in electron transfer processes. Martius and Nitz-Litzow, (1954) presented evidence that Vitamin K₁ was a hydrogen carrier mediating between NADH₂ and cytochrome b in a phosphorylating pathway as alternative to what they considered the non-phosphorylating pathway through the flavoprotein. The lack of evidence for the presence of vitamin K in mitochondrial preparations (Green and Lester, 1959) and the finding by Colpa-Boonstra and Slater (1958) that Vitamin K₃ (menadione) did not act as an electron
carrier in this position suggested seriously weakened Martius' hypothesis. More recently Anderson and Dallam (1959) have put forward evidence that tends to support Martius' theory. They have found that the restoration of the depressed phosphorylation in ultra-violet treated mitochondria requires cytochrome c only in the succinate oxidase system, but both cytochrome c and Vitamin K₁ in the NADH₂ oxidase system. This suggests that a factor similar to Vitamin K₁ might be required for the first phosphorylation site, peculiar to the NADH₂ oxidase system.

The previously mentioned work of Brodie has implicated Vitamin K₁ in the electron transfer and phosphorylation processes of Mycobacterium phlei (Brodie and Russell, 1961). Ultra-violet light treatment reduces the ability of M. phlei to oxidise a substrate and to esterify inorganic phosphate (Brodie et al. 1957; Brodie and Ballantine, 1960, 1960a). Both of these activities can be restored by the addition of the naturally occurring naphthoquinone in M. phlei, or by Vitamin K₁, or the naphthotocopherol isolated from the actively phosphorylating bacteria (Brodie et al. 1960). Any naphthoquinone which will cyclise to a β-chroman will also restore both activities. If the naphthoquinone will not form a β-chroman only oxidation is restored.

The difficulty of isolating, assaying and studying the reactions and properties of the vitamins K in biological
systems is considerable because of interference from the generally higher concentrations of ubiquinone and plastoquinone.

Other quinonoid compounds have been isolated from biological organisms. Fuller et al. (1961) described a quinonoid entity in Chlorobium with an absorption maximum at 262μm. Crane (1962) observed a quinonoid material in lipid extracts of spinach chloroplasts with an absorption maximum of 245μm, behaving differently, chromatographically from ubiquinone or plastoquinone. The same group (Henninger et al. 1963) and a Liverpool group (Bucke et al. 1964) have demonstrated the presence of α-tocopheryl quinone in plant tissues. Glover and Threlfall (1962) isolated a new quinone, rhodoquinone, from Rhodospirillum rubrum. This appears to be structurally similar to ubiquinone, a methoxy group being replaced by a hydroxyl.
CHAPTER II

STANDARD METHODS OF PREPARATION AND ASSAY.

This chapter deals largely with the routine methods used during the course of this investigation. Slight modifications to methods previously described in the literature are included in this section. Newly developed methods and major modifications to existing methods are described in the appropriate chapters.

Purification of solvents.

Water for all solutions was distilled twice in all-glass Quickfit apparatus to remove traces of metal ions which could affect electron transfer or phosphorylation activities.

40 - 60°C (B.Pt.) light petroleum, used to extract mitochondrial preparations was redistilled. Methanol, used to arrest enzymic reactions and for partitioning with mitochondrial petrol extracts was redistilled.

Spectroscopically pure ethanol was prepared by refluxing the ethanol with potassium hydroxide (40 gm./litre) and zinc dust (20 gm./litre) for at least two hours and then distilling over the ethanol. Analar ether and benzene were used for spectrophotometry.
Table II.1 **Comparison of the Waring Blender and mechanical mortar and pestle methods of heart muscle preparation.**

<table>
<thead>
<tr>
<th>Method of Preparation</th>
<th>Fragmentation time</th>
<th>Protein yield/g. tissue</th>
<th>Q0₂</th>
<th>Q0₂ with cyt.c.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waring Blender</td>
<td>1.25 mins.</td>
<td>1.5-2 mg.</td>
<td>c200</td>
<td>c.600</td>
</tr>
<tr>
<td>Mortar &amp; Pestle</td>
<td>1 hour.</td>
<td>6.1 mg.</td>
<td>244</td>
<td>522</td>
</tr>
<tr>
<td>Mortar &amp; Pestle</td>
<td>2 hours</td>
<td>7.3 mg.</td>
<td>21</td>
<td>96</td>
</tr>
</tbody>
</table>
Enzyme preparations.

1) Kalin and Hartree type pig heart muscle preparation:
These were made by a modification of the method described by Keilin and Hartree (1939). 400 gm. of fresh pig heart muscle, freed of all fatty and connective tissue, were minced in a Kenwood Chef mincer and the mince was washed in ice cold tap water for about three hours with a half hourly change of water. The mince was collected by straining through muslin and treated in one of two ways to break up muscle tissues and cells. In the earlier part of the investigation cells were broken up in a Waring Blender top drive macerator with 400 ml. of 0.1 M, pH 7.4 phosphate buffer for 1 1/4 mins. (Pumphrey and Redfearn, 1960). At a later date a Pascall mechanical mortar and pestle was obtained and this was used instead of the Waring Blender. The procedure used was based on that devised by King (1961a). 500 gm. of mince were ground for one hour with 500 ml. 0.02 M, pH 7.4 phosphate buffer and 200 gm. of acid washed sand. Grinding for two hours as suggested by King (1961a) considerably reduced the activity of the preparation (Table II.1).

The homogenate from either method was centrifuged at 1,800g for 20 mins. The resulting cloudy red supernatant was centrifuged for 30 mins. at 20,000g. The brown
sediment was resuspended in 0.1 M, pH 7.4 phosphate buffer (about 100 ml.) using a Potter Elvehjem homogenisor with a Teflon pestle. The suspension was recentrifuged for 30 mins. at 20,000g and the mitochondrial pellet was resuspended in the phosphate buffer to a protein concentration of about 35 - 40 mg./ml. protein and stored at 0°C.

The obvious advantage of the mechanical grinder method is the four times greater yield of enzyme with a QO₂ for succinate comparable with that obtained by the Waring Blender method. No difference in properties of the two enzyme preparations was observed in any of the enzyme systems employed in this investigation apart from a smaller reactivation of succinate oxidase activity by added cytochrome c in the mortar and pestle preparation. The Waring Blender type preparations could usually be reactivated to the extent of about 300 - 400% whereas the corresponding reactivation for the mortar and pestle preparation was generally in the region of 200% but varied considerably.

2) Cytochrome c deficient heart muscle preparations: These were prepared by the modification described by Tsou (1952) for a normal heart muscle preparation. After the usual washing procedures, the mince was washed 3 times for 1 hour and once overnight in ice-cold, 0.15 M, pH 7.4 phosphate buffer. The grinding and centrifugation procedures were
as described for a normal preparation. Addition of cytochrome c resulted in a 400 - 1000% increase in activity.

3) Cytochrome c preparations: Ox heart muscle was freed from fatty and connective tissue and minced in a Kenwood Chef. One kilogram of mince was homogenised in a Waring Blender top drive macerator with one litre of 0.145 N trichloroacetic acid. The homogenate was allowed to stand 2 - 3 hours at room temperature. It was then strained through muslin and the cloudy strainings were adjusted to pH 7.3 with 10% NaOH. Solid ammonium sulphate (500 gm./litre) was added slowly and with stirring until it all dissolved. The mixture was then filtered through fluted filter papers. More ammonium sulphate was added to the clear pink filtrate and the mixture was left overnight at 0°C. Any further precipitated protein was removed by filtration. 20% trichloroacetic acid was added to the filtrate (25 ml./litre) and the resulting brick red precipitate of cytochrome c was immediately centrifuged down at 1,800g for ten mins. The clear supernatant was discarded and the cytochrome c was washed by resuspending it in saturated ammonium sulphate and recentrifuging. The washed precipitate was dialysed against running tap water overnight through cellophane dialysis tubing in order to
remove ammonium sulphate. The next day the dialysed solution was filtered to remove denatured cytochrome c present as a dark brown precipitate. The clear red solution of cytochrome c was standardised spectrophotometrically and stored at -20°C.

Cytochrome c purchased from Boehringer and Soehne GmbH, Mannheim, was also used.

4) **Tightly coupled rat liver mitochondria:** The method was a modification of that used by Schneider (1948) described by Weinbach (1961). It was important during this preparation to maintain all solutions and instruments between 0 and 4°C. All procedures were carried out in a cold room. Double glass distilled water was used for all solutions. This is absolutely essential in this preparation as the presence of heavy metal ions readily uncouples this preparation. The preparation was made from the liver of a hooded Norway rat, from a strain inbred in the Department of Biochemistry, University of Liverpool and continued in this laboratory. These rats have low vitamin A storage in the liver. This is important in so far as vitamin A interferes with ubiquinone microassay.

The liver of a freshly killed rat (killed by chloroform euthanasia) was perfused with ice-cold 0.25 M sucrose, adjusted to pH 7.4 with 10% sodium hydroxide. This was
done by cutting the aorta and injecting the solution into the inferior vena cava with a hypodermic syringe. The liver was blotted dry of blood and placed in 0.25 M sucrose. It was stirred in the sucrose and washed similarly twice more. It was then diced to roughly quarter inch cubes.

Earlier in this investigation the mitochondria were liberated by placing two or three cubes in the mortar of a Potter Elvehjem homogenisor with a few ml. of 0.25 M sucrose. The pestle was driven by a high power, top drive, mechanical stirrer until an even suspension was obtained. This method was superseded by the discovery that tightly coupled mitochondria could be released using an Ultra Turrax high-speed macerator.

3 or 4 cubes of liver were placed in a polyvinyl tube with about 5ml. of sucrose and the macerator was turned on for 1 second only. This was sufficient to break up the tissue and cells. Any further maceration caused uncoupling by breaking up the mitochondria and local production of high temperatures. This method was found to be quicker and more convenient than, and just as consistent as the Potter Elvehjem homogenisor method.

The homogenate was, if necessary, diluted to about 60 ml. with 0.25 M sucrose and centrifuged for about
10 mins. in a Martin Christ Junior centrifuge at speed 2 (about 1,700g). This precipitated most of the tissue pieces, whole cells, blood cells and cell particles other than mitochondria and microsomes. The cloudy pink supernatant was then centrifuged at 8,500g for 10 mins. The supernatant from this spin, which was pink and slightly cloudy, was discarded. The sediment was in three portions:

i) an upper fluffy layer of broken 'light' mitochondria, which was discarded. This layer had very low respiratory control and P:O ratios.

ii) a middle yellowish layer of intact mitochondria, which was scraped from the third layer.

iii) a layer of blood cells, which was discarded.

The middle layer was suspended in a small amount of 0.25 M sucrose using a Potter-Elvehjem homogenisor and recentrifuged at 8,500 g for 10 mins. to wash the mitochondria. The mitochondrial pellet, after separating the layers in the last stage, was resuspended in 0.25 M sucrose to a protein concentration of about 20 mg./ml. The preparations had respiratory control ratios of between 3 and 10. P:O ratios measured polarographically were:

Succinate oxidase ................ .. 1.6 - 2.0
β-hydroxybutyrate oxidase ......... 2.2 - 2.9
Choline oxidase ................... 1.4 - 1.7
Fig. II.1 Demonstration of respiratory control for succinate oxidase in rat liver mitochondria.

The mitochondria were suspended in an isotonic assay solution comprising:
- Sucrose --- 0.05M.
- MgCl₂ --- 0.025M.
- KCl ------- 0.04M.
- Na/K phosphate buffer ------- 0.02M, pH 7.4

In this particular assay the respiratory control ratio is 5.5 and the P:O ratio was 2.05.
Respiratory control and P:O ratios for each preparation were determined before any investigation. The tracing (Fig. II.1) shows a typical polarographic determination of respiratory control for succinate oxidase. The respiratory control ratio was taken as the ratio of the activity after adding a limiting amount of ADP (usually about 0.33 mg.) and the activity on returning to the resting state. The polarographic equipment used is described later. P:O ratios can be determined polarographically from a knowledge of the amount of phosphate acceptor added, and a measurement of the oxygen utilisation obtained from figures for oxygen solubility.

Measurement of enzyme activities.

The diluted enzyme preparation was incubated for 20 mins. at 37°C prior to all enzymic assays, in order to activate succinate dehydrogenase. (Thorn, 1962.)

1) Succinate oxidase: Polarographic and manometric methods were used for succinate oxidase measurement. The polarographic technique is dealt with later. In the manometric method, which was performed at 37°C, the following reaction mixture was employed:

\[
\begin{align*}
\text{IN MAIN} & \\
\text{Phosphate buffer, pH 7.4} & \quad \text{Final concn. 0.07M} \\
\text{Sodium succinate} & \quad \text{" " 0.01M} \\
\text{Cytochrome c} & \quad \text{" " 2 x 10^{-5}} \\
\text{Enzyme suspension, 0.5-1.0 mg. protein} & \\
\text{WATER TO 3 ml.} & 
\end{align*}
\]
IN CENTRE WELL 0.1 ml. 20% KOH+ filter paper.

The manometers were equilibrated for 7 mins. and the taps closed. Readings were normally taken every 5 mins. for half an hour, and every 10 mins. for a further half hour. The rate of uptake of oxygen was recorded as the QO₂ in μlitres of oxygen/mg.protein/hour.

2) Oxidation of other metabolites: Sodium β-hydroxybutyrate, sodium α-keto-glutarate or sodium hydrogen malate oxidases could similarly be measured polargraphically or manometrically as for succinate oxidase, by substituting a similar concentration of the appropriate substrate for succinate. Choline oxidase activity of rat liver mitochondria was determined similarly using 0.01 M choline chloride as substrate.

3) NADH₂ oxidase: Nicotinamide adenine dinucleotide (reduced) oxidation was measured spectrophotometrically in a Unicam SP 500 or an Optica CF 4 recording spectrophotometer at room temperature.

Reaction mixture:

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concn. 0.07 M</th>
<th>Final concn. 1.1 x 10⁻⁴ M</th>
<th>Final concn. 2.0 x 10⁻⁵ M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer, pH 7.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADH₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome c</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enzyme suspension</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water to 3ml.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The blank cell contains buffer and enzyme. The reaction was started by adding enzyme to the test cell and the rate of NADH₂ oxidation was measured by following the decrease in extinction at 340 nm. NADH₂ oxidation rate was calculated using a molecular extinction coefficient for NADH₂ of 6.22 x 10⁻³ at 340 nm.

NADH₂ oxidase was also measured polarographically.

4) Cytochrome oxidase: This was measured manometrically at 37°C.

Reaction mixture:—

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer, pH 7.4</td>
<td>Final concn. 0.07 M</td>
</tr>
<tr>
<td>p-phenylene diamine</td>
<td>0.04 M</td>
</tr>
<tr>
<td>Cytochrome c</td>
<td>A series of concns. from about 7 x 10⁻⁷ M to 2 x 10⁻⁵ M</td>
</tr>
<tr>
<td>Enzyme suspension</td>
<td>0.5 - 1.0 mg. protein</td>
</tr>
<tr>
<td>Water to 3 ml.</td>
<td></td>
</tr>
</tbody>
</table>

A series of cytochrome c concentrations was used to correct for autoxidation of p-phenylene diamine by extrapolating oxygen uptake to an infinite concentration of cytochrome c.

5) Succinate dehydrogenase; Succinate dehydrogenase was measured using several different electron acceptors. Phenol-Indo - 2,6-dichloro-phenol, Ferricyanide, methylene blue, 2,6-dichlorophenolindophenol (DCIP) and phenazine methosulphate (PMS) were employed. These electron acceptors react at different points along
the respiratory chain and are discussed at some length in Chapters III & IV. Methylene blue and phenazine methosulphate reductases were measured manometrically at 37°C, DOPIP reductase spectrophotometrically at room temperature, and ferricyanide reductase potentiometrically at 20°C, (Chapter III).

Reaction mixtures for methylene blue or PMS reductase:

\[
\begin{align*}
\text{Phosphate buffer,} & \quad \text{Final concn. 0.07 M} \\
\text{IN MAIN} & \\
\text{Sodium succinate} & \quad " \quad 0.01 M \\
\text{KCN} & \quad " \quad 2 \times 10^{-3} \text{ M} \\
\text{FLASK.} & \\
\text{Methylene blue or PMS} & \quad " \quad 0.33 \text{ mg./ml} \\
\text{Enzyme suspension} & \quad " \quad 0.67 \text{ mg./ml} \\
\text{Water to 3 ml.} & \\
\text{IN CENTRE WELL} & \quad 0.1 \text{ ml. 20\% KOH + filter paper.} \\
\end{align*}
\]

Manometry as for succinate oxidase. \(Q_{O_2}\) values were halved to correct for the fact that the reduced electron acceptors take up one oxygen atom per electron originating from succinate.

\[
\begin{align*}
\text{DOPIP reductase reaction mixture:} & \\
\text{Phosphate buffer, pH 7.4} & \quad \text{Final concn. 0.07 M} \\
\text{Sodium succinate} & \quad " \quad 0.01 M \\
\text{KCN} & \quad 2 \times 10^{-3} \text{ M} \\
\text{DOPIP} & \quad 6.7 \times 10^{-5} \text{ M} \\
\end{align*}
\]
Enzyme suspension about 0.05 mg. protein
Water to 3 ml.

The reduction was followed by measuring the decrease in extinction at 600 m\(\mu\) on reduction of DCPIP.

The method used for measuring ferricyanide reductase is described in Chapter III.

3) Succinate - cytochrome c reductase: Succinate-cytochrome c reductase was determined spectrophotometrically at room temperature on either the Optica CF 4 or the Unicam SP 600.

**Reaction mixture:**

- Phosphate buffer, pH 7.4
- Sodium succinate
- Cytochrome c
- Potassium cyanide
- Enzyme suspension, about 0.05 mg. protein
- Water to 3 ml.

The blank cell contained buffer and enzyme. Cyanide was added immediately before the assay was commenced as this tends to inactivate cytochrome c. The reaction was started by addition of enzyme to the test cell. The rate of cytochrome c reduction was measured by following the increase in extinction at 550 m\(\mu\), which occurs on the reduction of cytochrome c. Readings were taken on the Unicam SP 600 at 15 sec. intervals for a convenient period. Cytochrome c reductase activities were calculated using

\[ \alpha_{\text{red}} - \alpha_{\text{ox.}} = 1.91 \times 10^7 \text{ cm}^2/\text{mole at 550 m}\(\mu\).\]
Fig. II.2 Standard curve for protein estimation.
Other determinations.

1) **Protein determination:** The protein concentration of enzyme preparations was determined by the method of Chance and Redfearn (1961). The mitochondrial preparation was diluted to between 1 and 5 mg. protein/ml. and 1 ml. of the diluted particles was mixed with 0.5 ml. of water and 1.5 ml. of biuret reagent (Gornall, Bardawill and David, 1949) and two drops of 40% sodium cholate. The mixture was shaken and incubated at room temperature for 30 mins. together with a blank containing all but enzyme preparation. The extinction at 540 nm was determined on a Unicam SP 600 or 500. The protein concentration was obtained from a calibration curve obtained using standard bovine serum albumin. Up to a value of about 4 mg./ml. the variation of protein concentration with $E_{540}$ is almost linear (Fig. II.2). Above 5 mg./ml. there is little or no variation in $E_{540}$ as the reagents present are exhausted and can form no more biuret.

2) **Assay of cytochrome c in a stock solution:** Cytochrome c concentration was determined spectrophotometrically using a Unicam SP 600.

**Reaction mixture:**

- A suitable volume of stock solution of cytochrome c
- 0.1 ml. of 0.01 M $K_3Fe(CN)_6$
- 0.1 M phosphate buffer, pH 7.4 to 3 ml.
The extinction of the mixture at 550 nm was measured against a buffer blank. About 0.5 mg. of sodium dithionite was added to the test cell and mixed in. The extinction at 550 nm was measured again. Cytochrome c concentrations were calculated using

$$\alpha_{\text{red.}} - \alpha_{\text{ox.}} = 1.91 \times 10^7 \text{ cm}^2/\text{mole}.$$ 

Polarography.

Polarographic methods were used for measuring the activity of oxidase reactions. The principle of the method was discovered by Daneel (1897) who found that in an electrolytic cell there was approximate proportionality between the current passed and the concentration of dissolved oxygen. Davies and Brink (1942) devised an electrode assembly which could measure oxygen tension in an aqueous solution or local oxygen tension in animal tissues. Many modifications of this assembly have been employed, the most important of which are the vibrating electrode system of Chance and Williams (1955b) and the static electrode - rotating solution arrangement originally described by Packer (1957). Chappell (1961) used a Clark oxygen electrode adapted to measuring mitochondrial respiration. This is commercially available (E.I.L.) as a unit comprising platinum and silver / silver chloride electrodes. The
Fig. II.3 Oxygen electrode: bench lay-out for polarographic determination of oxygen concentration.
Fig. II.4 Oxygen electrode:- circuit diagrams.

A) Sensitivity controls, i) Fine, ii) Coarse.
B.) On / off switch.
C.) Circuit determining switch, i) Check polarising voltage circuit, ii) Switch in 'RUN' position.
D.) Polarising voltage control.
E.) Matching resistances.
Fig. II.5  Oxygen electrode: diagram of reaction vessel and electrode assembly.

A.) Immersible magnetic stirrer.
B.) Rubber supporting piece.
C.) Polythene coated stirring flea.
D.) Rubber ring securing the electrode assembly polythene membrane.
dissolved oxygen of the test solution equilibrates across a polythene membrane with 0.5 M KCl electrolyte. This electrode was the one employed in the investigations described in this thesis. The change in current passing caused by changes in oxygen tension was measured by determining the voltage across a resistor. The voltage change was amplified by an E.I.L. Vibron Electrometer (Model 33B) which then gave sufficient current to activate a recorder (Kent, Multelec, Mk III). The bench layout is shown in Fig. II.3. The platinum wire is maintained at 600mV by a torch battery placed across the electrodes. The silver wire was at earth. A 500 ohm variable resistance placed across the battery was used to set the polarising voltage. The circuit diagrams and cell employed are diagrammatically illustrated in Figs. II.4 and II.5.

The oxygen uptake is calculated from the tracing from a knowledge of the oxygen solubility in the assay solution at the temperature of the assay (Chappell, 1964). One of the most useful polarographic techniques is the determination of P:O ratios. This is simply performed (Fig. II.1) by calculating the oxygen uptake for the period of active respiration after addition of a known amount of phosphate acceptor system.
CHAPTER III

THE SUCCINATE-FERRICYANIDE REDUCTASE SYSTEM
OF MITOCHONDRIAL PREPARATIONS

Introduction

Artificial electron acceptors have been widely used to study the components of a particular section of the respiratory chain. The use of ferricyanide as an electron acceptor has been a particularly valuable application of this technique. The reduction of ferricyanide by succinate in rat liver mitochondria has been used to isolate the first phosphorylation step of the succinate oxidase system (Estabrook, 1961). The observation by Green (1961) that ubiquinone -{(10) will mediate succinate-ferricyanide reductase in a similar manner to the mediation of methylene blue reductase by ubiquinone homologues (Redfearn, 1961b), suggested that this system might be useful for studying ubiquinone function.

The ferrocyanide/ferricyanide system was first used to study dehydrogenase by Quastel and Wheatley (1938). The overall reaction for the process is:—
but the number of intermediate steps in the reaction is still uncertain.

Two standard methods of measuring ferricyanide reduction exist. Quastel and Wheatley (1938) used a manometric method which depended upon the release of carbon dioxide from bicarbonate buffer by the hydrogen ions released during the reduction of ferricyanide. However, the more usually employed method (Pressman, 1955; Estabrook, 1961) is the spectrophotometric observation of the decrease in $E_{420}$ (the absorption maximum of potassium ferricyanide) on reduction of ferricyanide to ferrocyanide.

Although Singer and Kearney (1957) concluded that the best assay for succinate dehydrogenase employed phenazine methosulphate (PMS) reduction, the purified enzyme reacted with ferricyanide at 30-40% of the maximal rate obtained using PMS. (Kearney and Singer, 1956). On the other hand, ferricyanide reduction is a more sensitive assay than PMS reduction. Unfortunately, the use of ferricyanide as an electron acceptor has two important limitations. Ferricyanide oxidises sulphydryl groups and is consequently
<table>
<thead>
<tr>
<th>Fe(II) Concentration</th>
<th>Substrate</th>
<th>How Measured</th>
</tr>
</thead>
<tbody>
<tr>
<td>System</td>
<td>Rat Liver</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cytochrome</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oxidase Prep.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mitochondria</td>
<td></td>
</tr>
<tr>
<td>Reference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cross et al. (1949)</td>
<td>33 mM</td>
<td>Manometrically &amp;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Colorimetrically</td>
</tr>
<tr>
<td></td>
<td>2 mM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>17 mM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.95 mM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5-0.7 mM</td>
<td></td>
</tr>
<tr>
<td>Le Bain &amp; Lardy (1942)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Copenhagen &amp; Lardy (1955)</td>
<td>17 mM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.95 mM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.80 mM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.75 mM</td>
<td></td>
</tr>
<tr>
<td>Malley &amp; Lardy (1955)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pressman (1955)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estabrook (1961)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table III.1. Summary of Fe2+ ratios obtained by different workers using ferricyanide as a terminal electron acceptor.
an inhibitor of succinate dehydrogenase. (Barron and Singer, 1945). (This is less important in a particulate heart muscle preparation than in the study of the primary dehydrogenase). The other limitation is that ferricyanide may be reacting with the respiratory chain at more than one level. Slater (1948, 1949) first suggested multiple sites of ferricyanide reduction based on the observation that only 35% inhibition of ferricyanide reduction by succinate occurred, by an amount of 2:3 - dimercapto propanol (BAL) which would cause complete inhibition of succinate oxidation in a heart muscle preparation. There has been little work done since this time on the reaction of ferricyanide with the heart-muscle preparation, but a large amount of data has been accumulated for rat-liver mitochondria.

The use of ferricyanide as an electron acceptor in rat liver mitochondria is particularly advantageous as respiratory control has been observed for the first phosphorylation site (Estabrook, 1961, 1962). However, there has been no attempt to standardise the procedure and the data is extremely confused. Table III.1 shows the P:2e ratios obtained by several investigators in relationship to the concentration of ferricyanide they used. The cyclophorase system is a particulate system obtained from rat
liver mince which will catalyse the metabolism of pyruvate round the whole of the Krebs cycle. It is significant that in the manometric method a range of ferricyanide concentrations from 2-33 mM has been used and the P:O ratio obtained with NADH₂ linked substrates has never exceeded one. On the other hand spectrophotometric determinations never used a ferricyanide concentration more than 0.95 mM and the corresponding P:2e ratios were in the region of 2. This suggests the possibility that ferricyanide might react before the second phosphorylation site at high concentrations and after it at low concentrations. What is more Pressman (1955) found that the spectrophotometric method was, in contrast to the manometric method (Copenhaver and Lardy, 1952) sensitive to antimycin A. Pressman (1955) did not consider that ferricyanide concentration accounted for the discrepancy between the two techniques, but did not present any evidence to this effect. Estabrook (1961) obtained a similar (90%) inhibition by antimycin A and concluded that variation of ferricyanide concentration could affect the site of action and would account for the discrepancy.

Recently, Lardy's group (Walter, 1964; Walter & Lardy, 1964) have suggested that the lack of antimycin A inhibition in the manometric determination is due to inactivation of
antimycin A by high ferricyanide concentrations. This inactivation only occurred when ferricyanide and antimycin were preincubated in the absence of rat liver mitochondria, as the reaction between mitochondria and antimycin A is considerably greater than the inactivation of antimycin A by ferricyanide, and the ferricyanide does not appear to affect the enzyme bound antimycin A. It is difficult to reconsider the data in light of this claim as the order of addition of components is not usually stated. It is unlikely, however, that the enzyme will be withheld from the incubation mixture long enough to cause a significant decrease in the inhibitory power of antimycin A. Furthermore, it is difficult to reconcile this observation with a previous statement by Lardy (Copenhaver and Lardy, 1952) that "incubation of antimycin A with ferricyanide did not diminish the ability of the antibiotic to inhibit succinate oxidation." On the evidence presented in this earlier paper he concluded that the site of ferricyanide reduction was on the substrate side of the antimycin A sensitive region but has now reversed this conclusion and placed it on the oxygen side.

There seems to be little doubt that ferricyanide can react at two sites in the NADH$_2$ oxidase system of a heart muscle preparation. Singer (1961) demonstrated that, in
beef sarcosomal particles, the curve relating reciprocal ferricyanide concentration to reciprocal activity of ferricyanide reduction by NADH$_2$, showed a definite break. The two phases of the curve represent:

i) a reaction primarily at cytochrome c at a low ferricyanide concentration, and

ii) at the flavoprotein level at high ferricyanide concentration.

Singer also demonstrated that the reaction at cytochrome c is non-competitively inhibited by antimycin A or Amytal as these inhibitors prevent electron flux to cytochrome c. This reaction moreover was competitively activated by cyanide and azide as these inhibitors increase electron flux to ferricyanide. At high concentrations of ferricyanide, little inhibition by Amytal or antimycin A occurred as the large majority of the reaction occurred before their sites of inhibition. The biphasic nature of the plot disappeared when Singer (1961) attempted to purify the NADH$_2$ dehydrogenase flavoprotein - suggesting that the cytochrome c site of action has been removed.

In order to try and resolve the problem of the sites of interaction of ferricyanide with the succinate oxidase system a potentiometric method of measuring succinate-ferricyanide reductase activity has been developed which
Fig. III.1  Diagram of apparatus used for potentiometric determination of ferricyanide concentration.

A.) Calomel reference electrode.
B.) Platinum electrode.
C.) Stirrer.
proved to be more versatile than the manometric and spectrophotometric methods, which are limited in their application. The spectrophotometric method can tolerate only a limited ferricyanide and enzyme concentration in producing a readable and accurate rate of ferricyanide reduction. The manometric method limits investigation of the lower range of ferricyanide concentrations and, with the necessary corrections to be made for CO₂ retention, is rather tedious when used for serial determinations. It is also impossible to make an impromptu addition using the manometric technique. It was, therefore, decided to investigate the possibility of measuring ferricyanide reduction by observation of the change in oxidation-reduction potential of the system. Such a method would eliminate the above-mentioned limitations. Although a potentiometric method has been used to study Hill reaction activity of chloroplast preparations (Spikes et al., 1950, 1954) the technique does not appear to have been used in the study of mitochondrial electron transfer.

**Experimental**

Fig. III.1 shows the apparatus designed for the recording of the change in oxidation-reduction potential. It consisted of a platinum and calomel electrode assembly, the potential across which is observed on a millivoltmeter.
Fig. III.2  Tracing obtained during potentiometric recording of ferricyanide reduction.
Fig. III.3  Calibration curve for potentiometric determination of ferricyanide reduction.

% ferricyanide is given on the abscissa, % ferrocyanide is 100 - %ferricyanide. Total ferricyanide + ferrocyanide concentration is 0.5 M. These were dissolved in 0.1 M, pH 7.4 Na/K phosphate buffer containing 3.3 x 10^{-3} M sodium azide and 1 mg./ml. protein of heart muscle preparation.
and recorded on a potentiometric recorder. The electrode assembly was immersed in the cell containing the reaction mixture and the cell was suspended in a constant temperature water bath. The reaction mixture was stirred by a vibrating rod. The potential/time curves obtained on the reduction of ferricyanide had the characteristic shape of the Nernst equation (Fig. III.2). The conversion of this sigmoid curve to the straight line ferricyanide reduced/time plot was done by a modification of the procedure of Spikes et al. (1950). A calibration curve was drawn (Fig. III.3), with ferricyanide and ferrocyanide dissolved in 0.1 M, pH 7.4 phosphate buffer, containing about 1 mg. protein/ml., heart muscle preparation, in ratios varying from 100% ferricyanide to 100% ferrocyanide. The potential difference of these reference solutions was recorded and the calibration curve drawn. The mid-point potential, when the mixture contains 50% ferricyanide/50% ferrocyanide gives a potential difference of 0.16V. From the Nernst equation:

\[ E_{\text{observed}} = E_0 + \frac{RT}{F} \log_e \frac{[\text{Fe(CN)}_6^{3-}]}{[\text{Fe(CN)}_6^{4-}]} \]

\((R = \text{gas constant}, \; F = \text{Faraday}, \; T = \text{absolute temp.})\)

The difference between the standard redox potential of the ferricyanide-ferrocyanide system and the potential of the
Fig. III.4  Linear rate of reduction of ferricyanide, obtained by applying calibration curve (Fig. III.3) to tracing on Fig. III.2.
Fig. III.5 Effect of ferricyanide concentration on the rate of reduction of ferricyanide.

Curve A in text is: –•–•
Curve B in text is: – – • •
Curve C in text is: – – – –

Ferricyanide reduction rate is in μmoles of ferricyanide reduced/minute/mg protein.
calomel electrode is 0.16V as the final term in the equation becomes zero at equal concentrations of ferricyanide and ferrocyanide. Using this value a series of conversion figures can be calculated from the Nernst equation. These agree very closely with the calibration curve. Fig. III. 4 shows the curve obtained on applying the calibration curve to the sigmoid Fig. III.2. This shows that the rate of reduction was constant down to a very low ferricyanide level. The effect at low ferricyanide concentrations could be due to reduction of ferricyanide by impurities of mitochondrial components. A quick estimate of reduction rate may be made from the sigmoid curve, the time taken from the start to 0.16V being the time for half reduction of the ferricyanide.

Results

Effect of Ferricyanide Concentration on the Activity of Succinate-Ferricyanide Reductase: the rate of reduction of ferricyanide by succinate in a Keilin and Hartree pig heart muscle preparation was measured over a range of ferricyanide concentrations from 0.1 to 0.8 mM. (Fig. III.5, Curve A). The order of additions was i) buffer, ii) water, iii) heart muscle preparation, iv) sodium azide, v) ferricyanide. The azide is added to prevent oxidation of succinate by way of the cytochrome
oxidase system. The reaction was started by the addition of succinate. It was observed that in the range up to 0.3 mM ferricyanide, there was a linear increase in succinate ferricyanide reductase activity. Between 0.3 and 0.5 mM there was little increase in the rate of ferricyanide reduction, whilst from 0.5 mM to 0.8 mM there is a further linear increase in activity which can be extrapolated to the origin. It was considered that this biphasic curve was evidence for the postulated two sites of action for ferricyanide in the succinate oxidase system. If, as in the NADH₂-ferricyanide reductase system (Singer, 1961) and as suggested by Estabrook (1961, 1962) for the succinate system, the second site of action is at the cytochrome c level, addition of antimycin A to the reaction mixture should effectually prevent any reaction of ferricyanide at this level. A similar set of activity determinations over a range of ferricyanide concentrations was performed (Fig. III.5, Curve B) in the presence of sufficient antimycin A to completely inhibit succinate oxidase activity. Antimycin A and heart muscle preparation were added before ferricyanide in order to minimise antimycin A inactivation by ferricyanide (Walter, 1964). A fairly linear rate of increase in activity was obtained over the complete range investigated. This was similar to the
Fig. III.6  Double reciprocal plot of data presented in Fig. III.5.
Fig. III.7  The effect of 4-heptyl-2-hydroxyquinoline N-oxide on the rate of succinate-ferricyanide reductase.

--- was performed with 0.6 mM ferricyanide,

--- was performed with 0.2 mM ferricyanide.

Final concentration of AHQO was 5µg./ml. in each case.
extrapolated linear relationship above 0.5 mM which was observed in the absence of antimycin A. This suggests that above 0.5 mM the large majority of the reaction is occurring before the site of antimycin A sensitivity. The difference between these two curves (Curve C, Fig. III.5) has a maximum at 0.2 mM which probably corresponds to optimum ferricyanide concentration for reaction at the second site.

Fig. III.6 shows the data of Fig. III.5 plotted as reciprocal ferricyanide concentration/reciprocal activity (Lineweaver-Burke) curves. This gave a very similar picture to the data of Singer (1961) for NADH₂-ferricyanide reductase. The $K_m$ value for the reaction of ferricyanide with the cytochrome c level was 0.48 mM (compared with the value of 0.58 mM calculated from data of Singer (1961) and 2.5 mM for the reaction with the flavoprotein.

To ascertain that the effect was not merely due to the inactivation of antimycin A by ferricyanide, the inhibition of succinate-ferricyanide reductase by 4-heptyl-2-hydroxyquinoline N-oxide (Jackson and Lightbown, 1956) which inhibits at a site identical or close to the antimycin site, was investigated at representative concentrations of 0.2 and 0.6 mM ferricyanide (Fig. III.7). At the higher concentration, with most of the activity at the
Fig. III.8  **Effect of enzyme concentration on the activity of succinate-ferricyanide reductase.**

- was performed with 0.6 mM ferricyanide.
- was performed with 0.2 mM ferricyanide.

Enzyme concentration in mg./ml. protein.
Fig. III.9  Variation of succinate-ferricyanide reductase activity with pH.

- - - - - was performed with 0.6 mM ferricyanide.
- - - - - - - was performed with 0.2 mM ferricyanide.

From pH 4 - pH 5 0.07M acetate buffer was used.
From pH 6 - pH 7.8 0.07M phosphate buffer was used.
From pH 9 - pH 10 0.07M borate buffer was used.
flavoprotein level only 30% inhibition occurs, at the
lower concentration, activity mainly at the second site,
about 90% inhibition occurs. This suggests that the
antimycin results are not due to inactivation of antimycin
A by high ferricyanide concentrations.

**Effect of Enzyme Concentration:** the effect of enzyme
concentration on the activity of succinate-ferricyanide
reductase in a heart muscle preparation is shown in Fig.
III.8. Determinations were made at 0.2 and 0.6 mM
ferricyanide. The absolute activity increases linearly
over the whole range of enzyme concentration investigated
in both cases. This means that the specific activity
remains the same at any enzyme concentration likely to
be employed in this assay.

**Variation of Activity with pH:** the possibility that two
sites of reaction exist for ferricyanide in the succinate-
ferricyanide system, prompted the investigation of the
variation of succinate-ferricyanide reductase activity
with pH at ferricyanide concentrations representative
of both sites of action. Fig. III.9 shows the pH/
activity curves for the reaction at concentrations of 0.2
and 0.6 mM ferricyanide. At the high concentrations of
ferricyanide there is relatively high activity between
Table III.2 Mediation of succinate-ferricyanide reductase by ubiquinone homologues.

Ubiquinone homologues were added in 0.02 ml of ethanol.
pH 5 and 7 with increased activity between pH 7.5 and 9. A pH optimum exists in the region of pH 8.2. At the lower concentration a definite optimum exists at pH 7.6. This suggests that at low and high pH values there is little activity between the two postulated sites of reaction.

Effect of ubiquinone homologues and cytochrome c on succinate-ferricyanide reductase: the stimulation of succinate-ferricyanide reductase in a heart muscle preparation by ubiquinone homologues, first observed by Green (1961) with ubiquinone-(10) has been investigated. The results are shown in Tables III.2a and III.2b. As with the mediation of methylene blue reductase (Redfearn, 1961 b) only the lower ubiquinone homologues are stimulatory, the most active being ubiquinone (5). The pattern of stimulation at both concentrations of ferricyanide is very similar. This suggests that both pathways could be mediated in the same manner. This is borne out by the observation that in the presence of ubiquinone homologues, antimycin had no effect on the activity even at low ferricyanide concentrations. The ubiquinone concentration was too low to be causing reversal of the inhibition by its lipophilic action (Takemori and King, 1964).

Investigation of the effect of cytochrome c addition
to the system produced further evidence for the dual reaction mechanism. At the lower concentration (0.2 mM ferricyanide) where reaction rate is limited to a certain extent by cytochrome c availability, cytochrome c addition caused considerable increase in the rate of reduction, whereas at the higher ferricyanide concentration (0.6 mM), cytochrome c addition had little effect. This suggests that at high ferricyanide concentrations the cytochrome c site is not involved in the electron transport pathway.

Discussion.

The biphasic nature of the curve illustrating the rate of reduction of ferricyanide concentration, as a function of ferricyanide concentration, and the disappearance of the biphasic property on antimycin A treatment, add strength to the idea that ferricyanide can act at two levels in the succinate oxidase respiratory chain as suggested previously by Giuditta and Singer (1959) and Estabrook (1962). This view has been reinforced by the data presented for the pH/activity relationship of the enzyme system and for the stimulations caused by cytochrome c and ubiquinone homologues.

The fact that at low concentrations of ferricyanide the activity is stimulated by cytochrome c suggests that
the second site of reaction is with the cytochrome c site. The first site of reaction on the other hand is probably with a non-haem iron moiety closely associated with the flavoprotein. This can be deduced from data, presented in the next chapter, on the inhibitory effects of the metal chelating agent, thenoyltrifluoroacetone, on the succinate-ferricyanide reductase system. The inhibitory pattern suggests that the first ferricyanide reaction site is similar to the phenazine methosulphate reaction site. This is almost certainly with the metal component of the flavoprotein (Singer et al., 1957). This agrees with Singer's observation (Singer and Kearney, 1957) that ferricyanide can be reduced directly by the primary dehydrogenase, in contrast to methylene blue and indophenol which cannot. It is probable that ferricyanide reacts directly with the flavin in the NADH₂ system, as the activity at high ferricyanide concentrations is not affected by Amytal (Singer, 1961).

The mediation by added ubiquinone homologues at both concentrations of ferricyanide investigated, most probably occurred by reduction of the quinone by the factor in the respiratory chain responsible for the reduction of the endogenous quinone. The reduced ubiquinone is re-oxidised by the ferricyanide. The fact that this occurs
**Fig. III.10** Probable sites of ferricyanide reaction with in the succinate oxidase system in relationship to the sites of action of thenoyltrifluoroacetone, ANTIMYCIN A₈ and sodium azide.
even at low ferricyanide concentrations suggests that ubiquinone reacts more strongly at the flavoprotein level than does the low ferricyanide concentration. Fig. III.10 shows the probable reactions of ferricyanide and added ubiquinone with the respiratory chain in relation to the major inhibitory sites of thenoyltrifluoroacetone, antimycin A and azide.

It is difficult to explain why, if the initial ferricyanide concentrations determine the reaction velocity, why this should remain linear during the reaction time as ferricyanide concentration goes down. It is probable that the rate of reaction is determined by the total ferricyanide plus ferrocyanide rather than the absolute amount of ferricyanide in the medium.

Although these results are interesting in their own right, they are obviously even more important when considering data presented on the rate of ferricyanide reduction in mitochondrial preparations and the site(s) of reaction must be unambiguously determined before data can be interpreted.

Unfortunately, it was not possible to apply this technique to phosphorylating mitochondria as the added azide uncoupled oxidative phosphorylation by its inhibitory effect on the ATP - P\textsubscript{i} exchange reaction. Cyanide
cannot replace azide as this inhibitor inactivates a platinum electrode.
CHAPTER IV
THE ROLE OF NON-HAEM IRON IN THE REDUCTION
AND OXIDATION OF UBIQUINONE IN THE RESPIRATORY CHAIN.

Introduction

Despite the attractive pictures of electron transfer painted by some workers, the location of the site of action of ubiquinone has yet to be incontrovertibly demonstrated. The evidence suggesting that ubiquinone acts between the succinate dehydrogenase segment and the cytochrome segment of the respiratory chain has been discussed at some length in Chapter I. There is an increasing amount of evidence however that ubiquinone does not become reduced directly by the succinate dehydrogenase flavoprotein.

The first piece of evidence was the discovery by Doeg et al. (1960) that succinate dehydrogenase prepared according to the method devised by Singer et al. (1956) was unable to reduce ubiquinone. Although Szarkowska and Petrysryn (1961) claimed that ubiquinone would be reduced by succinate dehydrogenase prepared in two different ways (Wang et al., 1956; Keilin and King, 1960),
<table>
<thead>
<tr>
<th>Method</th>
<th>Reference</th>
<th>Ability to reduce HMG</th>
<th>Reactivation of alkali-inactivated heart muscle preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Extraction of acetone powder of heart mitochondria by Tris buffer, pH 8.9</td>
<td>Singer et al. (1956)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>2. Butanol treatment of Keilin-Hartree preparation preincubated with succinate and KCN.</td>
<td>Wang et al. (1956)</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>3. As 2 without KCN.</td>
<td>Keilin and King (1960)</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>4. Direct extraction of HMG with phosphate/borate buffer at pH 9.5</td>
<td>King (1961, 1961a)</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
more recent work by King (1963) supports the idea that a direct reduction of the quinone does not occur. The probable reason for this discrepancy is cleavage of the electron transfer chain at different points during the preparation of the enzyme. It is probable that the enzyme prepared by Szarkowska and Petrysryn (1961) was 'contaminated' by some component removed from the other preparations. It is likely that their enzyme resembled the succinate-ubiquinone reductase of Ziegler and Doeg (1959, 1962).

In addition to a variable ability to reduce ubiquinone these preparations of succinate dehydrogenase also exhibited a variation in ability to reconstitute the succinate oxidase activity of an alkali inactivated heart muscle preparation. This was investigated by King (1962). He prepared succinate dehydrogenase by four methods (Table IV.1) and attempted to use each enzyme to reconstitute succinate oxidase in an alkali inactivated heart muscle preparation. Although only preparation (3) was active in reconstitution of succinate oxidase, all four were able to reduce phenazine methosulphate. Preparation (2) was most probably inactivated by the action of cyanide on the enzyme. Preparations (1) and (4) could be made in the active form by preincubation with succinate (and to a lesser extent with other
reducing agents). King also observed differences in the electron paramagnetic resonance (EPR) spectra of the active and inactive forms of the enzyme (vide infra). It is apparent that the oxidation-reduction state of the components during solubilisation determines the position of cleavage. The available evidence suggests that non-haem iron is the missing component of the inactive enzyme.

Iron is indigenously present in the mitochondrion in three forms. In beef heart mitochondria about 50% of the iron is associated with the haem residues of the cytochromes. (Doeg and Ziegler, 1962.) The other 50%, not associated with haem, is termed non-haem iron (NHI). This can be further subdivided into two portions. The first form is closely associated with the primary succinate dehydrogenase, which, according to Ziegler accounts for half the non-haem iron in the succinate-ubiquinone reductase segment of the respiratory chain. Non-haem iron also exists as an apparently independent entity in the succinate- and NADH₂- ubiquinone reductase segments of the respiratory chain (Green and Wharton, 1963; Hatefi et al., 1962, 1962a; Ziegler and Doeg, 1959; Rieske and Zaugg, 1962).

The presence of iron associated with soluble
succinate dehydrogenase was first observed by Kearney et al., (1955). There has been a great deal of controversy concerning the amounts of non-haem iron associated with succinate dehydrogenase. The most reliable estimates have been reached independently by Singer's group (Singer et al., 1956) and Wang's group (Wang et al., 1956) using different methods for isolating succinate dehydrogenase. The figure they reached was 4 molecules NHI/1 molecule flavin/200,000 molecular weight in a freshly made preparation and half the amount of iron in an aged one.

The group working with D.E. Green attempted to solubilise the enzyme by other methods. The preparations they obtained varied quite widely in their iron content (Green and Beinert, 1955; Green, et al., 1956). They claimed that several different forms of succinate dehydrogenase existed containing different amounts of non-haem iron. This was challenged by Singer's group, (Singer et al., 1957) on the basis that they had found no difference in the composition and properties of succinate dehydrogenase isolated from different sources and concluded that the differences were more likely to be due to the breaking of the respiratory chain at different points. This would seem to be a likely explanation as the preparations of Green's group could sometimes react with...
externally added cytochrome c, but at other times were unable to.

In comparison with these preparations of unreliable properties, Singer's succinic dehydrogenase appears to have enjoyed more consistent composition and properties. As has previously been mentioned, ageing of this preparation is characterised by halving of its non-haem iron content. An interesting observation was that this change in composition was accompanied by halving of the activity of the enzyme to phenazine methosulphate and ferricyanide reduction. According to Singer these electron acceptors are able to react with the iron moiety of the enzyme. On the other hand little or no loss in the activity of the enzyme to dye oxidation by fumarate - the reverse reaction of succinic dehydrogenase - was noted. The electron donors used were reduced flavin mononucleotide or leucodimethylsafranin. (Singer, et al., 1957). This fits in with a different level of action of the two classes of dye, and is illustrated diagrammatically in Figure IV.1.

\[
\begin{align*}
\text{Succinate} & \xrightarrow{FP} \text{NHI} \xrightarrow{(\text{Fe(CN)}_6)^{''}} O_2 \\
\text{fumarate} & \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad \q
A further pointer to the arrangement of components in this region of the respiratory chain is obtained from experiments using other artificial electron acceptors. Methylene blue and dichlorophenolindophenol, which, in the intact system are generally considered to react with succinate dehydrogenase, unlike iMS and ferricyanide, do not react with the soluble enzyme. These unreactive electron acceptors may be reacting with ubiquinone or a further component between the flavoenzyme and the quinone, in the intact electron transfer system. This is illustrated diagrammatically in Fig. IV.2.

The possibility of the existence of such a compound and its nature is discussed in the 'Results and Discussion' section of this chapter.

A role for non-haem iron as an electron carrier in the succinate dehydrogenase segment of the respiratory chain has now been fairly conclusively demonstrated.
Although Massey (1957) was unsuccessful in an attempt to demonstrate reduction in the steady state oxidation-reduction level of non-haem iron on addition of succinate he was unwilling to rule out a rôle for metal as an electron carrier on this evidence alone. He suggested that the rate of oxidation of the iron by the next component in the sequence, might be fast enough to prevent any significant reduction.

Since then however Beinert has used the technique of electron paramagnetic (EPR) spectrometry to demonstrate the reduction of non-haem iron in the mitochondrion (Beinert and Sands, 1959, 1960; Sands and Beinert, 1960; Beinert and Lee, 1961). This technique is based on the observation (Zavoisky, 1945) that magnetic moments associated with unpaired electrons in paramagnetic substances give rise to a resonance absorption in a magnetic field. Any such substance produces an absorption with a characteristic 'g' value ('g' is gyromagnetic ratio = electron spin/magnetic moment) and this value is characteristic (but not unique) of a particular radical in a particular environment. Transition elements give rise to such an absorption and the reduction of iron can be studied by this technique. Sands and Beinert (1960) demonstrated a signal at g = 4.3 caused by ferric iron which could be
reduced by NADH₂. Beinert and Lee (1961) showed another signal at $g = 1.44$ caused by a component which could be reduced by succinate and NADH₂. They concluded that this was due to iron being reduced at a site other than that yielding the $g = 4.3$ signal. They also observed that this was reduced concomitantly with flavin and copper. Each of these signals could be reversed by oxygen or ferricyanide. This was convincing evidence that non-haem iron could undergo oxidation-reduction.

More recently, Rieske et al. (1964a) have demonstrated an EPR signal at $g = 1.40$ in the ubiquinone-cytochrome $c$ reductase segment (complex III). This signal, on purification and further segmentation of the chain, became distributed in exactly the same manner as a non-haem iron protein found in the complex. They demonstrated that this component could be enzymically reduced by ubiquinol or oxidised by ferricytochrome $c$, and tentatively determined a standard redox potential for the component of $0.18V$.

Another approach which has been used in the investigation of the role of metals in electron transfer has been the use of metal chelating agents. These have been used in the hope that they will chelate the protein bound non-haem iron of the electron transfer system and prevent its oxidation-reduction. If the iron were
### Table IV.2 Effect of chelating agents on the succinate oxidase activity of mouse kidney mitochondria.

*After Ames et al. (1946).*

<table>
<thead>
<tr>
<th>Chelating agent</th>
<th>Concentration</th>
<th>%Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-hydroxyquinoline</td>
<td>$1.4 \times 10^{-4}$ M</td>
<td>5</td>
</tr>
<tr>
<td>Sodium thioglycollate</td>
<td>$2.0 \times 10^{-3}$ M</td>
<td>25</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>$2.0 \times 10^{-3}$ M</td>
<td>61</td>
</tr>
<tr>
<td>Sodium pyrophosphate</td>
<td>$2.0 \times 10^{-3}$ M</td>
<td>43</td>
</tr>
<tr>
<td>αα dipyridyl</td>
<td>$2.0 \times 10^{-3}$ M</td>
<td>37</td>
</tr>
</tbody>
</table>
involved in the direct sequence of electron transfer
this should result in an inhibition of enzyme activities.

The first systematic study of the effects of chelating agents on electron transfer was made by Ames, et al. (1946) who showed that the succinate oxidase activity of mouse kidney mitochondria was inhibited by the chelating agents shown in Table IV.2

Using his succinate dehydrogenase preparation, Singer et al., (1958) found differences in the inhibitory powers of chelating agents depending upon whether the enzyme was the 2 or 4 atoms of iron variety. The aged enzyme which contained only two atoms of iron was unaffected by any of the chelating agents he investigated.

On the other hand, the four iron atom enzyme could be inhibited by β-globulin, α-dipyridyl, 9-oxyquinoline and 1:10 phenanthroline. In agreement with the scheme suggested earlier (Fig. IV.2) the reduction of fumarate by reduced flavinmononucleotide in either of these enzymes was unaffected by any of the chelating agents investigated.

Much later, Tappel (1960) investigated the effect of lipid soluble chelating agents on the reactions of the respiratory chain. He found that the most efficient metal chelating agent inhibitors of succinate oxidation
were 2-thienoyltrifluoroacetone and 4.7.diphenyl 1.10 phenanthroline. He did not attempt to locate their sites of action. One of the more interesting features to emerge from this work is the differential effects of chelating agents on the succinate and NADH\textsubscript{2} oxidase systems. The two chelating agents investigated in most detail in this study (TTA and 7 Iodo-5.chloro-oxine) react quite strongly with the succinate dehydrogenase system but are relatively ineffective in the inhibition of NADH\textsubscript{2} oxidase. On the other hand 1:10 phenanthroline, which inhibits NADH\textsubscript{2} oxidase has only a small effect on succinate oxidase (Redfearn et al., 1964).

Ziegler (1961) has demonstrated that 30\% of the iron of mitochondrial subfractions can be reduced by succinate, and 25 to 45\% by NADH\textsubscript{2}. These values are practically mutually exclusive as the two substrates could summate to reduce 60–70\% of the non-haem iron. Chance in the discussion of this paper pointed out that this was much lower than the concomitant reduction of ubiquinone, and suggested that not all of the non-haem iron could be implicated in the electron transfer pathway. It thus appears that the non-haem iron entities reducible by NADH\textsubscript{2} or succinate are both spatially and structurally distinct.
Fig. IV.3  The mechanism of iron chelation by thenoyltrifluoroacetone.
By studying the effects of chelating agents on the reactions of the succinate oxidase system it was hoped to demonstrate that the mechanism of their inhibition was, in fact, chelation of the non-haem iron associated with the respiratory chain, to locate the site(s) of action of metal chelating agents in the respiratory chain and to shed some light onto the reactions of ubiquinone with the components of the respiratory chain.

Results and Discussion.

The Structure and chemical nature of Thenoyltrifluoroacetone: \( \beta \)-diketones of the structure

\[
\begin{align*}
\begin{array}{c}
\text{X} - \text{C} - \text{CH}_2 - \text{C} - \text{CF}_3
\end{array}
\end{align*}
\]

have been successfully used for the separation of metal ions in solution (Reid and Calvin, 1950). The mechanism of the separation is selective chelation of one of the metals in solution. The formation of the co-ordination compound with ferric iron is illustrated in Fig. IV.3.

The function of the trifluoromethyl radical is to increase the acidity of the enol form without destroying the resonance of the chelate form. This is the probable mechanism of the inhibition of succinate oxidase activity in a heart muscle preparation by 2-thenoyltrifluoroacetone (4,4,4-Trifluoro-\( \beta \)-Thienyl)-1,3-butanedione,
Fig. IV.4  Effect of thenoyltrifluoroacetone concentration on the activities of succinate oxidase and succinate-phenazine methosulphate reductase in a heart muscle preparation.

'A' is succinate oxidase.

'B' is succinate-phenazine methosulphate reductase.
Fig. IV.3) with the exception that the mitochondrial iron, which can undergo chelation is certainly protein bound.

Preliminary experiments showed that succinic oxidase activity was inhibited to a much greater extent than was succinate phenazine methosulphate reductase activity (Fig. IV.4).

A concentration of 1 mM TTA, for instance produced an 80% inhibition of succinate oxidase but only a 25% inhibition of FMS reductase. This concentration was chosen as convenient to study the effect of the inhibitor on succinate oxidation reactions because of the wide difference in effect on these two systems, and because there is only a small amount of variation in inhibition with quite large changes in conditions.

In an attempt to locate the site of action of the inhibitor, the effect of 1 mM TTA on the various partial reactions of the succinate oxidase system of a pig heart muscle preparation was investigated. In general this was done by measuring the reduction of artificial electron acceptors, known to react at different loci along the respiratory chain, in the presence and absence of 1 mM TTA. The inhibitor was preincubated with the enzyme for five minutes at 37°C before the enzyme assays were commenced. The inhibitor was added in a small
Table IV.3  The effect of thenoyltrifluoroacetone on some manometrically measured enzymic activities of a heart muscle preparation.

Enzyme activities measured as described in Chapter II.
quantity (not more than 0.03 ml. of redistilled methanol). The results obtained in those systems which could be measured manometrically at 37°C are detailed in Table IV.3.

These results show that cytochrome c oxidase activity is unaffected by TTA. This means that the inhibitor has no effect on the copper present in the cytochrome oxidase region of the respiratory chain, and claimed by Green (1961a) to play an oxidation-reduction role in this region of the respiratory chain. The most dramatic observation is confirmation of the original observation that there is a large difference between the inhibition of the succinate oxidase and succinate phenazine methosulphate reductase activities. This suggests that there are at least two sites of action of TTA in the succinate oxidase system, which coincide with the site of non-haem iron deduced from Singer's observations (p. 82) and the site 'x' in Fig. IV.2. It is probable then that non-haem iron exists associated with the flavin and also independently as the next component in the series. The pattern of inhibition is depicted in Fig. IV.5.

\[ \text{Succinate} \quad \barwedge \quad \barwedge \quad \rightarrow \quad O_2 \]

Fig IV.5
A less significant difference in the extent of inhibition of succinate oxidase and succinate methylene-blue reductase activities occurs. This suggests a site of inhibition on the oxygen side of the methylene blue reductase point of reaction. The scheme in Fig. IV.6 shows the proposed sites of action.

It is also of considerable interest with respect to ubiquinone function, to note that ubiquinone mediated methylene blue reductase and methylene-blue reductase itself are inhibited to similar extents. This indicates that the electron acceptor and the quinone are acting at either adjacent or identical points.

It is now possible to clarify the picture a little more according to the accepted sites of action of methylene blue and phenazine methosulphate with the respiratory chain. (Fig. IV.7.)
Table IV.4. Effect of thenoyltrifluoroacetone on some spectrophotometrically and potentiometrically measured enzyme activities of a heart muscle preparation.

<table>
<thead>
<tr>
<th>Enzyme System</th>
<th>No. of expts.</th>
<th>% inhibition by $10^{-3}$ TTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate-cytochrome c reductase</td>
<td>3</td>
<td>83</td>
</tr>
<tr>
<td>Succinate-indophenol reductase</td>
<td>3</td>
<td>73</td>
</tr>
<tr>
<td>Succinate-indophenol reductase + ubiquinone-(15)</td>
<td>3</td>
<td>76</td>
</tr>
<tr>
<td>Succinate-indophenol reductase + PMS</td>
<td>3</td>
<td>21</td>
</tr>
<tr>
<td>Succinate-ferricyanide (0.2 mM) reductase</td>
<td>6</td>
<td>69</td>
</tr>
<tr>
<td>Succinate-ferricyanide (0.6 mM) reductase</td>
<td>6</td>
<td>30</td>
</tr>
<tr>
<td>NADH$_2$ oxidase</td>
<td>3</td>
<td>12</td>
</tr>
</tbody>
</table>
It is not possible to compare directly the inhibition by TTA of enzyme systems measured manometrically at 37°C with those measured spectrophotometrically at room temperature (also using a considerably smaller enzyme concentration). However the effects of 1 mM TTA on these latter reactions can be compared with each other to see if the results are compatible with the scheme previously compiled from the manometric determinations. A series of assays were consequently performed spectrophotometrically (with the exception of ferricyanide reductase - see Chapter III) using a variety of artificial electron acceptors. The results obtained are detailed in Table IV.4.

Using these systems a very similar picture to that previously noted is observed, as indophenol is generally considered to react with the electron transfer chain at the same site as methylene blue, and at the higher concentration, ferricyanide is most probably reacting at the same site as PMS (see Chapter III). At the lower concentration of ferricyanide the probable site of action is in the neighbourhood of cytochrome c (Whittaker and Redfearn, 1964).

It is probable that the low level of inhibition of the NADH oxidase system represents inhibition occurring
Fig. IV.8  The probable sites of reaction of thienyltrifluoroacetone in relation to the reactions of some artificial electron acceptors with the respiratory chain.
Table IV.5  Effect of trifluoroacetylacetone on the partial reactions of succinate oxidase.

Measurement of enzymic activities as described in Chapter II.

<table>
<thead>
<tr>
<th>Enzyme system</th>
<th>No. of Expts.</th>
<th>% Inhibition by 1 mM TFAA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate oxidase</td>
<td>5</td>
<td>31</td>
</tr>
<tr>
<td>MyB. reductase</td>
<td>3</td>
<td>23</td>
</tr>
<tr>
<td>M.B. reductase + UQ-(15)</td>
<td>3</td>
<td>24</td>
</tr>
<tr>
<td>PMS reductase</td>
<td>3</td>
<td>10</td>
</tr>
</tbody>
</table>
Table IV.6  
**Effect of compounds related to thenoyltrifluoroacetone on the succinate oxidase activity of a heart muscle preparation.**

The compounds tested were added in ethanolic solution.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl acetone</td>
<td>$10^{-2} \text{ M}$</td>
<td>0</td>
</tr>
<tr>
<td>Acetyl thiophen</td>
<td>$10^{-2} \text{ M}$</td>
<td>0</td>
</tr>
<tr>
<td>Benzoyl acetone</td>
<td>$10^{-3} \text{ M}$</td>
<td>49</td>
</tr>
<tr>
<td>Thienoyl acetone</td>
<td>$10^{-3} \text{ M}$</td>
<td>38</td>
</tr>
<tr>
<td>Trifluoroacetophenone</td>
<td>$10^{-2} \text{ M}$</td>
<td>0</td>
</tr>
</tbody>
</table>
at one site only, this is possibly at the third site of action in the succinate oxidase system.

The final detailed diagram illustrating the various acceptors, inhibitors and added factors, with the respiratory chain is shown in Fig. IV.8.

The effect on succinate oxidation of compounds structurally related to TTA.

**Trifluoroacetylacetone.**

This compound is, apart from the lack of a 2-thienyl residue, identical to TTA. It chelates iron in a similar fashion to TTA. The dissociation constants of the two complexes are comparable. Trifluoroacetylacetone inhibits the same reactions as TTA but to a proportionally smaller extent. This is shown in Table IV.5.

The inhibition produced by trifluoroacetylacetone at $10^{-3}$ M is between 34 and 43% of that produced by an equivalent amount of TTA. The difference probably arises in the readiness with which chelates are formed. The thiophen ring serves to lower the basic strength of the diketone ion because of its electrophilic nature, thus making co-ordination more likely.

Table IV.6 shows the effect of other related compounds on succinate oxidase activity.

Acetyl acetone had variable effects on the reactions
inhibited by TTA. It was at first considered that this compound had the effect of stimulating oxygen uptake of phenazine methosulphate and methylene blue reductase determinations. However this was caused by a non-enzymic gaseous uptake caused by the reaction of the electron acceptor with acetyl acetone. No consistent effect on succinic oxidase was observed. This is because only a very unstable complex is formed between acetyl-acetone and iron. Acetyl thiophen represents the thienyl portion of the TTA molecule and has no effect on succinate oxidase activity. This is because this compound is not a metal chelating agent. Benzoyl acetone and thenoyl acetone both chelate iron in a similar manner to TTA and cause some inhibition of succinate oxidase activity. The fact that benzoyl acetone is an inhibitor disagrees with the work of Tappel (1960) who did not find any significant inhibition. Trifluoroacetophenone was found to be uninhibitory suggesting that the trifluoromethyl radical does not per se contribute towards the inhibition by TTA. This suggests that the mechanism of inhibition is almost certainly metal chelation. The component most likely to be chelated is the non-haem iron of the respiratory chain.
Effect of TTA/iron complex on succinate oxidase: The complex, \((TTA)_3Fe\), was prepared by treating an ethanolic solution of thenoyltrifluoroacetone with a large excess of ferric chloride solution. The complex was formed as deep purple precipitate. This was filtered off and washed thoroughly with water. The complex was recrystallised five times from ethanol. The crystals were very small deep red hexagons.

The complex was estimated as 97% pure \((TTA)_3Fe\) based on extinction at 460 μ (Khopkar and De, 1960).

Rather variable results were obtained on treatment of a heart muscle preparation with this compound. On some occasions no inhibition at all occurred, at other times a fairly high level of inhibition occurred but the level was never very consistent.

0.33 mM complex (equivalent to a content of 1 mM TTA) caused about 25% inhibition of succinate oxidase, when a large number of polarographic assays was made. This is much smaller than that caused by 1 mM TTA. However if the inhibitory mechanism is chelation of non-haem iron this result is rather disconcerting, bearing in mind the stability of the complex. If dissociation were to have occurred this inhibition could be caused by \(5 \times 10^{-5}\) M TTA (see Fig. IV.4). This would represent a mere 5% dissociation. To test this theory, it was
decided to carry out a spectrophotometric examination of the complex under the conditions of the determinations. On changing from an ethanolic to a dilute aqueous solution there is a decrease in the $E_{338}$ band (characteristic of the complex) and an increase in $E_{267}$ (characteristic of uncombined TTA) equivalent to roughly ten per cent. Dissociation of the complex producing adequate free TTA to cause the inhibition observed. This suggests that the complex itself has no inhibitory action on succinate oxidase activity.

The most likely reason for the dissociation of such a stable complex in aqueous solution is that the following equilibrium is probably in existence under the conditions employed.

$$(\text{TTA})_3\text{Fe} + 3\text{H}^+ \rightleftharpoons 3\text{TTA} + \text{Fe}^{+++} \rightleftharpoons \text{Fe(OH)}_3 \downarrow$$

Precipitation of only a slight amount of dissociated iron would tend to draw the equilibrium over to the right, leaving free TTA in solution.

The observation that $(\text{TTA})_3\text{Fe}$ is uninhibitory supports the view that the mechanism of inhibition is chelation of non-haem iron.

Formation of the TTA/iron complex in the heart muscle preparation: Several initial experiments were performed to attempt to verify that the TTA/iron complex is formed
Fig. IV.9 Spectrophotometric demonstration of the chelation of iron in a heart muscle preparation by TTA.

A. \[3.3 \times 10^{-5} \text{M TTA against a water blank.}\]

B. \[\text{As 'A' + 1.3mg. of heart muscle preparation protein./3ml. Blank, 1.3 mg./3ml. HMP protein.}\]

C. \[1.2 \times 10^{-5} \text{M (TTA)}_{3}\text{Fe. against a water blank.}\]
when the mitochondria are incubated with TTA. A measure of success was obtained when the complex was extracted with ether or benzene from a TTA treated preparation. These experiments were not wholly satisfactory however, as there was no assurance that iron liberated by the solvent had not caused the complex formation. For a very short period however a Unicam SP 800 recording spectrophotometer was made available and the formation was demonstrated by direct spectrophotometry (Fig. IV.9). The TTA in buffer curve has a maximum at 267 m\(\mu\). When heart muscle preparation is added to the test and blank cells a new large peak occurs with a maximum at 338 m\(\mu\) the absorption maximum of the TTA/iron complex. This is convincing evidence that the inhibition by TTA is caused by iron chelation. The extinction at 338 m\(\mu\) suggests that 26 m\(\mu\)moles/mg. protein of mitochondrial iron have been chelated. This is a higher figure than that obtained by Green and Wharton (1965) for non-haem iron content of mitochondria.

Stability of the mitochondrial TTA/iron complex: Three observations suggest that the complex formed between the non-haem iron of the respiratory chain and TTA is not exceptionally stable. The first point is the fact that
### Table IV.7 Effect of other chelating agents on the activity of succinate oxidase in a heart muscle preparation.

<table>
<thead>
<tr>
<th>Chelating agent</th>
<th>Concentration</th>
<th>Inhibition</th>
<th>Lipid Solubility</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-globulin</td>
<td>0.5 mg./ml.</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Cupferron</td>
<td>$3.3 \times 10^{-3}$ M</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Dithizone</td>
<td>$3.3 \times 10^{-4}$ M</td>
<td>40%</td>
<td>+</td>
</tr>
<tr>
<td>7-Iodo-5-chlorooxine</td>
<td>$2.7 \times 10^{-5}$ M</td>
<td>85%</td>
<td>+</td>
</tr>
<tr>
<td>1,40-phenanthroline</td>
<td>$10^{63}$ M</td>
<td>0</td>
<td>low</td>
</tr>
</tbody>
</table>
spinning down and resuspending the washed particles with buffer will almost completely reactivate succinate oxidase activity. Very similarly dialysis overnight against 0.1 M phosphate buffer, removed the TTA and reactivated succinate oxidase. Also dilution of a preparation incubated with TTA produces an inhibition characteristic of the diluted solution rather than maintaining the inhibition observed prior to dilution.

All these observations point to the fact that the complex formed is not exceptionally stable. The TTA can be dissociated with relative ease from the respiratory chain.

The effect of other chelating agents: The effects of other chelating agents on the succinate oxidase activity of the heart muscle preparation were investigated to ascertain that succinate oxidase activity in this preparation could be inhibited by chelating agents other than TTA. Table IV.7 shows the effects of other chelating agents on succinate oxidase in relationship to the lipid solubility of their iron chelates.

Tappel (1960) also showed a considerable level of inhibition of succinate oxidase activity by the following chelating agents all of which are to some extent soluble in lipid solvents: 2-furoyltrifluoroacetone; 4,7,
diphenyl-2,9, dimethyl 1:10 phenanthroline; 4,7, diphenyl-
1:10, phenanthroline; 2,9, dimethyl-1:10, phenanthroline;
αα-dipyridyl.

The probable reason for what appears to be the necessity of lipid solubility of the chelating agent is that the inhibitor must penetrate the large amount of lipid present in the mitochondrion, in order to reach the sites of action. Green (1961) has shown that mitochondria contain some 25-30% of lipid and even suggests that the reactions of the terminal electron transfer systems occur in a lipid medium.

Inhibition of succinate oxidation by 7, Iodo-5chloro-8 hydroxyquinoline: The extremely strong inhibition of succinate oxidase system by 7, Iodo-5chloro-8 hydroxyquinoline (IC10) deemed it worthy of a further short study to compare its properties with those of TTA especially as these two compounds also shared the property of having little effect on NADH₂ oxidase. The mechanism of iron chelation by this oxine derivative is
Fig. IV.11  Effect of concentration of 7-Iodo-5-chloro-8-hydroxyquinoline inhibition of succinate oxidase.
<table>
<thead>
<tr>
<th>System</th>
<th>Concentration of IC10</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate-MB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>reductase</td>
<td>2.7 x 10^{-5} M</td>
<td>80%</td>
</tr>
<tr>
<td>Succinate-FMS</td>
<td>&quot;</td>
<td>0</td>
</tr>
<tr>
<td>reductase</td>
<td>&quot;</td>
<td>0</td>
</tr>
<tr>
<td>Cytochrome oxidase</td>
<td>&quot;</td>
<td>0</td>
</tr>
</tbody>
</table>

Table IV 8. **Effect of 7-Iodo-5-chloro-oxine on the partial reactions of succinate oxidase activity in a heart muscle preparation.**

IC10 added in ethanolic solution. Enzyme activities measured as described on Chapter II.
illustrated in Fig. IV.10.

The most striking point concerning the inhibition of succinate oxidase by IC10 was the interesting variation of the level of inhibition with the concentration of the inhibitor as shown in Fig. IV.11. There appeared to be a very low optimal concentration to give a maximal inhibition. This was roughly $2.7 \times 10^{-5}$ M. Above this there is a rapid decrease in inhibition from 85-60% on doubling the concentration. Further increase in concentration does not appear to cause an increased inhibition. It is likely that this pattern is caused by the extremely small solubility of the chelating agent in an aqueous medium. Above $2.7 \times 10^{-5}$ M precipitation of the inhibitor probably occurs. $2.7 \times 10^{-5}$ M probably represents a supersaturated solution as this gives the maximum inhibition possible under the conditions of the experiment.

Using similar conditions, the effect of the inhibitor on the partial reactions of succinate oxidase was studied. The results are shown in Table IV:8.

Although IC10 is a powerful inhibitor of succinate oxidase it appears to be more specific in its reaction with the respiratory chain than does TTA. It appears to block only what is postulated as the second site of
Fig. IV.12  Relief of IC10 inhibition by copper sulphate.
Succinate oxidase activity measured polarographically at 20°C.
action of TTA, i.e. that one between the sites of reaction of phenazine methosulphate and methylene blue with the respiratory chain (Fig. IV.7).

An interesting observation (Fig. IV.12) is that the inhibition by IC10 can be partially overcome by the addition of a small amount of copper sulphate (0.2μ moles). This amount of copper causes roughly 50% diminution of the amount of inhibition. The copper relieves the inhibition by forming a complex with the chelating agent preventing co-ordination with the non-haem iron of the chain. A further addition of copper caused inactivation presumably by the heavy metal effect on the sulphhydril groups of the mitochondrial protein. Several attempts have been made to reactivate the TTA inhibited heart muscle preparation in this manner but these were unsuccessful - possibly due to the instability of complex at pH 7.4.

Conclusions.

The large number of metal chelating agents that will inhibit the succinate oxidase system of the heart muscle preparation, and the fact that closely related compounds which are not chelating agents have no effect, leave very little doubt that the inhibition by these
compounds is due to chelation of metal components essential to the uninterrupted transfer of electrons along the electron transfer chain from succinate to oxygen. The most abundant metals in the mitochondrion which can undergo oxidation and reduction are copper and iron. The copper is confined to the cytochrome oxidase portion of the chain and as this is unaffected by any of the metal chelating agents examined, it is unlikely to be copper chelation which causes the inhibition.

Iron is present in the mitochondrion in two major forms. The first form, haem iron, is associated with the haem residues of the cytochromes, the second form is protein bound iron and does not affect cytochrome oxidase activity. Initially, therefore, by a process of elimination, it can be deduced that the action of TTA is on protein bound non-haem iron. The results presented here have added weight to this deduction.

A large proportion of the non-haem iron is located in the succinate-ubiquinone reductase region of the respiratory chain. (Doeg and Ziegler, 1962.) This is, hardly surprisingly therefore, where most of the inhibition caused by metal chelating agents is located. The region between the flavoprotein (succinate dehydrogenase) and the cytochrome system is the least resolved portion
of the electron transfer pathway. It contains the components non-haem iron, ubiquinone and cytochrome b, all of which have failed to show the relatively uncomplicated kinetics displayed by the other members of the respiratory chain (Massey, V. 1957, Redfearn and Pumphrey 1960, Chance and Redfearn, 1961, Chance and Williams, 1956).

This investigation was undertaken partly in the hope of simplifying the picture of the order of reaction of components in the electron transfer sequence in this region. The picture which has emerged, however, if true, presents a more complicated picture than was visualised in the first place.

The probability has been shown of at least two, and most probably three sites for non-haem iron in the succinate oxidase system. All of these sites are sensitive to TTA action but only one to ICIO action, and in fact all three are insensitive to other chelating agents (e.g. cupferron, β-globulin). Different proteins binding the iron at each site of its action could be responsible for the different reaction of the sites to chelating agents.

It is now possible to speculate as to the involvement of non-haem iron in the succinate oxidase system.
The proposed sequence is illustrated in Fig. IV.13.

![Diagram](image)

Cytochrome b has been omitted from the scheme because no new data on its position has been presented and its position is still uncertain. This scheme would make the reactions in this region of the respiratory chain:

1) \( \text{FpH}_2 + 2\text{Pr.} \text{Fe}^{++} \rightarrow 2\text{Pr.} \text{Fe}^{++} + \text{Fp} + 2\text{H}^+ \)
2) \( 2\text{Pr.} \text{Fe}^{++} + 2\text{Pr.} \text{Fe}_b^{++} \rightarrow 2\text{Pr.} \text{Fe}^{++} + 2\text{Pr.} \text{Fe}_b^{++} \)
3) \( 2\text{Pr.} \text{Fe}_b^{++} + \text{UQ}_{\text{one}} + 2\text{H}^+ \rightarrow 2\text{Pr.} \text{Fe}_b^{++} + \text{UQ}_{\text{ol}} \)
4) \( \text{UQ}_{\text{ol}} + 2\text{Pr.} \text{Fe}_c^{++} \rightarrow \text{UQ}_{\text{one}} + 2\text{Pr.} \text{Fe}_c^{++} + 2\text{H}^+ + 2e \)
5) \( 2\text{Pr.} \text{Fe}_c^{++} + 2\text{cyt.} \text{c}_1 \text{.Fe}^{+++} \rightarrow 2\text{Pr.} \text{Fe}_c^{++} + 2\text{cyt.} \text{c}_1 \text{.Fe}^{++} \)

The reason for non-involvement of the NADH\(_2\) reduced non-haem iron in TTA chelation is rather surprising considering the extent of the inhibition of succinate oxidase. It can only be pointed out once again that 1:10 phenanthroline is a potent inhibitor of this system and is relatively inactive in the succinate portion of the chain. It is quite obvious that the nature of
the protein binding the iron and the amount of protection afforded to the iron by either the protein structure or the lipid matrix in the physical region of its involvement determines the susceptibility of each site to co-ordination with a particular chelating agent.

The interactions of ubiquinone with the respiratory chain components acting in this region appear to be more complicated than previously imagined. If ubiquinone is on the direct pathway of electron transfer, as the argument in this chapter has assumed, it is apparently reduced by and reoxidised by different species of non-haem iron. On the other hand it is not yet certain where ubiquinone is acting. Redfearn (1961, 1961a) has detailed the three most likely methods of interaction of the quinone with the respiratory chain. The actual interaction with non-haem iron will, of course, depend upon which of these mechanisms is the correct one, but the data presented here do not help in the decision as to which one is.

The pathway preferred by Redfearn is the one in which ubiquinone is located on a branch pathway, linking the flavoprotein with a site on the substrate side of the Antimycin A sensitive region. The data presented here, in this circumstance, would place the sites of action...
of non-haem iron before the bifurcation and after the rejoining of the pathways (Fig. IV.14)

The level of inhibition in this case would be independent of whether electron transfer was via the direct or branch pathway.
CHAPTER V
THE STEADY-STATE OXIDATION-REDUCTION LEVELS OF MITOCHONDRIAL UBIQUINONE UNDER DIFFERENT METABOLIC CONDITIONS.

Introduction
The discovery (Crane et al., 1957, Pumphrey et al., 1958) that ubiquinone in submitochondrial heart muscle particles underwent oxidation on exposure of the preparation to oxygen and reduction on treatment with substrate and cyanide, suggested a role for ubiquinone in electron transfer processes. Most of the early work on ubiquinone was done on non-phosphorylating submitochondrial particles and although the results have implicated ubiquinone in electron transfer, the role of the quinone has never been conclusively demonstrated. Crane et al., (1957) were of the opinion that the ubiquinone of beef heart mitochondria was reduced by succinate but not NADH₂ and the reduction was antimycin A sensitive. Pumphrey et al., (1958) and Pumphrey and Redfearn (1959) showed that both succinate and NADH₂ reduced ubiquinone in a heart muscle preparation and that the reduction was insensitive to antimycin A. These results were confirmed by Chance and Redfearn (1961) who demonstrated
the antimycin insensitivity of the reduction using a double beam spectrophotometer, and Hatefi et al., (1959) who, at this stage, still held that the quinone reduction was antimycin sensitive, but conceded that NADH₂ could also reduce ubiquinone. Although its function is uncertain, it is apparent that ubiquinone is an electron carrier in some capacity.

It is possible nevertheless that fundamental changes in the electron transfer system may have occurred in non-phosphorylating particles. The problem of the reactions of ubiquinone in phosphorylating mitochondria was first investigated by Hatefi (1959), who measured the oxidation-reduction level of ubiquinone in phosphorylating beef heart mitochondria. Lardy and Wellman (1952) had previously demonstrated that the optimal respiratory rates only occurred when inorganic phosphate and a phosphate acceptor system (e.g. ADP, AMP, hexokinase system) are present. Chance and Williams (1956) examined the oxidation-reduction levels of the components of the electron transfer system in the state where these conditions were fulfilled (active state) and in the state when no phosphate acceptor system was present (controlled state). NAD, flavin and cytochromes b and c show a shift towards increased oxidation on transition from the
Hatefi (1959) investigated the reaction of ubiquinone from this viewpoint. He measured the ubiquinone oxidation-reduction level by heat denaturation and lipid solvent extraction of the quinone. He measured the changes occurring at 275 nm and suggested that both NAD and ubiquinone showed a shift towards greater reduction on addition of either phosphate or arsenate to the reaction mixture. This was not a general anion effect because sulphate, chloride, nitrate, borate or pyrophosphate had no effect. The results of Chance and Williams (1956) had not shown this. Addition of ADP or another phosphate acceptor system reversed the reduction caused by phosphate but not the one caused by arsenate. He suggested that this dual effect was caused by formation of a high-energy phosphate before transphosphorylation of ADP.

Redfearn and Pumphrey (1960a), using a technique involving denaturation by ice-cold methanol and petrol extraction, examined the oxidation-reduction levels of ubiquinone in different metabolic states of rat liver mitochondria. They found that between 10-20% of the intramitochondrial ubiquinone appeared to be enzymically inactive. Omission of ADP from the reaction mixture always resulted in an increased reduction of ubiquinone,
qualitatively similar to the results of Hatefi (1959). On the other hand, and in contrast to Hatefi (1959), when inorganic phosphate was omitted and replaced by tris-HCl buffer there was an increased reduction of the same magnitude as that brought about by the omission of ADP. The average decrease in steady state reduction of ubiquinone on transition from controlled to active state was 66% reduced to 46% for NAD linked substrates and 84% to 78% for succinate. Both Hatefi (1959) and Redfearn and Pumphrey (1960) suggested that on the basis of their results ubiquinone might form a high-energy intermediate of oxidative phosphorylation.

It was decided to use this technique to investigate the effects of inhibitors and uncoupling agents on the redox state of ubiquinone in rat liver mitochondria. Certain modifications of the method used previously (Pumphrey and Redfearn, 1960; Redfearn and Pumphrey, 1960) are described.

Since the majority of this work was performed, Szarkowska and Klingenberg (1963) have challenged the validity of the extraction technique for measuring oxidation-reduction states of ubiquinone, although Szarkowska and Drabikowska (1963) have since used the technique to measure ubiquinone reduction by glycerol-
1-phosphate. These criticisms are discussed in light of further investigation of the extraction technique.

**Experimental.**

**Ubiquinone/Ubiquinol determination.** The technique used to determine the oxidation-reduction state of the mitochondrial ubiquinone, involved slight modifications of the method described in detail by Pumphrey (1960).

The incubations were performed in a 10 ml. glass-stoppered test-tube at 20°C. In order to reduce the possibility of reaction after methanol addition and before complete denaturation has taken place, the flask containing the methanol (1 mg./ml. pyrogallol) was surrounded by crushed solid CO₂. The temperature of the methanol was usually in the region of -60°C. The reaction mixture was normally made up to 2 ml. 4 ml. of the -60°C methanol/pyrogallol was quickly added from a syringe with only glass and metal accessories. The resulting temperature was always in the region -10° to 0°C. The mixture was rapidly agitated for 1-2 secs. on a Vortex Junior mixer and 5 ml. of 40-60°C boiling range, light petroleum was added immediately. The mixture was thoroughly shaken for 1 min. on the Vortex mixer and the petrol and aqueous layers were separated by a short spin on a Martin Christ Junior centrifuge. The petrol layer was transferred, using a Pasteur pipette, to
another stoppered tube. The aqueous layer was extracted with another 3 ml. of the light petroleum. The extracts were bulked and shaken with 3 ml. 95% methanol for 30 secs. The volume of added methanol was increased from 2 to 3 ml. when it was found that increased turbidity occurred on addition of sodium borohydride to the ethanolic solution used for spectrophotometric assay (see also Pumphrey, 1960). This partition removes phospholipids which are relatively soluble in methanol and leaves ubinuinone, which is not, in the petrol layer. The petrol was removed from the methanol water layer with a Pasteur pipette, and evaporated to dryness in a 10 ml. beaker in a vacuum dessicator. Heating of the extract was avoided to minimise ubiquinol autoxidation. The lipid residue, usually of a yellowish colour, was immediately dissolved in spectrscopically pure ethanol. Complete solution was ensured by gentle warming to about 30-40°C.

The ultra-violet absorption spectrum of the ethanolic solution was measured immediately on a Unicam SP 500 or an Optica CF 4 recording spectrophotometer. A single crystal of sodium borohydride was added to the solution in order to reduce the ubinuinone to ubiquinol, and the spectrum was measured again after 1 minute. The spectra
<table>
<thead>
<tr>
<th>Oxidation technique</th>
<th>Apparent ubiquinone concentration (µ moles/gm.protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.41 mg/ml. FeCl₃ to petrol layer</td>
<td>1.69</td>
</tr>
<tr>
<td>0.20 mg./ml. K₃Fe(CN)₆ to petrol layer</td>
<td>1.65</td>
</tr>
<tr>
<td>5.0 mg/ml. NaOH preincubation</td>
<td>1.55</td>
</tr>
<tr>
<td>0.25 mg/ml. HAuCl₃ to petrol layer</td>
<td>1.87</td>
</tr>
</tbody>
</table>

Table V.1. Apparent ubiquinone concentration of rat liver mitochondria using different oxidation techniques.

Extraction technique described in text.
obtained were similar to those of Fig.18 and those of Redfearn (1961c). The ubiquinone concentration of the extract was calculated, using an extinction value, \((\alpha_{\text{ox}} - \alpha_{\text{red}}) 275\text{m}\text{m} = 12,250\) (Pumphrey, 1960). For a long series of assays on the same mitochondrial preparation, only the \(E_{275}\) values of the extract and reduced extract in ethanolic solution were determined.

Measurement of total ubiquinone. The procedure used for measuring total ubiquinone was similar to that described above. In rat liver mitochondrial preparations a part of the ubiquinone is maintained in the reduced form due to the presence of endogenous substrates, so that either before or after extraction the ubiquinone must all be oxidised. The first method used was treatment of the mitochondria with alkali prior to denaturation and extraction, but possibly due to degradation of the quinone with alkali (Lester et al., 1959) the results obtained were rather variable. Table V.1 shows the apparent concentrations of ubiquinone measured by this method and by oxidising the quinone after extraction by ferric chloride, potassium ferricyanide or chloroauric acid. The values obtained were slightly in excess of those found by Redfearn (1961a, 1961c) but lower than those of Szarkowska and Klingenberg (1963).
Table V 2. The steady state oxidation-reduction level of ubiquinone in rat liver mitochondria

Extraction technique described in text.
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Steady state % reduction of ubiquinone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resting State</td>
</tr>
<tr>
<td>Endogenous</td>
<td>46</td>
</tr>
<tr>
<td>Succinate</td>
<td>77</td>
</tr>
<tr>
<td>β-hydroxybutyrate</td>
<td>66</td>
</tr>
<tr>
<td>Choline</td>
<td>77</td>
</tr>
<tr>
<td>Malate</td>
<td>71</td>
</tr>
<tr>
<td>α-ketoglutarate</td>
<td>70</td>
</tr>
</tbody>
</table>

Table V.3. Redox States of Ubiquinone in Tightly Coupled Rat Liver Mitochondria.

R.S. = resting state. Extraction technique as in text.
The figure of 2.00 m moles ubiquinone/g. protein was obtained by the latter workers. The chloroauric acid addition to the petrol layer was adopted for the purposes of this investigation and furnished quite consistent results.

Results

Estimation of the oxidation-reduction level of ubiquinone in different metabolic states of rat liver mitochondria: Chance and Williams (1956) describe the five basic states of phosphorylating mitochondria, in which the components of the electron transfer system maintain characteristic oxidation-reduction, steady state levels. It was decided to investigate the steady state oxidation-reduction level of ubiquinone in these conditions (Table V.2). The results show that although there is more reduction of the quinone in the controlled state, by succinate than by \( \beta \)-hydroxybutyrate, the large reversal of this reduction on transition to the active state, as noted by Chance for cytochromes \( b \) and \( c \), flavin and NAD, only occurs when \( \beta \)-hydroxybutyrate is the substrate. The other significant fact, noted previously by Redfearn and Humphrey (1960a) is that 10-15% of the ubiquinone appears to be enzymically inactive - unable to be reduced or oxidised in conditions favourable to complete reduction or oxidation of electron transfer component. This is
in contrast to the results obtained by Chance and Williams (1956) for the other carriers which are completely oxidised in State 2 and completely reduced in State 5. If the results for States 2 and 5 are considered together it is unlikely that autoxidation of the quinone can account for this apparent, partial, enzymic activity. A partial compartmentation of the quinone appears to be ruled out as addition of $\beta$-hydroxybutyrate and succinate simultaneously, did not significantly increase the amount of quinone reduction that occurred.

In order to get further insight into the reactions of ubiquinone in phosphorylating electron transfer processes of rat liver mitochondria, the effects of uncoupling agents, metabolic inhibitors, and other substrates, on the steady-state oxidation-reduction levels of ubiquinone, were investigated (Table V.3). $\beta$-hydroxybutyrate, $\alpha$-Ketoglutarate and malate are NAD linked substrates. Choline is oxidised by a specific liver mitochondrial flavoprotein which is reoxidised by way of the respiratory chain (Packer et al., 1960). As expected the oxidation of all NAD linked substrates produced similar patterns of oxidation-reduction levels of ubiquinone. Choline oxidation on the other hand, with regard to its influence on ubiquinone steady-states, produced a
Fig. V. 1. The effect of dinitrophenol on the respiratory control and P:O ratios of rat liver mitochondria oxidising β-hydroxybutyrate.
Fig. V.2. **The effect of calcium chloride on the β-hydroxybutyrate oxidase system of rat liver mitochondria.**  

β-hydroxybutyrate oxidase measurement is described in Chapter II.
similar pattern to succinate oxidation. The changes in oxidation level on transition from controlled to active states were observed once again. This change was always greater for NAD linked substrates than for succinate or choline oxidations.

The reaction of ubiquinone to uncoupling agents is of interest. A concentration of 2,4-dinitrophenol producing complete uncoupling produces a quinone oxidation-reduction level generally similar to that observed in the active state. Addition of calcium chloride sufficient to uncouple oxidative phosphorylation produced very variable results, as though the uncoupling procedure was more drastic. The effects of these uncouplers on \( \beta \)-hydroxybutyrate oxidation also emphasise this difference. Dinitrophenol in increasing concentrations causes decreasing p:o ratio and respiratory control ratio. (Fig. V.1). Low concentrations of \( \text{CaCl}_2 \) (Fig. V.2) cause a temporary uncoupling which is quickly overcome in the presence of magnesium. There is little or no alteration in P:o or respiratory control ratios of the resultant activity. An all or none uncoupling occurs at about 5mM \( \text{CaCl}_2 \).

The results of amytal addition in the NAD linked substrate oxidations and the effects of KCN on all the
### Table V 4. Redox states of ubiquinone in frozen and thawed mitochondria.

Extraction technique described in text.
systems support the idea that a part of the quinone is enzymically inactive. Amytal, at the concentration used, caused little change in the oxidation-reduction state of the quinone.

The effects of freezing and thawing of the mitochondria on the changes in steady states of ubiquinone is shown in Table V.4. This treatment appears in some ways to emulate the effects of calcium addition. The resultant reactions to inhibitors appear to resemble those of the heart muscle preparation (Redfearn and Pumphrey, 1960), although even after freezing and thawing a small quantity of the quinone seems to be enzymically irreducible and a small quantity unoxidisable. Uncoupling treatments such as dinitrophenol or calcium chloride additions do not have any effect on the ubiquinone oxidation-reduction levels of frozen and thawed mitochondria.

It was at this stage of the investigation that Szarkowska and Klingenberg (1965) criticised the extraction procedure devised by Pumphrey and Redfearn (1960) on the basis of combined extraction and direct spectrophotometric methods. They found that a far greater oxidation of ubiquinone occurred on transition from the controlled to the active states when measured by the direct spectrophotometric technique rather than by the
chemical extraction technique. They attributed this to the inability of the methanol to catch the active state, this being due to uncoupling of phosphorylation before complete denaturation occurs. It is felt that this criticism is invalid as the results described here approximate more nearly to Klingenberg's spectrophotometric results than do the results of his modified extraction technique. What is more significant is the difference between the succinate and NAD linked substrate results. If uncoupling were to cause an apparent decrease in the redox difference of the quinone between the active and the controlled states this might be expected to occur equally in both systems, whereas the difference in the succinate system was invariably less than the corresponding difference in the NAD-linked substrate oxidase system. The other possibility which it was considered might account for this discrepancy was that as the succinate dehydrogenase system is less sensitive to denaturing treatments than the NADH$_2$ dehydrogenase system, some reduction by succinate might occur after methanol addition and before complete denaturation has taken place. This possibility was investigated in two ways. An attempt was made to prevent any ubiquinone reduction by succinate after methanol addition by adding
Table V 5. **Effect of inclusion of exaloacetate in the denaturation mixture on the apparent steady state oxidation-reduction level of ubiquinone in rat liver mitochondria.**

Extraction technique described in text.
<table>
<thead>
<tr>
<th>Conditions</th>
<th>Denaturation mixture</th>
<th>% Reduction of UQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMP</td>
<td>MeOH/pyrogallol</td>
<td>Taken as 0</td>
</tr>
<tr>
<td>HMP + KCN</td>
<td>&quot; &quot;</td>
<td>8</td>
</tr>
<tr>
<td>HMP + KCN + succinate</td>
<td>&quot; &quot;</td>
<td>94</td>
</tr>
<tr>
<td>HMP + succinate</td>
<td>&quot; &quot;</td>
<td>89</td>
</tr>
<tr>
<td>HMP</td>
<td>MeOH/pyrogallol/succinate</td>
<td>5</td>
</tr>
<tr>
<td>HMP + KCN</td>
<td>&quot; &quot;</td>
<td>6</td>
</tr>
</tbody>
</table>

Table V 6. Demonstration of the lack of enzymic oxidation or reduction of mitochondrial ubiquinone after methanol pyrogallol denaturation.
oxaloacetate simultaneously (Table V.5). It was impossible to demonstrate any increase in redox state difference between the active and controlled states using this technique. A more rigorous test was made by the simultaneous addition of succinate and -60°C methanol/pyrogallol to a heart muscle preparation in which the ubiquinone was in the completely oxidised state (Table V.6). There was no evidence of reduction of the quinone by succinate after methanol addition.

Further attempts were made to isolate the site of ubiquinone action in the phosphorylating electron transfer system. The first idea pursued was to attempt to inhibit electron transfer using quinone reagents which could block oxidation-reduction of quinones. The quinone reagents investigated were hydroxyammonium chloride, ethyl cyanoacetate and benzoyl, phenyl-hydrazone. Each of these compounds displayed some inhibition of succinate oxidase of a rat liver mitochondrial preparation but this inhibition appeared in each case to be much less specific than quinone reaction.

As an alternative to this technique it was decided to attempt to investigate quinone function in a system which had any electron transfer not coupled to phosphorylation blocked, as Redfearn (1961) and Green (1961)
suggested different roles for the quinone in phosphorylating and non-phosphorylating electron transfer systems. This study was prompted by the discovery (Neubert et al., 1963) that NAD caused an inhibition of succinate oxidation in sub-mitochondrial preparations and that this inhibition affected only that part of the succinate oxidation which was not coupled to phosphorylation. They suggest that there is a site in succinate dehydrogenase which binds NAD or one of its derivatives to act as a control mechanism for succinate oxidation. This phenomenon was investigated using a sonically prepared rat liver submitochondrial preparation. A similar inhibition with similar properties to that described by Neubert et al., (1963) was obtained, but the inhibition produced appeared to be due to accumulation of oxaloacetate formed from succinate by succinate and malate dehydrogenases in conjunction with NAD (Keilin and Hartree, 1940). This was deduced from the fact that succinate preincubation was essential for the inhibition, NADH₂ even in the presence of amytal reversed the inhibition and cysteine sulphinic acid, which stoichiometrically removes oxaloacetate by transamination and desulphination (Kearney and Singer, 1956) prevents the inhibition. The phenomenon seemed so complex that investigation
of ubiquinone function in the presence of NAD was abandoned.

Discussion.

It is difficult to criticise the spectrophotometric results obtained by Szarkowska and Klingenberg (1963) without access to the double beam spectrophotometer they used in their assays. It is only possible to suggest that at a wavelength of 275 m\text{\normalfont \textmu}m, where much interference occurs from mitochondrial protein and fumarate production, the possibility that absorption changes are caused by some other component cannot be strictly ruled out. Nevertheless the results described in this investigation approximate more closely to Klingenberg's spectrophotometric results than do his own results obtained by his 'improved' extraction technique. In the heart muscle preparation the chemical extraction technique has been equated to the direct spectrophotometric technique (Chance and Redfearn, 1961).

The demonstration that simultaneous addition of succinate and methanol resulted in no reduction of the oxidised ubiquinone of a heart muscle preparation suggests that the results presented here are not affected by reduction after methanol addition. This result is also supported in the case of rat liver mitochondria by the
finding that oxaloacetate added with the methanol does not result in a lower reduction of ubiquinone in the active state of succinate oxidase, as would be expected if uncoupling occurred at a significant time before denaturation. On the other hand it is possible that oxaloacetate does not act any more quickly than the methanol. However, considering these observations with the consistent reproducibility of the results it is probable that these are valid. It is interesting and encouraging to note that a modification of the extraction technique of Redfearn and Pumphrey (1960), substantially similar to the one used in this investigation, has been used by Hoffmann et al., (1964, 1964a) to determine the level and Redox Quotient of ubiquinone in guinea pig heart in vivo, and has been found to be quite reliable.

The effects produced by inhibitors are generally what one might expect. Addition of amytal in the presence of NAD linked substrates results in about 80-90% oxidation of the quinone. With succinate as substrate amytal has very little affect on the quinone oxidation-reduction level. 4mM amytal causes considerable inhibition of succinate oxidase associated with phosphorylation but little inhibition of uncoupled oxidation (Pumphrey and Redfearn, 1962, 1963). Cyanide
in the presence of any substrate results in about 90% reduction of the quinone due to inhibition of ubiquinol oxidation via the cytochrome system. It is difficult to speculate about the function of the enzymically inactive remainder. In view of the stoichiometric excess of ubiquinone it is probable that ubiquinone has a more refined function than as a straight oxidation-reduction carrier. It may be that a portion of the quinone is associated in some manner with the maintenance of intra- and extra-mitochondrial metabolic balance. If such were the case isolation of the mitochondria might destroy the functional link between this portion and portions directly implicated in the respiratory sequence. Chance (1961) has in fact suggested that the bulk of the ubiquinone is on a side pathway for the reduction of NAD by succinate.

It is equally difficult to explain a change in redox state of ubiquinone in transition from controlled to active states. Chance and Williams (1956) have suggested an explanation for the phenomenon based on their concept of the phosphorylation reaction sequence:-

\[
\begin{align*}
    A & + I \rightarrow AI \\
    AI & + BH_2 \rightarrow B + AH_2 \rightarrow I \\
    AH_2 & \leftarrow I + X \rightarrow AH_2 + X \leftarrow I \\
    X & \leftarrow I + ADP + Pi \rightarrow ATP + X + I
\end{align*}
\]
As has already been described the component 'I' has been introduced to explain the respiratory control phenomenon and by virtue of its function in phosphorylation it acts as a respiratory inhibitor. In the absence of a phosphate acceptor system, \( X \sim I \) and \( AH_2 \sim I \) will accumulate. On the addition of ADP the control of the phosphorylation sequence is removed and the inhibited reduced form of the respiratory carrier is released and can be reoxidised. It is more difficult to imagine the fate under these circumstances of components not directly concerned in the phosphorylation sequence, it is possible that by virtue of an uninhibited oxidation of all the components, they too might show an increased oxidation level on state 4 to 3 transition.

The results presented here suggest that ubiquinone is more intimately involved in the NADH\(_2\) oxidase than the succinate oxidase system. This could imply that in part ubiquinone is concerned in the first phosphorylation site of the NADH\(_2\) oxidase system. Green and Wharton (1963) described the composition of the four segments of the respiratory chain, finding ubiquinone in the three segments (I, III and IV) which are considered to display a phosphorylation site in the intact system. This may be purely coincidental, nevertheless it seems
especially peculiar that ubiquinone should be found in segment IV (cytochrome oxidase) in which up to now there has been no suggestion of an enzymic function for ubiquinone. This leads to the speculative suggestion that at each site of phosphorylation ubiquinone may be involved. Evidence will be presented in Chapter VI suggesting that added ubiquinone may possibly undergo oxidation-reduction in the cytochrome c region of the respiratory chain, although as can be seen from the results presented in this Chapter there is no evidence for any enzymically active quinone after the antimycin A sensitive region.

Uncoupling treatments produce fairly characteristic effects on the oxidation-reduction state of the quinone. Freezing and thawing and calcium treatment are most likely producing a state of affairs in part analogous to the Keilin and Hartree preparation. Dinitrophenol uncoupling is obviously by another mechanism. The postulated mode of action of dinitrophenol (Slater, 1961) as combining with the naturally occurring electron transfer inhibitor 'I', preventing the build up of the inhibited reduced form of the carrier, would result in a metabolic state in at least some respects similar to the active state. This is borne out by the observation that the oxidation-reduction levels of the quinone in both uncoupled and
active states is similar.

The picture of the electron transfer system emerging from this investigation is, unfortunately perhaps, less clear than at the beginning. It is possible that the system is far more complex than previously imagined. The idea of the electron transfer system as a string of components undergoing oxidation-reduction is rapidly becoming outmoded when the structure and interactions of the mitochondrial system are considered. Because of this the interpretations placed on the data presented in this chapter are somewhat speculative and certainly open to criticism. On the other hand the components' interactions are probably so complex that the data are inexplicable on our current concept of the electron transfer system.
CHAPTER VI

THE ACTIVATION OF ELECTRON TRANSFER BY UBIQUINONE IN CYTOCHROME c DEFICIENT MITOCHONDRIAL PREPARATIONS.

Introduction

The reactions of intramitochondrial ubiquinone have already been considered at some length. The ease of extraction of the quinone and the relatively simple synthesis of ubiquinone homologues (Isler et al., 1961; Folkers et al., 1961) meant that the oxidation-reduction of added ubiquinone homologues by mitochondrial systems could be examined in some detail. The most extensive study on the reactions of added ubiquinone has been made by Ramasarma and Lester (1960) using beef heart mitochondria. The ubiquinone homologues were added in ethanol with a trace of iso-octane or cyclohexane to provide sufficient emulsification of the quinone for the reaction to proceed optimally. They found that the added quinone reacted similarly to the endogenous quinone (Redfearn and Rumphrey, 1960) with regard to the effects of inhibitors.

Whilst working on the mediation of succinate-ferri-cyanide reductase by ubiquinone homologues (Chapter III) a stimulation of succinate oxidase activity by ubiquinone
was observed. This reaction of added ubiquinone had not been observed previously and as there was no obvious reason why such a stimulation should occur, it was decided to investigate the nature of this effect in the hope of obtaining a clearer view of the reaction of ubiquinone with the respiratory chain. It has been shown that the stimulation is an activation of the respiration inhibited by the loss of a portion of its cytochrome c during the preparative procedures. This chapter describes the investigation of the mechanism of this activation.

Results

The most surprising feature of this observed stimulation was that it had not been noted before. An investigation of the effect of added ubiquinone-(15) on several heart muscle preparations was made to determine whether this stimulation was merely a feature of the particular preparation in use. This investigation gave the first clue to the nature of the stimulation, as it was found that only preparations which could be stimulated by added cytochrome c could be stimulated by the added quinone. In the hope of enhancing the effect, cytochrome c deficient pig heart muscle submitochondrial
The effect of ubiquinone-(10) and cytochrome c addition to a cytochrome c deficient heart muscle preparation oxidising succinate.

Succinate oxidase measurement is described in Chapter II.
Fig. V I. 2. The effect of ubiquinone-(5) concentration on the stimulation of succinate oxidase of a cytochrome c deficient heart muscle preparation.

Ubiquinone-(5) was added in a small volume (less than 0.03 ml.) of ethanol.
fragments were made by the methods of Tsou (1952) and King (1961a). These were found to display the quinone reactivation effect. Fig. VI.1 shows the effect on succinate oxidase activity, of adding ubiquinone-(10) and cytochrome c to one of these preparations. Added ubiquinone stimulated activity by about 100% (in some preparations this was often as much as 200-250%). After addition of ubiquinone, cytochrome c caused a further stimulation. The reverse did not hold however as ubiquinone and cytochrome c together would not produce a higher activity than cytochrome c alone. This suggested that the two mechanisms were complementary to some extent. Fig. VI.1 also shows that the increased activity was also sensitive to antimycin A. The activity was also found to be sensitive to sodium azide. This eliminates the possibility that the mechanism of the stimulation is autoxidation of ubiquinol after reduction by succinate at some point in the respiratory chain.

The effect of ubiquinone concentration on the stimulation is shown in Fig. VI.2. A maximal stimulation by ubiquinone-(10) was obtained at a concentration of 2 x 10^{-5} M ubiquinone-(10). Above this concentration there is slight inhibition of succinate oxidase activity. Specificity of stimulation: The effect of different homologues of ubiquinone on the stimulation of succinate
Table VI.1. **Effect of ubiquinone homologues on the succinate oxidase activity of a cytochrome c deficient heart muscle preparation.**

Ubiquinone homologues added in a small amount of ethanol.

Succinate oxidase measured polarographically at 20°C as described on Chapter II.

<table>
<thead>
<tr>
<th>Homologue $10^{-4}$M</th>
<th>% Stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ubiquinone-(5)</td>
<td>102</td>
</tr>
<tr>
<td>Ubiquinone-(10)</td>
<td>120</td>
</tr>
<tr>
<td>Ubiquinone-(15)</td>
<td>47</td>
</tr>
<tr>
<td>Ubiquinone-(20)</td>
<td>9</td>
</tr>
<tr>
<td>Ubiquinones-(25)-(50)</td>
<td>0</td>
</tr>
</tbody>
</table>
oxidase activity of a cytochrome c deficient preparation has been investigated. The results are shown in Table VI.1. It was found that ubiquinone-(10) was the most active homologue followed by ubiquinones-(5) and -(15). Homologues above ubiquinone-(20) were inactive. The lower homologues are probably most active because of their higher water solubility than the homologues with longer side chains. It was attempted to cause the stimulation with ubiquinone-(50) by emulsifying the quinone by sonication with the phospholipid, asolectin. Unfortunately it has been impossible to bring about the stimulation with ubiquinone-(50) under any conditions. It is interesting to compare the pattern of stimulations by the different homologues with the data of Fynn and Redfearn (1964) for the adsorption of quinone homologues by heart muscle preparations. These are similar in the fact that the lower homologues are most active and are adsorbed to the greatest extent.

The fact that the lower soluble homologues of ubiquinone were active in the stimulation, whereas the higher, insoluble homologues were not, suggested the idea that the effect might possibly be a non-specific quinone effect. To test this possibility some quinones of variable degree of relationship with ubiquinone were investigated for their effects on the succinate oxidase
Table VI 2. Effect of ubiquinone homologues on the NADH<sub>2</sub> oxidase activity of a cytochrome c deficient heart muscle preparation.

Ubiquinones homologues added in a small volume of ethanol.

NADH<sub>2</sub> oxidase measured polarographically at 20°C as described in Chapter II.

<table>
<thead>
<tr>
<th>Homologue 10&lt;sup&gt;-4&lt;/sup&gt;M</th>
<th>% Stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ubiquinone-(5)</td>
<td>78</td>
</tr>
<tr>
<td>Ubiquinone-(10)</td>
<td>40</td>
</tr>
<tr>
<td>Ubiquinone-(15)</td>
<td>33</td>
</tr>
<tr>
<td>Ubiquinone-(20)</td>
<td>11</td>
</tr>
<tr>
<td>Ubiquinones-(25)-(50)</td>
<td>0</td>
</tr>
</tbody>
</table>
Fig. VI.4  The effect of sodium cholate on the stimulation by ubiquinone-(10) of succinate oxidase in a cytochrome c deficient heart muscle preparation.

Succinate oxidase measured as described in Chapter II.
activity of a cytochrome c deficient heart muscle preparation. Fig. VI.3 shows the various quinones investigated. None of these quinones, other than the ubiquinone homologues showed stimulation of succinate oxidase activity. Lower homologues of vitamin K₂ were inhibitory.

Table VI.2 shows the effects of ubiquinone homologues on NADH₂ oxidase activity of a cytochrome c deficient preparation. The stimulations appear to be smaller than the corresponding ones for the succinate oxidase system, an inactivation of the NADH₂ dehydrogenase component partially balancing the stimulatory effect.

Properties of the stimulation: Fig. VI.4 shows the effect of sodium cholate on the ubiquinone-(10) stimulation of succinate oxidase in a cytochrome c deficient heart muscle preparation. If added before ubiquinone no stimulation occurs and if added after ubiquinone the stimulation is reversed. This treatment has no effect on the stimulation caused by cytochrome c. Another interesting observation was that after freezing and thawing of the heart muscle preparation, no ubiquinone stimulation occurred. These effects were interpreted as the treatments destroying a binding site for the quinone on the mitochondrion. The effect of freezing and thawing possibly accounts for the fact that the phenomenon had
The effect of temperature on the stimulation by ubiquinone-(10) of succinate oxidase in a cytochrome c deficient heart muscle preparation.
Fig. VI. 6. The effect of temperature on the adsorption of ubiquinone-(10) by a cytochrome c deficient heart muscle preparation.
not been noted previously, as some of the early heart muscle preparations used in this investigation were stored at -20°C. This procedure was abandoned when it was found that the frozen preparations tended to sediment extremely quickly.

Another puzzling feature noted early in the investigation of this phenomenon was the inability to obtain a stimulation by ubiquinone-(10) when succinate oxidase was measured manometrically. This was resolved when the manometric technique and the polarographic technique were standardised by performing both at 20°C. A considerable stimulation of succinate oxidase activity was then manometrically observable. This prompted an investigation of the variation of the stimulation with temperature. Fig. VI.5 shows that an optimal stimulation is produced at 20°C. If the preparation was incubated at 37°C for 10 mins. and then returned to 20°C the stimulation did not appear to have been impaired. It was considered that this temperature optimum might be due to variation in absorption of the quinone at different temperatures. To investigate this possibility quinone adsorption was measured over a range of temperatures (Fig. VI. 6) using the method described by Fynn and Redfearn (1964) with the exception that the incubations and centrifugations were performed at controlled
Fig. VI. 7. The effect of cytochrome c on succinate oxidase activity of a cytochrome c deficient heart muscle preparation in the presence and absence of added ubiquinone-(10).

no added UQ (10)

plus $4 \times 10^{-5}M$ UQ (10)
Fig. VI. 8.  Double-reciprocal plot of data presented in Fig. VI. 7.

no added UQ (10)

plus 4 x 10^{-5}M UQ (10)
temperature. An optimal adsorption was observed at 20°C, and the variation in adsorption seemed to be sufficient to account for the large variation in succinate oxidase stimulation with temperature. This is good evidence that the stimulatory quinone must be adsorbed. Furthermore, it was demonstrated that the stimulatory ubiquinone-(10) was adsorbed by showing that stimulation of succinate oxidase persists after repeated centrifugation and washing of the particles with 0.1 M phosphate buffer after preincubation with the quinone.

Possibility of reaction of added ubiquinone at the cytochrome c level: The first possibility that came to mind to explain the stimulation was that the added ubiquinone was by-passing the site left vacant by the removal of cytochrome c. Succinate oxidase activity was measured in the presence of, and absence of a fixed concentration of ubiquinone-(10) (Fig. VI.7). This suggests once again that ubiquinone cannot cause an increase in succinate oxidase activity of a cytochrome c saturated preparation. What is more interesting is the double reciprocal plot of the data presented in Fig. VI.7. This is shown in Fig. VI.8. The two curves cut the ordinate in the same position suggesting that ubiquinone and cytochrome c competitively activate succinate oxidase activity.
Fig. VI. 9.  **Stimulation of ascorbate oxidase by ubiquinone-(20)**

Reaction mixture is as for succinate oxidase (Chapter II) plus \(10^{-2}\)M sodium ascorbate. Succinate and cytochrome c are omitted.
If the mechanism of the stimulation were a by-pass of the cytochrome $c$ site one would expect that the lower homologues of ubiquinone should mediate ascorbate oxidase activity, which reacts via the cytochrome oxidase pathway. Fig. VI.9 shows that ascorbate oxidase is stimulated considerably by ubiquinone-(20) and that this activity is completely sensitive to azide and insensitive to antimycin $A$. Ubiquinones-(5–15) caused an even greater stimulation but in no case were these stimulations completely azide sensitive. On the other hand the stimulation also persists after freezing and thawing. If this mechanism is the correct one it means that the reduction of the quinone is the reaction which is sensitive to freezing and thawing.

If added ubiquinone were to act as a cytochrome $c$ site by-pass in this type of preparation, one would expect that the added quinone would become reduced at two sites in the respiratory chain, the previously defined site before the antimycin $A$ sensitive region and the site at the cytochrome $c$ level. This should result in some degree of antimycin $A$ sensitivity of ubiquinone reduction by succinate contrary to previous findings (Redfearn and Humphrey, 1960). A good deal of difficulty was encountered in the methodology of measuring the
Fig. VI. 10. The effect of antimycin A on the reduction of added ubiquinone-(10) by succinate in a cytochrome c deficient heart muscle preparation.

Reaction mixture contains: KH$_2$PO$_4$/Na$_2$HPO$_4$ buffer, pH 7.4, 0.07M; Na succinate, 10$^{-2}$M; ubiquinone-(10), 2.5 x 10$^{-5}$M; KCN, 2 x 10$^{-3}$M; heart muscle preparation, 0.05 mg. protein. Total volume, 3ml.

Reaction measured spectrophotometrically at 275 μm at 17°C.
Fig. VI.11  The effect of protamine sulphate on the stimulation by ubiquinone-(10) of succinate oxidase in a cytochrome c deficient heart muscle preparation.

Succinate oxidase measured as described in Chapter II.
Cytochrome c added ....... 0.3 mg.
Ubiquinone-(10) added .... 0.1 mg.
Protamine sulphate added . 2.0 mg.
reduction of added ubiquinone spectrophotometrically, as in many of the determinations interference occurred due to increasing turbidity of the suspension on the reduction of the quinone. The results obtained were variable, but on some occasions considerable inhibition of the reduction of ubiquinone-(10) by antimycin A was obtained. In the recording shown in Fig. VI.10, approximately 50% inhibition of the reduction occurred. With a preparation that had been frozen and thawed however, no such inhibition was ever observed. This suggests that in the cytochrome c deficient preparation, ubiquinone-(10) can become reduced after the antimycin A sensitive region and that this reduction is sensitive to freezing and thawing.

It is known that the addition of protamine sulphate to a cytochrome c deficient preparation destroys the potentiality for the preparation to become stimulated by cytochrome c. (Person and Fine, 1960, 1961). The reactivation of a cytochrome c deficient preparation by cytochrome c can, in fact, be reversed by this basic protein. The mechanism of this effect is not certain. The effect of protamine treatment on the ubiquinone stimulation was investigated. (Fig. VI.11). It was found that the ubiquinone stimulation of succinate
Fig. VI.12  **Diagramatic illustration of the inter-chain conductor theory to explain the stimulation by ubiquinone homologues of succinate oxidase in a cytochrome c deficient heart muscle preparation.**
oxidase was unaffected by this treatment. This suggests that either the ubiquinone cannot be removed from the site of reduction, by protamine, in the same way as can cytochrome c, or that the ubiquinone is being reduced and oxidised at a different locus.

Added ubiquinone as an interchain conductor: Because of the apparently conflicting data obtained when considered from the point of view of ubiquinone as a cytochrome c by-pass, an alternative mechanism was looked for. The only other explanation of the phenomenon which could be imagined was that the added ubiquinone was acting as an inter-chain carrier. It was conceived that the added quinone could become reduced by an electron transfer chain which has had its cytochrome c removed and reoxidized by way of the cytochrome system of a chain which has not lost its cytochrome c. If the ubiquinol oxidase portion of the respiratory chain was not the limiting reaction sequence, it is possible that channelling of electrons from several succinate-ubiquinone reductase segments into a single, unblocked ubiquinol oxidase chain could result in the stimulation (Fig. VI.12). This is perhaps the more attractive theory as it involves ubiquinone as an interchain carrier at the site normally attributed to the endogenous quinone. It is
Table VI.3 The effect of ubiquinone-(10) on a cytochrome c deficient rat liver mitochondrial preparations.

Succinate oxidase measured as described in Chapter II.

Additions: cyt. c, 0.3 mg.; ADP, 3 mg.; ubiquinone-(10), 0.1 mg.; 2,4-dinitrophenol, 0.07 mM. \( \Delta Q_2 \) given in \( \mu \text{L} O_2/\text{mg. protein/hour.} \)

<table>
<thead>
<tr>
<th>Addition</th>
<th>( \Delta Q_2 ) Succinate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \mu \text{L} O_2 )</td>
</tr>
<tr>
<td>-</td>
<td>31</td>
</tr>
<tr>
<td>cyt. c</td>
<td>124</td>
</tr>
<tr>
<td>cyt. c + ADP</td>
<td>187</td>
</tr>
<tr>
<td>UQ-(10)</td>
<td>76</td>
</tr>
<tr>
<td>UQ-(10) + ADP</td>
<td>76</td>
</tr>
<tr>
<td>ADP</td>
<td>46</td>
</tr>
<tr>
<td>dnp</td>
<td>80</td>
</tr>
<tr>
<td>dnp + UQ-(10)</td>
<td>124</td>
</tr>
<tr>
<td>dnp + UQ-(10) + cyt. c</td>
<td>237</td>
</tr>
<tr>
<td>dnp + cyt. c</td>
<td>230</td>
</tr>
<tr>
<td>UQ-(10) + cyt. c</td>
<td>140</td>
</tr>
</tbody>
</table>
difficult to devise an experiment that will either confirm or refute this possibility. Nevertheless, two experiments have been performed, the significance of which is open to discussion. Both experiments tried to emulate the conditions of a cytochrome c deficient preparation by partially blocking ubiquinol oxidation. Antimycin A or sodium azide were added to a cytochrome c saturated preparation in a quantity which produced roughly 50% inhibition. Added ubiquinone homologues had no stimulatory effects under these conditions.

Cytochrome c deficient rat liver mitochondria: To determine whether the stimulation of succinate oxidase by added ubiquinone homologues was only a feature of cytochrome c deficient heart mitochondrial particles, the action of ubiquinone-(10) on rat liver mitochondria made deficient in cytochrome c by treatment with saline (Jacobs and Sanadi, 1960) was investigated. Table VI.3 shows that ubiquinone-(10) can cause a similar stimulation in these mitochondria, although this had to be distinguished from the uncoupling effects of ubiquinone homologues (Jacobs and Crane, 1960). Ubiquinone stimulation always occurred even after complete uncoupling with dinitrophenol.
Discussion.

The only previous activation of succinate oxidase activity by ubiquinone noted, is that of the acetone extracted preparation. (Lester and Fleischer, 1959; Redfearn, 1961a). That is not the mechanism of action of the activation under investigation here is clear, as no ubiquinone is removed during the preparative procedures and full activity can be obtained in the absence of ubiquinone if sufficient cytochrome c is present. After eliminating this possibility it was possible to think of only two other explanations of the effect both of which have been outlined in the 'Results' section. Both of these mechanisms require some new way of thinking with regard to ubiquinone function for there has been no concrete evidence previously that ubiquinone can either mediate between electron transfer chains or react at the cytochrome c level. One is led to considering the possibility that this observation has some physiological significance, by the apparently absolute specificity of the stimulation for the ubiquinone molecule. The most amazing fact in this respect is that plastoquinone-(10) which differs in only the three minor substituents of the quinone nucleus (Chapter I) is inactive. It is difficult to understand why the emulsified ubiquinone-(50) is not effective in this stimulation when
ubiquinone-(50) in this state will cause an appreciable stimulation of an acetone extracted preparation. It is obvious that a greater quinone solubility is essential to bring about the reactivation of the cytochrome c deficient preparation.

There does not appear to be any doubt, however, that the stimulatory quinones are adsorbed by the heart muscle preparation as the stimulated activity is maintained after spinning down and washing of the particles. What is more there appears to be some kind of sensitive binding site for the quinone which is reversibly inactivated by a higher or lower temperature than 20°C. This binding site also appears to be irreversibly inactivated by treatment with sodium cholate or deoxycholate or by freezing and thawing.

It has not proved possible to completely resolve the mechanism of this reactivation, but the data accumulated, although not conclusive, favours the idea that the added ubiquinone homologues can react at the level of cytochrome c, by-passing the deficient site. Evidence has been presented that ubiquinol can become oxidised by way of the cytochrome oxidase segment, as ascorbate oxidation is substantially stimulated by the lower ubiquinone homologues. This has never been noted
previously although Jacobs and Crane (1961) have shown that tetrachlorohydroquinone can become oxidised by this pathway, in cytochrome c depleted rat liver mitochondria. Some evidence that added ubiquinone can also become reduced in this region has also been presented, by the finding that the reduction of added quinone by succinate measured spectrophotometrically by the decrease in $E_{275}$ can under some circumstances be partially inhibited by antimycin A. No such inhibition occurs in a frozen and thawed preparation suggesting that it is the mechanism of ubiquinone reduction at this site that is sensitive to freezing and thawing.

The fact that protamine sulphate completely prevents cytochrome c stimulation, with little effect on the ubiquinone stimulation suggests however that this may not be the mechanism of the stimulation and that if such a mechanism were working, it would mean that ubiquinone was becoming reduced at a site positionally different from the site of cytochrome c reduction. The function of any ubiquinone acting as an oxidation-reduction carrier at this level would be rather difficult to define. No evidence has been obtained for any endogenous, enzymically active quinone at this point either in rat liver mitochondria (Chapter V) or heart muscle preparation (Redfearn and Pumphrey, 1960), although Griffiths
and Wharton (1961) have reported that the cytochrome oxidase segment of the respiratory chain contains a concentration of ubiquinone large enough for it to act as a respiratory carrier.

Another difficulty to be overcome if ubiquinone played the part of an oxidation-reduction carrier in this region of the respiratory chain, would be the non-sequential order of component redox potentials that this would involve. The order in this region of the chain would be:

\[ \text{cyt.} c_1 (0.24) \to \text{cyt.} c (0.26) \to \text{cyts. } a + a_3 (0.29) \to O_2 \]

\[ \to UQ (0.098) \]

This can be overcome in the cytochrome c deficient preparation by virtue of the fact that the missing cytochrome c would result in a large percentage reduction of cytochrome c$_1$ which could result in a lowering of its absolute redox potential to well below the potential of the added ubiquinone, which will be largely oxidised, producing favourable conditions for quinone reduction. The redox difference between ubiquinone and the cytochrome \( a + a_3 \) complex, is favourable to the reduction of the cytochrome components and should present no problem in reoxidation of the quinone. The difficulty in ubiquinone reduction could account for the fact that added
ubiquinone never produces the activity produced with cytochrome c alone.

The alternative theory, involving ubiquinone as an inter-chain connector is also extremely interesting and does not present the same difficulties with regard to physiological significance as does the by-pass mechanism. The possibility that ubiquinone is an inter-chain carrier was discussed by Redfearn (1961, 1961a). It is possible that the added quinone could increase the electron flux by such a route. The experiments designed to test this hypothesis, as has been observed, are open to criticism, as the mechanisms of reaction of antimycin A and azide with the respiratory chain are still uncertain. If the inter-chain conductor theory is the mechanism of ubiquinone stimulation, it is by virtue of stoichiometric removal of cytochrome c from some chains and not from others. To produce such a reactivation in a preparation inhibited in its succinate oxidase activity to the extent of about 50% by antimycin A or azide would require a reaction of these inhibitors in a 1:1 stoichiometric fashion with the respiratory chain. If this occurred the resulting situation would be analogous to the cytochrome c deficient preparation and ubiquinone stimulation might be expected. It is possible that these
inhibitors do not react in this manner and consequently
the fact that no ubiquinone stimulation was observed in
these circumstances is only indicative that this is not
the stimulatory mechanism.

The position is not absolutely resolved. It would
be interesting to investigate the cytochrome \( c \) deficient
preparation by double beam spectrophotometry in an attempt
to find a cross-over point between cytochromes \( c \perp \) and \( a \),
due to the inhibitory action of the missing cytochrome
\( c \), which if the mechanism of the reactivation is the
cytochrome \( c \) by-pass, might be expected to disappear on
the addition of the stimulatory ubiquinone homologues.
At present the evidence suggests that the mechanism of
stimulation is a by-pass of the cytochrome \( c \) site, but
the interchain conductor theory cannot be ruled out.
CHAPTER VII

GENERAL DISCUSSION.

The mitochondrial preparations used during this investigation have been Keilin and Hartree type pig heart muscle preparations and rat liver mitochondrial preparations. Although there is no evidence for any important differences in the components of these two systems it seems inevitable that the different preparative procedures cause some deviation from the physiological state, characteristic of each preparation. It is probable that rat liver mitochondria represent a condition as close to that of the physiologically existing mitochondria as is possible by current preparative methods. It seems likely that mitochondria such as these, with P:O ratios approaching the expected values and with good respiratory control ratios will ultimately provide the answer to the phosphorylation reaction sequence and to the mechanism of coupling of electron transfer to oxidative phosphorylation. For studies on the electron transfer system alone however, the presence of these complicating mechanisms is often undesirable. The Keilin and Hartree (1939) pig heart muscle preparation is an ideal preparation from this point of view, as it is relatively easy to prepare and
maintains stable respiratory activity for a period of up to two weeks. After this time bacterial action has rendered the preparation unreliable. When dealing with such a preparation it is important to consider the results in light of what structural and enzymic changes have occurred from the physiological state. The obvious changes which do occur, as seen merely from a study of the enzymic activities which have been lost, are the removal of cytochrome c to some extent, and practically complete removal of NAD and the NAD linked dehydrogenases. Other changes, such as differences in ubiquinone and cytochrome b function only become obvious when the kinetics of the reactions of the components are examined.

In connection with the interaction of the electron transfer components in any of the preparations used by investigators in the electron transfer field, perhaps the two most important problems remaining to be solved are the sequence of the components in the segment of the respiratory chain between succinate and cytochrome c, and the possibility of reactions between distinct electron transfer chains. In this latter respect it is possible that the term 'electron transfer chain' has become too readily used without stopping to consider the possible interactions between individual pathways.
A criticism of Green's postulated elementary particle, discussed at some length in Chapter I, is that very little scope remains on such a basis for inter-chain reactions. If as he claims each particle is a single electron transfer chain, the only means of communication between the chains would be by way of the mesolayer of the membrane structure. However, Green's approach to the study of mitochondrial electron transfer is commendable in so far as he attempts to correlate mitochondrial structure with its enzymic activity. Quite obviously the electron transfer system is a highly ordered system and must be accompanied by a similarly ordered structural organisation.

The knob like structures on the inner surface of the mitochondrial membrane, which were first noted by Fernandez-Moran (1962), must have looked like the answer to a prayer for those investigators who were convinced of the existence of an elementary electron transfer structure. If as Sjöstrand et al. (1964), and Stasny and Crane (1964) consider, these particles are sweated out during the negative staining procedure, the knobs have proved to be a rather disconcerting red-herring. It would be foolish to disregard all the work on this topic, nevertheless, since both Blair et al. (1963)
and Chance and Parsons (1963) have isolated mitochondrial subfractions, with characteristic compositions, which they thought were the knobs. Green's fraction (Blair et al., 1963), in fact, contained particles with almost stoichiometric amounts of the electron transfer components, high specific activity, and with a particle molecular weight of the order of that expected for an elementary particle. Chance's subfraction was a little less ambitious and merely contained cytochromes $c_1$ and $b$ and possibly some substrate dehydrogenases. These observations and the regular size and recurring pattern of the knobs must represent some form of structural organisation of the components on the mitochondrial membrane. Similarly the reaction of the four segments of the respiratory chain (Green and Wharton, 1963) to give a particle indistinguishable from that produced by degradative procedures is ample evidence for the existence of some elementary structure. The picture of mitochondrial structure obtained during this investigation is one of an ordered array of individual but interdependent electron transfer sequences. There does not appear to be much difference between this idea and the oxysome concept suggested by Chance et al., (1963).

The work described in Chapter III defining dual
reaction sites for ferricyanide in the succinate-ferricyanide reductase system of a heart muscle preparation is further evidence for an enzymic difference between rat liver mitochondria and submitochondrial heart particles. Walter and Lardy (1964) are of the opinion that there is only one site of ferricyanide action in the intact rat liver mitochondrion. It is possible that the normal site of action of ferricyanide in the intact mitochondrion is at the cytochrome c level and that disruption of mitochondria exposes a further site for ferricyanide reduction at high ferricyanide concentrations favourable to the reduction of this electron acceptor. This is most probably at the level of the non-haem iron of the succinate-ubiquinone reductase segment of the respiratory chain. There is little evidence that other artificial electron acceptors can act at two sites. Phenazine methosulphate reacts directly with soluble succinate dehydrogenase at such a rate that reaction of this acceptor with a further site is precluded. Methylene blue and dichlorophenolindophenol do not react with the primary dehydrogenase but with a component further along the sequence (non-haem iron?). It is possible that methylene blue might also react with a component after the antimycin A sensitive region as well as at this site
as Thorn (1956) has presented evidence that methylene blue reductase is partially sensitive to antimycin A. Such a site should not distract from the validity of the results presented in Chapter IV.

The study of the components acting after flavoprotein in the succinate oxidase system, has been complicated by the discovery of ubiquinone and the possibility that non-haem iron is a respiratory carrier. The kinetics of non-haem iron oxidation and reduction have not, as yet, been studied in any detail, whilst the kinetics of cytochrome b and ubiquinone reactions have not provided any obvious answer to the problem of the order of electron transfer in this region of the respiratory chain. Chapter IV attempts to clarify the role of non-haem iron as an electron carrier. The results presented do not absolutely eliminate the possibility that non-haem iron chelation causes respiratory inhibition by virtue of the prevention of some other activity than oxidation-reduction of the component. When considered in the light of the data of Beinert and his co-workers (Beinert and Sands, 1959, 1960; Sands and Beinert, 1959; Beinert and Lee, 1961) and Rieske et al., (1964), who demonstrated oxidation-reduction of non-haem iron by electron paramagnetic resonance, it does not seem to be
too great an assumption that prevention of oxidation-reduction of the iron is the inhibitory mechanism.

Three conditions must be fulfilled before iron can be considered to be active as an electron carrier in the direct sequence of electron transfer. The iron must undergo oxidation-reduction in a similar manner to the other components of the system, it must be present in the mitochondrion in an amount comparable to that of the other respiratory carriers, and complete removal of the iron should inactivate the system with reactivation able to be caused by replacing the component. The EPR data shows that the first condition is fulfilled in so far as demonstrating that the non-haem iron does undergo oxidation and reduction, although at present it does not tell us a great deal about the rate of these reactions compared with the rate of succinate oxidation. It has not as yet, been possible to stoichiometrically remove the iron from the mitochondrion, but the condition outlined above has been partially verified by the observation that complete inhibition of electron transfer can be brought about using a metal chelating agent. A further fulfilment of this condition is the observation that iodochloro-oxine inhibition can be reversed by addition of copper to the inhibited particles. Unfortunately
it has not been possible to reverse thenoyl trifluoroacetone inhibition by the same procedure. The other condition is easily satisfied. Green and Wharton (1963) have summarised the data of several workers in their department and describe non-haem iron concentrations as high as or higher than the other respiratory components in the three respiratory chain segments in which work in Chapter IV suggests there are sites of non-haem iron activity. The postulated sites of action also agree with sites postulated for the oxidation-reduction of non-haem iron by EPR studies.

All of these observations lead to the conclusion that different non-haem iron components of the respiratory chain are almost as numerous as the haem-iron cytochromes. The cytochrome species are generally distinguished by the characteristic nature of their haem prosthetic groups. If, as seems likely, however, the non-haem iron is directly protein bound, the distinguishable features of the different non-haem iron species are most likely to be the mode of binding of the iron to the protein, the protein structure and the general mitochondrial environment of the iron. That such differences do occur is apparent from the different reactions of the different non-haem iron species to chelating agents
(Chapter IV) and the inability of succinate to reduce that portion of the iron which is reducible by NADH$_2$ (Ziegler, 1961), although this latter observation might merely be due to spatial separation of the two compartments of a single species. The electron paramagnetic resonance technique seems to be a very promising tool in the study of non-haem iron function, as it appears to be able to distinguish between the different non-haem iron species. It is probable that a refinement of this technique will ultimately produce a picture of the kinetics of oxidation and reduction of the non-haem iron entities of the respiratory chain. What will be more difficult to determine will be the interaction of non-haem iron with other components in this region. Green (1961) thinks that ubiquinone is on the direct sequence of electron transfer, Chance (1961) and Redfearn (1961) think it is not. Chance and Williams (1956) think$^6$ that cytochrome $b$ is on the direct electron transfer sequence, Slater (1958) and Green (1959) do not.

The position of cytochrome $b$ in the respiratory chain has long been a subject of disagreement. Perhaps the most reliable data are the direct spectrophotometric studies of Chance and Williams (1956) who consider that in the phosphorylating electron transfer system cytochrome
b is in the direct sequence. It is possible that a fuller investigation of non-haem iron and ubiquinone function will leave the niche in the respiratory system into which cytochrome b will fall.

Ubiquinone shows peculiar kinetics (Redfearn and Pumphrey, 1960) peculiar steady state oxidation reduction values (Chapter V) and a peculiarly high concentration. On these grounds it is highly unlikely that ubiquinone is merely acting as an electron carrier in the direct sequence of electron transfer. Further evidence has been found recently by Redfearn (1964) which supports this contention. He has extracted heart muscle preparations with acetone to such an extent that no ubiquinone remains which can be identified by petrol extraction (Pumphrey and Redfearn, 1960) or by alkali saponification. (Mervyn and Morton, 1959). These preparations still retain considerable succinate oxidase activity which is sensitive to the usual electron transfer inhibitors.

Unlike non-haem iron, the bulk of the quinone is reducible by succinate or NADH₂, so any scheme devised for electron transfer must incorporate a link between ubiquinone and the succinate and NADH₂ oxidase systems. On the other hand, there appears to be a proportion (10-15%) of the mitochondrial ubiquinone which is enzymi-
Fig. VII.1  General scheme postulated for electron transfer processes in mammalian mitochondria.
cally non-involved in electron transfer processes. Although it is not impossible, it seems unlikely that ubiquinone can be involved in some function which does not entail oxidation-reduction of the quinone. It is more likely that this represents a part of the quinone whose oxidation and reduction has been prevented by some change occurring during the isolation of the mitochondria. A speculative suggestion is that this portion of the quinone might be a means of regulating intra- and extramitochondrial oxidation-reduction levels. It seems possible that the bulk of the quinone is, in the intact system, acting between electron transfer chains to improve the efficiency of the electron transfer array. The scheme shown in Fig. VII.1 is speculative in many ways, but attempts to define the roles of non-haem iron and ubiquinone in the respiratory chain with respect to the other respiratory components. It is possible that the mediatory quinone at the non-haem iron level (UQ<sub>en</sub>) could cope with any partial physiological blockage of the ubiquinol oxidase system by transfer of electrons to a neighbouring chain. It is highly unlikely that a blockage on the scale of that experienced in the cytochrome c deficient preparation is normally encountered and this would result in an overloading of the interchain
conduction system. Added ubiquinone homologues might help cope with such an increased load. This is possibly the mechanism of the effect discussed in Chapter VI. A more likely explanation appears to be the cytochrome c by-pass mechanism also discussed in Chapter VI. This is incorporated in Fig. VII.1, UQex acting as a by-pass or cross link at the cytochrome c level. The physiological significance of such a pathway is doubtful, but cannot be ruled out when the absolute specificity of the stimulation is observed. UQreg represents the postulated ubiquinone fraction which acts as an intra- and extra-mitochondrial mediator which has lost its enzymic connection with the respiratory chain in the isolated mitochondrion.

This scheme could explain cytochrome b kinetics in phosphorylating mitochondria and non-phosphorylating mitochondrial fragments, if the functional linkage between cytochrome b and the respiratory chain were to be destroyed during the uncoupling treatment. This would also agree with the finding (Redfearn, 1961a) that ubiquinone is reduced much more slowly in the phosphorylating respiratory chain than in a non-phosphorylating one. Destruction of the functional link between cytochrome b and the respiratory chain could cause an
increased electron flux through ubiquinone.

Non-haem iron has not yet been postulated as playing any other role in electron transfer processes than as an electron carrier. Some related function cannot be ruled out as yet. Ubiquinone on the other hand, by virtue of its peculiar structure and similarity to vitamin \( K_1 \) has been suggested as an oxidative phosphorylation intermediate. Theoretical considerations by Wessels (1954) and Harrison (1958) and practical experiments by Wieland (1958) and Clark et al. (1961) have shown that a quinol phosphate could be split by oxidation, the released phosphate being transferred to another phosphate to form pyrophosphate. This was important in showing that the energy of a quinol phosphate could be trapped by oxidation in a high energy phosphate compound. In the mitochondrion ubiquinone is the obvious choice for the role of quinol phosphate formation. Ultra violet light irradiation, which destroys ubiquinone, has been shown to inactivate oxidative phosphorylation. Fynn (1962), demonstrated that ubiquinone could not restore the activity although Anderson and Dallam (1959) had shown that vitamin \( K_1 \) could.

Chmielewska (1960) proposed a scheme in which a cyclised quinone can act as a phosphate carrier during
phosphorylation. This could correspond to ubiquromenol or some closely related compound. It was postulated that the reductive phosphate esterification of ubiquinone could give ubiquromenol phosphate, which on subsequent oxidation could transfer the phosphate to ADP and reform ubiquinone. It is tempting to think that the presence of ubiquinone in the three segments of the respiratory chain recognised to have phosphorylation sites in the intact system, is significant in this respect. A multiple function for ubiquinone would certainly agree with the high ubiquinone concentration.

Before the complete picture of the intact electron transfer system can be understood, it may be necessary to wait for the introduction of techniques which can be used to study respiratory function in whole cells, tissues and even intact organs. Hess (1961) studied the reactions of respiratory components in intact Ehrlich ascites tumour cells in relationship to physiological respiratory control mechanisms. He followed the reactions of respiratory components by spectrophotometric, fluorimetric and amperometric methods. Very recently Hoffman et al., (1964, 1964a) have studied the reactions of ubiquinone in intact guinea pig hearts, to different metabolic conditions. Although these investigations did not provide
any startling results it is evident that only the surface has been scraped, of what can be done in this direction. At present methodological limitations are set on the potentiality of these techniques. Until these are overcome the most promising techniques would be to study non-haem iron function by EPR spectrometry and ubiquinone function by a study of the reactions of ubiquinone other than oxidation-reduction by chemical and direct spectro-photometric methods.
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After Green and Wharton (1963).
Inhibition of Succinate Oxidation in a Heart-Muscle Preparation by Thenoyltrifluoroacetone

By P. A. Whittaker and E. R. Redfearn.

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Thenoyltrifluoroacetone [4,4,4-trifluoro-1-(2-thienyl)-1,3-butane-dione], a powerful metal-chelating agent, has been shown to be a potent inhibitor of succinate oxidation in mitochondrial preparations (Zeigler, 1961; Doeg, 1961; Green, 1961). The mechanism of the inhibition was assumed to be chelation of non-haem iron, which was believed to play an essential role in electron transfer.

We have studied the inhibitory action of thenoyltrifluoroacetone and related substances on the succinate oxidase system of heart-muscle preparations in an attempt to elucidate the site of action of the inhibitor and to clarify the role of metals in the mitochondrial electron-transfer system.

At 1 mM final concentration, thenoyltrifluoroacetone inhibited succinate oxidase, 86%; succinate-cytochrome c reductase, 83%; succinate-methylene blue reductase, 67%; succinate–methylene blue reductase mediated by ubiquinone, 68%; succinate–phenazine methosulphate reductase, 23%; succinate–methylene blue reductase mediated by phenazine methosulphate, 27%; cytochrome oxidase activity was not inhibited.

These results suggest that there are at least two sites of action of thenoyltrifluoroacetone. One is before the site of action of phenazine methosulphate (approx. 25% inhibition), and the second is between the sites of action of phenazine methosulphate and methylene blue (approx. 70% inhibition of the residual activity). There may also be a third site of action beyond these points as succinate oxidase was inhibited to a greater extent than succinate methylene blue reductase.

Trifluoroacetyleaceton, which is also a metal-chelating agent, inhibited the same systems similarly but to a proportionally smaller extent. Acetyl thiophen, on the other hand, which is not a metal-chelating agent, did not inhibit these enzyme systems.

Further evidence that thenoyltrifluoroacetone was inhibiting by virtue of its metal-chelating property was obtained by demonstrating that the thenoyltrifluoroacetone-iron complex had no effect on the enzyme activities.

In addition to confirming the essential role of metals in electron transport, these results also emphasize the difference in properties of the particle-bound and soluble forms of succinic dehydrogenase. In contrast to the particle-bound enzyme, the reduction of phenazine methosulphate by soluble succinic dehydrogenase is not inhibited by thenoyltrifluoroacetone (Zeigler, 1961) and non-haem iron does not appear to play a functional role (Massey, 1958). In fact, it may be necessary to investigate in greater detail the relationship between the soluble and particle-bound forms of the enzyme. King (1962) has already shown that phenazine methosulphate reduction is not necessarily a criterion that soluble succinic dehydrogenase will be active in the reconstitution of the respiratory chain.

This work was done during the tenure of a Medical Research Council Scholarship by one of us (P. A. W.).

The Activation of Electron Transport by Ubiquinone in Cytochrome c-Deficient Mitochondrial Preparations

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It has been shown previously that ubiquinone added to mitochondrial preparations undergoes oxidation-reduction reactions similar to those of the endogenous quinone (Ramasarma & Lester, 1960). The possibility that ubiquinone may be acting at more than one site in the respiratory chain has been revealed in experiments in which added ubiquinone homologues stimulated electron transport activity in mitochondrial preparations which were relatively deficient in cytochrome c.

Cytochrome c-deficient heart-muscle preparations were made from pig heart by the methods of Tsou (1952) and King (1961). Addition of ubiquinone homologues to the cytochrome c-deficient preparation resulted in an appreciable stimulation (up to 120%) of succinate oxidase activity. Addition of cytochrome c brought about a further stimulation of activity but only up to a level which could be achieved with cytochrome c alone. The stimulated activity was completely sensitive to antimycin A. Addition of ubiquinone homologues to preparations which showed no requirement for added cytochrome c did not result in an increased activity. The optimal temperature for the effect was about 20°; there was no effect at 37°. Freezing and thawing the preparation or treatment with surface-active agents, such as deoxycholate, abolished the stimulating effect of ubiquinone homologues but did not affect the stimulation by cytochrome c.

The effect was also demonstrated in rat-liver mitochondria in which the cytochrome c had been removed by the procedure of Jacobs & Sanadi (1960).

The most effective ubiquinone homologue was UQ (10) but UQ (5), UQ (15) and UQ (20) were also active; the higher homologues, UQ (25) to UQ (50) were inactive. The activity was probably due to the ubiquinone homologue becoming adsorbed on the mitochondrial particles (Fynn & Redfearn, 1964) since the stimulating effect persisted after repeated centrifugation and washing of the particles.

Other quinones tested included 2-methyl 3,5-dimethoxy-1,4-benzoquinone, 2-methyl-3,6-dimethoxy-1,4-benzoquinone, 2,3-dimethyl-5,6-dimethoxy 1,4-benzoquinone, tetramethyl-1,4 benzoquinone and vitamin K₃ homologues but these were either inactive or inhibitory.

This stimulating effect of ubiquinone homologues, which has also been observed in the NADH oxidase system, appears to be due to the quinone acting in the cytochrome c region of the chain. This possibility was supported by finding that the reduction of exogenous UQ (10) measured spectrophotometrically at 275 mµ was partially inhibited by antinycin A.

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Potentiometric Measurement of Ferricyanide Reduction by Succinate in Mitochondrial Preparations

By P. A. Whittaker and E. R. Redfearn. (Department of Biochemistry, University of Leicester)

The limitations and unreliability of the usual spectrophotometric method for the measurement of ferricyanide reduction by succinate in mitochondrial preparations have been adequately documented (Singer & Kearney, 1957; Estabrook, 1961). The major difficulties are the limited range of ferricyanide concentration which can be employed and the uncertainty regarding the site or sites of interaction of the acceptor with the respiratory chain. To overcome these difficulties, it was decided to explore the possibility of a method for measuring the rate of change of oxidation-reduction potential of the electron acceptor. Potentiometric methods have been used for studying Hill reaction activities in chloroplasts (Spikes, Lumry, Eyring & Wayrynen, 1950; Spikes, Lumry, Rieske & Marcus, 1954) but do not appear to have been used for mitochondrial electron transport reactions.

The apparatus consisted of a platinum and calomel electrode assembly, a millivoltmeter and a potentiometric recorder. The potential/time curves had an S-shape characteristic of the Nernst equation and these were interpreted as described by Spikes et al. (1950).

The rate of reduction of potassium ferricyanide by succinate in a pig heart-muscle preparation (King, 1961) was measured over a range of concentrations of ferricyanide. In the range up to 0·3 mM, there was a steady increase in succinate-ferricyanide reductase activity followed by a fairly flat region between 0·3-0·5 mM. From 0·5 to 0·8 mM there was an almost linear relationship between activity and ferricyanide concentration which could be extrapolated back to the origin. If the experiment was repeated in the presence of antimycin A a fairly linear relationship over the complete range of ferricyanide concentrations was obtained. This corresponded to the extrapolated linear relationship above 0·5 mM found in the first experiment. Subtraction of the two curves gave a curve with a maximum at 0·2 mM; this represents the optimal concentration for activity at a site on the oxygen side of the antimycin A-sensitive component. This site is probably at the level of cytochrome c as already suggested by Estabrook (1961). Above 0·5 mM, the principle site of interaction of ferricyanide is on the substrate side of antimycin A. The reduction of ferricyanide was inhibited by thienoyltrifluoroacetone (1 mM) which indicates that the site of interaction is not at the flavoprotein but at some point after the non-haem iron (Doeg, 1961; Whittaker & Redfearn, 1963). Other experiments have indicated that it acts at or after the ubiquinone site.

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INFORMATION EXCHANGE NO. 1

Oxidative Phosphorylation
and Terminal Electron Transport

FURTHER OBSERVATIONS ON THE ACTIVATION OF ELECTRON TRANSPORT
BY UBIQUINONE (COENZYME Q) IN CYTOCHROME c-DEFICIENT MITOCHONDRIAL

E. R. Redfearn and P. A. Whittaker
We wish to reply to Green and Rieske's criticism (Memo No. 181) of our paper (Memo No. 153) on the activation of electron transport by ubiquinone (coenzyme Q) homologues.

Green and Rieske do not accept the thesis that added ubiquinone may serve as an electron carrier between cytochrome $c_1$ and cytochrome $c$ but favour the view that ubiquinone "increases the turnover efficiency of residual cytochrome $c$ in a cytochrome $c$-limited particle". They do not suggest a mechanism for the latter effect but they put forward five facts which purport to argue against the first possibility.

We shall deal with each of these in turn.

(1) The fact that added ubiquinone does not induce any response which is not duplicated by cytochrome $c$ does not invalidate the concept that the quinone is acting in the cytochrome $c$ region. The observation that ubiquinone cannot duplicate the full restorative effect of cytochrome $c$ could be explained in terms of either an unfavourable equilibrium due to the low redox potential of the quinone compared with that of cytochrome $c$ or to some structural damage which has already occurred during the preparation of the particles. In reply to the third point, we did
not state that ubiquinone is an essential component at this site but merely that added ubiquinone may act as an electron carrier in bridging the gap caused by the deficiency in cytochrome c. The fact "there is not a single instance in the respiratory chain of an essential component that is replaceable by an entirely different member of the chain" is not a convincing argument. In any case, since cytochrome c and ubiquinone are the only components which are readily removable and replaceable it is quite probable that this type of phenomenon is restricted to cytochrome c and ubiquinone.

(2) As we have already pointed out, we do not claim that endogenous ubiquinone can act at the cytochrome c site but merely that added ubiquinone may do so.

(3) We can only repeat that the oxygen uptake of a particle preparation supplemented with ubiquinone-10 and cytochrome c is completely antimycin sensitive.

(4) The low potential of ubiquinone compared with that of cytochrome c does not rule out the possibility of interaction but as already pointed out in (1) it may explain why ubiquinone does not reproduce the full restorative effect of cytochrome c.

(5) Further investigation of the specificity indicates that the reaction is absolutely specific for ubiquinone homologues. The lower homologues of plastoquinone (PQ-10, PQ-15), thymoquinone and hydroxythymoquinone were completely ineffective. If the mechanism suggested by Green and Rieske is correct it is surprising that the plastoquinone homologues,
which have the same lipophilic side-chains as the active ubiquinones, are inactive.

None of the facts presented by Green and Rieske explains two of the most important aspects of the activation phenomenon. These are (i) that the reduction of the added ubiquinone is partially inhibited by antimycin A and (ii) that the activation is not obtained if the particles are frozen and thawed or if they are treated with surface-active agents; these treatments however, have little or no effect on the cytochrome c activation.

The interpretation of our results is admittedly difficult. It could be suggested that the activation of electron transport by ubiquinone homologues represents an additional site of action of the quinone which has become lost during the preparation of the particles. One cannot reject this suggestion out of hand since we do not yet understand the nature of the changes which occur during the preparation of derivative particles from intact mitochondria. Alternatively, the activation may be regarded as a non-physiological process in which a lower homologue of ubiquinone is able to replace partially the function of cytochrome c.

It is difficult to visualize how ubiquinone can increase the turnover efficiency of residual cytochrome c as suggested by Green and Rieske particularly in view of restrictions on chain interaction imposed by the elementary particle hypothesis. At the joint meeting of the Swedish and British Biochemical Societies held in Stockholm in May 1964, Whittaker and Redfearn presented a possible mechanism of the activation
phenomenon based on the assumption that the added ubiquinone homologue acted as an electron conductor between neighbouring respiratory chains and thus increased the effective utilization of the residual cytochrome $c$. This type of mechanism has also been suggested to us more recently by Dr. Peter Nicholls. However, it is difficult to reconcile the results of the antimycin and freezing-thawing experiments with this mechanism and we are compelled to consider the hypothesis that the added ubiquinone can in certain circumstances act in the cytochrome $c$ region of the chain.
SUMMARY.

In this thesis a study has been made of the interaction of the components of the mitochondrial respiratory chain with particular reference to ubiquinone (Coenzyme Q). The results may be summarized as follows:

i) A potentiometric method of measuring the succinate ferricyanide reductase activity of a heart muscle preparation has been described. It has been used to define two sites of reaction of the electron acceptor, potassium ferricyanide with the succinate oxidase respiratory chain. Above 0.6 mM, the reaction of ferricyanide is mainly at the succinate dehydrogenase-non-haem iron level, below this concentration of ferricyanide the main activity is at the cytochrome c level.

ii) Non-haem iron function as an electron carrier has been investigated using metal chelating agents as inhibitors of the enzymic reactions of the succinate oxidase system of heart muscle preparations. Three sites of involvement have been tentatively identified. These agree with those suggested by other workers approaching the problem from different angles. The formation of a metal chelate in heart muscle preparation treated with the chelating agent, 2-thenoyltrifluoroacetone has been demonstrated.
iii) The function of the lipid cofactor, ubiquinone, in electron transfer processes has been investigated in tightly coupled rat liver mitochondria. Examination of the oxidation-reduction state of the quinone in different metabolic conditions, and in the presence of different uncoupling agents and electron transfer inhibitors by rapid chemical extraction of the intramitochondrial ubiquinone has reinforced the idea that ubiquinone is an electron carrier. The possibility that ubiquinone is also involved in oxidative phosphorylation has not been eliminated.

iv) A newly discovered stimulation of succinate oxidase by ubiquinone homologues in cytochrome c deficient heart muscle preparations is described. The results suggested two possible explanations (a) that the added quinone might by-pass the cytochrome c deficient site or (b) that the electron flux through residual cytochrome c might be increased by the boosting of an inter-chain conductor pathway by the added quinone.
THE ACTIVATION OF ELECTRON TRANSFER IN MITOCHONDRIA (CONSIDER O.)

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It was found by Saiki and Katchalsky (1959) that the rate and extent of reduction of endogenous ubiquinone (coenzyme Q) by succinate
and malate was decreased by antimycin A but that the addition of ubiquinone
was blocked by this inhibitor. On the basis of these findings the site
of ubiquinone in the respiratory chain was assigned to a position between
complexes II and III in the chain.

However, the activation and reduction of added ubiquinone in heart
mitochondria by antimycin A were unaffected.

We have now found that the lower homologues of ubiquinone can bring
about a rate-doubling increase in the activity of succinate-
and malate-deciticient mitochondria preparations. The data obtained
indicate that the activation is due to the added ubiquinone acting at the
level of cytochrome a in the respiratory chain.

Cytochrome a-deciticient heart-muscle preparations were made from pig
heart by the method of Nunn (1953) and King (1961). The effects of
adding ubiquinones-10 and cytochrome a on the activity of succinate oxidase

INFORMATION EXCHANGE
It was shown by Pumphrey & Redfearn (1959) that the rate and extent of reduction of endogenous ubiquinone (coenzyme Q) by succinate or NADH₂ was unaffected by antimycin A but that the oxidation of ubiquinol was blocked by this inhibitor. On the basis of these findings the site of ubiquinone in the respiratory chain was assigned to a position between the succinate and NADH₂ dehydrogenase flavoproteins and the antimycin A-sensitive region (Redfearn & Pumphrey, 1959, 1960). Ramasarma & Lester (1960) studied the oxidation and reduction of added ubiquinone in beef heart muscle mitochondria and found that it reacted similarly to the endogenous quinone with regard to antimycin A inhibition.

We have now found that the lower homologues of ubiquinone can bring about a substantial increase in the activities of succinate NADH₂ oxidase in cytochrome c-deficient mitochondrial preparations. The data obtained suggest that the activation is due to the added ubiquinone acting at the level of cytochrome c in the respiratory chain.

Cytochrome c-deficient heart-muscle preparations were made from pig heart by the methods of Tsou (1952) and King (1961). The effects of adding ubiquinone-(10) and cytochrome c on the activity of succinate oxidase
are shown in Fig. 1. The quinone stimulated the activity by about 100%. Addition of cytochrome c brought about a further stimulation but this did not exceed the stimulation produced by cytochrome c alone. Addition of ubiquinone after cytochrome c had no effect.

The ubiquinone stimulation had several marked properties. Maximal stimulation was obtained with a quinone concentration of $2 \times 10^{-5}M$. It was completely inhibited by antimycin A and by sodium azide. This rules out the possibility that the mechanism of stimulation is autoxidation of ubiquinol after reduction by the respiratory chain. The stimulatory effect shows a temperature optimum at $20^\circ$ (Fig. 2). This may be related to the optimum conditions for the adsorption of the quinone by the heart-muscle particles (Iynn & Redfearn, 1964).

The stimulation is not obtained if the heart-muscle preparation is frozen and thawed or treated with surface-active agents such as deoxycholate. These treatments, however, had little or no effect on the cytochrome c stimulation.

A similar stimulation with similar properties was demonstrated in rat liver mitochondria made deficient in cytochrome c by treatment with saline (Jacobs & Sanadi, 1960).

Other ubiquinone homologues behaved similarly as shown in Table 1. The greatest stimulation was produced by ubiquinone-(10) followed by ubiquinones-(5) and -(15). Homologues above ubiquinone-(20) had no effect. The stimulatory effect of the ubiquinone homologues shows a pattern similar to the adsorption of ubiquinones by heart-muscle particles.
demonstrated by Fynn & Redfearn (1964). Evidence that the stimulation was due to adsorbed quinone was obtained by demonstrating that it persisted after repeated washing and centrifugation of the particles.

A variety of related quinones was investigated to test the specificity of the reaction. As shown in Fig. 3, only the ubiquinones were stimulatory. Other related quinones, including those which were active in mediating methylene blue reduction by succinate (Redfearn, 1961) were inactive, while vitamin K₂ homologues were inhibitory.

The stimulating effect of ubiquinone homologues, which has also been observed in the NADH₂ oxidase system, is probably due to the quinone acting in the cytochrome c region of the respiratory chain in addition to its previously established site of action between the flavoproteins and the antimycin A-sensitive region. If this were true then there should be a partial inhibition by antimycin A of the reduction of added ubiquinone by succinate. This was found to be the case (Fig. 4). The rate of reduction of ubiquinone-(10) was inhibited by about 50% by antimycin A. This inhibition could be eliminated by freezing and thawing the particles; this is in line with the elimination of the stimulatory effect on electron transport after freezing and thawing.

Further evidence that added ubiquinone acts in the cytochrome c region was obtained in an experiment in which succinate oxidase activity was measured in the presence of increasing amounts of cytochrome c both in the presence and absence of a fixed concentration of ubiquinone-(10) (Fig. 5). This demonstrates the complementary nature of the activations
produced by ubiquinone and cytochrome c. A double reciprocal plot of the data (Fig. 6) suggests that succinate oxidase is competitively activated by ubiquinone and cytochrome c.

These results clearly indicate that certain homologues of ubiquinone added to mitochondrial particles are capable of reacting at more than one site in the respiratory chain. The possible physiological significance of this fact is not yet understood.

Acknowledgements

We are indebted to the U.S. Public Health Service for a grant (AM-06858-01) in support of this work and to the Medical Research Council for the award of a Research Scholarship to one of us (P.A.W.).

References

Table 1. Stimulation of succinate oxidase activity by ubiquinone homologues.

<table>
<thead>
<tr>
<th>Homologue</th>
<th>Stimulation %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ubiquinone-(5)</td>
<td>102</td>
</tr>
<tr>
<td>Ubiquinone-(10)</td>
<td>120</td>
</tr>
<tr>
<td>Ubiquinone-(15)</td>
<td>47</td>
</tr>
<tr>
<td>Ubiquinone-(20)</td>
<td>9</td>
</tr>
<tr>
<td>Ubiquinone-(25) to -(50)</td>
<td>0</td>
</tr>
</tbody>
</table>

Succinate oxidase measured as described in Fig. 1.

Concentration of added ubiquinone homologues, $10^{-4}$M.
Fig. 1. Stimulation of succinate oxidase by ubiquinone-(10). Oxygen uptake measured polarographically at 20°C. Reaction mixture contained KH$_2$PO$_4$/Na$_2$HPO$_4$ buffer, pH 7.4, 0.07M; Na succinate 10$^{-2}$M; ubiquinone-(10), 4 x 10$^{-5}$M; cyt. c, 7.7 x 10$^{-6}$M; heart-muscle preparation, 1 mg. protein. Total volume, 3 ml. Antimycin A (20 μg in 0.02 ml. ethanol) added where indicated.
Fig. 2. Effect of temperature on succinate oxidase stimulation by ubiquinone-(10).

Succinate oxidase measured as described in Fig. 1.
Fig. 3. Effect of related quinones on succinate oxidase activity.

(a): Ubiquinone homologues

\[ n = 5 - 20: \text{stimulatory} \]
\[ n = 25 - 50: \text{inactive} \]

(b), (c), (d), (e): inactive

(f): inhibitory.
Fig. 4. The effect of antimycin A on the reduction of added ubiquinone-(10) by succinate.

Reaction mixture contained, KH$_2$PO$_4$/Na$_2$HPO$_4$ buffer, pH 7.4, 0.07M; Na succinate, 10$^{-2}$M; ubiquinone-(10), 2.5 x 10$^{-5}$M; KCN, 2 x 10$^{-3}$M; heart-muscle preparation, 0.05 mg protein.

Total volume, 3ml. Reaction measured spectrophotometrically at 275 m$\mu$ at 17$^\circ$C.
Fig. 5. The effect of cytochrome c on succinate oxidase activity in the presence and absence of added ubiquinone-(10).

- ○ ○ ○, no added UQ (10).
- ● ● ●, plus $4 \times 10^{-5}$ M UQ (10).

Assay procedure as described in Fig. 1.
Fig. 6. Double-reciprocal plot of data present in Fig. 5.

- , no added UQ (10).
- , plus $4 \times 10^{-5} \text{M UQ (10)}$.
THE INTERACTION OF ELECTRON CARRIERS IN THE MITOCHONDRIAL NADH₂ AND SUCCINATE OXIDASE SYSTEMS

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I. Introduction

The respiratory chain is a highly organized array of enzymes and electron carriers located in the lipoprotein membranes of mitochondria. Its function is to transfer electrons from the metabolites of the tricarboxylic acid cycle to oxygen with the concomitant conservation of the energy released in the form of ATP. Most of the oxidizable substrates utilise nicotinamide adenine dinucleotide (NAD) as the immediate hydrogen acceptor, but the hydrogens from succinate are transferred directly to a flavoprotein. It is now generally accepted that the NADH and succinate oxidase systems are not functionally separate chains, but that the two dehydrogenase segments join a common pathway at the cytochrome level (1, 2). Recent work (3) has indicated that the dehydrogenase flavoproteins do not react directly with the cytochrome b or ubiquinone of the common pathway but that the interaction is mediated by non-heme iron and possibly other components located in the two dehydrogenase segments. It seems likely also (4, 5) that all the electron transfer chains in mitochondria or sub-mitochondrial particles are functionally inter-connected although the elementary particle hypothesis recently proposed (6) would appear to eliminate or at least to limit this type of interaction. Inter-chain communication would also imply interaction between particles but previous attempts (7, 8, 9) to demonstrate such an interaction have given equivocal results.

In this paper we shall present some of the results of a study of the nature and function of the additional components of the NADH2 and
succinate dehydrogenase segments of the respiratory chain and also discuss the problem of interaction between particles.

II. The role of non-heme iron in the succinate oxidase system

A. Introduction

The presence of iron in excess of that associated with heme proteins has been demonstrated in mitochondria and sub-mitochondrial particles (10, 11). Thus beef heart mitochondria, and the electron transfer particle (ETP) have been shown to have 3.3 and 5.9 micromoles of non-heme iron per g. of mitochondrial protein respectively (12). Green and his coworkers have shown that there is a considerable amount of non-heme iron in each complex of the electron transfer chain with the exception of complex IV (cytochrome oxidase). Thus complex I (NADH₂-coenzyme Q reductase) has a non-heme iron concentration of 26 micromoles/g. protein or 17 atoms/mole flavin (13), complex II (succinate coenzyme Q reductase), 6.0 micromoles/g. protein or 8 atoms/mole flavin (14) and complex III (reduced coenzyme Q-cytochrome c reductase), 10.0 micromoles/g. protein (15) which is of the same order of magnitude as the cytochromes. Green (2) has suggested that the non-heme iron in each of these locations is functional as an electron carrier and that each site can be blocked by a specific inhibitor reacting with the metal. Thus the iron in the succinate dehydrogenase segment is inhibited by thenoyltrifluoroacetone, that in the NADH₂-dehydrogenase segment, by Amytal, while the site in coenzyme Q-cytochrome c reductase complex is inhibited by antimycin A.
Convincing evidence for an oxidation-reduction function for non-heme iron has been obtained by Beinert and coworkers using the technique of electron paramagnetic resonance (EPR) (16, 17). They showed that a signal characteristic of ferric iron disappears on the reduction of mitochondrial preparations or complexes I and II with succinate or NADH. However, this signal accounted for only a small part (approx. 5%) of the total non-heme iron.

B. The effects of metal-chelating agents

1. Thienyltrifluoroacetone (4,4,4 trifluoro[2-thienyl]-1,3-butanedicarboxaldehyde).

β-Diketones with the structure XCOCH₂COCF₃ have been successfully used for the separation of metal ions in solution. The mechanism of the separation is the selective chelation of one of the metals (Fig. 1). The function of the trifluoromethyl group is to increase the acidity of the enol form without destroying the resonance of the chelate form. Thienyl-trifluoroacetone has been shown to be a potent inhibitor of the succinate oxidase system and the mechanism of inhibition assumed to be chelation of non-heme iron (18, 19).

In an attempt to locate the sites of action of the inhibitor, the effect of TTA on the partial reactions of the succinate oxidase system was studied. This was done by measuring the reduction of artificial electron acceptors, known to react at different loci in the respiratory chain, in the presence and absence of 1 mM TTA. The inhibitor was preincubated with the enzyme preparation for 5 min. at 37°C before commencing the assays.
The results (Table 1) indicate that there are possibly three sites of action of TTA in the succinate oxidase system (20). One is before the site of action of phenazine methosulfate, the second is between the sites of action of phenazine methosulfate and methylene blue and the third beyond the site of action of methylene blue but before cytochrome oxidase since the activity of the latter was not inhibited (Fig. 2). The principal site of inhibition appears to be between the succinate dehydrogenase flavoprotein and ubiquinone. This is demonstrated by the finding that succinate-methylene blue reduction mediated by phenazine methosulfate is inhibited by only 23%. But when the reaction is mediated by ubiquinone it was inhibited by 67%.

Similar results were obtained using cytochrome c, 2,6-dichlorophenol-indophenol and potassium ferricyanide as the electron acceptors. The former two determinations were performed spectrophotometrically and the latter potentiometrically (21) at 20°C. The results were given in Table 2. The close similarity to the manometric determinations is evident since the succinate-cytochrome c reductase activity can be compared with succinate oxidase (because the lack of inhibition of cytochrome oxidase), indophenol is generally considered to act at the same site as methylene blue and ferricyanide (at 0.6mM) probably reacts in the same region as phenazine methosulfate. At a low concentration (0.2mM) ferricyanide acts at the cytochrome c level (21) and this explains the much higher inhibition obtained at this concentration.
The results confirm and extend the previous work of Tappel (18) and Ziegler (19). The first site of inhibition suggests that TTA is combining with the non-heme iron associated with the succinate dehydrogenase flavoprotein (22) although according to Ziegler (19) the primary flavoprotein-phenazine methosulfate reductase is not inhibited by TTA. On the other hand, earlier work by Singer (23) had shown that the dehydrogenase containing 4 iron atoms per flavin molecule was inhibited by metal chelating agents, such as α,α-dipyridyl and α-phenanthroline, but the aged preparation containing only 2 iron atoms per flavin was not inhibited. Thus the site of action of phenazine methosulfate may depend upon the particular state of the succinate dehydrogenase preparation. King (24) has already shown that phenazine methosulfate reduction is not necessarily a criterion that soluble succinate dehydrogenase will be active in the reconstitution of the alkali-treated preparations. Thus the physiologically active dehydrogenase probably contains iron atoms which react with TTA.

The second and principal site of inhibition by TTA probably represents a functionally-independent non-heme iron component mediating electron transfer between the flavoprotein complex and ubiquinone or cytochromes, as described by Ziegler (19).

The third site of action of TTA between ubiquinone and cytochrome c probably corresponds to the non-heme component situated between cytochromes b and c₁ described recently by Rieske et al. (25).

It was necessary to demonstrate that the inhibition by TTA was, in fact, due to metal-chelation. This was done by examining the effects of a number of structurally-related compounds. These included trifluoroacetylacetone, benzoyle acetone, thienoyle acetone and acetyl acetone, all of which
are chelating agents. With the exception of acetyl acetone, all inhibited succinate oxidase. Acetyl thiophen and trifluoroacetophenone which do not have chelating properties, did not inhibit. This rules out the possibility that the thiophen ring and the trifluoromethyl group have inhibitory effects per se.

The effect of the (TTA)₃Fe complex on the succinate oxidase system was also studied. The complex was prepared by treating an ethanolic solution of TTA with a large excess of ferric chloride solution. The complex which formed as a deep purple precipitate was recrystallized five times from ethanol to give small deep red hexagons. The complex was estimated as 97% pure (TTA)₃Fe based on the extinction at 460nm (26). Rather variable results were obtained on treating a heart-muscle preparation with this compound. The small amount of inhibition which was sometimes obtained was eventually attributed to the dissociation of the complex thus supporting inhibition by a metal-chelating mechanism.

Attempts were made to demonstrate an actual combination of TTA with iron in the preparation. TTA-treated preparations were extracted with organic solvents such as ether and benzene, and spectrophotometric examination of the extracts indicated the presence of (TTA)₃Fe complex. This corresponded to 1.2 μatoms of iron per g. protein. According to Green and Wharton (12) the total non-heme content of beef heart mitochondria is 3.3 μatoms Fe/g. protein.
2. The effects of other chelating agents on succinate oxidase activity

The effects of other chelating agents on the succinate oxidase activity of heart-muscle preparations were studied (Table 3). It will be seen that 7-iodo-5-chloro-8-hydroxyquinoline and diphenylthiocarbazone, both of which have lipid-soluble metal chelates were inhibitory whereas the others, which have water-soluble metal chelates, had no effect. Tappel (18) has also shown that lipid-soluble metal-chelating agents inhibited succinate oxidase. It appears therefore that chelation of the non-heme iron in the succinate-ubiquinone segment requires that the chelate and probably the chelating agent itself should be lipid soluble. This is particularly interesting when we consider the comparable site in the NADH system since TTA and other lipid-soluble chelating agents do not inhibit NADH oxidase to any great extent.

XIII. The NADH dehydrogenase segment

A. Introduction

The NADH and succinate dehydrogenase segments of the respiratory chain have a number of important differences. Apart from the fact that coupled phosphorylation occurs between NADH and the NADH dehydrogenase whereas there is no ATP synthesis in the corresponding part of the succinate oxidase system, the behaviour towards inhibitors is different. Thus barbiturates (e.g. Amytal and Secomal) (27) and rotenone (28) are potent inhibitors of NADH oxidation but they have no effect on succinate oxidation. On the other hand, certain chelating agents like themayl trifluoroacetone and
7-iodo-5-chloro-8-hydroxyquinoline are, as already shown, potent inhibitors of succinate oxidation but have little effect on the NADH system. Another difference is the great relative instability of the NADH system compared with the succinate system towards organic solvents (29) surface-active agents (30), urea (31) and heat treatment (32).

These observations suggest that the NADH dehydrogenase segment of the respiratory chain may be structurally different from the corresponding part of the succinate oxidase system. Thus there may be differences in the nature or accessibility of the non-heme iron components.

2. Inhibition with metal-chelating agents

Of a number of chelating agents tested as possible inhibitors of the NADH oxidase system, only thenoyltrifluoroacetone and 2-phenanthroline had appreciable inhibitory effects. However, an approximately $10^3$-fold concentration of TTA was required to inhibit NADH oxidase to the same extent as the succinate oxidase system. On the other hand, 2-phenanthroline proved to be a more potent inhibitor than TTA. The inhibitory effect of 2-phenanthroline on the NADH oxidase system was conveniently studied polarographically at 20°C. Under these conditions, an immediate inhibitory effect on NADH oxidase can be demonstrated but there is no effect on succinate oxidase. The effect of an increasing concentration of 2-phenanthroline on the NADH oxidase activity of a heart-muscle preparation is shown in Fig. 3. At low concentrations (approx. $10^{-4}$M), 2-phenanthroline produces an appreciable stimulation of activity but when the concentration is increased ( $>3.5 \times 10^{-4}$M) the activity is inhibited. A stimulatory effect of low concentrations of 2-phenanthroline and other chelating agents
on NADH oxidase activity has been noted previously (33). If a non-inhibitory concentration (6.6 x 10^{-5} M) of Amytal is also included in the reaction mixture, \( \text{g-phenanthroline} \) then has a more potent inhibitory effect and the stimulatory effect is eliminated (Fig. 3). Thus Amytal and \( \text{g-phenanthroline} \) appear to act synergistically.

Further experiments suggested that metal-chelation was probably not the principal mechanism of inhibition by \( \text{g-phenanthroline} \). Fig. 4 shows the relationship between the enzyme concentration and NADH oxidase activity at different levels of \( \text{g-phenanthroline} \) concentration. The activity is proportional to the enzyme concentration in all cases indicating that the inhibitor is not attaching itself to a specific component of the system. Again, if the reaction mixture of an inhibited preparation is diluted, the activity increases to a level corresponding to the new final concentration of the inhibitor.

Incubation of the preparation with \( \text{g-phenanthroline} \) at 37°C prior to assay increased the inhibitory effect. After a short incubation period (5 min.) the inhibitory effect is reversed by the addition of \( \text{Zn}^{2+} \) ions but after a longer incubation it is irreversible. Also, when the effects of \( \text{g-phenanthroline} \) and TTA on NADH oxidase are studied together, it can be shown that a TTA inhibited preparation can still be stimulated by low concentrations of \( \text{g-phenanthroline} \) but inhibited to a greater extent by higher concentrations. This suggests that TTA is inhibiting mainly in the common part of the chain while the \( \text{g-phenanthroline} \) acts mainly at a site in the NADH-dehydrogenase segment. These observations suggest that
g-phenanthroline exerts its effects by causing some structural change in
the system, perhaps similar to those of urea (31) or heat treatment (32).

C. Inhibition by rotenone

The fish poison, rotenone, is a potent inhibitor of NADH$_2$ oxidase
but it is without effect on succinate oxidase (28). Ernster et al. (34, 35)
have shown that it acts similarly to Amytal in its effects on electron
transport but unlike Amytal it does not affect the energy transfer reactions.
Rotenone also differs from Amytal in that it becomes tightly bound to the
mitochondrial particles and is presumably titrating a component on the
oxygen-side of the NADH$_2$ dehydrogenase flavoprotein. Rotenone is therefore
an inhibitor of considerable interest which may help towards an understanding
of the structure and function of the NADH$_2$ dehydrogenase segment of the
respiratory chain. For this reason we have made a detailed study of the
inhibitory effects of rotenone.

Rotenone at a concentration of 0.07-0.1 µg/mg. protein was found to
inhibit completely the NADH$_2$ oxidase of a heart-muscle preparation. A
curious feature of rotenone inhibition revealed in the polarographic deter-
mination of enzymic activity is that there is a lag period before the full
effect of the inhibitor is established (Fig. 5). Thus after 5 minutes
incubation at 20° with 0.06 µg. rotenone/mg. protein there is an immediate
inhibition to about 60% of the original activity but there is a lag of about
10 minutes after the NADH$_2$ addition before the activity is completely
inhibited. On increasing the concentration of rotenone the lag period
following the NADH₂ addition becomes progressively less until at a concentra-
tion of the order 0.2 μg/mg. protein it disappears completely. The lag
period persists even after preincubation of the enzyme preparation with
rotenone for long periods and after other treatments as shown in Fig. 5.

Rotenone did not have an appreciable inhibitory effect on the
NADH₂-ferricyanide reductase system (measured potentiometrically). At a
concentration of 0.2 μg/mg. protein, which in that particular preparation
was three times that needed for complete inhibition of NADH₂ oxidase,
NADH₂-ferricyanide reductase was inhibited only 25%. This suggests that
the main site of action of rotenone is on the oxygen-side of the flavoprotein.

Estabrook and his coworkers (36) have shown that the sulphydryl
group alkylating agent, N-ethyl-maleimide inhibited NADH₂ oxidase activity
but had no effect on NADH₂-ferricyanide reductase. We have studied the
effect of N-ethyl-maleimide on NADH₂ oxidase activity (Fig. 6) and shown
that inhibition increases rapidly to about 40% inhibition at a concentration
of about 1mM but thereafter, there is a slow increase in inhibition and even
at 8mM the inhibition does not exceed 60%. In these experiments the
inhibitor was incubated for 10 minutes at 20° before measuring the enzyme
activity; longer incubation periods did not increase the inhibitory effect.
In Estabrook’s experiments incubation of ETP with 1mM-N-ethyl-maleimide
gave a 95% inhibition of NADH₂ oxidase (36).

Experiments were carried out to test the effect of rotenone on
an enzyme which had been previously treated with N-ethyl-maleimide. It
will be seen (Fig. 7) that N-ethyl-maleimide had a marked potentiating
effect on the inhibition by rotenone.
IV. The interaction of mitochondrial particles

A. Introduction

Several attempts have been made to reconstitute electron transport activity by mixing together particles in which electron transferring ability had been blocked at different points in the chain. Thus Slater (7) could not demonstrate an interaction between particles which had been inhibited with p-aminophenylarsenoxide and particles which had been treated with NAL in the presence of air. Tissieres (8), on the other hand, was able to demonstrate spectrascopically the interaction between particles from Neurospora strain poky and alkali-treated Neurospora wild-type or heart-muscle particles. In other particles, which were deficient in cytochrome $c$, succinate oxidase could be reconstructed only in the presence of added cytochrome $c$.

Thorn (9) has shown that when heart-muscle particles which had been titrated with sufficient antimycin A to give complete inhibition were mixed with untreated particles, the succinate oxidase activity was higher than the activity of the untreated particles. This was interpreted as being due to a redistribution of the antimycin A over all the antimycin-inhibitable factor. More recently Ernst et al. (35) have attempted to restore activity by mixing together rotenone- and antimycin-treated particles but no reactivation was obtained.

B. Reactivation of inhibited particles

We have demonstrated the reactivation of succinate and NADH$_2$ oxidase activities in the following combinations of various types of treated
heart-muscle particles:

(1) Alkali-treated (37) + antimycin-treated.
(1i) Antimycin-treated + acetone-extracted. (Fig. 8.)
(1ii) Antimycin-treated + rotenone-treated. (Fig. 9.)
(1iv) Rotenone-treated + ether-extracted.

A study of the reactivation of antimycin-inhibited and acetone-
extracted particles showed that the succinate oxidase activity increased
with the time that the particles had been incubated together (Table 4).

When alkali-treated particles were mixed with antimycin-inhibited
particles and the reduction of the endogenous ubiquinone by succinate was
measured (38) it was found that only 44% of the total quinone was reduced.
This suggested that only the ubiquinone in the antimycin-treated particles
was being reduced.

These results are consistent with Thorn's (9) suggestion that
reactivation of antimycin-treated particles occurs by a redistribution of
the inhibitor. This redistribution would imply that antimycin dissociated
from an inhibited particle would have to diffuse through the medium and
become attached to the antimycin-site on an uninhibited particle. To test
this the possibility of this mechanism we carried out the following
experiments:

(1) An untreated preparation (19.8 mg. protein) in 0.1M phosphate buffer
(9 ml.) was dialysed against a solution of antimycin (10.8 mg.) in the
same buffer (100 ml.) for 16 hours at 0-4°C. It was found that the
succinate oxidase activity of the preparation was completely inhibited
thus showing that antimycin had passed freely through the "Fickian" membrane.
Another portion of the heart-muscle preparation (19.8 mg. protein) in 0.1M phosphate buffer (9 ml.) which had been titrated with antimycin (0.05 μg./mg. protein) to 95% inhibition was dialysed under the same conditions against 170 ml. of untreated preparation with the same protein concentration. No reactivation of the inhibited preparation occurred. It should be noted that it was previously demonstrated that the inhibited preparation would have to lose only about 15% of the antimycin to give 75% of the original activity.

(ii) A heart-muscle preparation was sonicated briefly and submitted to differential centrifugation at 10,000, 20,000 and 100,000g for 15, 30 and 30 minutes respectively. The 25,000g particles were discarded. The 10,000g particles were titrated with antimycin to 95% inhibition and mixed with an equal amount of protein of the untreated 100,000g particles and left at 0° for 16 hours. It was intended that the mixture should then be subjected to differential centrifugation as before but it was found that all the particles sedimented after 15 minutes at 10,000g. The same result was obtained when the centrifugation was preceded by a brief (15-30 seconds) sonication. It was found also that when the 100,000g particles themselves were left to stand for 16 hours, complete sedimentation was obtained after a 10,000g spin.

These experiments suggest that if a redistribution of antimycin A does take place then it must do so by an aggregation or coalescence of the particles. On the other hand, this type of combination of the particles would also allow an explanation of the reactivation in terms of an interaction between electron transfer chains of neighbouring aggregated or fused particles.
V. Conclusions

With regard to the role of non-heme iron in the respiratory chain, our results have confirmed that it probably functions in the succinate dehydrogenase segment and in other parts of the chain but we have been unable to obtain unequivocal evidence, using metal-chelating agents, that it plays a functional role in the NADH dehydrogenase segment. The effects of the various inhibitors and treatments on the NADH dehydrogenase segment are difficult to explain in terms of action on an individual component of the electron transfer system. It seems more likely that inhibition is a result of the effect of a structural disorganization of the system. The fact that a lower concentration of the inhibitor sometimes produces a stimulatory effect would also favor this explanation. The selective sensitivity of the NADH dehydrogenase segment may form the basis of an in vivo physiological control mechanism. For example, steroid hormones which have indeed been shown to inhibit NADH oxidase in vitro (42), may exert such a control in vivo by reversibly altering the structural configuration at this part of the chain. Several examples of structural changes induced by steroid hormones with concomitant alterations in enzymic activity have already been discussed (43).

The precise mechanism of the reactivation of inhibited particles has not been worked out but it seems to involve the aggregation or coalescence of particles. It would be of great interest to elucidate whether, under these circumstances, intercommunication between electron transfer chains could occur since this would have an important bearing on the present controversy concerning the structural organization of the electron transport system (6, 44).
We are indebted to the U.S. Public Health Service for a grant (NH-06558-01) in support of this work and to the Medical Research Council for the award of a Research Scholarship to one of us (F.A.W.). We also wish to thank Miss Margaret Albrow and Miss Jennifer Cook for their technical assistance.
REFERENCES


41. E.R. Redfearn and V. Jones, Unpublished work.
Table 1.

Inhibition of the succinate oxidase system by thienyltrifluoroacetone.

Enzyme activities measured manometrically at 37°.

<table>
<thead>
<tr>
<th>Enzyme Activity</th>
<th>Percentage inhibition by 1mM TTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate oxidase</td>
<td>86</td>
</tr>
<tr>
<td>Succinate-methylene blue reductase</td>
<td>67</td>
</tr>
<tr>
<td>Succinate-methylene blue reductase mediated by</td>
<td>68</td>
</tr>
<tr>
<td>ubiquinone-(15)</td>
<td></td>
</tr>
<tr>
<td>Succinate-methylene blue reductase mediated by</td>
<td>27</td>
</tr>
<tr>
<td>phenazine methosulfate</td>
<td></td>
</tr>
<tr>
<td>Succinate-phenazine methosulfate reductase</td>
<td>23</td>
</tr>
<tr>
<td>Cytochrome oxidase</td>
<td>0</td>
</tr>
</tbody>
</table>

Enzyme activities measured as previously described (5, 39, 40).
Table 2.

Inhibition of the succinate oxidase system by thenoyltrifluoroacetone. Enzyme activities measured spectrophotometrically at 20°.

<table>
<thead>
<tr>
<th>Enzyme activity</th>
<th>Percentage inhibition by 1 mM TTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Succinate-cytochrome c reductase</td>
<td>83</td>
</tr>
<tr>
<td>Succinate-indophenol reductase</td>
<td>73</td>
</tr>
<tr>
<td>Succinate-indophenol reductase mediated by ubiquinone (15)</td>
<td>76</td>
</tr>
<tr>
<td>Succinate-indophenol reductase mediated by phenazine methosulfate</td>
<td>71</td>
</tr>
<tr>
<td>Succinate-ferricyanide reductase (0.2 mM ferricyanide)</td>
<td>69</td>
</tr>
<tr>
<td>Succinate-ferricyanide reductase (0.6 mM ferricyanide)</td>
<td>39</td>
</tr>
<tr>
<td>NADH oxidase</td>
<td>12</td>
</tr>
</tbody>
</table>

Enzyme activities measured as previously described (39, 21, 41).
Table 3.

The effect of other chelating agents on succinate oxidase activity.

<table>
<thead>
<tr>
<th>Chelating agent</th>
<th>Concentration</th>
<th>Percentage inhibition</th>
<th>Lipid Solubility of chelate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cupferren</td>
<td>$3.3 \times 10^{-3}M$</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Diphenylthiocarbamone</td>
<td>$3.3 \times 10^{-4}M$</td>
<td>40</td>
<td>+</td>
</tr>
<tr>
<td>7-Iodo-5-chloroquinoline</td>
<td>$2.7 \times 10^{-5}M$</td>
<td>85</td>
<td>+</td>
</tr>
<tr>
<td>2-phenanthroline</td>
<td>$10^{-3}M$</td>
<td>0</td>
<td>-</td>
</tr>
</tbody>
</table>

Succinate oxidase measured as previously described (5).
Table 4

Effect of time on the reactivation of NADH$_2$ oxidase in inhibited preparations

<table>
<thead>
<tr>
<th>Preparation</th>
<th>NADH$_2$ oxidase activity (μmol oxygen/min/mg. protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>uninhibited preparation</td>
<td>0.294</td>
</tr>
<tr>
<td>acetone-extracted preparation</td>
<td>0</td>
</tr>
<tr>
<td>antimycin preparation</td>
<td>0.014</td>
</tr>
<tr>
<td>mixture of 4mg. acetone-extracted + 1mg. antimycin-inhibited</td>
<td></td>
</tr>
<tr>
<td>(i) immediately after mixing</td>
<td>0.002</td>
</tr>
<tr>
<td>(ii) after 10 min. at 20°</td>
<td>0.006</td>
</tr>
<tr>
<td>(iii) &quot; 40 &quot; &quot; &quot;</td>
<td>0.027</td>
</tr>
<tr>
<td>(iv) &quot; 90 &quot; &quot; &quot;</td>
<td>0.038</td>
</tr>
</tbody>
</table>

NADH$_2$ oxidase measured polarographically at 20°C.
Fig. 1. The chelation of iron by thenoyltrifluoroacetone.

Fig. 2. Sites of action of thenoyltrifluoroacetone in the respiratory chain.

Fig. 3. Effect of α-phenanthroline on NADH$_2$ oxidase activity in the absence and presence of Amytal. O—O, α-phenanthroline alone; O—O, in the presence of 0.66 x 10$^{-4}$M Amytal. NADH$_2$ oxidase determined polarographically at 20°. α-Phenanthroline added to the preparation (0.7 mg. protein) in 10 μl ethanol and the mixture incubated for 10 mins. before assay.

Fig. 4. NADH$_2$ oxidase activity of a heart-muscle preparation in the presence of different amounts of α-phenanthroline. Assay conditions as in Fig. 3. O—O, control (no α-phenanthroline addition); O—O, 0.5mM α-phenanthroline (inhibition, 35%); Δ—Δ, 1mM (inhibition, 66%); Δ—Δ, 2.5mM (inhibition, 92%).

Fig. 5. Rotenone inhibition of NADH$_2$ oxidase activity of a heart-muscle preparation. 1, control; 2, in presence of 0.064 μg. rotenone/mg. protein added immediately before assay; 3, as in 2 but mixture incubated for 25 min. at 20° before assay; 4, preparation incubated with NADH$_2$ for 1 min. at 20° before rotenone addition.

Fig. 6. Effect of N-ethylmaleimide on NADH$_2$ oxidase activity of a heart-muscle preparation. The preparation (1 mg. protein) incubated with inhibitor for 10 min. at 20° before assay.
Fig. 7. Potentiation of rotenone inhibition by N-ethyl-maleimide.
Preparation (1 mg. protein) incubated with N-ethyl-maleimide for 5 min. before rotenone inhibition and for a further period of 5 min. before assay.
C, control; 1, rotenone (0.12 μg.); 2, rotenone (0.12 μg.) + maleimide (1mM); 3, rotenone (0.03 μg.) + maleimide (1mM); 4, rotenone (0.03 μg.);
5, N-ethyl-maleimide (1mM).

Fig. 8. Reactivation of NADH₂ oxidase activity of an antinycin A-inhibited heart-muscle preparation by an acetone-extracted preparation.
1, Preparation (1.35 mg. protein) inhibited with antinycin (0.06 μg./mg. protein) + preparation (2.8 mg. protein) extracted with acetone; 2, acetone-extracted preparation (2.8 mg. protein); 3, antinycin-inhibited preparation (1.35 mg. protein).

Fig. 9. Reactivation of NADH₂ oxidase activity of antinycin- and rotenone-inhibited heart-muscle preparations.
1, Preparation (1.35 mg. protein) inhibited with antinycin (0.06 μg./mg. protein);
2, preparation (1.35 mg. protein) inhibited with rotenone (0.2 μg./mg. protein).
Where indicated by the arrow, the antinycin-inhibited preparation was added to the rotenone-inhibited preparation.
NADH$_2$ Oxidase Activity, %

$\omega$-phenanthrolone concn. (mM)

Fig. 3
Concn. of heart-muscle prepn. (mg. protein)
Fig. 6

% Inhibition

N-ethylmaleimide concn. (mM.)