THE LOCUS OF ACTION OF TRIALKYL TIN COMPOUNDS
IN YEAST MITOCHONDRIA.

A Thesis submitted to the University of Warwick in fulfilment of the Degree of Doctor of Philosophy.

The research described in this thesis was carried out in the Department of Molecular Sciences, University of Warwick.

K. Cain.
August, 1976.
'...... one finds it hard to accept that it would have been beyond
the wit of all the biochemists engaged in studying the coupling
mechanism in oxidative phosphorylation and photosynthetic phosphorylation
during the last forty years to demonstrate a single relevant direct
functional interaction or structural complexation between components of
the redox and ATPase systems, ......'

Abbreviations.

The abbreviations used in this thesis are as used by the Biochemical Journal, the exceptions or special abbreviations are listed below.

TET  triethyl tin sulphate.
$^{115}$Sn TET  $^{115}$Sn labelled triethyl tin chloride.
DBT  dibutyl tin dichloride.
DBCT  dibutyl chloromethyl tin chloride.
$H^3$-DBCT  tritium labelled DBCT.
BA  bongkrekic acid.
ATR  atractyloside.
CATR  carboxyatractyloside.
$H^3$-BA  tritium labelled bongkrekic acid.
$H^3$-ATR  tritium labelled atractyloside.
p  denotes presence or absence of mitochondrial DNA, eg. $p^+$, $p^0$.
CCCP  carbonyl cyanide-m-chloro-phenylhydrazone.
DNP  dinitrophenol.
mt-DNA  mitochondrial DNA.
OS-ATPase  oligomycin sensitive ATPase.
$F_o$  membrane components of the OS-ATPase.
$P_i$-ATPase  cold labile insensitive to oligomycin ATPase component of the OS-ATPase.
$P_i$-OSCP depleted particles  submitochondrial particles depleted of $P_i$-ATPase and oligomycin sensitive conferring protein.
OSCP  oligomycin sensitive conferring protein.
OL$^R$  oligomycin resistant mutant.
VEN$^R$  venturicidin resistant mutant.
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TET\(^R\) triethyl tin resistant mutant.

1799 1,1,5,5-trifluoromethyl-1,5-hydroxy-pentan-3-one.

1799\(^R\) 1799 resistant mutant.

Adn. adenine nucleotide.

EDTA ethylenediamine-tetra-acetic acid.

DCCD dicyclohexyl-carbodiimide.
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SUMMARY.

Trialkyl tin compounds have been shown to be potent inhibitors of the yeast mitochondrial OS-ATPase complex in both the membrane bound form and purified soluble preparation. Binding studies with $^{113}$Sn labelled triethyl tin have shown that the inhibitory properties of triethyl tin are due to the presence of a high affinity binding site which is not competed for by oligomycin or venturicidin. The concentration of the binding site in the purified enzyme is 6mole/mole enzyme, the binding site has been shown to be located on the $F_0$ component of the OS-ATPase complex.

A new radioactive affinity label (DBCT) for trialkyl tin compounds has been synthesized, binding experiments revealed that DBCT is a covalent inhibitor of the OS-ATPase. Extraction and isolation experiments have shown that DBCT binds to a small lipophilic, apparently non-protein component of the mitochondrial membrane. The significance of these findings are discussed in relation to current ideas on oxidative phosphorylation.

A biochemical genetic study has shown that triethyl tin may have another mode of action which is related to the transport of Adn nucleotides across the mitochondrial membrane.
CHAPTER 1.

Introduction.

The Respiratory Chain.

The prerequisite requirement for the maintenance of life in all living organisms is a source of energy, obtained from catabolic chemical reactions. It is impractical and inefficient for every energy requiring reaction in the cell to be directly linked to an energy producing one. Consequently, a common freely mobile energy currency has evolved in the form of ATP which can be transported within the cell from the specialised centres of production, namely the glycolytic and respiratory chain-oxidative phosphorylation pathways. Under aerobic conditions the most prolific producer and conserver of energy is the respiratory chain-oxidative phosphorylation pathway, approximately 90% of the energy made available in glucose oxidation is obtained from oxidative phosphorylation. Oxidative phosphorylation describes the process by which energy produced by the respiratory chain is conserved in the terminal high energy bond of ATP, the mechanisms and enzymes involved in this process are in essence very similar in procaryotic cells and the mitochondria of eucaryotes. The work presented in this thesis is an investigation into the mode of action of the potent fungicides, the trialkyl tin salts which inhibit the mitochondrial ATPase synthetase complex (E.C. 3.6.13.) in the yeast Saccharomyces cerevisiae. The advantages of using yeast mitochondria as opposed to beef heart or rat liver (the frequently used sources of mitochondria) will be discussed later in this introduction. The purpose of the ATPase synthetase in oxidative phosphorylation is to convert
energy from the respiratory chain into ATP, before discussing the mechanisms involved in this process, it is worth briefly outlining the structure and functioning of the respiratory chain.

The respiratory chain can be said to start with the NADH dehydrogenase which receives reducing equivalents from a variety of NAD linked dehydrogenases. Electrons are transferred stepwise down the chain to the terminal oxidase resulting in the reduction of oxygen to water. Current ideas to the components and their sequence in the chain are shown in Fig. 1.1., several techniques have been utilised to derive this scheme. They include measurements of the individual components redox potentials, kinetic determinations of the reaction sequences, studies on the donor and acceptor specificity of isolated components and the interaction of inhibitors with the respiratory chain. Chemical fractionation studies show that the chain can be split into four complexes (Hatefi, Fowler & Griffiths, 1962a, b; Ziegler & Doeg, 1952; Hatefi, Haavik, Fowler & Griffiths, 1962) which have the partial reactions shown in Fig. 1.2., and can be recombined stoichiometrically to give a reconstituted respiratory chain which behaves in a similar manner in its responses to inhibitors as the respiratory chain found in intact mitochondria. The stoichiometry of the reconstitution, that is $1:1:1:1$ suggested a rigid orientation of the complexes in the mitochondrial membrane. However, later work, reviewed by Green and Tzagoloff (1966) indicated that the original estimates of the M.W.'s of the complexes were wrong due to the presence of structural proteins which were not needed for catalytic activity, thus each chain in beef heart contained three molecules of complex IV per complex I, furthermore the proportions of complexes in the chain is only constant for that
Fig. 11. The electron transfer components of the respiratory chain arranged as a continuous sequence from NADH to oxygen. The components on the substrate side of site I being Fp, lipoate dehydrogenase; FpD1, NADH dehydrogenase flavoprotein, and the iron sulphur proteins designated C-1, C-3 and C-4. On the oxygen side of site I are FpS, the succinate dehydrogenase flavoprotein and associated iron-sulphur proteins (Fe-S); UQ, ubiquinone; FpD2, the fluorescent flavoprotein; C-2, iron sulphur protein; cytochromes b and b'. Between sites II and III there are the cytochromes, c, c, a, and a3 with associated Cu. Inhibitors of the respiratory chain are shown at their believed site of action. (Adapted from Chance, 1972).
I = NADH-ubiquinone reductase complex which catalyses the reaction \( \text{NADH} + Q + H^+ \rightarrow \text{NAD}^+ + QH_2 \)

II = Succinic ubiquinone reductase complex which catalyses the reaction \( \text{Succinate} + Q \rightarrow \text{Fumarate} + QH_2 \)

III = Ubiquinol cytochrome c reductase complex which catalyses the reaction \( \text{QH}_2 + 2 \text{ferrocyt.c.} \rightarrow \text{Q} + 2 \text{ferrocyt.c.} \)

IV = Cytochrome c oxidase complex which catalyses the reaction \( 2 \text{ferrocyt.c.} + 2H^+ + \frac{1}{2} O_2 \rightarrow 2 \text{ferricyt.c.} + H_2O \)

Fig. 1.2. The four complexes and their sequential arrangement in the respiratory chain (Hatefi, Haavik, Fowler, & Griffiths, 1962).
tissue, thus in bacteria and yeast there are extensive alterations in the molecular proportions of the component complexes. The conclusion was that the complexes are linked by mobile carriers.

Redox potential measurements on both 'in situ' and isolated components of the chain have enabled the presentation of the electron carriers on a potential diagram (Fig.1.3.). Chance (1972) in a review puts forward the concept of three groups of electron carriers which have fixed mid-potential and are linked with one another by means of energy-transducing carriers of variable potential. The energy transducing carriers for II and III being cytochromes $b^\alpha$ and $a^\alpha$ which are ATP-dependant, the carrier for site I is not yet known, the function of the carriers is to serve as 'redox' potential buffer pools and thereby fix the potential at which the energy transducing components operate. The respiratory chain therefore acts by an energy transduction process that operates through a series of quasi-equilibrium steps. In the case of $b^\alpha$ for example there are four species; oxidised high mid potential, oxidised low mid potential, reduced high mid potential and reduced low mid potential. $b^\alpha$ in its low mid potential form can only react with group II carriers and in its high mid potential form, only with group III carriers.

Thermodynamically the passage of electrons from NADH to $O_2$ produces sufficient energy to synthetise 3 molecules of ATP, synthesis taking place at three separate regions in the respiratory chain which are also the site of action of the respiratory chain inhibitors amyntal, rotenone, pericidin $A$, antimycin $A$, hydroxyquinoline-N-oxide, cyanide and carbon monoxide (Fig.1.1.). The actual site of ATP synthesis is now believed
Fig. 3. The electron carriers of the respiratory chain arranged as groups of fixed potential (I, II and III) and individual components of variable mid-potential ($b_m$, ($a_3)_T$ and ?). The other components are the same as in Fig.1.1., from Chance (1972).
to reside in the multi-enzyme ATP synthetase complex, under the right conditions this complex can behave as an ATPase, hydrolysing ATP to ADP and Pi. It has therefore become accepted dogma to refer to ATP synthesis as being carried out by the ATPase located on the inner mitochondrial membrane, the structure and functioning of this enzyme will be reviewed later in this chapter. It is important however, to consider the problem of how energy from the respiratory chain is made available to the ATPase in such a form that it can synthesise ATP.

Oxidative Phosphorylation.

Oxidative phosphorylation is the process by which respiratory chain energy is made available to the ATPase so that it can produce ATP. Currently, three theories are available to describe the phenomenon.

1. The Chemical Intermediate Theory (Slater, 1953).
3. The Conformational Theory (Boyer, 1964) and a now expanded version known as the Electromechanochemical model (Green and Ji, 1972).

All three theories have one thing in common in that the respiratory chain generates a high energy intermediate which can drive ATP synthesis, NADH transhydrogenation and cation translocation (Fig. 1.4.). ATP added to the system can drive reversed electron transport in the respiratory chain, suggesting a reversible connection between the respiratory chain and the ATPase complex. Similarly, transhydrogenation and cation translocation can be driven by ATP hydrolysis, moreover ATP synthesis, transhydrogenation and cation translocation all compete for the energy made available by the respiratory chain. All these reactions can be
Fig. 1.4. Three schemes which have been suggested for the interrelationships between the respiratory chain, ATP synthesis, proton translocation and metal cation translocation in mitochondria (Chance et al., 1967; Slater, 1967; Mitchell, 1969).
inhibited by compounds such as oligomycin and the trialkyl tin salts which are known to specifically inhibit the ATPase complex. Clearly, the ATPase complex must play a major role in the oxidative phosphorylation process. The three theories differ in one respect, the nature of the high energy intermediate, consequently, their interpretation of the mode of action of inhibitors like oligomycin and the trialkyl tins are markedly different.

The Chemical Intermediate and Chemiosmotic Theories.

The controversy between these two theories has raged for the last 15 years, and recent reviews by Deamer (1969), Greville (1969) and Harold (1972) have attempted to weigh up the pros and cons of the two ideas. Even though no definite conclusion has been reached, it is certain that this controversy has stimulated research in this area with a resultant improvement in our knowledge.

Simply, the chemical intermediate theory states that the respiratory chain generates high energy chemical intermediates which can be used by the appropriate reactions, this can be written as follows:

\[
\begin{align*}
A_{\text{red}} + B_{\text{ox}} + I & \rightarrow A_{\text{ox}} - I + B_{\text{red}} \quad \text{(1)} \\
A_{\text{ox}} - I + X & \rightarrow A_{\text{ox}} + X - I \quad \text{(2)} \\
X - I + P_i & \rightarrow X - P + I \quad \text{(3)} \\
X - P + ADP & \rightarrow X + ATP \quad \text{(4)}
\end{align*}
\]

the \(-\) refers to a high energy bond probably anhydride in nature, A and B are adjacent redox carriers of the respiratory chain. Oligomycin is presumed to block X\(-\)I synthesis, the result being a feedback inhibition on respiration, uncouplers are supposed to catalyse dissipation of the
high energy intermediates thereby stimulating respiration and also ATP hydrolysis activity. Reversed electron transport is supposed to be driven by $X^-I$ generated by ATP hydrolysis, transhydrogenation and cation translocation also being driven by $X^-I$. Evidence for the intermediate theory is largely based on the actions of inhibitors like oligomycin and partial reactions requiring energy which can be shown to be obtained neither from ATP or the respiratory chain. Unfortunately, no intermediate has ever been isolated, although glycolytic synthesis of ATP uses high energy phosphorylated intermediates to transfer phosphate to ADP. In addition, oxidative phosphorylation seems to require a vesicle system to work, a fact irreconcilable with the intermediate hypothesis. Furthermore, the swelling and shrinking movements observed in phosphorylating mitochondria are not easily explained by the intermediate theory nor is the fact that a great range of widely differing chemical structures will uncouple respiration from phosphorylation. Mitchell's theory attempts to satisfy these observations.

Mitchell's hypothesis relies on a number of premises, firstly, the inner membrane of the mitochondrion containing the respiratory chain and phosphorylation system is impermeable to ions particularly protons and is a non conducting barrier, although systems exist which can transport protons and certain other ions. Secondly, the respiratory chain components are arrayed in loops of proton and electron carriers (Fig. 1.5.), there being three loops from NADH to $O_2$ and two from succinate to $O_2$. The net result of the loops is to pump two protons out of the mitochondrion per loop. Thus, this may be regarded as distributing the elements of water, that is, two $H^+$ and two $OH^-$ ions between the inner
Fig. 1.5. Pathway of proton and electron transfer during oxidation of NADH, according to the chemiosmotic theory. From Harold (1972).
and outer phases of the mitochondrion, this generates a pH and electrical potential gradient resulting in a force (the protonmotive force or PMP) which tends to drive the translocated protons back into the mitochondrial matrix. Thirdly, an anisotropic reversible ATPase exists which catalyses the reaction:

\[
\text{ATP} + H_2O + 2H^+_{\text{in}} \rightarrow \text{ADP} + P_i + 2H^+_{\text{out}}
\]

\[
K_{eq} = \frac{[\text{ADP}][P_i]}{[\text{ATP}][H^+_{\text{in}}]^2} = \frac{[\text{H}^+_{\text{out}}]^2}{[\text{H}^+_{\text{in}}]^2}
\]

In this system the active centre of the ATPase is inaccessible to water and the dehydration of ADP and Pi is accomplished by the removal of H\(^+\) into the inner compartment and OH\(^-\) in the opposite direction. It can be seen from the equation that the direction of the ATP reaction (i.e. ATP hydrolysis or synthesis) is determined by the \((H^+)_{\text{in}}/(H^+)_{\text{out}}\) ratio, thus any reaction removing \((H^+)_{\text{in}}\) will drive the ATPase in the direction of ATP synthesis. This situation is considered to be achieved by the proton translocating respiratory chain. Recent reviews by Racker (1970a; 1970b) outline the evidence available which show the requirement for the P\(_1\)-ATPase in oxidative phosphorylation. Mitchell proposes that P\(_1\)-ATPase is the reversible proton translocating ATPase required for this hypothesis. Fig.1.6., shows two possible schemes for the ATPase, OH and O\(^2-\) being translocated by ionizable groups IOH and XH (COOH and XHCOO\(^-\) respectively), thus OH\(^-\) or O\(^2-\) would be translocated to the outer phase of the membrane as XH and IO\(^-\) or X\(^-\) and IO\(^-\) respectively.

On the outer phase of the membrane an X-I anhydride could produce an anhydride X-I and release the translocated protons to the outer phase. Mitchell further suggests that X-I undergoes a transition to a high energy intermediate X-I which would then react as follows:
Fig. 1.6. Alternative proton-translocating ATPase systems suggested by Mitchell, effecting the synthesis of ATP in a mitochondrion. (C) and (D) indicate how the transport of OH\(^-\) and O\(^2-\), (A) and (B) respectively, might be accomplished by hypothetical ionizable groups XH and I-OH. Hy, X-I hydrolase; Sy, X-I synthetase; In, inner compartment (matrix); Out, outer compartment.
ATPase I  X~I + ADP + POH ----→ ATP + X-H + IO^- + H^+
ATPase II  X~I + ADP + POH ----→ ATP + X^- + IO^- + 2H^+

The above reactions would be catalysed by an X—I synthetase, Mitchell proposes the F1-ATPase as the synthetase and the hydrolase as the F0 components. Of the the two ATPase systems put forward, ATPase II is the more likely as it fits with the 2H+/ATP stoichiometry found by Mitchell and Moyle (1965; 1968). The ATPase schemes do not take into account the ionization of the ATP, ADP and Pi. Fig.1.7a., shows a possible scheme for ATPase I in which the phosphate carrier and adenine nucleotide carrier (Adn carrier) are also included, phosphate transport is not electrogenic, thus the H_2PO_4^-/H^+ symport is also included, this idea then satisfies the 2H^+/ATP ratio. A more recent scheme for the ATPase not involving high energy intermediates is shown in Fig.1.7b.

The evidence for the chemiosmotic theory has been reviewed in detail by Greville (1969) and more recently by Mitchell (1976) and it is beyond the scope of this discussion to review all the necessary literature supporting the theory. Summarising, it can be said; 1. the electron transport chain does transport protons (Mitchell, 1967; Jagendorf, 1967), whether or not it does so by the Mitchell 'loops' is debatable; 2. the inner membrane is relatively impermeable to protons; 3. the requirement for a vesicle for phosphorylation is an arguable point due to the intrinsic difficulty in deciding whether or not a vesicle is present; 4. the theory convincingly explains uncoupler action as being due to an intrinsic property of uncouplers to ferry protons across the membrane and thus short circuits the pH gradient. However, it is interesting to note that recently Hanstein and Hatefi (1974) produced evidence for a specific uncoupler binding site, a result difficult
Fig. 1.7. Two alternative schemes for Mitchell's proton translocating ATPase. a) ATPase I as in Fig. 1.6, but taking into account the phosphate carrier (PC) and Adn nucleotide translocase (AdnT). b) ATPase scheme without intermediates. From Greville (1969) and Mitchell (1973).
to rationalise in chemiosmotic terms. Although there is a lot of evidence for Mitchell's theory it can be argued that phenomena associated with the theory such as the 'proton pump' could be powered by the chemical intermediates $X\sim I$ and $X\sim P$. A final solution to the controversy must lie in convincing experiments such as phosphorylation in a non vesicular environment or alternatively, conclusive evidence of the Mitchell 'loops'.

The Conformational/Electromechanochemical Theory.

The conformational theory was first proposed by Boyer (1964) and then extended by Green and Ji (1972) into the electromechanochemical theory (see Green, 1973 for review). Briefly, it envisages four premises: 1. there is a transducing unit called the supermolecule which is capable of performing all the coupling operations; 2. the supermolecule can reversibly transduce electrical, mechanical or chemical energy; 3. electron transport and ATP hydrolysis generate charged pairs; 4. interaction between exergonic and endergonic reaction centres involves electric fields and induced polarisation. Basically, the supermolecule consists of two elements, one is polarised by the separation of electrons and protons in the electron transport chain, this polarised element reacts with a depolarising component, the ATPase, in such a way that a conformational strain is induced in the enzyme, which is sufficient to drive ATP synthesis. After ATP formation, the ATPase reverts to a non strained conformation which can then depolarise the polarizing element of the supermolecule. The credulity of the conformational theory rests on evidence of direct complexation between the ATPase and redox centres, and as Mitchell (1976) has pointed out, this does not exist.
The Mitochondrial ATPase Complex.

It is generally accepted that the ATPase complex is the site of ATP synthesis, whether or not ATP synthesis observed in situ is just the reversal of the ATP hydrolysis observed in vitro is a moot point which is still not resolved. The ATPase complex is probably the most complex multisubunit enzyme known, performing in situ a number of reactions such as ATP-Pi exchange, ATP hydrolysis and synthesis, and also affected by a variety of inhibitors e.g. oligomycin, venturicidin, DCCD, mercurials, Dio 9 and substituted trialkyl tins. The inhibitor studies have proved to be very useful in trying to determine subunit/activity relationships in the complex, and obviously will continue to be so. The object of this thesis being exactly that. It is necessary to have some understanding of the ATPase complex and the following section is directed towards presenting a picture, which although incomplete, is the more incontrovertible one in terms of hard experimental evidence. A scrutiny of the recent reviews by Beechey (1974), Pedersen (1975), Tzagoloff, Rubin and Sierra (1973), Senior (1973), Beechey and Cattell (1973), Beechey (1974) and Catterall and Pedersen (1974), should give the reader a good background to the current ideas in the field.

Early work (Pullman et al., 1960 and Ponfasky et al., 1960) showed that treatment of beef heart mitochondria in a Nossal shaker resulted in particulate and soluble factors, both of which were necessary to reconstitute oxidative phosphorylation. The particulate fraction catalysed various electron transfer reactions and had no ATPase activity, whereas the soluble fraction designated as a coupling factor 1 i.e. F₁-ATPase, catalysed ATP hydrolysis. F₁-ATPase is cold labile and has no ATP-Pi exchange activity, it can be used to
reconstitute oxidative phosphorylation in $P_1$-ATPase depleted membranes. Penefsky and Warner (1965) showed that purified $P_1$-ATPase produced a sedimentation pattern consistent with a compact symmetrical molecule of 11.9S units, the action of low temperature and salts resulted in the dissociation of the complex into two or more components with different sedimentation coefficients. Morphologically speaking, $P_1$-ATPase appears to be a spherical molecule approximately 9nm in diameter. Various workers suggested that the spheres (IMS) seen on the inner membrane of the mitochondrion were in fact $P_1$-ATPase. Kagawa and Racker (1966) tritiated $P_1$-ATPase chemically and reconstituted $P_1$-ATPase depleted membranes with the labelled enzyme. The reconstituted particles had ATP-Pi exchange activity and showed IMS in the electron microscope, urea treatment removed all the radioactivity, coupled with the disappearance of the IMS. Racker and Horstman (1967) also showed that SU particles which are $P_1$-ATPase depleted membranes do not possess IMS, reconstitution of the membranes restores ATP-Pi exchange activity and IMS. Moreover, the size of the IMS is consistent with the size of soluble $P_1$-ATPase.

The early work of Penefsky and Warner (1965) ascribed a molecular weight of 284,000 daltons to $P_1$-ATPase, however subsequent work by a number of investigators has led to a generally accepted value of 340,000-384,000 daltons for the enzyme isolated from rat liver, beef heart and S. cerevisiae mitochondria (Lambeth et al., 1971; Lambeth and Lardy, 1971; Caterall and Pedersen, 1971; Senior and Brooks, 1971; Tzagoloff and Meagher, 1971). Electrophoresis of $P_1$-ATPase in sodium dodecyl sulphate reveals up to 5 different subunits as shown in Table 1.1a. The two larger subunits have similar molecular weights and appear in approximately equal
Table 1.1  Apparent molecular weights of ATPase subunits.

### a) $F_1$-ATPase

<table>
<thead>
<tr>
<th></th>
<th>Apparent Molecular Weight</th>
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<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Rat liver</td>
<td>62,500</td>
</tr>
<tr>
<td>(Caterall &amp; Pedersen, 1971)</td>
<td></td>
</tr>
<tr>
<td>Beef heart</td>
<td>53,000</td>
</tr>
<tr>
<td>(Senior &amp; Brooks, 1971)</td>
<td></td>
</tr>
<tr>
<td>Yeast</td>
<td>58,500</td>
</tr>
<tr>
<td>(Tzagoloff &amp; Meagher, 1971)</td>
<td></td>
</tr>
</tbody>
</table>

### b) OS-ATPase

<table>
<thead>
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<th></th>
<th>Beef heart</th>
<th>Beef heart</th>
<th>Yeast</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Swanljung et al, 1973)</td>
<td>(Capaldi, 1973)</td>
<td>(Tzagoloff &amp; Meagher, 1971)</td>
<td></td>
</tr>
<tr>
<td>178,000$^1$</td>
<td>126,000$^1$</td>
<td>84,000</td>
<td>73,000$^1$</td>
</tr>
<tr>
<td>126,000$^1$</td>
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<td>55,000</td>
</tr>
<tr>
<td>58,000</td>
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<td>8,000</td>
<td>8,000</td>
<td>7,500</td>
<td>8,000</td>
</tr>
</tbody>
</table>

$^1$ Thought to be either aggregates or impurities.
Table 1.2. Preparations of OS-ATPase

<table>
<thead>
<tr>
<th>Authors</th>
<th>Source</th>
<th>Method of Preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tzagoloff et al (1968)</td>
<td>Beef heart</td>
<td>Extraction with deoxycholate, cholate, fractionation with ammonium sulphate.</td>
</tr>
<tr>
<td>Kagawa and Racker (1966)</td>
<td></td>
<td>Extraction with cholate, fractionation with ammonium sulphate.</td>
</tr>
<tr>
<td>Swanljung and Emster (1971)</td>
<td></td>
<td>Extraction with deoxycholate, gel filtration through Sepharose.</td>
</tr>
<tr>
<td>Swanljung et al (1973)</td>
<td></td>
<td>Extraction with Triton X-100, affinity chromatography on Sepharose 4B containing F$_{1}$-ATPase inhibitor protein.</td>
</tr>
<tr>
<td>Tzagoloff (1969)</td>
<td>Yeast</td>
<td>Extraction with deoxycholate, cholate, fractionation with ammonium sulphate.</td>
</tr>
<tr>
<td>Tzagoloff and Meagher (1971)</td>
<td></td>
<td>Triton X-100 extraction, density gradient centrifugation in Triton X-100</td>
</tr>
</tbody>
</table>
amounts, together they account for the larger part of the complex (approximately 90%) as estimated by the uptake of stain in polyacrylamide gels. The third largest subunit is present in appreciable amounts, but the amounts of the two smaller subunits are somewhat variable. Senior (1973) has suggested a subunit arrangement of $A_3B_3C_3D_3E_3$ for beef heart, based on observations that electron micrographs (Kagawa and Racker 1966; Racker and Horstman 1967; Tzagoloff and Meagher, 1971.) show a hexagonal array of subunits (presumably the A and B), the arrangement would fit a molecular weight of 340,000-384,000 daltons and would also be consistent with the gel staining results. However, it is worth pointing out that the ability to take up dye is not necessarily an estimate of the quantity present, subunit 9 of the OS-ATPase for example stains poorly (Tzagoloff, Rubin and Sierra, 1973). Other complications are introduced by the presence of a sixth subunit in some preparations (Pullman et al, 1960; Pullman and Monroy, 1963; Horstman and Racker, 1970) known as the $F_0$-ATPase inhibitor. All five subunits of the $F_0$-ATPase have been purified and the amino acid analyses carried out (Knowles and Penefsky, 1972; Brooks and Senior, 1972). $F_0$-ATPase catalyses ATP hydrolysis in the presence of $\text{Mg}^{2+}$ with specific activities around 60 umole/mg/min, the ATPase activity is not sensitive to oligomycin although it is sensitive to aurovertin (Kagawa and Racker, 1966), azide (Slater, 1955) and $F_0$-ATPase antibody (Fessenden and Racker, 1966), all of which inhibit oxidative phosphorylation.

The $F_1$-ATPase complex is present in the membrane as part of the larger OS-ATPase complex. Several groups of workers have purified the OS-ATPase from beef heart, rat liver and yeast (see Table 1.2). All the available methods of purification involve solubilization of the membrane with a
detergent and then appropriate fractionation procedures. The purest preparation is that of Tzagoloff and Meagher (1971) from yeast which has a specific activity of around 30 μmole/mg/min. It contains approximately 10% phospholipid, full activity of the enzyme being dependent on the presence of exogenous lipid. The Tzagoloff preparation is a comparatively easy purification method when compared to those methods available for beef heart (Table 1.2.). Apparently, Triton X-100 has a high degree of specificity for the yeast OS-ATPase. The Tzagoloff enzyme is cold stable unlike F$_1$-ATPase and is inhibited by oligomycin, it does not possess ATP-Pi exchange activity unlike the preparation of Ryrie (1975). In general OS-ATPase preparations require phospholipid for maximal activity, largely due to the fact that detergent extraction procedures used in their preparation delipidate the enzyme. The Tzagoloff enzyme appears as a homogenous complex which in electron micrographs appears as an oval particle, molecular weight estimates by sedimentation velocity analysis show a value of 468,000 daltons (after correcting for 10% phospholipid). Gel electrophoresis of the enzyme under dissociating conditions reveal eight stained bands and one component which stains poorly and has a low molecular weight. Tzagoloff and Meagher (1971) have shown that bands 1, 2, 3, 4 and 8 correspond to the subunits A, B, C, D and E of F$_1$-ATPase (Table 1.1.). Bands 5, 6 and 9 are part of the membrane components (F$_0$) of the enzyme, in addition band 8 is comprised of two subunits, one corresponding to subunit E of the F$_1$-ATPase and the other to the oligomycin sensitive conferring protein (OSCP) first isolated by MacLennan and Tzagoloff (1968). OSCP is necessary for the reconstitution of oligomycin sensitive ATPase and binds F$_1$-ATPase to the membrane (Tzagoloff 1970, Tzagoloff, Rubin and Sierra, 1973). OSCP does not bind
oligomycin and Beechey (1974) has suggested a more apt name would be ATPase binding protein.

The $F_0$ components have one thing in common in that they are all extremely hydrophobic and are mitochondrially synthesized (Tzagoloff, Rubin and Sierra, 1973). Extraction of submitochondrial particles with chloroform-methanol (2:1) predominantly removes subunit 9 which is the most hydrophobic protein of the $F_0$ components and contains a very high percentage of non-polar amino acids (Tzagoloff, Rubin and Sierra, 1973). Furthermore, subunit 9 has been shown to aggregate to give a hexamer with an apparent molecular weight of 45,000 daltons, treatment with alkali or organic solvents results in the monomeric form being produced. The other membrane components have not been purified although they can be easily extracted with acidic chloroform methanol (Sierra and Tzagoloff, 1973).

Until recently the beef heart OS-ATPase preparations described in the literature (Table 1.2) have not equaled the Tzagoloff preparation from yeast. However, Hatefi et al. (1974) have demonstrated a preparation (Complex V) for beef heart which has an equivalent number of subunits to that of the yeast OS-ATPase. The enzyme is characterised by being activated by phospholipids and exhibiting sensitivity to a variety of inhibitors of oxidative phosphorylation. Of more interest, is the fact that the enzyme exhibits ATP-Pi exchange without the need of the special reconstitution systems described by Kagawa and Racker (1971) and Racker (1973) which require the formation of enzyme-phospholipid vesicles from membrane fragments (extracted with cholate and ammonium sulphate), $F_1$-ATPase, OSCP and phospholipids. The Hatefi preparation will carry out ATP-Pi
exchange simply by mixing the enzyme with phospholipid. A modified version of the Hatefi preparation has been presented by Serrano, Kanner and Racker (1976), this enzyme preparation has the advantage over the Hatefi preparation in that it is quicker to carry out. Otherwise, it appears essentially similar to Complex V.

Catalytically the OS-ATPase can be divided into two components, the $F_1$-ATPase and $F_0$+ OSCP components. Only $F_1$-ATPase has any known catalytic function, $F_0$ does not appear to have any catalytic activity, although, as discussed later, it does have an important structural and regulatory role. The pure soluble $F_1$-ATPase has only one recorded catalytic function, and that is the hydrolysis of ATP. The hydrolysis requires the presence of Mg$^{2+}$, although other metal ions such as Co$^{2+}$, Mn$^{2+}$ and Fe$^{2+}$ will also support hydrolysis (Pedersen, 1973). The Km values for ATP in the rat liver and beef heart enzymes are between 0.79 and 1.25 mM at pH values between 7.4 and 8.0 (Catterall and Pedersen, 1974; Van de Stadt et al., 1973; Hammes and Hilborn, 1971). Several authors have reported that the $F_1$-ATPase has a much greater affinity for ATP when it is membrane bound (Hammes and Hilborn, 1970; Mitchell and Moyle, 1971; Pedersen, 1973) as shown by lower Km(ATP) values. It may be that the membrane bound ATPase has a different conformation from the soluble enzyme. Although it is generally agreed that ADP inhibits the $F_1$-ATPase in both the soluble and membrane bound forms, the values reported for the $K_i$(ADP) differ widely from laboratory to laboratory. Pullman et al. (1960), for example, quote a value of around 10mM for the $K_i$(ADP) in the purified $F_1$-ATPase, whereas Hammes and Hilborn (1971) record a value of 30 pM. The only values for the OS-ATPase have been obtained by Tzagoloff et al. (1968), who recorded a value of 10mM for producing 50% inhibition.
The investigation of ADP binding to the $F_1$-ATPase has produced conflicting results, in the case of the beef enzyme two sites have been detected by Hilborn and Hammes (1973). One is a high affinity site of high specificity for ADP ($K_d = 0.28 \mu M$ with a $Mg^{2+}$ concentration of 2mM and a $K_d$ of 11 $\mu M$ when EDTA is present) the other site is of lower affinity ($K_d = 47 \mu M$) and of the same order as is the $K_4(ADP)$ value for ATPase inhibition recorded by the same authors (Hammes and Hilborn, 1971). This binding site was also relatively insensitive to the presence or absence of $Mg^{2+}$. In contrast, Catterall and Pedersen (1972; 1974) have only found one ADP binding site in rat liver $F_1$-ATPase ($K_d = 0.94 \mu M$ with no $Mg^{2+}$ and $K_d = 2.0 \mu M$ with 5mM $Mg^{2+}$), the affinity of this site is similar to the $K_m(ADP)$ value for oxidative phosphorylation. It would appear that the $F_1$-ATPase in rat liver has only one ADP binding site, alternatively the conditions used by Catterall and Pedersen may not have been suitable for detecting the lower affinity binding site. The results for the beef enzyme suggest that the low affinity binding site participates in ATP hydrolysis and the high affinity site in ATP synthesis. The latter suggestion for two separate sites, one for synthesis and the other for hydrolysis is substantiated by findings that $F_1$-ATPase inhibitor (Pullman and Monroy, 1963), AMP-PNP (Penefsky, 1974) and quercetin (Lang and Racker, 1974) will inhibit ATP dependent reaction but not oxidative phosphorylation. Similarly, aurovertin is a more effective inhibitor of oxidative phosphorylation than it is of ATP supported energy-linked reactions and ATPase activity (Roberton et al. 1968; Lardy et al. 1964; Connelly and Lardy, 1964; Lee and Ernst, 1968). Chang and Penefsky suggest that there are two sites for aurovertin on $F_1$-ATPase, one is responsible for ATPase inhibition, the other effecting inhibition of ATP synthesis. Aurovertin decreases the
ADP binding in the absence of Mg\(^{2+}\) (Catterall and Pedersen, 1974). However, in the presence of Mg\(^{2+}\) aurovertin increases the ADP binding. Thus, it would appear that aurovertin inhibition is due to a direct interaction with the ADP binding site involved in oxidative phosphorylation.

The actual catalytic site on the F\(_1\)-ATPase has not so far been delineated. Of interest, is the work of Radda and co-workers (Ferguson, Lloyd, Lyons, and Radda, 1975; Ferguson, Lloyd, and Radda, 1975) with the inhibitor 4-chloro-7-nitrobenzofurazan (Nbf-Cl). Their work has shown that a specific tyrosine residue on the F\(_1\)-ATPase complex is essential for activity, inactivation of this tyrosine residue with Nbf-Cl leads to the loss of activity. This surprising result indicates that either there is one single catalytic site, or that a complex set of cooperative subunit interactions is an integral part of the catalytic mechanism.

The F\(_o\) component does not appear to have any catalytic activity. However, it does have an important function in that it binds F\(_1\)-ATPase to the membrane. The membrane bound OS-ATPase complex then has an ATPase activity which is sensitive to oligomycin (Lardy et al. 1958), venturicidin (Walter et al. 1967), DCCD (Beechey et al. 1967) and the trialkyl tin compounds (Aldridge and Street, 1964). The complexation of F\(_1\)-ATPase with F\(_o\) and OSCP leads to a modification of the F\(_1\)-ATPase as witnessed by the fact that the ATPase activity of the purest OS-ATPase is only 30 \(\mu\)mole/mg/min whereas ATPase activities of 60 \(\mu\)mole/mg/min have been recorded for the F\(_1\)-ATPase. Of the inhibitors acting on the OS-ATPase only DCCD has been unquestionably located to a particular subunit of F\(_o\). Beechey and co-workers in an elegant series of papers (Cattell et al. 1971; Beechey et al. 1967; Roberton et al. 1968)
Cattell et al. (1970) showed that DCCD binds to a subunit of $F_0$ which is extremely hydrophobic and has a molecular weight of approximately 10,000 daltons. The DCCD binding protein has been suggested to be subunit 9 of the OS-ATPase, but as Beechey (1974) points out, the evidence for this is not conclusive. Partis (1975) has presented evidence that the DCCD binding protein in yeast is equivalent to subunit 9. Evidence for oligomycin acting on $F_0$ comes largely from experiments with $F_1$-ATPase and OSCP depleted membranes (Mitchell, 1973; 1975; Mitchell and Moyle, 1974). In such preparations the vesicles have a high permeability to protons, the addition of oligomycin or reconstitution with $F_1$-ATPase and OSCP (Racker, 1967) effectively renders the membrane impermeable to protons. Mitchell believes that these experiments clearly prove that the function of the $F_0$ is to provide a proton channel through the membrane. Dawson and Selwyn (1975) have shown that tributyl tin will also reduce the abnormal proton conductance of $F_1$-ATPase and OSCP depleted membranes to a more normal level. Although these experiments can be taken to indicate that the function of the $F_0$ components is to transport protons to the catalytic site of the $F_1$-ATPase, an alternative explanation is that the $F_0$ catalyses the intermediate reactions described for the chemical intermediate theory of oxidative phosphorylation. The basic argument of how the OS-ATPase can synthesize ATP is not solved, however, one thing is clear, the OS-ATPase is necessary for ATP synthesis and $F_1$-ATPase on its own can only hydrolyse ATP.

A puzzling aspect of the OS-ATPase is the size and complexity of the molecule, it seems strange that such a large complex should only catalyze ATP hydrolysis and the transmembrane movement of protons. The possibility that it catalyses other reactions such as ion transport cannot be ruled out.
Evidence from this laboratory (Cain, Griffiths and Lancashire, 1974) has shown that the adenine nucleotide translocase may be functionally related to the OS-ATPase by a common subunit. Vignais et al. (1975) have produced evidence that ADP transported into the mitochondrion is first phosphorylated to ATP before being released into the matrix. A finding which suggests that at the very least the adenine nucleotide translocase is in very close association with the OS-ATPase. Obviously, the exact function / structure / activity relationships of the subunits must await more profound methods of subunit isolation than those currently available. Only when we can purify the subunits to a high degree, will it be possible to carry out unambiguous reconstitution experiments to determine their actual function. It must be pointed out that the reconstitution experiments pioneered by Racker suffer from a basic defect in that many of the components he uses are not highly purified (see Serrano, Kanner and Racker, 1976, for example). Consequently, it is virtually impossible to assign definite functions to particular subunits.

A Genetic Approach to Oxidative Phosphorylation.

Auxotrophic mutants have long been a very useful tool in the study of the elucidation of many biochemical pathways. As outlined in the preceding sections of this chapter, the chemical transformations occurring in oxidative phosphorylation have been difficult to resolve with the usual biochemical techniques. The fundamental problem is that the catalytic units are often an integral part of the membrane structure. This observation has prompted a number of laboratories to tackle the problem of energy conservation by means of a mutant approach. Much of the work to date has been carried out on the eucaryot, the yeast.
Kovac (1974) has reviewed the advantages of yeast as a suitable organism for biochemical genetic studies of oxidative phosphorylation. Briefly, its main advantages are: 1. it possesses mitochondria with properties very similar to mammalian mitochondria; 2. its biochemistry, genetics and cytology are known in great detail; 3. it can survive major genetic lesions affecting mitochondria, by virtue of the fact that it can grow on fermentable substrates. The most suitable yeast for these studies is the strain Saccharomyces cerevisiae, largely because its biochemistry, genetics and cytology are the most well documented. The methods of isolation and screening for suitable mutants have been described by Kovac (1974) and will not be discussed. The basic approach has been to make use of the fact that mitochondria are semi-autonomous organelles possessing their own DNA (mt DNA) and protein synthesizing systems. Moreover, the mitochondrial protein synthesizing system can be specifically inhibited by chloramphenicol and erythromycin (Wheeldon and Lehninger, 1966; Lamb et al., 1968) but not by cycloheximide which inhibits the cytoplasmic synthesizing system. This has enabled several workers to determine which components of the mitochondria are synthesized on the mitoribosomes. The available evidence (see Schatz and Mason, 1974; Tzagoloff, Rubin and Sierra, 1973) indicates that three subunits of cytochrome oxidase, two subunits of ubiquinone-cytochrome C reductase and the four membrane subunits of the ATPase system are mitochondrially synthesized. A general assumption is that mt DNA codes for the mitochondrially synthesized proteins. Consequently, any mutation affecting oxidative phosphorylation which is mitochondrially coded will most likely result in a protein modification of a mitochondrially synthesized subunit.
The first types of mutant to be actively investigated were the respiratory deficient mutants known as petites. Petites can form spontaneously and can be characterised by the fact they will form smaller than normal size colonies on solid medium containing glucose as a carbon and energy source (Ephrussi, 1953). Alternatively, petites can be induced by the action of chelating drugs such as ethidium bromide (see Lloyd, 1974 for review). The major characteristics of petite mutants are: 1. the absence of cyanide sensitive respiration; 2. the presence of an active glycolytic pathway; 3. an inability to grow on non-fermentable substrates. Petite mutations have been found to be due to a major deletion of the mt DNA (see Borst, 1972 for review), in some cases the whole genome may be deleted. Moreover, cytoplasmic petites do not possess a functional mitochondrial protein synthesizing system (Borst, 1972).

Perlman and Mahler (1970) and Mahler (1971) in a detailed study of a petite mutant showed that the mitochondria resembled those of wild type in shape, size and buoyant density. Levels of L-malate dehydrogenase, NADP dependent isocitrate dehydrogenase and ATPase were comparable to those of the wild type mitochondria. However, the ATPase was cold stable and oligomycin insensitive, but was inhibited by the F$_1$-ATPase inhibitor from beef heart. The respiratory chain of the mitochondria had the usual pleitropic mutations associated with the petite mutation. Kovac and Weissova (1968) also showed that petite mitochondria had oligomycin insensitive ATPase. The work of Ercoli (1972) and Tramploff and Meagher (1971) has shown that the ATPase present in petite mitochondria is F$_1$-ATPase and that the F$_0$ components are absent. Mitochondria of petite mutants do not catalyse an ATP-Pi exchange reaction and are not energised by ATP as shown by the lack of
fluorescence with ANS (Kovac, Groot and Racker, 1972). The conclusion from these experiments is that the $F_0$ subunits are necessary for the ATPase to participate in energy conservation. On the other hand, the mitochondrial membrane is impermeable to protons and would on the basis of the chemiosmotic theory, be capable of energy conservation.

Although the apparent lesions in petite mitochondria can be rationalised on the basis of the known biogenesis data, conflicting data has been reported on other important components involved in the energy conservation processes. In particular, there is disagreement about the translocase for adenine nucleotides. Perkins et al. (1972) and Haslam et al. (1972) produced evidence that the adenine nucleotide translocase is not present in petite mitochondria, they concluded that "the products of the mitochondrial protein synthesizing system, probably coded by mitochondrial DNA, are required for the normal function of the adenine nucleotide transporter". However, Kolarov et al. (1972), have concluded that the translocase is present in petite mitochondria. In addition, Kolarov and Klingenberg (1974) have shown that carboxyatractyloside (CAT), a potent inhibitor of the adenine nucleotide translocase, binds to petite mitochondria with a similar affinity to that exhibited by wild life mitochondria. An important difference in the mutant mitochondria was that the number of CAT binding sites was approximately four fold less than in the wild type strain. A similar result for atracyloside is demonstrated in chapter 5 of this thesis, essentially equivalent results for both atracyloside and bonkreic acid have been obtained by Vignais and coworkers (personal communication). It has been suggested (Kolarov and Klingenberg, 1974) that the decrease in CAT and ADP binding sites in the petite mitochondria is due to other membranes...
contaminating the mitochondria. Alternatively, the diminished number of sites may reflect a significant membrane change in the mitochondria. The op\textsubscript{1} mutant (see Kovac, 1974 for review) is a nuclear mutant which can respire on non-fermentable substrates but not grow on them. In this mutant the adenine nucleotide translocase is extensively modified, indicating that a nuclear gene has a positive involvement in the translocase. On the other hand, work presented in this thesis (Cain et al. 1974) points to a requirement for mitochondrial genes in the normal functioning of the translocase.

A different approach to the use of mutants in biochemical genetic studies has been adopted by Griffiths and coworkers in this laboratory. The technique used has been to produce drug-resistant mutants according to the following rationale (Griffiths et al. 1972): 1. inhibitors of mitochondrial energy conservation reactions have specific inhibitor sites associated with specific protein subunits of the oxidative phosphorylation complex; 2. ideally the inhibitor should not be metabolised and should have a mode of action predominantly, if not exclusively on the inner membrane of the mitochondrion; 3. the inhibitor should specifically inhibit aerobic growth on non-fermentable substrates, but have no effect on fermentive growth; 4. inhibitor resistant mutants should exhibit modified sensitivity at the mitochondrial, sub-mitochondrial levels and also ideally in the purified enzyme preparation.

The demonstration that the mutation is cytoplasmically inherited and located on mt DNA is good \textit{a priori} evidence that a mitochondrial gene product that is a component of the oxidative phosphorylation has been modified. The demonstration of mitochondrial inheritance of the resistance mutation is of key importance in assessing whether a modification of the drug receptor site
or a detoxifying/permeability phenomenon has occurred. A large number
of inhibitor resistant mutants have been isolated (Griffiths et al. 1972;
Griffiths, 1975). The first mutants to be isolated were those resistant
Two classes were differentiated: Class I mutants showed cross resistance to
aurovertin, Dio-9, venturicidin, triethyltin, antimycin A, bis-hexafluoro-
acetonyl acetone (1799), uncoupling agents and protein synthesizing inhibitors
such as chloramphenicol, mikamycin, erythomycin and cycloheximide; Class II
mutants were specifically resistant to oligomycin and structurally related
antibiotics (rutamycin, ossamycin, pekimycin) and exhibited no cross-
resistance to venturicidin, triethyltin, DCCD, aurovertin or uncoupling agents.
The majority of the Class I mutants and one of the Class II mutants lost
resistance at the non-permissive temperature of 20°C, and can be subclassified
on this basis. The Class I mutants exhibited normal growth yields whereas
the Class II strains had growth yields which were 15-20% less than those
of the parental strain. The morphology of the mutant mitochondria appeared
to be unaltered (Watson and Linnane, 1972). The genetic analysis of Class I
mutants revealed a complex involvement of both nuclear and cytoplasmic genes
(Avner and Griffiths, 1973). The resistance phenomenon in the Class I
mutants could be the result of one or more of the following parameters:
1. permeability barriers to the inhibitors; 2. the presence of a cytoplasmic
detoxifying mechanism; 3. mitochondrial detoxification and non specific
binding; 4. modification of the inhibitor binding site in the OS-ATPase.
The Class II mutants are all cytoplasmically determined with the mutated
loci residing in mt DNA. The resistance phenomenon is conferred by at least
two groups of distinct non-allelic cytoplasmic determinants which map on the
mitochondrial genome at two loci designated OL_I and OL_{II}. 
Biochemical experiments (Houghton et al. 1974; Griffiths et al. 1974) on the Class II mutants revealed that the ATPase, ATP-Pi exchange and ADP stimulated respiration in isolated mitochondria were all less sensitive to oligomycin. Moreover, the purified OS-ATPase also exhibited the decreased sensitivity, the theory of Swanljung et al. (1972) that ergosterol levels were changed in the mitochondria and that this was responsible for the resistance phenomenon has been disproved (Griffiths et al. 1974).

Similar studies on triethyl tin resistant mutants (Lancashire and Griffiths, 1971; Griffiths, 1974; Lancashire and Griffiths, 1975) have revealed the presence of both Class I and Class II resistant mutants. Class I mutants are cross resistant to oligomycin, uncoupling agents and mitochondrial protein synthesizing inhibitors. Class II mutants are specifically resistant to triethyl tin and show no cross resistance to oligomycin or other agents. A third class (Class III) has been defined on a basis of cross resistance to "1799" only. Class II mutants are cytoplasmic and the resistance determinant is deleted by the action of ethidium bromide. Recombination studies show that the triethyl tin resistance loci (T₁) is not allelic with OL₁ and OL₂₁. Unlike the oligomycin resistant mutants, the triethyl tin resistant mutants have limited resistance at the mitochondrial level (see Chapter 5 of this thesis).

Class II triethyl tin resistant mutants show a cross resistance to venturicidin. Studies with venturicidin resistant mutants (Griffiths et al. 1975) reveal similar results to oligomycin and triethyl tin mutants. Class II mutants are mitochondrially coded and can be divided into those cross resistant to oligomycin (VEN₁OL₁₁) and those cross resistant to
triethyl tin (VEN\textsuperscript{TET\textsuperscript{R}}). The VEN\textsuperscript{R}OLY\textsuperscript{R} mutants show modified sensitivity to venturicidin and oligomycin at both the whole cell level and also at the mitochondrial ATPase level. Recombination studies (Lancashire and Griffiths, 1975) show that the VEN\textsuperscript{R}OLY\textsuperscript{R} mutants map at a new mitochondrial locus that is closely linked to OL\textsubscript{I} and has therefore been termed the OL\textsubscript{III} locus. The VEN\textsuperscript{R}TET\textsuperscript{R} mutants map at the T\textsubscript{I} locus and are probably identical to the Class II triethyl tin resistant mutants.

Griffiths (1975) has interpreted these results as showing that oligomycin binding in the ATPase complex is effected by three binding points. Venturicidin is complexed by two attachment points, one of which it has in common with oligomycin, the other attachment point is equivalent to the triethyl tin binding site. Strong evidence for this hypothesis comes from experiments with the aglycone of venturicidin, which has lost the rhamnose residue. This modification produces an oligomycin like structure. Experiments with OLY\textsuperscript{R}, VEN\textsuperscript{R}OLY\textsuperscript{R} and VEN\textsuperscript{R}TET\textsuperscript{R} mutants on both in vivo and in vitro sensitivity to the aglycone reveal that the capability to interact with the T\textsubscript{I} locus is lost. These findings provide strong evidence for separate interaction sites for oligomycin, venturicidin and triethyl tin. Of interest is the finding that cytoplasmic mutants resistant to uncouplers and ionophores can be produced (Griffiths, 1975). This finding is difficult to rationalise in terms of chemiosmotic dogma. At the present time no resistant mutant has been correlated with a protein modification, although recently Tzagoloff et al. (1976) have produced evidence that OL\textsubscript{I} mutants have an aberrant subunit 9 which cannot form a hexamer.
Chapter 2.

The action of trialkyl tin compounds on the OS-ATPase of the yeast inner mitochondrial membrane.

Introduction.

Aldridge and Cremer (1955) first showed that triethyl tin sulphate was a potent inhibitor of oxidative phosphorylation in rat liver mitochondria, the triethyl derivative was more potent than the diethyl analogue. A number of papers from Aldridge's group (Aldridge, 1958; Aldridge and Thredfall, 1961; Aldridge and Street, 1964) demonstrated that trialkyl tin compounds inhibited ADP stimulated respiration, DNP stimulated respiration, $^{32}$Pi-ATP exchange, DNP stimulated ATPase (i.e. OS-ATPase) activity in rat liver mitochondria, in addition, low concentrations had a weak uncoupler like action. Sone and Hagihara (1964) and Stockdale, Dawson and Selwyn (1970) reported similar actions but did not reproduce the inhibition of DNP stimulated respiration. The apparent discrepancy between Aldridge and other workers concerning the inhibition of DNP stimulated respiration was resolved by Coleman and Palmer (1971). These workers showed that the inhibition of DNP stimulated respiration was dependent on an acid pH and a Cl$^-$ media, at the more alkaline pH's and in the absence of Cl$^-$ there was no inhibition (Coleman and Palmer, 1971; Rose and Aldridge, 1972). Further work by Dawson and Selwyn (1974) confirmed these findings, showing that trialkyl tins catalysed a Cl$^-$/OH$^-$ antiport across the inner mitochondrial membrane which produced the inhibition of DNP stimulated respiration.

The marked pH dependence of the ATPase inhibitory effect of triethyl
tin described by Coleman and Palmer (1971) was originally thought to be a reflection of trialkyl tin binding being pH dependent. However, Dawson and Selwyn (1974), showed that there was in fact a very pH dependent complex formed between the trialkyl tin and the inorganic phosphate used in the assay media. The oligomycin like action of trialkyl tins has been correlated with a high affinity binding site in rat liver mitochondria (Aldridge and Street, 1970, 1971; Rose and Aldridge, 1972.). Dawson and Selwyn (1975) have put forward evidence for the oligomycin like action to be due to a binding site complexed by trialkyl tins and located on the $F_{0}$ components of the OS-ATPase. However, their work is not direct evidence in that they have not correlated a binding site with the OS-ATPase components. The difficulty in their experiments is that there are not any good homogenous preparations for the beef heart and rat liver enzymes. In contrast the yeast OS-ATPase can be purified to a high degree of homogeneity and specific activity (Tsagoloff, Byington and Maclennan, 1968; Tsagoloff and Meagher, 1971.). The yeast OS-ATPase therefore offers a very good system for examining the site of action for trialkyl tins. This chapter examines trialkyl tin inhibition of the OS-ATPase in both membrane bound and soluble purified preparations. Subsequent chapters (3 & 4) will examine the site of action with the use of $^{113}$Sn labelled triethyl tin chloride and specially synthesised site directed affinity label, i.e., dibutyl chloromethyl tin chloride. A further probe into the site of action is described in Chapter 5, in which a biochemical genetic approach is used.
Materials and Methods.

Materials.

The yeast used in this study S. cerevisiae was the haploid strain D22 (αd2, r, α) donated by Dr. D. Wilkie. Trialkyl tin chlorides and triethyl tin sulphate were obtained as described by Lancashire (1974). Oligomycin and Triton X-100 were purchased from Sigma Chemical company, venturicidin was a gift from Dr. I. D. Fleming (Glaxo Laboratories, Stoke Poges, U.K.) All other chemicals were Analar grade purity. All solutions were made up in triple glass distilled water which had been deionised with Chelex-100.

Growth of yeast.

Batch culturing of yeast was carried out in 10L of culture medium in New Brunswick Fermentors, under vigorous stirring (400 r.p.m.) and aeration (10L/min). The growth media contained 0.5% (w/v) yeast extract, 0.1% (w/v) peptone, 0.01% (w/v) adenine sulphate, mineral salts (Wickerham, 1946) 1.0% (v/v) ethanol and tributyl citrate (0.01%, v/v) as an anti-foaming agent. A 1% (v/v) inoculum of late logarithmic cells was used and the batch culture harvested at the late logarithmic stage of growth (36-40h.), cells were collected in a Mistral 6L centrifuge by spinning at 2,000 r.p.m for 10min in 12L centrifuge bottles. Collected cells were washed twice in cold distilled water by centrifuging as above. The washed cells were resuspended in an equal volume of mitochondria isolation media (0.5M sorbitol, 20mM Tris-Cl, pH 7.5, 1mM EDTA) containing 0.1% (w/v) bovine serum albumin.
Preparation and Isolation of Mitochondria and Sub-Mitochondrial Particles.

All the following operations were carried out at 0-4°C. Yeast cells were disrupted by shaking cell suspensions with glass beads (0.45-0.5mm) for 30s. in a cooled Braun homogeniser (B.Braun, Melsungen) at 4,000r.p.m. The glass beads were washed between shakes with 10ml of media. The cell debris and glass beads were pelleted by spinning the cell homogenate in the G.S.A head of the Sorval R.C.2-B centrifuge at 3,000r.p.m for 10min. The pellet was discarded and the supernatant recentrifuged, this centrifugation procedure was repeated until no pellet was observed (usually 3 centrifugations were needed). The mitochondria were sedimented from the supernatant by centrifuging in the S.S.34 rotor at 14,000r.p.m for 20min. The mitochondrial pellet was resuspended to approximately 30mg/ml protein concentration in isolation media.

The mitochondrial suspension produced in this manner was then layered onto a discontinuous sucrose gradient (14ml each of 15%, 30%, 50% and 70% (w/v) sucrose solutions containing 20mM Tris-Cl-, pH 7.5, 1mM EDTA) and centrifuged in a Beckman S.W.25.2 rotor at 23,000r.p.m for 3h. The mitochondrial band at a density of 1.20g/cm³ was collected and diluted in mitochondria isolation media (without bovine serum albumin) and centrifuged as described above to pellet the mitochondria. This final mitochondrial pellet was resuspended to 20mg/ml protein concentration and represents the purified mitochondrial fraction.

Sub-mitochondrial particles were prepared by sonicating mitochondria (20mg/ml) for 1min (4 x 15s. bursts, allowing time for cooling between each sonication) with an M.S.E 60-W sonicator at full power. The
sonicated suspension was diluted with 2 volumes of buffer and centrifuged in the S.3-34 head at 14,000 for 15min to sediment unbroken and large mitochondrial fragments. The supernatant was then centrifuged at 35,000 r.p.m for 30min (i.e. 100,000g for 30 min), the submitochondrial pellet was resuspended in 0.25M sucrose, 20mM Tris-Cl\(^-\), pH 7.5, 1mM EDTA and washed by centrifugation at 100,000g for 30min. The final pellet was resuspended to approximately 20mg/ml and this represented the purified submitochondrial particles.

Preparation of Purified OS-ATPase.

A partially purified soluble OS-ATPase was prepared as described by Tzagoloff and Meagher (1971) by extracting submitochondrial particles at 6mg/ml with 0.5\% (w/v) Triton X-100, the extracted membranes (designated Triton X-100 extracted particles) were sedimented at 100,000g for 1h. The supernatant from this spin, the Triton X-100 extract represents the partially purified OS-ATPase (N.B. submitochondrial particles which were prepared from non-gradient purified mitochondria contained endogenous lipid which after extraction with Triton X-100 and centrifugation was seen as a white floating contaminating layer. In this case the supernatant represented the Triton X-100 extract. Purified submitochondrial particles do not exhibit this lipid layer on Triton X-100 extraction, consequently all submitochondrial particle preparations described in this thesis were prepared from gradient purified mitochondria.). Highly purified OS-ATPase was prepared as described by Tzagoloff and Meagher (1971) by centrifuging approximately 5ml of Triton X-100 extract on a 50ml continuous sucrose gradient (5\%-20\% (w/v) sucrose, 20mM Tris-Cl\(^-\), pH 7.5, 0.1\% (w/v) Triton X-100) in the Beckman S.W.25.2 rotor at 23,000 r.p.m for 18h. The gradient
after centrifugation was fractionated into twelve 4.5 ml fractions. Each fraction was assayed for protein and ATPase activity. The most active fractions were pooled and represent the purified OS-ATPase enzyme (see results section for more details of specific activity and inhibitor sensitivity). F₁-ATPase was prepared as described by Tzagoloff, MacLennan and Byington (1968).

**ATPase and Protein assay Methods.**

ATPase activity was as described by Griffiths and Houghton (1974) at 30°C, in 1.0 ml reaction medium containing 5 mM ATP, 2 mM MgCl₂, 50 mM Tris-maleate (pH 6.0-7.5) or 50 mM Tris-Cl⁻ (pH 7.5-9.5) and approximately 100 μg of protein (the amount of protein used was adjusted to give control values for inorganic phosphate released no greater than 2 μmole, this was necessary to ensure that substrate limiting conditions did not arise.). Assays were normally carried out at pH 9.5 except where indicated. Inhibitors were preincubated with protein for 10 min before adding the ATP, reactions were stopped by the addition of 1.0 ml of trichloroacetic acid (10%, w/v) and the precipitated protein removed by centrifugation, 0.5 ml samples were assayed for inorganic phosphate by the method of King (1932). Inhibitors were added in ethanol (no more than 1%, v/v final concentration) and no effect on the ATPase was observed at these concentrations. All assays were corrected for zero-time non-enzymic inorganic phosphate release. Specific activity (designated S.A.) is expressed as μmole of inorganic phosphate released per mg of protein per min (μmole/mg/min) and I₅₀ value as the concentration of inhibitor per mg of protein reducing the S.A. of a non-inhibited control by 50%.
Mitochondrial and purified enzyme protein concentrations were estimated by the method of Lowry, Rosebrough, Farr and Randall (1951). In the case of Triton X-100 enzymes, controls contained appropriate concentrations of Triton X-100.

Results and Discussion.

Trialkyltins are potent inhibitors of oxidative phosphorylation in mammalian systems, and work in this laboratory (Lancashire and Griffiths, 1975; Skipton, 1974) has shown that the potent fungicide effects in yeast are due to a direct action on mitochondrial oxidative phosphorylation. Triethyl tin inhibits ADP stimulated but not DNP stimulated respiration at an $I_{50}$ value of 13nmole/mg of protein (Skipton, 1974). It is therefore apparent that trialkyltin compounds inhibit yeast mitochondria in a similar manner to that exhibited in rat liver mitochondria. The inference from these findings is that the probable site of action for trialkyltin compounds in yeast is the OS-ATPase. Although it must be pointed out that there is another possible site of action on the adenine nucleotide translocase system which is examined in Chapter 5.

The general properties of the membrane bound OS-ATPase of yeast mitochondria were described by Kovac (1968) and Somlo (1968), the ATPase activity is sensitive to low levels of oligomycin and appears to be very similar to the beef heart enzyme. None of the mitochondrial preparations (i.e. mitochondria and submitochondrial particles) used in this work showed any stimulation with uncoupler a result in agreement with Kovac (1968). The lack of stimulation with uncoupler can be attributed to the difficulties experienced in preparing well coupled yeast mitochondria.
and also due to the fact that yeast mitochondria appear to be less well coupled than many mammalian systems (see Lloyd, 1974, Chapter 2 for review). In Fig. 2.1, the effects of some trialkyl tin compounds on the ATPase of submitochondrial particles is shown. Triethyl, tripropyl, and tributyl tin exhibit a very potent inhibitory effect with \( I_{50} \) values of 2, 3 and 5nmole/mg respectively. These values compare favourably with control experiments with oligomycin in which \( I_{50} \) values of approximately 3-4nmole/mg were recorded. Conversely, tetrabutyl and trimethyl tin are relatively impotent as ATPase inhibitors. Aldridge and Street (1971) have proposed that triethyl tin binding was due to penta-coordination taking place in the inhibitor site, the penta-coordination necessary for binding being dependent on the conformation of the alkyl tin compounds concerned. Consequently, tetrabutyl tin would be expected to be relatively ineffective due to the presence of its fourth alkyl group sterically hindering the penta-coordination. Trimethyl tin has also been shown to be relatively inactive in rat liver by Stockdale, Dawson and Selwyn (1970), in their study tripropyl and tributyl were very similar in potency. Aldridge and Street (1970) showed that trimethyl tin binding affinity was ten times less than that of triethyl tin in rat liver mitochondria, the poor efficacy of trimethyl tin as an inhibitor in both yeast and rat liver would appear to derive from a poor interaction with the trialkyl tin binding site. It would appear that efficient interaction with the trialkyl tin inhibitor site requires a certain minimum size for the alkyl group. It is of interest in this context that the larger the alkyl group the more potent the \( 37^\text{Cl} / \text{OH}^\text{−} \) exchange effect (Stockdale, Dawson and Selwyn, 1970). Thus, a trialkyl tin compound's potency as an ATPase inhibitor is correlated with its potency in mediating the \( \text{Cl}^\text{−} / \text{OH}^\text{−} \) exchange, for
Fig. 2.1. Inhibition of OS-ATPase in submitochondrial particles by alkyl tin compounds.

ATPase assays were carried out according to Griffiths and Houghton (1974) as described in the methods. S.A. = 5.0 μmole /mg/min; △, tributyl tin chloride; ○, tripropyl tin chloride; ●, triethyl tin sulphate; ▲, trimethyl tin chloride; ■, tetra- butyl tin chloride.
example trimethyl tin chloride is also the least potent in mediating 
Cl⁻/OH⁻ exchange.

One of the advantages of yeast mitochondria is the relative ease in 
which the OS-ATPase can be purified from its membrane bound form. A 
typical purification scheme is shown in Table 2.1.

Table 2.1. Purification of OS-ATPase by 0.5% (w/v) Triton X-100 extraction.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Protein (mg)</th>
<th>S.A.</th>
<th>ATPase units</th>
<th>Triethyl tin sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondria *</td>
<td>180</td>
<td>4.5</td>
<td>810</td>
<td>+ ve</td>
</tr>
<tr>
<td>Submitochondrial</td>
<td>90</td>
<td>5.5</td>
<td>495</td>
<td>+ ve</td>
</tr>
<tr>
<td>particles</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triton X-100 extract</td>
<td>45</td>
<td>9.7</td>
<td>436</td>
<td>+ ve</td>
</tr>
<tr>
<td>Triton X-100 extracted particles</td>
<td>45</td>
<td>1.1</td>
<td>50</td>
<td>- ve</td>
</tr>
<tr>
<td>OS-ATPase</td>
<td>10.8</td>
<td>30.0</td>
<td>324</td>
<td>+ ve</td>
</tr>
</tbody>
</table>

* The protein lost here is due to the removal of mitochondrial debris 
and matrix enzymes.

The sensitivity of the various preparations produced in the purification 
scheme is shown in Fig. 2.2., it can be seen that as the OS-ATPase is purified 
there is a gradual increase in the 50 nmole/mg in mitochondria 
to 4.5nmole/mg in the purified enzyme. Although there is an approximately 
6 fold increase in specific activity on purification of the OS-ATPase,
Fig. 2.2., Inhibition of various preparations of yeast mitochondrial OS-ATPase by triethyl tin sulphate.

Assay conditions were as described in Fig. 2.1.; ○—○, mitochondria; •—•, submitochondrial particles; ▲—▲, Triton X-100 extract; ■—■, OS-ATPase. The numbers in ( ) refer to the specific activities for that particular preparation.
there is only an approximately 3-fold increase in the $I_{50}$ value. As a 6-fold purification would indicate an increase in that order of actual ATPase units per mg of protein, one would also expect an equivalent increase in the number of inhibitor sites (if the OS-ATPase is the site of action) and therefore an equivalent increase in the $I_{50}$ value. This apparent discrepancy is not exhibited in the binding experiments described in Chapter 3, however, the binding experiments do provide an answer to this question and this is discussed in Chapter 3 in more detail.

Throughout this work the purification of the enzyme was carried out at 0.5% instead of the usual 0.25% Triton X-100. The reason for this is that preliminary experiments with 0.25% extractions revealed that the extracted membrane had a specific activity of approximately 2.5 of which approximately 40% was sensitive to triethyl tin, increasing the concentration to 0.5% dropped the specific activity of the extracted membranes to around 1.5 and the activity was insensitive to triethyl tin. In the Chapters (3&4) describing the locus of action of trialkyl tins, it was essential to extract all the triethyl tin sensitive ATPase. The purified OS-ATPase prepared by 0.5% extraction and gradient purification was exactly the same in terms of its responses to inhibitors to enzyme prepared by 0.25% extraction, and S.D.S gels revealed the usual pattern of subunits described by Tsagoloff, Sierra and Rubin (1973).

A typical OS-ATPase gradient is shown in Fig.23. Analysis of the gradient shows that maximum activity is obtained in the presence of exogenous phospholipid (azolectin). A property common to other OS-ATPase preparations described in the literature (Kagawa and Racker, 1966; Kopazyk
Fig. 2.3., Sucrose density purification of OS-ATPase. Preparation of the Triton X-100 extract and conditions for sucrose density centrifugation were as described in the methods. [▲], specific activity without azolectin; [■], specific activity with 30 mg/mg azolectin.
there is only an approximately 3 fold increase in the $I_{50}$ value. As a 6 fold purification would indicate an increase in that order of actual ATPase units per mg of protein, one would also expect an equivalent increase in the number of inhibitor sites (if the OS-ATPase is the site of action) and therefore an equivalent increase in the $I_{50}$ value. This apparent discrepancy is not exhibited in the binding experiments described in Chapter 3, however, the binding experiments do provide an answer to this question and this is discussed in Chapter 3 in more detail.

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A typical OS-ATPase gradient is shown in Fig. 2.3., analysis of the gradient shows that maximum activity is obtained in the presence of exogenous phospholipid (azolectin). A property common to other OS-ATPase preparations described in the literature (Kagawa and Racker, 1966; Kopazyk
et al, 1968; Tzagoloff et al, 1968; Svanljung et al, 1975; Cunningham and George, 1975). Partis (1975) has demonstrated that the effect of exogenous phospholipid on the yeast OS-ATPase essentially lowers the $K_m$ and increases the $V_{max}$ for ATP, a similar result has been obtained in beef heart by Svanljung et al. (1975). Moreover, the pH characteristics of the enzyme are unchanged, the added phospholipid does not appear to be necessary for inhibitor sensitivity although it can adversely modify the $I_{50}$ value. This point is demonstrated in Fig.2.4. and Fig.2.5., in the former, the activation of the ATPase activity by azolectin is demonstrated and is shown to be a saturable phenomenon. The latter figure shows that the unstimulated enzyme is fully sensitive to triethyl tin, addition of 10 and 30 mg/mg of azolectin produces maximal activation of the ATPase activity and increases the $I_{50}$ value from 4.5 to 10 and 17 nmole/mg respectively. An explanation for these results is that the enzyme requires a lipid environment for maximum activity but not for the inhibitor sensitivity, exogenous lipid in excess of this requirement for the correct environment effectively sequesters the inhibitor. This conclusion is backed up by the experiments of Partis (1975) which showed that small amounts of added exogenous lipid can stimulate the OS-ATPase and not effect the inhibitor sensitivity for oligomycin and DCCD. However, it must be pointed out that although exogenous phospholipid does not play a role in inhibitor sensitivity, there may well be a requirement for tightly bound phospholipid in producing an inhibitor sensitive enzyme, a conclusion in agreement with the observed fact that the enzyme as prepared contains 10% endogenous phospholipid (Tzagoloff and Meagher, 1971). In inhibitor sensitivity experiments with the pure enzyme, it is convenient and more reliable to do the assays on the unstimulated enzyme as even a
Fig. 2.4. Activation of OS-ATPase activity with azolectin.

OS-ATPase was prepared and assayed as described in the methods.
**Fig. 2.5.** Effect of exogenous phospholipid on the sensitivity of OS-ATPase to triethyl tin chloride.

- • , no azolectin, specific activity = 13 μmole/mg/min;
- ■, 10mg/mg azolectin, specific activity = 30 μmole/mg/min;
- ▲, 30mg/mg azolectin, specific activity = 30 μmole/mg/min.
small excess of exogenous phospholipid can alter the \textit{I}_{50} \text{ value} substantially. Consequently, all inhibitor experiments were normally carried out in the absence of azolectin, controls were always carried out with azolectin to ensure that the enzyme was fully activated and specific activities for the OS-ATPase normally refer to the activated enzyme.

A comparison of the inhibitory effects of various trialkyl tin salts on the purified OS-ATPase is shown in Fig. 2.6., it can be seen that the purified enzyme has similar properties to its membrane-bound form. Triethyl tin sulphate, tripropyl tin chloride and tributyl tin chloride have similar potencies whereas the trimethyl tin compound is relatively inactive.

Experiments on \textit{F}_{1}-\textit{ATPase} revealed that triethyl tin did not inhibit even at concentrations as high as 500mmole/mg, the enzyme was sensitive to the \textit{F}_{1}-\textit{ATPase} inhibitor aurovertin. This result on the yeast \textit{F}_{1}-\textit{ATPase} is in agreement with the data on beef heart enzyme in which several workers have reported that the trialkyl tin compounds are inactive against the enzyme (Tzagoloff, McClellan & Byington, 1968; Kagawa and Racker, 1966; Selwyn et al, 1972). The obvious interpretation of this result is that the \textit{F}_{0} components of the OS-ATPase contain the inhibitory site, however, lack of inhibition does not necessarily mean that the inhibitory site is not on the \textit{F}_{1}-\textit{ATPase}. In order to unequivocably locate the inhibitory site on the \textit{F}_{0} components it is necessary to demonstrate the presence of a binding site (see Chapter 3). However, the finding that only the OS-ATPase is inhibited gives support to the hypothesis that the \textit{F}_{0} at the very least is involved in the inhibitory process and work
Fig. 2.6. Sensitivity of OS-ATPase to trialkyl tin compounds.

Assays were carried out as described in the methods without azolectin. □ — , tripropyl tin chloride; • — , triethyl tin sulphate; ▼ ▼ ▼ ▼ , tributyl tin chloride; ▲ — , trimethyl tin chloride. Specific activity was 30µmole/mg/min with azolectin.
presented in Chapter 3 shows that the actual binding site for trialkyl tin compounds is the $F_0$.

It is apparent from this chapter that in yeast mitochondria the site of action for trialkyl tin compounds is the OS-ATPase, however, it should be noted that several workers (Kovac, Bednarova & Greksak, 1968; Somlo, 1968) have suggested that yeast mitochondria have two OS-ATPases on the inner mitochondrial membrane. There theories are based on the fact that there appears to be two pH optima for the enzyme, one around 6.5-7.0 and the other at 9.5, moreover the pH 9.5 activity is more sensitive to oligomycin. Griffiths and Houghton (1974) showed that in oligomycin resistant mutants of D22, the pH activity at 6.5 was greater than in the parental strain, in both mutant and parental strains the pH 9.5 activity was inhibited by lower concentrations of oligomycin than those needed for inhibiting the pH 6.5 activity. In addition the ATPase resistance of the mutant was exhibited at all pH's tested. In Fig. 2.7, a pH profile for submitochondrial particles is shown, there is the typical shoulder at pH 6.5 described by Kovac et al (1968) and the major activity peaking at 9.0 to 9.5, repeating the pH curve in the presence of a constant inhibitory concentration of triethyl tin reveals that the activity at more alkaline pH is more sensitive to the inhibitor. The pH profile of the purified OS-ATPase (Fig. 2.8.) is similar to the membrane bound ATPase, although there does not appear to be a well defined shoulder at pH 6.5, again the triethyl tin is more effective at alkaline pH. There are a number of possible explanations for these results; a) There are two ATPases with different pH optima and inhibitor sensitivity, with regard to this possibility, it should be noted that in the work of Griffiths and Houghton (1974), the mutant ATPase exhibited decreased
ATPase assays were carried out as described in the methods. Tris-maleate was used for pH 6.0 to 7.0 and Tris-Cl for 7.5 to 9.5. O—O, no triethyl tin; □—□, 7.8 nmole/mg triethyl tin sulphate.

**Fig. 2.7.** The effect of pH on the ATPase activity of submitochondrial particles.
Moreover, Sone, Furya and Hagihara (1968) have reported that in the yeast Endomyces magnusii, the ATPase is stimulated by the maleate ion at acidic pH; b) Binding of the inhibitor increases with pH resulting in greater inhibition, the experiments in Chapter 3 on triethyl tin binding show that this is not the case; c) The observed pH changes are resulting from a conformational transformation in the enzyme which effects only ATPase activity and not the inhibitor site. Thus the inhibitor site would exert its effect at all pH's and the seemingly greater inhibition at alkaline pH is due to the greater ATPase activity.

Conclusions.

The work presented in this chapter provides strong evidence for the hypothesis that the trialkyl tin inhibitory site is located on the OS-ATPase $F_0$ component.
CHAPTER 5.

Localisation of the site of action of trialkyl tin salts.

Introduction.

As discussed in Chapters 1 and 2, trialkyl tin salts inhibit oxidative phosphorylation in at least two ways. The first as described by Aldridge and Street (1964), Sone and Hagihara (1964), Rose and Aldridge (1972) and Stockdale, Dawson and Selwyn (1970) is an inhibition of ADP stimulated respiration, DNP stimulated ATPase and ATP-Pi exchange in either chloride or non chloride media, indicating that the site of action is similar to oligomycin (Slater and Welle, 1969) and DCCD (Beechey et al., 1966, 1967; 1968) in that it interacts with the OS-ATPase of the inner mitochondrial membrane. The experiments described in Chapter 2 bear out this earlier work showing that in yeast the trialkyl tins inhibit the OS-ATPase in both the membrane bound form and the highly purified complex described by Tzagoloff, Rubin and Sierra (1973), no inhibition of the $F_1$-ATPase is observed. Essentially similar results have been demonstrated in beef heart by Tzagoloff, McClennan and Byington (1968).

The second mode of action of trialkyl tin salts is only manifested in a chloride medium, in which the trialkyl tins catalyse a $Cl^-/OH^-\)$ exchange resulting in an uncoupler like action and swelling of mitochondria (Stockdale, Dawson and Selwyn, 1970). The exchange reaction was shown to be distinct from the oligomycin like action and later work by Harris, Bangham and Zukovic (1973) suggested the $Cl^-/OH^-\)$ exchange reaction was a specific carrier property of trialkyl tin salts. Skillen (1976) has shown that the $Cl^-/OH^-\)$ exchange reaction mediated by trialkyl tins is affected
by inhibitors of ATP hydrolysis in intact mitochondria, moreover the concentrations producing maximal Cl⁻/OH⁻ exchange are similar to those needed for the oligomycin like action. Therefore, there may well be a relationship between the two reported actions of the trialkyl tins.

To date, the site of action of the trialkyl tins in the oxidative phosphorylation complex has not been located. Binding studies with triethyl tin in rat liver mitochondria (Aldridge and Street, 1970) revealed the presence of two binding sites of high and low affinity, the oligomycin like effect of triethyl tin was correlated with the high affinity binding site which appears to be of the order of 0.8 mmole/mg of protein. However, the high affinity binding site was not correlated with a specific component of the oxidative phosphorylation system. Recent experiments with coupling factor deficient particles in beef heart (Dawson and Selwyn, 1975) suggest that tributyl tin is like DCCD and oligomycin in interacting with the hydrophobic components of the OS-ATPase.

The experiments outlined in this Chapter describe binding studies with ¹¹⁳Sn labelled triethyl tin on both the membrane bound and purified OS-ATPase complex. The results provide direct evidence for the oligomycin like action of triethyl tin to be a result of an interaction with the hydrophobic Fₒ components of the OS-ATPase.

Materials and Methods.

Materials

¹¹⁳Sn labelled triethyl tin chloride (8.6 mCi/mole) was purchased from the Radiochemical Centre, Amersham, Bucks., U.K., and was used as an ethanolic solution. Spectropore dialysis membrane (M.W. cut off,
6,000-8,000) was purchased from Raven Scientific Limited, Haverhill, Suffolk, U.K., and washed for several hours in distilled water before use. All other reagents were either A.R. grade or as described in Chapter 2.

Methods.

Growth of yeast, preparation of mitochondria, submitochondrial particles, Triton X-100 extracted particles, Triton X-100 extract, preparation of purified OS-ATPase and assays of ATPase activity and protein determinations were as described in Chapter 2.

Preparation of submitochondrial particles depleted of P$_1$-ATPase and OSCP as described by Tzagoloff (1971) and hereafter referred to as P$_1$-OSCP depleted particles, were prepared as follows: Submitochondrial particles (4ml) at a concentration of 20mg/ml were mixed with an equal volume of 6M sodium bromide and centrifuged at 100,000g for 30min. The treated particles were resuspended in 8ml of 0.25M sucrose, 20mM Tris-Cl pH 7.5, 1mM EDTA, and recentrifuged at 100,000g for 30min. The pellet was resuspended in 4ml of buffer and 4ml of 1N ammonium hydroxide added, the particles were stirred at 5°C for 10min before adjusting the pH to 7.5 with hydrochloric acid. The particles were then centrifuged as before, the pellet was resuspended in buffer and centrifuged as before. The pellet from this final centrifugation was suspended to a final protein concentration of 20mg/ml and this represented the P$_1$-OSCP depleted particles.

$^{113}$Sn labelled triethyl tin was assayed by liquid scintillation counting in a Packard 4325 scintillation counter, essentially as described by Dawson and Selwyn (1974). Samples in aqueous solution (1ml final volume) were counted in 9ml of a Triton X-100 based scintillant cocktail.
(1 litre toluene, 0·5 litre Triton X-100, 7g Butyl PBD). Standards were counted with each batch of samples and at least 4000 counts above background were recorded for each sample, under these conditions approximately 60% efficiency of counting was achieved.

Binding studies with $^{113}$Sn labelled triethyl tin chloride.

Binding of $^{113}$Sn labelled triethyl tin to submitochondrial particles, Triton X-100 extracted particles and $F_1$-OSCP depleted particles was carried out by incubating $^{113}$Sn labelled triethyl tin (0·04μm-20μm) with 1-2mg of protein in 5·0ml of buffer (0·5M sorbitol, 10mM Hepes-KOH, pH 7·5) in cellulose nitrate tubes (Beckman 40·5 tubes) at 5°C for 30min. Particles were sedimented at 100,000g for 30min in the 40·3 rotor of the Beckman ultracentrifuge. Aliquots of supernatant were collected for scintillation counting, the remaining supernatant was discarded and any surplus supernatant on the centrifuge tube walls carefully removed with blotting paper. The pellet was then solubilised overnight with 1ml of 2% (w/v) Triton X-100 before counting. Results were corrected for a blank run containing only protein and buffer, no binding of $^{113}$Sn labelled triethyl tin to the cellulose tubes was detected. Control experiments showed the $^{113}$Sn labelled triethyl tin chloride to have the same inhibitory properties as the unlabelled triethyl tin sulphate.

Binding of $^{113}$Sn labelled triethyl tin to purified OS-ATPase was carried out in a multi-chambered dialysis unit which was a development of a unit commercially available from Cole Palmer, 7425 North Oak, Park Avenue, Chicago, Illinois 60648, U.S.A. The unit consisted of two lucite discs (9·5cm in diameter by 1cm), each disc had 16 accurately milled chambers (10mm in diameter by 7mm) spaced at equal intervals.
(chamber centre to centre distance 17mm) round the perimeter of the
disc (chamber centre to disc edge distance 10mm). The two discs bolt
together with dialysis membrane sandwiched between them forming 16
paired dialysis chambers. Each chamber has a sampling hole (2mm in
diameter) tapped to receive a short nylon screw which forms a tight
leakproof seal. A spindle pierces the centre of the completed
assembly which when attached to a suitable electric motor allows
the whole assembly to be rotated about its centre in the vertical
plane. When the cells are filled (0.5ml) and sealed, rotation of the
apparatus causes the small air bubble produced on sealing to rotate
in the opposite direction thereby mixing the contents of the cell.
The lucite discs and sealing screws were all treated with Silicolad,
Clay Adams, Becton Dickinson and Company, Parsippany, New Jersey
07054, U.S.A., to minimise any binding of the ligand used to the
cell surfaces. Under these conditions there was no significant
binding of $^{113}$Sn labelled triethyl tin to the cells or Spectropore
dialysis membrane. In a typical experiment 150µg of purified OS-ATPase
in 0.36M sucrose, 20mM Tris-Cl, pH 7.5, 0.1% (v/v) Triton X-100 buffer
(0.5ml) were placed in one side of the paired cells (Bound ligand cell),
the opposite cells (Free ligand cell) are filled with the same buffer
(0.5ml) containing varying concentrations of $^{113}$Sn labelled triethyl
tin (0.2um-46um). The cells are then rotated at 20 r.p.m. for 24hours
at 5°C., at the end of the experiment both Bound and Free ligand cells
are sampled and assayed for $^{113}$Sn labelled triethyl tin. Control
experiments showed that there was no loss of activity or change in
inhibitor sensitivity of OS-ATPase during this period. In addition
control cells were run at the same time containing everything but protein,
these cells were always sampled before the end of the experiment to
verify that complete equilibration of the ligand across the dialysis membrane had been achieved.

Results and Discussion.

The experiments in Chapter 2 indicated that triethyl tin was more effective as an inhibitor of the OS-ATPase at pH 9.5 than 7.0. The whole problem of the effect of pH on trialkyl tin inhibitory actions has been investigated by a number of workers. Coleman and Palmer (1971) showed that the inhibition of DNP stimulated ATPase in rat liver mitochondria by triethyl tin was markedly pH dependent, being effective above 7.0 and ineffective below. They proposed that this was due to the changing affinity of the binding group for triethyl tin as the degree of dissociation altered. This finding supported earlier work by Rose (1970), who showed that each rat haemoglobin molecule binds two molecules of triethyl tin with a $K_d$ of 3.3$\mu$M, the binding was pH dependent, there being lower affinity at pH values below 8.0. Photoxidation studies pointed to two histidines being involved in the binding site as shown in Fig. 3.1, similar results were obtained with a protein derived from guinea pig liver. These results correlated well with the work of Janssen, Luijten and Van der Kerk (1964) who showed that trialkyl tin salts form stable penta-coordinate complexes with imidazole in non
aqueous solvents. As Aldridge and Street (1970) had showed a high affinity site in rat liver mitochondria with a $K_d$ of 2$nM$, which is very similar to that found in the rat haemoglobin molecule, Hose put forward the hypothesis that the triethyl tin binding site in rat liver mitochondria was also two histidines. Aldridge and Street produced no data as regards the effect of pH on triethyl tin binding in rat liver mitochondria. However, Dawson and Selwyn (1974) showed that the Coleman and Palmer results in rat liver mitochondria could be explained by Pi forming complexes with the triethyl tin which were markedly pH dependent.

In addition binding studies with $^{113}$Sn labelled triethyl tin showed very little pH dependence over the range 6.8 to 7.6, in fact the high affinity binding site showed a decrease at pH 8.0.

In the experiment shown in Fig.3.2, the effect of pH on the binding of $^{113}$Sn labelled triethyl tin to submitochondrial particles is demonstrated. As discussed by Dawson and Selwyn (1974) a low concentration of triethyl tin will titrate predominately the high affinity binding sites whilst a high concentration produces a situation in which the low affinity site greatly predominates. It can be seen from the figure that the high affinity binding hardly changes over the pH range 6.5 to 7.5, above pH 8.0 there is less binding due to either less binding sites or decreased affinity. Similar results are shown for the low affinity site. Plotting the results in terms of bound/free (B/F) ratios reveals that the triethyl tin high affinity binding sites decrease above pH 8.0 whereas the low affinity site does not appear to be affected. Thus, the greater potency of triethyl tin as an inhibitor of GS-ATPase at alkaline pH does not appear to be a result of increased binding to the inhibition site. This would suggest that either there are two ATPases present, one
Sub-mitochondrial particles (1mg protein) prepared from *S. cerevisiae* were incubated for 30min at 5°C with constant amounts of $^{113}$Sn TET at varying pH in 0.5M sorbitol, 10mM HEPES buffer (5ml). The sub-mitochondrial particles were then centrifuged at 100,000g for 30min, a 1ml aliquot was taken from the supernatant and assayed for free $^{113}$Sn TET. The remainder of the supernatant was discarded and the pellet solubilised in 1ml of 2% Triton X-100. Both solubilised pellets and supernatants were assayed for $^{113}$Sn TET by scintillation counting in 9ml of Triton X-100 scintillant as described in the methods.
Fig. 3.3. The effect of pH on $^{113}$Sn TET binding to sub-mitochondrial particles prepared from *S. cerevisiae*.

The protocol for this experiment is the same as in Fig. 3.2. In this figure the results are expressed in terms of a bound/free (B/F) ratio as described by Scatchard (1949).

- $\square$ 0.42 nmole $^{113}$Sn TET/assay
- $\blacksquare$ 42 nmole $^{113}$Sn TET/assay
one with a pH optimum at around 7.0 which is less sensitive to triethyl tin and one which has a pH optimum at approximately 9.5 and is more sensitive to the inhibitor. A similar conclusion has been reached by Houghton, Lancashire and Griffiths (1974) in studies on oligomycin resistant mutants of yeast, the pH profile of the resistant mutants was altered in the pH 7.0 region. Alternatively, there could be one ATPase present, in which a conformational change occurs going from pH 7.0 to 9.5 which not only allows a greater specific activity but also a much more efficient inhibition to be imposed by the triethyl tin binding site.

In Fig. 3.4., the effect of washing on triethyl tin binding is shown, in this experiment submitochondrial particles were incubated with a low concentration of $^{113}$Sn labelled triethyl tin, this experiment is therefore predominantly investigating the effects of washing on the high affinity site. It is apparent that the binding of triethyl tin to submitochondrial particles, is a dissociable phenomenon, as several washes with the binding buffer virtually removed all of the bound $^{113}$Sn labelled triethyl tin. The triethyl tin binding is therefore not due to the formation of a covalent bond, and in this respect differs from DCCD, which has been shown by Beechey et al., (1967, 1968) to be covalently bound to a subunit of $F_0$.

The shape of the binding curve for $^{113}$Sn labelled triethyl tin binding to submitochondrial particles (Fig. 3.5.) suggests that there are more than one binding site present. This conclusion is borne out by plotting the data as described by Scatchard (1949). The Scatchard analysis (Fig. 3.6.) reveals that there are clearly two classes of binding site with different
Sub-mitochondrial particles prepared from S. cerevisiae.

Five assay tubes containing sub-mitochondrial particles (1 mg), 0.5M sorbitol, 10mM HEPES pH 7.5 buffer (5 ml) and 0.42 nmole $^{113}$Sn TET were incubated as described in Fig. 3. 2., after centrifuging the supernatants were decanted. One pellet was assayed for radioactivity as previously described, the remaining pellets were resuspended in 5 ml of incubation buffer and recentrifuged. This procedure was repeated a further three times, i.e. until the last pellet, had been resuspended and washed four times, was assayed for radioactivity.

Fig. 3.4. Effect of washing on $^{113}$Sn TET binding to sub-mitochondrial particles prepared from S. cerevisiae.
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Fig. 3.5. Binding of $^{113}$Sn TET to sub-mitochondrial particles prepared from S. cerevisiae.

Sub-mitochondrial particles (1mg) were incubated with increasing amounts of $^{113}$Sn TET in 0.5M sorbitol, 10mM HEPES, pH 7.5 (5ml) buffer for 30min at 5°C. Centrifuging and assaying of radioactivity in the supernatant and pellet were carried out as previously described.
Fig. 3.6. Scatchard plot of $^{113}$Sn TET binding to sub-mitochondrial particles.

The above figure is a Scatchard analysis of the data presented in Fig. 3.5.

<table>
<thead>
<tr>
<th>Affinity Site</th>
<th>$K_d$</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>High affinity</td>
<td>$4.3 \times 10^{-7}$M</td>
<td>1.4 nmole/mg</td>
</tr>
<tr>
<td>Low affinity</td>
<td>$4.0 \times 10^{-5}$M</td>
<td>60 nmole/mg</td>
</tr>
</tbody>
</table>
binding affinities. The high affinity binding site is of the order of
7.7 n mole/mg of protein concentration with a $K_d$ of 0.6 $\mu$M (Table 3.1.)
and the low affinity site has a concentration of approximately 70 n mole/
mole/mg of protein and a $K_d$ of 45 $\mu$M (Table 3.1.). These values are in good
agreement with those of Aldridge and Street (1970) for rat liver
mitochondria (high affinity site = 0.8 n mole/mg of protein and $K_d$ of
2 $\mu$M; low affinity site = 66 n mole/mg of protein and $K_d$ of 80 $\mu$M). The
high affinity site clearly correlates with the $I_{50}$ value of 2 n mole/mg of
protein (Chapter 2) for ATPase inhibition. This is also in agreement
with Aldridge and Street’s conclusions (1970, 1971) that the high
affinity site in rat liver mitochondria represented the binding site
responsible for the oligomycin like effects of triethyl tin. The low
affinity site probably represents non specific binding to the phospholipids
of the membrane. The latter conclusion is substantiated by Aldridge and
Street (1964), who in a survey of triethyl tin binding to a variety of
molecules, using a colorimetric assay with diphenylthiocarbazone, showed
that triethyl tin had some affinity for certain phospholipids such as
lecithin but no affinity for a wide range of biologically important
domponds.

The obvious advantage of yeast mitochondria over rat liver mitochondria
is the relative ease in which the OS-ATPase can be extracted from the
inner mitochondrial membrane by Triton X-100 extraction. In Fig.3.7.,
the binding of $^{113}$Sn labelled triethyl tin to particles which have had
the OS-ATPase removed by 0.5% (w/v) Triton X-100 extraction is shown.
The portion of the curve shown represents low concentrations of triethyl
tin which will demonstrate the effect of OS-ATPase extraction on
Table 3.1. Binding of $^{113}$Sn TET to Mitochondrial OS-ATPase.

$^{113}$Sn TET binding to various mitochondrial preparations was carried out as described in the methods section. The results are expressed where appropriate as ± standard deviation (number of observations).

<table>
<thead>
<tr>
<th>Preparation</th>
<th>High Affinity site</th>
<th>Low Affinity site</th>
<th>S.A. of TET sensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_d$</td>
<td>No. of sites</td>
<td>$K_d$</td>
</tr>
<tr>
<td></td>
<td>µM</td>
<td>nmole/mg</td>
<td>µM</td>
</tr>
<tr>
<td>Submitochondrial particles</td>
<td>$59 ± 2$</td>
<td>(4)</td>
<td>$45 ± 9$</td>
</tr>
<tr>
<td>Triton X-100 extracted particles</td>
<td>$28 ± 29$</td>
<td>(4)</td>
<td>$53 ± 4$</td>
</tr>
<tr>
<td>OS-ATPase</td>
<td>$6.9$</td>
<td>(2)</td>
<td>NOT PRESENT</td>
</tr>
<tr>
<td>$P_i$-OSCP depleted particles</td>
<td>$1.2$</td>
<td>(1)</td>
<td>$41$</td>
</tr>
</tbody>
</table>

* In this case the residual ATPase after Triton X-100 extraction was insensitive to triethyl tin.
Fig. 3.7. Binding of $^{113}$Sn TET to Triton X-100 extracted sub-mitochondrial particles.

Sub-mitochondrial particles were extracted with 0.5% Triton X-100 as described in the methods. Binding of $^{113}$Sn TET to the extracted particles was carried out in an identical manner to that described for sub-mitochondrial particles. $\bullet$ = Control curve, i.e. $^{113}$Sn TET binding to sub-mitochondrial particles; $\bigcirc$ = $^{113}$Sn TET binding to Triton X-100 extracted particles.
predominantly the high affinity sites. The figure clearly shows a marked decrease in high affinity binding. A Scatchard analysis (Fig.3.8.) confirms this conclusion, the high affinity sites are apparently reduced in number, whereas, the low affinity sites are relatively unaffected. The concentration of high affinity sites (0.5 nmole/mg, Table 3.1.) represents a 3 fold decrease in the number of sites, this correlates with the approximately 3 fold difference in specific activity between the extracted particles and submitochondrial particles and also the 2 fold increase in specific activity of the Triton X-100 extract. The low affinity sites (55 nmole/mg) are not greatly affected, the Kd is higher at 76μM, this probably represents a truer estimate of binding affinity, as the contribution from the high affinity binding sites has been greatly reduced. The small decrease in the number of binding sites can be attributed to some delipidation of the membrane caused by the Triton X-100 extraction.

The effect of removing F1-ATPase and OSCP from the membrane on triethyl tin binding is shown in Fig.3.9 & 10. Depleting the membrane of F1-ATPase and OSCP does not remove the high affinity binding site, in fact there is an increased number of sites (Table 3.1.), representing the increased number of F0 components in the depleted membrane, the Kd is 1.2μM which is similar to that for submitochondrial particles. The low affinity sites are virtually unaffected. The overall conclusion from this experiment is that F1-ATPase and OSCP does not contain a high affinity binding site for triethyl tin, a conclusion in agreement with the lack of inhibition of F1-ATPase by triethyl tin.
Fig. 3.8. Scatchard plot of $^{113}$Sn TET binding to sub-mitochondrial particles extracted with 0.5% Triton X-100.

The above figure is a Scatchard analysis of the data presented in Fig. 3.7. $\circ$ $^{113}$Sn TET binding to Triton X-100 extracted particles; control Scatchard plot of $^{113}$Sn TET binding to sub-mitochondrial particles.

<table>
<thead>
<tr>
<th>Site</th>
<th>$K_d$ (M)</th>
<th>$n$ (nmole/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High affinity site</td>
<td>$3.3 \times 10^{-7}$</td>
<td>0.4</td>
</tr>
<tr>
<td>Low affinity site</td>
<td>$1.2 \times 10^{-4}$</td>
<td>70</td>
</tr>
</tbody>
</table>
Fig. 3.9. Binding of $^{113}$Sn TET to sub-mitochondrial particles stripped of $F_1$-ATPase and OSCP.

Sub-mitochondrial particles were depleted of $F_1$-ATPase and OSCP by NaBr/NH$_4$OH extraction as described in the methods. Binding of $^{113}$Sn TET to the depleted particles was carried out in an identical manner to that described for sub-mitochondrial particles. $$\longrightarrow$$, control curve, i.e $^{113}$Sn TET binding to sub-mitochondrial particles; $\bullet--\bullet$, $^{113}$Sn TET binding to depleted particles.
Fig. 3.10. Scatchard plot of $^{113}$Sn TET binding to sub-mitochondrial particles stripped of $F_1$-ATPase and OSCP.

The above figure is a Scatchard analysis of the data presented in Fig. 3.9. --- $^{113}$Sn TET binding to depleted particles; --- ---, control Scatchard plot of $^{113}$Sn TET binding to sub-mitochondrial particles.

<table>
<thead>
<tr>
<th></th>
<th>High affinity site</th>
<th>Low affinity site</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_d$</td>
<td>$1.2 \times 10^{-5}\text{M}$</td>
<td>$4.1 \times 10^{-5}\text{M}$</td>
</tr>
<tr>
<td>$n$</td>
<td>$6.5 \text{nmole/mg}$</td>
<td>$102 \text{nmole/mg}$</td>
</tr>
</tbody>
</table>
Fig. 3.10. Scatchard plot of $^{113}$Sn TET binding to sub-mitochondrial particles stripped of $P_i$-ATPase and OSCP.

The above figure is a Scatchard analysis of the data presented in Fig. 3.9. o---o, $^{113}$Sn TET binding to depleted particles; ---, control Scatchard plot of $^{113}$Sn TET binding to sub-mitochondrial particles.

**Table:**

<table>
<thead>
<tr>
<th>Affinity Site</th>
<th>$K_d$  $x 10^{-6}$ M</th>
<th>$n$  nmole/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>High affinity site</td>
<td>$1.2 x 10^{-6}$ M</td>
<td>65</td>
</tr>
<tr>
<td>Low affinity site</td>
<td>$4.1 x 10^{-6}$ M</td>
<td>102</td>
</tr>
</tbody>
</table>
Examining the binding of $^{113}$Sn labelled triethyl tin (Fig. 3.11 & 12.) to the purified OS-ATPase, reveals only one class of binding site of high affinity. The $K_4$ of which is $7\mu M$ (Table 3.1.) and the concentration $11.9$ nmole/mg. Thus there is an approximately 5-6 fold increase in the number of high affinity binding sites in purifying the enzyme from submitochondrial particles to the purified OS-ATPase complex. This correlates with the observed increase in specific activity. The correlation between the $I_{50}$ values for inhibition of the membrane bound ATPase of submitochondrial particles and the purified OS-ATPase (2 nmole/mg and 4 nmole/mg respectively) is not so good. However, the binding data provides a good explanation for this apparent discrepancy. In submitochondrial particles the amount of inhibition will depend on the proportion of applied triethyl tin which actually complexes with the high affinity binding site, however, even at low triethyl concentrations the low affinity site will bind triethyl tin. The pH binding data indicates that at pH 9.5 the competition effect of the low affinity site would be even greater. The net result would be to decrease the actual amount of triethyl tin which is interacting with high affinity sites. Consequently, the concentration of triethyl tin needed to actually inhibit the ATPase in submitochondrial particles is in reality lower than the observed $I_{50}$ value. As there is no low affinity site in the purified OS-ATPase this problem does not arise. These conclusions are backed up by the experiments described in Chapter 2 in which applied azolectin can markedly increase the $I_{50}$ value for triethyl tin on the purified OS-ATPase. In essence the azolectin is an excess of low affinity binding sites which compete for the triethyl tin.
Fig. 3.11. Binding of $^{113}$Sn TET to purified OS-ATPase.

Purified OS-ATPase was prepared as described in the methods, essentially as outlined by Tzagoloff, Rubin and Sierra (1973). Binding was carried out by equilibrium dialysis in a multi-chambered dialysis unit. Full details of this apparatus are described in the methods section.
High affinity site  
Low affinity site

\[ K_d = 6.9 \times 10^{-6} \text{M} \]
\[ n = 1.1 \times 10^9 \text{n mole/mg} \]

**Fig. 3.12.** Scatchard plot of \(^{113}\text{Sn TET} \) binding to purified OS-ATPase.

Experimental details are as for Fig. 3.11.
The increased $K_d$ of the high affinity site in the purified OS-ATPase complex may be due to a modified conformation in the detergent dispersed enzyme. The enzyme 'in situ' is integrated into a highly ordered membrane structure, so it is not unlikely that the conformation of the complex in Triton X-100 is slightly different from that found in the membrane.

Although triethyl tin appears to act like oligomycin, there is no proof available to suggest that the two inhibitors act at the same site or even on the same subunit of the OS-ATPase. Biochemical genetic studies carried out in this laboratory (see Griffiths, 1975, for review) have shown that there are separate interaction sites for triethyl tin, oligomycin and venturicidin. The experiments described in Fig.3.13, provide direct evidence for these conclusions. Preincubation of submitochondrial particles with venturicidin and oligomycin concentrations approximately 10 fold greater than those of used to inhibit the ATPase by 50% do not effect the high affinity site ($K_d = 0.5\mu M$ and concentration $= 1.4$ nmole/mg of protein). Other experiments presented in Chapter 4 show that the triethyl tin site can be competed for with other trialkyl tin compounds, thus, the oligomycin like action of triethyl tin is not due to a common binding site. It must however be pointed out that this does not preclude there being binding sites for triethyl tin and oligomycin on the same protein subunit.

**Conclusion**

The results presented in this chapter have shown that the OS-ATPase/synthetase complex of the yeast inner mitochondrial membrane contains a specific binding site of high affinity for triethyl tin. The binding
Fig. 3.13, The effect of venturicidin and oligomycin on $^{113}$Sn TET binding to submitochondrial particles.

Experimental details were as for Fig. 3.2, except that the submitochondrial particles were preincubated with inhibitor for 30 min before adding $^{113}$Sn TET.
site is present in both the membrane bound and highly purified detergent dispersed form. Neither, $F_1$-ATPase or OSCP contribute to the triethyl tin binding site and the conclusion is that high affinity binding site is located in the hydrophobic $F_0$ components of the OS-ATPase. The binding site is distinct from two other inhibitors (oligomycin and venturicidin) which act in a very similar manner.

The question of which subunit of $F_0$ binds triethyl tin cannot be answered from present data and the experiments in Chapter 4 with a specific affinity label DBCT (dibutyl chloromethyl tin chloride) have been directed towards solving this problem. However, the data does show that every mole of OS-ATPase (assuming a M.W. of 486,000 daltons) contains 6 moles of triethyl tin binding site. It is therefore of interest that Beechey (1975) asserts that there are 2–3 moles of DCCD binding site per mole of OS-ATPase in beef heart. Partis (1975) has presented evidence that the subunit 9 of $F_0$ is the DCCD binding protein in yeast. Moreover, yeast subunit 9 has a molecular weight of 7,800 daltons, but this is normally aggregated as a hexamer of 45,000 daltons molecular weight (see Tzagoloff, Rubin and Sierra, 1973, for review). Beechey believes that the subunit 9 hexamer is in a 1:1 stoichiometry with the OS-ATPase. Subunit 9 would therefore appear to have the requirements for the triethyl tin binding site, each subunit complexing one triethyl tin, giving a total of six per OS-ATPase molecule.
CHAPTER 4.

Studies on a new affinity label for the OS-ATPase.

Introduction.

The major problem facing biochemists in the study of the OS-ATPase is the determination of structure/function relationships for the various subunits of the complex. Obviously, the identification of specific inhibitor binding sites with particular subunits of the complex would provide valuable information for solving this problem. Unfortunately, no great progress has been made in identifying inhibitor binding sites. As discussed in Chapter 1, oligomycin appears to interact with $P_0$ in such a manner that proton transfer is inhibited, however an actual binding site on a subunit or subunits has not been demonstrated. In the case of DCCD the situation is clearer by virtue of the covalent nature of DCCD inhibition. Beechey and co-workers (Beechey et al., 1967; Roberton et al., 1968; Cattell et al., 1970; Cattell et al., 1971.) showed that DCCD irreversibly inhibited the OS-ATPase and ATP dependent reactions in beef heart. Further investigation showed that the DCCD was bound covalently to a small protein of 10,000 daltons molecular weight. The protein is extremely hydrophobic and can be extracted only with chloroform-methanol (2:1), a property which is very similar to that exhibited by subunit 9 of the OS-ATPase complex. However as Beechey (1974) points out, the identification of the DCCD binding protein with subunit 9 is very speculative. On the other hand, Stekhoven et al. (1972) have shown that the DCCD binding protein is present in the beef heart OS-ATPase complex. Partis (1975) has demonstrated that in yeast DCCD binds to a subunit in the OS-ATPase which has all the
properties of subunit 9 including the fact that it is synthesized on mitoribosomes.

In the case of triethyl tin the work presented in this thesis (Chapters 2 and 3) has shown that the $P_0$ component of the OS-ATPase contains the specific binding site for the inhibitor. In order to further localise the site of action of trialkyl tin compounds, a new approach was adopted. The objective was to find an affinity labelled derivative of the trialkyl tin compounds, a likely candidate for this was dibutyl chloromethyl tin chloride (DBCT). The rationale being that the reactive chloromethyl group should form a covalent bond at the inhibitor binding site. Preliminary experiments with a sample of DBCT obtained from Dr. J. Price, Tin Research Institute, revealed that the compound was a potent inhibitor of the OS-ATPase. This chapter describes the synthesis of radioactively labelled DBCT, the inhibitory properties of DBCT and the isolation and localisation of the DBCT binding component.

Methods.

a) General.

Growth of yeast, preparation of mitochondria, submitochondrial particles, $P_1$-OSCP depleted particles, OS-ATPase, assay of ATPase activity and binding experiments were prepared as described in Chapters 2 and 3.

Preparation of beef heart mitochondria was essentially as described by Low and Vallin (1963), the mitochondria being resuspended in 0.25M sucrose, 20mM Tris-Cl, 1mM EDTA, pH 7.5 at a concentration of approximately
35 mg/ml. Submitochondrial particles from beef heart were prepared as described for yeast submitochondrial particles. Inhibition of ADP stimulated respiration in beef heart mitochondria was carried out as shown in the legends to the figures. Assays of ATP and succinate driven transhydrogenase were as described by Beechey et al. (1967). The assay of ATP driven reduction of NAD⁺ by succinate was carried out as described by Low and Vallin (1963) with the modifications described by Beechey et al. (1967).

b) Preparation of radioactively labelled DBCT.

The method chosen for the preparation was as described by Seyferth and Rochow (1955), the equation for the reaction being:

\[
(C_4H_9)_2SnCl_2 + CH_2N_2 \rightarrow (C_4H_9)_2CH_2ClSnCl
\]

dibutyl tin dichloride diazomethane dibutyl chloromethyl tin chloride (DBCT)

Obviously, the inclusion of tritium atoms in the diazomethane i.e. \(CH_2N_2\) would result in radioactively labelled DBCT. Gassman and Greenlee (1973) described a method for deuterating diazomethane, consequently any method for preparing deuterium labelled diazomethane should be equally applicable to preparing tritium labelled diazomethane.

1. Preparation of tritium labelled diazomethane.

Initially a trial run was carried out with \(D_2O\) to investigate the best conditions for labelling. The reaction is as follows:

\[
NaOH
\]

\[
CH_2N_2 \rightarrow CD_2N_2
\]

\(D_2O\) (or \(T_2O\))

diazomethane labelled diazomethane.
Diazomethane was prepared as described in Practical Organic Chemistry (Vogel, 1956) by heating with vigorous stirring a solution containing 6g of KOH, 10ml water, 35ml carbitol and 10ml ether. When the ether began to distill over (70-75°C) a solution (125ml) of ether containing 21.5g p-tolysulphonylmethyl nitrosamide was added dropwise from a dropping funnel over a period of 15min. The rate of addition being adjusted to match that of the ethereal diazomethane solution which was distilling over into the receiving flasks. The ethereal diazomethane solution was collected in two flasks containing 10 and 35ml of ether respectively cooled in dry ice (-70°C). After the addition of the p-tolysulphonylmethyl nitrosamide solution a further 20ml of ether was added until the distillate was colourless. The contents of the two receiving flasks were combined and the ethereal solution of diazomethane dried over sodium carbonate in a round bottom flask fitted with a rubber bung and drying tube. The amount of diazomethane was estimated by decolourising a known volume of the ethereal solution with a known amount of benzoic acid. The excess benzoic acid was titrated with N/10 NaOH, consequently the exact amount of diazomethane could be calculated. In this experiment 150ml of the ethereal solution contained 30 mMoles of diazomethane.

Deuteration was carried out by stirring vigorously 150ml of the diazomethane solution with 40mg of NaOH and 1ml of D₂O in a loosely stoppered flask for 3hr at 0°C. The deuterated diazomethane solution was then dried over sodium carbonate. The extent of incorporation of the deuterium into the diazomethane was estimated as follows:
1. 10ml of the deuterated solution was just neutralized with benzoic acid
to give methyl benzoate; 2. the methyl benzoate solution was transferred
to a separating funnel and washed twice with 2 vol of 5% NaHCO₃ solution,
then 1 vol of dichloromethane was added; 3. the ether and dichloromethane
were evaporated and more dichloromethane added; 4. the dichloromethane
was again evaporated, this treatment with dichloromethane was repeated
until all the ether had been removed. The deuterated methyl benzoate
was then examined by 60 MHz proton NMR. Integration of the spectrum
shown in Fig. 4.1, reveals that there are 2 and 3 protons at 8·3 and 7·1
ppm respectively which are the aromatic protons and 2 protons at 3·8ppm.
corresponding to -OCH₂D. Thus the diazomethane is at least 50% deuterated.
A sample of the deuterated methyl benzoate was also analysed by mass spect­
rometry by the Physico-Chemical Measurement Unit, Harwell, and the following
results were obtained; CH₃ = 44%, CDH₂ = 40%, CD₂H = 14%, CD₃ = 2%. Thus
the mass spectrometry data confirms the NMR results that over 50% of the
diazomethane was deuterated.

Preparation of tritiated diazomethane was conducted in exactly the
same manner to that described for deuterated diazomethane. Tritiation
was carried out with 0·8ml of T₂O (4 Ci), 40mg NaOH and 100ml of
diazomethane solution (ie. 40 mMole) at 0°C for 3hrs. The ethereal
solution was then dried over sodium carbonate at -70°C.

NB. 1. Diazomethane is extremely toxic and also potentially explosive.
2. For safety reasons all work should be carried out behind safety
screens in a fume cupboard.
3. Diazomethane has been known to explode on contact with sharp
surfaces. Consequently all glassware was fire polished and quickfit
apparatus was not used.
Fig. 4.1 «« 60 MHz spectrum of deuterated methyl benzoate.
2. Preparation of unlabelled and tritium labelled DBCT.

The DBT precursor used for this reaction was first purified by fractional distillation. The white crystalline solid had a melting point of 40-42°C which compares favourably with published data (Poller, 1970), an I.R spectrum was identical with that described by Noel, Lesbre and de Roch, 1956. A 90 MHz proton NMR spectrum of DBT is shown in Fig.4.2a, integration of the spectrum reveals that the three multiplets each account for 6 protons. The spectrum is unusual in that one would expect the two butyl groups to produce 4 multiplets corresponding to 6 protons and 3 sets of 4 protons. However there are a number of isotopes of tin, some of which have a nuclear magnetic moment, consequently the unusual spectrum for DBT may be due to some spin-spin interaction between the proton and tin nuclei.

Preparation of unlabelled DBCT was carried out in a 250ml three necked flask equipped with a reflux condenser plus drying tube. The flask contained 5g of DBT in 75ml of anhydrous ether and a teflon stirrer bar, a dry inert nitrogen atmosphere was maintained throughout the reaction. The central neck of the flask was connected by means of suba-seal fittings and glass tubing to the round bottom flask containing 33 mmole of diazomethane in 120ml of ether. The contents of the three necked flask were stirred vigorously at -70°C in a dry ice bath, dry nitrogen was then used to force the diazomethane solution from the round bottom flask to the three necked reaction vessel. The diazomethane solution is decolourized on contact with the DBT solution. After adding all the diazomethane solution, the reaction mixture was bright yellow in colour and was stirred for 1hr at -70°C, stirring was then continued.
2. Preparation of unlabelled and tritium labelled DBCT.

The DBT precursor used for this reaction was first purified by fractional distillation. The white crystalline solid had a melting point of 40-42°C which compares favourably with published data (Poller, 1970), an I.R spectrum was identical with that described by Noel, Lesbre and de Roch, 1956. A 90 MHz proton NMR spectrum of DBT is shown in Fig.4.2a, integration of the spectrum reveals that the three multiplets each account for 6 protons. The spectrum is unusual in that one would expect the two butyl groups to produce 4 multiplets corresponding to 6 protons and 3 sets of 4 protons. However there are a number of isotopes of tin, some of which have a nuclear magnetic moment, consequently the unusual spectrum for DBT may be due to some spin-spin interaction between the proton and tin nuclei.

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Fig. 4.2. 90 MHz proton NMR spectra of dibutyl tin dichloride and dibutyl chloromethyl tin chloride.

The compounds were dissolved in CDCl₃ for the NMR spectra (a) Redistilled dibutyl tin dichloride. (b) DIBUTYL CHLOROMETHYL TIN CHLORIDE obtained from Dr. J. Price, Tin Research Institute.
while allowing the solution to reach room temperature. The stirring was continued until the solution was colourless, the solution was then stored overnight at -70°C. Overnight a small white precipitate formed which was probably unreacted diazomethane decomposing to polymethylene, this precipitate was discarded. The ether was evaporated on a rotary evaporator, the resulting viscous liquid was fractionally distilled on a micro-distillation apparatus and yielded two fractions. Fraction 1 (1.29 gm) distilled at 48-68°C (0.3 mmHg) and fraction 2 (0.587 gm) distilled at 70°C (0.3 mm Hg). Proton NMR spectra showed that fraction 2 was predominantly DBT. Fraction 1 revealed an NMR spectrum similar to that shown in Fig. 4.2b, redistilling fraction 1 produced one fraction (0.976 gm) distilling at 102-106°C (0.38 mm Hg), the boiling point of this fraction is in good agreement with the value reported for DBCT by Seyferth and Rochow (1954) of 106-110°C (0.3 mm Hg). The proton NMR spectrum of the spectrum, shown in Fig. 4.3a, integrates to give the right number of protons for DBCT, the multiplet at 3.5 ppm corresponds to the two protons of the -CH₂Cl group, the remaining multiplets account for the 18 protons of the two butyl groups. Redistilling the DBCT sample obtained from the Tin Research Institute produced one fraction, the NMR spectrum of which was identical to that shown in Fig. 4.3a. It is apparent from these spectra that the Tin Research Institute sample contained approximately 50% of the precursor DBT, and that the synthesized DBCT sample contained no DBT precursor. The latter results are important, because of the fact, as shown later in this chapter, that DBT will inhibit the OS-ATPase, consequently all inhibition studies were carried out with the highly purified sample shown in Fig. 4.3a. It is interesting to note that the effect of introducing the chloromethyl group into DBT to produce DBCT,
Fig. 4.3. 90 MHz proton NMR spectra of dibutyl chloromethyl tin chloride and tritium labelled dibutyl chloromethyl tin chloride.

The compounds were dissolved in CDCl₃ for the NMR spectra (a) Dibutyl chloromethyl tin chloride, synthesized as described in the methods. (b) Tritium labelled dibutyl chloromethyl tin chloride, synthesized as described in the methods.
causes the three multiplets to appear as two multiplets of 12 and 6 protons respectively.

The labelled DBCT was prepared in an identical manner to that described for the unlabelled compound except that tritiated diazomethane was used. The purified fraction distilled at 100°C (0.25 mm Hg) and the 90 MHz proton NMR spectrum shown in Fig. 4.3b., reveals that the tritiated DBCT (H³-

Results.

a) inhibitory properties of DBCT.

i. Yeast OS-ATPase

The effect of DBCT on the OS-ATPase of submitochondrial particles is shown in Fig. 4.4., it can be seen that DBCT is as potent an inhibitor of the ATPase activity as triethyl tin. The I₅₀ value is 2 nmole/mg, surprisingly the DBT compound is also a potent inhibitor with an I₅₀ value of around 6 nmole/mg. These results outline the importance of ensuring that the synthesized DBCT was free from the DBT precursor. DBCT inhibits both the membrane bound enzyme, the partially purified enzyme, the Triton X-100 extract and also the highly purified OS-ATPase enzyme (Fig. 4.5.). The F₄-ATPase was not inhibited by 500 nmoles/mg of DBCT. The inhibitory effects show the same pH dependency as that exhibited by
Fig. 4.4. Comparison of DBCT with other alkyl tin compounds as an inhibitor of the CS-ATPase in submitochondrial particles.

Assay methods were as described in Chapter 2. O—O DBCT; ●—●, triethyl tin sulphate; □—□, DEDT; ■—■, tributyl tin chloride. Specific activity of the ATPase was 5.0 μmole/mg/min.
Fig. 4.5. Inhibition of various preparations of yeast mitochondrial OS-ATPase by DBCT.

Assay conditions and preparation of the membrane bound and solubilized enzymes were as described in Chapter 2.

- - O, submitochondrial particles, specific activity = 6.0 μmoles/mg/min; O, Triton X-100 extract, specific activity = 9.0 μmoles/mg/min; O- O, purified OS-ATPase, specific activity = 27.0 μmoles/mg/min.
triethyl tin (Fig.4.6). The presence of excess azolectin will also markedly affect the sensitivity of the OS-ATPase to DBCT (Fig.4.7.), however, the sequestering effect of the azolectin is more pronounced with DBCT than with triethyl tin. Thus 10/mg/mg azolectin increases the $I_{50}$ value for triethyl tin from 4.5 to 10 nmole/mg, whereas the same concentration of azolectin increases the $I_{50}$ value for DBCT from 5 to 35 nmole/mg. It is apparent from these results that DBCT is a potent inhibitor of the OS-ATPase and that the inhibition is identical to that produced by triethyl tin.

ii. Inhibition of energy linked reactions in beef heart mitochondria.

Although yeast is an ideal source for preparing highly purified OS-ATPase, it is unsuitable for examining energy linked reactions such as the transhydrogenase reaction, due to the fact that it does not possess site I (see Lloyd, 1974, pp 99 for review). Various energy linked reactions can be easily studied in beef heart mitochondria and submitochondrial particles. These reactions are useful in that they can be used to determine the exact site of action of an inhibitor of oxidative phosphorylation.

In Fig.4.8., the effect of DBCT on mitochondrial respiration is demonstrated, at a concentration of 5-3 nmole/mg approximately 65% inhibition of the ADP stimulated respiration is produced. Neither the unstimulated state 4 respiration or DNP stimulated oxygen uptake is affected by the inhibitor. Triethyl tin has a similar effect at approximately the same concentrations.
**Fig. 4.6.** The effect of pH on the DBCT inhibition of OS-ATPase in submitochondrial particles. ATPase assays were carried out as described in Fig. 2.7.

- ○, no DBCT; ▲▲, 6.2 nmole/mg DBCT.
Fig. 4.7: Effect of excess azolectin on the sensitivity of Os-ATPase to DBCT.

Assays were carried out as described in Chapter 2. • – • no azolectin, specific activity = 13 μmole/mg/min; ▲ – ▲ 10mg/mg azolectin, specific activity = 30 μmole/mg/min.
1.0M pyruvate and rate (S; 10ul of 30mM ADP (ADP) and 5ul of DNP (DNP) as indicated on the traces. Trace 1 was a control experiment with 5ul of ethanol added at A. In traces 2 and 3, one of the concentrations of pyruvate and malate (8) was reduced by 50% or 5ul of 20mM DNP (DNP) and 5ul of 50mM ADP (ADP) were added at B. In trace 4, 100mM KCl, 1mM MgCl₂, 7.5 mM MgO₄, pH 7.5 buffer at a temperature of 30°C. Heart mitochondria were measured with a Clarke oxygen electrode in 2ml of 0.25M sucrose, 10mM TrisCl.

---

The effect of adding triethyltin in 5ul of ethanol at D (2.6 nmole) and E (7.8 nmole).

---

In traces 2 and 3, one of the concentrations of pyruvate and malate (8) was reduced by 50% or 5ul of 20mM DNP (DNP) and 5ul of 50mM ADP (ADP) were added at B. In trace 4, 100mM KCl, 1mM MgCl₂, 7.5 mM MgO₄, pH 7.5 buffer at a temperature of 30°C. Heart mitochondria were measured with a Clarke oxygen electrode in 2ml of 0.25M sucrose, 10mM TrisCl.
The OS-ATPase of beef heart submitochondrial particles is inhibited by low concentrations of DBCT and triethyl tin (Fig. 4.9). DBCT has an $I_{50}$ value of 0.1 nmole/mg and triethyl tin produces 50% inhibition at 2 nmole/mg. The remarkable potency of DBCT compared to triethyl tin as an inhibitor of the beef heart ATPase is surprising. In yeast the two inhibitors have a similar potency (i.e., approximately 2 nmole/mg), a possible explanation is that the beef heart has a more ordered subunit structure, such that interactions with one subunit can allosterically affect other functional subunits. This result is even more remarkable when the energy linked reactions are examined.

In the case of the ATP driven reduction of NADP⁺ by NADH, DBCT will inhibit the reaction at low concentrations (Fig. 4.10.), the non-energy linked reaction is not inhibited. DBCT has an $I_{50}$ value of 0.9 nmole/mg, oligomycin 0.3 nmole/mg and triethyl tin approximately 2.3 nmole/mg for this reaction (Fig. 4.11). The triethyl tin is again less potent than the DBCT but the $I_{50}$ values for triethyl tin on the ATPase and the ATP driven transhydrogenase reactions are very similar, the $I_{50}$ value for oligomycin inhibition of the transhydrogenase reaction is in good agreement with the value reported by Robertson et al. (1968) of approximately 0.5 nmole/mg.

In the case of the succinate driven reduction of NADP⁺ by NADH, neither DBCT, triethyl tin nor oligomycin had any effect, whereas the uncoupler FCCP totally inhibited the reaction. Thus DBCT, like oligomycin, has no effect on the transhydrogenation reaction when it is driven by energy derived direct from the respiratory chain.
Inhibition of OS-ATPase in beef heart submitochondrial particles by DBCT and triethyl tin sulphate.
ATPase assays were carried out as described in Chapter 2.
Fig. 4.10. DBCT inhibition of ATP driven transhydrogenase in beef heart submitochondrial particles.

Assays were carried out as described by Beechey et al. (1967) with 1 mg of submitochondrial particles in 3 ml of buffer containing 0.25 M sucrose, 5 mM MgCl₂, 50 mM Tris Cl⁻, pH 8.0, 1 μM KCN, 330 μM NADP⁺, 66 μM NAD⁺, 300 μg of yeast alcohol dehydrogenase and 1.33 mM ATP. Submitochondrial particles were preincubated for 3 min at 30°C with or without inhibitor before adding NADP⁺; 3 min later the ATP was added. Reduction of NADP⁺ was followed at 340 nm in a Unicam S.P.1800 spectrophotometer. Figures in ( ) refer to concentrations of DBCT (n mole/mg).
Fig. 4.11. Comparison of oligomycin, DBCT and triethyl tin as inhibitors of the ATP driven transhydrogenase.

Assays were carried out as in Fig. 4.10., •—•, oligomycin; ■—■, DBCT; ▲—▲, triethyl tin sulphate.
Fig. 4.12. DBCT inhibition of ATP driven reduction of NAD\(^+\) by succinate in beef heart submitochondrial particles.

Assays were carried out as described by Beechey et al., (1967) with 1 mg of submitochondrial particles in 3 ml of medium containing 0.25 M sucrose, 6 mM MgCl\(_2\), 50 mM Tris-Cl\(^-\), pH 8.0, 1 mM KCN, 1 mM NAD\(^+\), 5 mM succinate and 2 mM ATP at a temp. of 30°C. Submitochondrial particles were preincubated with or without inhibitor for 5 min before adding the ATP. A. control, no inhibitor; B. = 0.3 nmole/mg DBCT; C. = 1.2 nmole/mg DBCT; D. = 2.4 nmole/mg DBCT.
Fig. 4.13. Comparison of DBCT, triethyl tin and oligomycin as inhibitors of the ATP driven reduction of NAD$^+$ by succinate.

Assays were carried out as described in Fig. 4.12.

- •, oligomycin; ■ - ■, DBCT; ⃝ - ⃝, triethyl tin sulphate.
The ATP driven reduction of NAD⁺ by succinate is markedly affected by DBCT (Fig.4.12.). The reduction is progressively inhibited by increasing amounts of DBCT, moreover a 'lag phase' develops before the reduction begins. The duration of the 'lag phase' is proportional to the amount of DBCT added, this 'lag phase' was also observed with triethyl tin but not with oligomycin. The I₅₀ values for DBCT, triethyl tin and oligomycin were 0.9, 2.3 and 0.15nmole/mg respectively (Fig.4.13.), these values are similar to those obtained for the transhydrogenase reaction.

The results on the energy linked reactions show that DBCT has very similar actions to oligomycin and triethyl tin. Ernster and Lee (1964) proposed the scheme shown in Fig.4.14., to explain the action of oligomycin on these reactions, DCCD has also been reported to act in a similar manner (Roberton et al., 1968).

\[ \text{Succinate} \rightarrow \text{O} \]
\[ \text{NADH} \]
\[ \text{X} + \text{I} \]
\[ \text{X} \sim \text{I} \]
\[ \text{X-P} \]
\[ \text{ADP} \]
\[ \text{ATP} \]

\textbf{Fig.4.14.} Postulated site of action of DCCD and oligomycin.
In this scheme, the ATP driven partial reactions are visualised as being energised by \( \Delta G \) which is derived from ATP hydrolysis. The production of \( \Delta G \) from ATP is catalysed by the \( \Delta G \)-ATPase and involves the intermediate \( \Delta G \). The reaction producing \( \Delta G \) from \( \Delta G \) is proposed to be inhibited by oligomycin and DCCD. Obviously from this scheme, concentrations inhibiting the ATPase should produce equal inhibition of the energy linked reactions. In the case of oligomycin and DCCD (Roberton et al., 1966) this appears to be the case. With triethyl tin there is a similar correlation, however the DBCT results show that the ATPase reaction is approximately 9 times more sensitive to the inhibitor than the ATP driven reactions. A possible explanation is that there are two intermediates between \( \Delta G \) and the respiratory chain. One intermediate would be \( \Delta G \) which could be formed from \( \Delta G \) and \( \Delta G \), resulting in the release of \( \Delta G \). \( \Delta G \) could then energise transhydrogenase reactions. The other intermediate (designated \( \Delta G \)) would be formed in an analogous manner by the interaction of \( \Delta G \) and \( \Delta G \), again releasing phosphate. The reactions producing the intermediates could be considered to be in competition with each other. In the case of \( \Delta G \), the rate of formation of the intermediate would depend upon the rate at which the intermediate was used up. Thus in a transhydrogenase reaction \( \Delta G \) would be continuously removed, hence the \( \Delta G \) would be diverted to produce \( \Delta G \). In the case of \( \Delta G \), \( \Delta G \) could be considered to be a mobile pool of redox components which can be oxidized and reduced by the respiratory chain. In the ATPase assay the dominant reaction would be \( \Delta G \) formation. Thus DBCT, oligomycin and triethyl tin could be considered to inhibit both the formation of \( \Delta G \) and \( \Delta G \), the only difference being that DBCT is a much more potent inhibitor of \( \Delta G \) formation than of \( \Delta G \) production.
In this scheme, the ATP driven partial reactions are visualised as being energised by $X^{-}\text{I}$ which is derived from ATP hydrolysis. The production of $X^{-}\text{I}$ from ATP is catalysed by the $\text{O}_{2}\text{-ATPase}$ and involves the intermediate $X^{-}\text{P}$. The reaction producing $X^{-}\text{I}$ from $X^{-}\text{P}$ is proposed to be inhibited by oligomycin and DCCD. Obviously from this scheme, concentrations inhibiting the ATPase should produce equal inhibition of the energy linked reactions. In the case of oligomycin and DCCD (Roberton et al., 1966) this appears to be the case. With triethyl tin there is a similar correlation, however the DBCT results show that the ATPase reaction is approximately 9 times more sensitive to the inhibitor than the ATP driven reactions. A possible explanation is that there are two intermediates between $X^{-}\text{P}$ and the respiratory chain. One intermediate would be $X^{-}\text{I}$ which could be formed from $X^{-}\text{P}$ and I, resulting in the release of Pi, $X^{-}\text{I}$ could then energise trans-hydrogenase reactions. The other intermediate (designated $X^{-}\text{Y}$) would be formed in an analogous manner by the interaction of $X^{-}\text{P}$ and Y, again releasing phosphate. The reactions producing the intermediates could be considered to be in competition with each other. In the case of $X^{-}\text{I}$, the rate of formation of the intermediate would depend upon the rate at which the intermediate was used up. Thus in a transhydrogenase reaction $X^{-}\text{I}$ would be continuously removed, hence the $X^{-}\text{P}$ would be diverted to produce $X^{-}\text{I}$. In the case of $X^{-}\text{Y}$, Y could be considered to be a mobile pool of redox components which can be oxidized and reduced by the respiratory chain. In the ATPase assay the dominant reaction would be $X^{-}\text{Y}$ formation. Thus DBCT, oligomycin and triethyl tin could be considered to inhibit both the formation of $X^{-}\text{I}$ and $X^{-}\text{Y}$, the only difference being that DBCT is a much more potent inhibitor of $X^{-}\text{Y}$ formation than of $X^{-}\text{I}$ production.
b) Binding of DBCT to yeast submitochondrial particles.

Fig. 4.15 shows a binding curve for H^3-DBCT to submitochondrial particles, the curve can be considered to be produced by the presence of two non-identical binding sites. A comparison of the binding curves for DBCT and triethyl tin (Fig. 3.5.) reveals that the higher affinity sites are predominantly titrated at low concentrations of ligand, at high ligand concentration the lower affinity sites are also titrated. The number of high affinity sites in each case can be estimated from the curves, in the case of triethyl tin the value is approximately 1.5nmole/mg, a value which is substantiated by the Scatchard analysis (Fig. 3.6.). In the case of DBCT the value for the high affinity sites is much higher, in the region of 10nmole/mg. If DBCT is an affinity label it should be impossible to remove the covalently bound DBCT. This hypothesis was examined in two ways. In the first experiments, H^3-DBCT was incubated with submitochondrial particles for 30min. The submitochondrial particles were then washed by repeated centrifugation and resuspension in binding buffer. The results of the washing procedure are shown in Table 4.1., in experiment A the particles were incubated with a low concentration of DBCT and in experiment B the particles were incubated with a large excess of H^3-DBCT. In the case of the low H^3-DBCT concentration, approximately 70% of the bound DBCT cannot be removed by washing, with excess DBCT a much larger proportion of the bound H^3-DBCT (approximately 60%) is removed. These results can be explained by considering the DBCT to be binding to two sites, one site is binding DBCT covalently or at the very least with a very high affinity, the other binding site is of a much lower affinity and the bound DBCT can be removed by the washing procedure. The high affinity (covalent) binding is not removed by washing, the concentration of the high affinity sites was estimated by incubating particles with excess H^3-DBCT (20nmole/mg) for
Fig. 4.15. Binding of $H^3$-DBCT to yeast submitochondrial particles.

Binding assays were carried out as described for $^{113}$Sn triethyl tin binding in Chapter 3.
Table 4.1. The effect of washing on $^3$H-DBCT binding to yeast submitochondrial particles.

Binding assays were carried out as described in Chapter 3. The sedimented particles were washed by repeated resuspension and sedimentation in binding buffer. Two concentrations of $^3$H-DBCT were employed: A. a sub-$I_{50}$ level of 0.31 nmole/mg; B. an excess concentration of 31 nmole/mg, i.e. fifteen times the $I_{50}$ value.

<table>
<thead>
<tr>
<th>Number of nmole/mg Expressed as %</th>
<th>Amount bound</th>
<th>Number of nmole/mg Expressed as %</th>
<th>Amount bound</th>
</tr>
</thead>
<tbody>
<tr>
<td>washes</td>
<td>0' control</td>
<td>washes</td>
<td>0' control</td>
</tr>
<tr>
<td>'0'</td>
<td>0.23</td>
<td>100</td>
<td>0'</td>
</tr>
<tr>
<td>'1'</td>
<td>0.20</td>
<td>89</td>
<td>'1'</td>
</tr>
<tr>
<td>'2'</td>
<td>0.19</td>
<td>82</td>
<td>'2'</td>
</tr>
<tr>
<td>'3'</td>
<td>0.15</td>
<td>65</td>
<td>'3'</td>
</tr>
<tr>
<td>'4'</td>
<td>0.16</td>
<td>67</td>
<td>'4'</td>
</tr>
</tbody>
</table>


18hr at 0°C to ensure that all the DBCT high affinity sites had been
titrated. The particles were then washed 5 times with binding buffer, for five
different preparations a value of 8.64 nmole/mg ± 1.3 was obtained, moreover,
under these conditions a further 5 washes with buffer containing 1mg/ml
bovine serum albumin failed to remove any more of the bound DBCT. Triethyl
tin in comparison is totally removed by just five washes of buffer. The
washing experiments clearly indicate that the high affinity (covalent) binding
sites for DBCT in the inner mitochondrial membrane are of the order of 8-10
nmole/mg. The binding of DBCT to these sites can be considered to be as
either:

(1) \[ B + L_1 \overset{K_1}{\longrightarrow} BL_1 \]
[ B = binding site \]
[ L_1 = DBCT \]

(2) \[ B + L_1 \overset{K_1}{\underset{K_2}{\longrightarrow}} BL_1 \]

where \( K_1 \) is much greater than \( K_2 \).

Evidence that equation (1), i.e. a covalent interaction is taking place
is demonstrated by the experiment described in Table 4.2. In this experiment
a significant proportion of bound DBCT survives TCA precipitation, it is
unlikely that a dissociable type of binding would survive such drastic
conditions.

Table 4.2. The effect of TCA precipitation on \( H^3\)-DBCT binding.

Submitochondrial particles were incubated with \( H^3\)-DBCT for 30 min at 0°C in
3ml of binding buffer, 2ml of 10% TCA were then added and the precipitate
separated by filtration through a Whatman GF/F glass fibre filter. After
washing with 5ml of water the filters were dried and assayed for radioactivity
by scintillation counting.

<table>
<thead>
<tr>
<th>( H^3)-DBCT applied (nmole/mg)</th>
<th>TCA precipitated DBCT (nmole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
<td>2.16</td>
</tr>
<tr>
<td>17.6</td>
<td>3.24</td>
</tr>
</tbody>
</table>
Table 4.3. The effect of inhibitors on triethyl tin binding to yeast submitochondrial particles.

Binding assays were carried out as described in Chapter 3, except that the submitochondrial particles were preincubated with the indicated inhibitors for 30 min before adding the $^{113}$Sn labelled triethyl tin chloride.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration (n mole/mg)</th>
<th>$^{113}$Sn triethyl tin concentration (n mole/mg)</th>
<th>% inhibition of binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>oligomycin</td>
<td>4.6</td>
<td>0.44 n mole/mg</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>13.2</td>
<td>&quot;</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>18.4</td>
<td>&quot;</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>23.0</td>
<td>&quot;</td>
<td>0</td>
</tr>
<tr>
<td>venturicidin</td>
<td>4.0</td>
<td>&quot;</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>12.0</td>
<td>&quot;</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>16.0</td>
<td>&quot;</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>20.0</td>
<td>&quot;</td>
<td>0</td>
</tr>
<tr>
<td>tripropyl tin chloride</td>
<td>47.0</td>
<td>0.60 n mole/mg</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>141.0</td>
<td>&quot;</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>235.0</td>
<td>&quot;</td>
<td>74</td>
</tr>
<tr>
<td>DBCT</td>
<td>20.0</td>
<td>&quot;</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>40.0</td>
<td>&quot;</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>80.0</td>
<td>&quot;</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>120.0</td>
<td>&quot;</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>200.0</td>
<td>&quot;</td>
<td>56</td>
</tr>
<tr>
<td>trimethyl tin chloride</td>
<td>17.0</td>
<td>&quot;</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>85.0</td>
<td>&quot;</td>
<td>11</td>
</tr>
</tbody>
</table>
If the DBCT and triethyl tin binding sites are identical there should be evidence of competition between the two ligands for the site according to equation 4.

$$H_2 L_2 \rightleftharpoons L_2 + B + L_1 \rightarrow B L_1$$

where $B$ = binding site, $L_2$ = triethyl tin and $L_1$ = DBCT.

In Table 4.3, submitochondrial particles were preincubated with various inhibitors for 30 min before adding a low concentration of $^{113}$Sn triethyl tin (i.e. to predominantly titrate the high affinity sites). Only DBCT, tripropyl and tributyl tin inhibited the binding. If these effects were a straight competition phenomenon it would be expected that the competing ligands would be effective at concentrations which were equimolar with respect to triethyl tin. Furthermore, triethyl tin does not inhibit DBCT binding even in large excess (Table 4.4). Equation 4 predicts that even though DBCT is a covalent interaction there should still be competition with triethyl tin. It would therefore appear that DBCT and triethyl tin do not bind to the same site. However, the inhibition of $^{113}$Sn triethyl tin binding by DBCT would seem to indicate that there is an interaction between the DBCT and triethyl tin binding site.

Summarising, DBCT can be considered to be a specific inhibitor of the OS-ATPase. Its effects are similar to those demonstrated by its analogue triethyl tin. However, DBCT inhibition is brought about by an essentially irreversible binding phenomenon which titrates a component of the inner mitochondrial membrane. The concentration of this component is between 6-10 nmole/mg and it appears to be essential for the normal functioning of ATP dependent reactions. The DBCT binding site is not equivalent to the triethyl tin high affinity site described in Chapter 3, although there does appear to be an interaction between the two sites.
Table 4.4. The effect of trialkyl tin compounds on $H^3$-DBCT binding to yeast submitochondrial particles.

Binding assays were carried out as described in Fig. 4.15, except that the submitochondrial particles were either preincubated for 30 min with the inhibitor before adding the $H^3$-DBCT (column A), or alternatively the inhibitors were added at the same time as the $H^3$-DBCT (column B).

<table>
<thead>
<tr>
<th>$H^3$-DBCT bound (nmole/mg)</th>
<th>INHIBITOR</th>
<th>concentration (nmole/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>B</td>
<td>NO INHIBITOR</td>
</tr>
<tr>
<td>0.44</td>
<td>0.41</td>
<td></td>
</tr>
<tr>
<td>0.44</td>
<td>0.39</td>
<td>triethyl tin sulphate</td>
</tr>
<tr>
<td>0.46</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>0.44</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>0.45</td>
<td>0.42</td>
<td></td>
</tr>
<tr>
<td>0.44</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>0.43</td>
<td>0.42</td>
<td></td>
</tr>
<tr>
<td>0.42</td>
<td>0.43</td>
<td></td>
</tr>
<tr>
<td>0.40</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>0.42</td>
<td>0.44</td>
<td>tributyl tin chloride</td>
</tr>
<tr>
<td>0.44</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>0.40</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>0.40</td>
<td>0.43</td>
<td>tripropyl tin chloride</td>
</tr>
<tr>
<td></td>
<td>0.42</td>
<td></td>
</tr>
<tr>
<td>0.39</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>0.39</td>
<td>0.41</td>
<td></td>
</tr>
</tbody>
</table>
The following series of experiments have been largely carried out on a preparation which will be designated SMP-DBCT. This preparation was routinely prepared by incubating yeast submitochondrial particles (1 mg/ml) with an excess DBCT concentration (20-30 nmole/mg) for 18 hr at 0°C. The particles were then washed repeatedly with 0.25M sucrose, 20mM Tris-Cl, pH 7.5 until all the unbound DBCT had been removed (usually 5 washes were sufficient). These treated particles then have between 8-10 nmole/mg DBCT covalently bound, moreover, the ATPase activity is 95-100% inhibited. In analogous experiments with beef heart particles all the ATP dependent reactions are totally inhibited, whereas other functions such as the succinate driven transhydrogenase reaction have normal activity. Thus a component of the OS-ATPase essential for ATPase activity is irrevocably tagged with H₃-DBCT.

In Table 4.5, an SMP-DBCT preparation has been extracted with Triton X-100 in the manner used to extract the OS-ATPase (see Chapter 2). Nearly 70% of the bound DBCT is extracted by the Triton X-100, approximately 30% of the DBCT is left behind in the extracted membrane. This result is similar to the effects of Triton X-100 extraction on the ¹¹³Sn triethyl tin high binding site shown in Chapter 3. Obviously, the removal of the OS-ATPase from the membrane is accompanied by the removal of the major part of the DBCT binding components.

The question arising from this Triton X-100 extraction experiment, is how many of the DBCT binding sites are associated with the OS-ATPase? Two experiments were carried out to investigate this problem. The first
The objective of the experiment described in this table was to determine the effect of Triton X-100 extraction on the covalently bound $H^3$-DBCT. SMP-DBCT were prepared as discussed in the results, 0.5% W/V Triton X-100 extraction was carried out as described in Chapter 2.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>DBCT bound Total (nmole)</th>
<th>Protein (nmole/mg)</th>
<th>ATPase activity *% Inhibited</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMP-DBCT</td>
<td>173</td>
<td>8</td>
<td>21.6</td>
</tr>
<tr>
<td>Triton X-100 extract.</td>
<td>117</td>
<td>15</td>
<td>7.8</td>
</tr>
<tr>
<td>Triton X-100 extracted particles.</td>
<td>56</td>
<td>4</td>
<td>13.8</td>
</tr>
</tbody>
</table>

* As compared to a control experiment with uninhibited submitochondrial particles.
Fig. 4.16. Precipitation of a DBCT labelled component from a DBCT labelled Triton x-100 extract with antisera specific to OS-ATPase.

Aliquots (500μg) of DBCT labelled Triton x-100 extract prepared as previously described were incubated with increasing amounts of antisera specific to purified OS-ATPase in 0.25M sucrose, 20mM Tris-Cl, pH 7.5 buffer (1ml final volume) at 30°C for 60min. The resultant precipitate was centrifuged at 100,000g for 20min in the Beckman 40-3 head, using cellulose nitrate tubes (approx. volume 1.3ml) with micro-adaptors. The supernatants were assayed for radioactivity as previously described. The results are expressed as a % of a control experiment in which no antisera was present.
experiment is described in Fig. 4.16., in this experiment an antisera specific to the OS-ATPase was used to precipitate the enzyme. The titration curve reveals that a maximum of 70% of the DBCT sites are precipitated with the OS-ATPase thereby representing 49% of the sites originally located in the submitochondrial particles. Control experiments with preimmune antisera revealed that at a concentration of 10 mg/mg only 10% of the bound H³-DBCT was precipitated. Consequently, the precipitated DBCT binding components must be associated with the OS-ATPase or at the very least are associated with structures which contain enough OS-ATPase subunits to be antigenically active with respect to the antisera.

A second approach to the identification of the DBCT binding sites with the OS-ATPase, is to run the labelled Triton X-100 on a sucrose density gradient as used to prepare the purified OS-ATPase. This is demonstrated in Fig. 4.17., the DBCT appearing in the various fractions is bound DBCT as witnessed by the fact that the radioactivity is TCA precipitable. The interesting thing about the gradient is that the DBCT is found not only in the region of the gradient where the maximum ATPase activity is, but also at the top of the gradient where the ATPase activity is low and the protein concentration high. The amount of H³-DBCT bound to the OS-ATPase is between 3-4 nmole/nmole of OS-ATPase, a result similar to the 6 nmole/nmole result for the triethyl tin binding to the purified OS-ATPase. However, the H³-DBCT bound to the OS-ATPase represents

* Antisera specific to the OS-ATPase was obtained from rabbits immunised with 8mg of purified OS-ATPase suspended in Freund's adjuvant as described by Schatz, Peneffsky and Racker (1967).
Fig. 4.17. Sucrose density gradient purification of a Triton X-100 extract labelled with $^{3}H$-DBCT.

A Triton X-100 extract of SMP-DBCT was prepared as discussed in Table 4.5, and the extract run on a sucrose density gradient for purifying OS-ATPase as described in Chapter 2. The gradient was fractionated and each fraction assayed for radioactivity, protein and ATPase activity. A shows the amount of $^{3}H$-DBCT bound in each fraction; $\frac{\Delta}{\Pi}$, the ATPase activity of each $^{3}H$-DBCT fraction relative to a control gradient of an unlabelled Triton X-100 extract.
approximately 30% of the total bound $^3$DBCT found on the gradient. The purification scheme for the Os-ATPase preparation shown in Table 2.1, reveals that 25% of the Triton X-100 extract protein contains 75% of the ATPase activity. Thus 30% of the bound $^3$DBCT on the gradient is associated with intact functional OS-ATPase. However, the antisera experiment precipitated 70% of the bound $^3$DBCT, consequently one must conclude that 40% of the precipitated counts must contain components antigenically active to the antisera. Partis (1975) has shown that the $F_0$ components in the membrane can be removed by Triton X-100 extraction from $F_1$-OSCP depleted particles. Moreover, a major portion (70%) of the extracted $F_0$ components are precipitated with antisera. This would suggest that the Triton X-100 extract contains complete intact OS-ATPase complexes and a large pool of $F_0$ type components. The $F_0$ components may just represent dissociated OS-ATPase molecules or alternatively they may in themselves be independent structural entities which interact with the OS-ATPase. The $F_0$ type components would have a lower molecular weight than OS-ATPase and would appear at the top of an OS-ATPase gradient, consequently on a molecular weight basis for $F_0$ of approximately 120,000 daltons, the bound $^3$DBCT would have a specific activity of around 1.5 n mole/n mole $F_0$. However, this conclusion is very speculative as the exact nature of the protein found at the top of the gradient has not been delineated.

The $^3$DBCT binding component is not associated with $F_1$-ATPase and OSCP as shown in the experiment described in Table 4.6. In part A the particles were labelled with $^3$DBCT before extraction, the $F_1$-OSCP depleted particles have an increased number of $^3$DBCT molecules bound
In this experiment, the influence of $F_1$-ATPase and OSCP on the binding of $H^3$-DBCT to submitochondrial particles was examined. Submitochondrial particles were stripped of $F_1$-ATPase and OSCP by NaBr/NH$_4$OH extraction as described in Chapter 3. In A the particles were first labelled with $H^3$-DBCT before extraction, in B the particles were stripped of $F_1$-ATPase and OSCP and then labelled with $H^3$-DBCT in the same way as the SMP-DBCT were prepared.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>$H^3$-DBCT bound</th>
<th>Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total(nmole)</td>
<td>nmole/mg</td>
</tr>
<tr>
<td>SMP-DBCT</td>
<td>500</td>
<td>10·4</td>
</tr>
<tr>
<td>A. Supernatants from NaBr/NH$_4$OH extractions.</td>
<td>122</td>
<td>5·9</td>
</tr>
<tr>
<td>$F_1$-OSCP depleted particles.</td>
<td>223</td>
<td>13</td>
</tr>
<tr>
<td>B. $F_1$-OSCP depleted particles.</td>
<td>75</td>
<td>12</td>
</tr>
<tr>
<td>Triton X-100 extract of $F_1$-OSCP depleted particles.</td>
<td>26·5</td>
<td>17·7</td>
</tr>
<tr>
<td>Triton X-100 extracted $F_1$-OSCP depleted particles.</td>
<td>48·5</td>
<td>10</td>
</tr>
</tbody>
</table>

Protein lost through procedure = 10·15 mg.
reflecting the increased $P_0$ concentration. The $H^3$-DBCT found in the supernatants from the extraction is due to contaminating membrane fragments rather than a binding component being present in $F_1$-ATPase and OSCP. This is due to the fact that after 6M NaBr extraction, the stripped particles float (due to the high salt concentration) on the supernatant after centrifugation, consequently it is difficult to remove the supernatant without also taking some of the membrane fraction. Part B of the experiment shows that stripping the particles of $F_1$-ATPase and OSCP and then labelling with $H^3$-DBCT also results in an increased amount of bound $H^3$-DBCT. It is apparent from these experiments that $F_1$-ATPase and OSCP are not directly involved in the binding of $H^3$-DBCT to the membrane. It is also of interest that $P_0$ can still be removed by Triton X-100 extraction after $F_1$-ATPase and OSCP have been removed (Table 4.6B).

One of the more useful properties of the DCCD binding protein is the fact it can be extracted from the membrane with chloroform-methanol (2:1). Consequently, an attempt was made to adopt the purification scheme devised by Cattell et al. (1971) for the DCCD binding protein to the DBCT binding site. Table 4.7 shows that the DBCT binding component is readily extracted with chloroform-methanol, in this experiment 84% of the bound $H^3$-DBCT was extracted, the average % of bound radioactivity extracted for 3 experiments was $90 \pm 4.32%$. The actual removal of the membranes from the chloroform-methanol extract involves filtering through glass wool which invariably leads to some loss of the extract. This fact in conjunction with obvious phase partition effects indicates that the figure of 90% for the amount of $H^3$-DBCT extracted from the membrane is probably on the low side, and chloroform-methanol extraction in fact...
Table 4.7., Purification of DBCT binding component.

SMP-DBCT were extracted with chloroform-methanol (2:1) and purified according to the method of Cattell et al. (1971). Protein determinations in organic solvents were carried out by the method of Lowry as modified by Hess and Lewin (1965).

<table>
<thead>
<tr>
<th>Fraction</th>
<th>total cpm</th>
<th>total DBCT</th>
<th>Protein</th>
<th>H&lt;sup&gt;3&lt;/sup&gt;-DBCT</th>
<th>% H&lt;sup&gt;3&lt;/sup&gt;-DBCT</th>
<th>% Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMP-DBCT</td>
<td>5,926,284</td>
<td>454</td>
<td>56.7</td>
<td>8.0</td>
<td>100</td>
<td>100.0</td>
</tr>
<tr>
<td>C/M extract</td>
<td>4,914,000</td>
<td>378</td>
<td>9.4</td>
<td>40.2</td>
<td>84</td>
<td>16.6</td>
</tr>
<tr>
<td>Washed C/M extract</td>
<td>4,407,000</td>
<td>339</td>
<td>1.6</td>
<td>211.8</td>
<td>75</td>
<td>3.0</td>
</tr>
<tr>
<td>Ether supernatant</td>
<td>3,094,000</td>
<td>238</td>
<td>0.44</td>
<td>540.0</td>
<td>53</td>
<td>0.8</td>
</tr>
<tr>
<td>Ether precipitate</td>
<td>132,000</td>
<td>102</td>
<td>1.2</td>
<td>8.5</td>
<td>2</td>
<td>2.1</td>
</tr>
</tbody>
</table>
removes all of the bound $H^3$-DBCT.

The next step in the purification scheme is an aqueous wash to remove all non proteolipid protein. A very small proportion of the counts are removed whereas in this case a large proportion of the protein is removed. The amount of protein removed by the washing procedure appears to be variable, for example in another preparation the washing removed only 20% of the protein and 5% of the radioactivity. It would appear that there is no direct correlation between the protein removed and the radioactivity loss. A similar series of results is seen in the work of Cattell et al. (1971). The ether precipitation step is reported to precipitate only protein (Kuntzel et al., 1975), it can be seen that approximately 80% of the protein is precipitated but only 3% of the radioactivity. This result is substantially different from the DCCD binding component as described by Cattell et al. (1971), with the DCCD binding component approximately 65% of the radioactivity is precipitated with 70% of the protein. The $H^3$-UBCT binding component remains in the ether supernatant.

In Fig. 4.18, a sample of the washed chloroform-methanol extract was analysed by thin layer chromatography, it can be seen that only one peak of radioactivity corresponding to a spot visualised with iodine is resolved, which has a vastly different K.F value when compared to the free $H^3$-UBCT. It can be concluded that the $H^3$-UBCT bound to the membrane of submitochondrial particles is covalently attached to only one component. This component does not run with any of the common mitochondrial phospholipids which are also extracted with chloroform-
Fig. 4.18. Thin layer chromatography of a chloroform-methanol extract of SMF-DECT.

A washed chloroform-methanol extract of SMF-DECT was run on a 0.5 mm Silica Gel plate in a CHCl₃/CH₃OH/HCl/H₂O (130:50:1-0.62:7.2) solvent system. An aliquot of H₂-DECT was run as a control. The spots were visualised with iodine vapour and the plate sectioned and assayed for radioactivity by scintillation counting.
Fig. 4.19. Thin layer chromatography of various phospholipids compared with the chloroform-methanol extract of SMP-DBCT.

Thin layer chromatography was carried out as in Fig. 4.18., PE, phosphatidylethanolamine; DPG, diphosphatidylglycerol; C/M washed chloroform-methanol extract of SMP-DBCT; PC, phosphatidylcholine; phosphatidic acid; phosphatidyl serine.
methanol (Fig. 4.19, Cattell et al. 1971; Folch, Lees and Sloane-Stanley, 1957),
either does it migrate with subunit-9 (Partis, 1975). However, gel
electrophoresis of the chloroform-methanol extract (Fig. 4.20.) shows that
the radioactive component migrates with the bromophenol blue marker dye,
a characteristic of subunit-9 and the DCCD binding protein. However, it
should be pointed out that any small molecule of less than 10,000 daltons
molecular weight would run at the front with the bromophenol blue marker.
Purification of the labelled component by preparative thin layer chromatog-
raphy reveals one component which has the same R.F. value as that shown in
Fig. 4.18., moreover this runs in gradient gel electrophoresis as one band
with an apparent molecular weight of 6,000-8,000 daltons (E. Zanders and
K. Cain, unpublished results). The purified component does not give a
significant Folin’s reaction and would appear not to be a protein, although
this does not rule out that it is associated with a protein or proteins.
In Fig. 4.21., it can be seen that the $^3$H-DBCT component is apparently
associated with a protein which has a molecular weight of approximately
45,000 daltons.

Discussion and Conclusion.

It is apparent from the work described in this chapter that DBCT offers
a new approach to the intrinsic problem of structure/function relationships
in the OS-ATPase complex. DBCT is a potent and apparently specific inhibitor
of reactions which are dependent on the OS-ATPase. It is very similar in
its actions to DCCD and oligomycin. Unlike oligomycin it is apparently a
covalent inhibitor, moreover labelling of mitochondrial membranes results
in only one labelled component which can be totally extracted with chloroform-
methanol. At the present time the identity of this component is unknown, although
Fig. 4.20. Gel electrophoresis of chloroform-methanol extract of SMP-DBCT.

A washed chloroform-methanol extract of SMP-DBCT was co-electrophoresed with an antisera precipitate of a triton extract prepared from submitochondrial particles which had been isolated from cells grown in the presence of H$^2$-leucine, cycloheximide and chloramphenicol as described by Tzagoloff (1971). Polyacrylamide gel electrophoresis was as described by Weber and Osbourne (1969).
Fig. 4.21. Slab gel electrophoresis of Triton X-100 extract of SMP-DBCT.

Slab gel electrophoresis was carried out as described by Douglas and Butow (1976). The indicated fractions of the gel were sliced after staining and assayed for radioactivity.
the evidence points to it not being a protein. However, the OS-ATPase
gradient and antisera experiments provide strong evidence for it being
directly involved in the OS-ATPase complex, but a number of anomalies
stand out from the results. Firstly the OS-ATPase appears to have between
3-6 nmole of H\textsuperscript{3}-DBCT associated with each nmole of enzyme. This figure
agrees quite well with the triethyl tin data presented in Chapter 3.
However, the H\textsuperscript{3}-DBCT associated with the OS-ATPase represents only 30\% of
the total amount of bound inhibitor. This could be explained by
incomplete extraction and dissociation with Triton X-100, however this
would not explain the fact that as the membrane contains a pool of
H\textsuperscript{3}-DBCT binding sites of between 8-10 nmole/mg one would expect to see
a much larger number of DBCT molecules associated with the OS-ATPase.
It should be pointed out that this phenomenon is also present in beef
heart (K.Cain, unpublished results) and in E.coli (M.D.Partis, unpublished
results). A possible explanation is that the membrane contains additional
F\textsubscript{0} components which are not directly associated with an F\textsubscript{1}-ATPase. These
F\textsubscript{0} molecules could have other functions such as ion transport and interact
with the OS-ATPase 'proper' possibly being energised in the process. This
type of scheme could explain the differential sensitivity observed in
the beef heart transhydrogenase and ATPase reactions. Interestingly,
some recent work in our laboratory (K.Cain and E.Zanders unpublished
results) has shown that all the fractions of the OS-ATPase contain all the
subunits present in the OS-ATPase. Thus the decrease in the ATPase
specific activity seen in the less dense fractions of the gradient is
either due to a mixture of dissociated OS-ATPase molecules, or alternatively
there is a small amount of OS-ATPase which is masked by an excess of
F\textsubscript{0} type components.
An alternative suggestion for these results is that $H^3$-DBCT is labelling a mobile chemical intermediate. Evidence for this hypothesis has been produced by several workers in our laboratory and this will be discussed in a general discussion in Chapter 6. It is apparent that the identification of the $H^3$-DBCT binding component should produce a new insight on not only trialkyl mechanisms of action but also in the functioning of the US-ATPase and oxidative phosphorylation reactions themselves.
CHAPTER 5.

The relationship between the OS-ATPase and the ADP-ATP translocase system.

Introduction.

As discussed in Chapter 1, an alternative approach to the investigation of the OS-ATPase complex is to make use of specific cytoplasmically determined mutants which are resistant to OS-ATPase inhibitors. A detailed biochemical genetic study with oligomycin resistant mutants has shown that the in vivo resistance is due to a modification in the OS-ATPase complex (Avner and Griffiths, 1970, 1973; Houghton et al., 1974, Griffiths et al., 1974; Tzagoloff et al., 1976). Similar studies with triethyl tin resistant mutants (Lancashire and Griffiths, 1975) have revealed two classes of mutant, the Class 2 mutants are specifically resistant to triethyl tin and the uncoupling agent '1799', and are cytoplasmically determined. Consequently, the OS-ATPase of triethyl tin resistant mutants should exhibit a decreased sensitivity to the inhibitor. This chapter in part examines the ATPase of triethyl tin resistant mutants. A report by Harris et al., (1973) showed that triethyl tin inhibited ADP/ATP translocation in rat liver mitochondria. A genetic study prompted by this result revealed that the specific triethyl tin resistant mutants were also cross resistant to bongkrekic acid, an inhibitor of the ADP/ATP translocase (Cain et al., 1974). The biochemical analysis of this involvement with translocase is the subject of this chapter (some of this data has already been published, Cain et al., 1974).
Methods and Materials.

Materials.

(\(^{14}\))ADP was obtained from the Radiochemical Centre, Amersham, Bucks., U.K. Bongkrekic acid (BA) was a gift from Dr. W. Berends, Technical University, Delft, The Netherlands. Atractyloside (ATR) and carboxy-atactyloside (CATR) were purchased from Boehringer, Mannheim, Germany. Cellulose filters 0.45 \(\mu\)M HAWP 02500 were obtained from Millipore. Tritiated BA (\(^3\)H-BA) was a gift from P.V. Vignais, Grenoble and had a specific activity of 45,000 dpm/nmole.

Methods.

a) General

Growth of yeast, isolation of Braun shaker mitochondria, ATPase assays and protein determinations were carried out as described in Chapter 2. In the case of translocase assays, intact mitochondria with good respiratory control are required. Consequently, intact yeast mitochondria were prepared from protoplasts isolated by the snail enzyme digestion method (Watson et al., 1970) from cells harvested in mid-exponential phase.

b) Translocase assays.

Translocase assays were normally carried out by the (\(^{14}\))ADP uptake method of Pfaff and Klingenberg (1968). The reaction was normally carried out in an incubation medium containing 10 mM-Hepes-KOH, 0.5 M sorbitol, 1 mM EGTA, pH 7.0 and 0.5-1.0 mg of mitochondrial protein. After a defined preincubation (see results) with or without
inhibitors, the reaction was initiated by rapid mixing with 0.5ml of incubation buffer containing \(^{14}\text{C}\) ADP at the same temperature as the reaction mixture. The reaction was then terminated after a set time (usually 30sec) by rapid filtration through a Millipore filter (0.45 µM) followed by washing with ice-cold buffer. The filtration was carried out in special holders which were individually linked to a negative pressure reservoir maintained by a vacuum pump, consequently a large negative pressure could be applied to any filter holder enabling very rapid filtration (2 sec) and reaction termination times to be achieved. Radioactivity was estimated by drying the filter and assaying by scintillation counting.

c) Preparation of \(H^3\) labelled atractyloside (\(H^3\)-ATR)

The method devised for the synthesis of \(H^3\)-ATP was based on an observation of Defaye et al. (1972) which showed that CATR could be decarboxylated by pyrolytic conversion at 165°C (see Fig. 5.1. for structures) to ATR. Approximately 50 mg of CATR were heated in a sealed tube in the presence of 0.5 Ci of \(T^2\)O for 1 hr at 165°C. The pyrolysis products (CATR*) were dissolved in 50% ethanol (10ml) and the resultant solution evaporated to dryness before redissolving in 1 ml of 50% ethanol. Purification and analysis of \(H^3\)-ATR from this CATR* solution were carried out by paper chromatography and electrophoresis as described by Defaye et al. (1972). Purification was achieved by preparative descending paper chromatography run in a n-butanol/acetic acid/water solvent (4:1:5). Analytical runs had shown the R.F values for ATR, CATR and CATR* (as visualised by spraying with a solution of 0.5g of vanillin in 50% orthophosphoric acid and heating for 2 min at 100°C) were 0.5, 0.45 and 0.5.
R = H = Atractyloside
R = COOH = Carboxyatractyloside

Bongkrekic acid.

Fig. 5.1. Inhibitors of ADP/ATP translocation in mitochondria
Consequently, after running the preparative chromatogram, a thin strip was cut out, visualised with vanillin and assayed for radioactivity. The relevant portion of the chromatogram was then eluted with 50% ethanol as shown in Fig. 5.2a., the eluted fraction was then run on a second preparative chromatogram. This second preparative run produced only one peak with a very low background. The specific activity of the eluted peak was $2.37 \times 10^4$ dpm/nmole. Paper electrophoresis showed that the radioactive fraction had an R.F. value identical to ATR and that there was no contaminating CATR (Fig. 5.2b). Further proof that the radioactive compound was $^3\text{H}$-ATR was obtained by biological assay, Bruni et al. (1962) showed that the inhibition of ADP stimulated respiration in rat liver mitochondria by ATR can be reversed with an increased ADP concentration, however CATR inhibition (Vignais et al. 1971) is unaffected by increasing the ADP concentration. The experiments in Fig 5.3. show that ATR inhibits the ADP stimulation of respiration produced by 100 $\mu$M ADP, adding 1 mM ADP overcomes the inhibition, the labelled compound behaves exactly like ATR. Conversely, CATR inhibition is not affected by increased ADP concentration. It was concluded that the labelled compound was in fact $^3\text{H}$-ATR. Since this preparation was devised, a similar preparation has been published by Brandolin et al., 1974 which confirms the validity of this method of synthesis.

Results and Discussion.

a) Sensitivity of triethyl tin resistant mutants to triethyl tin at the ATPase level.

The characteristics of some of the triethyl tin resistant mutants
Fig. 5.2. Chromatographic purification and analysis of $^{3}$-ATR.

a) Preparative chromatography as described in methods, eluted from 1st run, eluted from 2nd run.

b) Paper electrophoresis of ATR, CATR and $^{3}$-ATR was carried out as described by Defaye et al. (1972) and the spots visualised with a vanillin spray (0.5g in 50% orthophosphoric acid).
Rat liver mitochondria were prepared according to the method of Chappell and Hansford (1969). Each incubation mixture contained 8.7 mg of mitochondria in 3 ml of medium containing, 0.25 M sucrose, 20 mM Tris-Cl, pH 7.4, 5 mM MgCl₂, 10 mM KCl, 5 mM KH₂PO₄. The following additions were made, the figures in ( ) giving the final concentration. A. potassium glutamate (15 mM); B. ADP (100 mM); C. CCCP (0.82 mM); D. ATR (0.39 mM); E. ADP (1000 mM); F. H³-ATR (0.39 mM); G. CATR (0.39 mM).
isolated by Lancashire (1974) are shown in Table 5.1, previous studies have revealed that whole cell respiration in D22/EC1, D22/EC2, D22/EC9, D22/EC10, D22/EC16 and D22/EC23 are all resistant to inhibition by triethyl tin (Lancashire and Griffiths, 1975). This would discount the possibility that the triethyl tin resistance is due to an inducible detoxifying mechanism. Skipton (1974) has shown that state 3 respiration in D22/EC2 mitochondria is slightly resistant to triethyl tin. In Fig. 5.4, a number of triethyl tin mutants were investigated with respect to the sensitivity of the mitochondrial ATPase to triethyl tin. It can be seen from the figure that only D22/EC6 a Class 2(b) mutant showed any resistance to the inhibitor, approximately a two fold decrease in sensitivity was observed. The Class 1(b) mutant D22/EC11 showed no significant difference, D22/EC1 and D22/EC2 exhibited a small decrease in sensitivity. These findings are difficult to reconcile with the known inhibitory properties of triethyl tin and the in vivo resistance characteristics. D22/EC6 for example is more than 20 fold resistant to triethyl tin at the in vivo level (Lancashire, 1974). The experiments carried out by Griffiths and Houghton (1974) on oligomycin mutants showed that D22/A21 which was more than 10 fold resistant to oligomycin at the in vivo level, exhibits approximately an 8 fold decrease in sensitivity at the ATPase level. This would suggest that the failure to see significant resistance with the triethyl tin mutants is due to the ATPase assay not adequately reflecting the mode of action of triethyl tin. A similar pattern of results has been shown for venturicidin resistant mutants (Griffiths, Houghton, Lancashire and Meadows, 1975), consequently the finding that the triethyl tin resistant mutants were cross resistant to BA prompted the work described in the next section.
Table 5.1. Resistance and genetic characteristics of triethyl tin resistant mutants.

Resistance (R) and Sensitivity (S) of D22 mutants to inhibitors.

<table>
<thead>
<tr>
<th>Class</th>
<th>Strain</th>
<th>Triethyl tin</th>
<th>Oligomycin</th>
<th>BA</th>
<th>Inheritance</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>D22</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td></td>
</tr>
<tr>
<td>1(a)</td>
<td>D22/EC16</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>Nuclear</td>
</tr>
<tr>
<td>1(b)</td>
<td>D22/EC11</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>Nuclear</td>
</tr>
<tr>
<td>2(a)</td>
<td>D22/EC1</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>Cytoplasmic</td>
</tr>
<tr>
<td>2(b)</td>
<td>D22/EC2</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D22/EC12</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D22/EC6</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D22/EB16</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td></td>
</tr>
<tr>
<td></td>
<td>D22/EB8</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td></td>
</tr>
</tbody>
</table>

This table is abstracted from Cain et al. (1974) and Lancashire and Griffiths (1975).
Fig. 5.4. Comparison of ATPase sensitivity to triethyl tin in various triethyl tin resistant mutants.

ATPase activity was assayed in Braun shaker prepared mitochondria, isolated and gradient purified from the indicated strains as previously described in Chapter 2. The S.E. bars where indicated refer to the mean of 4 preparations, otherwise the points are the results of 2 preparations. • D22, S.A. = 5.4 ± 0.4; • D22/EC11, S.A. = 6.9 ± 1.25; ▲ D22/EC6, S.A. = 4.6 ± 0.25; □ D22/EC1, S.A. = 4.6; ○ D22/EC2, S.A. = 5.7.
b) ADP/ATP translocation in yeast mitochondria.

i. Introduction.

The demonstration of a specific carrier for ADP and ATP in the mitochondrion was as a result of early work with the inhibitor ATR which showed that it inhibited state 3 respiration in a manner which was competitive with respect to ADP (see Vignais, 1976, for review). Subsequent experiments have shown that ATR inhibits the translocation of ADP into the matrix space of the mitochondrion and thereby indirectly inhibits state 3 respiration (Klingenberg and Pfaff, 1966). The finding that ATR inhibited the external ADP binding to mitochondria (Bruni et al., 1964) suggested that a transporter was involved in ADP/ATP translocation. Since this early work, it has been shown that ADP or ATP is transported into the matrix by an exchange diffusion reaction, such that an ADP molecule entering the matrix is balanced by an ATP molecule leaving the matrix space (Pfaff and Klingenberg, 1968). The exchange stoichiometry is one to one (Pfaff, Klingenberg and Heldt, 1965; Duée and Vignais, 1965), and the process obeys Michaelis Menten type kinetics (Pfaff and Klingenberg, 1968; Duée and Vignais, 1969) with a Km value for external ADP of between 1 to 10μM (Duée and Vignais, Pfaff, Heldt and Klingenberg, 1969). The translocation process has a high specificity for ADP and ATP and they will compete with each other (Pfaff and Klingenberg, 1968; Duée and Vignais, 1969; Kemp and Groot, 1967; Souverijn et al., 1970). The Km for external ATP appears to be dependent upon the energy state of the mitochondria (Souverijn et al., 1973). The exchange is an extremely rapid phenomenon, the Vmax is reached within 10sec at 20°C in rat liver mitochondria and is of the order of 200nmole/mg/min, at 0°C the reaction is slower and the
Vmax is 7 nmole/mg/min (Klingenberg, 1970). Consequently, new rapid methods of terminating and separating mitochondria from the assay mixtures were developed (see Klingenberg, 1970, for review).

The main role of the translocase is to regulate aden metabolism in the mitochondrion, thus under phosphorylating conditions it will import ADP and export ATP. The exchange is an electrogenic phenomenon, that is $ADP^3-$ for $ATP^4-$, charge compensation taking place by means of Pi transport (Duee and Vignais, 1969). Thus N-ethyl maleimide (NEM), an inhibitor of Pi transport will inhibit the exchange under phosphorylating conditions (McGiven, Grebe and Klingenberg, 1971). The driving force for the translocation is believed to be the membrane potential (Vignais, 1976).

In addition to ATR, there are two other important specific inhibitors, CATR and BA. CATR differs from ATR only in the addition of an extra -COOH group, this is sufficient to alter the mode of action, such that CATR is a non competitive inhibitor of translocation (Vignais, Vignais and Defaye, 1973) ATR and CATR are non penetrant inhibitors, and it is assumed that they bind to the outside of the inner membrane. BA is a non competitive inhibitor of translocation and is believed to act on the inside of the mitochondrial membrane (Henderson and Lardy, 1970; Klingenberg, Grebe and Heldt, 1970; Kemp et al., 1971).

II. Development of assay methods for ADP translocation in yeast mitochondria.

In this study the objective was to determine whether or not triethyl tin resistant mutants had a modified sensitivity to BA at the translocase level. It was therefore necessary to ascertain the correct conditions
to investigate the effect of BA. The following experiments were carried out on the parental D22 strain. Obviously when comparing the inhibitor sensitivity of one strain with another it is preferable to measure initial velocities. Fig. 5.5., shows that the rate of the reaction is markedly affected by temperature, there is a sharp increase in activity around 12°C. The specific activity increased more than 10-fold on raising the temperature from 0 - 27°C, a result similar to that found in rat liver mitochondria (Klingenberg, 1970). Activity determinations at 20°C and higher were therefore subject to large errors as equilibrium was reached within 10 sec. The Km ADP values at 5°C and 20°C were 2.8µM and 0.85µM respectively (Fig. 5.6.), the value of 2.8µM is similar to that found in rat liver mitochondria, and Lauquin and Vignais (1973) have shown the Km ADP value in the yeast Candida Utilis is 1.8µM. The increased affinity for ADP at 20°C presumably reflects an altered conformational structure, probably produced by a phase transition in the membrane phospholipids. At a temperature of 5°C and (14C) ADP concentration of 10µM, the ADP uptake was linear for 60 sec, consequently all inhibition experiments were carried out at 5°C and 10µM (14C) ADP concentration, normally the reaction was terminated after 30 sec by millipore filtration. These standard conditions for the translocase assay were all carried out with the forward uptake method of Pfaff and Klingenberg (1968). However, it should be noted that assaying the ADP translocation by another method, namely the reverse exchange process (Pfaff and Klingenberg, 1968) has revealed essentially the same results. In this assay the mitochondria are prelabelled with (14C) ADP and the reaction is initiated by the addition of unlabelled nucleotide, thus the reaction is measuring the release of labelled nucleotide rather than uptake. Under these
Fig. 5.5. *Km* determination for ADP translocation.

Assays were carried out with varying concentrations of (\(^{14}\text{C}\)) 
ADP (6,000 cpm/m mole specific activity) and stopped by 
Millipore filtration after 30 sec as described in the methods. 
*Km* determinations were carried out at 5\(^{\circ}\)C and 20\(^{\circ}\)C, *Km* ADP 
at 5\(^{\circ}\)C = 0·85 \(\mu\)M and *Km* ADP at 20\(^{\circ}\)C = 2·8 \(\mu\)M.
Fig. 5.6 Effect of temperature on ADP translocation.
Assays were carried out as previously described with a 10uM (14C) ADP concentration at the indicated temperatures.  
conditions, the Km value for external ADP was 1.9mM, and again at 50°C the reaction was linear for 60sec. The reverse exchange assay also revealed that there is a spontaneous leakage of nucleotides from the mitochondria after preparation. Within 3hr 15% of the nucleotides are lost, after 5hr over half the nucleotide pool has leaked out. This result outlines an important criterion adopted for the assay, that is, the mitochondria were always used within 3hr of preparation. The leakage phenomenon has also been observed in Candida Utilis mitochondria (Lauquin and Vignais, 1973). The rate of ADP translocation did not show a significant pH dependence between 6.5 and 8.5 and consequently all assays were carried out at pH 7.0, a similar result has been demonstrated for rat liver mitochondria (Vignais, Vignais, Lauquin and Morel, 1973).

iii. Sensitivity of D22 and D22/EC6 to BA and ATR.

The effect of ATR on the ADP uptake is shown in Fig.5.7., the I50 value is 7nmole/mg in the D22 parental strain. In the case of the triethyl tin resistant mutant D22/EC6 which has a four fold resistance to BA in vivo, the I50 value is only slightly modified at approximately 41nmole/mg. It should be pointed out that although ATR is a potent inhibitor of ADP uptake in mitochondria from S.cerevisiae and Candida Utilis (Lauquin and Vignais, 1973) it does not kill the intact cell, presumably due to the fact that it does not penetrate the cell.

Fig.5.8., shows that BA inhibits the D22 strain at an I50 value of 4nmole/mg, the D22/EC6 strain requires 13nmole/mg to produce the same degree of inhibition. Thus translocase from the mutant has a 3-fold decrease in sensitivity to BA, a value which is in good agreement with the in vivo results.
Fig 5.7. Inhibition of ADP translocation by ATR in D22 and D22/EC6.

Assays were initiated with 10μM (14C) ADP at 5°C and stopped by filtration after 30sec as described in methods. ATR was preincubated for 10min before adding the (14C) ADP solution. The results are expressed as mean of three preparations ± S.E.O—O, D22 parental strain; D22/EC6 triethyl tin resistant mutant; O——O.
Fig. 5.8. Inhibition of ADP translocation by BA in D22 and D22/EC6.
Assays were carried out as described in Fig. 5.7, except that the mitochondria were preincubated for 20 min with BA before initiating the reaction. The results are for the mean ± S.E. of 4 preparations for D22/EC6 ●—●, and 3 preparations for D22 O—O.
The demonstration of a modified BA interaction site in mitochondria coupled with the genetic evidence (Cain et al., 1974) that the resistance phenomenon is mitochondrially coded provides strong evidence that a subunit of the ADP translocase is determined by mt DNA as has also been suggested by Haslam et al. (1973). An alternative suggestion is that the triethyl tin interaction site and BA interaction site occupy a common subunit which is common to the OS-ATPase and ADP translocase complexes. However, a number of factors cast doubt on this conclusion. Firstly, I have not been able to inhibit the ADP translocase system with triethyl tin, under a variety of conditions such as assaying at pH values from 6.5 to 8.0 and long preincubation times, there has been no significant inhibition with triethyl tin even with concentrations as high as 88nmole/mg. It is hard to understand how Harris et al. (1973) found triethyl tin to be a translocase inhibitor in rat liver mitochondria when the yeast system is unaffected. A possible explanation is that triethyl tin will work in rat liver mitochondria but not in yeast, this appears unlikely as every inhibitor of translocation in rat liver mitochondria has been found to be effective in yeast (Vignais, 1976). A second alternative is that the ADP uptake observed by Harris was not ADP translocation, this argument is strengthened by the fact that no evidence was presented that the ADP uptake observed was sensitive to ATR. Secondly, if the triethyl tin binding site was equivalent to the BA interaction site, one would expect some effect on triethyl tin binding when BA was added. Fig.5.9 shows that triethyl tin binding is unaffected by BA at a concentration of 20nmole/mg. This would seem to rule out a direct involvement between the triethyl tin and BA interaction sites.
Fig. 5.9. Competition for $^{113}$Sn triethyl tin binding by bongkrekic acid.
Assays were carried out as described in Fig. 3.13. ---, no inhibitor; ——, 20nmole/mg BA. K, for high affinity site, $5.0 \times 10^{-7}$ and number of sites = 114nmole/mg.
iv. \( H^3 \)-Atractyloside binding to yeast mitochondria.

It is apparent from the literature (Vignais, 1976) that the ATR binding site is an integral part of the functioning Adn translocase. Consequently, any information concerning the ATR binding site should be useful in understanding the molecular mechanisms of ADP/ATP translocation. Unfortunately a potentially powerful tool, the biochemical genetic approach, is seriously inhibited by the fact that ATR does not inhibit whole cell growth in yeast, thereby making the problem of producing ATR resistant mutants virtually insoluble. As a result details about the genetic history of the ATR binding site must come from indirect methods. In this context, the petite mutation can prove useful. Work in this laboratory (Griffiths, Lancashire and Zanders, 1975) has revealed that treatment of resistant mutants with ethidium bromide can produce a variety of petite phenotypes. Of special interest are the petites produced from D22/69, this strain is resistant to venturicidin, triethyl tin, 1799 and rhodamine 6G (R6G) an inhibitor of ADP translocation (Gear, 1975). This strain exhibits normal sensitivity to oligomycin, chloramphenicol and erythromycin, phenotypically and genetically this strain appears to be very similar to D22/EC6 (Griffiths et al., 1975; Lancashire and Griffiths, 1975). Table 5.2 shows the phenotypes of the various petites isolated from D22/69 as described by Griffiths, Lancashire and Zanders (1975).

It can be seen that the resistance marker for triethyl tin, venturicidin, R6G and 1799 is not located on mt DNA, consequently a biochemical analysis of the Adn translocase in a \( p^0v^0 \) and \( p^0v^R \) would give useful
information on the biogenesis of the Adn translocator.

*Table 5.2. Ethidium bromide induced petites.*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Phenotype of diploid strain (× D41)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D22/69</td>
<td>p⁺v⁺R</td>
<td>VEN⁺ TET⁺ R6G⁺ 1799⁺</td>
</tr>
<tr>
<td>parental</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D22/69/D1</td>
<td>p⁻v⁺R</td>
<td>VEN⁺ TET⁺ R6G⁺ 1799⁺</td>
</tr>
<tr>
<td>D22/69/D3</td>
<td>p⁻v⁻o</td>
<td>VEN⁻ TET⁻ R6G⁻ 1799⁻</td>
</tr>
<tr>
<td>D22/69/D4</td>
<td>p⁻v⁺R</td>
<td>VEN⁺ TET⁺ R6G⁺ 1799⁺</td>
</tr>
<tr>
<td>D22/69/D8</td>
<td>p⁻v⁻R</td>
<td>VEN⁻ TET⁻ R6G⁻ 1799⁻</td>
</tr>
</tbody>
</table>

* Abstracted from Griffiths, Lancashire and Zanders, 1975.

The two strains chosen were D3 and D4, translocase assays were carried out on snail enzyme prepared mitochondria as described previously. However, no detectable translocase activity was found, this would tend to suggest that the petite phenotype results in a loss of ADP translocation. Groot et al (1975) showed that in the p⁻ petite RD11A an ATR and BA sensitive uptake could be induced by preincubating the freshly isolated mitochondria with a high concentration of ATP, they concluded that the low levels of ADP translocation normally seen in petites are due to a very low content of endogenous nucleotides. The ATP preincubation step was not carried out in these
experiments, however, $^{3}\text{H}^{3}$-ATR binding can be used as an assay for the Adn translocator and is a convenient tool which can be applied to petite mitochondria.

The characteristics of $^{3}\text{H}^{3}$-ATR binding in normal parental mitochondria are shown in Fig.5.10., the binding data can be resolved by Scatchard analysis into two binding sites. The binding site with highest affinity ($K_d = 4 \times 10^{-6}M$) is present at a concentration of approximately 0.5 nmole/mg, the lower affinity site ($K_d = 1 \times 10^{-6}M$) is present at approximately 1.0 nmole/mg concentration. The high affinity site is completely abolished by the addition of ADP a finding which is in good agreement with the data on rat liver mitochondria (Vignais, Vignais, Lauquin and Morel, 1973). The high affinity binding site is however virtually non-existent in submitochondrial particles (Fig.5.11.), this result can be explained by the fact that ADP is a non penetrant inhibitor and submitochondrial particles are believed to be inside out vesicles. Consequently, the ATR cannot reach its binding site, moreover, it would appear that Braun shaker prepared mitochondria are also largely inside out vesicles (Fig.5.11.). in fact $^{3}\text{H}^{3}$-ATR binding to Braun shaker mitochondria appears to be variable, as sometimes the preparation does exhibit a proportion of the high affinity binding site. it is apparent that $^{3}\text{H}^{3}$-ATR binding could be used as a convenient probe for the functional integrity of mitochondrial preparations.

Binding of $^{3}\text{H}^{3}$-ATR to the $p^0v^0$ petite $\psi 3$ (Fig.5.12) reveals that the number of high affinity binding sites/mg of protein is markedly decreased (from 0.5 to 0.1 nmole/mg), however, the affinity is similar to
Binding assays were carried out as previously described in 0.5M sorbitol, 10mM Hepes-KOH, 1mM EDTA, pH 7.0 buffer. □— □, without ADP; □— □, with 200uM ADP; H.A.F = high affinity site; L.A.F = low affinity site.

Fig. 5.10. $^{3}H$-Atractyloside binding to snail enzyme prepared mitochondria.

Binding assays were carried out as previously described in 0.5M sorbitol, 10mM Hepes-KOH, 1mM EDTA, pH 7.0 buffer. □— □, without ADP; □— □, with 200uM ADP; H.A.F = high affinity site; L.A.F = low affinity site.
Fig. 5.11. $H^3$-Atractyloside binding to various mitochondrial preparations.

Binding assays were as described in Fig. 5.10. ○—○, snail enzyme prepared mitochondria; △—△, Braun shaker prepared mitochondria, □—□, submitochondrial particles.
Fig. 5.12. $^3$H-Atractyloside binding to a $^{30}O_p$ petite mutant.

Binding assays were carried out as in Fig. 5.10. ■■, no ADP;
△△, with 200μM ADP.
the wild type and it can still be removed by AW. Essentially similar results were obtained with the p°R petite. There is therefore no difference in the two types of petite, it is however, highly significant that the number of high affinity binding sites is decreased 5 fold in the petite phenotype. It could be argued that this is due to disruption of the petite mitochondria on preparation forming submitochondrial like vesicles. However, G Lauquin and P.V.Vignais (personal communication) have produced similar results with H3-ATR binding to petite mitochondria, moreover, H3-BA binding which is a penetrant inhibitor is also markedly reduced in the petite strain. Thus it would appear that petite mitochondria have a significantly diminished number of Adn translocase units (as demonstrated by H3-ATR binding) per mg of protein. The fact that the characteristics of the H3-ATR binding are similar to the wild type p+ strain would suggest that some component synthesized by the mitochondrial protein synthesizing system is necessary for the normal integration of the Adn translocase into the mitochondrial membrane.

v. H3-BA binding to yeast mitochondria.

A possible explanation for the decreased sensitivity to BA in the triethyl tin resistant mutant is that BA and triethyl tin bind to a common subunit of the OS-ATPase. If this hypothesis is true, one would expect that extraction of OS-ATPase with Triton X-100 would remove the

* This work was carried out in the laboratory of Dr.P.V.Vignais, Grenoble, France, I have subsequently confirmed the findings presented here since I returned to Warwick.
binding site from the membrane. In Fig. 5.13 and 5.14, $H^3$-BA binding has been carried out on a variety of mitochondrial preparations. It can be seen that there is a high affinity binding site for BA in both mitochondria and submitochondrial particles, there are approximately 40% less sites in mitochondria than in submitochondrial particles. However, the affinity for $H^3$-BA is 3 fold lower in the submitochondrial particles. The increased number of sites in the submitochondrial particles reflects the increased proportion of membrane components, the decreased affinity could be due to a conformational distortion of the binding site. This conclusion is not unlikely as Lauquin and Vignais (personal communication) have found that ADP which normally increases BA binding affinity in mitochondria does not significantly affect the binding affinity in submitochondrial particles. Extraction of the OS-ATPase does not markedly affect either the binding affinity or the number of sites. Moreover, equilibrium dialysis binding techniques revealed that there was no BA binding to the Triton X-100 extract. The conclusion is therefore that the BA binding site is not in the OS-ATPase complex. However, removal of $F_1$-ATPase and OSCP although not significantly affecting the number of sites, results in an approximately 10 fold increase in binding affinity. It would therefore appear that although $F_1$-ATPase and OSCP do not bind BA directly they have an interaction with the Adn translocase, a conclusion which is in agreement with the work of Vignais, Vignais and Doussiere (1975).

Conclusions

The biogenesis of the Adn translocase is at present under some controversy. Kovac and Hrusovska (1968) have shown that the nuclear mutant op^ has a
Fig. 5.13. Binding of $^3$H-BA to various mitochondrial preparations.

Binding assays were carried out as previously described for $^3$H-ATR binding assays. ●●, submitochondrial particles; , mitochondria; ○○, F$_1$-OSCP depleted particles; △△, Triton X-100 extracted membranes.
Fig. 5.14. Scatchard analysis of H$_2$-HA binding to various mitochondrial preparations.

The data from Fig. 5.13 were analysed by the method of Scatchard (1949). Submitochondrial particles, $K_d=1.2 \times 10^{-7}$ M and $n=0.5$ nmole/mg; mitochondria, $K_d=3.8 \times 10^{-8}$ M and $n=0.3$ nmole/mg; $F_1$-OSCP depleted particles, $K_d=2.5 \times 10^{-8}$ M and $n=0.4$ nmole/mg; Triton X-100 extracted membranes, $K_d=1.5 \times 10^{-7}$ M and $n=0.6$ nmole/mg.
vastly modified translocase. Also Kolarov et al. (1972) and Groot et al. (1975) have all produced evidence that the Adn translocase is preserved in petite mitochondria. These results would suggest that the biogenesis of the Adn translocase is nuclear coded and that synthesis takes place on the cytoribosomes. However, the data presented here on the triethyl tin resistant mutant would suggest a cytoplasmic involvement. A similar conclusion has been reached by Haslam et al. (1973) and Perkins et al. (1972) who reported that the presence of erythromycin or loss of mt DNA by mutation profoundly altered the Adn translocator. Additionally, the H^3-ATR data presented in this chapter indicates that the number of high affinity binding sites is markedly decreased. Attempts at producing translocase mutants by producing BA resistant mutants have led to the production of nuclear determined mutants (Perkins, Haslam, Klyce and Linnane, 1973) suggesting that the Adn translocase is nuclear coded. It is apparent that the nuclear genetic system plays an important role in the normal functioning of the Adn translocator. The role of the cytoplasmic synthesizing system is more difficult to understand. It may be that there is an interaction protein between the OS-ATPase complex and the Adn translocase subunits which is cytoplasmically inherited. Modification of this protein could therefore be reflected by a modified sensitivity to triethyl tin and BA. It is also apparent from the BA binding data in this chapter, that although the OS-ATPase does not contain the BA binding site it is in close association, such that modifications in OS-ATPase can result in modified binding parameters. This interaction phenomenon is obviously worthy of further experimental work, a good method of tackling this problem could be to follow the biogenesis of BA binding in standard anaerobic to aerobic and repressed to derepressed experiments.
CHAPTER 6.

General Discussion and Conclusions.

It is apparent from this thesis that trialkyl tin compounds are potent inhibitors of the mitochondrial ATPase complex (E.C.3.6.13). Using the triethyl tin analogue, the inhibitory site of action has been localised on the OS-ATPase complex as described by Tzagoloff et al. (1973). Moreover, the inhibitory site would appear to be located on the $F_o$ component of the complex, there is no inhibition whatsoever of the purified $F_1$-ATPase. The inhibition data shows that triethyl tin is an oligomycin like inhibitor, with inhibition characteristics in yeast similar to those exhibited in mammalian mitochondria.

$^{113}$Sn triethyl tin binding has shown definitively that there is a high affinity binding site of the following constants, $K_d = 0.5\mu M$ and $n = 1.7\text{n mole/mg}$. A comparison of the binding data and inhibition results shows that the high affinity site is of the correct order to account for the inhibition observed. A similar conclusion has been arrived at by Aldridge and coworkers in their studies on triethyl tin binding to rat liver mitochondria (Aldridge and Rose, 1969; Aldridge and Street, 1971; Rose and Aldridge, 1972.) who concluded that the inhibitory power of triethyl tin required a binding site in the order of $K_d = 0.1\mu M$. The only other binding site observed in these studies was of a much lower affinity and a much greater site concentration ($70 \text{n mole/mg}$). There seems little doubt that the high affinity binding site observed in this work is responsible for the inhibition of ATPase activity. The Triton
X-100 extraction results show that the major part of the binding sites are removed with the OS-ATPase, the extraction procedure does however leave approximately 30% of the high affinity binding sites in the membrane. A probable explanation for the unextracted sites, is that the extraction procedure does not completely remove all of the OS-ATPase.

An alternative explanation for the unextracted sites can be put forward, based upon the work of Aldridge and Street (1970). In their binding experiments they also investigated the binding of the less potent trimethyl tin to rat liver mitochondria, in the case of trimethyl tin, three classes of binding site were observed. The binding constants for trimethyl tin binding to rat liver mitochondria were $n_1 = 0.8\text{n mole/mg}$, $K_d = 70\mu M$; $n_2 = 120\text{n mole/mg}$, $K_d = 0.1\mu M$; $n_3 = 0.0042\text{n mole/mg}$, $K_d = 0.25\mu M$. The binding affinity of $n_1$ would be characteristic for the inhibitory site for trimethyl tin. The interesting thing is that there is a third site of a higher affinity than the site responsible for inhibition. This site was only identified because of the generally lower binding affinity exhibited by trimethyl tin, in the case of triethyl tin which has a much higher binding affinity, it would be experimentally difficult to identify the higher affinity site (ie if it were greater than 0.5μM). Consequently, the apparent single class of high affinity recorded in this work may reflect two classes of high affinity binding site. One class of sites would represent the interaction site for the inhibition of ATPase activity, this site is therefore extracted by Triton X-100 and is localised in the purified OS-ATPase. The nature and function of the other high affinity site may be reflected by the biochemical genetic experiments described in Chapter 5. Thus the decreased
sensitivity to triethyl tin and BA exhibited by D22/EC6 may be due to a modification in this higher affinity site. In this context it is worth noting that the high affinity site concentration for BA in the submitochondrial particles is around 0.5nmole/mg and that is equivalent to the concentration of triethyl tin sites after Triton X-100 extraction.

However, it is apparent that the inhibition of the purified OS-ATPase is due to the high affinity binding site found in equilibrium binding experiments. Moreover, this binding site is located on the F₀ component. The current knowledge on the structure and function of the OS-ATPase would point to the subunit 9 hexamer as the binding site for triethyl tin. Regardless of speculations as to the identity of the binding site, the stoichiometry of binding shows that there are 6 moles of binding site per mole of OS-ATPase.

The development of DBCT described in this work has produced some surprising results. DBCT has the properties of a site directed inhibitor which competes for the triethyl tin binding site. However, unlike triethyl tin it is a covalent inhibitor. Moreover, its properties are confined to the reactions in which the OS-ATPase or ATP synthetase complex participates. Thus it has no effect on pyruvate/malate respiration in coupled mitochondria or DNP stimulated respiration. Furthermore, the F₁-ATPase is unaffected, there therefore seems little doubt that DBCT is a specific covalent inhibitor of oxidative phosphorylation.

The binding and extraction experiments show a number of significant
facts:
1) DBCT covalently titrates a large 'pool' of components in the membrane at a concentration of 8-10nmole/mg.
2) This 'pool' of components appears to be a single species.
3) There are approximately 4 - 5 times more DBCT binding components than triethyl tin sites.
4) The purified OS-ATPase has a roughly equivalent number of DBCT sites and triethyl tin binding sites; moreover, antisera precipitation experiments reveal that a significant proportion of the binding sites are closely associated with the OS-ATPase.
5) The binding component is of low molecular weight and is also associated with the 45,000 M.W. subunit 9 hexamer.

A possible explanation for these results is that DBCT is titrating a mobile coupling factor which interacts with the OS-ATPase and respiratory chain. Evidence for this hypothesis has been produced in this laboratory since the work for this thesis was completed. Firstly, DBCT inhibition of ATP dependent reactions in beef heart and ATPase inhibition in beef heart and yeast can be specifically reversed by the action of reduced lipoic acid (Griffiths, Cain and Hyams, 1976), this action is not mimicked by a variety of SH reagents, in particular reduced lipoamide has no effect. This reversal will take place in particles specifically labelled with DBCT and then washed several times as described in Chapter 2. Thus the reversal is not a 'scavenging' effect, and indicates that the lipoic acid is replacing the component which has been inactivated by the DBCT. Even more important is the finding (Partis and Griffiths, unpublished results) that there is a
large pool of lipoic acid in the OS-ATPase at levels equivalent to the DBCT interaction sites. DBCT readily covalently reacts with lipoic acid and the DBCT-lipoate runs in T.L.C systems with the same R.F. value as the purified DBCT binding component (Partis, Cain and Griffiths, unpublished results). A structural analogue of lipoic acid, 8-methyl lipoic does not produce reversal of DBCT inhibition, more important it will inhibit the ATPase reaction (Hyams and Griffiths, unpublished results). It is apparent that lipoic acid plays an important role in the energy conservation reactions involved with the OS-ATPase. This has led to a new theory of oxidative phosphorylation (Griffiths, 1976), the essential details of which are shown in Fig.6.1. At the present time further work is being carried out in our laboratory to investigate this hypothesis.
Fig. 6.1. Role of lipoic acid in oxidative phosphorylation. This scheme is abstracted from Griffiths (1976).

1. Respiratory chain linked to lipoic acid pool via disulphide reductase.
2. Energy coupling factor (ECF) containing mobile pool of lipoic acid and X.
3. ATP synthetase (OS-ATPase) which contains the interaction sites for lipoic acid and X.
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