STUDIES ON LIPID INVOLVEMENT IN THE MECHANISM OF ENERGY COUPLING

by

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What in the midst lay but the Tower itself?
The round, squat turret, blind as the fool's heart,
Built of brown stone, without a counterpart
In the whole world. The tempest's mocking elf
Points to the shipman this the unseen shelf
He strikes on, only when the timbers start.

Not see? because of night perhaps? - why, day
Came back again for that! before it left,
The dying sunset kindled through a cleft:
The hills, like giants at a hunting, lay,
Chin upon hand, to see the game at bay,-
"Now stab and end the creature - to the heft! "

Not hear? when noise was everywhere! it tolled
Increasing like a bell. Names in my ears
Of all the lost adventurers my peers, -
How such a one was strong, and such was bold,
And such was fortunate, yet each of old
Lost, lost! one moment knelled the woe of years.

There they stood, ranged along the hillsides, met
To view the last of me, a living frame
For one more picture! in a sheet of flame
I saw them and I knew them all. And yet
Dauntless the slug-horn to my lips I set,
And blew.

'Childe Roland to the Dark Tower came'.

Ts'ui gathering together

Sui following

Chien development

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ABBREVIATIONS AND NOMENCLATURE

This thesis follows the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature. Non-standard abbreviations and nomenclature used in this thesis are defined below.

ATPase	adenosine triphosphatase, ATP phosphorylase EC 3.6.1.3
AMP-PNP	adenylyl imidodiphosphate
CCCP	carbonyl cyanide m-chlorophenyl hydrazone
DBCT	dibutylchloromethyltin chloride
DBT	dibutyltin dichloride
DCCD	dicyclohexylcarbodiimide
DNP	2,4-dinitrophenol
er	endoplasmic reticulum
esr	electron spin resonance
ETP_H	electron transport particles derived from heart mitochondria
FCCP	carbonyl cyanide p-trifluoromethoxyphenyl hydrazone
F_1	the hydrophilic catalytic portion of the mitochondrial ATPase involved in energy coupling
F_O	the hydrophilic membrane bound portion of the mitochondrial ATPase involved in energy coupling
$F_1.F_O$ ATPase	the isolated mitochondrial ATPase: sensitive to DCCD and/or oligomycin
$lip(SH)_2$	dihydrolipoic acid
lip(S-S)	oxidised lipoic acid α-DL 68 thioctic acid
NMR	nuclear magnetic resonance
OSCP	oligomycin sensitivity conferring protein
OS ATPase	oligomycin sensitive ATPase (from yeast)
PCA	perchloric acid
PCP	pentachlorophenol
p $lip(SH)_2$	a polymerised form of lipoic acid derived from dihydrolipoic acid
p lip(S-S)	a polymerised form of lipoic acid derived from α-DL 68 thioctic acid
SMP	submitochondrial particles
S-13	2',5'-dichloro-3-tert-butyl-4'-nitrosalicylanilide
TBT	tributyltin chloride
TCA	trichloroacetic acid
TET	triethyltin sulphate

$TF_1.F_O$	an F_1F_O ATPase derived from the thermophilic bacterium PS3
TPT	tripropyltin chloride
TTFB	4,5,6,7-tetrachloro-2'-trifluoromethylbenzimidazole
UQn	ubiquinone n
VE 2283	a complex organotin compound in which the tin is pentacovalent (see Appendix A)
1799	bishexafluoroacetonylacetone
'$\sim$'	the high energy state of the coupling membrane generated by electron transport or ATP hydrolysis in coupled systems

Δp	the chemiosmotic 'proton motive force'
$\Delta\tilde{\mu}H^+$	the chemiosmotic 'proton motive force'
$\Delta\psi$	the chemiosmotic osmotic membrane potential
$\Delta\psi'$	membrane potential including terms for fixed charges
$-Z\Delta pH$	$-RT \ln_e \Delta$ pH
P/O	ratio of moles phosphate esterified in ATP per mole O_2 consumed
H^+/O ratio	no. of H^+ ions ejected by the respiratory chain during the passage of $2e^-$ to O_2: substrate undefined
$H^+/2e^-$ ratio	no. of H^+ ions ejected by the respiratory chain during the passage of $2e^-$ through a number (undefined) of coupling sites
H^+/site ratio	no. of H^+ ions ejected by the respiratory chain during the passage of $2e^-$ through a (defined) coupling site
H^+/ATP_{enz} ratio	no. of H^+ ions translocated by the ATPase per ATP hydrolysed or synthesised
$H^+/ATP_{overall}$ ratio	no. of H^+ ions translocated by the ATPase and Pi and adenine nucleotide porters acting together during oxidative phosphorylation when the rate of ATP synthesis equals the extramitochondrial rate of ATP hydrolysis (i.e. in the steady state)
$H^+/\sim$ ratio	no. of H^+ ions translocated per 'high energy phosphate bond' equivalent: this is equal to H^+/site and $H^+/ATP_{overall}$
charge/$\sim$ ratio	no. of +ve charges translocated per high energy phosphate bond equivalent: this is equal to the charge/site ratio

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The results presented in this thesis were obtained between June, 1976 and October, 1978. The experiments described in Tables 3.3, 3.10-12, 5.4 and 5.7 were done in co-operation with R.L. Hyams in this laboratory, who also carried out the radioactive extractions described in Tables 3.4, 3.5, 5.9 and Figures 5.5-7. Those described in Tables 3.15-16 were co-experiments with Dr. M.D. Partis. Many of the experiments described in Chapters 7 and 8 were devised and carried out in co-operation with Dr. E. Bertoli. The ^{31}P NMR and mass spectrographic analysis of oleoyl phosphate described in Chapter 5 was carried out by M. Shabir.

SUMMARY

The interactions of derivatives of lipoic acid and unsaturated fatty acids with the mitochondrial energy coupling system was investigated. Derivatives of dihydrolipoic acid, oleoyl-S-lipoate and oleoyl phosphate were observed to stimulate ATP synthesis in a number of ATPase preparations derived from bovine heart and other mitochondria. This synthesis was sensitive to various inhibitors of oxidative phosphorylation such as oligomycin, efrapeptin, triorganotin halides and uncoupling agents.

A derivative of dihydrolipoic acid also stimulated an energy-linked transhydrogenase reaction in bovine heart SMP. This reaction has features in common with both ATP and respiration-driven transhydrogenase.

All these reactions were observed at a low frequency. The reasons for this were investigated as were the precise natures of the compounds stimulating ATP synthesis. No clear answers were obtained to either question, although suggestions were made.

The role of ubiquinone in energy coupling was also investigated, using solvent extraction studies and a low molecular weight homologue of ubiquinone, ubiquinone 3. These studies suggest that ubiquinone may have a role in energy coupling reactions other than that expected from its well-know role as a respiratory chain redox carrier, possibly in the control of various ATPase-linked reactions, e.g. ATP synthesis, ATP-Pi exchange, ATP-driven transhydrogenase.

The results presented here describing 'dihydrolipoate' driven ATP synthesis (tables 3.1-3.19 & fig.3.2) and oleoyl phosphate driven ATP synthesis and ATP-Pi exchange (tables 5.3-5.10 & figs.5.1-5.7) were most of those obtained showing these reactions over a two year period and represent ∿ 1% of the total number of attempts to demonstrate them (see pp 88-89,212-213).

The remaining results presented in this thesis are usually representative of 2-6 experiments performed in duplicate.

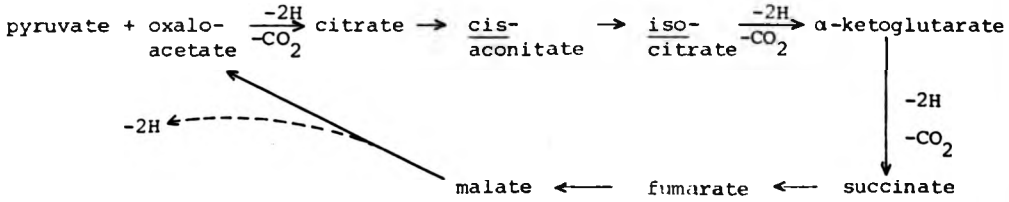
CHAPTER 1

COMPONENTS OF THE RESPIRATORY CHAIN

The major source of energy used for metabolic processes in cells comes from the oxidation of low molecular weight carbon compounds by the enzymes of the tricarboxylic acid cycle (TCA) cycle). The final steps of this oxidative process are the transport of reducing equivalents (electrons & hydrogen) via a series of carriers leading to the reduction of molecular oxygen to water. This releases large amounts of free energy which can be used to condense phosphate (Pi) into adenosine triphosphate (ATP). This energy is released upon hydrolysis of ATP to adenosine diphosphate (ADP). ATP thus becomes the energy currency of the cell. This oxidative process has been termed 'oxidative phosphorylation'. A full historical survey of the origins of the study of oxidative phosphorylation is not within the scope of this introduction; however, some outline of the early work involved in the recognition and elucidation of this phenomenon is required to understand recent work in the area. For further reviews see (21-26).

The study of respiration and oxidative phosphorylation perhaps began in 1913 when Warburg found that respiration was associated with insoluble granular elements of cellular structure that could be recovered by filtration of tissue dispersions (1). These insoluble elements were later termed mitochondria. The major part of the groundwork prerequisite to the study of oxidative phosphorylation was carried out in the 1930's. The recognition that oxidation and phosphorylation might be linked in some way was made by Engelhardt in 1930, who showed that the esterification of Pi by lysed erythrocytes occurred during respiration (2,3). Lohmann had, in 1931, discovered ATP in muscle, but thought it peculiar to this tissue (4). Its real significance was not realised until 1937-8 when Warburg and Meyerhof showed that ATP formation

was linked to the oxidation of glyceraldehyde-3-phosphate and phosphopyruvate. Hans Krebs, working on previous suggestions and observations of Szent-Gyorgyi and Martius and Knoop proposed that pyruvate could be oxidised to 3 molecules of CO_2 by a cycle of reactions, later known as the



tricarboxylic acid, citric acid or Krebs cycle.

Lehninger later showed that the TCA cycle was the main pathway for the oxidation C_2 units derived from the breakdown of fatty acids by the ß-oxidation pathway. Bretscher later demonstrated that C_2 units derived from the catabolism of ketone bodies are also oxidised via the cycle. The TCA cycle then occupies a cardinal position in cellular oxidative processes.

In 1937, Kalckar showed an oxygen dependent phosphorylation of glucose, glycerol and adenosine monophosphate (AMP) during the oxidation of citrate and other substrates in kidney homogenates (5). The oxygen dependency of this phosphorylation precluded it occurring by glycolysis. He later demonstrated that respiration dependent ATP synthesis could take place in cell free tissue preparations (6). Quantitative evaluations of the amount of Pi esterified in ATP per oxygen consumed (P:O ratios) were first made by Belitzer and Tsibakowa, working on muscle (7) and Ochoa working with cell free brain and heart preparations (8-10), who reported P:O ratios of 2-3:1. They proposed that phosphorylation occurred not only during the dehydrogenation of substrate (substrate level phosphorylation) but also during the transport of hydrogen (or electrons) to water. Ochoa recognised that the presence of ATPases would interfere with the determination of P:O ratios; to minimise

this he used an assay in which the concentration of ATP was kept constant by an enzymic regenerating system. With this system, average P:O ratios of 3:1 were obtained for the complete oxidation of pyruvate to CO_2 + H_2O. Further investigation of these reactions required the isolation of functional, intact mitochondria.

Early attempts to isolate mitochondria by differential centrifugation of tissue homogenates had failed because the mitochondria obtained were extensively osmolysed (11). Intact functional mitochondria were eventually obtained by Hogeboom, Schneider and Palade in 1948 (12). This led to the demonstration by Friedkin and Lehninger that mitochondrial phosphorylation was coupled to the oxidation of reduced nicotinamide adenine dinucleotide (NADH) by the respiratory chain (13). The P:O ratios obtained though were low, due to the relative impermeability of mitochondria to NADH. Lehninger (14) and Jacob and Sanadi (15) later demonstrated that, after treatment of mitochondria with hypotonic salt solutions, NADH enters easily and P:O ratios up to 2.6:1 could be obtained. Isolation of mitochondria led to the demonstration that most cell fatty acid oxidase activity and TCA cycle activity of rat liver tissue was localised in the mitochondria, whereas none of its glycolytic activity was (32).

Friedkin and Lehninger also demonstrated that the oxidation of ascorbate via cytochrome c in rat liver mitochondria led to the esterification of ^{32}Pi into ATP (13). This was later confirmed by Judah, using non-radiolabelled Pi, who obtained P:O ratios close to 1 (6). Maley and Lardy found that adrenalin or 3,4-dihydroxyphenylalanine could replace ascorbate (17). Lehninger (14) and Slater (18) demonstrated that chemically reduced external cytochrome c was oxidised by mitochondria and that this oxidation was coupled to ATP synthesis in a reaction inhibited by a known inhibitor (uncoupler, see Chapter 2 and Appendix A) of oxidative phosphorylation 2,4-dinitrophenol (DNP). P:O ratios of

0.5 - 0.8 were obtained. These studies confirmed that there was a phsophorylation 'site' between cytochrome c and oxygen. This site was termed site 3. Sites 1 and 2 were also identified. Copenhaver and Lardy, using a mitochondria preparation with electron transport blocked by the inhibitor antimycin A (see Appendix A), showed that phosphorylation driven by NADH could still occur if ferricyanide was used as an electron acceptor (19). P:$Fe(CN)_6^{3-}$ ratios of 1 were obtained. The region of the respiratory chain between NADH and $Fe(CN)_6^{3-}$ was said to contain 'site 1'. Slater obtained P:O ratios of 1 and 2 with succinate and β-hydroxy butyrate (respectively) if cytochrome c was used as an electron acceptor. The region succinate → cyt c contains 'site 2'. We can turn now from an historical survey to a description of the known structure and functions of mitochondria.

Mitochondria (mitochondrion singular) are intracellular organelles with a characteristic double membrane structure. The outer membrane is separated from the inner by a well defined 'intermembrane space'. The inner membrane is folded into cristae, dramatically increasing its surface area. The number and size of these cristae is dependent on the respiratory activity of the tissue containing the mitochondria. In tissues of high respiratory activity, e.g. myocardium, insect flight muscle, avian salt gland, the cristae are large and densely packed, whereas in liver they are smaller and less densely packed. The inner matrix space, that bounded by the inner membrane, varies in volume with its ionic content, and as a result the inner membrane occurs in various configurations. These have been described as 'orthodox','condensed', 'energised' and 'energy twisted', but these terms are only generalisations of types. Cristae can and do adopt a bewildering array of shapes, ranging from simple fingerlike projections to complex paracrystalline arrays that appear to occupy the whole matrix space. Attempts have been made to relate changes in cristal structure to mitochondrial respiratory

states. This has led to certain proposals about the nature of energy coupling in mitochondria (27-29) (see Chapter 2).

The number and morphology of mitochondria in each cell is highly variable. Yeast cells appear to contain about 10 mitochondria, mammalian cells, several hundred, while the giant amoeba, Chaos chaos, contains 500,000. These mitochondria appear in electron micrographs to vary somewhat in shape and size but they are roughly cylindrical with axes of 1.5 μm and 0.5 μm and volumes of $\sim$ 0.8 μm^3. Isolated preparations of mitochondria certainly have these dimensions but these appear different to mitochondria observed in situ. Using phase contrast light microscopy, Shelton has observed mitochondria in cultured fibroblasts and noticed that over a few hours they adopt an array of shapes. Mitochondria would appear to rutpure and reform into circles, V and Y shapes and many other filamentous forms (30). Many similar observations on other tissues have been made (24). This suggests that in vitro mitochondria do not possess this fixed cylindrical morphology.

Recent work by Skulachev and co-workers (33) and Davison and Garland (34) has gone some way to reconcile the electron and light microscopical views of mitochondrial shape. They have demonstrated by serial sectioning of tissues that apparently isolated mitochondria form weaving interlinked reticula through the cells. The more metabolically active the cells, the more complex the reticulum. These heart cells contain not several hundred mitochondria but a few (possibly one) giant reticulate mitochondria. It is salutory to reflect, then, that most work on mitochondria has been done on severely disrupted systems. Certain authors have suggested that this complex structure might impose restrictions on the mechanisms of energy conservation in such systems (98).

The inner and outer mitochondrial membranes are quite distinct structurally and functionally, the former being the most complex and metabolically active. The outer membrane is usually isolated from the

inner by its rupture, followed by differential centrifugation. Parsons et al. (36,24) used a method which involved large amplitude mitochondrial swelling in a phosphate, bovine serum albumin buffer system. This gave an outermembrane preparation selectively enriched in cytochrome b_5. This was later shown to be not due to contamination by smooth endoplasmic reticulum (er),which also contains b_5,as the b_5 species are electrophoretically distinct (38). Sotocasa et al. isolated outer membrane from mitochondria subjected to shrinking swelling cycles followed by sonication. This membrane fraction contained 90% of the mitochondrial rotenone insensitive NADH-cyt c oxidase activity (39). Schnaitman et al. concluded that monoamine oxidase and kynurenine hydroxylase were also concentrated in the outer membrane (40). Monoamine oxidase activity is frequently used as a marker for the outer membrane.

The outer membrane is freely permeable to water, low molecular weight solutes (e.g. sucrose) ions and also some compounds of considerably higher M.W. such as ferritin and various dextrans.

Electron microscopy has revealed that the outer membrane is studded with spherical substructures (41); projections from the membrane have not been generally observed (42), cf. inner membrane, but see (43). Its general structure appears to be rather similar to that of smooth er and there have been suggestions that the two are contiguous in vivo. Parsons has reported that the outer membrane contains hollow cylinders 60 Å tall and 60 Å wide in a regular array with centre to centre spacing of 80 Å (44), but this may be due to artefacts produced by the specific staining technique used,as other workers have not observed this structure.

The inner membrane is very different to the outer, not only in the range and type of enzymic functions contained within it but in its protein lipid ratio (3-4:1 in bovine heart compared to 1:3 for outer

membrane and smooth er from the same tissue). Mitochondria from other tissues, e.g. liver, have lower ratios but still higher than those of the outer membrane and smooth er.

Much presumptive evidence had accumulated in the 1940's, supporting the idea that the enzymes of the respiratory chain were contained only in the inner membrane (45-47). It was not till 1963, however, that definitive work by Klingenberg (24) and Vogel (24) established this.

The inner membrane is now known to contain many of the enzymes of the TCA cycle; the enzymes of the respiratory chain, the ATP synthase and related activities; specific transport systems for ions and metabolites, e.g. Na^+, K^+, dicarboxylic acids, phosphate, adenine nucleotides, and protons. The inner membrane is highly impermeable to most of these compounds, and these specific transport systems allow the vectorial transport of them to take place.

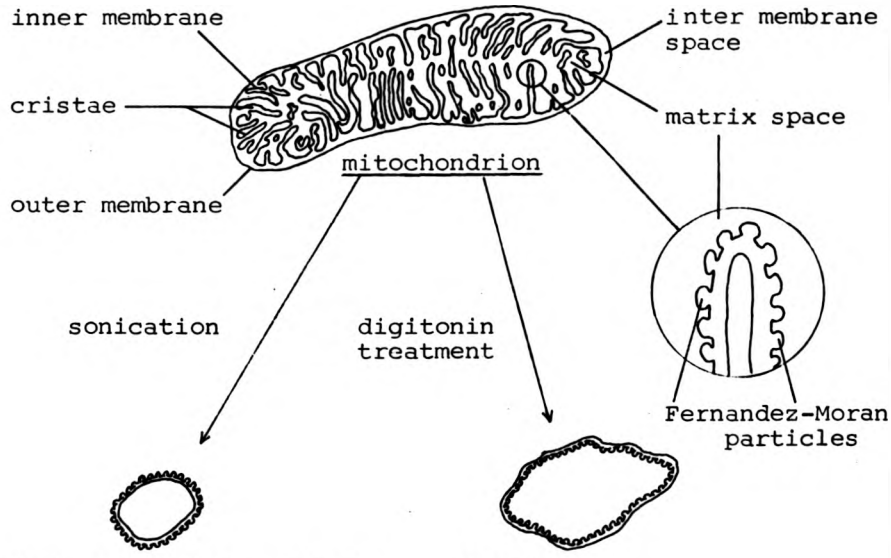
The enzymes on or in the inner membrane have been classified into three types (24) These are (a) those dissociable only by surfactants, These are firmly bound and include cytochrome bc_1 complex, succinate dehydrogenase, NADH dehydrogenase and parts of the ATPase. (b) Those dissociable by sonication. These are considered 'tightly enclosed' and include NADPH dehydrogenase, malate, isocitrate, glutamate and lipoate dehydrogenase, various transaminases and phosphate transferases and parts of the ATPase. (c) Those dissociable ionically in a hypotonic solution. These are loosely bound and include 'secondary phosphate transferases', adenylate kinase, creatine kinase, an ATP-ADP exchangease and cytochrome c: many of the enzymes in this group may be of matrix space origin.

Proteins are distributed asymmetrically across the inner membrane this distribution is considered important to various theories of energy conservation. This is dealt with in Chapter 2.

The inner membrane is usually isolated as submitochondrial particles

(SMP) or electron transport particles (ETP) from sonicated mitochondria or as mitoplasts made by stripping away the outer membrane with digitonin.

Fig. 1.1 Mitochondria, submitochondrial particles and mitoplasts



submitochondrial particle.

Sonication shears off the outer membrane and disrupts the inner which reseals into smaller vesicles, mainly with an inside-out morphology.

mitoplast.

Digitonin disrupts the outer membrane which can be removed from the largely intact inner by differential centrifugation leaving vesicles of right side out morphology.

These various particulate preparations vary somewhat in protein constitution, mainly because of the different preparation methods used (see (51) for review).

Electron microscopy has not given a clear picture of inner membrane structure. Fernández-Moran observed arrays of spherical or polyhedral particles protruding from the inner membrane (52). These were occasionally attached to the membrane by a thin stalk. The size of the substructures was variable but was usually ∿ 150 Å in diameter. This would give cristae a minimum thickness of 300 Å. The particles were observed in cristal fragments, negatively stained with phosphotungstate.

They were not observable in mitochondria stained *in situ* which appear to contain spherical substructures embedded in the inner membrane. Later electron microscopical studies led Fernández-Moran *etal*. to conclude that cristae were composed of thousands of these substructures, termed electron transport particles (53-4). These were composed of three parts (a) spherical or polyhedral headpiece, 80-100 Å diameter; (b) a cylindrical stalk 50 Å long, 30-40 Å diameter; (c) a base piece, dimensions 40-110 Å. The isolated particle appeared in electron micrographs to be a studded prolate ellipsoid of short axis 120 Å and long, 180 Å. Although similar particles have been observed in mitochondria from many tissues (24, 44), many workers suggest that they are artefacts produced by the staining techniques used for visualisation under electron microscopy. Indeed, Fleischer *et al*. (55) made microdensitometer tracings of cristae from intact mitochondria which gave them a thickness of 48.8 Å ± 6.5 Å (300 Å would be the minimum expected for two opposing arrays of electron transport particles). Attempts have been made, however, to identify the function of the spherical headpieces. Racker *et al*. found that urea treated bovine heart SMP lost ATPase activity and the phosphotungstate visualisable headpiece particles, suggesting that they were the seat of ATPase activity (56). Subsequently, Kagawa and Racker demonstrated that addition of ATPase to salt inactivated SMP led to the restoration of the particles in electron micrographs (57). This view has been challenged by Green, who claimed that if urea was removed from urea treated SMP by dialysis, both ATPase activity and the particles reappeared without the need for any addition of free ATPase.

The structure along cristal membranes is little studied now as most structural investigations have been devoted to transmembrane protein distributions. The 2D structure is considered to be of prime importance to the mechanism of energy transduction by Green in his

'paired moving charge' theory (59).

The mitochondrial matrix space possesses a distinct enzyme population including some TCA cycle enzymes and accessory systems, L-glutamate dehydrogenase, certain transaminases linking carbohydrate and protein metabolism to the TCA cycle and the enzymes of the β-oxidation sequence for fatty acid breakdown. Hackenbrock has calculated that the proteins in the matirx space are in a 56% solution (60), that is, they cannot be considered to be in true aqueous solution. On the basis of this and electron microscopical studies, Hackenbrock has suggested that the matrix protein is organised into an ultra-structural reticulate network, continuous with the inner membrane. Matrix protein may be as much as 67% of the total mitochondrial protein (61).

The intermembrane or intracristal space contains about 7% of mitochondrial protein, and possibly most of the mitochondrial adenylate kinase activity (61).

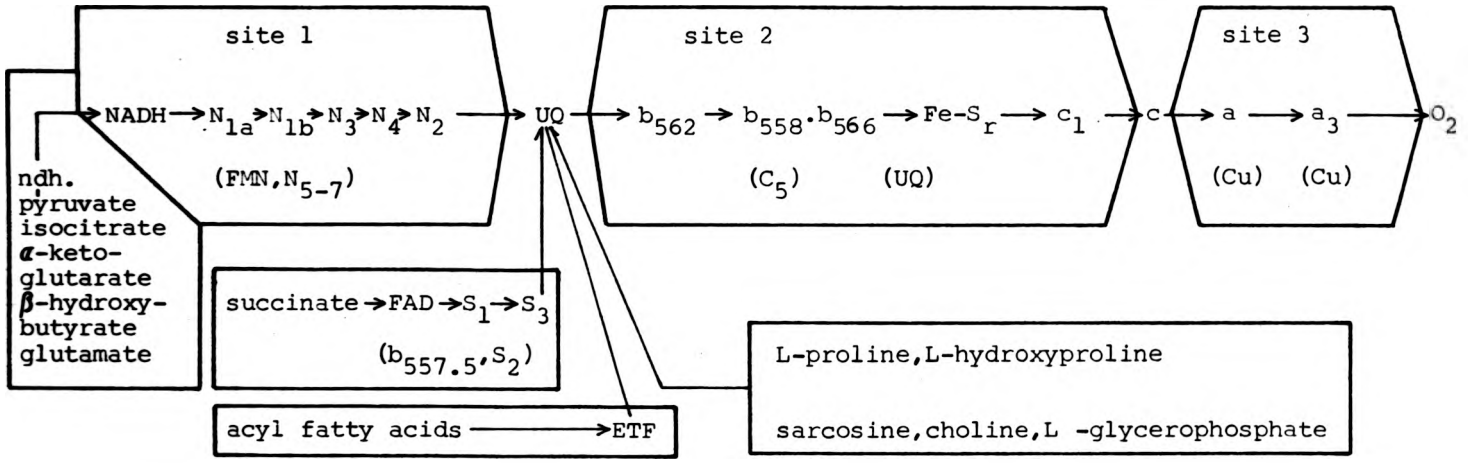
The respiratory chain

This is a series of inner membrane associated redox carriers directly responsible for catalysing the oxidation of various metabolites by molecular oxygen. This, however, is not its only function as it is, with certain other enzymic groupings (e.g. ATP synthetase, energy linked transhydrogenase) responsible for the transduction of free energy released by these redox reactions into other forms, notably ATP, ion gradients, conformational changes of inner membrane proteins and a high energy state of the membrane '∿' (see Chapter 2).

The respiratory chain is a complex array of proteins, lipids and active metallic groupings (see Fig. 1.2).

The bovine heart mitochondrial respiratory chain was fractionated into four fractional complexes by the pioneering work of Hatefi et al. (83, 84), viz.

Fig 1.2. The Bovine Heart Mitochondrial Respiratory Chain.



N_{1-7} Fe-S centres of complex I
S_{1-3} " " " " II
Fe-S_r 'Rieske' Fe-S centre, complex III
C_5 Fe-S " " III
ETF Electron transferring flavoprotein
ndh. NADH linked substrate dehydrogenases

Components represented in brackets are those whose position, or involvement in, the respiratory chain is not yet defined.

Complex I NADH ubiquinone reductase: $NADH + UQ + H^+ \rightarrow NAD + UQH_2$

Complex II succinate ubiquinone reductase: $succinate + UQ \rightarrow fumarate + UQH_2$

Complex III UQH_2-cytochrome c reductase: $UQH_2 + 2ferricytochrome\ c \rightarrow UQ + 2ferrocytochrome\ c$

Complex IV cytochrome(c) oxidase: $2ferrocytochrome\ c + 2H^+ + \frac{1}{2}O_2 \rightarrow 2\ ferricytochrome\ c + H_2O$

The individual complexes will be dealt with in greater detail later in this chapter, but it will first be worth considering some of their chemical components separately.

The cytochromes

These were first discovered by a Scottish physician, C.A. MacMunn, in 1886 who had noticed the widespread distribution of two coloured pigments, histohaematin and myohaematin in tissues by hand spectroscopy (63-4). As the names suggest, he thought they contained haem. He assigned them a respiratory function connected with oxygen transfer. His work was, though, ignored for 20 years, mainly because of criticism by the chemist Hoppe-Seyler. The 'rediscovery' of cytochromes was largely due to the work of Keilin. Keilin had noticed the apparently universal occurrence of a pigment, which he termed cytochrome, in insect tissues (65). This pigment exhibited reversible redox changes as part of its function. He later demonstrated that 'cytochrome' consisted of three spectroscopically distinct components, a, b, c (67). He also obtained evidence that cytochrome b was closest to the substrate side of the reactions mediated through it, and that there existed an entity sensitive to CO, CN^- and H_2S that linked cytochromes to oxygen. He termed this 'cytochrome oxidase' and suggested that it contained copper. By 1940, Keilin had shown that cytochrome oxidase was identical to or contained cyt a_3, that the cytochromes probably acted as a linear chain of electron carriers and that the terminal portion of this chain consisted of cyt c and cytochrome oxidase (68). Warburg had

earlier identified a CO sensitive 'Atmungsferment', later shown to be identical with cytochrome oxidase (69).

Detailed analysis of the cytochromes owes much to the work of Britton Chance and associates, who developed the techniques of dual beam and dual wavelength spectroscopy (70-1). Careful analysis of mitochondrial absorption spectra has permitted the detection of interactions between cytochromes, cytochromes and copper ions, cytochromes and energy transfer inhibitors (73) and 'high' and 'low' energy forms of cytochromes (74).

Mitochondria contain other components having characteristic absorption spectra. These include flavoproteins, nonhaem iron proteins, ubiquinone and copper ions (75-6).

Other techniques have also proved useful in identifying the redox carriers of the respiratory chain. These include electron spin resonance spectroscopy, particularly useful in the study of Fe-S centres and also cytochromes, copper ions and flavoproteins (80). Low temperature esr studies have revealed several 'new' redox active Fe-S centres in the respiratory chain (81).

The kinetic characteristics of redox centres possibly involved in the chain has been investigated by careful titrations of these with oxygen, revealing at least seven redox centres on the oxygen side of the antimycin A block (82).

The cytochromes consist of a haem-based prosthetic group linked to an apoprotein moiety. The haem centres are the redox active electron transferring groups. Most have their haem Fe as a low spin hexaco-ordinate complex, two co-ordinate bonds being donated by protein cysteine-SH groups adding across the vinyl groups of the haem.

The mammalian respiratory chain contains three main types of cytochrome, b, c and a.

The b group of cytochromes are characterised by relatively low potentials, suitable for their roles as substrate,and dehydrogenase proximal components of the electron transfer chain. The haems are hexa-co-ordinated and will not react with O_2,CO or CN^-. Most authors agree that there are at least 3 b type cytochromes in the respiratory chain, one in complex II and two in complex III (77-9).

The c type cytochromes are generally central electron carriers lying between the b type cytochromes and the terminal oxidases in redox potential. Mammalian respiratory chains usually contain two,c_1 and c. One, c, is usually freely diffusible in the membrane and is easily extractable. The other is more firmly embedded in the matrix of the membrane. Yeast mitochondria possess two cyt c species (as well as c_1), iso-1 (major) and iso-2 (minor), whose proteins differ in 22 amino acids.

The a type cytochromes are involved in the terminal oxidase of the respiratory chain. Mammalian systems have two, a and a_3. The differences between these are unclear. Cytochrome a_3 has a penta-co-ordinated haem, as one ligand is required to react with oxygen. Cyanide, N_3^-, H_2S and CO can also react with this.

Iron sulphur centres (nonhaem iron centres)

Submitochondrial particles contain ∿ 8 x more nonhaem iron than haem (cytochrome) iron (24). Most of this iron is associated with labile sulphur atoms. These nonhaem irons are now known as iron-sulphur centres (Fe-S centres) and can be defined as protein prosthetic groups having 2-4 Fe atoms complexed with 2-4 atoms of inorganic sulphur and 2-4 sulphur atoms from cysteine. These iron sulphur proteins often contain more than one Fe-S centre. These can occupy a wide range of redox potentials. Each centre can take up 1 e^- and will then exhibit electron spin resonance. Esr spectroscopy has been the major research tool used to elucidate the function of these components in the respiratory chain.

The respiratory chain contains at least 9 Fe-S centres most associated with the early steps of NADH and succinate oxidation (81).

Complex I contains 4 well defined Fe-S centres, 1, 2, 3 and 4 (86-7). All appear to be kinetically competent to participate in electron transport between NADH and ubiquinone. Centre 1 has been split into two components, a and b (81).

Fe-S centre	1	2	3	4
E_m value. component a	-380±20mV	-20±20mV	-240±20mV	-410±20mV
component b	-240±20mV			

The redox potentials for centres 2 and 3 reported by Onishi (81) (see above) have been disputed by Beinert and co-workers (87). The kinetic sequence of reduction by NADH appears to be 1, then 3 and 4, then 2.

Three Fe-S centres have been identified as being associated with succinate dehydrogenase, S-1, S-2, S-3, of redox potential O, -400 mV, + 60 mV (± 10 mV) respectively (88-9). S-1 and S-3 have been kinetically implicated in electron transport between succinate and UQ; the function of S-2 (not reduced by succinate) is unknown.

Complex III appears to contain one Fe-S centre associated with a 26,000 M.W. protein of redox potential + 220 mV. It is not clear whether it is an obligatory electron carrier but recent reports suggest that it is (90).

Two new Fe-S flavoproteins have been identified by Ruzicka and Beinert (150) and by Hatefi and coworkers (84). The former has been implicated in electron transport processes between the ß-oxidation of fatty acids and the respiratory chain, and may be identical to the centre-S described by Onishi (91). The latter, which copurifies with complex V ATPase has, as yet, no described function and may be identical with the former preparation; both appear to contain FAD as prosthetic groups.

Flavoproteins

The respiratory chain contains other well defined flavoproteins, notably those associated with the NADH and succinate dehydrogenases (see sections on complexes I and II).

Lipids

Although mitochondria generally have lower lipid:protein ratios than other cellular membrane assemblies, lipids still play an essential role in their structure and function. Indeed, one (ubiquinone) is an essential redox component of the respiratory chain. Mitochondria from different tissues have broadly similar lipid compositions. Liver mitochondria are 21-30% lipid dry weight, 90% of which is phospholipid. Heart mitochondria are similar, pig being 28% lipid, 91% of which is phospholipid (93); ox being ∿ 26% lipid, 88% of which is phospholipid (94).

The lipid composition of inner and outer membranes differs widely, particularly in terms of ubiquinone (entirely localised in the inner membrane) and phospholipid content.

Table 1.1 Phospholipid composition of inner and outer mitochondrial membranes

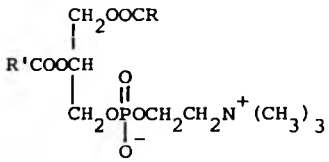
phospholipid	mitochondria	inner membrane	outer membrane	microsomes
% phospholipid (wt. of total dry wt.)	14.4	21.4	45.1	28.0
cardiolipin	22.5	21.5	3.2	0.5
phosphatidyl inositol	7.0	4.2	13.5	13.4
phosphatidyl serine	ND	ND	ND	4.5
phosphatidyl ethanolamine	28.4	27.7	25.3	18.3
phosphatidyl choline	40.0	44.5	55.2	62.8
unidentified	2.3	2.2	2.5	1.1

Data modified after (92).

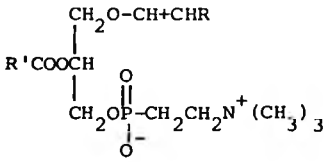
The similarity in composition between outer membrane and microsomes has led to the suggestion that the former may have a microsomal origin (92); indeed, continuity between them has been observed in goldfish brain mitochondria (92).

Phospholipids

Lecithin is the predominant mitochondrial phospholipid, comprising up to 40% of total phospholipid. Heart and liver lecithin contain large amounts of lecithin plasmalogen; its function is unknown.

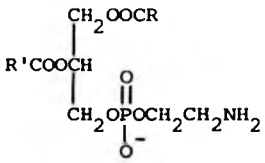


lecithin (phosphatidyl choline)

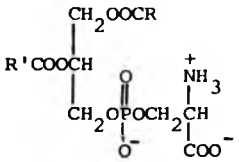


lecithin plasmalogen (phosphatidal choline)

Phosphatidyl serine and ethanolamine comprise 30% of mitochondrial phospholipid; much of this may be in the plasmalogen form (95).

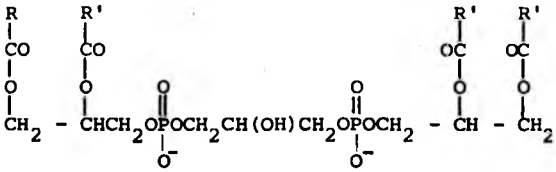


phosphatidyl ethanolamine



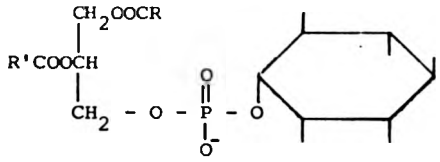
phosphatidyl serine

Mitochondria contain an unique phospholipid, cardiolipin, which can comprise as much as 20% of mitochondrial phospholipid; it is almost entirely localised in the inner membrane.



Cardiolipin. RR'R"R"' are fatty acyl groups, usually unsaturated.

The other major mitochondrial phospholipid is phosphatidyl inositol. It is mainly found in the outer membrane, and can compose as much as 15% of mitochondrial phospholipid (99).



phosphatidyl inositol

Mitochondrial phospholipids are rich in unsaturated fatty acids, particularly oleic, linoleic, arachidonic and docosahexaenoic. At least 55-65% of all mitochondrial fatty acids contain one double bond, while as much as 40-50% contain 2 or more.

Research has clearly demonstrated that phospholipids are essential to many mitochondrial functions.

The particulate succinate dehydrogenase isolated by Zeigler and Doeg (101) contains 20% w/w phospholipid which is not required if UQ 2 is used as electron acceptor, but is if UQ 10 is used. This is probably due to the insolubility of UQ 10 in aqueous systems. Acetone extraction will remove 85% of this phospholipid leading to an inactivation of the enzyme (102). Phospholipids will restore activity. Phosphatidyl inositol, phosphatidyl ethanolamine, cardiolipin and Tween 80 (a nonionic detergent) restore ∿ 50% activity, while lecithin restores only ∿ 25% (103). The residual activity of succinate dehydrogenase after acetone extraction is not inhibited by theonyltrifluoroacetone (TTFA), an inhibitor of the native enzyme. Readdition of phospholipids restored sensitivity to TTFA.

A phospholipid requirement for NADH dehydrogenase activity was first demonstrated by Fleischer et al. (104) who found that phospholipase A treatment inhibited NADH-UQ reductase activity. This activity could be restored by phospholipids. These results were extended by Machinist and Singer who showed that most bovine heart SMP NADH-UQ reductase activity could be removed by short incubations with low levels of phospholipase A (105). This inactivation could be reversed

by phospholipids (except cardiolipin cf. to succinate dehydrogenase). Prolonged phospholipase A treatment led to an irreversible inactivation of the dehydrogenase and its solubilisation from the membrane. This has led to the suggestion that phospholipid is important in the attachment of the dehydrogenase to the rest of the respiratory chain. Awasthi et al. have shown it to be necessary to hydrolyse cardiolipin to release the dehydrogenase.

Preparations of cytochrome oxidase consist of 10-33% phospholipid (w/w). Cardiolipin seems to be concentrated in cytochrome oxidase, especially in the 'tightly bound' lipid fraction, of which it may be ∿ 70% w/w. Greenlees and Wainio demonstrated that phospholipids would restore activity to deoxycholate delipidated cytochrome oxidase preparations; phosphatidyl serine was the most effective (107). Acetone extraction also inactivates the enzyme; phospholipid restores the activity. Both lysolecithin (108) and phosphatidyl ethanolamine (102) have been reported to be the most effective in restoring activity. Phospholipase A treatment seems to have varying effects on cytochrome oxidase activity, depending on the source of the enzyme, inhibiting that of a rat brain homogenate (109) while having no effect on rat liver mitochondrial activity (110). Cytochrome oxidase has been prepared depleted in phospholipids, notably by the methods of Tzagoloff and MacLennan (111) and Crane and Sun (92). Both preparations have low activity in the absence of phospholipids which restore it on readdition with little specificity for phospholipid. These methods do not remove the 'tightly bound' cardiolipin, removal of which completely inactivates the enzyme. Maximal activity is only restored to the oxidase if it and phospholipid are mixed in high concentration, suggesting particle-particle interaction is required for maximal activity. Similar observations have been reported for complexes I, II and III (92). Phospholipid appears to be required for the interaction

between oxidase and cyt c. Morrison et al. have suggested that the true substrate for the enzyme is not cyt c but a phospholipid-cyt c complex (113).

Energy linked processes and phospholipids

It is not known if phospholipids play any direct role in energy conservation but there are indications that the ATPase requires phospholipids for maximal activity (116). The P:O ratio in ageing rat liver mitochondria decreases with the breakdown of membrane phospholipids, probably due to the uncoupling effect of the free fatty acids released by this breakdown (114-5). Vignais et al. have reported that phosphatidyl inositol was specifically associated with a KCl soluble protein factor required for the ATP-driven contraction of aged rat liver mitochondria (117). The requirement for phospholipid was absolutely specific for cofactor-like levels of phosphatidyl inositol.

Neutral lipids

The bulk of the remaining mitochondrial lipid is loosely classified as neutral lipid and consists mainly of neutral fats, triglycerides, ubiquinone, cholesterol and lower amounts of tocopherol and ubichromenal. These comprise about 10% of total mitochondrial lipids.

Cholesterol

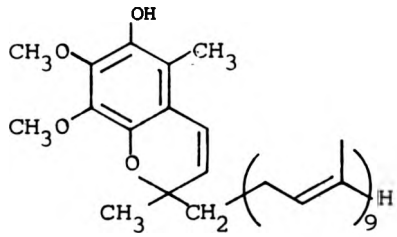
Cholesterol is a well known membrane component; although its function is not clear it has been suggested that it acts to stabilise phospholipid bilayers (100). This requires equimolar amounts of phospholipid and cholesterol, however, so it is unlikely to be its role in mitochondria. It does not appear to be required for electron transport. Digitonin fragmentation of mitochondria is considered to be due to its affinity for cholesterol (118). Cholesterol is found in higher concentration in the outer rather than the inner membrane (hence digitonins action).

Ubiquinone

The ubiquinones are a series of 2,3-dimethoxy-5-methyl-6-isoprenyl benzoquinones that can undergo redox reactions. They are essential members of the respiratory chain and lie either between the dehydrogenases and the b type cytochromes or between cyt b and cyt c_1 (79, 120, 121). A more detailed description of the ubiquinones and their function can be found in the introduction to Chapter 7.

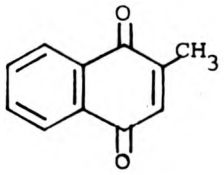
Ubichromenal

This is a cyclised derivative of UQ 10, consistently found in small amounts in mitochondria (125). It does not appear to have any role in electron transport or energy coupling and is probably merely a breakdown product of ubiquinone.

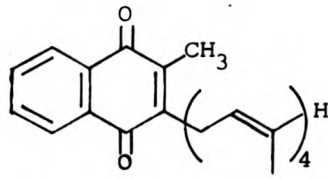


Vitamin K, menaquinone

There is conflicting evidence as to whether mitochondria contain vitamin K. Bioassays have given both a positive (126) (60 μmoles K/mg mitochondria protein) and a negative response (127).



menadione, vitamin K_3



menaquinone, vitamin K_2

It may be involved in electron transport in mammalian NADH oxidase systems as it is in many bacterial ones (128). In bacterial systems, u.v. light destroys vitamin K and therefore NADH oxidase activity. Similar effects are obtained in mammalian NADH oxidases. Solvent

extraction does not generally lead to a requirement for vitamin K in these systems; such a requirement has been reported after a series of ether extractions, however (92). A role for K in phosphate transfer has been proposed, based on studies of K deficient chicks which display low phosphorylation efficiencies (130). This work has been challenged, however, and the roles, if any, of vitamin K in electron transport and energy transfer remain obscure (131).

Nenniquinone

This is a benzoquinone found in mitochondria. Its structure is unclear. It is more polar than ubiquinone and present to 5-10% of the amount of ubiquinone (132). It may be a modified ubiquinone.

The tocopherols

Four roles for these in mitochondria have been proposed: (a) as an antioxidant, a well known general membrane role of tocopherols; (b) as a component of NADH oxidase (133); (c) as a cofactor for oxidative phosphorylation (134); (d) as a specific stabilising agent for α-ketoglutarate dehydrogenase (135).

Tocopherol's role as an antioxidant in mitochondria is open to some doubt as ubiquinol is present in much higher concentration and is better at preventing lipid peroxidation. Nason and Lehman reported that tocopherol restored NADH-cyt c reductase activity to isooctane extracted mitochondria (133). This was probably due to a general 'lipid activation' effect. They later reported a specific tocopherol requirement for NADH dehydrogenase activity in an NADH dehydrogenase preparation obtained from digitonin treated mitochondria (136). A redox function is unlikely, though, as tocopherol undergoes no redox or concentration changes in heavily aerated mitochondria.

Slater *et al* suggested a tocopheryl quinol phosphate could act as a cofactor for oxidative phosphorylation. Phosphate transfer from this to ADP was observed *in vitro* but not *in vivo*, however (134).

Schwarz attributed the loss of respiratory activity in tissue slices from tocopherol deficient chicks to a decline of α-ketoglutarate dehydrogenase activity (135). This is probably due to a lack of protection from oxidation of the enzyme.

Lipid R_{283}

This is a mitochondrial neutral lipid of similar chromatographic but not chemical properties to the tocopherols. It may be a precursor of ubiquinone. It is present at low levels and is unlikely to be an electron carrier.

Carotenoids

These are present in mammalian mitochondria. They derive entirely from the diet. This presence may not be due to a mere portioning of lipids as mitochondrial lipid levels do not generally mirror dietary lipid levels (137). Their function, if any, is unknown.

Triglycerides

Free triglycerides are present at very low levels in mitochondria. Little is known about their functions.

The respiratory chain complexes

The respiratory chain has been fractionated into four complexes. These have been extensively investigated following their preliminary isolation by Hatefi et al. (83-4).

Complex I: NADH-ubiquinone reductase

Complex I is a large lipoprotein enzyme complex, first extracted from bovine heart mitochondria by Hatefi, Haavick and Griffiths (138-9). It has the following properties:-

(a) it catalyses a rapid rotenone sensitive reduction of UQ 1 by NADH,

(b) it catalyses a rapid NADH-ferricyanide oxidoreductase activity but reacts slowly with other e^- acceptors,

(c) NADH induces multiple esr signals in complex I of similar line shape and E'_o values to those in SMP's,

(d) other types of NADH hydrogenase can be prepared from complex I,

(e) it reconstitutes NADH oxidase with complex III, IV and cytochrome c,

(f) energisation and ATP synthesis can be coupled to NADH-UQ 1 oxidase activity when complex I is reconstituted with the ATP synthase in phospholipid vesicles.

There have been other preparations of complex I-like enzymes, notably the types I and II NADH dehydrogenases, but they do not share all its properties (143).

Type I (high M.W.) soluble NADH hydrogenases

These were first prepared by Ringler et al. (140) and by Huang and Pharo (141). They appear similar to complex I but lack phospholipid and rotenone sensitive UQ 1 reductase activity. It has been thought that they only differ from complex I in their phospholipid content, though this may not be so. Hare and Crane have provided evidence that type I dehydrogenases differ from complex I in that they lack a 16,000 M.W. Fe-S protein (142).

Type II (low M.W.) soluble NADH dehydrogenases

Degradation of the type I dehydrogenases by heat, acid and ethanol, proteolytic enzymes, urea and other chaotropes causes the loss of their original activities and the appearance of new ones, notably a rotenone insensitive NADH-UQ 1 oxidoreductase (143). Their properties have been extensively reviewed (145-7).

The reduction of UQ 1 by NADH in complex I exhibits a phospholipid dependence. SMP's lose this activity after brief exposure to phospholipase A. Activity can be restored by addition of phospholipid. More prolonged exposure leads to an irreversible inactivation. Lipid extraction of complex I has to be carried out under anaerobic reducing conditions if reconstitution of rotenone sensitive electron transport activity by addition of phospholipids is required. Both lecithin and cardiolipin will restore activity singly but both are needed to restore it to in vivo rates (148).

Rotenone is generally thought to inhibit electron transport through complex I at about a 1:1 molar level with the enzyme (based on FMN content) (139). There have been reports, though, that there may be 2 rotenone binding sites per NADH dehydrogenase in bovine heart SMP (149).

Prosthetic groups of complex I

There is one, flavin mononucleotide (FMN). FAD has also been reported present but this has been shown to be due to contamination by an Fe containing flavoprotein associated with β-oxidation pathways in mitochondria (150). FMN does not appear to be covalently bound to the complex and can indeed by partially removed without any effect on NADH-ferricyanide oxidoreductase activity (151). There is no firm evidence that it is involved in complex I's activities at all. There appear to be 1 - 1.2 FMN/mole complex I (143).

Fe-S centres

Complex I and the type I dehydrogenases contain several Fe-S groups. Most workers agree on four containing 4 Fe each (87, 139, 151, 140, 153, 141). Recent work by Onishi and coworkers suggests that there may be as many as 7 Fe-S centres per complex I (152).

SH groups

Complex I contains several SH groups. These have been classified by Gutmann et al. into five distinct groups on the basis of the preparation used (complex I, type I and II dehydrogenase, SMP) and their sensitivity to p-chloromercuribenzoic acid (154). It is not clear whether they play any direct role in electron transport. Complex I NADH-UQ 1 reductase activity is 90% inhibited by 6 μM p-chloromercuibenzoate. Its NADH-ferricyanide oxidoreductase is unaffected by concentrations as high as 200 μM (155).

Ubiquinone

Complex I contains 4.2 - 4.5 nmoles UQ/mg protein. Recent work has suggested that complex I may contain a specific ubiquinone binding protein (124) (see introduction to Chapter 7).

Molecular weight and subunit composition

Estimations of molecular weight vary; most values are in the region of 670,000-800,000. This variation is probably due to differing amounts of bound lipid in different complex I preparations and possibly also due to minor differences in subunit structure.

Table 1.2 Subunit composition of complex I

Hare and Crane preparation (156)	Ragan preparation (157)	Stoicheiometry[1]
74,000	75,000	0.95 ± 0.04
53,000	53,000	2.24 ± 0.04
42,000	42,000 }	2.12 ± 0.15
37,000	39,000	
	33,000	0.90 ± 0.05
	29,000	0.91 ± 0.03
	26,000	1.0
27,000	25,000	
	23,500	
23,000	22,000	
	20,500	
19,000	18,000	
16,000	15,500	
	8,000	
	5,000	

[1] mol per 850,000 g (M.W. based on FMN content)

Gel profiles very similar, but Hare and Crane did not assign M.W. to all peak shoulders.

Complex I and energy conservation

Complex I contains an energy coupling site, site I. The mechanism of energy coupling in general is unclear (see Chapter 2). Phospholipid vesicles that contain complex I as the sole protein component accumulate protons during NADH-UQ 1 reductase activity in a manner sensitive to rotenone and uncouplers. The $H/2e^-$ ratio for this is 1.4:1 (158). These vesicles, which have ∿ 85% of their complex I in a SMP-like configuration, also catalyse the uptake of the lipophilic

anion, tetraphenyl boron, further supporting the idea that their insides become more + ve during electron transport. NADH-UQ 1 reductase activity in these phospholipid vesicles is greatly stimulated by uncouplers or valinomycin + nigericin + K^+. These observations have given rise to the suggestions that complex I is or contains a proton motive redox loop or a proton pump.

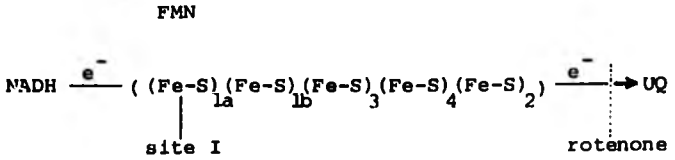
Complex I has also been incorporated into vesicles containing the ATP synthase enzyme. These vesicles catalyse ATP-^{32}Pi exchange, ATP linked proton uptake and NADH-UQ 1 oxidoreductase linked to ATP synthesis (159). This latter reaction was sensitive to rutamycin and various uncouplers. This experiment does not unequivocally demonstrate the existence of an osmotically linked coupling site in complex I as the ATP synthase preparation contained a 'hydrophobic protein' preparation that is known to be substantially contaminated by other respiratory chain components and activities.

Organisation of complex I in the membrane

Complex I is transmembraneous. This would theoretically allow it to act as a Mitchellian proton translocating redox loop (160) or as a redox linked proton pump. Present information on the transmembrane distribution of redox active centres in complex I makes the former possibility unlikely.

Mechanism of electron flow in complex I

Little is known about this. Electron transfer reactions are not easy to study because of their rapidity. Onishi has suggested that esr evidence supports the following sequence for electron flow in complex I (152).



Complex II: Succinate-ubiquinone oxidoreductase

Complex II is probably the least studied of the four complexes. It is the only one that does not contain a coupling site. It catalyses the following reaction:-

$$\text{succinate} + UQ \longrightarrow \text{fumarate} + UQH_2$$

The natural electron acceptor is ubiquinone but ferricyanide, phenazine methosulphate and 2,6-dichloroindophenol are all used to assay the purified enzyme. Succinate dehydrogenase comprises ∿ 50% by weight of complex II. Complex II contains 4-5 nmoles flavin, 36-38 nmoles nonhaem iron, 32-38 nmoles acid labile sulphur and 4.5 - 4.8 nmoles cyt b per mg protein and is 20% by weight lipid. It contains no ubiquinone (84). The flavin, nonhaem Fe and inorganic sulphide are all found in the succinate dehydrogenase part of the molecule.

Succinate dehydrogenase

This is a water soluble enzyme of M.W. 97,000 ± 4%. It is composed of two subunits, M.W. 70,000 ± 7% and 27,000 ± 5% (84). The enzyme contains 1 mole FAD per mole; this is covalently linked to the N3 nitrogen of a protein histidine's imidazole ring *via* the 8 Me group of the isoalloxazine ring in FAD. The flavin appears to be entirely bound to the 70,000 M.W. subunit.

There are three Fe-S centres in succinate dehydrogenase. Their distribution is unclear but available data suggests one subunit contains 2 Fe_2S_2 centres while the other contains one Fe_4S_4 centre (402, 406).

Both membrane bound and soluble succinate dehydrogenase undergo a reversible (de)activation. Activators include succinate, malonate, fumarate, Pi, SO_4^{2-}, ITP, IDP, formate, $FMNH_2$, various chaotropic anions such as ClO_4^-, I^-, Br^- and NO_3^-. The enzyme is inhibited by thiol reagents, oxaloacetate, malonate, fumarate, methylene succinate, maleate, HCO_3^-, glyoxylate, acetoacetate, glycolate and formate. Oxaloacetate

appears to be involved in *in vivo* regulation of succinate dehydrogenase (405).

Protein components of complex II

Early examinations of the polypeptide components of complex II revealed 7 different polypeptides (403, 338). More recent studies, however, reveal 10 (404). Four of these are major components while the other 6 appear to be due to contamination by complex III. The major subunits are of 70,000, 24,000, 13,500 and 7,000 M.W. The 70,000 and 24,000 M.W. subunits contain flavin and Fe-S centres and appear identical to the 70,000 and 27,000 components of succinate dehydrogenase (84). The 2 smaller subunits are very hydrophobic. The 13,500 one is the link betwen succinate dehydrogenase and the respiratory chain. The b cytochrome of complex II is contained in one of these subunits.

The b cytochrome of complex II

This was first described by Davis *et al.* and was designated $b_{557.5}$. It appears to be confined exclusively to complex II. It is not reduced by succinate but if reduced by dithionite is rapidly oxidised by fumarate and ubiquinone. Its role in electron transfer is unclear (402).

Complex III: b-c_1 complex; ubiquinone-cytochrome c reductase

This complex has been isolated by a number of groups (23, 161-3). Much interest has surrounded its purification and properties as it contains a coupling site and is much smaller than complex I. It was hoped that this would make study of energy coupling in complex III easier than in complex I; this has not proved to be the case.

Protein components of complex III

There are four main groups of protein components, the b cytochromes, cyt c_1, an Fe-S protein and the colourless 'core protein'; the last mentioned contains more than one protein (164-6). Cytochromes c (162) and b (170 have been isolated in relatively pure form. SDS polyacrylamide gel electrophoresis under dissociating conditions reveals 7 or 8 subunits (164).

Table 1.3 Polypeptide components of complex III

Band	Baum et al. (161)	Dasgupta and Rieske (162)	Gellerfors and Nelson (163)
I	core protein	core protein I (46,000)	core protein (50,000)
II	core protein	core protein II (43,000)	core protein (42,000)
IIA			cyt b dimer? (34,000)
III	cyt c_1	cyt c_1 I (29,000)	cyt c_1 (29,000)
IV	Fe-S protein	Fe-S protein (25,000)	Fe-S protein (25,000)
V	cyt c_1	cyt b (15,000)	cyt b (14,500)
VI	cyt b	cyt c_1 (14,000)	cyt c_1 (13,000)
VII	no assignment	antimycin A core protein (11,500)	antimycin A binding site (10,000)
VIII	unidentified (7,000-8,000) from Rieske (164)		

From this table it can be seen that cyt c_1 appears to be in two bands, M.W. 29,000 and 13-14,000. Baum et al. tried to explain this on the basis of a monomer-dimer transition (161). Yu et al., however, showed that highly purified cyt c_1 had a M.W. of 40,000 and that only band III contained haem (168). They suggested that the low M.W. 'cyt c_1' might be bound to the high M.W. molecule by disulphide bridges and that it might be a glycoprotein. A similar dimeric structure has been suggested for cyt f in chloroplasts (169). Complex III appears to contain ∿ 15% phospholipid (w/w).

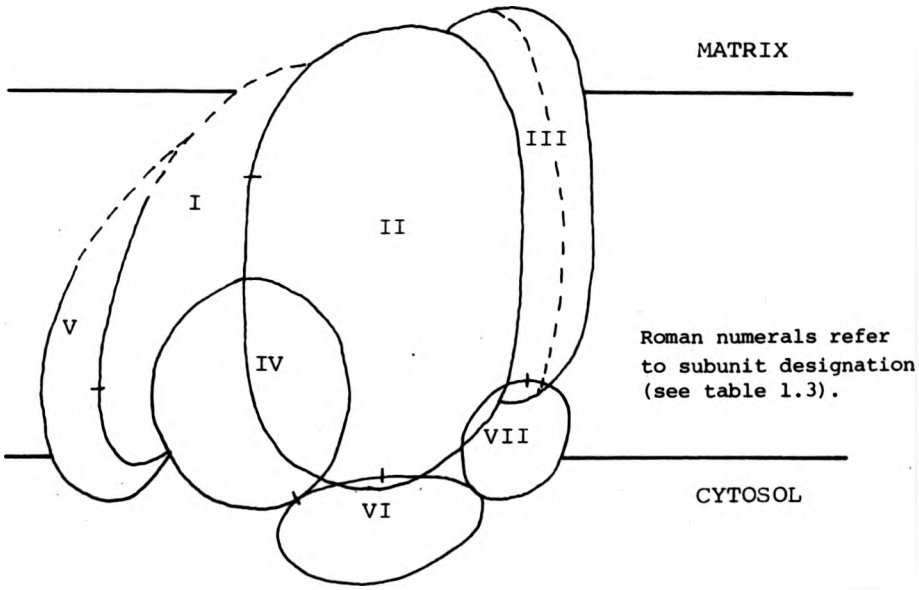
Molecular weight and shape of complex III

Minimum M.W. estimates of 250,000 have been made on the basis of sedimentation characteristics. Light scanning techniques give 262-288,000 Complex III appears to be roughly spherical in shape on electron micrographs after both negative staining and freeze fracture techniques have been used. Its diameter is ∿ 75-90 Å and the molecule appears to

contain a cleft 25 Å deep and 15 Å in diameter. This may be the antimycin A or the ubiquinone binding site (164), but it could just be an artefact of electron microscopical visualisation.

The three dimensional structure of the complex has been investigated, using a combination of cross-linking studies (233) and surface polypeptide labelling with ^{35}S diazobenzene sulphonate (234), by Capaldi et al.

Figure 1.3 Three dimensional structure of complex III



This structure has certain implications for the mechanism of energy transduction in the complex. All the known redox active centres lie on the cytosolic side of the membrane (V, (Fe-S) IV and VI (c_1)). (The haem group of cyt b, III, also lies on this side of the membrane). This makes transmembrane electron flow, and thus a strict chemiosmotic mechanism of energy transduction in complex III unlikely.

Function of components of complex III

Complex III acts in vivo as a single unit but this is best understood or described in terms of complex arrangements of mutually interacting components.

The b cytochromes

These have been the most studied components of complex III because of their multiplicity of forms and their possible relation to energy coupling (78).

Chance originally proposed that there were three distinct forms, b_{556}, b_{562}, b_{566} (172). He later redesignated b_{556} as b_{558}. That classical b cytochrome, i.e. the one reducible by succinate is b_{562}, b_{558} and b_{566} to one b cytochrome, b_T; b_{562} was termed b_K (173-4). Other groups have reported that the reducibility of all the b cytochromes increases in the presence of ATP (175-7). It is unclear if $b_{558/566}$ are two distinct cytochromes or one cytochrome with 2 α-absorption bands. Present evidence supports the former view (178). Wikstrom has proposed a cyt b stoicheiometry of 1:3:2 (558 : 562 ; 566) (78) whereas Flatmark and Pederson have reported 1:5:5 (177). Weiss and Ziganke have reported the isolation of two cytochrome b species distinct in amino acid composition in Neurospora crassa mitochondria (164). Phelps and Crane have reported the spectroscopic resolution of four cytochrome b associated peaks at 558, 561-562, 564 and 567 nm.

The apparent multiplicity of cyt b species has led to the suggestion that mitochondria contain dual or branched respiratory chains (180). One containing b_{565} + b_{558} contains a functional coupling site in complex III; the other, containing b_{562}, does not. The proposal has been criticized, however, and the reported observations supporting the hypothesis were probably obtained because the SMP preparation used contained a mixed population of coupled and uncoupled particles (78).

The antimycin A binding site in complex III: effects of antimycin A on complex III

(a) UQH_2 - ferricytochrome c reductase blocked by 1 mol antimycin A/ mol c_1

(b) complex III → dissociation into b + c_1 fragments by chaotropic agents inhibited by antimycin A stoicheiometrically

(c) causes changes in spectral and redox properties of the b cytochromes

The nature of the antimycin A binding site remains obscure. Storey has suggested that a b_{562}/antimycin A complex analogous to the a_3/CO complex in CO inhibited cytochrome oxidase is formed (181). Fluorescence quenching analysis of the complex II-antimycin A complex indicated that this is unlikely (182).

Antimycin A can chelate Fe. It was thought that it might therefore act at the level of the Fe-S protein of complex III. The idea has now been discarded as antimycin will inhibit the chaotrope induced dissociation of complex II, depleted of the Fe-S protein (183). Dasgupta and Rieske synthesised a tritiated affinity label for antimycin A, ^3H deformamido azido antimycin A. This inhibited poorly but bound to an 11,500 M.W. component in an antimycin A sensitive manner (162).

Fe-S centre of complex III

This was first isolated as a succinylated derivative by Rieske et al. (166). Esr studies indicate it to be on the oxygen side of the antimycin A block. Lee and Slater have suggested that complex III contains 2 esr detectable Fe-S centres (184). Analysis of complex III, however, reveals only one Fe/molecule. Lee and Slater's results may be due to a partially denatured complex III preparation containing native and denatured Fe-S centres.

The function of the Fe-S centre is unclear. Its necessity for electron transport has been questioned (185). Suggestions for its function have included (a) that it is the anitmycin A binding site (see above, however), (b) that it is involved in transmembrane H^+ transport (186), (c) that it controls electron flow between cyt b and cyt c_1 (187), (d) that is has a role in energy coupling (188). Redox potentiometric measurements make (b) unlikely: (a) can be dismissed on evidence previously presented. Suggestion (d) is also unlikely as Fe chelators that inhibit energy coupling have no effect on complex III

Fe-S centre's esr spectra. Recent evidence suggests that it may indeed play a role in electron transport or in the control of it between b and c (400).

Mechanism of electron transport in complex III

This is still not understood. There are two main hypotheses, those of Rieske (190) and of Wikstrom and Berden (191). Present evidence does not enable us to choose between them. Mitchell has used Wikstrom and Berden's scheme as the basis for his 'protonmotive Q cycle' hypothesis (342).

Complex IV: cytochrome oxidase, cytochrome c oxidase

Cytochrome oxidase catalyses the reduction of molecular oxygen to water. There have been numerous methods of preparing cytochrome oxidase; all depend on disrupting the inner mitochondrial membrane, usually with cholate and deoxycholate (75, 107, 192, 194-5, 197). Of these early preparations, that giving the highest activity, lowest M.W. and least bound lipid was that of Griffiths and Wharton (75). These preparations all have a M.W. $\sim$ 200,000 and contain 2 haem residues and 2 Cu atoms.

Copper in cytochrome oxidase

Keilin originally thought that cytochrome oxidase was not a haemoprotein but a copper containing protein (68). He later discarded this idea when it was shown that the oxidase was a haemoprotein. It was not until 25 years later that firm evidence showing that the enzyme contained copper was presented by Takemori (196) and Griffiths and Wharton (198-9). There was much debate about whether this copper was intrinsic to the enzyme's activity. Yonetani, on the basis of studies with bathocuproine sulphonate, a Cu chelator, claimed that it was not (200). Later studies showed that not all the copper was accessible to this chelator, however. Esr spectroscopy has proved of great value in the study of cytochrome oxidase copper (201-4). Beinert and Palmer

demonstrated that Cu in excess of 1:1 with haem Fe in complex IV has a different hyperfine esr structure to that of intrinsic (1:1) copper (203-4). This excess copper has no relationship to the enzyme's activity. The role of intrinsic Cu, although now known to be essential to activity, is not understood. Only 25% of intrinsic Cu is esr detectable. This Cu is easily reduced by ferrocytochrome c and reoxidised by O_2 in a CN^-, N_3^- and CO sensitive manner (205). Sulphydryl groups may be involved in the binding of intrinsic copper.

Protein components of complex IV

Estimates of the number of subunits in bovine heart cytochrome oxidase have varied between 2 (222) and 7 (210). It is likely that functional cytochrome oxidase has 7 subunits.

Table 1.4 Polypeptide components of cytochrome oxidase: molecular weight

component	bovine heart					yeast
	(206)	(207)	(208)	(209)	(210)	(211)
I	40	40	37	47.5	40	40
II	25	21		20	21	33
III	19		19	14.5	14.8	23.5
IV	14	15	14	14.5	13.5	14.5
V	10	13	10	13	11.6	13
VI	8	12		11	9.5	12.5
VII					7.6	4.7

Figures refer to M.W. in thousands

Yu and Yu estimated the stoicheiometry of the subunits to be 1 per complex. This gives a minimum M.W. of ∿ 120,000 for cytochrome oxidase (210). Early estimates of 200,000 for complex IV's M.W. are now thought to be due to aggregation of the enzyme. Yu and Yu also purified various polypeptides from cytochrome oxidase. Component II appears to contain ∿ 40 nmoles Cu/mg protein (stoicheiometry of ∿ 0.8:1). Cu may be found elsewhere in complex IV as not all Cu atoms in it have similar properties (212). Copper may be associated with haem binding subunit (213). Two

subunits, I and V, may be involved in haem binding; V contains haem but not copper (214). The subunits are probably linked by cysteine disulphide bonds (215-7).

Electron microscopy of cytochrome oxidase/phospholipid vesicles indicates that the molecule spans the bilayer (long axis 83 Å) (222). Antibody studies suggest that it is aggregated in the membrane in hexamers or octamers of the 120,000 M.W. unit (223). Hackenbrock has demonstrated that the complex is highly mobile in the lateral phase of the membrane and that it may be associated with the F_o portion of the ATPase as well as cyt c (224).

Nonpenetrant protein labelling reagents indicate that the molecule is 'plugged through' the inner membrane (225). Studies by Eytan et al., using p-diazonium benzene sulphonate,indicate that subunits II, V and VI lie on the cytosol side of the membrane, subunit III lies on the matrix side of the membrane and subunits IV and I are buried in the membrane (226). A similar position for II had already been suggested by Kornblatt et al., based on studies using N-ethyl maleimide (226).

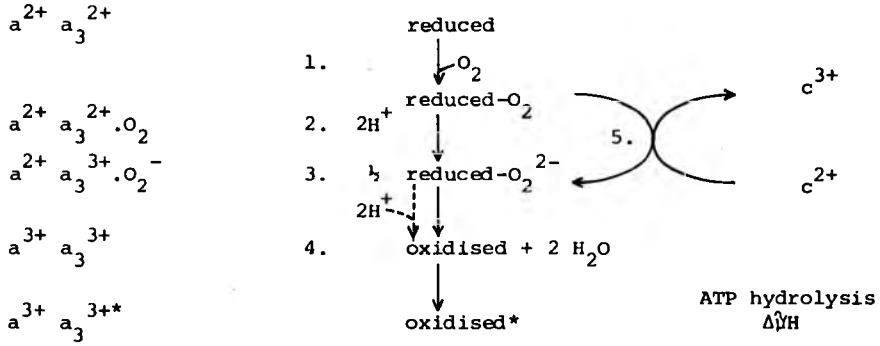
The cytochrome c binding site

The site of interaction , cyt c with the complex, is unclear. Various methods based on using affinity label derivatives of cyt c have given a wide range of answers, i.e. subunit II (228); III and others (229-30); VI and VII (231). Recent work by Seiter et al. suggests that cyt c binds to subunit IV (232). This result seems at odds with the transmembrane position of IV suggested by Eytan et al. (226). The apparent contradiction has yet to be resolved.

Mechanism of action of cytochrome oxidase

Cytochrome oxidase reduces molecular oxygen to water. The reactions are very rapid and thus difficult to study. At least four groupings are involved, cyt a, cyt a_3 and 2 Cu atoms (copper's role remains obscure).

Low temperature, 'freeze clench techniques' introduced by Chance et al. indicate that the enzyme cycles between a fully oxidised form $a^{2+}a_3^{3+}O_2$ and the half reduced peroxy form (218). More recent work suggests that this peroxy form may be a mixture of a^{2+}, $a_3^{3+}O_2^{2-}$ and $a^{3+}a_3^{2+}O_2^{-}$ (2-9). The reaction would proceed as outlined below:-



* energised form of the enzyme

This scheme has many features in common with that described by Nicholls and Pederson (220). The stabilisation of the enzyme at high phosphorylation potentials would limit respiration (221). Such a kinetic component to respiratory control (in addition to equilibrium types of control at coupling sites 1 and 2) may be necessary at cytochrome oxidase to ensure irreversibility and hence rapid electron flow in the respiratory state.

Cytochrome oxidase also contains coupling site 3. This is currently the subject of intense controversy and is discussed in Chapter 2.

Adenosine triphosphate (ATP) synthase; ATPase; Complex V

The enzyme responsible for the synthesis of ATP during oxidative phosphorylation is the ATP synthase. It is located in the inner membrane and protrudes out of it into the matrix space. It is a complex multisubunit enzyme of M.W. 450-500,000. It has been termed complex V by Hatefi and coworkers (350). It catalyses a number of reactions in situ; these include:-

(i) ATP synthesis: $ADP + Pi + nH^+ \longrightarrow ATP + H_2O$

(ii) ATP hydrolysis: $ATP + H_2O \longrightarrow ADP + Pi + nH^+$

n is pH dependent and is $\sim$ 0.7 at pH 7.0.

(iii) exchange reactions: these are a series of reactions in which atoms from H_2O, Pi, ATP and ADP can be exchanged between each other. The most studied of these are ATP-Pi exchange, ATP-ADP exchange, ATP-H_2O and Pi-H_2O exchange.

The enzyme appears to be divided into two functionally dissimilar parts; the hydrophilic F_1 ATPase which protrudes from the inner membrane into the matrix space and the F_O portion of the ATPase which is highly hydrophobic in nature and is buried in the inner membrane. Both the F_1 ATPase and F_1F_O ATPases have been purified from the inner mitochondrial membrane. As well as differing in their subunit structure they also have different kinetic and catalytic properties and different sensitivities to inhibitors of ATP synthesis and hydrolysis (see Appendix A).

F_1 ATPase.- The F_1 ATPase can be removed from the inner membrane by a number of tecnhiques including sonication (409, 262) and chloroform treatment (236). The isolated enzyme displays only ATP hydrolase activity and is not sensitive to inhibitors of ATP synthase and ATPase activity such as oligomycin, venturicidin and trialkyltin compounds. The F_O subunits of the ATPase are necessary for the enzyme to display oligomycin sensitivity and ATP-Pi exchange reactions. The released F_1 ATPase appears to be a spherical molecule in ultracentrifugation (410) and electron microscopy experiments (22, 53). The enzyme prepared from bovine heart mitochondria has a M.W. in the region of 347-360,000 (411-2). It is a complex macromolecule containing five subunits termed α, β, γ, δ, ε in order of decreasing M.W. The stoicheiometry of these subunits in the complex is difficult to establish,and methods involving dye staining and amino acid frequency have been used (411). These indicate that there are

around 10 polypeptide components per F_1 molecule. There is no consensus on the stoicheiometry of subunit composition of F_1 ATPase (see Table 1.5) and estimates ranging from $\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$ to $\alpha_2\beta_2\gamma_1(\delta\epsilon)$? have been made (409-10).

Table 1.5 Molecular weight and subunit composition of F_1 ATPases

Subunit	Subunit molecular weight					
	bovine heart	yeast	rat liver	chloroplast	E. coli	B. stearothermophilis
α	54(3)	58	62(3)	62(2)	56(3)	56(3)
β	50(3)	54	57(3)	57(2)	51(3)	53(3)
γ	33(1)	38.5	36(1)	38(1)	32(1)	32(1)
δ	17(1)	31	12(1)	21(1)	20(1)	15.5(1)
ε	5.7(1)	12	7.5(1)	14(2)	13.2(1)	11(1)
inhibitor protein	∿10	-	-	-	-	-
M.W. range	347-360	-	360-384	3.25	360-390	380

M.W. expressed in thousands
Figures in brackets refer to stoicheiometry of subunit per F_1 ATPase

Certain F_1 ATPase preparations appear to contain an additional subunit of ∿10,000 M.W. This is the 'ATPase inhibitor protein' which may be responsible for the kinetic control of ATPase activity *in vivo* (414).

Kinetics of F_1 ATPase.- Estimates of the KmATP of F_1 ATPase vary widely between 0.2 - 1.25 mM. The KmATP changes if the F_1 is bound to the membrane. ADP is a competitive inhibitor of F_1 ATPase activity. The true substrate of the enzyme appears to be a MgATP complex (415). The enzyme will also hydrolyse a number of other nucleotide triphosphates including inosine and uridine triphosphate.

Bound nucleotides in F_1 ATPase.- Adenine nucleotides bind at a number of different sites on the F_1 ATPase. There appear to be two ADP binding sites per F_1 (414). However, Slater has reported that there are 5 moles

of tightly bound adenine nucleotide in isolated,purified F_1 (416). The relationship between the tightly bound adenine nucleotides and the two high affinity sites for ADP is not clear. Slater's 5 sites only undergo a slow exchange with exogenous adenine nucleotides, whereas the two high affinity sites for ADP show a rapid nucleotide exchange. Results presented by Penefsky and coworkers (411,414) go some way to reconciling these two sets of data. They found 3 moles of tightly bound adenine nucleotide/F_1, ∿2 moles ADP, ∿1 mole ATP and a little AMP. They also found two sites that undergo reversible binding of adenylimidodiphosphate AMP-PNP, an ATP analogue.

The presence of five adenine nucleotide binding sites in the F_1 ATPase raises questions as to their role(s) in ATP synthesis and ATP exchange. At least one site appears to be involved in adenine nucleotide induced conformational changes in F_1. It is not clear whether all 5 sites must be occupied for catalytic activity. There is no apparent difference in the rate of ATPase catalysed by native F_1 and adenine nucleotide depleted F_1. There is evidence to suggest that some of the sites may have a function in the control of the catalytic sites (417).

F_1F_O ATPases.- These are ATPases that possess the hydrophobic membrane bound subunits as well as the hydrophilic F_1 ATPase molecule. There are a number of preparations of these enzymes from different sources. The purest are probably those of Tzagoloff from yeast (418), Hatefi et al. from bovine heart (350, 244) and Kagawa and coworkers from the thermophilic bacterium, PS3 (419). Both the oligomycin sensitive ATPase (OSATPase) of Tzagoloff and the complex V ATPase of Hatefi are sensitive to the inhibitors oligomycin, venturicidin, trialkyltin halides and DCCD. The TF_1F_O ATPase of Kagawa is unaffected by oligomycin, a property it shares with most other bacterial ATPases, but it is sensitive to DCCD. All of these ATPases are purified from the inner membrane using chaotropic detergents such as Triton X-100, cholic acid and deoxycholic

acid and are consequently severely delipidated in their final purified form. They all require added phospholipid to express maximum ATPase activity.

Purified F_1F_O ATPases contain at least 8 different subunits (419) and possibly as many as twelve (244).

Table 1.6 Composition of F_1F_O ATPases: subunit M.W.

Yeast 'OSATPase' (418)		Bovine heart 'Complex V' (244)		PS3 'TF_1F_O' (419)		
		83				
		68				
58	α	53	α	56	α	(3)
54	β	50	β	53	β	(3)
		47				
		44				
38.5	γ	33	γ	32	γ	(1)
31	δ	31		19		(1)
29		24				
22		22.5		15.5	δ	(1)
18.5		15	δ	13.5		(2)
12 (x 2)	ε	13		11	ε	(1)
7.5		8-9	ε	5.4		(5)
Total M.W.	∿460	∿450-480		∿458		

M.W. in thousands: Greek letters denote F_1 ATPase subunits: bracketed figures indicate subunit stoicheiometry

Hatefi and coworkers have recently further purified Complex V to give a preparation containing 11 - 12 subunits (420). Most of these subunits have been tentatively described (see Table 1.7 on next page).

ATP-Pi exchange in F_1F_O ATPases.- Perhaps the most important difference between F_1 ATPases and F_1F_O ATPases is the ability of the latter to catalyze an ATP-Pi exchange reaction. Usually preparations of F_1F_O ATPases require added phospholipid to display the exchange activity. This has been thought to be because the phospholipid and ATPase combine to form protein phospholipid vesicles which allows the ATPase to form a

potential gradient across the lipid membrane that can be used to drive ATP-Pi exchange. This chemiosmotic view has been challenged by Hatefi, whose complex V contains very low levels of phospholipid (too low to form vesicles) but displays high levels of ATP-Pi exchange (244).

Table 1.7 Identification of subunits of Complex V ATPase

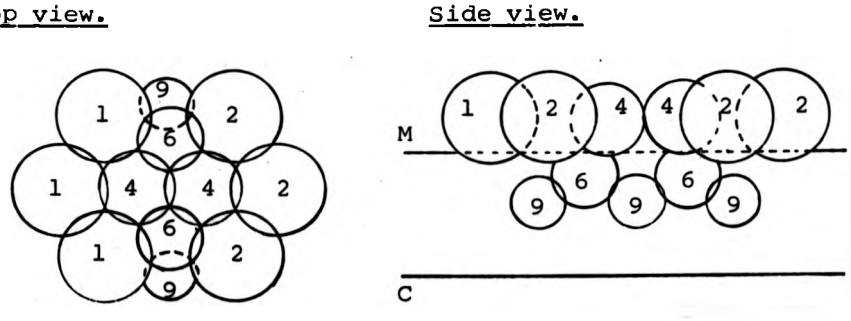
Subunit M.W. (in thousands)	Identity
53	α subunit F_1 ATPase
50	β subunit F_1 ATPase
33	γ subunit F_1 ATPase
30	uncoupler binding protein
23	F_0 component; pantotheine binding protein (421)
22	oligomycin sensitivity conferring protein (OSCP)
15	δ subunit F_1 ATPase
12	DCCD binding protein
8	coupling factor F_6 (422)
11-13	ATPase inhibitor? coupling factor B (387-8)
6	ε subunit F_1 ATPase

Function of the F_0 component of ATP synthase.- The subunits that compose the membrane sector of the ATPase, F_0, have not been shown to catalyse any biochemical reactions so far. They are necessary for the expression of oligomycin sensitivity and for ATP-Pi exchange activity. The F_0 sector is supposed to be the region in which the high energy state, '~', generated by coupled electron transport is transduced to '~P'. The chemical and conformational hypotheses of oxidative phosphorylation propose that '~' is a chemical bond or strained membrane state that is eventually used to produce '~P' and ATP. The F_0 sector then should catalyze a series of reactions in which '~' is transduced to '~P'. Chemiosmotic theory treats '~' as a protonmotive force, Δp, generated

across the coupling membrane. Here the F_O sector is considered to be a proton conducting 'well', spanning the inner membrane, which feeds protons to the active site of the F_1 ATPase. Submitochondrial particles depleted of F_1 ATPase leak protons, apparently through the F_O ATPase as oligomycin, which binds in the F_O sector, 'plugs' this proton leak (22). Experiments on a purified TF_O fraction from PS3 bacteria suggest a similar function in which TF_O acts as a proton well which is 'gated' (controlled) by TF_1 ATPase (423). Fuller discussion of chemical, chemiosmotic and conformational coupling hypotheses can be found in Chapter 2.

Three dimensional structure of F_1F_O ATPases.- Although the distribution of subunits between the F_1 and F_O portions of the ATPase has been known for some time (409), little has been known about the 3-dimensional arrangement of these subunits. A recent paper by Enns and Criddle has gone some way to describing this 3D structure (399). They cross-linked the ATPase subunits of a yeast OSATPase preparation with methyl-4-mercaptobutyriimidate and then subjected the enzyme to 2-dimensional polyacrylamide gel electrophoresis. The results of these are shown in Fig. 1.4.

Fig. 1.4 Three dimensional structure of F_1F_O ATPase, after (399)



numbers refer to subunit type, see (399)

the position of the other subunits of the ATPase complex (notably 3,5,7,8) is as yet undefined

Active catalytic groupings in F_1F_O ATPase.- Little is known about the chemical groupings of the ATPase involved in ATP synthesis or hydrolysis. STudies with tetranitromethane indicate that there is at least one tyrosine residue essential to ATPase activity per F_1 (408). Experiments with phenylglycoxal and butanedione indicate that the enzyme also contains two essential arginine residues. One of them appears to be located in the F_1 ATPase and concerned with ATP hydrolysis. The other appears to be located at or near the uncoupler binding site and involved in ATP-Pi exchange (424). Thiol groups may also be essential for ATP-Pi exchange activity as p-chloromercuribenzoate inhibits the reaction (244).

F_1F_O catalysed ATP synthesis.- Isolated preparations of F_1F_O ATPase do not catalyse ATP synthesis. There have been reports though that ATP synthesis can be observed when F_1F_O ATPases are reconstituted into lipid vesicles with systems capable of forming a proton gradient across the vesicles; these include complex I (143) and 'purple membrane' protein from Halobacterium halobium (425-6).

Relationship between ATPase, ATP-Pi exchange and ATP synthase activities.- It has generally been assumed that the ATPase reaction is a mere reversal of ATP synthase activity in coupling ATPases and that ATP-Pi exchange is a residual reflection of ATP synthase activity. There is a growing body of evidence, however, which strongly suggests that this is not the case. Inhibition studies with certain inhibitors, e.g. ribose ring opened ATP, suggests that the catalytic sites responsible for ATPase and ATP-Pi exchange activity are not those directly involved in ATP synthesis (427). Studies with antibodies to chloroplast F_1 also suggest that different sites are involved in ATP-Pi exchange and ATP synthesis (428).

Coupling factors and F_1F_O ATPases.- A number of protein factors from the inner membrane have been isolated which 'couple' electron transport

to energy-linked reactions in a series of depleted submitochondrial particles (22). These 'coupling factors' include factor F_1, factor A, factor A-X, all of which are the F_1 ATPase with or without certain other ATPase subunits. A number of other coupling factors are known which have no F_1 ATPase activity; these include factor B (387) and factor F_6 (422). Most of these 'factors' are considered to be subunits of the F_1F_O ATPase (see (51) for review).

A full understanding of the molecular mechanisms of ATP synthase activity is essential for the understanding of energy coupling in general. Hypotheses of energy coupling are dealt with in the next chapter.

CHAPTER 2 MECHANISMS OF ENERGY TRANSDUCTION

The mechanism by which redox energy released during the oxidation of respiratory chain substrates by the respiratory chain is utilised by the ATP synthase to phosphorylate ADP to ATP is unknown. It has been the subject of intense controversy for the last thirty years. Many hypothetical mechanisms have been proposed. These fall mainly into three broad categories (a) chemical, (b) conformational and (c) chemiosmotic hypothesis. They differ over the nature of the high energy state linking the processes of respiration and phosphorylation.

Chemical hypothesis.- This was first proposed by Slater and was based on Warburg's mechanism of glycolytic phosphorylation. Slater originally proposed that the following series of reactions described oxidative phosphorylation (407).

1. $AH_2 + B + C \rightleftharpoons A \sim C + BH_2$
2. $A \sim C + ADP + Pi \longrightarrow A + C + ATP$
3. $A \sim C + H_2O \longrightarrow A + C$

Reaction 3 describes 'uncoupling'. A and B are adjacent respiratory carriers. C is an additional grouping required for their interaction. This mechanism was later revised to its present form.

1. $AH_2 + B + C \longrightarrow A \sim C + BH_2$
2. $A \sim C + X \longrightarrow X \sim C + A$ (I replaces C in some terminologies)
3. $X \sim C + Pi \longrightarrow X \sim P + C$
4. $X \sim P + ADP \longrightarrow X + ATP$

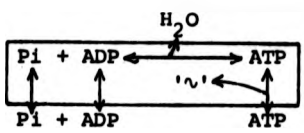
The nature of X and C is undefined.

The high energy state in this series of reactions would be $X \sim C$. This would be broken down by uncouplers, thus preventing ATP synthesis. This series of reactions would be fully reversible (62). The term 'chemical hypothesis' is also used to describe a whole series of mechanisms which may or may not follow Slater's

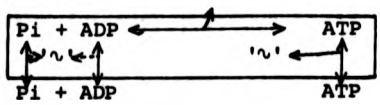
revised scheme. The common feature of all these mechanisms is that the high energy state ('∿' or X ∿ I) of the mitochondrial inner membrane is contained in a single covalent chemical bond (or a series of covalent bonds). X and C are usually considered to be small, mobile compounds dissolved in the lipid phase of the membrane.

Conformational hypothesis.- A mechanism of oxidative phosphorylation analogous to the conversion of chemical into mechanical energy during muscle contraction was proposed by Boyer (408). Here Boyer proposed that the energised state would be brought about by coupling oxidation to a conformational change in a protein. The energised protein would probably be a respiratory carrier or an associated structure and the conformational change could result from the formation or cleavage of a covalent bond. This mechanism is clearly only a modification of the chemical hypothesis in which proteins replace both the small discrete chemical species X and C. Boyer has now (apparently) abandoned this hypothesis in favour of one which involves conformational changes occurring through multiple non-covalent bonding at the catalytic site of the ATP synthase (31). The experimental basis for this latter hypothesis rests on Boyer's work on the various exchange reactions catalysed by the ATP synthase. ^{18}O exchange between Pi and water was found to be less sensitive to the action of uncouplers than ATP-^{32}Pi or ATP-$H_2{}^{18}O$ exchange. This led to the suggestion that energy may be used to promote release of ATP from the catalytic site (see Fig. 2.1, scheme A) (31). Later evidence amplified this viewpoint but also suggested that energy was used to facilitate the binding of Pi and/or ADP to the ATP synthase prior to the synthesis of ATP (Scheme B) (37).

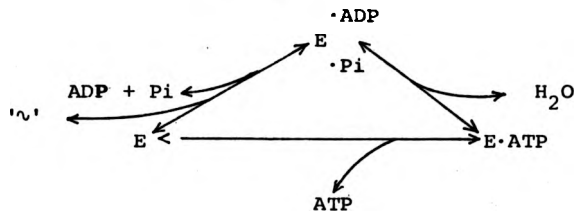
Scheme A (31)



Scheme B (37)

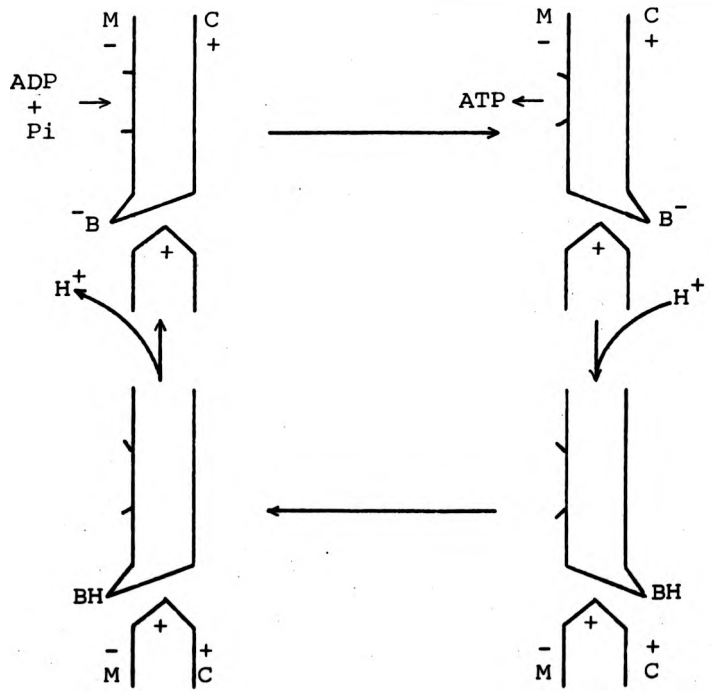


Adenine nucleotides are known to be bound both 'loosely' and 'tightly' to F_1 ATPase; Boyer has suggested that '∿' might only favour the 'tight' binding of ADP and Pi, viz.



Conformational models have also been proposed for the coupling of membrane potential to ATP synthesis or hydrolysis (48).

Fig. 2.1 Scheme for coupling membrane potential to ATP synthesis, after(48)



The attractive features of the scheme are that any H^+/ATP stoicheiometry could be accommodated by it, and that ATP synthesis could be driven by proton gradient or by a membrane potential. There is as yet, however, no firm experimental evidence for such a proposal.

Chemiosmotic hypothesis.- Certain practical considerations led some workers to doubt the applicability of the chemical hypothesis, based as it was on substrate level phosphorylation, to oxidative phosphorylation. The hypothetical intermediates X and C or any of their adducts had not been isolated, nor was there any clear indication of their nature although many hypothesis had been proposed (25). Oxidative phosphorylation appeared to have an obligatory requirement for an intact membrane structure (topologically closed), an observation not theoretically implicit in the chemical or conformational hypothesis and not readily explainable from their basic formulations. Respiration and phosphorylations could be uncoupled from each other by a series of compounds, e.g. dinitrophenol, dicoumarol, salicylate and azide. These are chemically dissimilar and it was difficult to imagine how such a group of compounds could all lead to the hydrolysis of X ∿ C (their proposed mode of action). These and other considerations led Peter Mitchell to propose his 'chemiosmotic' hypothesis in 1961 (49). This stated that phosphorylation was linked to electron transport by a protonic electrical potential across the inner membrane. This potential would be set up by the action of a proton motive respiratory chain which would pump protons out of the mitochondrial matrix. This potential could be collapsed through an 'anisotropic reversible protonmotive ATPase' with the concomitant synthesis of ATP. The high energy state of the membrane, X ∿ C or '∿', would not exist as a discrete chemical bond but as a potential difference across the membrane. Mitchell proposed that uncouplers had their mode of action by collapsing this potential as they all appeared to be weak acids or bases dissolvable in the lipid phase of the membrane in both their acid or base forms, and could thus cause a proton 'leak' through the membrane.

The hypothesis was published with no experimental evidence and was largely ignored until 1965 when evidence supporting it began to appear (301).

The formulation of the chemiosmotic hypothesis must be considered as one of the great landmarks of biochemistry, not because of its truth or otherwise but because it gave a framework within which experimentation on oxidative phosphorylation could be carried out. It made certain predictions about the nature of energy coupling which could be tested, a property sadly lacking from the other hypotheses available at the time, which really only suggested an (apparently vain) search for X and C. Since 1961 the hypothesis has undergone several modifications (50, 56, 261, 58, 66). Its latest form, essentially that outlined in (58, 66), is that which will be discussed here.

The chemiosmotic hypothesis has four fundamental postulates:-

1. The ATP synthase is a membrane located reversible proton-translocating ATPase having characteristic H^+/P stoicheiometry.

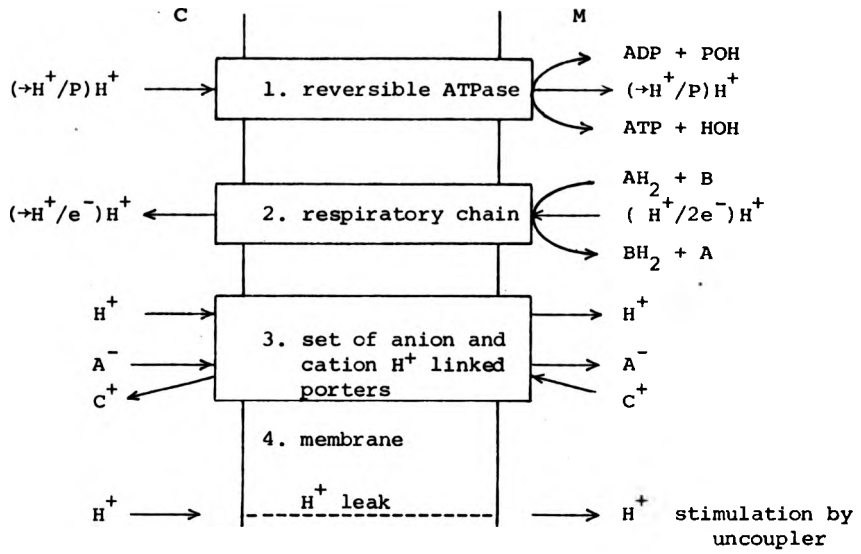
2. The respiratory chain is a membrane located vectorial metabolic proton translocating system, having a characteristic $H/2e^-$ stoicheiometry and having the same polarity of proton translocation across the membrane for normal forward redox activity as the ATPase has for ATP hydrolysis.

3. There are proton linked (or ^-OH linked) solute porter systems for osmotic stabilisation and metabolite transport.

4. Systems 1-3 are plugged through a topologically closed insulating membrane called the coupling membrane that has a nonaqueous osmotic barrier phase of low permeability to solutes in general and to H^+ and ^-OH ions in particular (see Fig. 2.2).

The redox system going forward (towards oxygen) creates a potential difference across the membrane by translocating protons. This potential difference would be similar to an electrical potential but not identical as the vector of its production is the proton not the electron. This has led Mitchell to propose the notion of 'proticity' (cf. electricity) (120).

Fig. 2.2 Four fundamental postulates of chemosmosis



The aqueous media on the inside (M) and the outside (C) of the inner mitochondrial membrane are quite different. Mitchell proposed that there is a significant protonic potential difference arising from the effective concentration difference of mobile protons as well as from their electric potential difference. Therefore, the total protonic or protonmotive potential difference Δp (or $\Delta\tilde{\mu}H^+$) must be given as the sum of the electric potential difference $\Delta\psi$ and a concentration potential difference that is equal to $-RT\ln_e \Delta pH$ or $-Z\Delta pH$, where $\Delta\psi$ is measured in mV. This gives

$$\Delta p = \Delta\psi - Z\Delta pH$$

This is the fundamental mathematical formulation of chemiosmotic theory: both $\Delta\psi$ and $-Z\Delta pH$ are osmotically derived terms, i.e. they depend for their magnitude on bulk concentrations of protons and other ions. $\Delta\psi$ is in effect the membrane potential of the system. One of the main functions of the solute porters (postulate 3) would be to regulate the contributions of $\Delta\psi$ and $-Z\Delta pH$ to Δp.

When the membrane is not leaky and most of the proton current generated by the redox system passes back through the reversible ATPase there will be stoicheiometric coupling between oxidoreduction and phosphorylation. The P/O (or $P/2e^-$) quotient of the overall process is given by the $\rightarrow H^+/2e^-$ quotient of the redox system divided by the $\rightarrow H^+/P$ quotient of the ATPase.

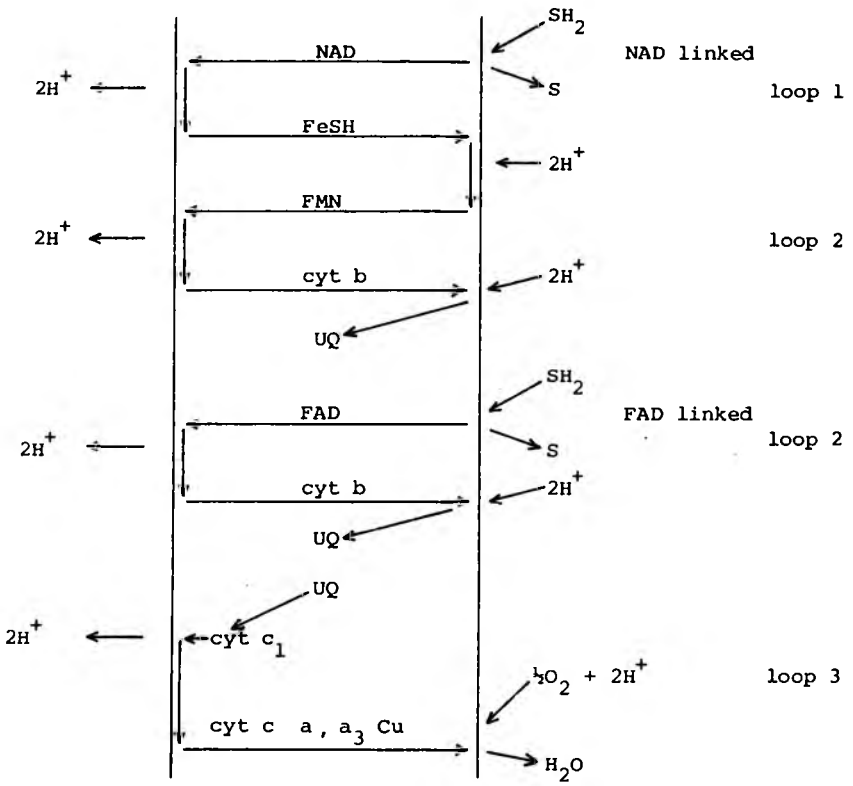
$$P/2e^- = \rightarrow H^+/2e^-/\rightarrow H^+/P$$

Assuming an $\rightarrow H^+/P$ of 2 and a $H^+/2e^-$ value of 2 at each site, a standard free energy of hydrolysis of ATP corresponding to a hydrolysis constant of 10^{-5} M^{-1}, Δp should be $\underset{\sim}{}210$ mV at 25° C at a phosphate concentration = 10 mM when ADP / ATP = 1. For an ADP / ATP = 20 and Pi = 10 mM, Δp will be $\underset{\sim}{}250$ mV. Respiratory control in mitochondria would be expected to occur at Δp values of ~ 250 mV.

A short circuiting proton conducting pathway across the membrane would collapse Δp and would release its control effect on redox and ATPase activity. It would also uncouple phosphorylation while allowing redox activity to accelerate. Mitchell proposed that uncouplers provide this short circuit by being lipid soluble weak acids (or bases), lipid soluble in both their protonated and anionic forms. Most uncouplers appear to be compounds of this type (58).

Chemiosmosis, while proposing a chemically simple respiratory chain, requires that it is physically highly organised across the plane of the membrane. Mitchell originally proposed that it was organised into 3 proton translocating loops (Fig. 2.3). Present evidence suggests that the respiratory chain is not folded into such loops (222). This is discussed later in this chapter. The lack of support that transmembrane spatial organisation studies on the inner membrane has given to the concepts of loops 2 and 3 has led Mitchell to propose an alternative to them, his 'protonmotive ubiquinone cycle' (342-3) (see Fig. 7.1 and associated discussion). It is worth pointing out that this hypothesis

Fig. 2.3 Proton translocating loops 1-3, after (58).

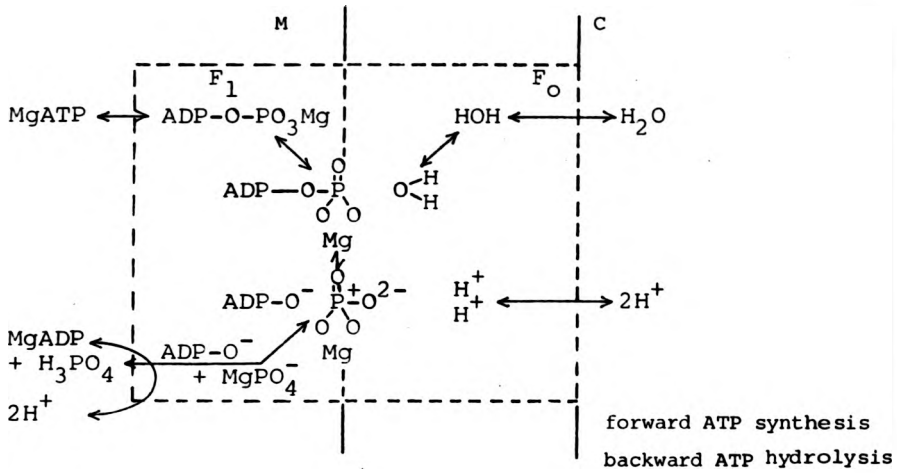


requires a certain distribution of b cytochromes across the membrane and that the available evidence suggests that this distribution is unlikely (222).

The chemiosmotic hypothesis is independent of the molecular mechanisms by which proton translocation may be stoicheiometrically coupled to ATPase of ATP synthase and to respiratory activity in the redox systems. It states that there are no intermediates common between the proton pumping respiratory chain and ATPase systems. This does not mean that there are no intermediates and/or conformational changes involved in either of these systems. However, the apparent lack of intermediates involved in ATP synthesis has led Mitchell to postulate that they do not exist (72).

Mitchell has proposed a mechanism for H^+ translocation by the ATPase involving the translocation of H_2O one way and the translocation of O^{2-} between H_2O and the terminal P atom of ATP the other. This produces a net translocation of $2H^+$ (Fig. 2.4).

Fig. 2.4 Proton translocation by the F_1F_O ATPase



Certain authors have been quick to point out that mechanistic considerations make this hypothesis highly unlikely (85, 48, 35) although Mitchell, with small modifications (H_2O enters from the matrix side) retains it (66).

Much experimental evidence has accrued since 1961 supporting the four basic postulates of the chemiosmotic hypothesis (see (58, 66) for list of references). The respiratory chain and the F_1F_O ATPase have been shown apparently translocate H^+ in the direction predicted. Mitchell and Moyle found that $2H^+$ were translocated out across the membrane for every $2e^-$ transversing each phosphorylation site (58). This electron transport between NADH and O_2 causes the extrusion of $6H^+$, between succinate and O_2 $4H^+$ and between an ascorbate (TMPD) couple and O_2 $2H^+$. Similar ratios were found in bacterial systems. Studies on the ATPase indicated that $2H^+$ were translocated for every ATP hydrolysed. Experimental

estimates of Δp gave values in the region of 200-230 mV in good agreement with the theoretical expected, 250 mV. Mitchell incorporated H^+/site and $\rightarrow H^+$/ATP ratios of 2 into his hypothesis. Later analysis of all these experimental findings has suggested that the situation might not be as simple as was first thought. These considerations are discussed later.

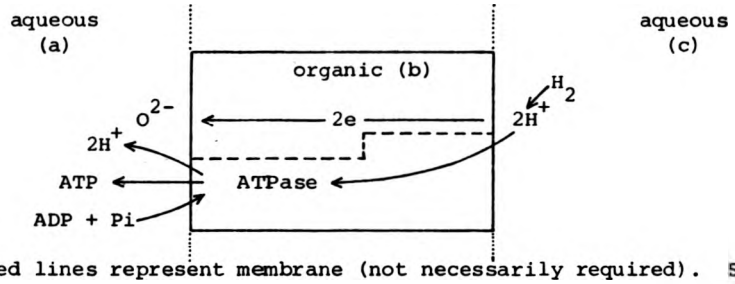
Whatever the fate of the 'chemiosmotic hypothesis' as proposed by Mitchell, its impact on and importance to all subsequent experimental and theoretical work in bioenergetics is difficult to overestimate. It has been more successful as a general framework within which bioenergetic phenomena can be described than any other single theory, and all subsequent hypotheses of energy coupling have to explain both its strengths (which are many) and its weaknesses, which are few but are of fundamental importance.

A number of hypotheses of energy coupling, which are broadly chemiosmotic in nature but disagree with Mitchell on the mechanism of proton translocation, have been proposed. These usually replace Mitchell's protonmotive redox loops by a redox linked proton pump (456). The mechanism by which protons are translocated through these 'pumps' is not defined.

Localised proton hypothesis.- At the same time as Mitchell first proposed his chemiosmotic hypothesis, an alternative model also involving protons as the active intermediaries between electron transport and ATP synthesis was proposed by R.J.P. Williams (95-6). He proposed that as the formation of ATP from ADP and Pi is a condensation, then ATP synthesis could be 'driven' by stabilizing water, ADP-OH + P-OH → ADP-OP + H_2O. He suggested that water was 'stabilized' by H^+ generated by electron transport through the respiratory chain. This stabilization of water would only be possible in a nonaqueous region of space and if done

by H^+, where H^+ was generated far from bulk water. He proposed that these events took place in a highly localised intramembrane space within which protons could exist at a very high activity.

Fig. 2.5 The localised proton model for ATP formation



Dotted lines represent membrane (not necessarily required). Solid lines represent a particle of high hydrophobicity within which ADP and Pi are condensed to ATP (by the ATPase).

The model above becomes chemiosmotic if,and only if,the membrane is present and all H^+ is released to phase (c) and all O_2^{2-} to phase (a).

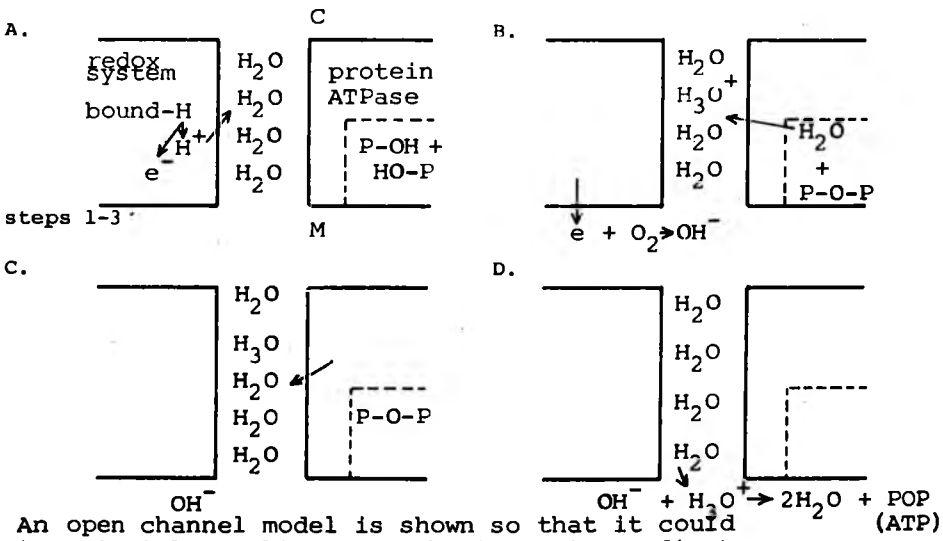
Williams argues that ATP synthesis would be controlled kinetically by the diffusion of $H^+(H_3O^+)$ from the active site (ATPase) which is hydrophobic in nature (or close to a hydrophobic site containing H^+) into the hydrophilic phase nearby (that is, to an aqueous phase or to bound charges localised near the hydrophobic (hydrophilic) interface). There are obvious similarities between the localised proton theory and chemiosmosis. Chemiosmosis would be a specific type of localised proton effect in which the diffusion of H^+ was controlled by the bulk (osmotic) concentrations of H^+ in the two aqueous phases ((a) and (c) in Fig. 2.5) topologically isolated from each other by the membrane. Williams has made several significant criticisms of chemiosmosis on theoretical grounds. These are discussed later in this chapter (97-98). Williams does not make specific proposals about the actual catalytic steps which produce ATP,as he believes that too little is known about the structure of bound ADP, Pi and ATP in ATP utilising and producing enzymes. He does, however,

propose a generalised model for a coupling mechanism between electron transport and ATP synthesis (Fig. 2.6).

Fig. 2.6 Indirect localised proton coupling model, after (97-8)

The model requires 3 sites, X, Y, Z. The reactions proceed →

		diagram
1.	ADP and Pi bound at X	A
2.	nH^+ generated at site Y (by electron transport apparatus)	A
3.	nH^+ diffuse to site Z. Site Y relaxes and can accept new reducing equivalents	A
4.	Binding of nH^+ at Z causes a conformational change in Z	B
5.	At X, ADP + Pi → ATP + H_2O (H_2O moves to Z to react with nH^+)	B
6.	Release of nH^+ or H_3O^+ from Z and release of ATP from Y	C
7.	Relaxation of Y and Z to receive next round of reactants	C-D



An open channel model is shown so that it could in principle be linked to chemiosmotic gradients although this is not required by the theory.

There is perhaps one other energy coupling hypothesis worth considering in some detail here; this is the 'paired moving charge' model proposed by D.E. Green (59).

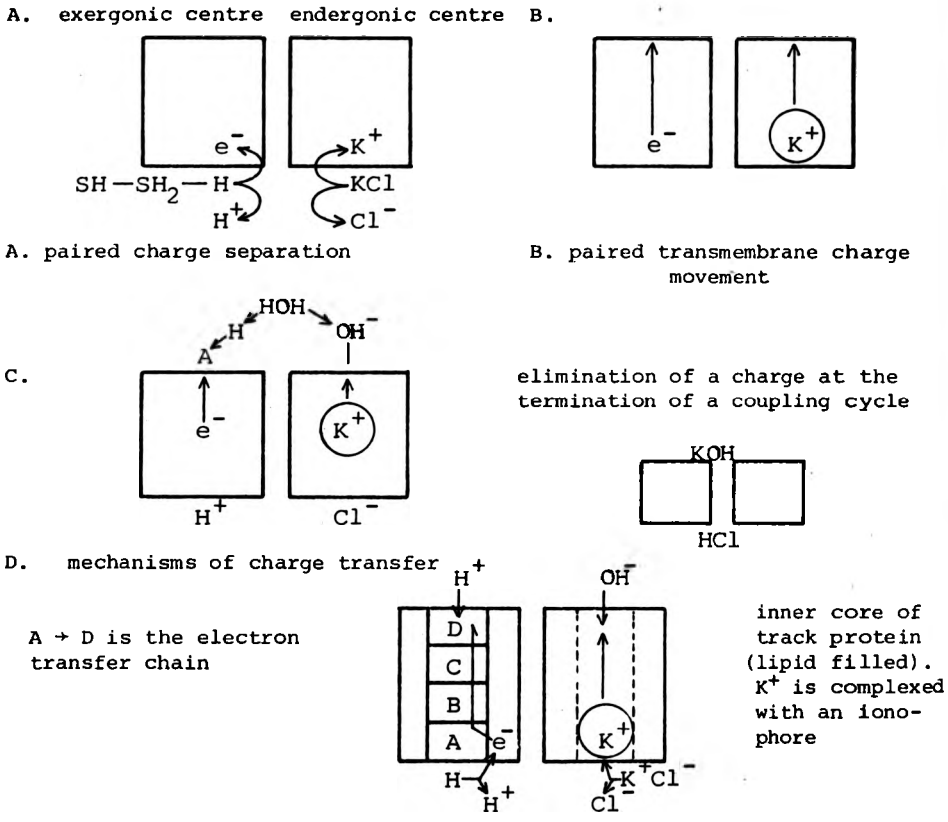
Paired moving charge (PMC) model.- This model has its roots in earlier hypotheses by Green (112) and Green and Ji (129) which were broadly

conformational in type. A brief description of it is not easy as Green's assumptions about the nature of the components of the inner mitochondrial membrane differ from most other workers in the field. He proposes that the electron transport components and the ATPase are arranged in ribbon structures in the membrane. These ribbon structures are composed on two 'continuums', the tripartite continuum and the electron transport continuum. The former contains all the systems required for coupled ATP synthesis, ATP hydrolysis, active transport of cations and the translocation of metabolites. The latter contains the four complexes of the respiratory chain. These four complexes and the ATPase are all multisubunit enzymes. Green proposes that among the components of these enzymes are a series of ionophoroproteins, ionophoroenzymes, track proteins and cation-binding proteins. It is these components that allow the charge pairing and charge movement essential to Green's hypothesis to take place.

Green proposes that there are two types of transmembrane charge flow involved in energy coupling. These are a symport charge flow (e^- and M^+ together) which favours active transport, and an antiport charge flow (e^- and A^- in opposite directions) which favours oxidative phosphorylation. He proposes a number of ground rules on which the PMC model is based (see Fig. 2.7 on following page).

The initial event in energy coupling is charge separation followed by paired charge movement and charge elimination. The charges move within tracks that traverse the membrane. The track may be the electron transport chain for e^- or the inner core of a track protein in the case of an ionophorous moving charge, K^+. ATP is generated by anionic forms of Pi and ADP, complexed with Mg^{2+} and ionophores to cationic forms, moving in symport with electron flow. Phosphate is used to phosphorylate AMP bound in the ATP synthase (144). The ADP produced by this then reacts with ADP to give bound AMP and 'free' ATP (Fig. 2.7).

Fig. 2.7 Ground rules for the PMC model



In the PMC model, control of coupling is not exerted on individual proteins but on proteins which are part of a continuum. The co-operative nature of the ribbon structure ensures that it is the ribbon as a unit which responds to the trigger action of the control mechanism. The mitochondrion would then behave as if it were one giant protein molecule that undergoes shape changes dictated by the ion fluxes in the membrane. Green proposes that there are two basic states of the membrane; the N^+ state induced by Mg^{2+} and the Ca^{2+} state induced by Ca^{2+} (Table 2.1).

Chemiosmosis and the PMC model both have a charge separation as the initial step in coupled processes; they differ, though, in several important aspects. In the 'PMC model charge' (a) separation is always paired, (b) coupling is designed to avoid rather than create a membrane

potential, (c) the flow of C^+ is directly coupled to e^- flow, (d) coupling does not require an osmotically intact membrane.

Fig. 2.8 PMC molecular mechanism for coupled ATP synthesis, after (59)

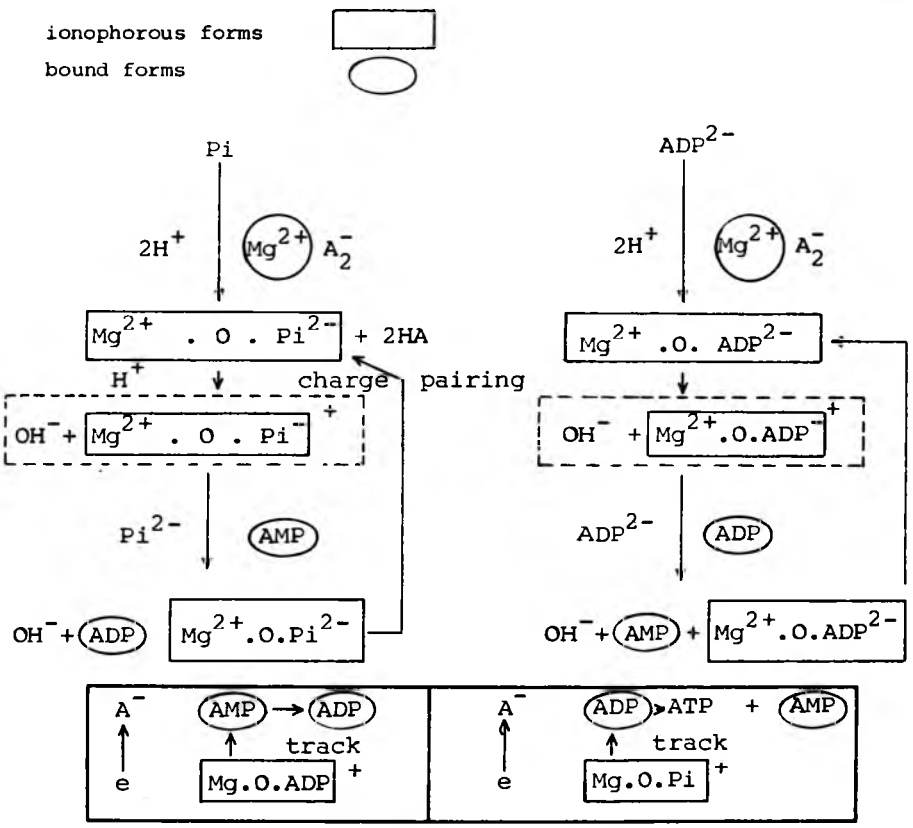


Table 2.1 Properties of the mitochondrion in the N^+ and Ca^{2+} state

	N^+ state	Ca^{2+} state
oxidative phosphorylation	+	o
coupled transhydrogenation	+	o
active transport of cations	+	o
cyclical transport of cations	o	+
respiratory control	+	o
permeability to sucrose	o	+
response to uncoupler	+	o
configuration	aggregated	orthodox

It is very difficult to assess the validity of the PMC model. Green appears to have isolated many of the ionophorous components and 'track proteins' that his theory requires. He also presents evidence from electron micrographs supporting his ideas on membrane structure (59, 189, 193, 227). There has been little or no evidence supporting his hypothesis published from outside his group, though it is fair to point out that this is largely because his ideas have been ignored. It is also worth noting that, if Green is right, then mitochondria and energy coupling processes are far more complicated than they have generally been thought to be.

The high energy state of the membrane '∿' generated by electron transport in coupled systems can be used to drive a number of reactions: these include ATP synthesis, nicotinamide nucleotide transhydrogenation, ion transport, mitochondrial shrinkage and swelling and reversed electron transport. The chemical and conformational hypotheses consider '∿' to be a chemical bond (or bonds), while the chemiosmotic hypothesis considers '∿' to be an electrochemical protonmotive force generated across the coupling membrane. It was one of the major theoretical triumphs of chemiosmosis that it predicted that mitochondria should display redox linked proton movements. These observations would not be expected from the chemical or conformational hypotheses in their earlier forms which later proposed that the observed proton movements were due to a '∿'-driven proton pump and that '∿' was in a close equilibrium with Δp. Δp would control ion transport processes across the inner membrane, but '∿' would be the primary high energy intermediate produced in the membrane.

The chemiosmotic hypothesis is the most testable of all current energy coupling hypotheses. The bulk of evidence appears to give support to it (see (58) for list of references). However, there is a (growing) body of experimental data and some theoretical considerations which strongly suggest that the hypothesis as presently stated should be modified or possibly dismissed. Some aspects of these criticisms of chemiosmosis will be reviewed.

The mechanism of uncoupling.- Uncoupling is the process by which the kinetic restraints acting between electron transport and ATP synthesis (or '∿' consuming processes)are released, dissipating '∿', usually as heat. A wide range of compounds possess this ability. A complete understanding of the mechanism of uncoupling would of necessity involve a similar understanding of the coupling mechanism. Investigation of the former should then throw light on the latter. Mitchell proposed that uncouplers are weak acids or bases, lipid soluble in both their protonated and unprotonated forms,and that they act by allowing a short circuiting of the proton gradient across the coupling membrane. Much evidence has been produced to support this hypothesis (58). Comparisons have been made of the effectiveness of uncouplers in mitochondria and in different artificial membrane systems. There appears to be a poor correlation between uncoupling in mitochondria and the uncouplers'ability to stimulate ion fluxes through black lipid membranes. There is a good correlation between uncouplers'uncoupling activity in mitochondria and their ability to collapse a proton gradient across liposome membranes containing artificial redox systems (433). Although there does appear to be a general correlation between uncoupling ability and the ability to transport H^+ across liposome membranes, this does not hold when the full range of uncouplers is tested for the ability to uncouple oxidative phosphorylation and collapse ΔpH across liposomes made from mitochondrial phospholipids. If this is done there is not an exact correlation between the two,which suggests that the ability to transport H^+ across lipid membranes is not the only factor involved in uncouplers' mode of action (397).

In chemiosmotic terms, uncouplers act by dissipating $-Z\Delta pH$. It is difficult then to understand quite how they manage to inhibit coupled reactions so completely. The proton motive force, Δp, is composed of two terms, $\Delta\psi$ and $-Z\Delta pH$. The contributions of $\Delta\psi$ and $-Z\Delta pH$ to Δp vary,

but it has been estimated that $-Z\Delta pH$ is usually < 50% of Δp and occasionally has a value of 0 (434, 453). $\Delta\psi$ is a term that maybe is related to ΔpH but does not directly relate to bulk concentrations of protons. Collapsing of ΔpH should leave $\Delta\psi$ intact; systems in which $\Delta\psi$ is $\simeq \Delta p$ should not be rapidly affected by uncouplers unless $\Delta\psi$ is somehow collapsed rapidly in response to ΔpH being collapsed or unless uncouplers directly affect $\Delta\psi$. This latter possibility may be what happens. Recent work by Green and coworkers suggests that uncouplers, singly and when paired with ionophores, are highly effective at transporting cations across phospholipid membranes (471-2). They could thus directly collapse $\Delta\psi$.

The chemical and conformational hypotheses propose that uncouplers act by dissipating '$\sim$' in the membrane possibly by hydrolysis of an anhydride-like intermediate. Williams suggests that uncouplers act by sequestering protons away from their intramembrane 'high activity' sites.

There is some evidence to suggest that uncouplers bind to protein components in the membrane.

Trinitrophenol penetrates the mitochondrial inner membrane only rather slowly, and is not an effective uncoupler in mitochondria. It is, however, a highly efficient uncoupler in SMP's,suggesting that it has to interact with a membrane site only accessible from the matrix side of the inner membrane (435).

Hanstein and Hatefi have synthesised an azido derivative of DNP, 2-azido-4-nitrophenol. This is a potent uncoupler which will in the presence of u.v. light covalently bind to protein groupings. They have demonstrated that this uncoupler appears to bind to two components in the mitochondrial membrane, an F_1 subunit and a 30,000 m.w. protein. This binding is specifically prevented by other uncouplers (DNP, PCP, CCCP, S13) but not by ATPase inhibitors. The 30,000 m.w. component is probably an F_O subunit (397, 420, 436). They suggest that this subunit

is responsible for, or involved in, the transduction of '∿' and that this may be a major locus of action for uncouplers in the mitochondrial inner membrane.

The precise mechanism of uncoupling thus remains obscure; see (397) for review.

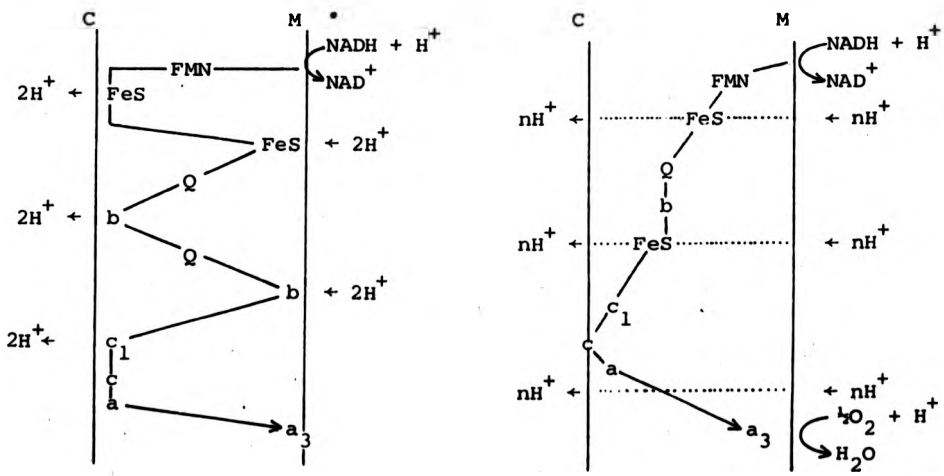
Function of the coupling membrane.- It is fundamentally implicit in the chemiosmotic hypothesis that a topologically closed, proton impermeable, membrane system is required so that a Δp may be maintained across it and collapsed through it to generate ATP. Coupled processes do not appear to take place in the absence of such as membrane (58, 261). There have in recent years,however, been a number of reports suggesting that such a membrane is not required for energy coupling to take place in mitochondria. Azzone and Masari reported that EDTA particles (SMP's stripped of F_1 by EDTA treatment) which do not have oxidative phosphorylation did have respiration-driven transhydrogenase activity (a coupled process utilising '∿'). These particles were osmotically leaky and were permeable to inulin and 15,000 MW dextrans. They concluded that the chemiosmotic terms $\Delta\psi$ and ΔpH, which are osmotically derived, play no direct role in energy conservation (430).

More interesting and possibly more important are the results reported by Komai et al. (431-2). Lysolecithin is a detergent and collapses membrane structure. Mitochondria treated with lysolecithin have no vesicular structure observable under the electron micrsocope. They do, however, retain coupled functions. These functions are insensitive to ionophores but retain sensitivity to uncouplers and ATPase inhibitors. Komai et al. claim that these results present clear evidence that topologically closed, proton impermeable, membrane structures are not essential for energy coupling processes to take place in mitochondria. Support for the idea that closed membrane vesicles are not essential prerequisites for energy coupling comes from the reported

purification of an ATP-Pi exchangease, complex V ATPase, by Hatefi and coworkers (350, 244). They claim that purified complex V, which has too low a phospholipid content to form protein-lipid vesicles, displays high ATP-Pi exchange rates. This reaction has generally been thought to be due to a reversal of ATPase activity generating ATP. Chemiosmotic theory proposes that it should therefore require a closed vesicular system in which to take place. Hatefi's results suggest that this may not be so. If Komai's and Hatefi's results are confirmed then they may invalidate postulate 4 of the chemiosmotic hypothesis.

The transmembrane distribution of respiratory chain redox carriers.- Chemiosmotic theory requires a specific transmembrane distribution of redox carriers to form the proton translocating loops 1, 2 and 3 (58) (Fig. 2.9A). Present evidence indicates that the actual transmembrane distribution would not allow transmembrane H^+ (or e^-) transfer via a series of loops (222) (Fig. 2.9B).

Fig. 2.9 Transmembrane distribution of respiratory chain redox centres, after (222)



A: distribution required for chemiosmotic loops 1, 2 and 3 (58)

B: Actual distribution (see also Fig. 2.10)

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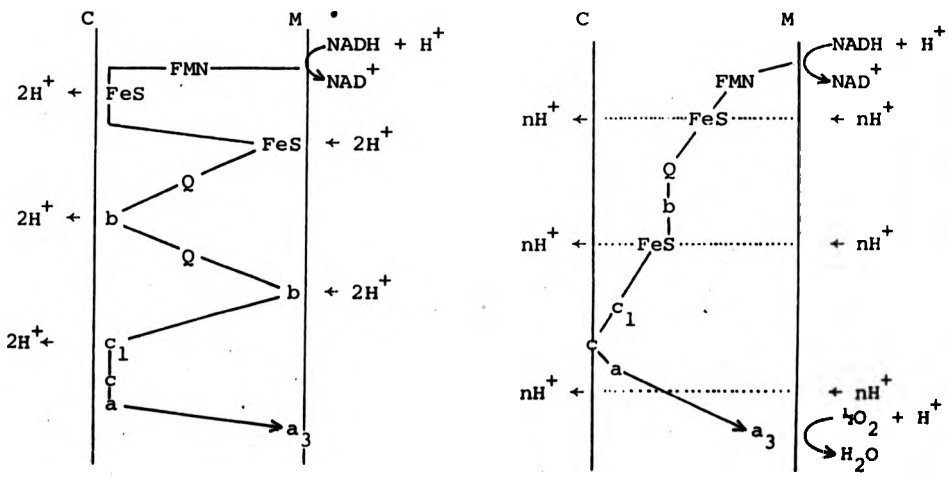
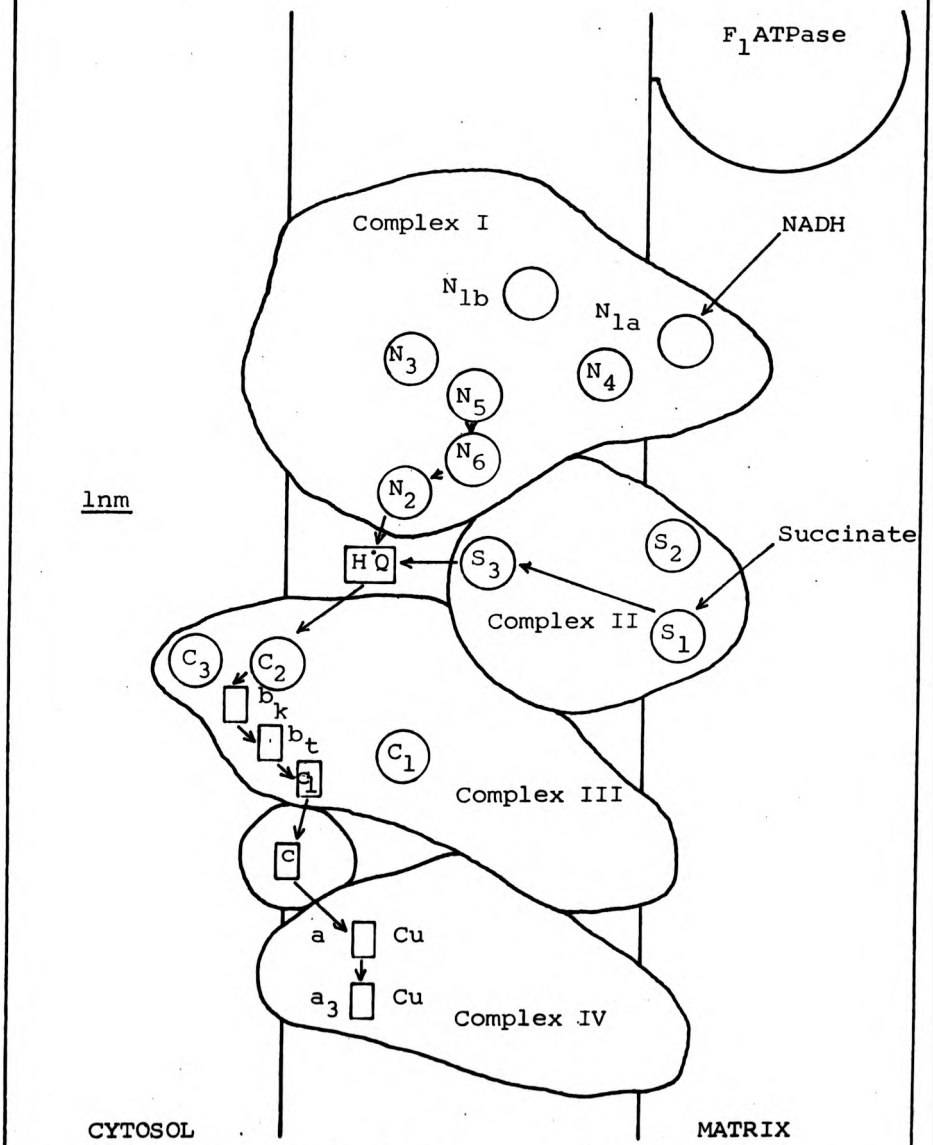


A: distribution required for chemiosmotic loops 1, 2 and 3 (58)

B: Actual distribution (see also Fig. 2.10)

Fig 2.10. Structural model of the respiratory chain components in the mitochondrion, after (437-8).



Mitchell has partially recognised this by discarding loops 2 and 3 and replacing them with a proton motive,loop 2-3, cycle (Fig. 7.1) (342-3). This, however, requires a certain distribution of the complex III b cytochrome across the inner membrane. Present evidence suggests that they lie at similar depths in the membrane and that transmembrane electron flow between them would be unlikely (Fig. 2.10).

Onishi and coworkers have used paramagnetic ions and e.s.r. techniques to investigate the transmembrane distribution of redox centres (437-8). These studies probably give the best picture currently available (Fig. 2.10). If this picture is correct then it precludes the existence of any proton translocating 'loops' across the membrane. It does not, however, exclude a chemiosmotic mechanism which uses proton pumps to generate ΔpH.

The role of the proton motive force and membrane potential in bioenergetic systems.- Chemiosmotic theory states that the respiratory chain generates a proton motive force, Δp, composed of two osmotic terms, a membrane potential, Δψ and a proton gradient term, $-RTLn_e \Delta pH$. Δp is collapsed through the ATPase generating ATP or through other endergonic systems, e.g. the energy linked nicotinamide nucleotide transhydrogenase. The rate and extent of coupled processes such as ATP synthesis should then be directly related to the magnitude of Δp. Much research effort has been devoted to measuring Δp,Δψ and ΔpH in bioenergetic systems. Assuming an H^+/ATP ratio of 2, then a Δp of ∿ 250 mV would be required to drive the synthesis of 1 ATP per coupling site (1-3). Measurements made by Mitchell and others indicate that a Δp of ∿ 200-250 may indeed exist across bioenergetic membranes (see refs. (58, 261) for extensive reviews and references relating to these investigations). There are workers, however, who do not accept that Δp and Δψ are of this magnitude and that they are directly related to the energy coupling capacity of the membrane systems tested.

The most tenacious and longstanding opponent of the idea that there is a significant metabolically dependent membrane potential in mitochondria is probably Henry Tedeschi. Tedeschi has measured mitochondrial inner membrane potential directly, using microelectrodes,and has concluded from these studies that it is small (9-15 mV), does not alter when the metabolic state of the mitochondria alters and that it is +ve inside (chemiosmosis requires that it is -ve inside) (449, 450). This work has been much criticised (22) but more recent work by Tedeschi,using a series of dyes to estimate the electrical potential of the membrane,largely confirms his earlier findings (451). Here he reports that $\Delta\psi$ is variable and usually very low, even in the presence of respiratory substrates. He concludes that the results indicate that a large membrane potential is not established on the initiation of metabolism and that it does not play a significant role in ADP phosphorylation. These results have generally been ignored or dismissed, a fate not worthy of the excellence of the reported work. Tedeschi considers that all observable membrane potentials are due to simple Gibbs-Donnan type effects and can be described by the Nernst equation (470).

Other workers have reported that the measurable inner mitochondrial membrane potential and proton motive force is too small to account for observable ATP synthesis (452).

While many workers would not accept this, there is a growing body of evidence suggesting that there is no strict relationship between Δp and the phosphorylation rate (453-5).

Proton translocation; H^+/ν ratios.- Experimental observation has apparently demonstrated that mitochondria and bacteria are able to eject protons into the suspending medium during respiration by a process closely linked to electron transport down the respiratory chain (58). There have been differing views as to how this proton translocation is

achieved. Mitchell proposed that it is due to the vectorial arrangement of alternating H^+ and e^- carriers in the inner membrane into chemiosmotic loops (261, 58) and into more complex cyclic loops (342-3). Some authors, while broadly accepting the four basic postulates of chemiosomosis, reject the loop hypothesis and consider the redox pump in more general terms with H^+ translocation driven by electron transport dependent pK changes or similar effects (456). Indirect proton pumps models have been postulated, driven by a 'high energy' intermediate generated by electron transport (457) or by an energy linked conformational change driving an electroneutral cation-H^+ exchange (458).

Mitchell and Moyle measured the $H^+/\sim$, ($H^+/2e^-$, H^+/site) ratios of the entire respiratory chain and found them to be in the region of $2H^+/\sim$ at each site, with no H^+ extrusion at the level of cytochrome oxidase (459). Much support has been forthcoming for the ejection of two H^+/site by the respiratory chain (58) and it has been incorporated into the theoretical framework of chemiosmotic H^+ ejection and ATP synthesis (72, 66).

This work has, however, been challenged, firstly by Pressman and coworkers who reported $K^+/\sim$ (equivalent to $H^+/\sim$) ratios 4-7 $K^+/\sim$ (460), then by Azzone and coworkers who reported $K^+/\sim$ ratios of $4K^+/\sim$ (461, 458, 448),and more recently by a large body of work emanating from the laboratories of Lehninger (462-6) and of Brand (467, 472) which reports charge/site ratios of 4 and H^+/site ratios of 3-4 H^+/site. These higher $H^+/\sim$ and charge/site ratios have been obtained by taking into consideration the movement of certain anions, notably phosphate which buffers H^+ ion movement in the experiments described by Mitchell and Moyle. Phosphate can be removed from the system,or phosphate transport prevented by blocking it with N-ethyl maleimide; this gives higher $H^+/\sim$ ratios (448, 466). This approach has been criticised by Moyle and

Mitchell (468), although it is fair to point out that these criticisms are far from convincing (466).

These charge/∿ ratios of 3-7 charges/∿ cannot be incorporated into a chemiosmotic theory requiring Mitchellian redox loops (58) or his mechanism of ATP synthesis (66). They can, however, be incorporated into one in which proton ejection is effected by redox linked proton pumps.

The conditions under which respiration-driven H^+ transport is observed are highly artificial; that is, compounds to collapse $\Delta\psi$ and compounds to prevent phosphate transport are usually present. The respiratory chain is often blocked in various places with various inhibitors. This has led some authors to question the physiological relevance of the observed H^+ movements (97, 98, 409). Archold et al. have reported that under physiological conditions there is no observable H^+ ejection by the mitochondrion during ATP synthesis (469). They also report that ATP synthesis is accompanied by the masking and unmasking of fixed charge sites on the inner membrane which may be important in regulating $\Delta\psi'$, which may control the rate of ATP synthesis.

The role of cytochrome oxidase.- The chemiosmotic hypothesis, as described by Mitchell (58), requires that cytochrome oxidase constitutes the electron translocating arm of loop 3 catalysing vectorial transport of e^- between cyt c, on the C side of the membrane and a_3 on the M side. A large body of evidence has accumulated supporting this idea (58, 222, 439). Recent work by Wikstrom and others has, however, fundamentally challenged this idea by suggesting that cytochrome oxidase acts as a proton pump, pumping H^+ from the M phase to the C phase (440-1). This, if it happened, would short-circuit any redox loop operating at this level. The experimental evidence supporting the idea has been vigorously contested by Mitchell and others (442-3). The actual role of cytochrome oxidase in proton transport remains uncertain, although

a growing body of evidence now exists suggesting that it does act as a proton pump,as proposed by Wikstrom (440-1, 444-8). If this is the case, any chemiosmotic mechanism proposed with redox loops as the method of H^+ translocation would be invalid.

Williams' criticisms of chemiosmosis.- Although Williams 'localised proton' model has some features in common with chemiosomosis, he has always had several severe theoretical criticisms of chemiosmosis. There is not space here to go into these in detail (see (95-8, 474-6) for a further discussion) but several of his most prominent criticisms can be summarised.

Mitochondria lie in the cell cytoplasm; this has a high buffering capacity. Williams says that attempts to form pH gradients against this buffering capacity represent an energy leak rather than a form of stored energy. This criticism has bever been answered thoroughly although claims have been made that it has (66, 97). Mitchell has suggested that H^+ diffusion away from the outer side of the inner membrane may be restricted so that it is not buffered by the cytoplasm. If this is so, then the basic mathematical formulation of chemiosmosis, $\Delta p = \Delta\psi - Z\Delta pH$, must fall as $\Delta\psi$ and ΔpH are osmotic terms that describe bulk concentrations of ions and protons.

As stated above,in chemiosmosis $\Delta p = \Delta\psi - Z\Delta pH$, that is, the general energy across the whole membrane is given by

$$\Delta G = \psi + \Delta pH$$

For extreme local models, Williams states

$$\Delta G = \Delta(\Delta G^{\circ}) + \psi'$$

where ψ' is the membrane potential due to fixed charges and $\Delta(\Delta G^{\circ})$ is the chemical potential of the system (not including any contribution from fixed charges). Williams claims that Δp does not give an adequate description of the free energy change of protons across the coupling site which is given by

$$\Delta G = \Delta(\Delta G^{o}) + \Delta\psi' + Z\Delta pH$$

$$\Delta G = \Delta p + \Delta(\Delta G^{o}) + \Delta\psi' - \Delta\psi$$

Chemiosmosis does not take account of the terms $\Delta\psi'$ and $\Delta(\Delta G^{o})$ which may make important contributions to the energy available to ATP synthesis (469, 473, 97, 98, 475). Williams also suggests that the natural elasticity of bioenergetic membranes makes energy conservation in bulk osmotic gradients of ions across the membrane unlikely (475-6).

There have been indications that thiol groups may be closely involved in the mechanism of energy coupling (318). Several mechanisms of oxidative phosphorylation which involve the formation of thioesters have been proposed, e.g. (385, 386, 377). Model reaction systems linking ATP synthesis to the oxidation of thiols have been constructed. These mimic some of the properties of oxidative phosphorylation remarkably (see (472) and following three papers).

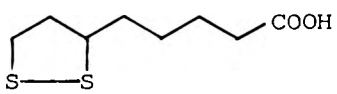
Griffiths has reported that derivatives of lipoic acid and oleic acid have the ability to drive ATP synthesis in ATPase systems (248). The involvement of these and other lipids in the energy coupling systems of the mitochondrial inner membrane was investigated.

CHAPTER 3

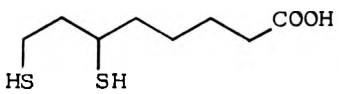
THE INTERACTION OF LIPOIC ACID AND RELATED COMPOUNDS WITH ENERGY COUPLING SYSTEMS

Introduction

Lipoic acid was first described as a growth requirement for micro-organisms and later as an 'acetate replacing factor', protogen A (360-2). It was eventually isolated in pure form but very low yield from a water insoluble extract of beef liver. The structure was later demonstrated to be 1,2-dithiolane-3-valeric acid, i.e.



also known as αDL 6,8-thioctic acid or lipoic acid. The disulphide bridge in the compound is easily reducible, giving dihydrolipoic acid.



Its described role as a coenzyme has so far been confined to a relatively small number of enzymes, most notably pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, lipoic acid reductase, transacetylase or succinylase, lipoamide oxidoreductase, lipoamidase and a lipoic acid activating system (363).

The α-keto acid dehydrogenases catalyse the following overall reaction in mammalian systems:-

$$RCOCOOH + HS - CoA + NAD^{+} \longrightarrow RCO \sim SCoA + CO_2 + NADH + H^{+}$$

They are the only known enzymes that contain a protein bound lipoic acid (bound as an amide linkage with a εNH_2 group from lysine). There are two distinct enzymes in mitochondria, pyruvate dehydrogenase and α-ketoglutarate dehydrogenase. The α-keto dehydrogenase exists as multienzyme complexes with a molecular weight of several millions (364).

Three types of enzyme have been isolated from these complexes:-

(i) a thiamin pyrophosphate containing decarboxylase;

(ii) a lipoic acid containing lipoic acid reductase transacylase;

(iii) an FAD containing lipoamide oxidoreductase.

The individual enzymes are linked by non-covalent bonds between lipoic acid reductase transacylase and the other two enzymes (there appear to be no bonds between the decarboxylase and lipoamide oxidoreductase)(365).

Pyruvate dehydrogenase obtained from Escherichia coli contains 12 molecules of pyruvate decarboxylase of M.W. 183,000 each; 6 molecules of lipoamide oxidoreductase, M.W. 112,000 each and 1 aggregate of lipoic acid reductase transacetylase, M.W. 1,600,000. Lipoamide oxidoreductase contains 2 moles FAD per mole enzyme. Lipoic acid reductase transacetylase contains one mole protein bound lipoic acid per 35,000 grams protein.

The α-ketoglutarate dehydrogenase complex differs from pyruvate dehydrogenase in several respects. Its lipoic acid reductase transsuccinylase (equivalent to transacetylase) has a molecular weight of 1,200,000 and the final amino acid sequence for protein bound lipoic acid is slightly different.

gly-asp-lipoyllys-ala — lipoic acid reductase transacetylase

thr-asp-lipoyllys-val-(val-leu)-glu-NH_2 — lipoic acid reductase transsuccinylase

Lipoic acid reductase transsuccinylase contains one mole lipoic acid per 84,000 grams protein.

The lipoamide oxidoreductases of the complexes appear to be identical. These are the most studied of all the lipoic acid utilising enzymes, they catalyse the following reaction:-

$$\underset{\text{(dihydrolipoamide)}}{\text{dihydrolipoate}} + NAD^+ \longleftarrow \underset{\text{(lipoamide)}}{\text{oxidised lipoic acid}} + NADH + H^+$$

They are also known by the generic term diaphorases and also as lipoamide dehydrogenases. Lipoamide dehydrogenase was first isolated by Straub (366) as a 'flavoprotein diaphorase'. The pure enzyme has a yellow colour and displays absorption and fluorescence spectra characteristic of a flavoprotein. The holoenzyme can be dissociated under appropriate conditions into a colourless apolipoprotein and a prosthetic group which was shown to be FAD (367-8). The enzyme contains ∿ 2 moles FAD/mole.

The bound lipoic acid contained in lipoic acid reductase transacetylase is stable and does not appear to exchange with free lipoic acid. This will only exchange into the apoprotein in the presence of a 'lipoic acid activating system' which catalyses the formation of an energy rich adenyl lipoate.

$$\text{lipoic acid} + \text{ATP} \rightleftharpoons \text{AMP lipoate} + \text{PPi}$$

$$\text{AMP lipoate} + \text{apoenzyme} \longrightarrow \text{holoenzyme} + \text{AMP}$$

Adenyl lipoate will not replace the activating system. There is also an enzyme, lipoamidase, which catalyses the hydrolysis of protein bound lipoic acid from the holoenzyme.

The major biochemical reactions so far known to involve lipoic acid can then be summarised:-

$$\text{RCOCOOH} + \text{TPP-E}_1 \longrightarrow \text{RCH(OH)} - \text{TPP-E}_1 + \text{CO}_2$$

$$\text{RCH(OH)} - \text{TPP-E}_1 + \text{lipS}_2 - \text{E}_2 \rightleftharpoons \text{RCO} - \text{S} - \text{lipSH} - \text{E}_2 + \text{TPP-E}_1$$

$$\text{RCOS} - \text{lipSH} - \text{E}_2 + \text{HSCoA} \rightleftharpoons \text{lip(SH)}_2 - \text{E}_2 + \text{RCO} \sim \text{SCoA}$$

$$\text{lip(SH)}_2 - \text{E}_2 + \text{FAD} - \text{E}_3 \rightleftharpoons \text{FADH}_2 - \text{E}_3 + \text{lipS}_2 - \text{E}_2$$

$$\text{FADH}_2 - \text{E}_3 + \text{NAD}^+ \rightleftharpoons \text{FAD} - \text{E}_3 + \text{NADH} + \text{H}^+$$

E_1: TPP containing decarboxylase; E_2: lipoic acid reductase transacylase; E_3: lipoamide oxido reductase. (For a more detailed discussion of these reactions, see discussion to Chapter 5).

The open structure of the dehydrogenase multienzyme complex and the fixed positions of the subunit enzymes make it difficult to envisage

how lipoic acid firmly bound to one of these enzymes can react with the active sites of the other two enzymes. This has led Reed to propose the following mechanism (369) (see Fig. 3.1).

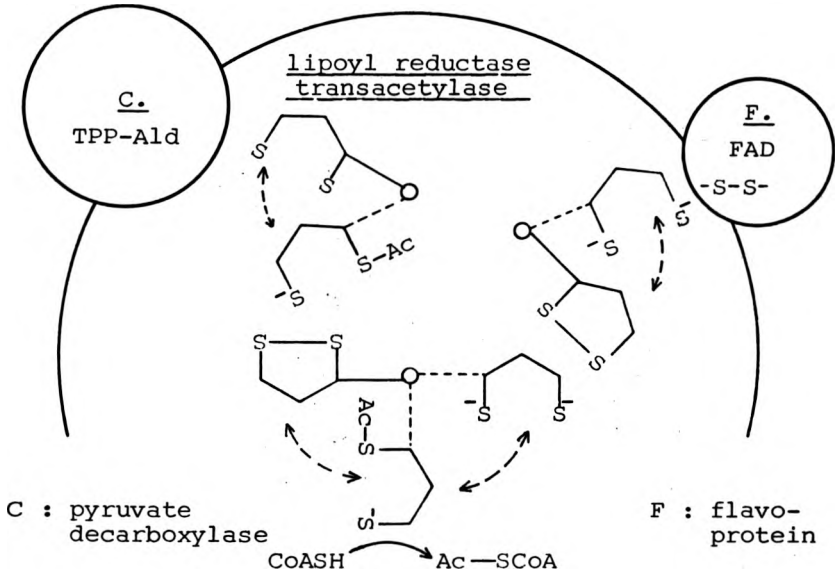


Figure 3.1

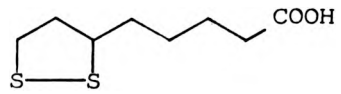
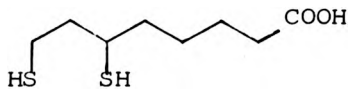
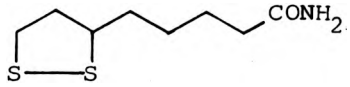
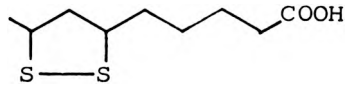
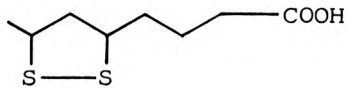
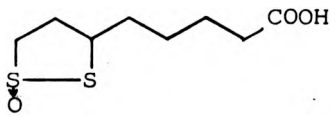
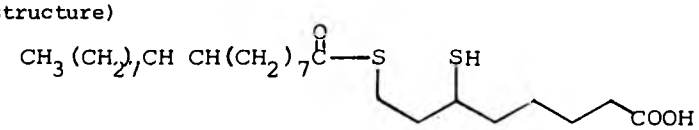
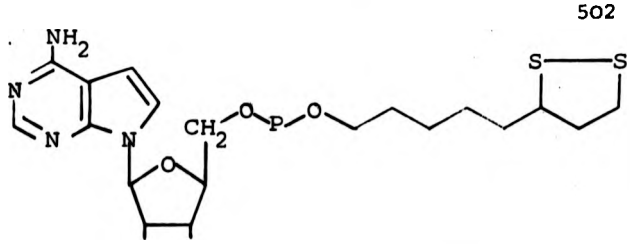
Conformational changes in the three enzymes and disulphide interchanges have also been proposed to explain interactions between the protein complexes (370).

Work in this laboratory on the mode of inhibition of energy linked reactions by dibutylchloromethyltin chloride suggested that the inhibitor titrated a lipophilic non-protein component of the mitochondrial inner membrane. This was later identified as either lipoic acid or a closely related compound (315,317,235,371). Dihydrolipoic acid was also shown to drive the synthesis of ATP in a variety of ATPases derived from mitochondria (248,372). Aspects of this 'dihydrolipoate'-driven ATP synthesis were investigated.

Materials

All chemicals used were of AnalaR or similar grade. Organic solvents of AnalaR grade were redistilled before use. 8-Methyl lipoate

Table 3.1 Lipoic acid and related compounds: structures

Name	Structure	M.W.
lipoic acid αDL 6,8-thioctic acid		206
dihydrolipoic acid		208
lipoamide		207
8-methyl lipoic acid		220
7-methyl thiheptic acid		192
β-lipoic acid (several forms)		222
oleoyl-S-lipoate (presumed structure)		472
adenyl lipoate		502

was a gift from Dr. J.R. Guest, Department of Microbiology, University of Sheffield. The uncoupler 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole, TTFB, was a kind gift from Professor B. Beechey, Shell Research Ltd., Sittingbourne, Kent. Oligomycin, fatty acyl chlorides, lipoic acid, dihydrolipoic acid, lipoamide, valinomycin, gramicidin D, fatty acids, carbonyl cyanide-M-chlorophenylhydrazone (CCCP) were obtained from the Sigma Chemical Company. Dicyclohexyl carbodiimide (DCCD) was obtained from B.D.H. Ltd. 1,1,5,5-trifluoromethyl-1,5-hydroxy-pentan-3-one ('1799') was a kind gift from Dr. P. Heytler. Nigericin and efrapeptin were obtained from Dr. R. Hamill, Eli Lilley & Co.

Methods

Freshly excised bovine hearts were brought on ice from a nearby slaughter house; all subsequent operations, preparation of submitochondrial particles and enzymes, were carried out at 4° C unless otherwise stated. Fat, connective tissue and ligaments were carefully trimmed from the muscle tissue which was then cut up in to 2 cm cubes and passed through a meat grinder. The resulting minced tissue was resuspended in 2 volumes ice cold 0.25 M sucrose, the pH was adjusted to pH 7.0-7.5 by addition of 1 M Tris base, and the suspension was homogenised in a Waring tissue blender for 40 seconds at top speed. The suspension was then pH'd to a stable pH 7.0-7.5 by the addition of 1 M Tris base with rapid stirring; care has to be taken at this stage to ensure that the pH is stabilised and is not above 7.5. The suspension was then centrifuged at 2,000 rpm in a Mistral 6L centrifuge 4 x 1.25 L rotor for 30 minutes at 4° C. The supernatant was passed through 4 layers of muslin and the pH adjusted to 7.5 with 1 M Tris base before being centrifuged at 10,000 rpm for 20 minutes in a Sorvall RC-2B centrifuge G.S.A. rotor, pre-cooled to 4° C. The supernatant from this spin was carefully removed by aspiration, as was the top light

layer of the pellet. Any fat lining the centrifuge bottle was then carefully removed by wiping with a tissue. The dark brown mitochondrial pellet was then resuspended and homogenised in a glass homogeniser fitted with a teflon pestle in 0.25 M sucrose; 10 mM Tris-Cl, pH 7.5, diluted to 0.5 - 1.0 litres in the same buffer and recentrifuged at 10,000 rpm for 20 minutes in a Sorvall RC-2B centrifuge G.S.A. rotor. The supernatant was removed as previously and the pellet resuspended in 0.25 M sucrose, 10 mM Tris-Cl and recentrifuged as above. The pellet from this centrifugation was resuspended in 0.25 M sucrose; 10 mM Tris-Cl, pH 7.5; 5 mM $MgCl_2$ as recentrifuged as above. The mitochondrial pellet was resuspended to 40 mg protein/ml in 0.25 M sucrose; 10 mM Tris-Cl, pH 7.5; 1 mM EDTA often 5 mM in succinate; 2 mM in ATP; 5 mM in $MgCl_2$ and either frozen immediately or kept at 4° C as required.

Submitochondrial particles were prepared by sonication of mitochondria. Frozen mitochondria were thawed rapidly and diluted to $\sim$ 10 mg/ml protein concentration and sonicated for 1 minute (4 bursts of 15 seconds interspersed by 30 second gaps) in an MSE 60W sonicator at maximum amplitude. The suspension was centrifuged at 15,000 rpm for 15 minutes in a Sorvall RC-2B centrifuge SS34 rotor. The supernatant was then centrifuged at 100,000 g for 30 minutes in a Beckman L2-50 centrifuge, 40, 50 Ti or 65 rotor. The pellet from this centrifugation was resuspended in 0.25 M sucrose; 10 mM Tris-Cl, pH 7.5; 1 mM EDTA and recentrifuged as above. The submitochondrial pellet was resuspended in the same buffer to a final protein concentration of $\sim$ 20 mg/ml.

Electron transport particles (ETP_H) were prepared by the same method as submitochondrial particles except that the sonication buffer contained 2 mM sodium succinate; 2 mM ADP; 5 mM $MgCl_2$.

Complex V ATPase was prepared as described by Hatefi and co-workers (350, 244), using bovine heart submitochondrial particles as a starting

material. SMP's were pelleted and resuspended in 0.66 M sucrose; 50 mM Tris-Cl, pH 8.0; 1 mM histidine to a protein concentration of 23 mg/ml. Potassium deoxycholate was added to a final concentration of 0.3 mg/mg protein from a stock solution 10% w/v, pH 9.0. Potassium chloride was then added to 72 g/L and allowed to dissolve at 4° C with stirring. The suspension was then centrifuged at 30,000 rpm for 30 minutes in a Beckman 30 rotor. The precipitate was discarded (or used for cytochrome oxidase preparations) while the reddish supernatant was diluted with 0.25 volumes of water and recentrifuged at 30,000 rpm for 40 minutes in the No. 30 rotor. The supernatant from this step was dialysed against 10-20 volumes 10 mM Tris-Cl, pH 8.0 for 3 hr at 4° C. The dialysate was centrifuged for 90 minutes at 30,000 rpm in the No. 30 rotor. The upper 2/3 of the supernatant from this step was removed from the loosely packed pellet, frozen in liquid nitrogen and stored until used. This supernatant was then passed through a Sephadex G25 column pre-equilibrated and eluted with 10 mM Tris-acetate, pH 7.5 at 100-130 ml per column of size 10 x 22 cm at 4° C. The pink column eluant was taken to 42% saturation with neutral saturated $(NH_4)_2SO_4$ at 4° C and centrifuged for 15 minutes at 70,000 g. The pellet from this step was resuspended in 0.25 M sucrose; 10 mM Tris-acetate, pH 8.0 to a concentration of 15-20 mg/ml protein. Potassium cholate was then added to 0.35 - 0.38 mg cholate/mg protein (from a 20% w/v stock solution, pH 7.9) followed by $(NH_4)_2SO_4$ to 25% saturation. The suspension was then centrifuged after 10 minutes stirring at 4° C at 40,000 rpm for 15 minutes in a No. 40 rotor. The precipitate was discarded and the supernatant taken to 42% saturation with saturated $(NH_4)_2SO_4$ at 4° C. The supernatant was centrifuged as before and the pellet collected, redissolved to ∿ 20 mg/ml in 0.60 M sucrose; 50 mM Tris-Cl, pH 8.0; 1 mM histidine, frozen in liquid nitrogen and stored until used. This is complex V ATPase.

'Proton translocating' ATPase was prepared essentially as described by Serrano et al. (245). Bovine heart mitochondria, 40 mg protein/ml in 0.25 M sucrose were diluted with water and 0.1 M sodium pyrophosphate, pH 7.4 to 20 mg protein/ml and 0.01 M sodium pyrophosphate and then sonicated for 2 minutes (20 second bursts interspersed by 30 seconds) in an M.S.E 60W sonicator at maximum amplitude before being centrifuged at 26,000 g for 15 minutes. The supernatant was then centrifuted at 105,000 g for 45 minutes. The pellet from this stage was resuspended in 10 mM Tris-SO_4, pH 7.5; 0.5 mM EDTA; 1 mM $MgSO_4$; 10% satd. $(NH_4)_2SO_4$; 1.5% cholic acid neturalised with NaOH to pH 7.5 (last addition) to a protein concentration of 25 mg/ml, Buffer systems described by Serrano et al. (245) all contain 0.5 mM DTT. This was omitted in any ATPase preparations with which dihydrolipoic acid or derivatives were to be used. The suspension was stirred @ 4° C for 7 minutes and centrifuged at 50,000 rpm for 50 minutes in a Beckman No. 60 rotor. The supernatant from this step was taken to 38% saturation by addition of 0.45 ml saturated $(NH_4)_2SO_4$/ ml buffer. This suspension was then centrifuged at 15,000 g for 15 minutes in a Sorvall SS34 rotor. The clear supernatant was taken to 42% saturation by addition of 0.127 ml saturated $(NH_4)_2SO_4$ per ml and recentrifuged as above. The pellet from this step contains 'proton translocating' ATPase and was resuspended in 50 mM sucrose; 10 mM Tris-SO_4; 0.5 mM EDTA; 1 mM $MgCl_2$, pH 7.5 to 20 mg protein/ml, giving a clear pale yellow solution which was frozen in liquid nitrogen and stored at -20° C until use.

Rat liver mitochondria.- Rats were killed by stunning and partial exsanguination. The abdomen was washed with 90% ethanol, the liver dissected out and washed in three changes of 0.25 M sucrose and transferred to a tared beaker. Approximately 15 g of liver tissue was homogenised in 40 ml of 0.25 sucrose; 1 mM EDTA with 2 to 3 passes of

a glass-teflon homogeniser. The homogenate was centrifuged twice at 800 g for 20 minutes and the pellets discarded. The supernatant was centrifuged at 9,000 g for 10 minutes and the supernatant and loosely packed pellet (upper layer) of microsomes discarded. The brown,hard packed pellet of mitochondria (lower layer) was resuspended in 0.25 M sucrose; 1 mM EDTA in a homogeniser and centrifuged at 9,000 g, the supernatant and upper layer (pink) of microsomes again being discarded. The mitochondrial pellet was washed again and finally resuspended to 20 mg protein/ml in 0.25 M sucrose; 10 mM Tris-Cl, pH 7.5; 1 mM EDTA.

Escherichia coli and Rhodospirillum rubrum were prepared as described in Chapter 5. A DCCD sensitive F_1F_O type ATPase enzyme was prepared from Escherichia coli vesicles by the method of Friedl et al. (351).

Oxidative phosphorylation.- Oxidative phosphorylation was assayed in a glucose-hexokinase trap system. Assays containing 0.5 mg - 1.0 mg enzyme in 1 ml of 0.25 M sucrose; 20 mM glucose; 20 mM Tris-Cl, pH 7.3; 2 mM $MgCl_2$- 0.2 mM EDTA; 4 mM K_2HPO_4 either 0.2 mM or 2 mM in ADP and several units of hexokinase (usually either Sigma type F-300 or C-300),ranging from 1-5 in the case of mitochondria or bacterial vesicles to 10-25 units in the case of purified ATPase enzymes,were preincubated with any inhibitors, e.g. rotenone, antimycin A, oligomycin, DCCD, uncouplers, ionophores, etc. for 5 minutes prior to initiation of assay in a wide-based phosphorylation tube in a shaking, heated water bath at 30° C. Assays were usually run for 10-30 minutes prior to termination. Assays were terminated by taking 0.2 ml aliquots into 0.2 ml 10% trichloroacetic acid (TCA) or 0.2 ml perchloric acid (PCA). Coagulated protein was removed by centrifugation in a bench centrifuge. Oxidative phosphorylation was measured either as the disappearance of phosphate from the medium or as the appearance of glucose-6-phosphate.

Estimation of glucose-6-phosphate.- ATP can act as a phosphate donor to glucose in the presence of the enzyme hexokinase (ATP:D-hexose-6-phosphotransferase EC 2.7.1.1) producing glucose-6-phosphate:-

$$\text{ATP} + \text{D-glucose} \xrightleftharpoons{\text{hexokinase}} \text{D-glucose-6-phosphate} + \text{ADP}$$

This reaction is used as the basis for the assay of ATP produced by oxidative phosphorylation. ATP is used by a glucose-hexokinase trap system to produce glucose-6-phosphate; the ADP released by this reaction can then be rephosphorylated by the ATP synthase system to produce ATP. This allows the ADP concentration of the assay to be kept low as ADP can recycle until the free phosphate concentration of the assay becomes limiting.

After oxidative phosphorylation was terminated by taking an 0.5 ml aliquot from the glucose-hexokinase trap system into 0.2 ml 1 M P.C.A. coagulated protein was removed by centrifugation. An 0.5 ml aliquot from the supernatant of this step was taken into sufficient 6 M KOH to neutralise it (75 µl). The tubes were kept at 0° C for 20 minutes to ensure complete precipitation of $KClO_4$ which was then removed by centrifugation. An 0.1 ml supernatant aliquot was taken into 1.9 ml 0.5 M Tris-Cl, pH 7.5, containing 0.5 µmoles $NADP^+$ and 2-3 units G6P dehydrogenase which converts G6P to glucono-δ-lactone-6-phosphate with the concomitant reduction of $NADP^+$ to NADPH. NADPH was estimated from its absorption at 340 nm: NADPH E_{mM} = 6.22 cm^2/µmole. The amount of ATP (added as NADPH was virtually 100%.

Estimation of inorganic phosphate.- Phosphate was determined by the method of Fiske and Subbarow (352). This method is based on the formation of a phosphate molybdate complex which can be assayed spectrophotometrically. Phosphate will, in acid conditions, form a phosphomolybdous complex with ammonium molybdate. This can be reduced by an appropriate reducing agent (1-amino-2-naphthol-4-sulphonic acid) to a phosphomolybdic complex which has a characteristic blue colour that can be assayed spectrophotometrically.

Assay Procedure.- Inorganic phosphate (10-500 nmoles) in 0.5 ml water is added to an assay tube containing 1.5 ml water and 0.2 ml 2.5% ammonium molybdate in 5 N H_2SO_4. The contents are mixed on a vortex shaker and 0.3 ml of a 0.2% 1-amino-2-naphthol-4-sulphonic acid; 12% $NaHSO_3$; 2.4% $Na_2SO_3.7H_2O$ solution (ANSA reagent) are added. The tubes are vortex

mixed and allowed to stand for 30 minutes at room temperature after which time their optical density at 691 nm is read against a blank containing water, molybdate reagent and ANSA reagent. The A_{691} values obtained can be used to construct a standard curve for phosphate. Free phosphate concentration in assays, e.g. ATPase assays, can be obtained by taking aliquots through the procedure above and comparing the A_{691} values to the standard curve

ATPase assay.- ATPase activity was determined by assaying phosphate released by the hydrolysis of ATP. Assays containing 100-200 µg mitochondrial protein or 1-10 µg purified ATPase protein were incubated in 1 ml 50 mM Tris-Cl, pH 8.5; 5 mM $MgCl_2$ (assays were carried out at pH 8.5 unless specified otherwise) for 5 minutes with effectors and inhibitors, if any are present in the assay, at 30° C prior to initiation of the assay with 5 µmoles ATP. Assays were terminated after 5-15 minutes. By addition of 0.5 ml 10% TCA. Coagulated protein was removed by centrifugation in a bench centrifuge and a 0.5 ml aliquot of the supernatant was taken into an assay to determine inorganic phosphate (Pi).

ATP-Pi exchange.- ATP-Pi exchange activity was determined as described by Stigall et al. (244). A solution was prepared: 50 mM Tris-Cl, pH 7.5; 500 mM sucrose; 30 mM $MgSO_4$; 40 mM potassium phosphate (pH 7.5) and 6 mg/ml bovine serum albumin. To 0.5 ml of this reaction medium at 30° C were added 10^6 cpm of carrier-free ^{32}Pi or ^{33}Pi and water to bring the volume to 0.90 - 0.95 ml. Mitochondrial protein, 1 mg (50-100 µl volume) was then added and the assay preincubated with any inhibitors or effectors for 5 minutes at 30° C. The reaction was started with 12 µmoles ATP, pH 7.5, and terminated after 5 minutes by addition of 0.5 ml 1 M PCA or 0.5 ml 10% TCA. Coagulated protein was removed by bench centrifugation and a 0.5 ml aliquot from the supernatant taken for determination of ^{32}P or ^{33}P labelled ATP by a method based on that described by Pullman (353). The 0.5 ml assay aliquot was taken into a 10-20 ml glass test tube containing 1.0 ml 5% ammonium molybdate in $5NH_2SO_4$; 1 ml acetone was then added and the mixture mixed vigorously on a vortex shaker. 0.1 ml Br water was added followed by 5mls water saturated isobutanol benzene (1:1). The mixture was vortex mixed for 30 secs and the upper layer removed by aspiration. The process was

repeated. 5 ml of water-saturated isobutanol were then added, the mixture vortexed and the upper layer removed by aspiration. The remaining aqueous layer was extracted twice by water-saturated diethylether (5 ml) which was also removed by aspiration. An aliquot from the remaining aqueous layer was taken for determination of ^{32}P or ^{33}P by liquid scintillation counting.

Radiolabelled glucose-6-phosphate was determined by the same method described for ^{32}P labelled ATP above as it partitions between isobutanol/benzene and water in the same way as ATP.

The use of benzene has now been terminated in this laboratory. Toluene has replaced benzene in the extraction procedures outlined above.

NADH-Ferricyanide reductase activity.- This was assayed by the method of Hatefi et al. (354) in 1 ml of a 50 mM Tris-Cl, pH 7.5 buffer containing 0.7 mM potassium ferricyanide; 0.25 mM NADH and 3-30 µg enzyme (depending on degree of activity, i.e. 3 µg complex I or 30 µg submitochondrial particles). The reaction was initiated with NADH and the ferricyanide reductase activity determined by following the absorbance change at 410 nm in a Pye Unicam SP1800 dual beam recording spectrophotometer using E_{mM} $K_3Fe(CN)_6$ ox-red = 1 at 410 nm.

Cytochrome c reductase.- Cytochrome c reductase was assayed by the method described by Hatefi et al. (354) in 1 ml of a buffer containing 20 mM potassium phosphate, pH 8.0; 2 mM sodium azide; 0.7 mg oxidised cytochrome c; 0.15 mg mixed phospholipid, 0.25 µmole NADH or 5 µmole sodium succinate. Assays were initiated by addition of 1-100 µg enzyme (depending on degree of purity). Activity was followed at 550 nm in a Pye-Unicam dual beam recording spectrophotometer. E_{mM} cyt c ox-red = 18.5 at 550 nm.

Energy linked transhydrogenase was assayed as described in the methods section to Chapter 6.

Protein determination.- Mitochondrial protein was estimated by the biuret method of Gornall et al (355). Purified enzyme protein was estimated by the Folin-Lowry method described by Lowry et al. (356).

Preparation of dihydrolipoic acid.- This was either obtained as such from the Sigma Chemical Company or was prepared from oxidised lipoic acid by sodium borohydride reduction.

Before use, all glassware used in the preparation of dihydrolipoic acid was washed in chloroform/methanol (2:1), methanol, then 200 mM EDTA followed by 0.5 M potassium phosphate before being rinsed in several changes of triple glass-distilled water and left to dry.

One gram oxidised lipoic acid was weighed out into a round bottomed 250 ml quickfit glass flask containing a small teflon coated magnetic follower. The oxidised lipoic acid was then dissolved by addition of 100 ml saturated sodium bicarbonate solution. A four-fold molar excess of sodium borohydride was then added over a period of 10 minutes to the flask with rapid stirring. The reaction mixture was then left stirring in the dark at RT for 1 hr. The pH was then taken to 1-2 by the addition of concentrated HCl_{aq}. This precipitated dihydrolipoic acid which was removed from the reaction mixture by extraction with 2 x 100 ml of redistilled 'AnalaR' chloroform (redistilled to remove ethanol). The chloroform extracts were pooled and evaporated in a rotary evaporator to remove the bulk of the chloroform. This gave a preparation of dihydrolipoic acid substantially free of oxidised lipoic acid. Further purification of dihydrolipoate was obtained by vacuum distillation at 50^{o} C, 0.001 mm Hg. This gives a preparation of dihydrolipoic acid free from any impurities (by TLC). Dihydrolipoic acid obtained in this way is a mobile colourless liquid. That obtained from Sigma or by borohydride reduction prior to vacuum distillation is a yellow visous liquid due to contamination by oxidised liploic acid and by lipoic acid polymers.

Dihydroplioate was stored in de-ionised borosilicate vials under nitrogen.

Preparation of dihydrolipoate solutions.- Unless otherwise described, dihydrolipoic acid solutions were normally prepared as follows. Dihydrolipoic acid was weighed out into a de-ionised glass or borosilicate vial: enough 0.25 M sucrose; 10 mM Tris-Cl, pH 7.5; 1 mM EDTA was added to give an 0.4 M (or 0.2 M) solution. Tris base (1 M) was then added until dissolution of dihydrolipoate was obtained; the final volume of the solution was adjusted to 0.2 M (or 0.1 M) by addition of the requisite volume of 0.25 M sucrose; 10 mM Tris-Cl, pH 7.5; 1 mM EDTA. The final pH of these solutions was usually 7.5 - 7.8. Dihydrolipoate solutions were stored at 4^{o} C or at -20^{o} C unless otherwise stated.

Preparation of oleoyl-S-lipoate.- Dihydrolipoic acid (0.5 mmole) was dissolved in 10 ml redistilled tetrahydrofuran + 10 ml triple distilled water. The pH was adjusted to 7.5 with 1 M NaOH. Oleoyl chloride (0.5 mmole) was then added to this reaction mixture with rapid stirring. The mixture was then stirred in the dark at room temperature for 3-4 hours; the pH was monitored and kept between pH 7.5-8 by addition of 1 M NaOH. The mixture was assayed for thiol content by the DTNB method. After 3-4 hours the reaction was adjudged to be complete (using the disappearance of thiol as the parameter). The reaction mixture was acidified to pH 2-3 by addition of 1 M HCl and extracted with 50 ml diethylether which was then removed from the reaction products by rotary evaporation. Oleoyl-S-lipoate was the major reaction product as indicated by TLC in a system containing toluene: ethyl formate:formic acid, 5:4:1. Further purification was carried out using preparative thin layer chromatography eluting with the same solvent system. Oleoyl-S-lipoate was dissolved in dimethylformamide and assayed by the acyl ester methods of Snyder and Stevens (243) or

Lipmann and Tuttle (242), as described in Chapter 5.

Other fatty acyl-S-lipoates were prepared in the same manner from the respective fatty acyl chlorides.

Fatty acyl lipoates were occasionally synthesised from the N-hydroxy-succinimide esters of the appropriate fatty acids (357-8).

Preparation of adenyl lipoate.- Adenyl lipoate was synthesised essentially as described by Reed et al. (359). Lipoic acid (288 mg) was dissolved in 1.5 ml redistilled acetonitrile and kept stirring on ice. DCCD (144 mg) was added as a solution in 0.5 ml acetonitrile. The reaction was stirred for 30 minutes at RT after which time dicyclohexyl urea was removed by filtration. Acetonitrile was removed by rotary evaporation leaving lipoic anhydride. This was redissolved in 2.5 ml acetonitrile and immediately added to a solution of AMP (500 mg) in 5.25 ml 32% aqueous pyridine. The mixture was stirred at RT for 50 minutes prior to addition of 5 ml ice cold water. The mixture was then extracted with 2 x 10 ml peroxide free diethyl ether and then with 2 x 10 ml $CHCl_3$. A light yellow precipitate was deposited from the aqueous layer after chloroform extraction. This was filtered off and washed in ice cold water and dried. The yield was rather low (10-20%). The product showed a single spot on paper chromatography in an isopropyl alcohol/water system (7:3) and displayed u.v. maxima at 259 nm and 332 nm.

Estimation of thiol groups.- Thiols were estimated by determination with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), using an extinction coefficient $E_{mM} = 13.6$ at 412 nm.

Results

'Dihydrolipoate'-driven ATP synthesis was a very rarely observed reaction. The results presented here are most of the dihydrolipoate-driven ATP syntheses observed over a two year period and represent about 1% of the total number of experimental attempts to demonstrate

this reaction. Possible reasons for the low frequency of observing this reaction are examined in the discussion section to this chapter. ATP synthesis was assayed in a glucose-hexokinase trap system. Here, ATP synthesised during (say) oxidative phosphorylation is used by hexokinase to act as a phosphate donor to glucose producing glucose-6-phosphate. ATP synthesis can then be estimated either by following the free phosphate content of the system or assaying the glucose-6-phosphate produced. The latter is done using the enzyme glucose-6-phosphate dehydrogenase which converts glucose-6-phosphate to glucono-δ-lactone-6-phosphate with the concomitant reduction of $NADP^+$ to NADPH. This NADPH produced can be assayed spectrophotometrically at 340 nm.

Table 3.2 shows apparent dihydrolipoate and oleoyl-S-lipoate-driven ATP synthesis in bovine heart mitochondria. These mitochondria have had their respiratory chains blocked with antimycin A and rotenone. One µmole dihydrolipoate appears to produce between 1.0 and 1.3 µmoles of ATP. The stoicheiometry of ATP synthesis-driven by oleoyl-S-lipoate is more difficult to establish as the acyl ester was added as a crude oil assumed to be 50% pure, the amount added was calculated on a weight basis to be about 2 or 5 µmoles. If it was 100% pure, this amount was 4-10 µmoles. This was, however, unlikely as TLC's show that it contained substantial amounts of lipoic acid The same is true for oleoyl-S-lipoate added in Tables 3.5,8,9. ATP synthesis, driven by oleoyl-S-lipoate calculated by the glucose-6-phosphate method was substantially higher than that calculated by the phosphate disappearance method. This may be due to a stimulation of mitochondrial myokinase activity. Absorbance changes observed in this assay were in the order of 0.09 A_{691} units/µmole ATP produced for the phosphate assay and 0.21 A_{340} units/µmole ATP produced for the glucose-6-phosphate assay. These are ΔA_{691} values of 25% and ΔA_{340} values of 34% over no enzyme and substrate controls.

Table 3.2 Dihydrolipoate and oleoyl-S-lipoate-driven ATP synthesis in bovine heart mitochondria

Additions	ATP synthesis µmoles/20 minutes	
	1	2
none	0	0
none + dihydrolipoate (1 µmole)	1.3	1.0
none + dihydrolipoate (2 µmole)	1.9	1.6
none + dihydrolipoate (1 µmole)[3]	1.1	1.2
none + dihydrolipoate (1 µmole) + oligomycin (5 µg)	0	0.15
none + oleoyl-S-lipoate (∿ 2 µmole)	0	2.7
none + oleoyl-S-lipoate (∿ 5 µmole)	1.5	3.1

[1] estimated by phosphate disappearance

[2] estimated by glucose-6-phosphate appearance; corrected for any myokinase activity

[3] containing 25 units Sigma C-300 hexokinase

Assays containing 1.4 mg mitochondrial protein were preincubated, with rotenone and antimycin A (1.38 µg/mg protein) and oligomycin, where indicated, for 5 minutes at 30° C in a shaking water bath in a 1.1 ml glucose hexokinase trap system, 2 mM in ADP, containing 25 units Sigma F-300 hexokinase, except where indicated. Assays were initiated by addition of dihydrolipoate (as a 0.2 M solution in 0.25 M sucrose; 10 mM Tris-Cl, pH 7.5; 1 mM EDTA) or oleoyl-S-lipoate (added as a crude oil in $CHCl_3$: molarity estimated by weight) and terminated and assayed for phosphate and glucose-6-phosphate as described in Table 3.4.

Dihydrolipoate-driven ATP synthesis was observed in preparations of F_1F_O ATPases from bovine heart mitochondria, Tables 3.3-7). ATP synthesis in these purified enzymes appeared to require the presence of an unsaturated fatty acid and oleoyl CoA in cofactor-like quantities. Both the 'proton translocating ATPase' of Serrano and Racker and Hatefi's complex V on occasion have catalysed dihydrolipoate-driven ATP synthesis. This synthesis appears to be roughly stoicheiometric with the amount of dihydrolipoate added. Table 3.3 shows ATP synthesis catalysed by 'proton translocating ATPase'. This was measured by three methods simultaneously, i.e. phosphate disappearance, glucose-6-phosphate appearance and ^{33}P glucose-6-phosphate appearance. There was generally a good correlation between the amounts of ATP produced indicated by these methods. Here 1 µmole of ATP produced gave ΔA_{691} of ∿ 0.1; ΔA_{340} of ∿ 0.18 and 271 cpm ^{33}P under the conditions of assay; this corresponds to Δ absorbances of ΔA_{691} 25%, ΔA_{340} 100% respectively, compared to substrate and enzyme blanks. Radioactive background counts and substrate and enzyme gave ∿ 38 cpm. Similarly ATP synthesis was observed in complex V preparations incubated with dihydrolipoate (Tables 3.4-5). Again this synthesis was assayed simultaneously by phosphate disappearance, glucose-6-phosphate appearance and ^{33}P labelled glucose-6-phosphate appearance. Under the conditions of assay used, 0.72 µmole ATP was equivalent to ΔA_{691} of 0.065, ΔA_{340} of 0.155 and ^{33}P cpm. 60 counts over a background of 31 (Table 3.4), corresponding to ΔA_{691} of 15% and ΔA_{340} of ∿ 95% compared to no enzyme and no substrate controls. In Table 3.5, 0.351 µmoles ATP was equivalent to ΔA_{691} of 0.045, ΔA_{340} of 0.07 and radioactive ^{33}P 116 cpm over background and no enzyme and no substrate controls. This corresponds to ΔA_{691} of ∿ 10%, ΔA_{340} of ∿ 33% compared to no substrate and no enzyme controls. Dihydrolipoate-driven ATP synthesis in these enzymes was sensitive to

Table 3.3 Dihydrolipoate-driven ATP synthesis in 'proton translocating ATPase

Additions	ATP synthesis µmoles/20 mins		
	1	2	3
Proton translocating ATPase + oleate + oleoyl CoA	0	0	0
Proton translocating ATPase + oleate + oleoyl CoA + dihydrolipoate (1 µmole)	1.2	1.12	1.15
Proton translocating ATPase + oleate + oleoyl CoA + dihydrolipoate (1 µmole)	0.96	0.50	1.36
Proton translocating ATPase + oleate + oleoyl CoA + dihydrolipoate (1 µmole)	0.96	1.12	1.22
Proton translocating ATPase + oleate + oleoyl CoA + dihydrolipoate (2 µmole)	1.98	1.60	2.34
Proton translocating ATPase + oleate + oleoyl CoA + dihydrolipoate (1 µmole)[4]	0.96	0.94	0.74
Proton translocating ATPase + oleate + oleoyl CoA + dihydrolipoate (1 µmole) + oligomycin (10 µg)	0	0	0.01

[1] estimated as phosphate disappearance

[2] estimated as glucose-6-phosphate appearance; values corrected for any myokinase activity

[3] estimated as ^{33}P labelled glucose-6-phosphate and ^{33}P labelled ATP

[4] containing 25 units Sigma C-300 $(NH_4)_2SO_4$ precipitated hexokinase

Assays containing 0.57 mg proton translocating ATPase, 5 nmoles oeloyl CoA; 15 nmoles oleate were preincubated with these and inhibitors, where indicated, for 5 minutes at 30° C in a 1 ml glucose-hexokinase trap system, 2 mM in ADP, containing 25 units of Sigma F-300 hexokinase except where indicated, in a shaking water bath. Assays were initiated by addition of dihydrolipoate (0.2 M in 0.25 M sucrose; 10 mM Tris-Cl, pH 7.5; 1 mM EDTA) and terminated after 20 minutes, as described in Table 3.4.

Table 3.4 Dihydrolipoate-driven ATP synthesis in 'complex V' ATPase from bovine heart mitochondria

Additions	ATP synthesis µmoles/20 mins		
	1	2	3
complex V + oleate + oleoyl CoA	0	0	0
complex V + oleate + oleoyl CoA + dihydrolipoate (1 µmole)	0.72	0.88	0.75
complex V + oleate + oleoyl CoA + dihydrolipoate (2 µmole)	1.68	1.29	1.45
complex V + oleate + oleoyl + CoA + dihydrolipoate (1 µmole)	1.02	0.83	0.60
complex V + oleate + oleoyl + CoA + dihydrolipoate (1 µmole) + oligomycin (10 µg)	0	0.14	0.02

1 estimated by phosphate disappearance

2 estimated by glucose-6-phosphate appearance ; values corrected for any myokinase activity

3 estimated as ^{33}P labelled glucose-6-phosphate and ^{33}P labelled ATP

Assays containing 1 mg complex V protein, 5 nmoles oleoyl CoA, 15 nmoles oleic acid were preincubated with inhibitors and effectors, where indicated, for 5 minutes at 30° C in a 1.2 ml glucose-hexokinase trap system, 2 mM in ADP, containing 20 units of Sigma C-300 hexokinase in a shaking water bath. Assays were initiated by addition of dihydrolipoate (as a 0.2 M solution in 0.25 M sucrose: 10 mM Tris: 1 mM EDTA, pH 7.5). Assays were terminated by (i) taking 0.1 ml aliquot into 0.1 ml 10% TCA and assaying for phosphate as described in the methods section to Chapter 3. (ii) Taking 0.5 ml into 0.25 ml 30% PCA and assaying for glucose-6-phosphate as described in the methods section to this chapter. (iii) Taking 0.5 ml into 0.25 ml 30% PCA, removing coagulated protein by centrifugation and taking 0.5 ml of the supernatant into 0.1 ml aqueous bromine water and 1.0 ml 5% ammonium molybdate in 5 NH_2SO_4 and assaying for ^{33}P labelled glucose-6-phosphate as described in the methods section to Chapter 3.

Table 3.5 Dihydrolipoate-driven ATP synthesis in complex V ATPase

Additions	ATP synthesis µmoles/25 min		
	1	2	3
complex V + oleate + oleoyl CoA	0	0	0
complex V + oleate + oleoyl CoA + dihydrolipoate (0.5 µmole)	0.35	0.337	0.34
complex V + oleate + oleoyl CoA + dihydrolipoate (1.0 µmole)	0.58	0.48	0.42
complex V + oleate + oleoyl CoA + dihydrolipoate (5 µmoles)	0	0	0.25
complex V + oleate + oleoyl CoA + dihydrolipoate (1 µmole) + oligomycin	0.35	0	0
complex V + oleate + oleoyl CoA + dihydrolipoate (1 µmole) + DCCD	0	0	0
complex V + oleate + oleoyl CoA + oleoyl-S-lipoate (∿ 2 µmoles)	0	2.12	2.37
complex V + oleate + oleoyl CoA + oleoyl-S-lipoate (∿ 5 µmoles)	0.66	3.80	3.94
complex V + oleate + oleoyl CoA + oleoyl-S-lipoate (∿ 2 µmoles) + oligomycin	0	0.19	0.04
complex V + oleate + oleoyl CoA + oleoyl-S-lipoate (∿ 2 µmoles) + DCCD	0	0.14	0.01

[1] estimated by phosphate disappearance

[2] estimated by glucose-6-phosphate appearance

[3] estimated as ^{33}P labelled glucose-6-phosphate and ATP. 1 ml phosphorylation buffer contains 50,000 cpm ^{33}P

Assays containing 0.5 mg complex V protein, 5 µg oleate and 5 µg oleoyl CoA were preincubated with 1 µg inhibitors, where indicated, for 5 minutes at 30° C in a shaking water bath in a 1 ml glucose-hexokinase trap system, 2 mM in ADP, containing 25 units Sigma F-300 hexokinase prior to initiation by addition of dihydrolipoate (as a 0.1 M solution in 0.25 M sucrose; 10 mM Tris-Cl, pH 7.5; 1 mM EDTA) or oleoyl-S-lipoate (as a crude oil in chloroform, molarity estimated by weight). Assays were terminated after 25 minutes and aliquots taken for phosphate, glucose-6-phosphate and ^{33}P G6P assay as described in Table 3.4

oligomycin (Table 3.3-4) and showed stoicheoimetries ranging between 0.351 - 1.2:1 μmoles ATP produced/μmole dihydrolipoate added. There was generally good correlation between the methods used to estimate ATP synthesis. Oleoyl-S-lipoate-driven ATP synthesis was also observed; this was sensitive to inhibition by oligomycin and DCCD (Table 3.5). It is rather difficult to estimate the stoicheiometry of ATP synthesis driven by oleoyl-S-lipoate under these conditions as the acyl lipoate was added as a crude oil.

The requirement for an unsaturated fatty acid in dihydrolipoate-driven ATP synthesis by purified F_1F_O ATPases appears to be specific for oleic acid, a *cis* Δ-9 monoenoic acid. ATP synthesis was not observed in the presence of other *cis* Δ-9 monoenoic acids such as palmitoleic, myristoleic and ricinoleic acid or the *trans* isomer of oleic acid, elaidic acid (Table 3.6) which appeared together with erucic acid to be fairly potent ihhibitors of dihydrolipoate-driven ATP synthesis in 'proton translocating ATPase' (Table 3.7). The ΔA_{691} observed in Table 3.6 was 0.07 (30% *cf.* to controls) and 0.11 (33% *cf.* to controls) in Table 3.7.

A time course assay on dihydrolipoate-driven ATP synthesis gave initial velocities for the reaction of ∿ 500 nmoles ATP synthesised/min/mg at 30° C in the presence of 2 μmoles dihydrolipoate and 200 nmoles ATP synthesised/min/mg at 30° C in the presence of 1 μmole dihydrolipoate (Fig. 3.2); ΔA_{691} of 0.08 corresponds to ∿ 1 μmole under the conditions of assay (ΔA_{691} of 25% *cf.* to controls). Oleoyl-S-lipoate-driven ATP synthesis was also observed in bovine heart SMP; elaidoyl-S-lipoate appeared to be unable to drive ATP synthesis (Tables 3.8-9).

Dihydrolipoate-driven ATP synthesis was also observed in rat liver mitochondria (Tables 3.10-13). This synthesis was sensitive to oligomycin and the uncouplers DNP and CCCP. Stoicheiometry ranging between 0.44 and 1.17:1 μmoles ATP produced/μmole dihydrolipoate added was observed in these experiments.

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The requirement for an unsaturated fatty acid in dihydrolipoate-driven ATP synthesis by purified F_1F_O ATPases appears to be specific for oleic acid, a *cis* Δ-9 monoenoic acid. ATP synthesis was not observed in the presence of other *cis* Δ-9 monoenoic acids such as palmitoleic, myristoleic and ricinoleic acid or the *trans* isomer of oleic acid, elaidic acid (Table 3.6) which appeared together with erucic acid to be fairly potent ihhibitors of dihydrolipoate-driven ATP synthesis in 'proton translocating ATPase' (Table 3.7). The ΔA_{691} observed in Table 3.6 was 0.07 (30% *cf.* to controls) and 0.11 (33% *cf.* to controls) in Table 3.7.

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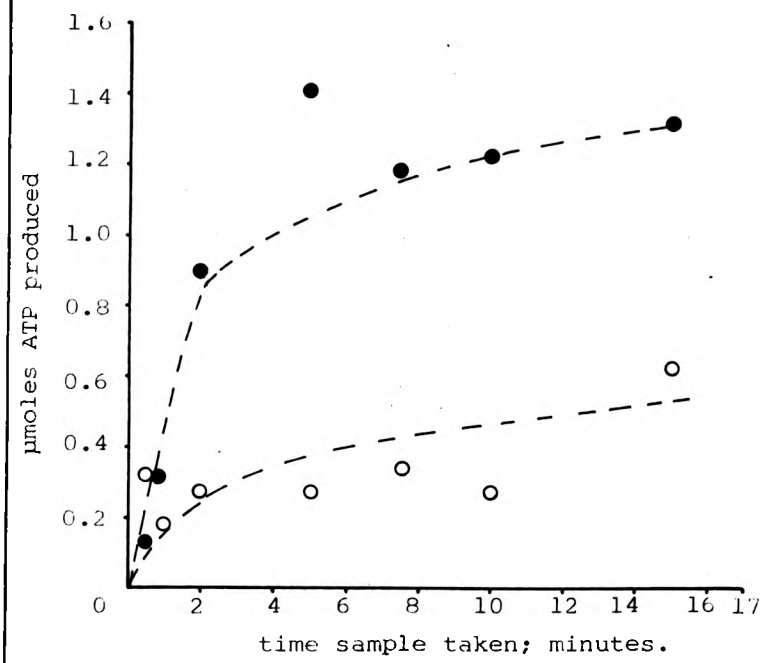


Fig 3.2 Dihydrolipoate driven ATP synthesis in complex V ATPase: time course.

Assays,containing 1mg complex V ATPase protein, were preincubated in 2mls of a phosphorylation buffer (see methods section chapter 3 for composition) 0.2mM in ADP;5mM in Pi containing 20 units sigma F-300 hexokinase,10 nmoles oleate,10 nmoles oleoyl CoA for 5 minutes at 30°C prior to initiation by dihydrolipoate. Samples (0.2ml) were taken at various times for Pi analysis as described in the methods section to ch.3.

●——● assay initiated with 2 µmoles $lip(SH)_2$.
o——o " " " 1 µmole $lip(SH)_2$.

Table 3.6 Dihydrolipoate-driven ATP synthesis in 'complex V' ATPase: fatty acid specificity

Additions	ATP synthesis μmoles/20 min
complex V ATPase	0
complex V + dihydrolipoate (1 μmole)	0
complex V + dihydrolipoate (1 μmole) + oleic acid	0
complex V + dihydrolipoate (1 μmole) + oleic acid + oleoyl CoA (10 nmole)	0.69
complex V + dihydrolipoate (1 μmole) + oleoyl CoA (10 nmole) + stearic acid	0
complex V + dihydrolipoate (1 μmole) + oleoyl CoA (10 nmole) + elaidic acid	0
complex V + dihydrolipoate (1 μmole) + oleoyl CoA (10 nmole) + erucic acid	0
complex V + dihydrolipoate (1 μmole) + oleoyl CoA (10 nmole) + linoleic acid	0
complex V + dihydrolipoate (1μmole) + oleoyl CoA (10 nmole) + linolenic acid	0
complex V + dihydrolipoate (1 μmole) + oleoyl CoA (10 nmole) + γ-linolenic acid	0
complex V + dihydrolipoate (1 μmole) + oleoyl CoA (10 nmole) + arachidonic acid	0
complex V + dihydrolipoate (1 μmole) + oleoyl CoA (10 nmole) + palmitoleic acid	0
complex V + dihydrolipoate (1 μmole) + oleoyl CoA (10 nmole) + ricinoleic acid	0
complex V + dihydrolipoate (1 μmole) + oleoyl CoA (10 nmole) + ricinelaidic acid	0
complex V + dihydrolipoate (1 μmole) + oleoyl CoA (10 nmole) + myristoleic acid	0

Assays containing 1 mg complex V ATPase were preincubated with oleoyl CoA (10 nmoles) and 5 μg fatty acids (except dihydrolipoate) for 5 minutes in a 1 ml glucose-hexokinase trap system, 0.2 mM in ADP, containing 25 units Sigma F-300 hexokinase at 30° C in a shaking water bath. Assays were initiated by addition of dihydrolipoate and terminated after 20 minutes and assayed for phosphate, as described in Chapter 3.

Table 3.6 Dihydrolipoate-driven ATP synthesis in 'complex V' ATPase: fatty acid specificity

Additions	ATP synthesis μmoles/20 min
complex V ATPase	0
complex V + dihydrolipoate (1 μmole)	0
complex V + dihydrolipoate (1 μmole) + oleic acid	0
complex V + dihydrolipoate (1 μmole) + oleic acid + oleoyl CoA (10 nmole)	0.69
complex V + dihydrolipoate (1 μmole) + oleoyl CoA (10 nmole) + stearic acid	0
complex V + dihydrolipoate (1 μmole) + oleoyl CoA (10 nmole) + elaidic acid	0
complex V + dihydrolipoate (1 μmole) + oleoyl CoA (10 nmole) + erucic acid	0
complex V + dihydrolipoate (1 μmole) + oleoyl CoA (10 nmole) + linoleic acid	0
complex V + dihydrolipoate (1μmole) + oleoyl CoA (10 nmole) + linolenic acid	0
complex V + dihydrolipoate (1 μmole) + oleoyl CoA (10 nmole) + γ-linolenic acid	0
complex V + dihydrolipoate (1 μmole) + oleoyl CoA (10 nmole) + arachidonic acid	0
complex V + dihydrolipoate (1 μmole) + oleoyl CoA (10 nmole) + palmitoleic acid	0
complex V + dihydrolipoate (1 μmole) + oleoyl CoA (10 nmole) + ricinoleic acid	0
complex V + dihydrolipoate (1 μmole) + oleoyl CoA (10 nmole) + ricinelaidic acid	0
complex V + dihydrolipoate (1 μmole) + oleoyl CoA (10 nmole) + myristoleic acid	0

Assays containing 1 mg complex V ATPase were preincubated with oleoyl CoA (10 nmoles) and 5 μg fatty acids (except dihydrolipoate) for 5 minutes in a 1 ml glucose-hexokinase trap system, 0.2 mM in ADP, containing 25 units Sigma F-300 hexokinase at 30° C in a shaking water bath. Assays were initiated by addition of dihydrolipoate and terminated after 20 minutes and assayed for phosphate, as described in Chapter 3.

Table 3.7 Inhibition of dihydrolipoate-driven ATP synthesis in 'proton translocating' ATPase by fatty acids

Addition	ATP synthesis µmoles/20 min
proton translocating ATPase	0
proton translocating ATPase + oleate + oleoyl CoA	0
proton translocating ATPase + dihydrolipoate + oleoyl CoA + oleate (15 nmole)	1.7
proton translocating ATPase + dihydrolipoate + oleoyl CoA + erucate (15 nmole)	0
proton translocating ATPase + dihydrolipoate + oleoyl CoA + oleate + elaidate (15 nmole)	0
proton translocating ATPase + dihydrolipoate + oleoyl CoA + oleate + erucate (15 nmole)	0
proton translocating ATPase + dihydrolipoate + oleoyl CoA + oleate + elaidate (1.5 nmole)	0
proton translocating ATPase + dihydrolipoate + oleoyl CoA + oleate + erucate (1.5 nmole)	0
proton translocating ATPase + dihydrolipoate + oleoyl CoA + oleate (30 nmole)	1.7
proton translocating ATPase + dihydrolipoate + oleoyl CoA + oleate + oligomycin (5 µg)	0

Assays containing 0.725 mg of proton translocating ATPase protein were preincubated with oleoyl CoA (10 nmoles) and fatty acids (15 nmoles, except where indicated) for 5 minutes at 30° C in a shaking water bath in a 1 ml glucose-hexokinase trap system, 0.2 mM in ADP containing 22 units of Sigma C-300 hexokinase. Assays were initiated by the addition of 2 µmoles dihydrolipoate as a 0.2 M solution in 0.25 M sucrose; 10 mM Tris-Cl, pH 7.5; 1 mM EDTA. Assays were terminated and phosphate determined as described in Table 3.4

Table 3.8 Oleoyl-S-lipoate-driven ATP synthesis in bovine heart SMP and 'complex V' ATPase

Additions	ATP synthesis µmoles/20 min	
submitochondrial particles (1.9 mg)	0[1]	0[1]
submitochondrial particles + oleoyl-S-lipoate (∿ 2 µmoles)	1.24	0.95
submitochondrial particles + oleoyl-S-lipoate (∿ 5 µmoles)	1.85	1.50
complex V (0.5 mg)	0	0
complex V + oleoyl-S-lipoate (∿ 5 µmoles)	1.75	1.09
complex V + oleoyl-S-lipoate (∿ 2 µmoles) + oligomycin (5 µg)	0.55	0

[1] parallel determinations by taking separate aliquots to test for phosphate disappearance

Assays containing either submitochondrial particles or complex V were preincubated with inhibitors (rotenone and antimycin in case of SMP's, 2 µg/mg) for 5 minutes in a 1 ml glucose hexokinase trap system, 2 mM in ADP containing 25 units Sigma F-300 hexokinase in a shaking water bath at 30° C. Assays were initiated by addition of solutions of oleoyl-S-lipoate in dimethylformamide and were terminated after 20 minutes by taking 0.2 ml aliquots into 0.2 ml 10% TCA and assaying for phosphate as described in Table 3.4.

Table 3.9 Oleoyl-S-lipoate-driven ATP synthesis in bovine heart SMP

Additions	ATP synthesis µmoles/20 min
none	0
oleoyl-S-lipoate (∿ 2 µmoles)	1.09
oleoyl-S-lipoate (∿ 5 µmoles)	2.08
oleoyl-S-lipoate (∿ 2 µmoles) + 5 µg oligomycin	0.16
elaidoyl-S-lipoate (1 µmole)	0
elaidoyl-S-lipoate (2 µmole)	0

Assays containing 2 mg bovine heart submitochondrial protein were preincubated with rotenone and antimycin (1.5 µg/mg SMP) and oligomycin, where indicated, for 5 minutes in a 1 ml glucose-hexokinase trap system, 2 mM in ADP, containing 12.5 units Sigma F-300 hexokinase, prior to initiation with solutions of fatty acyl lipoates in dimethylformamide. Assays were terminated after 20 minutes by taking 0.2 ml aliquots into 0.2 ml 10% TCA. Protein was removed by centrifugation and ATP synthesis was estimated as disappearance of phosphate by the method of Fiske and Subbarow, as described in the methods section of Chapter 3.

Table 3.10 Dihydrolipoate-driven ATP synthesis in rat liver mitochondria

Additions	ATP synthesis µmoles/20 min
rat liver mitochondria	0
rat liver mitochondria + succinate (20 µmoles)	2.32
rat liver mitochondria + antimycin A + dihydrolipoate (1 µmole)	0.44
rat liver mitochondria + antimycin A + dihydrolipoate (2 µmole)	0.907
rat liver mitochondria + antimycin A + dihydrolipoate (3 µmole)	0.974
rat liver mitochondria + antimycin A + dihydrolipoate (2 µmole) + oligomycin (1 µg)	0.168
rat liver mitochondria + antimycin A + dihydrolipoate (2 µmole) + DNP (1 µg)	0.100

Assays containing 1 mg mitochondrial protein, 1 µg rotenone, 1 µg antimycin A, oligomycin and DNP, where indicated, were preincubated in a 1 ml glucose-hexokinase trap system, 0.2mM in ADP, containing 28 units of Sigma C-300 hexokinase for 5 minutes at 30° C in a shaking water bath. Assays were initiated by addition of sodium succinate or dihydrolipoate (added as a 0.2 m solution in 0.25 M sucrose; 10 mM Tris-Cl, pH 7.5; 1 mM EDTA) and terminated after 20 minutes and assayed for phosphate as described in Table 3.11.

Table 3.11 Dihydrolipoate-driven ATP synthesis in rat liver mitochondria

Additions	ATP synthesis[1] µmoles/20 min
rat liver mitochondria	0
rat liver mitochondria + succinate	3.77
rat liver mitochondria + antimycin A + 'dihydrolipoate' 1	2.13
rat liver mitochondria + antimycin A + 'dihydrolipoate' 2	2.01
rat liver mitochondria + antimycin A + 'dihydrolipoate' 3	2.05
rat liver mitochondria + antimycin A + 'dihydrolipoate' 4	0.86
rat liver mitochondria + antimycin A + 'dihydrolipoate' 4 + oligomycin	0

[1] assayed by phosphate disappearance

Dihydrolipoates 1-4 as in Table 3.12

Assays containing 1 mg mitochondrial protein, 2 µg rotenone and antimycin A, 1 µg oligomycin, where indicated, were preincubated for 5 minutes in a shaking water bath at 30° C prior to initiation by addition of 10 moles sodium succinate, pH 7.5, or 2 µmoles (10 µl of 0.2 M solutions in 0.25 M sucrose; 10 mM Tris-Cl, pH 7.5; 1 mM EDTA) dihydrolipoate. Assays were terminated by taking 0.2 ml aliquots into 0.2 ml 10% ice cold TCA. Protein was removed by centrifugation and 0.2 ml of the supernatant was assayed for free phosphate by the method of Fiske and Subbarow, as described in the methods section to this chapter. Assays were carried out in a 1 ml glucose hexokinase trap system, 0.2 mM in ADP.

Table 3.12 Dihydrolipoate-driven ATP synthesis in rat liver mitochondria

Additions	ATP synthesis µmoles/15 min
rat liver mitochondria	0
rat liver mitochondria + succinate	2.05
rat liver mitochondria + antimycin A	0
rat liver mitochondria + antimycin A + dihydrolipoate 1	0.87
rat liver mitochondria + antimycin A + dihydrolipoate 2	0.59
rat liver mitochondria + antimycin A + dihydrolipoate 3	0.73
rat liver mitochondria + antimycin A + dihydrolipoate 4	0.17
rat liver mitochondria + antimycin A + dihydrolipoate 5	0
rat liver mitochondria + antimycin A + dihydrolipoate 1 + oligomycin (1 µg)	0.03
rat liver mitochondria + antimycin A + dihydrolipoate 1 + DNP (60 nmoles)	0.03
rat liver mitochondria + antimycin A + dihydrolipoate 1 + CCCP (2 µg)	0
rat liver mitochondria - antimycin A + succinate + 8-methyllipoic acid (5 µg)	1.42
rat liver mitochondria - antimycin A + succinate + 7-methylthiheptic acid (5 µg)	1.22

Dihydrolipoate 1-3 prepared from Sigma Grade II dihydrolipoic acid; dihydrolipoate 4 prepared from double distilled dihydrolipoic acid obtained initially by borohydride reduction of oxidised lipoic acid; dihydrolipoate 5 obtained by reduction of oxidised lipoic acid by sodium borohydride. All dihydrolipoates were added as 0.2 M solutions in 0.25 M sucrose- 10 mM Tris-Cl, pH 7.5; 1 mM EDTA.

Assays containing 1 mg rat liver mitochondrial protein and 2 µg rotenone were preincubated for 5 minutes at 30° C in a shaking water bath in a 1 ml glucose-hexokinase trap system, 0.2 mM in ADP, containing 28 units of Sigma C-300 hexokinase with 1 µg antimycin A and other inhibitors, where indicated. Assays were initiated by addition of sodium succinate or 2 µmoles dihydrolipoate, and terminated after 15 minutes and assayed for phosphate as described in Table 3.11.

Table 3.13 Dihydrolipoate-driven ATP synthesis in rat liver mitochondria

Additions	ATP synthesis µmoles/15 min
Rat liver mitochondria	0
rat liver mitochondria + succinate (20 µmoles)	2.09
rat liver mitochondria + antimycin A	0
rat liver mitochondria + antimycin A + dihydrolipoate 1	1.17
rat liver mitochondria + antimycin A + dihydrolipoate 2	1.06
rat liver mitochondria + antimycin A + dihydrolipoate 3	0.11
rat liver mitochondria + antimycin A + dihydrolipoate 5	0.04
rat liver mitochondria + antimycin A + dihydrolipoate 5A	0
rat liver mitochondria + antimycin A + dihydrolipoate 5B	0.40
rat liver mitochondria + antimycin A + dihydrlipoate 5C	0
rat liver mitochondria + antimycin A + dihydrolipoate 5D	0

Dihydrolipoates 1,2,3 and 5 as in Table 3.12; dihydrolipoates 5A, B, C and D were aliquots of dihydrolipoate 5, stored at R.T. in the dark and light and at 4° C in the dark and light respectively for 18 hr prior to experiment.

Assays containing 1 mg rat liver mitochondrial protein, 2 µg rotenone and 5 µg antimycin A, where indicated, were preincubated for 5 minutes at 30° C in a shaking water bath in a 1 ml glucose-hexokinase trap system, 0.2 mM in ADP, containing 28 units Sigma C-300 hexokinase, prior to initiation with sodium succinate or dihydrolipoate (2 µmoles). Assays were terminated after 15 minutes and phosphate content was determined as described in the legend to Table 3.11.

Table 3.14 Dark phosphorylation in Rhodospirillum rubrum chromatophores

Additions	ATP formed µmoles/20 min
chromatophores	0
chromatophores + dihydrolipoate (1 µmole)	0.69
chromatophores + dihydrolipoate (2 µmole)	1.61
chromatophores + oleoyl-S-lipoate (1 µmole)	0.70
chromatophores + elaidoyl-S-lipoate (1 µmole)	0
chromatophores + stearoyl-S-lipoate (1 µmole)	0
chromatophores + palmitoleoyl-S-lipoate (1 µmole)	0.45

Assays containing 1 mg chromatophore protein were preincubated with 5 µg HOQNO in a 1 ml glucose-hexokinase trap system, 0.2 mM in ADP, containing 25 units Sigma C-300 hexokinase for 5 minutes at 30° C in a shaking water bath. Assays were initiated by addition of dihydrolipoate or fatty acyl lipoate and were terminated after 20 minutes by taking a 0.2 ml assay aliquot into 0.2 ml 10% TCA. ATP was estimated by the disappearance of phosphate as described in the methods section of Chapter 3.

Table 3.15 Dihydrolipoate-driven ATP synthesis in a DCCD sensitive ATPase from *Escherichia coli*[1]: fatty acid specificity

Additions	ATP synthesis µmole Pi/20 minutes
none	0
dihydrolipoate + oleoyl CoA + oleate	2.52
dihydrolipoate + oleoyl CoA + elaidate	0
dihydrolipoate + oleoyl CoA + erucate	0
dihydrolipoate + oleoyl CoA + palmitate	0
dihydrolipoate + oleoyl CoA + oleate + erucate	0

[1] enzyme prepared by the method of Friedl *et al.* (351)

Assays containing 1 mg enzyme were preincubated with fatty acids (15 nmole) and oleoyl CoA (5 nmole) for 5 minutes at 30° C in a 1 ml glucose-hexokinase trap system, 0.2 mM in ADP, containing 25 units hexokinase. Assays were initiated with 3 µmole dihydrolipoate and terminated after 20 minutes by taking a 0.5 ml assay aliquot into 0.5 ml 10% TCA. ATP synthesis was estimated by phosphate disappearance; method as described in the methods section to Chapter 3.

Table 3.16 Fatty acyl lipoate-driven ATP synthesis in 'complex V' fatty acid specificity

Additions	ATP synthesis µmoles/20 min
none	0
oleoyl-S-lipoate	0.92
stearoyl-S-lipoate	0
elaidoyl-S-lipoate	0
erucoyl-S-lipoate	0
palmitoyl-S-lipoate	0
linoleoyl-S-lipoate	0
linolenoyl-S-lipoate	0
oleoyl-S-lipoate + erucoyl-S-lipoate (1 nmol)	0.50
oleoyl-S-lipoate + erucoyl-S-lipoate (5 nmol)	0.27
oleoyl-S-liqoate + erucoyl-S-lipoate (10 nmol)	0
oleoyl-S-lipoate + elaidoyl-S-lipoate (100 nmol)	0.27
oleoyl-S-lipoate + elaidoyl-S-lipoate (1 µmol)	0

Assays containing 0.5 mg protein were initiated by addition of 1 µmole fatty acyl lipoate, except where indicated. Incubations were carried out in a 1 ml glucose-hexokinase trap system, 0.2 mM in ADP, containing 25 units hexokinase, for 20 minutes at 30° C and were terminated by taking an 0.5 ml assay aliquot into 0.5 ml 10% TCA. ATP synthesis was estimated by the disappearance of phosphate as described in the methods section to Chapter 3.

Under the conditions of the assay used, 1 μmole ATP was equivalent to ΔA_{691} of ∿ 0.14 (ΔA_{691} of ∿ 25% *cf.* to controls). Chromatophores prepared from *Rhodospirillum rubrum* also displayed dihydrolipoate and oleoyl-S-lipoate-driven ATP synthesis (Table 3.14) although here 0.69 μmole ATP was only equivalent to ΔA_{691} of 0.032 (ΔA_{691} of ∿ 10% *cf.* to controls).

A chloroform released 'F_1' like ATPase from bovine heart submitochondrial particles did not appear to catalyse dihydrolipoate-driven ATP synthesis in the presence of a dihydrolipoate preparation that would drive ATP synthesis in bovine heart SMP in a parallel experiment (Table 3.17).

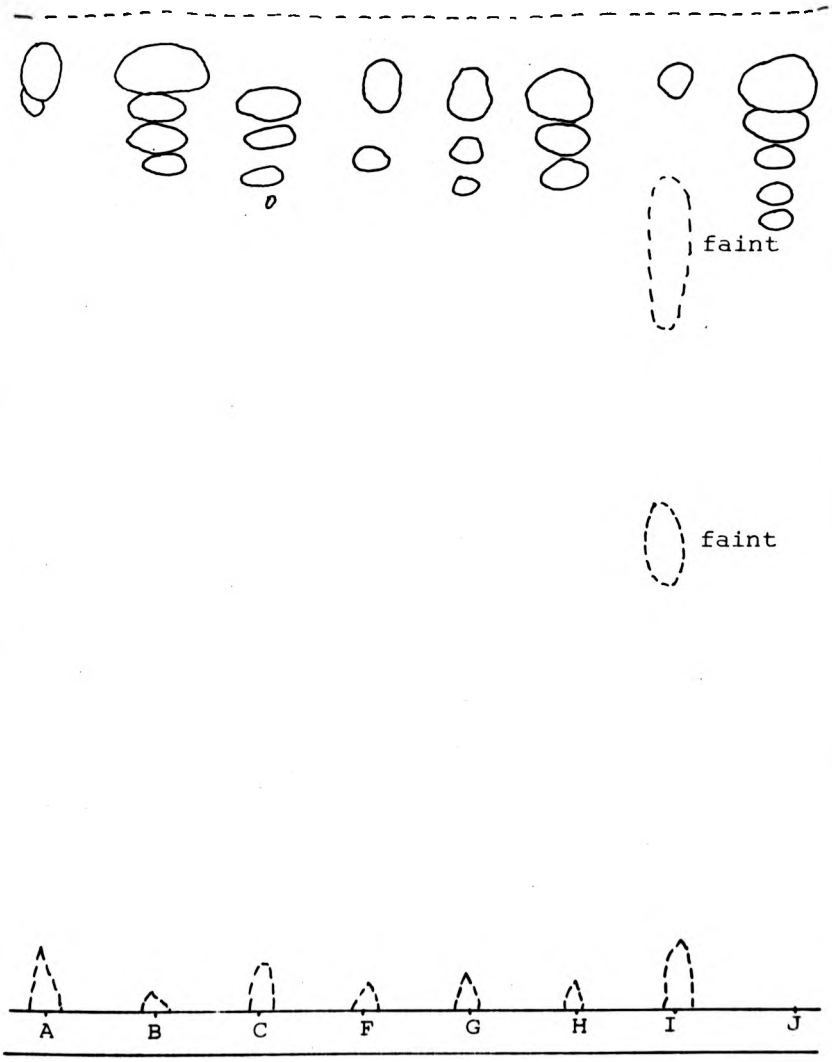
As stated before, dihydrolipoate-driven ATP synthesis was a rarely observed reaction. Possible reasons for this were investigated. It had been noticed, in the course of work, that certain solutions of dihydrolipoate had the ability to inhibit oxidative phosphorylation at fairly low concn. (Table 3.20). A number of solutions of lipoic acid were prepared and assayed over a period of time for the ability to drive ATP synthesis in bovine heart mitochondria (Table 3.19). The dihydrolipoates were also assayed for thiol content; their profiles in a chloroform/methanol/formic acid-silica gel TLC were also followed (Figs. 3.3-4). Those dihydrolipoates that appeared to drive ATP synthesis showed characteristic TLC profiles, lipoics D, E and I (Figs. 3.3-4). These profiles were similar to those observed in preparations of dihydrolipoic acid that stimulated an 'energy-linked' transhydrogenase reaction (see also Chapter 6). Possibly the most notable result obtained in this experiment was that obtained with dihydrolipoate I, whose thiol content fell from 100% of the theoretical expected to 0%, but still retained the ability to drive ATP synthesis. Dihydrolipoates that drove ATP synthesis had a very noticeable smell, best described as that of broken digestive biscuits.

Fig 3.3 Thin layer chromatograms of dihydrolipoates assayed for the ability to drive ATP synthesis.

T.L.C. of dihydrolipoates assayed for ATP synthesis on 12/5/77.See table 3.19 & also fig 3.4 &3.6

○ spots visualised with iodine vapour. ◌ visualised with H_2SO_4

Fig 3.4. Thin layer chromatograms of dihydrolipoates assayed for the ability to drive ATP synthesis.



Conditions as in fig 3.3. See also table 3.19

Table 3.17 Dihydrolipoate-driven ATP synthesis in bovine heart SMP and chloroform released F_1 ATPase

Additions	ATP synthesis µmoles/20 min
SMP	0
SMP + dihydrolipoate (2 µmoles)	0.546
$CHCl_3$ released 'F_1' ATPase	0
$CHCl_3$ released 'F_1' ATPase + dihydrolipoate (2 µmoles)	0
$CHCl_3$ released 'F_1' ATPase + oleoyl-S-lipoate (1 µmole)	0

Assays containing either 1 mg SMP protein, 1 µg rotenone and 1 µg antimycin A or 100 µg chloroform released 'F_1' ATPase protein were preincubated for 5 minutes at 37° C in a 1 ml glucose-hexokinase trap system, 0.2 mM in ADP, containing 25 units Sigma F-300 hexokinase, prior to initiation by addition of dihydrolipoate (as a 0.2 M solution in 0.25 M sucrose- 10 mM Tris-Cl, pH 7.5; 1 mM EDTA) or oleoyl-S-lipoate (as a 0.1 M solution in dimethylformamide). Assays were terminated after 20 minutes by taking an 0.2 nl aliquot into 0.2 ml 10% TCA. Protein was removed by centrifugation and 0.2 ml of the supernatant was assayed for phosphate as described in the methods section to Chapter 3.

Table 3.18 Effect of an 'inhibitory dihydrolipoate' on 'dihydrolipoate'-driven ATP synthesis in bovine heart mitochondria

Additions	ATP synthesis moles/20 min
mitochondria	0
mitochondria + pyruvate/malate (10 µmoles/1 µmole)	4.92
mitochondria + rotenone + antimycin A	0
mitochondria + rotenone + antimycin A + dihydrolipoate I (2 µmoles)	1.72
mitochondria + rotenone + antimycin A + dihydrolipoate B (2 µmoles)	0
mitochondria + rotenone + antimycin A + dihydrolipoate I (2 µmoles) + dihydrolipoate B (200 nmoles)	1.16
mitochondria + rotenone + antimycin A + dihydrolipoate I (200 nmoles) + dihydrolipoate B (2 µmoles)	0.15

Dihydrolipoate I and B are as described in the legend to Table 3.18.

Assays containing 1 mg mitochondrial protein were preincubated with 1 µg rotenone and, where indicated, for 5 minutes at 37° C in a shaking water bath in a 1 ml glucose-hexokinase trap system, 0.2 mM in ADP, containing 25 units Sigma F-300 hexokinase, prior to addition of dihydrolipoates. Similar quantities of dihydrolipoates (200 nmoles) were added 1 minute before larger amounts (2 µmoles). Assays were terminated after 20 minutes and phosphate content determined as described in Table 3.17.

Table 3.19 ATP synthesis in bovine heart mitochondria by an array of dihydrolipoates

Additions	ATP formed[1]	Thiol content[2]	Smell[3]	ATP formed[1]	Thiol content[2]	Smell[3]	ATP formed[1]	Thiol content[2]	Smell[3]
mitochondria	0			0			0		
mitochondria + pyruvate/malate	4.22			5.47			0.625		
mitochondria + rotenone + antimycin A	0			0			0		
mitochondria + rotenone + antimycin A+ dihydrolipoate A	0.16	0.006	b	0.15	0.109	b	0	0.109	b
as between *...* above + dihydrolipoate B	0	1.389	st	0	1.057	st	0	1.003	st
... + dihydrolipoate C	0	0.743	rt	0	0.615	rt	0.39	0.495	b
... + dihydrolipoate D	0.32	0	b	0.66	0	b	nt	nt	nt
... + dihydrolipoate E	0.16	0	b	0.22	0	b	nt	nt	nt
... + dihydrolipoate F	0	0	b	0	0.391	-	0.15	0	-
... + dihydrolipoate G	0.16	0.402	rt	0.42	0.169	-	0	0.093	-
... + dihydrolipoate H	0	0.717	rt	0	0	-	0	0.457	rt
... + dihydrolipoate I	1.01	1.059	st	1.55	0.220	st/b	1.09	0	b
... + dihydrolipoate J	nt	nt	nt	0.42	1.059	-	0	1.302	rt
Assay date		12th May, 1977			16th May, 1977			18th May, 1977	

[1] µmoles/20 minutes; [2] µmoles SH [3] smell - b: biscuit, st: sweet thiol, rt: rank thiol.

ATP synthesis was determined by phosphate disappearance in a 1 ml glucose-hexokinase trap system, 0.2 mM in ADP, containing 25 units Sigma F-300 hexokinase, essentially as described in Table 3.17. SH content assayed by DTNB titration, as described in the methods section to this chapter. Assays were initiated by addition of 1 µmole dihydrolipoate except dihydrolipoate J (2 µmoles) or 10 µmoles pyruvate/ 1 µmole malate, pH 7.5.

Table 3.20 Inhibition of oxidative phosphorylation by 'polymerised lipoate' solutions

Additions	Oxidative phosphorylation nmoles ATP formed/min/mg
none	0
10 µmoles pyruvate/1 µmole malate	157
10 µmoles pyruvate/1 µmole malate + 10 nmoles plip(s-s)	98
1o µmoles pyruvate/1 µmole malate + 100 nmoles plip(s-s)	0
10 µmoles sodium succinate	209
10 µmoles sodium succinate + 100 nmoles plip(s-s)	0

Assays containing 1 mg bovine heart mitochondria were preincubated with plip(s-s), where indicated, for 5 minutes at 30° C in a 1 ml glucose-hexokinase trap system, 0.2 mM in ADP, containing 5 units Sigma F-300 hexokinase. Assays were initiated by addition of pyruvate/malate or succinate and were terminated after 15 minutes incubation at 35° C in a shaking water bath by taking an 0.2 ml aliquot into 0.2 ml ice cold 10% TCA. ATP synthesis was estimated from phosphate disappearance by the method of Fiske and Subbarow, as described in the methods section to this Chapter.

Table 3.21 Inhibition of NADH → $Fe(CN)_6^{3-}$ reductase activity in bovine heart mitochondria and SMP's by plip (S-S)

Additions	Rate[1]	
	SMP	mitochondria
none	0	0
NADH	7.97	4.42
NADH + 200 nmole plip(s-s)[2]	0.47	0
NADH + 200 nmole plip(s-s)[3]	1.17	0.94
NADH + 170 nmole oleate[2]	7.97	4.42
NADH + 170 nmole oleate[3]	7.97	4.42
NADH + 100 µg '1799'[2]	7.97	nt
NADH + 10 ng DBCT[2]	7.97	nt

[1] µ moles ferricyanide reduced/min/mg

[2] addition during assay

[3] preincubated with enzyme prior to NADH addition

Assays containing 32 µg SMP protein or 26 µg mitochondrial protein were initiated by addition of 250 nmoles NADH. Fericyanide reductase activity was determined as described in the methods section to this Chapter. Activities quoted were corrected for any non-enzymic reduction of ferricyanide.

plip(s-s): polymerised lipoate, originally an ozidised lipoate solution, 0.2 M in methanol

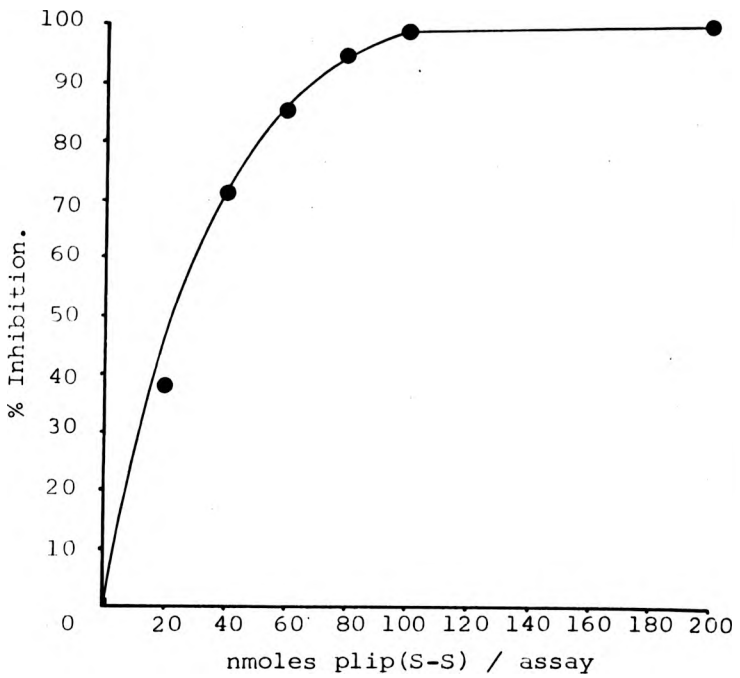


Fig 3.5. Inhibition of NADH — $Fe(CN)_6^{3-}$ reductase activity by plip(S-S).

Assays containing 32µg bovine heart SMP protein were performed as described in the methods section to chapter 3.

Specific activity: 5.156 µmoles $Fe(CN)_6^{3-}$ reduced / min / mg.

See also table 3.21.

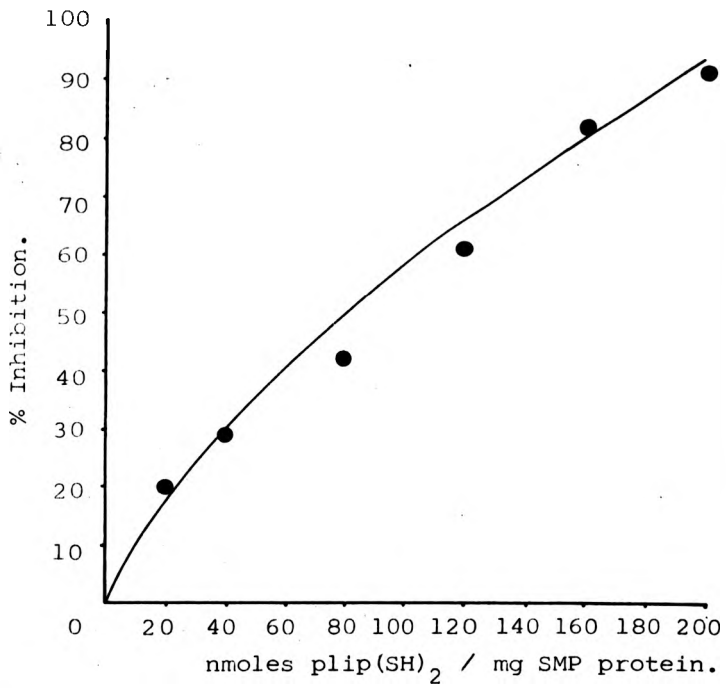
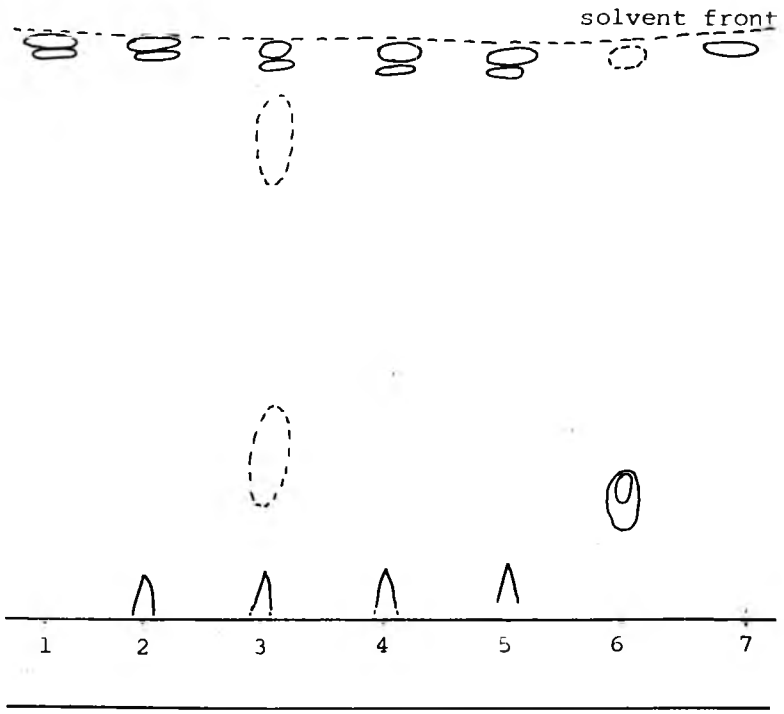


Fig 3.6. Inhibition of ATP driven transhydrogenase by plip$(SH)_2$.

Assays containing 1mg bovine heart SMP protein were preincubated with plip$(SH)_2$ for 5 minutes at 30°C before being assayed for ATP driven transhydrogenase activity as described in the methods section to chapter 6.

Specific activity of the uninhibited transhydrogenase = 57.4 nmoles $NADP^+$ reduced / min / mg SMP protein.

Fig 3.7. Thin layer chromatograms of (polymerised) lipoic acids.



Solvent system. $CHCl_3/CH_3OH/HCOOH$. 8 : 1 : 1.
See also table 3.22

spots visualised with iodine vapour.

spots visualised with H_2SO_4 and charring.

Not all lipoates that possessed this smell had the ability to drive ATP synthesis, but most of those that did drive synthesis did smell like this.

Dihydrolipoic acid and oxidised lipoic acid solutions have a strong tendency to photooxidise and polymerise. It was polymerised products of these two that were responsible for the inhibition of oxidative phosphorylation observed (Table 3.20). These compounds were further investigated to find their mode and/or locus of action. Polymersied lipoic acid inhibits electron transport through site 1 (Table 3.21; Fig. 3.5). The levels of polymerised lipoate required to inhibit electron transport were, however, substantially higher than those required to inhibit oxidative phosphorylation. A locus of action close to the coupling mechanism was suggested by their ability to inhibit ATP-driven energy linked transhydrogenase (Fig. 3.6) and to stimulate the coupled ATPase activity of bovine heart and rat liver mitochondria (Tables 3.22-23). Uncoupling agents will also stimulate coupled ATPase (by releasing respiratory control). Polymerised lipoate, however, differs from uncouplers in its ability to stimulate uncoupled mitochondrial ATPase activity (Table 3.23, Fig. 3.9). This stimulation of ATPase activity was not due to a partial release of F_1 ATPase as the stimulated activity displayed the same sensitivity to ATPase inhibitors as normal mitochondrial ATPase (Table 3.25; Figs. 3.11-12). Determinations of the Km ATP of mitochondrial ATPase in the presence of lipoate showed that the affinity for ATP was lowered but the v_{max} of the system was increased (Fig. 3.10).

The rarity of observed dihydrolipoate-driven ATP synthesis, coupled with the extreme complexity of lipoic acids solution chemistry, made identification of the components in dihydrolipoate solutions responsible for ATP synthesis very difficult. Lipoic acid has a rather

Table 3.22 Effect of various dihydrolipoates on ATPase activity in bovine heart mitochondria and SMP

Additions	ATPase			
	mitochondria		SMP	
	specific activity[1]	% activity	specific activity[1]	% activity
none	0.916	100	2.55	100
dihydrolipoate 1	0.924	101	2.59	101
dihydrolipoate 2	0.822	90	2.55	100
dihydrolipoate 3	1.814	198	2.76	108
dihydrolipoate 4	0.800	87	2.59	101
dihydrolipoate 5	0.962	105	3.10	121
dihydrolipoate 6	2.100	229	2.89	113

[1] µmoles phosphate released/min/mg

Assays containing either 100 µg mitochondrial protein or 57 µg SMP protein were preincubated with 20 nmoles dihydrolipoates for 5 minutes at 30° C in 1 ml 50 mM Tris-Cl, pH 8.5; 5 mM $MgCl_2$ prior to initiation of assay with 5 µmoles ATP. Assays were terminated with 0.5 ml 10% TCA and phosphate was determined as described in the methods section to Chapter 3.

See Fig. 3.7 for TLC of dihydrolipoates

Table 3.23 Stimulation of rat liver mitochondrial ATPase by polymerised lipoate

Additions	ATPase specific activity[1]	
	-plip(s-s)	+plip(s-s)
none	0.386	0.749
DNP	0.492	0.759
TTFB	0.606	0.914
Gramicidin D	0.633	0.820
Valinomycin	0.351	0.890

[1] µmoles phosphate released/min/mg

Assays containing 100 µg mitochondrial protein were preincubated with ionophores, uncouplers (1 µg/assay) and polymerised oxidised lipoate (plip(s-s)) for 5 minutes at 30° C prior to initiation by addition of 5 µmoles ATP. Assays were terminated after 5 minutes at 30° C by addition of 0.5 ml 10% TCA. Phosphate was determined as described in the methods section to this Chapter. Assays were incubated in 1 ml 50 mM Tris-Cl, pH 8.5; 5 mM $MgCl_2$.

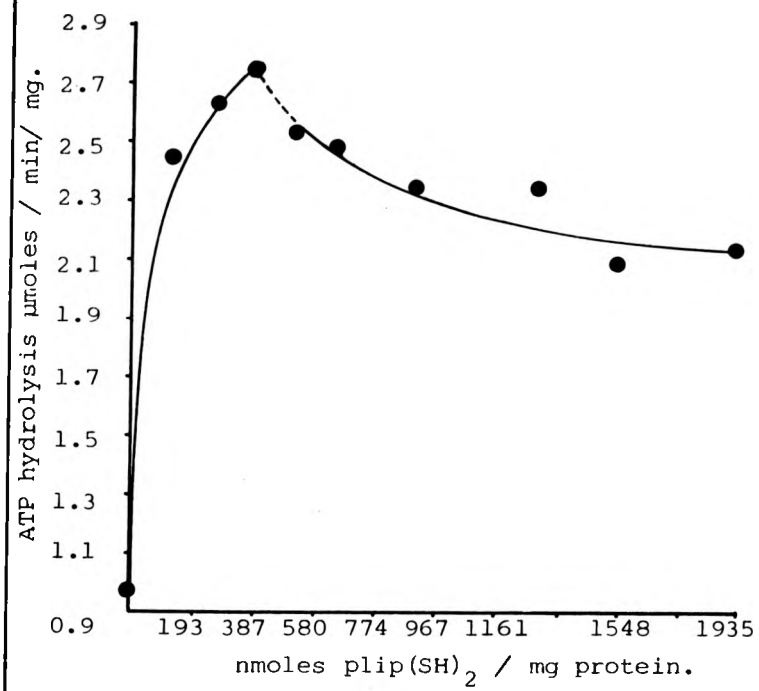


Fig 3.8. Stimulation of coupled bovine heart mitochondrial ATPase by plip(SH)$_2$.

Assays containing 155µg bovine heart mitochondrial protein in 0.9 ml 50mM Tris-Cl pH 8.5;5mM $MgCl_2$ were preincubated with plip(SH)$_2$ for 5 minutes at 30°C. Assays were initiated by the addition of 5 µmoles ATP and terminated after 5 minutes at 30°C by the addition of 0.5 mls ice cold 10% TCA.Free Pi and hence ATPase activity was determined as described in the methods section to chapter 3.

Table 3.24 Polymerised dihydrolipoate stimulated bovine heart mitochondrial ATPase: effect of uncouplers and ionophores

Additions	ATPase specific activity[1]	
	coupled ATPase[2]	uncoupled ATPase[3]
none	1.78	0.60
$plip(SH)_2$	3.34	0.97
$plip(SH)_2$ + FCCP	3.34	0.97
$plip(SH)_2$ + CCP	3.61	nt
$plip(SH)_2$ + 1799	3.44	nt
$plip(SH)_2$ + TTFB	3.34	0.97
$plip(SH)_2$ + DNP	3.61	0.97
$plip(SH)_2$ + PCP	3.70	nt
$plip(SH)_2$ + DBrP	3.70	nt
$plip(SH)_2$ + S13	3.70	0.90
$plip(SH)_2$ + valinomycin	4.01	0.97
$plip(SH)_2$ + nigericin	4.32	0.97
$plip(SH)_2$ + gramicidin	4.41	1.02

[1] moles phosphate released/min/mg

[2] coupled ATPase. ATPase activity in coupled mitochondria that display respiratory control and uncoupler stimulated ATPase

[3] uncoupled ATPase: ATPase in mitochondria lacking repsiratory control or an uncoupler stimulated ATPase

Assays containing either 100 µg coupled or uncoupled mitochondrial protein were preincubated with either 100 nmoles or 10 nmoles polymerised dihydrolipoate[4] and either 0.65 µg or 0.5 µg ionophores or uncouplers for 5 minutes at 30° C in 1 ml 50 mM Tris-Cl, pH 8.5; 5 mM $MgCl_2$ prior to initiation of assay with 5 µmoles ATP. Assays were terminated after 5 minutes with 0.5 ml 10% TCA; phosphate was determined as described in the methods section to this Chapter.

[4] nmoles polymerised dihydrolipoate, $plip(SH)_2$, expressed as nmoles dihydrolipoate expected from initial molarity of solution prior to polymerisation.

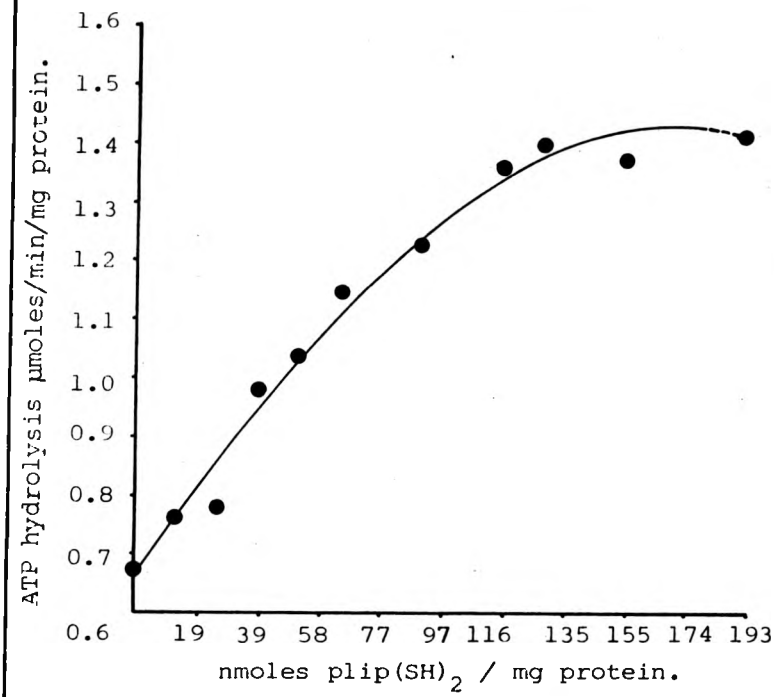


Fig 3.9 Stimulation of uncoupled bovine heart mitochondrial ATPase by plip(SH)$_2$.

Assays containing 155 µg bovine heart mitochondrial protein were preincubated with plip(SH)$_2$ for 5 minutes at 30^{o}C .Assays were performed as described in fig 3.8.

Table 3.25 Effect of inhibitors on 'polymerised lipoate' stimulated bovine heart mitochondrial ATPase

Additions	ATPase specific activity[1]
none	0.634
polymerised lipoate	1.02
polymerised lipoate + FCCP	1.08
polymerised lipoate + TTFB	1.02
polymerised lipoate + oligomycin	0.04
polymerised lipoate + venturicidin	0.12
polymerised lipoate + DBCT	0.01
polymerised lipoate + TET	0.03
polymerised lipoate + efrapeptin	0
polymerised lipoate + aurovertin	0.04

[1] µmoles Pi released/min/mg

Assays containing 100 µg mitochondrial protein were preincubated with 13 nmoles polymerised dihydrolipoate[2] for 5 minutes at 30° C prior to preincubation with 1 µg inhibitors and uncouplers for a further 5 minutes at 30° C in 1 ml 50 mM Tris-Cl, pH 8.5; 5 mM $MgCl_2$. Assays were initiated by addition of 5 µmoles ATP and incubated at 30° C for 5 minutes before termination by addition of 0.5 ml 10% TCA. Protein was removed by centrifugation and phosphate determined by the method of Fiske and Subbarow, as described in the methods section to this Chapter.

[2] nmoles of polymerised lipoate expressed as nmoles dihydrolipoate, i.e. with reference to original molarity of solution prior to its polymerisation (see discussion section to this Chapter).

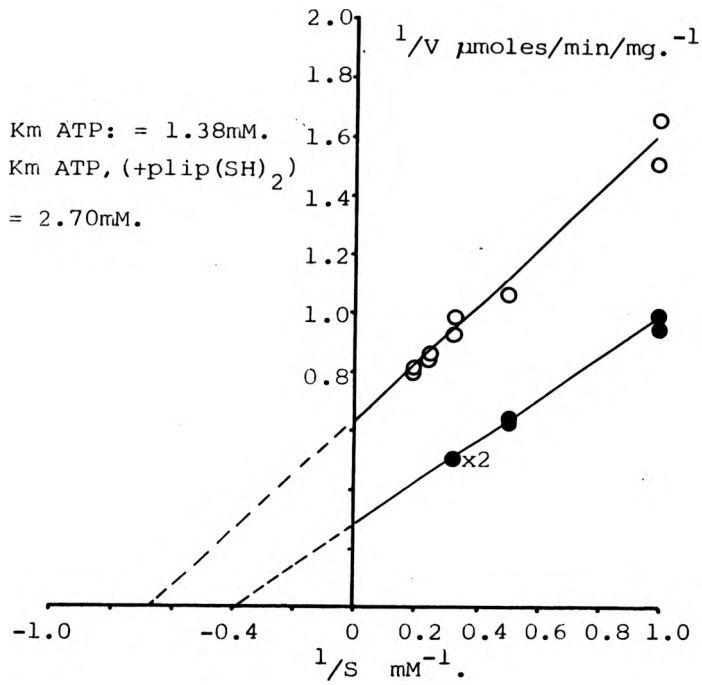


Fig 3.10.Bovine heart mitochondrial ATPase;Km: effect of plip(SH)$_2$.

ATPase assays containing 130µg bovine heart mitochondrial protein preincubated with 30 nmoles plip(SH)$_2$ for 5 minutes at 30°C were initiated with ATP and assayed essentialy as described in fig 3.8.

o—o -plip(SH)$_2$.

●—● +plip(SH)$_2$.

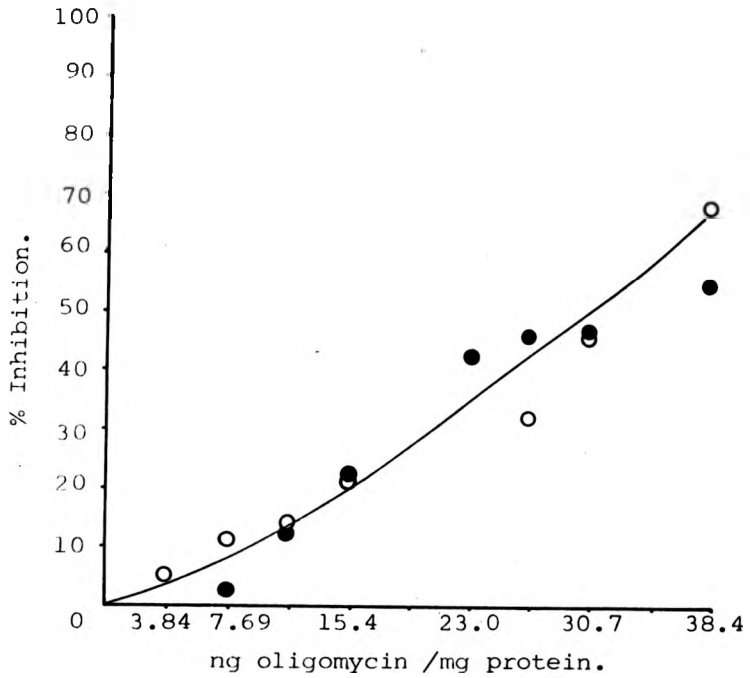


Fig 3.11. Oligomycin inhibition of bovine heart mitochondrial ATPase: effect of plip$(SH)_2$.

●—● bovine heart mitochondria

○—○ " " " preincubated with plip$(SH)_2$, 300 nmoles / mg protein.

Assays containing 130µg bovine heart mitochondrial protein were preincubated with oligomycin and plip$(SH)_2$, where indicated, for 5 minutes at 30°C prior to initiation of the assay with 5 µmoles ATP. Assays were terminated after 5 minutes at 30°C and ATPase activity was determined as described in the methods section to chapter3
Specific activity ATPase = 1.32 µmoles/min/mg.

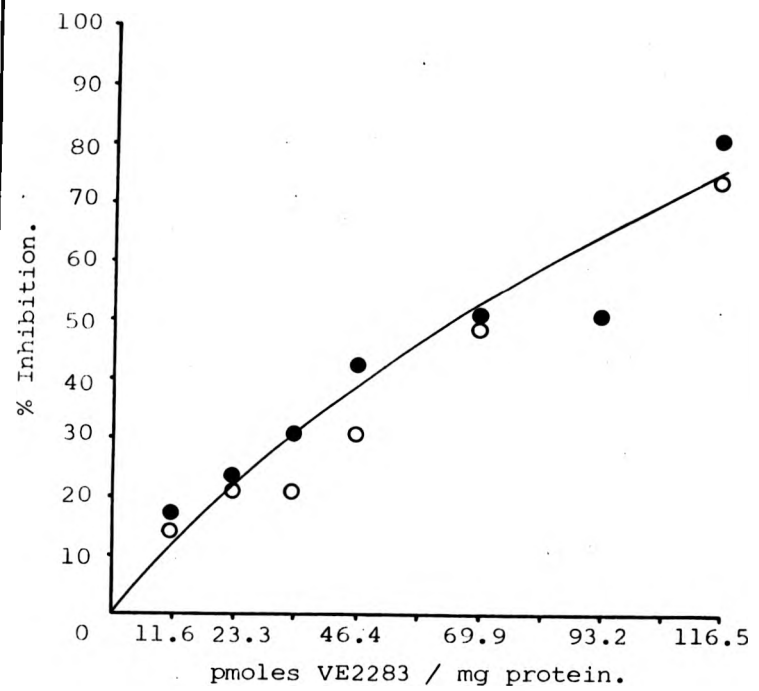


Fig 3.12. VE2283 inhibition of bovine heart mitochondrial ATPase: effect of plip$(SH)_2$.

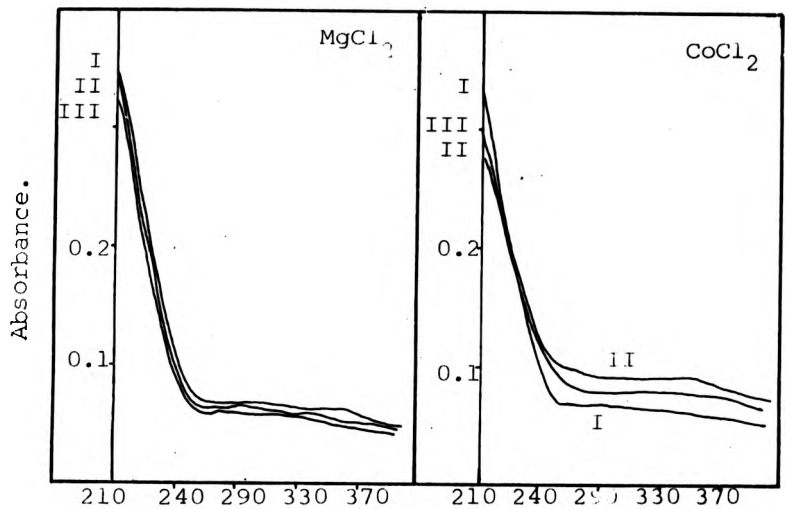
●—● bovine heart mitochondria.

o—o " " " preincubated with plip$(SH)_2$, 182 nmoles / mg protein.

Assays containing 220µg bovine heart mitochondrial protein were preincubated with VE2283 and plip$(SH)_2$, where indicated, for 5 minutes at 30°C. Assays were carried out as described in fig 3.11.

Specific activity ATPase = 0.86 µmoles ATP hydrolysed / min / mg.

Fig 3.13.Dihydrolipoate-metal ion u.v.spectra: Mn^{2+},Mg^{2+},Ca^{2+},K^{+},Na^{+},NH_4^{+},Co^{2+}.



I:0.4 µmole lip$(SH)_2$

II: as I + 0.2µmole $MgCl_2$.

III: as I + 0.4 " "

I:0.4 µmole lip$(SH)_2$

II: as I+ 0.1µmole $CoCl_2$

III: as I + 0.4 " "

Spectra were recorded in a Pye-Unicam SP1800 dual-beam recording spectrophotometer in 1ml 50mM Tris-Cl pH 7.5. Spectra were recorded against blanks containing 1 ml 50mM Tris-Cl and metal ions at a concentration equivalent to that in the sample cuvette.

Similar spectra were also observed with Mn^{2+},Ca^{2+}, K^{+},Na^{+} and NH_4^{+}.

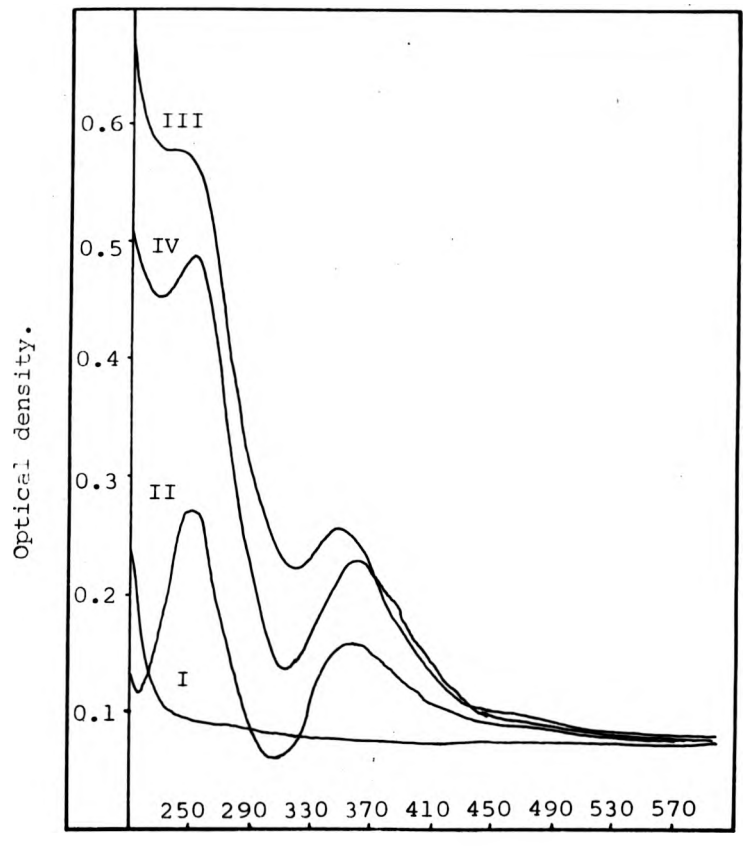


Fig 3.14 Dihydrolipoate-metal ion u.v. spectra:Fe^{3+}.

Spectra were recorded in a Pye-Unicam SP1800 dual beam spectrophotometer as described in fig 3.13.

I : 200 nmole $lip(SH)_2$.
II : " + 0.1 µmole $FeCl_3$.
III : " + 0.2 µmole $FeCl_3$.
IV : " + 0.4 µmole $FeCl_3$.

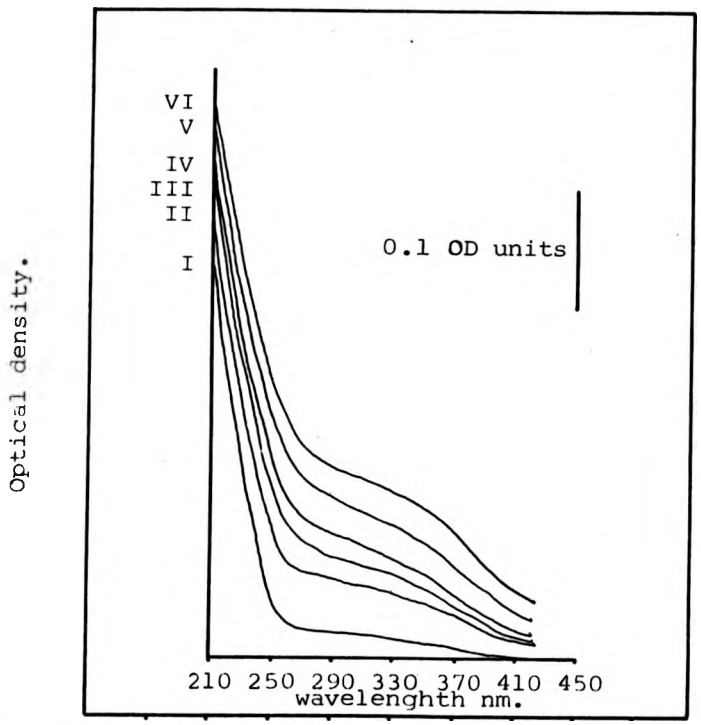


Fig 3.15 Dihydrolipoate-metal ion spectra: Fe^{2+}.

U.V. spectra were recorded in a Pye-Unicam SP1800 dual beam recording spectrophotometer as described in fig 3.13.

Spectrum I. 0.4 µmoles $lip(SH)_2$
" II. " + 0.1 µmoles $FeSO_4$.
" III. " + 0.2 " "
" IV. " + 0.3 " "
" V. " + 0.4 " "
" VI. " + 1.0 " "

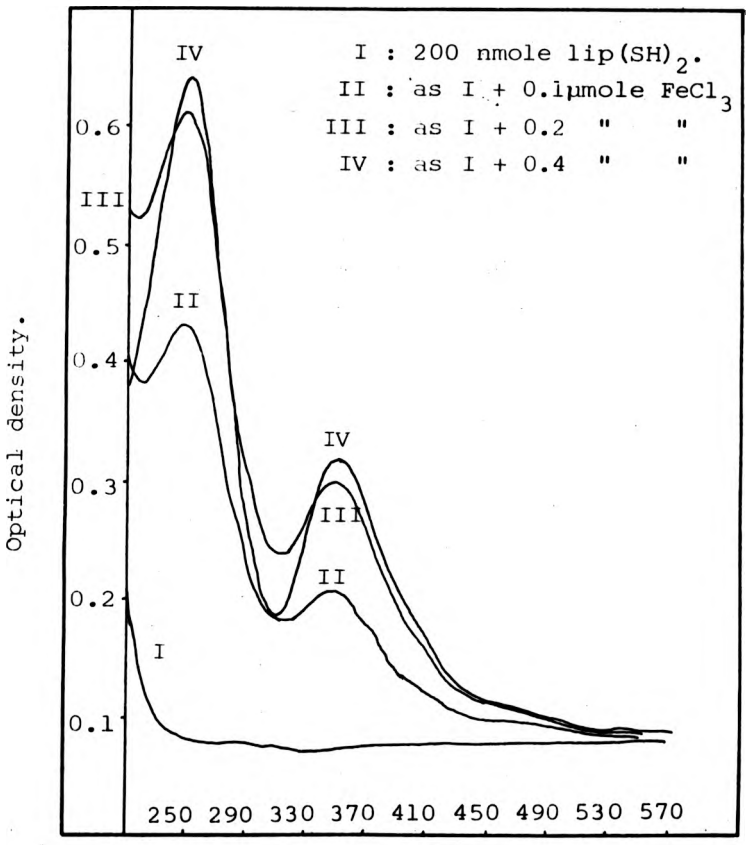


Fig 3.16 'Active' dihydrolipoate-Fe^{3+} u.v. spectra.

Spectra were recorded as described in fig 3.13. This sample of 'dihydrolipoate' drove ATP synthesis; stimulated ATP-Pi exchange and stimulated an energy linked transhydrogenase in bovine heart SMP.

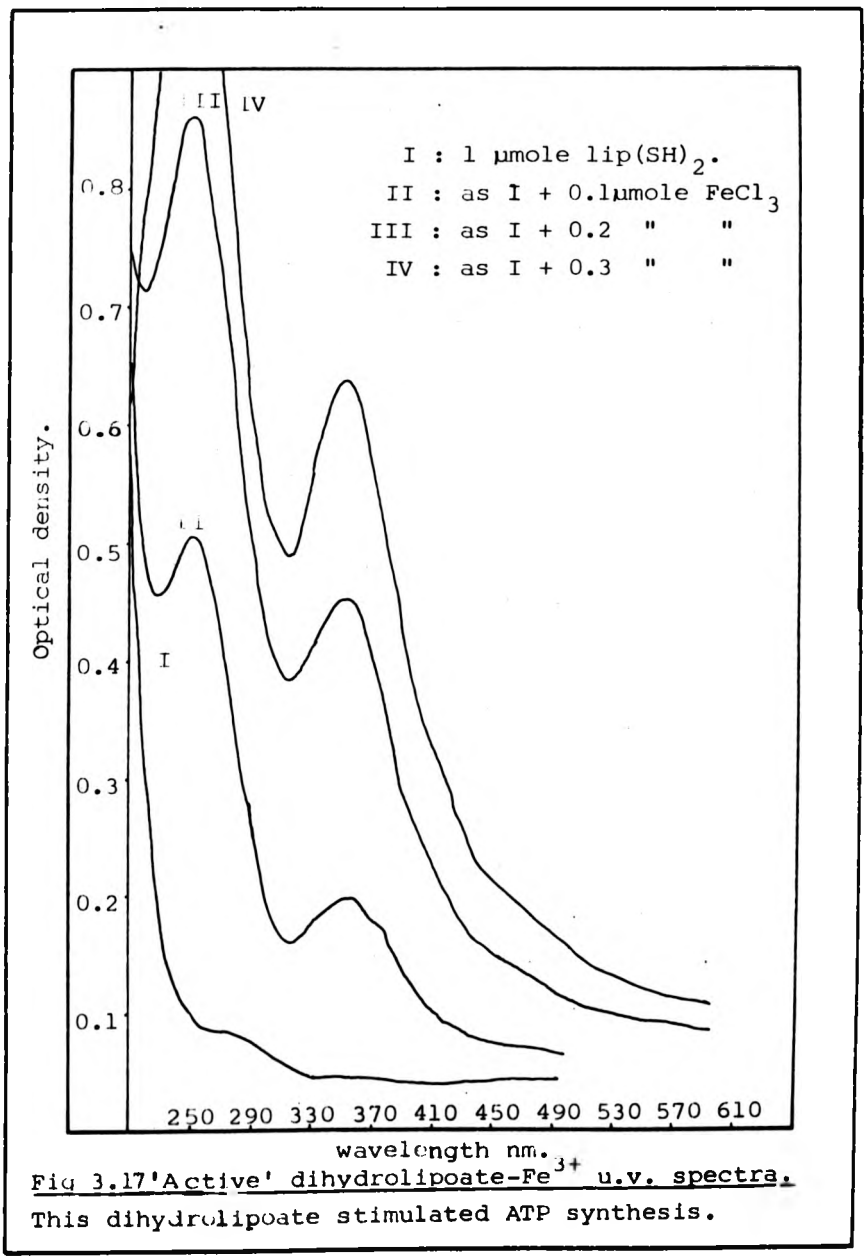


Fig 3.17 'Active' dihydrolipoate-Fe^{3+} u.v. spectra.
This dihydrolipoate stimulated ATP synthesis.

nondescript u.v. spectrum and virtually no fluorescence characteristics. Nuclear magnetic resonance spectroscopic analysis of solutions was not possible as the complex nature of the buffer system (sucrose; Tris-Cl; EDTA) made interpretation of spectra obtained impossible. Mass spectrometry only revealed the basic skeleton (M.W. 206) expected of lipoic acid.

Titration of dihydrolipoic acid with certain metal ions yields complexes which have characteristic u.v. spectra in the region 210-400 nm. Dihydrolipoic acid solutions were investigated, using various metal ions as 'probes' for structure or specific states. Metal ions such as Mn^{2+}, Mg^{2+}, Ca^{2+}, K^{+}, Na^{+}, NH_4^{+} induced no observable structural changes in dihydrolipoates u.v. spectrum in the 210-400 nm region (Fig. 3.13):metals such as Fe^{3+}, Fe^{2+}, Cr^{3+}, Co^{2+}, Cu^{2+}, Ni^{3+} did, however (Figs. 3.14-15). Of these, Fe^{3+} proved the most useful probe of dihydrolipoates 'active structure'. Solutions of dihydrolipoate that drove ATP synthesis and energy linked transhydrogenase reactions displayed characteristic Fe^{3+}-u.v. spectra (Figs. 3.16-17). These spectra may give some indication of the chemical nature of active dihydrolipoate.

Discussion

There are perhaps two central questions relating to the 'ATP synthesis' described in this chapter that must be asked. The first is is the observed synthesis real synthesis or an artefact of the assay? The second is if this synthesis is real, what relevance does it have to that observed in oxidative phosphorylation?

ATP synthesised during oxidative phosphrylation was routinely assayed ina glucose-hexokinase trap system which uses ATP to phosphorylate glucose producing glucose-6-phosphate. ATP synthesis can thus be assayed either by the disappearance of phosphate or the appearance of glucose-6-phosphate.

Phosphate was assayed by a method based on that described by Fiske and Subbarow (352). Here inorganic phosphate is complexed with molybdate in the presence of sulphuric acid, producing a phosphomolybdic complex $(NH_4)_3(PMo_{12}O_{40})aq$. This, when reduced to a molybdous form has a deep blue colour which can be assayed spectrophotometrically. The 'molybdenum blue' complex can be used to estimate inorganic phosphate quantitatively. The phosphomolybdic complex was reduced with 1-amino, 2-naphthol, 4-sulphonic acid.

When ATP is synthesised, Pi is removed from the assay. This is observed as a reduction in the amount of 'molybdenum blue' complex produced. Phosphorylation assays were customarily performed in buffers containing 4 µmoles-6 µmoles Pi/ml. The production of 1 µmole ATP in these conditions would produce a drop in the absorbance due to molybdenum blue of between ∿ 17-25%, depending on the original Pi concentration. Artefactual 'ATP syntheis' would be observed in this system if Pi was removed by a method other than esterification into ATP, or if the phosphomolybdic complex was formed with reduced efficiency, or if it were not reduced to the molybdous form with the same efficiency (relative to standard conditions, i.e. determination of Pi in the absence of enzymes or substrates). The source of these errors could be due to either non-enzymic or enzymic effects. No enzyme, control assays run in the presence of substrate show no diminution in the amount of detectable phosphate compared to standard assays, suggesting that non-enzymic effects (such as precipitation of a Pi-lipoate adduct) are not responsible for the diminution in the phosphate content observed. Indeed dihydrolipoate has the opposite effect, if any, increasing the amount of 'molybdenum blue' complex. This is because dihydrolipoate can reduce the complex to its molybdous blue' form. The amount of dihydrolipoate usually 'carried over' from the phosphorylation assay into the phosphate determination step is too

small to appreciably effect the amount of 'molybdenum blue' produced.

There are a number of possible (though not probable) enzymic effects that could result in an apparent ATP synthesis. Phosphate could be adduced directly to glucose by some lipoate mediated or stimulated enzyme system present in mitochondria, SMP's or mitochondrial F_1F_o ATPases. This enzyme system is unknown and if it exists would have the rather interesting properties of being inhibited by energy transfer inhibitors such as oligomycin (thought to be a specific inhibitor of the F_1F_o ATPase complex) and uncoupling agents such as S13, 1799, FCCP and DNP, and copurifying with the ATPase complex. As ATP was not determined directly (by chromatographic purification for example), there remains a possibility that this or a similar reaction was taking place. Phosphate could have been esterified into some other component of the assay system, e.g. sucrose, 'Tris'base or glucose, by an unknown enzymic contaminant stimulated or mediated by lipoate. Again this contaminant would have to be present in mitochondria and purified F_1F_o ATPase systems and be sensitive to energy transfer inhibitors and uncoupling agents. Such an enzymic system would not, however, explain the apparent production of glucose-6-phosphate (Tables 3.2-5) and can probably be discounted as a source of spurious 'ATP synthesis'.

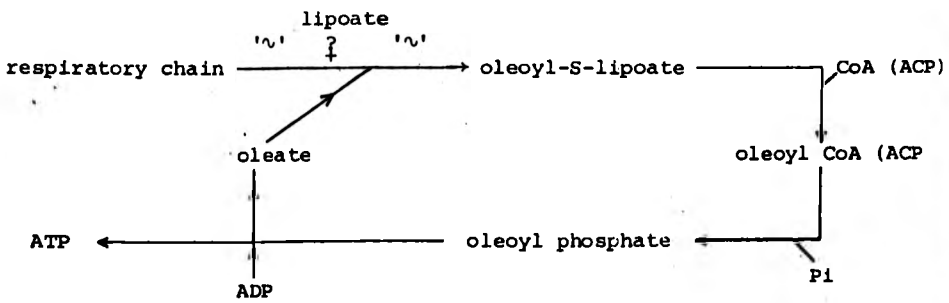
Mitochondria and hexokinase are contaminated by myokinase activities. These are enzymes which can convert ADP to ATP and AMP.

$$2\ ADP \overset{\text{myokinase}}{\rightleftharpoons} ATP + AMP$$

The glucose hexokinase trap systems used to estimate ATP synthesis were 2 mM or 0.2 mM in ADP; myokinase or dihydrolipoate stimulated myokinase activity could have produced $\sim$ 2 or $\sim$ 0.2 μmoles ATP by this mechanism. This could have been used to phosphorylate glucose, producing glucose-6-phosphate. Myokinase activity could thus give a spurious ATP synthesis as assayed by the appearance of glucose-6-phosphate. This could, however,

not explain the observed parallel disappearance of phosphate from the assay system (Tables 3.2-5) and can then be discounted as a source of major error in the assays. (For a further discussion of myokinase activity and possible artefactual systems associated with it in these assays, see the discussion section to Chapter 5). Control experiments have failed to demonstrate a dihydrolipoate stimulated myokinase activity in either mitochondria or hexokinase.

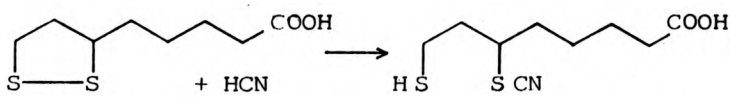
The observed ATP synthesis then cannot be ascribed to any simple artefact or contaminating enzyme system. This being so, we must assume that it is real ATP synthesis and attempt to relate it to the known ATP synthesising pathway in mitochondria, oxidative phosphorylation mediated *via* the ATP synthase enzyme. The observation of dihydrolipoate-driven ATP synthesis in mitochondria and submitochondrial particles, and the requirement for an unsaturated fatty acid and fatty acyl CoA as cofactors for synthesis in purified F_1F_O ATPase preparations (248) led to the suggestion that there might be a series of reactions analogous to those of substrate level phosphorylation associated with the energy transduction apparatus of bioenergetic membranes (235). Substrate level phosphorylation involves the generation of succinyl lipoate, its transacylation to CoA and the generation of GTP *via* a succinyl phosphate intermediate (these reactions are discussed in greater detail in the discussion to Chapter 5). Griffiths postulated the oleoyl cycle as a framework for further experimentation (235).



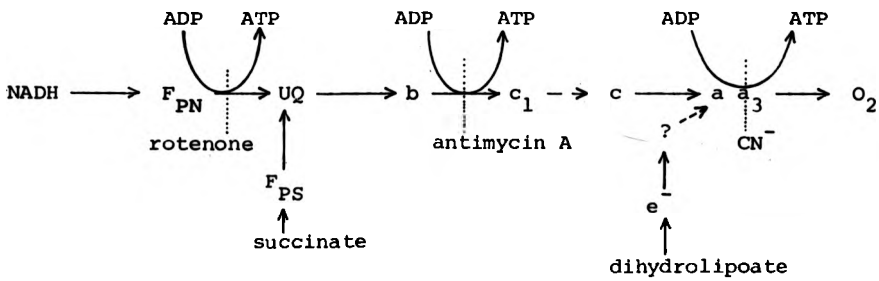
'Dihydrolipoate'-driven ATP synthesis was observed on a number of occasions in mitochondria, submitochondrial particles and photosynthetic bacteria chromatophores. Synthesis was not observed in purified F_1F_O ATPases (such as complex V, 'proton translocating' ATPase) with dihydrolipoate alone; here two further components had to be present in cofactor-like quantities. These were an unsaturated fatty acid and an unsaturated fatty acyl CoA. There appeared to be a specificity for oleic acid in these reactions (Tables 3.3-7; Fig. 3.2). Although other cis Δ-9 monoenoic acids (palmitoleic, ricinoleic acids) have been reported to substitute for oleic acid (320, 375), this effect was not observed. Oleoyl-CoA was the only CoA effective in these reactions, palmitoyl CoA, acetyl CoA and free HSCoA had no effect (see (248)). Various fatty acyl lipoates were synthesised;again there appeared to be a requirement for oleic acid in the fatty acyl moiety of these compounds (Tables 3.8-9,14,15) as stearoyl lipoate, elaidoyl lipoate, erucoyl lipoate and other fatty acyl lipoates did not drive ATP synthesis under any conditions in either vesicular ATPase or purified ATPase preparations. Certain fatty acids, notably elaidic acid, the transisomer of oleic acid, and erucic acid appeared to be potent inhibitors of dihydrolipoate-driven ATP synthesis (Tables 3.7,14,15). Similar results were obtained with elaidoyl and erucoyl derivatives on oleoyl phosphate-driven ATP synthesis (see Chapter 5).

Dihydrolipoate-driven ATP synthesis was observed with ATPase systems derived from a number of sources, bovine heart and rat liver mitochondria, Escherichia coli and Rhodospirillum rubrum. Assays performed with mitochondria, submitochondrial particles or bacterial vesicles were run in the presence of respiratory chain inhibitors such as rotenone and antimycin or hydroxy quinoline-N-oxide (in the case of bacterial vesicles). Particles that have their respiratory chain blocked with cyanide do not display dihydrolipoate-driven ATP synthesis. This could be for two

reasons; cyanide reacts with thiol groups, giving isothiocyanates



a lipoic acid, isothiocyanate derivative might inhibit dihydrolipoate requiring reactions; a similar inhibition has been observed with the 8-methyl derivative of lipoic acid. The effect of cyanide may be due to a reaction with cytochrome oxidase, however. Dihydrolipoate has the ability to reduce cytochrome c in solution (not shown) and might under certain conditions be able to supply redox energy to this portion of the respiratory chain in a manner like that observed for ascorbate/TMPD couples. If this was so, then there could be a redox chain mediated transfer of redox energy between dihydrolipoate and oxygen <u>via</u> cyt c and cytochrome oxidase. Redox energy passing through this area of the respiratory chain can be used by coupling site 3 to synthesise ATP from ADP and phosphate.



Cyanide would inhibit this reaction. This reaction should not take place in purified F_1F_O ATPase preparations as they should contain no cytochromes and other respiratory chain enzymes which strongly suggests that ATP synthesis does not occur <u>via</u> this pathway. Respiratory chain mediated dihydrolipoate-driven ATP synthesis cannot be completely ruled out, however, as both complex V and proton translocating ATPase contain low levels of respiratory chain components (particularly

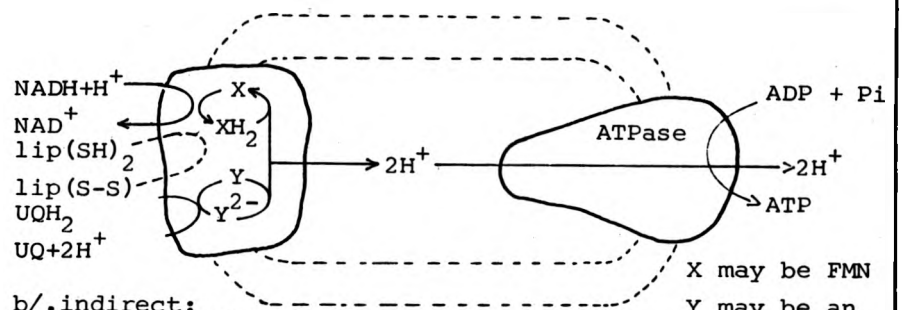
'proton translocating ATPase'), most notably complex I as assayed by NADH-ferricyanide reductase activity (a partial reaction of complex I (see (244),(245)). Complex I contains several types of thiol groups (see Chapter 1); it is not clear whether they are involved in the coupling site contained in this complex. The iron sulphur centres, however, probably are involved in the coupling mechanism, particularly centre 1a. The redox potentials of pyruvate/malate and NAD^+/NADH couples are -0.32 and -0.33 volts at pH 7 in aqueous solution, while that of lipoate ox/red is -0.29 under the same conditions: reduced lipoate would be quite able to tap into complex I at or around the level of the (Fe-S) centres. Redox active groupingsin hydrophobic environments (lipid-protein membranes) can occupy a wide range of redox potentials, depending on their microenvironment. It might be possible for dihydrolipoate to reduce NAD^+ under these conditions. It is interesting to note that diaphorase-like activities have been detected in complex V and proton translocating ATPase prepared in this laboratory (376). Reversal of this reaction would generate NADH from $lip(SH)_2$. This might then produce '$\sim$' via complex I which would, in turn, drive ATP synthesis via the ATP synthase complex (see Fig. 3.18 for details of these mechanisms).

The requirement for an unsaturated fatty acid and fatty acyl CoA for these reactions in purified ATPases makes these contamination explanations rather unlikely or very simplified, although these lipids may have a function in providing the correct hydrophobic environment for the enzymes. This is unlikely as the free fatty acid content of mitochondrial membranes is very low. Mechanisms of energy conservation at site I involving fatty acid groupings have been proposed, notably that by Weiss (377), which involves the reduction of a fatty acyl thioester to an aldehyde by NADH. Oxidation of this aldehyde by a non-haem iron centre gives a fatty acyl group which initially binds to a membrane base. As

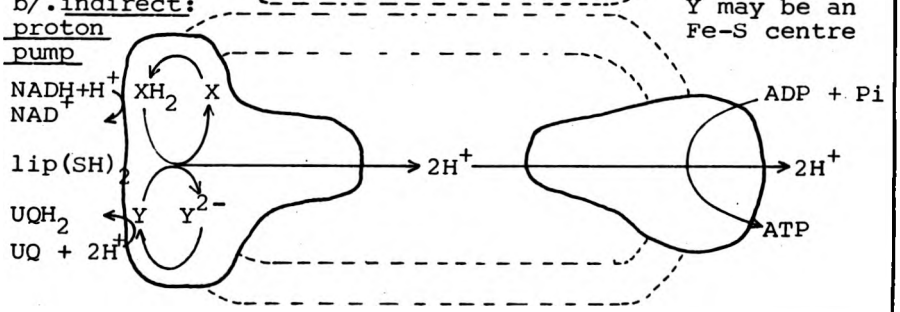
Fig 3.18. Dihydrolipoate driven ATP synthesis mediated by contaminating complex I in F_1F_o ATPase preparations.

1/. Chemiosmotic mechanism requires closed vesicles

a/. direct loop

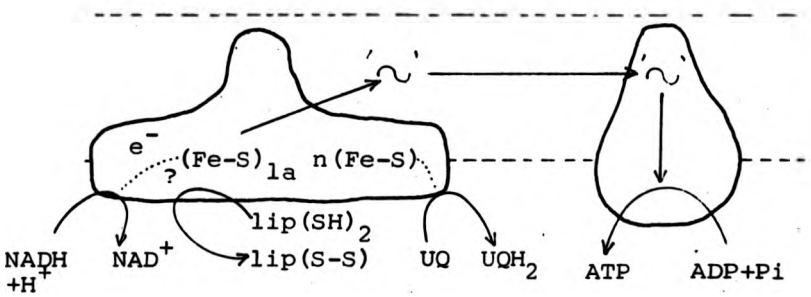


b/. indirect: proton pump

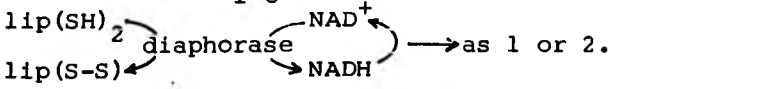


2/. Chemical, conformational or proton in membrane model.

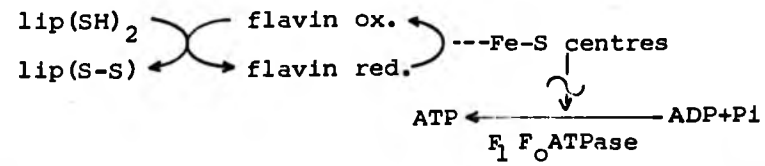
no requirement for closed vesicles



3/. Variant mechanism. Reversal of diaphorase like activity contaminating F_1F_o ATPase preparations.



4/. Variant mechanism. Reduction of complex I flavin.



non-haem iron reduces, entropy is lost through the stretching of a lipid bilayer attached to non-haem iron and the membrane to which the fatty acid chain binds. Simultaneously, energy is expended in separating a carboxyl from the protonated base. This charge separation induces a movement of protons and reactants,resulting in ATP formation. An essential step in this mechanism is the reduction of flavin by NADH; the reduced flavin then reduces the fatty acyl thioester to a fatty aldehyde form and the reaction proceeds as outlined above. Dihydrolipoate is fully capable of reducing flavins and could tap into this reaction pathway at this level.

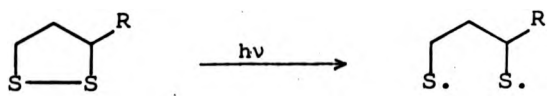
The requirement for a fatty acid can be explained in terms of the oleoyl cycle. Here lipoate (whether it is bound, 'free' or in some conjugate form is not defined) is acylated to oleoyl-S-lipoate by oleate or an oleoyl X (where X is an oleoyl carrier, possibly an oleoyl ubiquinone conjugate). Purified F_1F_o ATPases such as complex V and 'proton translocating' ATPases are prepared by cholate and deoxycholate fractionation of mitochondria followed by ammonium sulphate precipitation. All these procedures delipidate the enzymes being purified; indeed, the ATPases eventually obtained require added phospholipid for maximal activity. Oleoyl, lipooyl and similar lipid groupings would also be stripped from the ATPase complex by these detergent fractionation steps (assuming that they are not bound to the bulk protein of the complex). This could explain the requirement for fatty acids and fatty acyl CoA's in dihydrolipoate-driven ATP synthesis in these purified ATPases. The added lipids would replace (or reactivate) the natural cofactors of the reaction removed (or damaged) during the isolation of the ATPases. It is worth noting that dihydrolipoic acid and oleoyl CoA will under appropriate conditions nonenzymically form oleoyl-S-lipoate in solution.

Support for the concept of the 'oleoyl cycle' might be derived from the observed oleoyl-S-lipoate-driven ATP synthesis in F_1F_o ATPase enzymes.

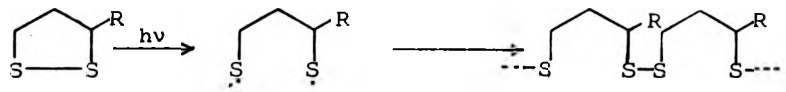
This synthesis had an apparent specificity for oleoyl-S-lipoate. However, other interpretations may be made of these results. Dihydrolipoate-driven ATP synthesis has a requirement for oleic acid (or a similar fatty acid) (Table 3.6); other fatty acids inhibit this reaction. Oleoyl-S-lipoate prepared by the methods outlined earlier in this chapter contains some free dihydrolipoate; it may be that this was responsible for the observed ATP synthesis. Dihydrolipoate itself may not be the actual species responsible for driving ATP synthesis (this is discussed in greater detail later in this chapter); oleoyl-S-lipoate may contain or mimic this 'active' form of lipoic acid which drives ATP synthesis under these conditions. If this were so, other fatty acyl-S-lipoates might be expected to contain this active form of lipoate which might drive ATP synthesis. This did not appear to be the case (Tables 3.9,14,16). This does not mean, however, that oleoyl-S-lipoate has the specific ability among fatty acyl lipoates to drive ATP synthesis. Certain fatty acids and fatty acyl lipoates have the ability to inhibit dihydrolipoate-driven ATP synthesis (Tables 3.7,15,16). This may be happening in assays containing fatty acyl lipoates other than oleoyl-S-lipoate.

Until this point, for simplicity's sake, the lipoic acid species resonsible for driving ATP synthesis has been referred to as dihydro-lipoate. This was because those solutions which drove ATP synthesis all originally contained dihydrolipoate. There is evidence to suggest that this is <u>not</u> the case. As stated in the first sentence of the results section of this chapter, 'dihydrolipoate-driven ATP synthesis' was a reaction only very rarely observed. It has never, in my experience, been observed in freshly prepared solutions of dihydrolipoic acid, only in solutions that have been 'aged' in some way, usually by 2-3 cycles of freeze-thawing over a period of days. The solution chemistry of lipoic acid is complex; dihydrolipoate oxidises to the disulphide form fairly rapidly in solution on standing, especially in the light. Solutions of

'dihydrolipoate' that drive ATP synthesis display a unique TLC profile; that is, they appear to contain a compound, that of Rf value 0.74 in chloroform:methanol:formic acid (8:1:1) TLC systems. This compares with a Rf value of 0.92 for authentic oxidised lipoic acid and 0.95 for dihydrolipoate. Polymeric and sulphoxide forms of lipoic acid display varying Rf values, generally decreasing with increasing molecular weight (see Figs. 3.3,4,7). Experiments have also shown that there is no strict correlation between the stoicheiometry of 'dihydrolipoate'-driven ATP synthesis and dihydrolipoate's thiol content (see Table 3.19). Indeed, samples of dihydrolipoate that have been shown to contain no free thiol groups still possesses the ability to drive ATP synthesis (see dihydrolipoate I, Table 3.19: Fig. 3.3). Solutions of lipoic acid capable of driving ATP synthesis have a rather peculiar smell that can only be described as that of broken biscuits. The smell is intense and appears to be associated with most of these active forms. The nature of this active form of lipoic acid has been investigated, but as yet no firm conclusions have been reached. This is largely because of the complex nature of lipoic acids solution chemistry. This chemistry is complex mainly because of the reactivity of the dithiolane ring structure and the number of valence states that sulphur can occupy. The strain energy of lipoic acid's dithiolane ring is small, being only 4-6 k cal/mole (373-4); its reactivity comes from the ease with which the S-S bond is broken, giving a reactive dithiylyl radical

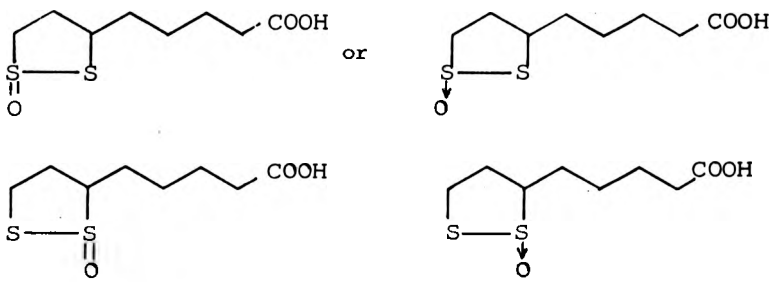


The energy required to break the S-S bond is small and can be supplied by light energy in the visible-ultraviolet range. Polymerisation is common in dithiolane compounds. These usually take the form of long linear polymers.

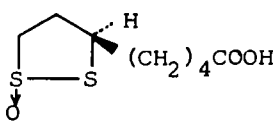


These polymers can be as much as 10-200 lipoate units in length (378). Similar polymerisations have been observed in dihydrolipoate solutions.

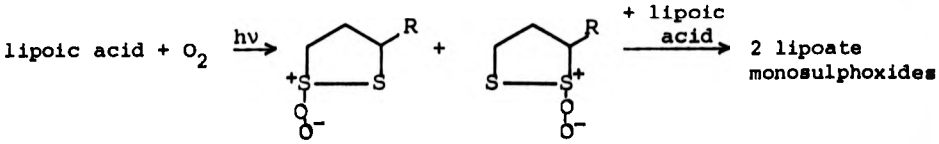
Sulphur can exhibit a number of valency states, most notably 2,4 6,8. Lipoic acid compounds often display the higher valency state in one or more of their sulphur atoms. The most widely known of these compounds are the monosulphoxides or thiosulphinates, also known as β-lipoic acid or 'protogen B'. They have the general structures:-



There are 8 isomeric forms of the lipoate monosulphoxides, 4 for the d and 4 for the l form of lipoic acid (380). β-lipoic acid was first isolated as an oxidation product of α-lipoic acid from bovine liver extracts (381) and has the structure:-

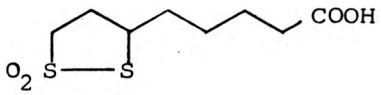


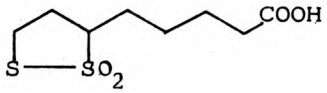
These compounds are formed by oxidation of lipoic acid by molecular oxygen in the presence of light.



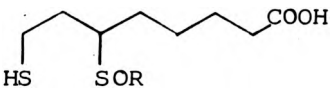
zwitterion intermediates

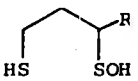
These intermediates can also give rise to a series of thiosulphonates of general structure.



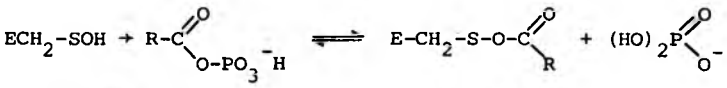


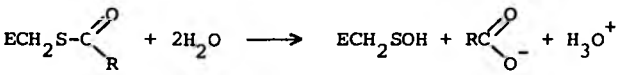
Further oxidation products, thiosulphonic acids, are also known. Another important, but little studied,group of oxidation compounds are the sulphenic acids, which have a general formula.



Photolysis of lipoic acid solutions produces the unstable 

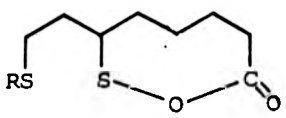
which later oxidises to the monosulphoxide. Sulphenic acids are known to play a role in enzyme catalysis. The sulphenic acid of cysteine $R\text{-}CH_2SOH$ may participate in acyl phosphatase activity via an intermediate acyl sulphenylate.



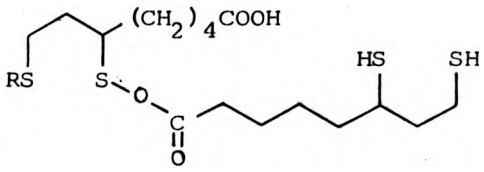


Glyceraldehyde-3-phosphate dehydrogenase catalyses the dehydrogenation of glyceraldehyde-3-phosphate. A thiol group derived from cysteine 149 is essential to this reaction. If it is oxidised to a sulphenate (-S-OH) the enzyme becomes an acyl phosphatase (382).

All the above mentioned compounds are likely breakdown products of dihydrolipoic acid in solution. Thiolactone compounds may also be formed in these solutions. These may have the structures:-



internal thiolactone
R = H or lipoic acid



external thiolactone
(acyl sulphenylate)

The internal ring thiolactones would be highly strained and thus rather unstable.

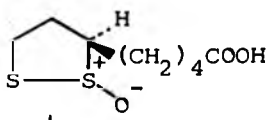
These lipoic acid compounds are all rather difficult to study in the complex solutions in which dihydrolipoate was originally made up. These solutions contain sucrose, Tris base and EDTA and make NMR studies of lipoic acid very difficult. It is unlikely that sucrose, Tris and EDTA play any direct part in the ATP synthesis observed as solutions of dihydrolipoate in dimethylformamide have also driven ATP synthesis, although with a much lower frequency than those in sucrose, Tris and EDTA buffers. Mass spectrometry of lipoic acid solutions yield only the basic skeleton of α-lipoic acid. Any unstable forms would break down under the ionisation conditions used. The u.v.-visible absorption spectrum of lipoic acid is rather nondescript, with a broad maximum at 333 nm. Dihydrolipoic acid solutions were titrated with metal ions to investigate possible structures. Metal ions such as Mn^{2+}, Mg^{2+}, Ca^{2+}, K^{+}, Na^{+}, NH_4^{+} induced no observable change in lipoic acid's u.v. spectra (Fig. 3.13). Metals such as Fe^{3+}, Fe^{2+}, Cr^{3+}, Co^{2+}, Co^{3+}, Ni^{3+}, Sn^{2+} did, however (Figs. 3.14,15). Of these, Fe^{3+} proved the most useful probe of dihydrolipoates 'active structure'. Solutions of dihydrolipoate that drove ATP synthesis and energy linked transhydrogenase displayed characteristic u.v.-Fe^{3+} spectra (Fig. 3.16,17). The chemical nature of lipoic acid in these complexes is unclear, but the spectra do display some similarity to that of β-lipoic acid (363). Preparations of complex V and the purple membrane of Halobacterium halobium (383) that supported dihydrolipoate-driven ATP synthesis displayed rather similar Fe^{3+}-u.v.-visible absorption spectra. It is worth noting that solutions of active

lipoate eventually precipitate as a white crystalline polymer which appears rather similar to polymers derived from oxidation products of α-DL lipoic acid.

During these investigations into the structure of 'active' lipoic acid, it was noticed that certain dihydrolipoate and lipoate solutions appeared to be fairly potent inhibitors of oxidative phosphorylation (Table 3.20). Investigation suggested that these inhibitory lipoates were linear chain polymers of lipoic acid (hereafter referred to as plip$(SH)_2$ or plip(S-S), depending on the nature of the original solution). These polymerised lipoates have a number of modes of action in mitochondria. They inhibit electron transport in complex I between NADH and ferricyanide (Table 3.21; Fig. 3.5) and also between succinate and oxygen (data not shown). They are more effective, however, at inhibiting energy linked reactions such as ATP-driven transhydrogenase (Fig. 3.6) and ATP-Pi exchange (data not shown). It should be pointed out that inhibition values expressed for plip$(SH)_2$ or plip(S-S) are quoted in terms of nmoles lip$(SH)_2$ or lip(S-S). This is done because the molecular weight of the polymers is not constant and each polymer may contain up to several hundred lipoate units, although 10 to 20 is more normal. The inhibition of oxidative phosphorylation, ATP-Pi exchange and ATP-driven transhydrogenase suggested that plip$(SH)_2$ or plip(S-S) might act at the locus of the F_1F_O ATPase. The polymerised lipoates do not inhibit the mitochondrial F_1F_O ATPase, however; they stimulate it (Table 3.22). Mitochondrial ATPase activity can be stimulated by uncoupling agents. This is thought to be due to release of the kinetic restraints imposed by respiration on the ATPase in coupled mitochondria. Polymerised lipoate can mimic this reaction, suggesting that it might act as an uncoupling agent (Tables 3.22-24; Fig. 3.8). However, plip$(SH)_2$ and plip(S-S) retained this ability to stimulate ATPase in uncoupled mitochondria

that is, mitochondria that display no uncoupler stimulated ATPase (Fig. 3.9; Table 3.24). Polymerised lipoate did not stimulate SMP ATPase (Table 3.22) or F_1 ATPase activity (data not shown). Double reciprocal plots of ATPase and stimulated ATPase activity reveal an increase in v_{max} of the ATPase and an increase in the K_{mATP} value (Fig. 3.10). The stimulated ATPase activity is not due to a release or partial release of F_1 ATPase as it exhibits the same sensitivity to F_1F_O ATPase inhibitors as unstimulated ATPase (Table 3.25; Figs. 3.11-12). Polymerised lipoate may act by activating (or deactivating) a thiol group involved in ATPase (or ATP synthase) activity by disulphide exchange.

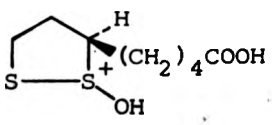
The scant evidence suggests that the active form of lipoic acid may be a thiolactone or oxidation product (sulphenate, sulphenylate) of dihydrolipoate. Other lipoic acid derivatives, lipoamide, dihydrolipoamides, 8-methyl lipoic acid, adenyl-lipoate and β-lipoic acid have not been observed to drive ATP synthesis under any conditions. Weiss has proposed a mechanism of oxidative phosphorylation in which a lipoate monosulphoxide and oleate play vital roles (384).



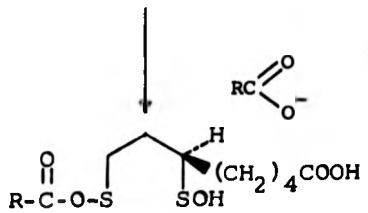
lipoic acid S-monoxide associated with ATPase enzyme (may be bound to protein via -COOH group)

H_3O^+

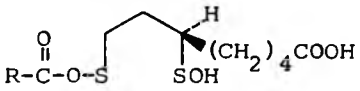
protonation within active site



protonated enzyme bound lipoic acid S-oxide

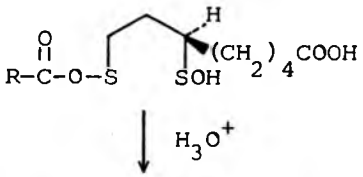


$RCOO^-$ could be oleate bound to the same enzyme and involved in the same active site



acyl sulphenylate which reacts with phosphate to give acyl phosphate. If the intermediate enzyme bound complex is required to have a reasonable lifetime prior to the appearance of a phosphate anion (and then ADP), the sulphenic acid group (SOH) might be stabilised by adding across the double bond in oleic acid, viz.

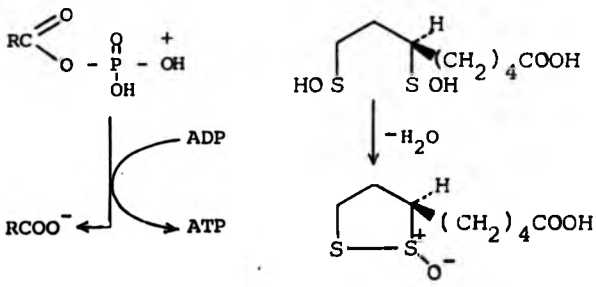
these are known reactions and are readily reversible



second protonation step

protonated sulphenylate

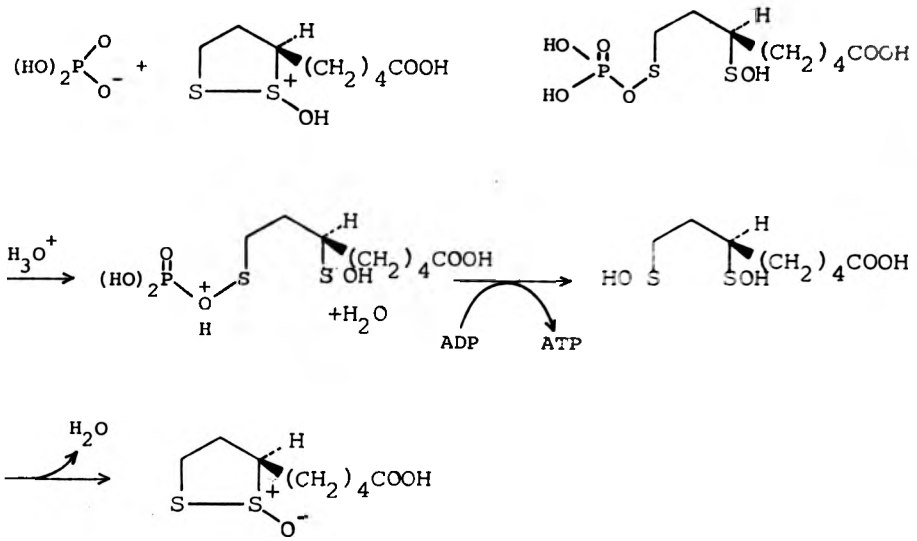
nucleophilic attack of phosphate



acylphosphate + bis (sulphenic acid)

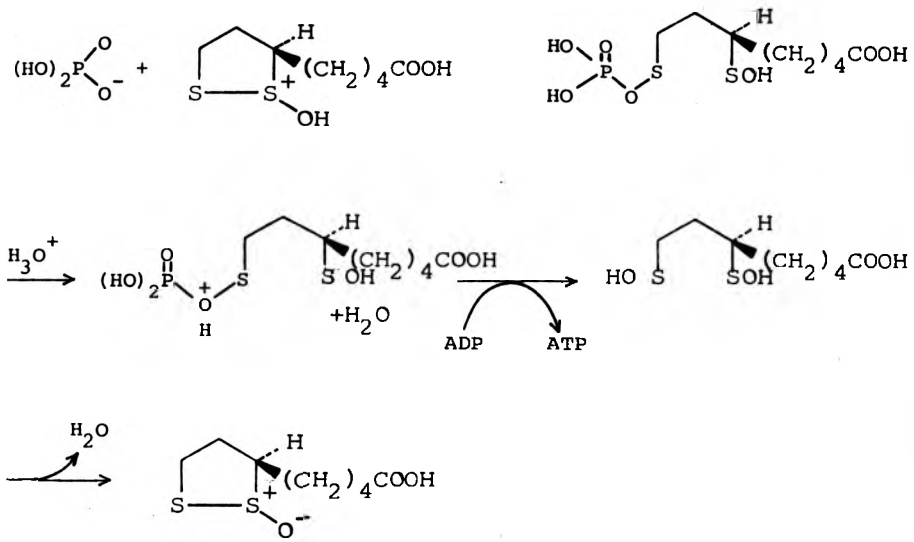
spontaneous reformation of lipoic monoxide + water

Weiss also suggested an alternative scheme in which acyl phosphate was not an obligatory intermediate, viz.



The former of these two mechanisms has obvious similarities to the 'oleoyl cycle'. Active lipoate and oleoyl lipoate might drive ATP synthesis by tapping into these cycles at some stage.

There have been several suggestions that vicinal dithiol compounds may be involved at the coupling sites of oxidative phosphorylation, notably those of Fluharty and Sanadi (385,386) who demonstrated that the dithiol reagents, γ-(p-arsenophenyl)-n-butyrate and Cd^{2+} acted like uncoupling agents. Further direct evidence was not forthcoming. Several proteinaceous oxidation phosphorylation factors have been isolated from the mitochondrial inner membrane (see (51) for review). One of these, Factor B, isolated by Sanadi and co-workers (387) contained two essential thiol groups. Hatefi et al. (388, 389) have purified a similar factor to a higher degree of homogeneity. This factor stimulates various energy linked reactions and also contains an essential dithiol (or ½ a dithiol) grouping. Similarly, McKinney et al. (390) have demonstrated that the ATPase coupling factor, CF_1, from chloroplast contains a thioredoxin-like activity buried deep in its structure. The active grouping in true thio-



The former of these two mechanisms has obvious similarities to the 'oleoyl cycle'. Active lipoate and oleoyl lipoate might drive ATP synthesis by tapping into these cycles at some stage.

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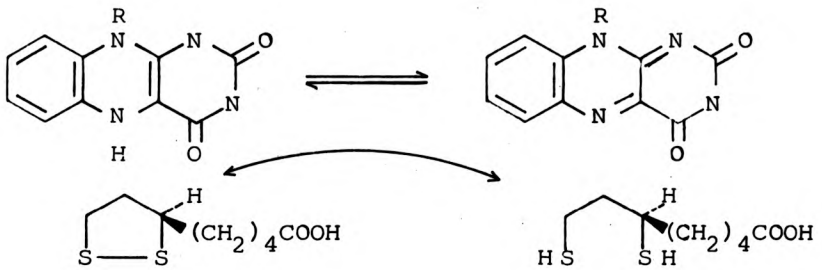
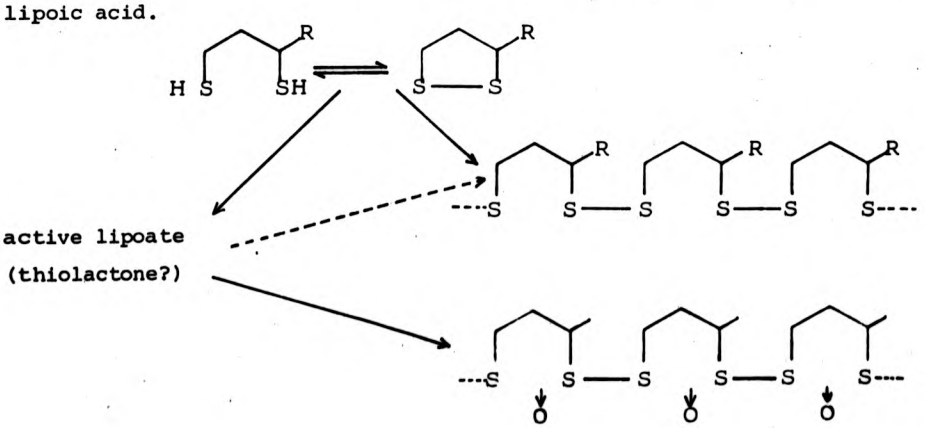
redoxins is a dithiol There is,then,evidence that a dithiol grouping has some function in the ATPase.

Flavins have also been reported to be components of the F_1F_o ATPase (84, 349); these are reducible by dithiol compounds.



The presence of both flavins and dithiols in the F_1F_o ATPase is strong circumstantial evidence that they have some role there, possibly in energy coupling. Active lipoic acid may exert its effect by supplying redox energy to this system of redox carriers.

The 'active' form of dihydrolipoate is unknown. There is evidence which suggests that it may be an unusual oxidation product of lipoic acid. It eventually polymerises to a compound similar to polymers observed during the oxidation of lipoic acid. The following scheme can be drawn, outlining the relationships between the different forms of lipoic acid.



CHAPTER 4

THE EFFECT OF FATTY ACIDS ON THE DICYCLOHEXYL-CARBODIIMIDE INDUCED INHIBITION OF MITOCHONDRIAL ATPases

Introduction

Studies on the nature of the inhibition of energy linked reactions by dibutylchloromethyl tin dichloride (DBCT) suggested that it titrated a mobile cofactor of these reactions in the inner mitochondrial membrane (315,396). Bovine heart submitochondrial particles incubated with sufficient DBCT to induce 95% inhibition of ATPase activity, when mixed with an equal amount of non-inhibited particles, recover up to 100% of their normal ATPase activity (50% would be the theoretical maximum expected)(235). This suggested that the DBCT binding component could diffuse off the ATPase complex or be replaced by an uninhibited component from a pool of such moieties in the membrane. Binding studies had shown that DBCT was bound to a non-proteinaceous lipophilic component of the innter mitochondrial membrane which might be lipoic acid, or a lipoic acid derivative(315).

Dihydrolipoic acid apparently exhibited a specific ability to reverse the effect of the presumed covalent inhibitor of energy linked reactions, DBCT (315,235). These observations lead to the suggestion that dihydrolipoic acid or a derivative of it might be directly involved in the mechanism of ATP synthesis in mitochondria.

Dicyclohexylcarbodiimide (DCCD), a well-known covalently binding inhibitor of energy linked reactions (394) also appeared to titrate some mobile component in the inner membrane, as DCCD inhibited bovine heart SMP recovered activity in similar experiments to those described above. Attention was thus focussed on a search for a compound, or groups of compounds, that might reverse the inhibition of energy linked reactions induced by DCCD which, by analogy to the apparent relationship between

DBCT inhibition and dihydrolipoate, might also be involved in the mechanism of ATP synthesis in mitochondria.

Materials

All chemicals used were of the highest purity available, usually 'AnalaR' or a similar grade. All organic solvents were of 'AnalaR' grade, redistilled. Fatty acids, fatty acid methyl esters, coenzyme A and derivatives, carnosine, anserine, thiamin monophosphate, glutathione, lipoic acid, carnitine and oligomycin were all obtained from the Sigma Chemical Company. Efrapeptin was obtained from Dr. R.L. Hamill of Eli Lilley & Co. Ltd.

Methods

Bovine heart mitochondria, submitochondrial particles and electron transport particles were prepared as described in Chapter 3.

DCCD inhibited 'proton translocating' ATPase was prepared from bovine heart SMP, incubated overnight at 4° C with sufficient DCCD to induce 100% inhibition of oxidative phosphorylation and F_1F_o ATPase activity (2-5 nmoles DCCD/mg SMP protein), essentially as described for the non-inhibited enzyme in Chapter 3.

Oxidative phosphorylation was assayed in a glucose-hexokinase trap system as described in Chapter 3.

ATPase activity was assayed essentially by the method of Griffiths and Houghton, as described in Chapter 3.

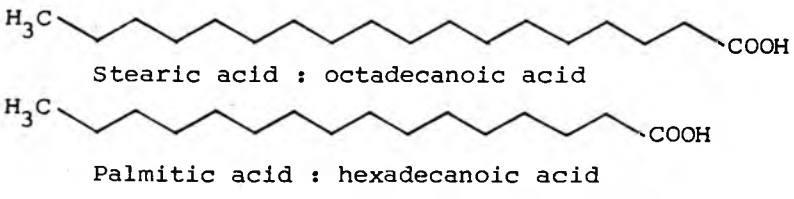
ATP-driven energy linked transhydrogenase activity was assayed as described in Chapter 6.

Protein was routinely assayed by a 'quick' biuret method (355) and by the Folin-reagent method of Lowry et al. (356).

Results

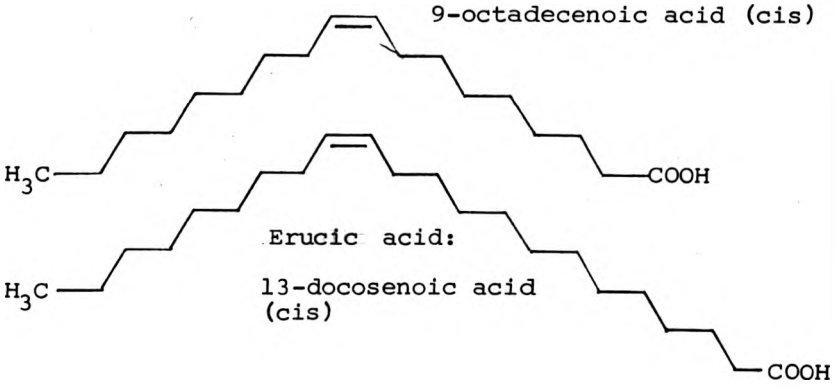
A wide spectrum of compounds was tested for the ability to reverse the DCCD induced inhibition of both submitochondrial particle ATPase and a purified ATPase preparation, the 'proton translocating' ATPase of

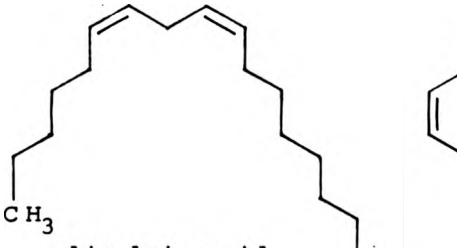
Fig 4.1. Structures of fatty acids.



Stearic acid : octadecanoic acid

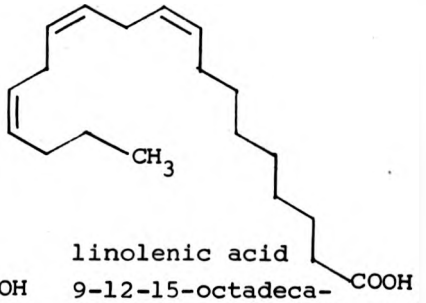
Palmitic acid : hexadecanoic acid





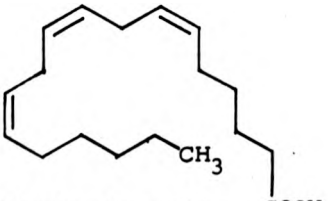
linoleic acid

9-12-octadecadienoic acid (all cis)



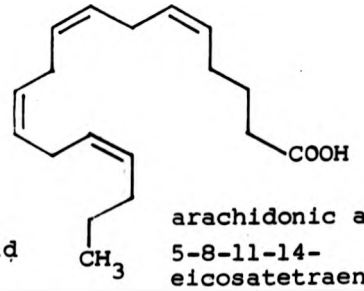
linolenic acid

9-12-15-octadeca-trienoic acid (all cis)



γ-linolenic acid

6-9-12-octadecatrienoic acid (all cis)



arachidonic acid

5-8-11-14-eicosatetraenoic acid (all cis)

Table 4.1 Effect of various compounds on DCCD inhibited proton translocating ATPase

Effector	ATPase activity µmoles/min/mg
None (DCCD inhibited)	0
Coenzyme A (100 µg)	0.18
Acetyl coenzyme A (100 µg)	0
Glutathione (100 µg)	0
Dihydrolipoate (400 nmoles)	0
DL carnitine (50 µg)	0
Carnosine (50 µg)	0
Anserine (100 µg)	0
Thiamin monophosphate (100 µg)	0
Methyl linolenate (100 µg)	1.70
Cardiolipin (100 µg)	0.30
Dihydrolipoate (400 nmoles) + coenzyme A (100 µg)	0
Dihydrolipoate (400 nmoles) + acetyl coenzyme A (50 µg)	0.96
Dihydrolipoate (400 nmoles) + glutathione (100 µg)	0
Dihydrolipoate (400 nmoles) + DL carnitine (50 µg)	0
Dihydrolipoate (400 nmoles) + carnosine (50 µg)	0
Dihydrolipoate (400 nmoles) + anserine (100 µg)	0
Dihydrolipoate (400 nmoles) + thiamin monophosphate (100 µg)	0
Dihydrolipoate (400 nmoles) + methyl linolenate (100 µg)	3.30
Dihydrolipoate (400 nmoles) + cardiolipin (100 µg)	0.45

Non-inhibited proton translocating ATPase (parallel preparations) sp Act usually 2-6 µmoles/min/mg.

Assays containing 90 µg enzyme protein suspended in 0.9 ml 0.25 M sucrose; 50 mM Tris-Cl, pH 9.0; 5 mM $MgCl_2$; 1 mM EDTA were preincubated with indicated amounts of effectors (added as ethanolic or aqueous solutions) for 5 minutes at 30° C. The reaction was initiated by the addition of 5 µmoles ATP and incubated for 5 minutes at 30° C. Assays were terminated after 5 minutes by the addition of 0.5 ml ice cold 10% T.C.A. Coagulated protein was removed by bench centrifugation and a 0.5 ml aliquot from the supernatant was taken for phosphate assay by the method of Fiske-Subbarow, as described in Chapter 3.

DCCD inhibited proton translocating ATPase was prepared by the method of Serrano and Racker (245) from DCCD inhibited SMP (5 nmoles DCCD/mg SMP protein).

Table 4.2 Effect of coenzyme A and derivatives on DCCD inhibited 'proton translocating' ATPase

Coenzyme A derivative	ATPase activity µmoles/min/mg
None (DCCD inhibited)	0
Coenzyme A (100 µg)	0.3
Acetyl coenzyme A (100 µg)	0
Oleoyl coenzyme A (20 µg)	2.87

Non-inhibited proton-translocating ATPase (parallel preparations) sp Act usually 2-6 µmoles/min/mg

---

Assays containing 100 µg enzyme protein in 0.9 ml 0.25 M sucrose; 50 mM Tris-Cl, pH 9.0; 5 mM $MgCl_2$; 1 mM EDTA were preincubated with effectors (added as aqueous solutions) for 5 minutes at 30° C and were initiated by the addition of 5 µmoles ATP. Assays were terminated after incubation for 5 minutes at 30° C by addition of 0.5 ml ice cold 10% T.C.A. Phosphate was assayed as described in Table 4.1.
DCCD inhibited proton translocating ATPase was prepared by the method of Serrano and Racker (245) from DCCD inhibited SMP (5 nmoles DCCD/mg SMP protein).

Table 4.3 Effect of various lipids on a partially DCCD inhibited preparation of 'proton translocating' ATPase

Effector	ATPase activity µmoles/min/mg
None (DCCD inhibited)	0.65
Methyl linolenate (100 µg)	2.30
Linolenic acid (100 µg)	1.80
Dihydrolipoic acid (400 nmoles)	0.65
Coenzyme A (100 µg)	0.90
Oleoyl -coenzyme A (100 µg)	2.10

Non-inhibited proton translocating ATPase (parallel preparations). sp Act usually 2-6 moles/min/mg.

Assays containing 100 µg enzyme protein suspended in 0.9 ml. 0.25 M sucrose; 50 mM Tris-Cl, pH 9.0; 5 mM $MgCl_2$; 1 mM EDTA were preincubated with effectors (added as ethanolic or aqueous solutions) for 5 minutes at 30° C. Assays were initiated with 5 µmoles ATP, terminated after 5 minutes at 30° C incubation with 0.5 ml 10% T.C.A. and assayed for phosphate as described in Table 4.1.

Serrano and Racker (245). A typical set of results from these experiments is listed in Table 4.1. It can be seen that two classes of compound appear to reverse the DCCD inhibition of ATPase activity; these are compounds of a generally lipid-like nature, and compounds related to coenzyme A. The former are the more effective. The lipids most effective as reversing agents are fatty acid methyl esters and free fatty acids. Coenzyme A and derivatives also partially reverse the inhibition, the most effective derivative tested being oleoyl-S-coenzyme A. This is, however, no more effective as a reversing agent than methyl oleate (Tables 4.2-3). All the fatty acyl methyl esters tested could reverse the DCCD inhibited ATPase activity at roughly the same levels (Table 4.4). This broad specificity was narrowed, though when free fatty acids were tested (as the sodium or 'Tris' salt) (Table 4.5). Here the most effective reversing agents were the unsaturated fatty acids, especially oleic acid. Saturated fatty acids such as palmitic, stearic and arachidic acid would also reverse the inhibition, but rather less effectively (Table 4.5 and Figures 4.4-5). Shorter chain length fatty acids such as caprylic acid and lipoic acid (both in the oxidised and reduced form) had no effect on the inhibited ATPase at the concentrations tested (Table 4.5).

Bovine heart submitochondrial particles (ETP_H), incubated with levels of DCCD which induced complete inhibition of oligomycin sensitive ATPase and ATP synthase activities were also treated with various fatty acids. Reversal of inhibited ATPase activity was observed with oleic and linolenic acids, oleic acid being slightly the more effective (Figure 4.3). Although ATPase activity was reversed, ATP synthase (Table 4.6), ATP-Pi exchange and ATP-driven energy linked transhydrogenase (Figure 4.2) activities were not restored by incubating DCCD inhibited ETP_H with levels of oleate which gave substantial reversal of ATPase activity.

The fatty acid induced ATPase activity of DCCD treated ETP_H and detergent purified ATPase preparations had altered sensitivities to some of the well-known inhibitors of ATPase and energy linked reactions, e.g.

Table 4.4 Effect of fatty acid methyl esters on a DCCD inhibited 'proton translocating' ATPase

Ester	ATPase activity µmoles/min/mg
None (DCCD inhibited)	0
Methyl linolenate (100 µg)	3.02
Methyl oleate (100 µg)	4.20
Methyl palmitoleate (100 µg)	3.52
Methyl heptadecanoate (100 µg)	4.13

Non-inhibited proton translocating ATPase (parallel preparations). sp Act usually 2-6 moles/min/mg.

Assays containing 100 µg enzyme protein in 0.9 ml 0.25 M sucrose; 50 mM Tris-Cl, pH 9.0; 5 mM $MgCl_2$; 1 mM EDTA were preincubated with methyl esters for 5 minutes at 30° C prior to initiation of assay with 5 µmoles ATP. Assays were terminated after 5 minutes incubation at 30° C by addition of 0.5 ml ice cold 10% T.C.A. Phosphate was determined as described in Table 4.1.

DCCD inhibited enzyme was prepared by the method of Serrano and Racker (245) from DCCD inhibited submitochondrial particles (5 nmoles DCCD/mg SMP protein).

Table 4.5 Effect of fatty acids on a DCCD inhibited preparation of 'proton translocating' ATPase

Additions	ATPase activity µmoles/min/mg
None (DCCD inhibited)	0
Caprylate (100 µg)	0
Lipoate (20 µg)	0
Palmitate (40 µg)	1.80
Stearate (40 µg)	1.74
Oleate (40 µg)	5.15
Linoleate (40 µg)	3.50
Linolenate (40 µg)	4.37
γ-linolenate (100 µg)	2.49
Arachidonate (40 µg)	5.05
Arachidic (100 µg)	1.61

Non-inhibited 'proton translocating' ATPase (parallel preparations) sp Act usually 2-6 µmoles/min/mg.

Assays containing 100 µg enzyme protein suspended in 0.9 ml 0.25 M sucrose; 50 mM Tris-Cl, pH 9.0; 5 mM $MgCl_2$; 1 mM EDTA were preincubated with fatty acids (added as the free acid in ethanolic solution: fatty acids added in dimethylformamide or as sodium salts in aqueous buffer systems have similar effect) for 5 minutes at 30° C prior to initiation of assay with 5 µmoles ATP. Assays are terminated after 5 minutes incubation at 30° C by addition of 0.5 ml 10% T.C.A. Phosphate was determined as described in Table 4.1.

DCCD inhibited enzyme was prepared by the method of Serrano and Racker (245) from DCCD inhibited SMP (5 nmoles DCCD/mg SMP protein).

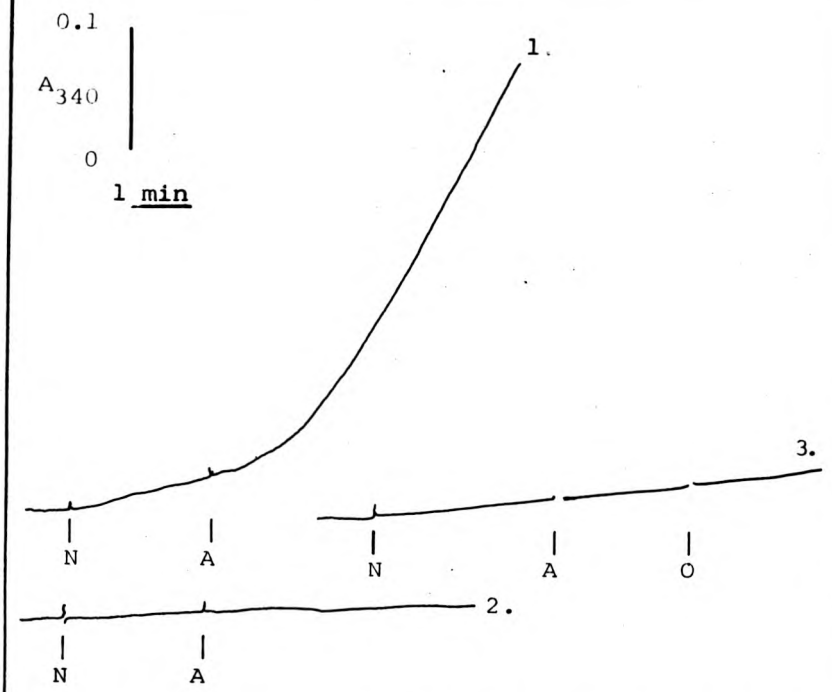


Fig 4.2 Effect of oleate on DCCD inhibited ATP-driven transhydrogenase in bovine heart SMP.

Transhydrogenase assays containing 1mg SMP protein were performed as described in fig 6.5.

N : 0.5 µmoles $NADP^+$. A : 5 µmoles ATP.
O : 50 nmoles oleate.

Assay 1. uninhibited SMP.
Assay 2. SMP preincubated with 4 nmoles DCCD for 10 minutes at 30°C.
Assay 3. as 2. oleate induces no reversal of activity if added before or after ATP even if pre-incubated with the enzyme for long periods.

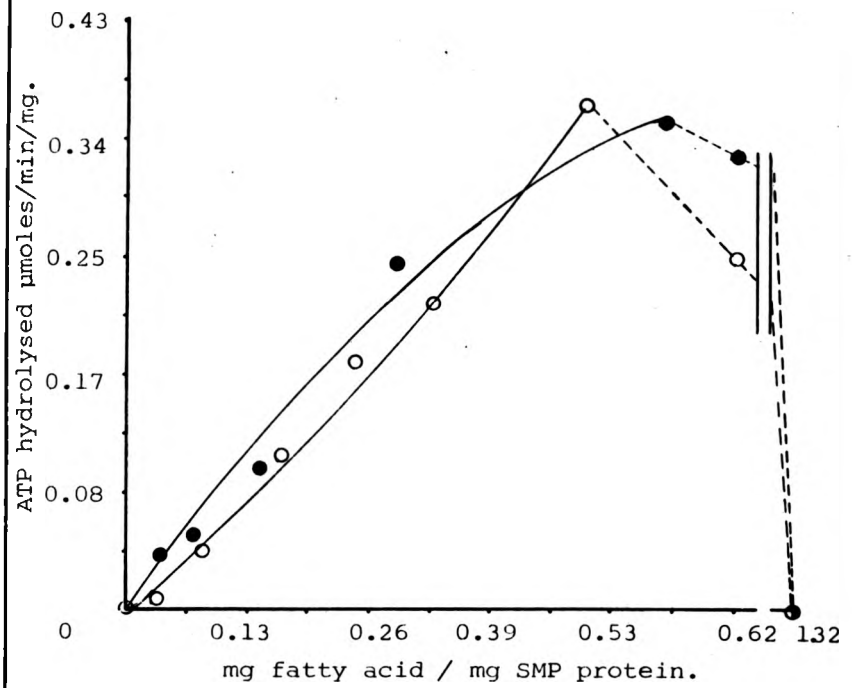


Fig 4.3. Effect of fatty acids on DCCD inhibited bovine heart SMP ATPase activity.

Assays containing 0.55mg SMP protein were preincubated with fatty acids (added as methanolic solutions) for 10 minutes at 30°C in 0.9ml 0.25M sucrose;50mM Tris-Cl pH 9.0;5mM $MgCl_2$;1mM EDTA prior to initiation by the addition of 5 µmoles ATP.Assays were terminated and ATPase activity determined as described in table 4.1.

o—o assays preincubated with oleate.

●—● " " " linolenate.

Table 4.6 Effect of oleic acid on DCCD inhibited bovine heart submitochondrial particles: oxidative phosphorylation

Additions	Rate nmoles Pi esterified/min/mg
ETP_H	0
ETP_H + sodium succinate	170
DCCDi ETP_H	0
DCCDi ETP_H + oleate (150 nmoles)	0
DCCDi ETP_H + oleate (300 nmoles)	0
DCCDi ETP_H + oleate (1 µmole)	0

Oxidative phosphorylation was assayed in a glucose-hexokinase trap system, 0.2 mM in ADP as described in Chapter 3. All assays contained 1 mg of ETP_H. ETP_H were inhibited with DCCD by incubation with 15 nmoles DCCD/mg ETP_H for 30 minutes at 4° C prior to commencement of assay. ETP_H were incubated in 1 ml phosphorylation buffer with oleate, where indicated, for 15 minutes prior to initiation of assay by addition of 10 µl 1 M sodium succinate, pH 7.3. Assay terminated after 15 minutes by removing a 200 µl aliquot into 200 µl 10% T.C.A. and assaying for phosphate disappearance from the assay by the method of Fiske and Subbarow, as described in the methods section to Chapter 3.

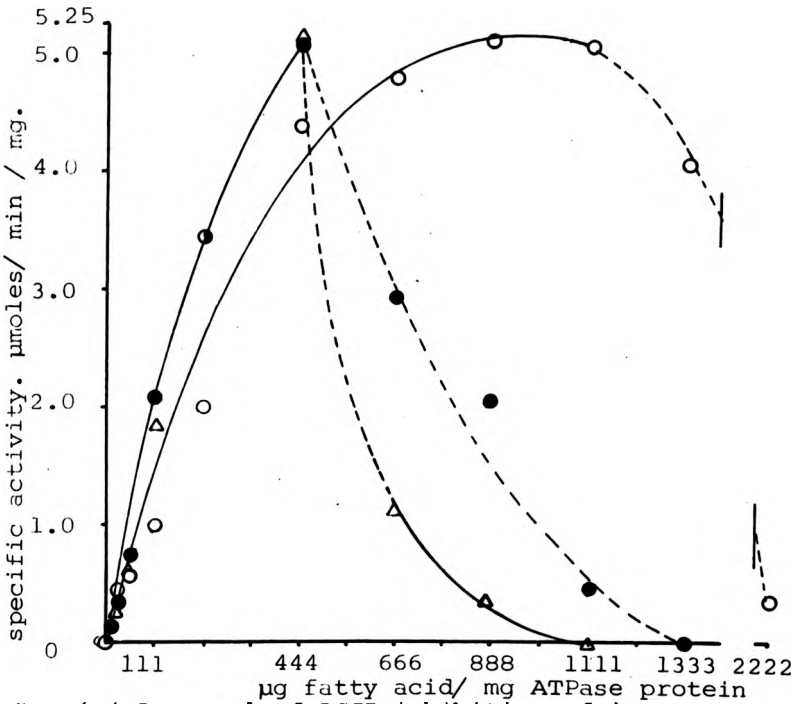


Fig 4.4 Reversal of DCCD inhibition of 'proton-translocating'ATPase activity by fatty acids:linolenate;arachidonate;oleate.

Assays containing 90µg DCCD inhibited 'proton translocating'ATPase in 0.9ml,0.25M sucrose;50mM Tris-Cl, pH 9.0;5mM $MgCl_2$;1mM EDTA;were preincubated with fatty acids for 5 minutes @ 30°C.Assays were initiated with 5µmoles ATP and incubated for 10 mins at 30°C prior to termination with 0.5ml ice-cold TCA. ATPase activity was determined as described in table 4.1.ATPase prepared as described in Fig 4.5.

O---O assays preincubated with linolenate.
●---● " " " arachidonate.
Δ---Δ " " " oleate.

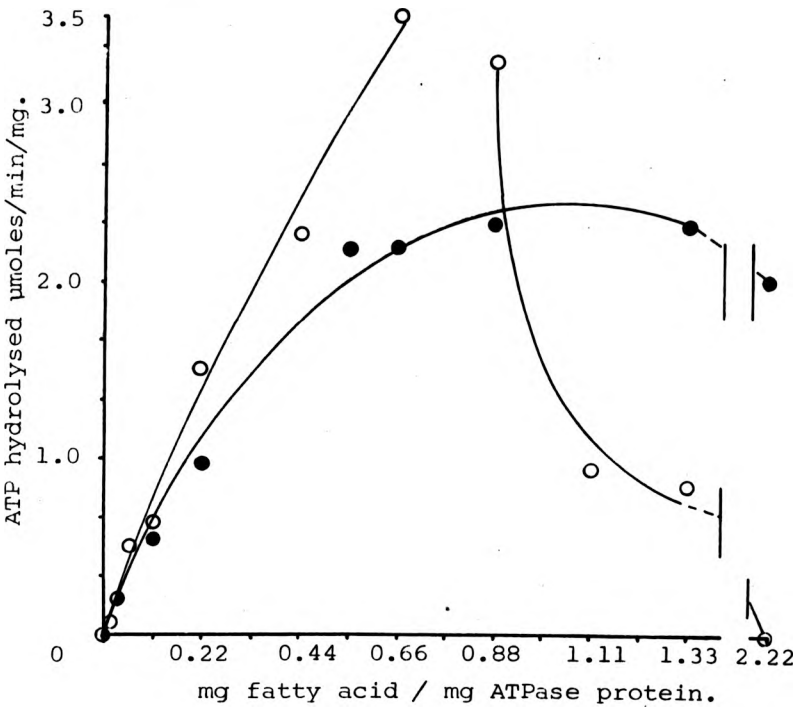


Fig 4.5 Reversal of DCCD inhibition of 'proton translocating' ATPase by fatty acids:linoleate: palmitate.

Preincubation conditions and ATPase activity determinations were as those described in fig 4.4.

o—o assays preincubated with linoleate.

●—● " " " palmitate.

DCCD inhibited 'proton translocating' ATPase was prepared as described in the methods section to ch.4.

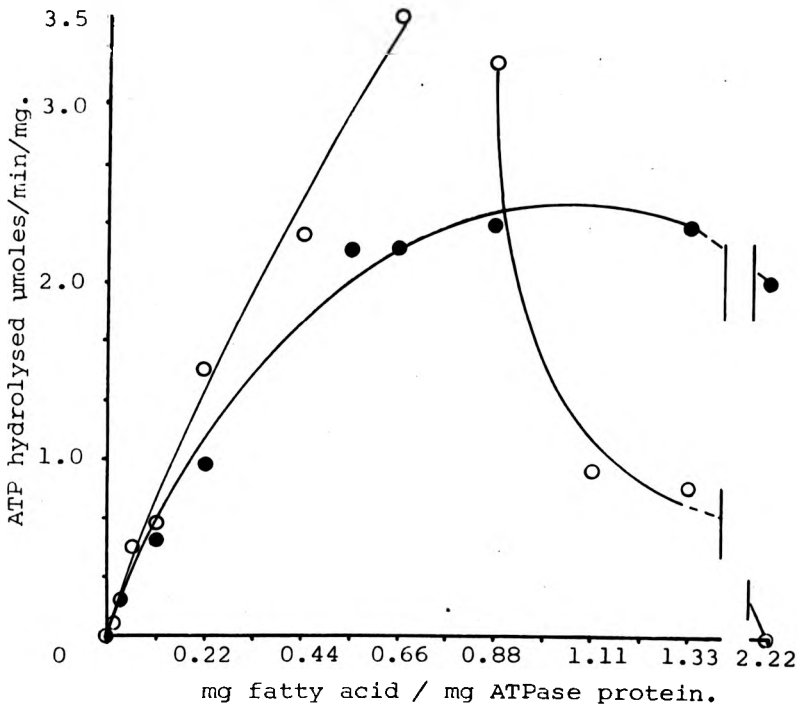


Fig 4.5 Reversal of DCCD inhibition of 'proton translocating' ATPase by fatty acids:linoleate: palmitate.

Preincubation conditions and ATPase activity determinations were as those described in fig 4.4.

O—O assays preincubated with linoleate.

●—● " " " palmitate.

DCCD inhibited 'proton translocating' ATPase was prepared as described in the methods section to ch.4.

oligomycin, efrapeptin, DBCT and DCCD itself (Tables 4.7-8). Oleate induced ATPase activity in DCCD treated 'proton translocating' ATPase is almost entirely insensitive to further additions of DCCD and DBCT even at levels 10-20 times greater than those required to give DCCD and DBCT inhibition of 'normal' ETP_H and proton translocating ATPase activities (Figure 4.6). The situation is somewhat different with oligomycin (Figure 4.7) and efrapeptin (Figure 4.8). Here the oleate restored activity is partially sensitive to high levels of oligomycin and rather more sensitive to efrapeptin. The oleate restored ATPase activity is, however, rather insensitive to all the inhibitors tested.

Discussion

Reversal of DCCD induced inhibition of SMP ATPase and 'proton translocating' ATPase activity was obtained by incubating the inhibited enzymes with fatty acids and fatty acid methyl esters. Fatty acids were the most effective compounds in relieving DCCD inhibition (Tables 4.1-5). Although all fatty acids of chain length > C_{16} tested could relieve the inhibition induced by DCCD, some were far more effective than others. The most effective were the C_{18} unsaturated fatty acids, oleic, linoleic and linolenic acid and the C_{20} polyunsaturated arachidonic acid (for structures, see Figure 4.1). Of these,oleic acid appeared to be marginally the most effective (Figures 4.3-5).

Although oleic acid could reverse the DCCD induced inhibition of mitochondrial ATPase, it was unable to reverse the inhibition of energy linked reactions such as oxidative phosphorylation (Table 4.6), ATP-driven energy linked transhydrogenase (Figure 4.2) and ATP-^{32}Pi exchange reactions (data not shown). This discrepancy of action can be explained by a number of possible mechanisms (Figure 4.9). These are:-

(a) Oleate causes F_1 ATPase to dissociate completely from F_O, thus apparently reversing DCCD inhibition.

(b) Oleate causes a dislocation of F_1 and F_O subunits, releasing an F_1-like activity.

Table 4.7 Reversal of DCCD inhibition of bovine heart SMP ATPase: effect of further inhibitors

Additions	ATPase activity µmoles/min/mg
SMP	2.31
DCCD inhibited SMP (5 nmoles DCCD/mg SMP protein)	0.03
DCCD inhibited SMP + oleate (70 µg)	2.06
DCCD inhibited SMP + oleate + tributyltin chloride (1 µg)	1.99
DCCD inhibited SMP + oleate + VE2283 (1 µg)	2.06
DCCD inhibited SMP + oleate + oligomycin (1 µg)	1.01
DCCD inhibited SMP + oleate + efrapeptin (1 µg)	0.61
DCCD inhibited SMP + oleate + DCCD (10 nmoles)	2.01

Assays containing 100 µg SMP in 1 ml 50 mM Tris-Cl, pH 8.5; 5 mM $MgCl_2$ were incubated with inhibitors for 5 minutes at 30° C: oleate was then added and preincubation was continued for 5 minutes at 30° C. Assays were initiated with 5 µmoles ATP and terminated after 5 minutes incubation at 30° C by addition of 0.5 ml 10% T.C.A. Phosphate was determined as described in Table 4.1.

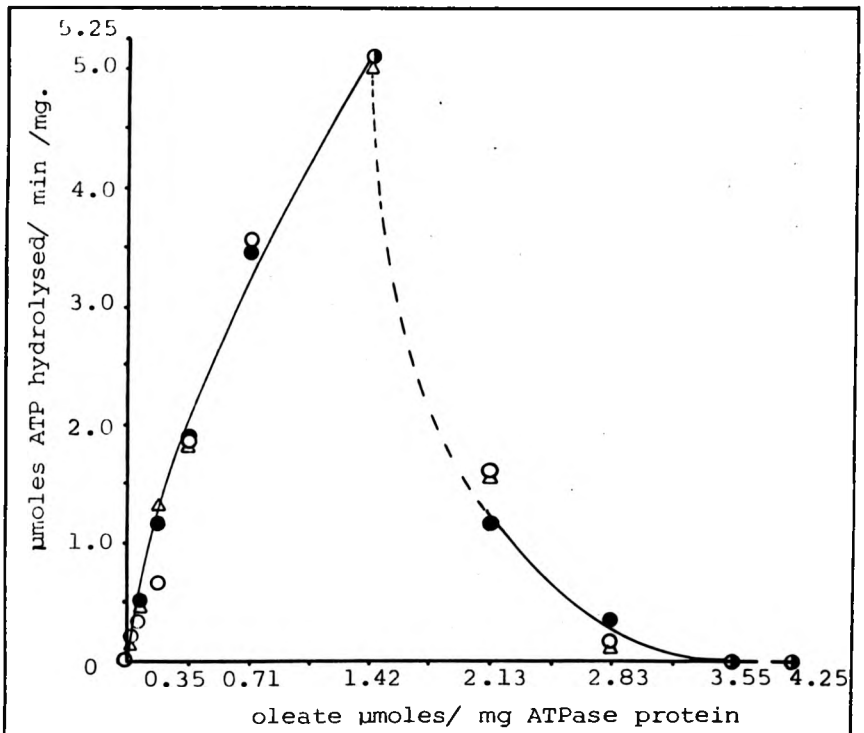


Fig 4.6 Reversal of DCCD inhibition of 'proton translocating'ATPase by oleate ;effect of further DCCD and DBCT.

Assays containing 100µg DCCD inhibited'proton translocating ATPase protein in 0.9ml 0.25M sucrose;50mM Tris-Cl,pH 9.0;1mM EDTA;were preincubated with i/oleate for 5 minutes @ 30°C then, ii/ DCCD or DBCT (10µg/ mg protein) for 5 minutes @ 30°C.Assays were initiated by the addition of 5µmoles ATP and incubated for 10 minutes @ 30°C prior to termination by the addition of 0.5mls ice cold 10% TCA.ATPase activity was determined as described in table 4.1.

O—O assays preincubated with oleate
●—● " " " " +DBCT.
Δ—Δ " " " " +DCCD.

Oleate was added as either a methanolic solution of the free acid or as the sodium salt (both produce the same effect.ATPase prepared as described in fig 4.5.

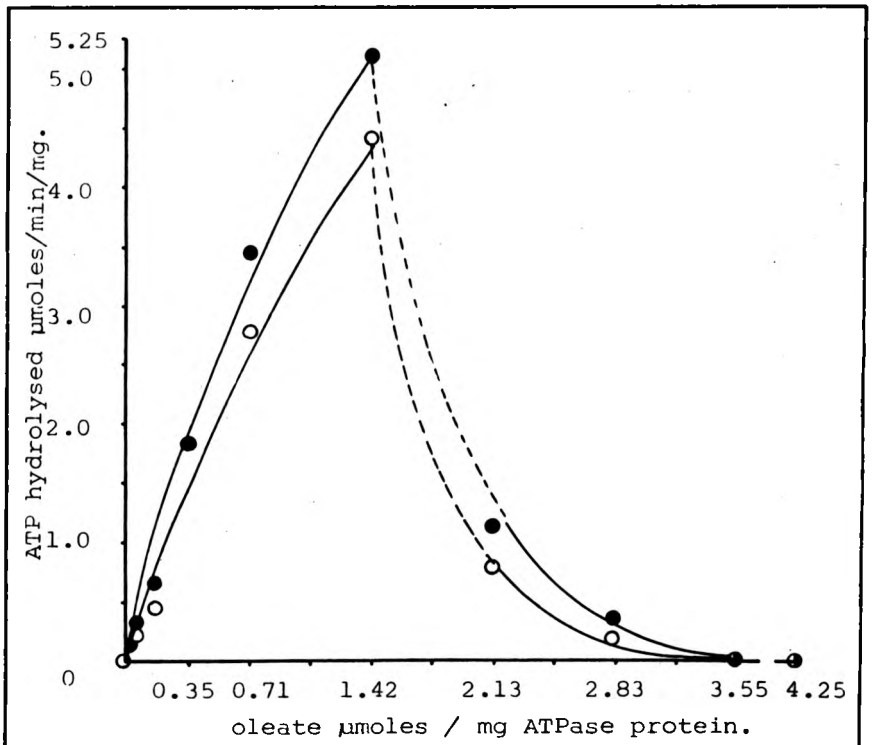


Fig 4.7. Reversal of DCCD inhibition of 'proton translocating' ATPase by oleate:effect of oligomycin.

Preincubation conditions and ATPase activity determinations were as those described in fig 4.6.

●——● assays preincubated with oleate.

o——o " " " " + oligomycin (10 µg / mg ATPase protein).

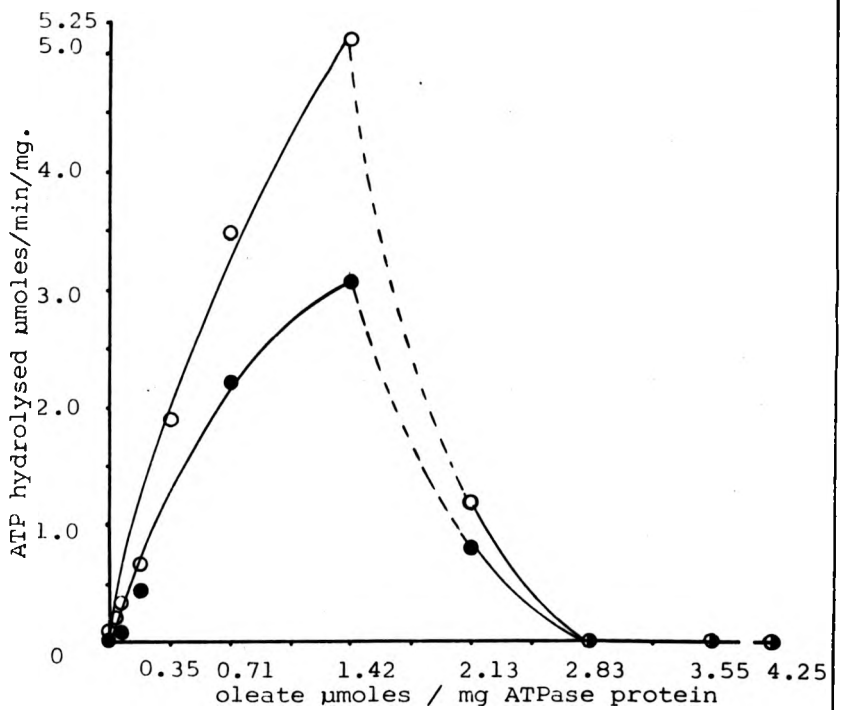


Fig 4.8. Reversal of DCCD inhibition of 'proton translocating'ATPase by oleate:effect of efrapeptin.

Preincubation conditions and ATPase activity determinations were carried out as described in fig 4.6.

O—O assays preincubated with oleate.

●—● " " " " + efrapeptin (10µg / mg ATPase protein).

Table 4.8 Sensitivity of fatty acid reversed DCCD inhibited ATPase activity of 'proton translocating' ATPase to further inhibition

Additions		+ oligo-mycin	+ efra-peptin	+ DCCD	+ DBCT
Oleate (10 µg)	1.82	1.30	1.15	1.80	1.87
Linoleate (10 µg)	1.43	nt	0.85	nt	nt
Linolenate (10 µg)	0.59	0.49	0.41	0.59	0.58
γ-linolenate (100 µg)	2.3	1.48	nt	nt	nt
Arachidonate (100 µg)	1.48	1.20	0.736	nt	nt
Palmitate (100 µg)	0.52	0.21	nt	nt	nt

DCCD inhibited proton translocating ATPase specific activity 0
Non-inhibited proton translocating ATPase (parallel preparations) sp. Act usually 2-6 µmoles/min/mg.

Assays containing 100 µg DCCD inhibited ATPase protein suspended in 0.9 ml 0.25 M sucrose; 50 mM Tris-Cl, pH 9.0; 5 mM $MgCl_2$; 1 mM EDTA were incubated with inhibitors (1 µg of indicated inhibitor per assay) where indicated for 5 minutes at 30° C. Fatty acid was then added and the preincubation continued for 5 minutes at 30° C. Assays were initiated by addition of 5 µmoles ATP and were terminated after incubation for 5 minutes at 30° C by the addition of 0.5 ml 10% T.C.A. Phosphate was determined as described in Table 4.2.

DCCD inhibited proton translocating ATPase was prepared by the method of Serrano and Racker (245) from DCCD inhibited SMP (5 nmoles DCCD/mg SMP protein).

(c) Oleate induces a true reversal of DCCD inhibition but also simultaneously uncouples energy linked reactions.

Mechanism (a) is unlikely as oleate treated DCCD inhibition does not release F_1 from the membrane (ATPase activity partitions with oleate treated DCCD inhibited SMP membranes in differential centrifugation experiments; results not shown). Mechanisms (b) and (c) are rather more difficult to distinguish between. Mechanism (b) supposes oleate induces a dislocation between the F_1 and F_O portions of the ATPase, resulting in a functional uncoupling of ATP consuming or producing reactions. Mechanism (c) postulates that oleate truly relieves DCCD inhibition, allowing the ATPase to generate a high energy membrane state ' ∿ ' but that free oleate acts in its well known role as an uncoupler of energy linked reactions (397), thus dissipating ' ∿ '. This would result in a reversal of ATPase activity, but apparently not of oxidative phosphorylation or ATP-driven transhydrogenase.

In order to throw light on the mode of relief of DCCD inhibition, the fatty acid induced ATPase activity was examined to ascertain whether it was sensitive to various inhibitors of 'normal' mitochondrial ATPase activity, viz. further DCCD, DBCT, oligomycin and efrapeptin. Oligomycin, DCCD and DBCT all inhibit the complete ATPase, that is, the F_1F_O ATPase complex. They do not inhibit free F_1 ATPase activity and can thus be used as markers for the structural integrity of the F_1F_O complex. Efrapeptin, a polypeptide fungal product, is a potent inhibitor of the isolated F_1. If the oleate induced ATPase activity is sensitive to efrapeptin but not to the other inhibitors, then it would be probable that F_1 ATPase is released or dislocated from the F_O portion of the enzyme.

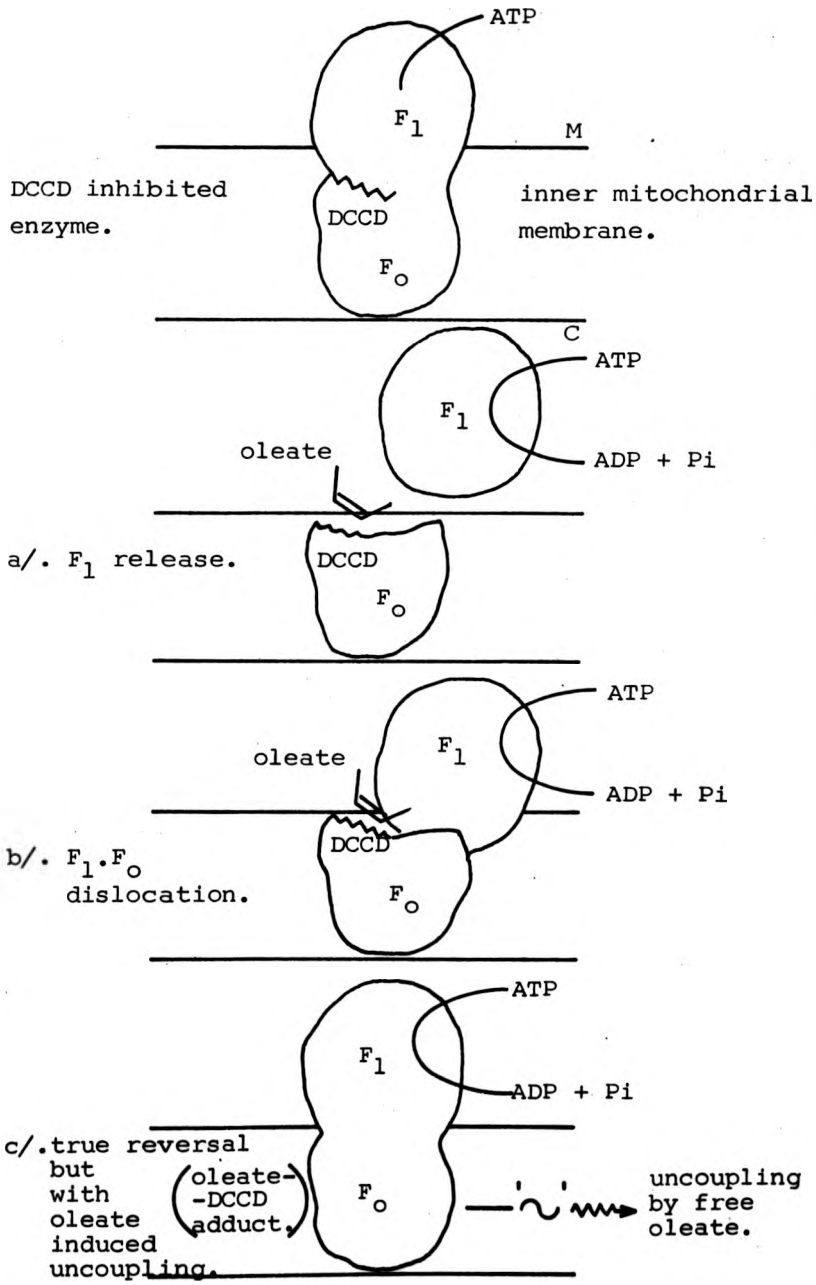
The results in Figures 4.6-8 show that the actual situation is somewhat more confused than this. DCCD and DBCT are unable to inhibit the oleate induced ATPase, whereas efrapeptin can, suggesting that F_1-like

activity has indeed been released. However, this activity is partially sensitive to oligomycin (Tables 4.7-8 and Figure 4.7). The activity is only partially sensitive to efrapeptin also: this is probably due to the high levels of fatty acid used, sequestering the inhibitors away from their sites of action. Essentially the same results are obtained if the inhibitors are incubated with the DCCD inhibited enzyme before or after oleate addition. A variant on mechanism (b) is that oleate does cause a dislocation of F_1 from F_O, but this dislocation is only local in that it disturbs the binding sites of DCCD and DBCT enough to release ATPase activity, but not enough to render it oligomycin insensitive. This would require a functional separation of the binding sites for DCCD, DBCT and oligomycin.

The DCCD induced inhibition of bovine heart mitochondrial ATPase can be correlated with the covalent binding of ^{14}C DCCD to a hydrophobic component of the inner membrane (393) which has been identified as a subunit of the ATPase complex, subunit 9 (393). Oligomycin also binds somewhere in the hydrophobic F_O sector of the ATPase. Studies by Criddle *et al.* (398) suggest that oligomycin might also bind to subunit 9. Alkyltin compounds such as DBCT also appear to act in a very similar manner to oligomycin but studies with radiolabelled triethyl tin (396) have shown that the tin binding site is distinct from the oligomycin binding site, and that this is also located on or around subunit 9. Thus all three binding sites are close together but are structurally different. This structural dissimilarity might then explain the apparent partial inhibitor sensitivity of oleate induced ATPase activity observed.

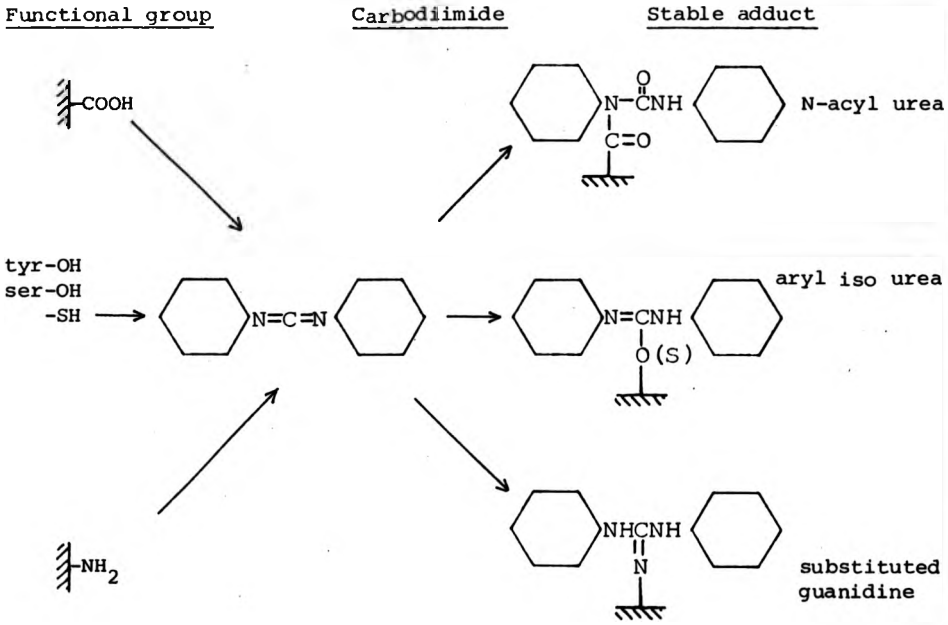
Although, in subunit 9, purified from bovine heart mitochondria incubated with ^{14}C DCCD, radioactivity co-purifies with the protein, it is far from clear to which grouping DCCD is bound in the proteolipid or indeed whether it exerts its function by primarily binding to a protein derived grouping at all. A number of possible binding scenarios

Fig 4.9. Possible mechanisms of oleate induced reversal of DCCD inhibition of ATP utilising and producing systems in bovine heart mitochondria.



are displayed in Figure 4.10. The most likely is that DCCD binds to a -COOH residue derived from glutamic or aspartic acid. This makes mechanism (c), true reversal of DCCD inhibition by oleate, unlikely as oleate has at best a rather unreactive carboxyl group which is unlikely to strip reacted DCCD away from its binding site.

Figure 4.10 Some possible stable covalent adducts of DCCD and protein functional groups, adapted after (395)



So it is likely that oleate causes a disruption or dislocation of the DCCD inhibited F_1F_O ATPase complex which releases a partial F_1F_O ATPase activity sensitive to efrapeptin and oligomycin, but insensitive to DCCD and DBCT rather than inducing a true reversal of DCCD inhibition and then uncoupling the energy transducing apparatus of the inner mitochondrial membrane.

CHAPTER 5

STUDIES ON THE ROLE OF ACYL PHOSPHATES IN ENERGY COUPLING

Introduction

Studies on dihydrolipoate-driven ATP synthesis in 'complex V' ATPase and 'proton-translocating' ATPase preparations from bovine heart mitochondria together with the ability of certain fatty acids to reverse DCCD inhibition of the ATPase activity of these enzymes indicated that fatty acids might play a role in mitochondrial ATP synthesis. Indeed, dihydrolipoate-driven ATP synthesis in these enzymes appeared to require the presence of both a cis- -9 monoenoic acid, such as oleic acid, and oleoyl-CoA in small cofactor like quantities. This led Griffiths to postulate that the terminal stages of ATP synthesis might involve a series of reactions analogous to those of substrate level phosphorylation. These latter reactions, the generation by oxidative decarboxylation of a keto acid, the generation of succinyl lipoate and its transacylation to coenzyme A and the generation of GTP (and hence ATP) via succinyl phosphate led to the hypothesis that the terminal steps of oxidative phosphorylation consisted of a series of similar reactions, termed the 'oleoyl cycle' (235) (see Fig. 6.21). In this the ATPase would catalyse a transphosphorylation from oleoyl phosphate to ADP in a manner similar to the transphosphorylation from succinyl phosphate to ATP (GTP) catalysed by succinyl thiokinase.

Oleoyl phosphate was synthesised and tested for its ability to stimulate ATP synthesis in a series of ATPase enzymes derived from mitochondria, Escherichia coli and photosynthetic bacteria vesicles.

Materials

All chemicals used were of 'AnalaR' or equivalent grades of purity. All organic solvents used were of AnalaR grade and redistilled. Oleoyl chloride and other fatty acyl chlorides were obtained from the Sigma Chemical Company, as were acetyl phosphate, methyl oleate and

oligomycin. Efrapeptin was obtained from Dr. R. Hamill of Eli Lilley Co. '1799' was a kind gift from Dr. P. Heytler. DBCT was synthesised here by Dr. K. Cain.

Methods

Bovine heart mitochondria, submitochondrial particles, electron transport particles, 'complex V' ATPase and 'proton translocating' ATPase were prepared as described in Chapter 3. Oxidative phosphorylation and ATPase was assayed as described in Chapter 3.

Chloroform released F_1 ATPase was prepared essentially after the method of Beechey et al. (236). Submitochondrial particles were suspended in a buffer containing 0.25 M sucrose; 10 mM Tris-Cl, pH 7.5; 1 mM EDTA to a final protein concentration of 20 mg/ml. Redistilled AnalaR chloroform (0.5 volumes) was added, and the two phases were vigorously mixed for 30 seconds using a vortex shaker. The emulsion was broken by centrifuging in a Sorval RC-2B centrifuge SS34 rotor at 2,000 rpm for 10 minutes. The upper 2/3 of the aqueous layer was then centrifuged at 40,000 rpm for 30-60 minutes in a Beckman L5-65 centrifuge 65 rotor. The supernatant contained the ATPase activity which usually had a specific activity in the region of 20-30 µmoles/min/mg and occasionally 50-70 µmoles/min/mg.

Chloroform released ATPases were prepared from E. coli vesicles; Rhodopseudomas capsulata and Rhodospirillum rubrum chromatophores by a similar procedure to that above.

Escherichai coli strain K12-W1485 was grown essentially as described by Herbert and Guest (237).

Cells were grown aerobically in baffle flasks in a shaking incubator for 18 hr @ 37° C and isolated by centrifugation at 5,000 g for 15 minutes in a Sorval RC2B centrifuge, GSA head. Membrane vesicles were then prepared from the cells, either by a disruption in a French press or or by a lysozyme and osmotic shock treatment. The French press method

was modified after that of Bragg et al. (238). After centrifugation, cells were resuspended in 50 mM potassium phosphate, pH 7.0 (1 g cells wet weight/5 ml buffer). They were then disrupted in a French pressure vessel at 16,000 psi. The suspension was then centrifuged at 17,000 g for 10 minutes to remove unbroken cells and larger fragments of cell debris. The supernatant was then centrifuged at 120,000 g for 2 hrs. The pellet was washed and resuspended in 50 mM Tris-Cl, pH 7.5; 10 mM $MgCl_2$. In the lysozyme method, cells were resuspended in 250 mM sucrose, 50 mM Tris-Cl, pH 8.0 and spheroplasts were prepared by lysozyme treatment of washed cells as described by Witholt et al. (239) and stabilised by the addition of 20 mM $MgCl_2$. The spheroplasts were washed by centrifugation at 10,000 g for 15 minutes and broken by osmotic shock by resuspension in 20 mM potassium phosphate, pH 8.0, containing DNAase (20 µg/ml). Unbroken cells and large fragments of cell debris were removed by centrifugation at 10,000 g for 15 minutes. Membrane vesicles were isolated by centrifugation of the 10,000 g supernatant at 100,000 g for 30 minutes. The vesicle pellet was then resuspended in the desired buffer. Both methods result in a population of vesicles which consists of a large proportion with 'inside out' morphology (238,240),that is,their F_1 ATPases protrude into the suspension medium.

The photosynthetic bacteria, Rhodospirillum rubrum and Rhodopseudomonas capsulata, were grown in a media containing (quantities in g/L), yeast extract, 0.5; L-glutamic acid, 4.0; dl-malic acid, 3.5; $CaCl_2$. 0.04; $MgSO_4$, 0.2; trisodium citrate, 0.8; KH_2PO_4, 0.12; K_2HPO_4, 0.18; pH was adjusted to 6.8 with 60% KOH. Bacteria grown in a 100 ml starter culture from stock slabs were inoculated into flasks containing 10 litres of the above medium and were grown anaerobically under strong white light illumination, with stirring for 4-5 days. Cells were isolated from the growth media by centrifugation at 2,000 rpm for 30

minutes in an MSE 'Mistral' centrifuge, 4 x 1.25L head. Cells were resuspended in 10 mM Tris-Cl, pH 7.8, and washed by centrifugation at 10,000 rpm for 15 minutes in a Sorval RC-2B centrifuge, SS34 rotor.

To prepare chromatophores, cells were resuspended in 10 mM Tris-Cl, pH 7.8, and sonicated in an MSE 60W sonicator at maximum amplitude for 2 minutes @ 4° C. The suspension was then centrifuged at 15,000 rpm for 10 minutes in a Sorval RC-2B centrifuge SS34 rotor. The supernatant was then centrifuged at 100,000 g for 1 hr. Chromatophores were resuspended in 10 mM Tris-Cl, pH 7.8, and used immediately or, if wanted for more than 24 hrs, were made 50% in glycerol and stored under nitrogen at -20° C.

Bacteriochlorophyll was determined by taking a 0.1 sample of chromatophores into 30 ml of distilled water and reading the absorbance at 880 nm (E_{mM} bacteriochlorophyll = 140 @ 880 nm).

Preparation of oleoyl phosphate.- Dry orthophosphoric acid (0.325 g) was weighed into a round bottomed, quickfit, 250 ml flask, to which was added 30 ml dry, redistilled acetonitrile. The flask contents were then stirred, using a magnetic stirrer and follower. Triethylamine (500 µl) was added to facilitate the dissolution of orthophosphoric acid. Oleoyl chloride (1 ml) was then added, and the reaction was left to stir for 1½ hours in the dark at room temperature. After this time 100 ml 1 M aqueous sodium chloride was added, followed by 70 ml redistilled AnalaR chloroform. The reaction mixture was shaken and transferred to a separating funnel where the chloroform and aqueous phases were allowed to separate. The aqueous phase was then re-extracted with 30 ml chloroform and the two chloroform phases were pooled. This was then shaken with water to remove any free phosphoric acid and, after separation, the chloroform layer was dried over anhydrous sodium sulphate for 10-20 minutes. The chloroform was then decanted from the sodium sulphate and filtered through chloroform washed Whatman No. 1 filter

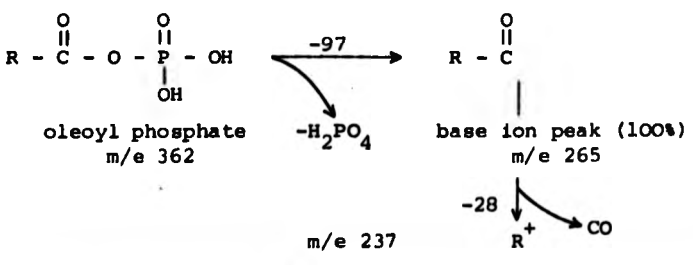
paper to remove any suspended sodium sulphate. The filtrate was then evaporated to dryness in a Buchi evaporator. The remaining material is crude oleoyl phosphate. Thin layer chromatography in a chloroform methanol (2:1) system shows it to consist of one spot. Purification using silica gel thin layer chromatography is difficult as oleoyl phosphate undergoes decomposition on silica gel rather easily.

Characteristaion of oleoyl phosphate.- Infra-red spectra of crude oleoyl phosphate show $\ddot{\nu}$ maxima at 1780 cm^{-1} for C=O of acyl phosphate, 1250-1300 cm^{-1} for P=O and 2660 cm^{-1} for hydrogen bonded OH strongly indicating that the crude product does contain substantial amounts of oleoyl phosphate.

The crude product was also assayed for its acyl ester content by the hydroxamate assay of Lipman and Tuttle (see methods) and its hydrolysable phosphate content (see methods). Both these methods, together and singly, showed that (a) crude oleoyl phosphate contained equimolar amounts of fatty acyl ester and hydrolysable phosphate, and (b) both the ester and phosphate contents were consistent with the 'crude' oleoyl phosphate being 90-100% pure (usually 95-100% pure).

The ^{31}P NMR spectrum of crude oleoyl phosphate shows a chemical shift at 6.38 ppm against a reference H_3PO_4 as zero consistent with the product being an acyl phosphate.

The mass spectrum of 'crude' oleoyl phosphate displays a very weak molecular ion peak, m/e 362. A base ion peak, due to loss of H_2PO_4 occurs at m/e 265 (100%). Further loss of CO results in an R^+ ion at m/e 237. These results are consistent with a breakdown pattern as follows:-



$R = CH_3(CH_2)_7CH = CH(CH_2)_7-$

The mass spectrum shows no signs of the higher molecular weight dioleoyl or trioleoyl phosphate esters whichmight possibly be produced by the synthetic method used (this is unlikely). This,together with the ester and phosphate content assays,indicate that the 'crude' oleoyl phosphate is largely pure. Silica gel thin layer chromatography in a chloroform: methanol:acetic acid: water (85:15:10:1) system shows that the only major contaminant is oleic acid. This however was usually only present at 1-5% (by weight) in freshly prepared oleoyl phosphate.

Oleoyl phosphate was also prepared by the method described by Johnson and Criddle (241). This entailed the mixing of 92% ortho-phosphoric acid with oleoyl chloride at 0° C and extracting the mixture after 15-30 minutes with diethyl ether. The diethyl ether was then evaporated to an oil and stored at -20° C. Although Johnson and Criddle report a yield of 80-90% oleoyl phosphate using this method, we found the product to be more usually 50-60% oleoyl phosphate, the rest being oleic acid. This is probably because 92% phosphoric acid contains substantial amounts of hygroscopic water which causes hydrolysis of oleoyl chloride to oleic acid and HCl.

The purest preparations of oleoyl phosphate are not oils at room temperature but waxy solids; preparations that are oils may contain either large amounts of residual solvent (it is rather difficult to remove all the chloroform from oleoyl phosphate by rotary evaporation) or may be a solution of oleoyl phosphate in oleic acid. This variation in substrate preparation is discussed more fully later (see discussion).

Hydroxamate assay for acyl esters.- Oleoyl phosphate and other fatty acyl esters (e.g.oleoyl-S-lipoate, erucoyl phosphate) were assayed, using a modified hydroxamate assay procedure based on the original method of Lipman and Tuttle (242). Tubes containing the acyl ester under test and 200 µl 10% sodium dodecyl sulphate (electrophoretic grade) were made up to a final volume of 500 µl with distilled water. Hydroxylamine reagent (0.5 ml) and acetate buffer (0.5 ml) were then added, the tubes

were then vortexed and allowed to stand for 10 minutes at room temperature. Then 0.5 ml HCl solution, 0.5 ml 12% trichloroacetic acid and 0.5 ml ferric chloride reagent were added with mixing. The assays were allowed to stand at room temperature for 15 minutes and their optical density at 500 nm was measured in a Unicam SP600 spectrophotometer or an MSE Servoplus spectrophotometer. The amount of acyl ester was then estimated from a standard curve constructed using either methyl oleate, acetyl phosphate or stearoyl oleate as a standard.

Hydroxylamine reagent was prepared for each assay from equal volumes of stock solutions of 28% hydroxylamine hydrochloride (w/v) and 14% sodium hydroxide (w/v). Acetate buffer consists of 0.1 M acetic acid and 0.1 M sodium acetate., 2:8. Ferric chloride reagent is 6% $FeCl_3.6H_2O$ in 0.1 M HCl (w/v). Hydrochloric acid solution is a dilution of the concentrated acid (36% HCl), 1:3 with water (v/v). Fatty acyl phosphates were assayed for hydrolysable phosphate content. Samples of 0.2 M solutions of fatty acyl phosphate dissolved in dimethylformamide were added to 0.5 ml water: 0.5 ml 60% perchloric acid was then added and the assay was incubated at 30° C for 30 minutes with frequent mixing. 10% SDS was added to aid dissolution of acyl phosphate if required. At the end of this period 0.5 ml samples were taken to assay for phosphate in a standard Fiske Subbarow phosphate assay. Samples were also taken for phosphate assay prior to addition of perchloric acid and immediately after it. Oleoyl phosphate preparations were generally found to contain little or no free inorganic phosphate.

Acyl ester content of oleoyl phosphate was occasionally estimated, using the method of Snyder and Stevens (243). Stock solutions of the following assay reagents were made:- reagent A - stock ferric perchlorate:; 5 grams ferric perchlorate dissolved in 10 ml 20% perchloric acid + 10 ml water diluted to 100 ml with cold absolute ethanol.

reagent B; alkaline hydroxamate, 4%: 2.0 g hydroxylamine dissolved in 2.5 ml water diluted to 50 ml with cold absolute ethanol; reagent C: ethanolic sodium hydroxide, 8%; 4 g sodium hydroxide dissolved in 2.5 ml water diluted to 50 ml with cold absolute ethanol. Assay reagents were prepared immediately prior to use:- reagent D - acid ferric perchlorate: 4 ml reagent A + 3 ml 70% perchloric acid diluted to 100 ml with cold absolute ethanol; reagent E - alkaline hydroxylamine: equal volumes of reagent B + C were mixed and the precipitated sodium chloride removed by centrifugation. The supernatant was used immediately.

Reagent E (1 ml) was added to a dry acyl ester sample (i.e. no solvent present) in a pyrex test tube. This was then heated at 65° C for two minutes in a water bath and then left to cool at room temperature for five minutes. Reagent D (2.5 ml) was then added with mixing and the assay was allowed to stand for 30 minutes at room temperature, prior to reading its optical density at 530 nm. Acyl ester was estimated by comparision to standard curves prepared with acetyl phosphate or methyl oleate, usually the latter.

Table 5.1 Structures of acyl phosphates

trivial name	systematic name	structure	MW
oleoyl phosphate.	cis Δ9 octadecanoyl phosphate	$CH_3(CH_2)_7CH{=}CH(CH_2)_7C(=O)O\text{-}P(=O)(OH)OH$ (cis)	362
elaidoyl phosphate.	trans Δ9 octadecenoyl phosphate	$CH_3(CH_2)_7CH{=}CH(CH_2)_7C(=O)O\text{-}P(=O)(OH)OH$ (trans)	362
erucoyl phosphate.	cis Δ13 docosaenoyl phosphate	$CH_3(CH_2)_7CH{=}CH(CH_2)_{11}C(=O)O\text{-}P(=O)(OH)OH$ (cis)	418
palmitoyl phosphate.	hexadecanoyl phosphate	$CH_2(CH_2)_{14}C(=O)O\text{-}P(=O)(OH)OH$	326

Results

Oleoyl phosphate was synthesised and characterised as described in the methods section of this chapter (see Table 5.1 for structures and molecular weights of oleoyl and other fatty acyl phosphates) and was normally found to be in the region of 90-100% pure acyl phosphate. Oleoyl phosphate was tested for its ability to reverse the DCCD induced inhibition of Serrano and Raker's 'proton translocating ATPase' (Table 5.2) and was found to be at least as effective as other oleoyl derivatives at reversing this inhibition. The restored ATPase activity was insensitive to further additions of DCCD, DBCT, TET and the uncoupler '1799', but was completely sensitive to efrapeptin, thus reinforcing the hypothesis proposed in Chapter 4 that oleoyl derivatives 'reverse' the DCCD induced inhibition of ATPase activity by a dislocation of the enzyme rather than by a true reversal.

Oleoyl phosphate was then tested for its ability to drive ATP synthesis in bovine heart submitochondrial particles in a glucose-hexokinase trap system. ATP synthesis was observed with an apparent stoicheiometry of 0.5 moles ATP produced per mole oleoyl phosphate added (Table 5.3 and Fig. 5.1). Stoicheiometry of ATP production was generally found to be in the region of 0.6 - 0.9 mole ATP produced per mole oleoyl phosphate added in experiments where any ATP synthesis was observed. This ATP synthesis in bovine heart submitochondrial particles was insensitive to the inhibitors of ATP synthesis, DBCT, TET, oligomycin and the uncoupler '1799' but sensitive to DCCD and efrapeptin (Table 5.4).

Oleoyl phosphate was then shown to drive ATP synthesis in two purified ATPase preparations; 'complex V' of Hatefi and coworkers (244) and the 'proton translocating' ATPase of Serrano and Racker (245). This synthesis was also sensitive to efrapeptin and DCCD but insensitive to oligomycin (Tables 5.5-6). The insensitivity of oleoyl phosphate-driven

Table 5.2 Effect of oleoyl phosphate on DCCD inhibited 'proton translocating' ATPase

Additions	ATP hydrolysis moles/min/mg	Total phosphate released (nmoles
DCCD inhibited proton translocating ATPase	0	0
DCCD inhibited proton translocating ATPase + oleoyl phosphate (50 nmoles)	0.34	18
DCCD inhibited proton translocating ATPase + oleoyl phosphate (125 nmoles)	2.29	124
DCCD inhibited proton translocating ATPase + oleoyl phosphate (250 nmoles)	2.92	157
DCCD inhibited proton translocating ATPase + oleoyl phosphate (125 nmoles) + DBCT	2.00	108
DCCD inihibited proton translocating ATPase + oleoyl phosphate (125 nmoles) + TET	2.11	114
DCCD inhibited proton translocating ATPase + oleoyl phosphate (125 nmoles) + DCCD	2.16	117
DCCD inhibited proton translocating ATPase + oleoyl phosphate (125 nmoles) + 1799	2.29	124
DCCD inhibited proton translocating ATPase + oleoyl phosphate (125 nmoles) + efrapeptin	0	0

Assays containing 90 µg enzyme were preincubated with oleoyl phosphate (as a 50 mM solution in dimethylformamide) and 2 µg effectors, were indicated, for 5 minutes at 30° C in 1 ml 50 mM Tris-Cl, pH 8.5; 5 mM $MgCl_2$ prior to addition of 5 µmoles ATP. Assays were terminated by addition of 0.5 ml 10% T.C.A. Phosphate was determined by the method of Fiske/Subbarow as described in Chapter 3. Parallel experiments indicate no oleoyl phosphatase activity in the absence of ATP.

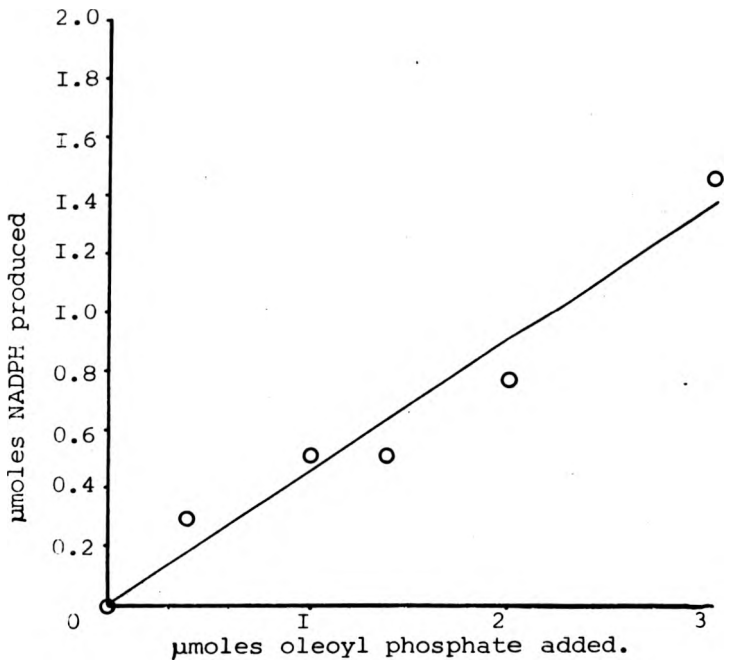
Table 5.3 Oleoyl phosphate-driven ATP synthesis in bovine heart submitochondrial particles: stoicheiometry of ATP production

Additions	ΔA_{340}	ATP formed[1] µmoles/20 mins
none	0[2]	0
SMP + azolectin	0[2]	0
SMP + oleoyl phosphate (0.4 µmoles)	0.06	0.29
SMP + oleoyl phosphate (1.0 µmoles)	0.105	0.51
SMP + oleoyl phosphate (1.4 µmoles)	0.105	0.51
SMP + oleoyl phosphate (2.0 µmoles)	0.160	0.77
SMP + oleoyl phosphate (3.0 µmoles)	0.305	1.47
SMP + oleoyl phosphate (1 µmole) + 2 µg aurovertin	0	0
SMP + oleoyl phosphate (1 µmole) + 5 µg DCCD	0	0
SMP + oleoyl phosphate (1 µmole) + 5 µg 1799	0.08	0.30
SMP + oleoyl phosphate (1 µmole) - ADP	0	0

[1] measured as NADPH

[2] assays read against blanks containing all assay components except enzyme or substrate (separate controls)

Assays containing 2 mg SMP's were preincubated with inhibitors, where indicated, for 5 minutes in a 1 ml glucose-hexokinase trap system, 2 mM in ADP at 30° C in the presence of 2 µg rotenone and 2 µg antimycin. Assays were initiated with oleoyl phosphate (as a 0.2 M suspension in 10 mg/ml azolectin/water). Assays were terminated after 20 minutes and assayed for ATP synthesis as described in Table 5.5.



Fig,5.I Oleoyl phosphate driven ATP synthesis in bovine heart submitochondrial particles: stoicheiometry of ATP production.

Assays were performed as described in table,5.3.

Table 5.4 Oleoyl phosphate-driven ATP synthesis in bovine heart submitochondrial particles: sensitivity to inhibitors of oxidative phosphorylation

Additions	ATP synthesis µmoles/15 minutes[1]	
	0.02^2	A_{340}
none		0
oleoyl phosphate (3 µmoles)	0.80	2.75
oleoyl phosphate (3 µmoles) + DBCT 2 µg	0.70	2.41
oleoyl phosphate (3 µmoles) + TET 4 µg	0.62	2.12
oleoyl phosphate (3 µmoles) + oligomycin 4 µg	0.75	2.58
oleoyl phosphate (3 µmoles) + DCCD 20 nmoles	0.02	0
oleoyl phosphate (3 µmoles) + '1799' 10 µg	0.50	1.69
oleoyl phosphate (3 µmoles) + efrapeptin 4 µg	0.07	0.17

[1] estimated as glucose-6-phosphate (NADPH)

[2] assays were measured against no substrate and no enzyme blanks (both separately

Assays containing 2 mg submitochondrial particles, 2 µg antimycin, 2 µg rotenone, were incubated with inhibitors where indicated for 5 minutes at 30° C in a 1 ml glucose-hexokinase trap system, 2 mM in ADP, for 5 minutes prior to addition of oleoyl phosphate. Assays were incubated for 15 minutes at 30° C and terminated by addition of 100 µl 3N HCl.Protein was removed by centrifugation and 0.1 ml supernatant was taken into 1.9 ml 0.5 M Tris-Cl, pH 7.5 and assayed for glucose-6-phosphate as described in Chapter 3.

Table 5.5 Oleoyl phosphate-driven ATP synthesis in 'complex V' ATPase

Addition	ΔA_{340}	NADPH produced µmoles/20 minutes	ATP synthesis µmoles/20 minutes
none	0		
azolectin	0		
complex V	0.09	0.434	-
complex V + oleoyl phosphate (1 µmole)	0.235	1.133	0.699
complex V + oleoyl phosphate (2 µmoles)	0.420	2.025	1.591
complex V + oleoyl phosphate (1 µmole) + 5 µg DCCD	0.12	0.578	0.144
complex V + oleoyl phosphate (1 µmole) + 5 µg '1799'	0.27	1.3022	0.868

Assays containing 0.5 mg complex V were incubated for 5 minutes at 30° C in 1 ml 250 mM sucrose; 20 mM Tris-Cl, pH 7.3; 20 mM glucose; 5 mM $MgCl_2$; 0.2 mM EDTA; 2 mM ADP plus 20 units of hexokinase, with inhibitors, where indicated. Assays were initiated by addition of an oleoyl phosphate azolectin suspension (∿ 0.2 M) and were incubated for 20 minutes at 30° C. Assays were terminated by the addition of 0.425 ml 1 M perchloric acid. Coagulated protein was removed by centrifugation in a Jobling bench centrifuge; 75 µl 6 M KOH was added to the supernatant which was then stood in ice for 20 minutes to allow complete precipitation of potassium perchlorate. This was then removed by centrifugation and 0.1 ml of the supernatant was added to 1.9 ml 0.5 M Tris-Cl, pH 7.5. To this, 0.5 µmole NADP and 1-2 units of glucose-6-phosphate dehydrogenase was added. The tubes were whirlimixed and allowed to stand for 15 minutes prior to reading their absorbance at 340 nm in a Pye-Unicam SP1800 dual beam spectrophotometer. Assays were measured against no enzyme and no substrate controls.

Table 5.6 Oleoyl phosphate-driven ATP synthesis in bovine heart mitochondrial F_1F_o ATPase complex preparations

Addition	ATP synthesis[1] µmoles/20 minutes
'complex V'	0
'complex V' + oleoyl phosphate (3 µmoles)	2.47
'complex V' + oleoyl phosphate (3 µmoles) + 2 µg efrapeptin	0
'proton translocating' ATPase	0
'proton translocating' ATPase + oleoyl phosphate (3 µmoles)	2.19
'proton translocating' ATPase + oleoyl phosphate (3 µmoles) + 2 µg oligomycin	2.19
'proton translocating' ATPase + oleoyl phosphate (3 µmoles) + 2 µg efrapeptin	0

[1] measured as NADPH

Assays, 2 mM in ADP, containing 1 mg ATPase protein were carried out as described in Table 5.4

Table 5.7 Oleoyl phosphate-driven ATP synthesis in a chloroform released F_1 ATPase from bovine heart SMP

Additions	ATP synthesis μmoles/20 minutes[1]
palmitoyl phosphate (1 μmole)	0
elaidoyl phosphate (1 μmole)	0
erucoyl phosphate (1 μmole)	0
oleoyl phosphate (1 μmole)	0.84
oleoyl phosphate (1 μmole + erucoyl phosphate (100 nmoles)	0
oleoyl phosphate (1 μmole) + elaidoyl phosphate (100 nmoles)	0
oleoyl phosphate (1 μmole) + palmitoyl phosphate (100 nmoles)	0.83
oleoyl phosphate (1 μmole) + palmitoyl phosphate (1000 nmoles)	0

Assays containing 100 μg $CHCl_3$ released ATPase were preincubated with indicated fatty acyl phosphates (second column) for 5 minutes at 30° C in a 1 ml glucose-hexokinase trap system, 0.2 mM in ADP. Assays were initiated by addition of acyl phosphates (as 0.2 M solutions in dimethylformamide and terminated after 20 minutes at 30° C by addition of perchloric acid. ATP was determined as described in Chapter 3.

[1] ATP synthesis quoted has been corrected for myokinase activity (∿ 0.1 μmoles ATP)

ATP synthesis to inhibitors of the ATPase such as oligomycin, DBCT and TET, all of which act on the membrane bound F_o fraction of the ATPase and its sensitivity to efrapeptin (an F_1 inhibitor) suggested that all the steps of the oleoyl phosphate → ATP reaction occur on the F_1 portion of the ATPase. A chloroform released F_1 ATPase from bovine heart submitochondrial particles was prepared according to the method of Beechey et al. (236). ATP synthesis was observed with oleoyl phosphate in this enzyme (Table 5.7). Other fatty acyl phosphates, elaidoyl, erucoyl and palmitoyl phosphates did not drive ATP synthesis (Table 5.7).

As dihydrolipoate-driven ATP synthesis in purified ATPase preparations was found to be very sensitive to certain fatty acids (especially erucic acid and elaidic acid) (see Chapter 3) oleoyl phosphate-driven ATP synthesis was examined to see if it displayed a similar sensitivity to other fatty acyl phosphates. Elaidoyl phosphate and erucoyl phosphate appeared to be extremely effective as inhibitors of the reaction, whereas palmitoyl phosphate was rather ineffective (Table 5.7). The free fatty acids, elaidic and erucic acid and elaidoyl lipoate and erucoyl lipoate (analagous to the postulated oleoyl lipoate intermediate) also strongly inhibit this reaction (Figs. 5.2-4). All the elaidoyl and erucoyl derivatives tested appeared to be extremely potent inhibitors of oleoyl phosphate driven ATP synthesis (Table 5.8), the phosphates being more effective than the lipoates, which in turn were more effective than the free fatty acids.

Chloroform released F_1 ATPase in common with other F_1 ATPase preparations displays no ATP-^{32}Pi exchange activity. As this exchange activity is thought to be indicative of residual ATP synthase activity, it might be thought that an F_1 ATPase displaying apparent ATP synthesis could also catalyse ATP-^{32}Pi exchange. Oleoyl phosphate was thus tested for its ability to stimulate ATP-^{32}Pi exchange in chloroform released F_1 ATPase from bovine heart submitochondrial particles. ATP-^{32}Pi

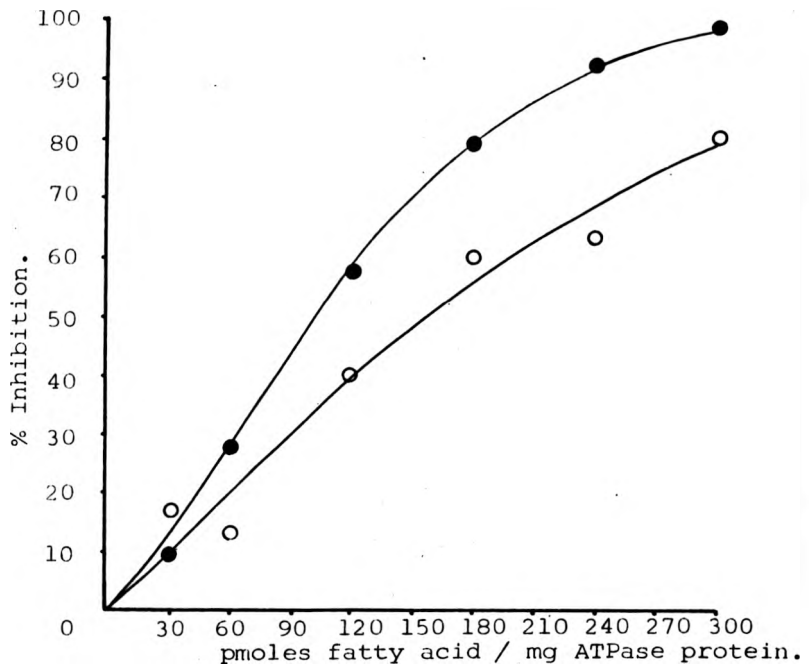


Fig 5.2. Inhibition of oleoyl phosphate driven ATP synthesis by erucic and elaidic acid.

Assays containing 100µg chloroform released ATPase protein from bovine heart SMP were preincubated with fatty acids, added as solutions in dimethylformamide, prior to initiation with 1 µmole oleoyl phosphate. ATP was estimated as described in table 5.8

●—● erucic acid

o—o elaidic acid

Preincubations with fatty acids were carried out for 5 minutes at 30°C.

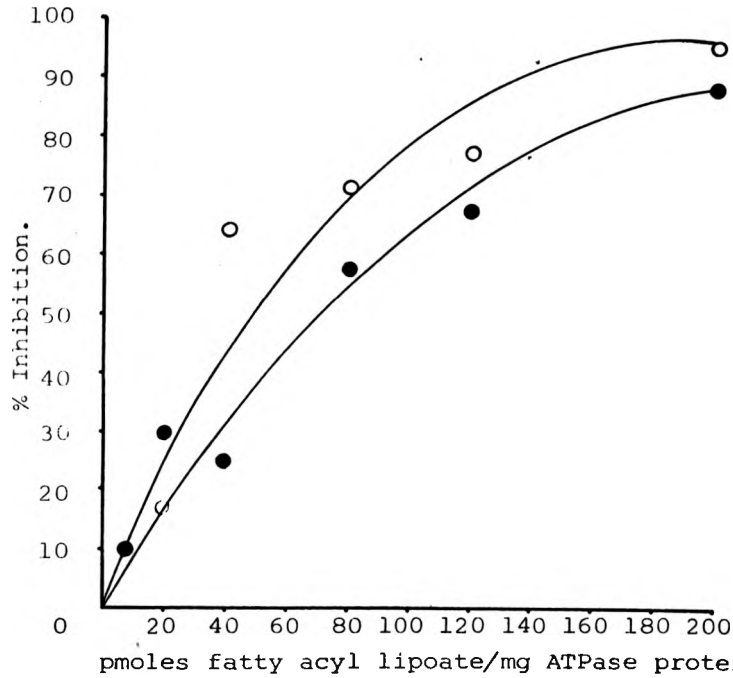


Fig 5.3. Inhibition of oleoyl phosphate driven ATP synthesis by fatty acyl lipoates.

●—● elaidoyl lipoate.
o—o erucoyl lipoate.

Assays containing 100µg chloroform released ATPase protein from bovine heart SMP, were preincubated with fatty acyl lipoates for 5 minutes at 30°C prior to addition of 1µmole oleoyl phosphate. Assays were incubated for 20 minutes at 30°C and terminated and ATP determined as described in table 5.8.

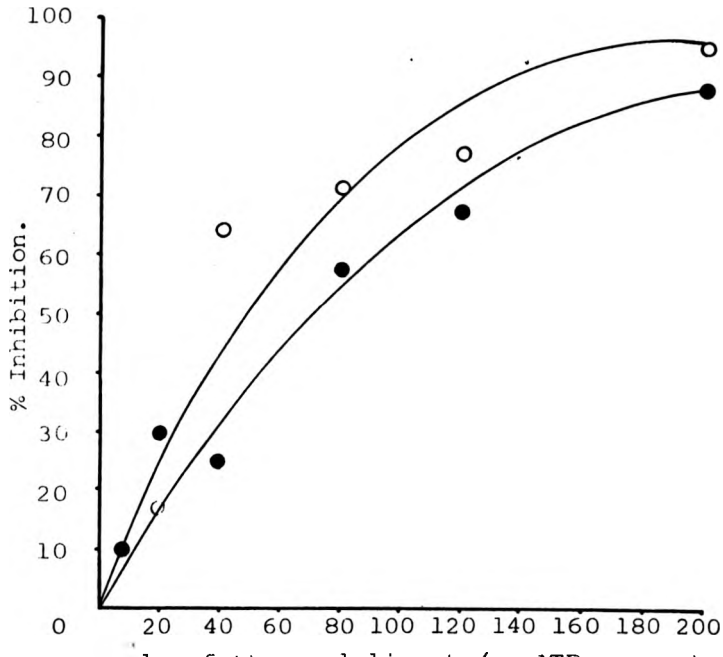


Fig 5.3. Inhibition of oleoyl phosphate driven ATP synthesis by fatty acyl lipoates.

●—● elaidoyl lipoate.
○—○ erucoyl lipoate.

Assays containing 100µg chloroform released ATPase protein from bovine heart SMP, were preincubated with fatty acyl lipoates for 5 minutes at 30°C prior to addition of 1µmole oleoyl phosphate. Assays were incubated for 20 minutes at 30°C and terminated and ATP determined as described in table 5.8.

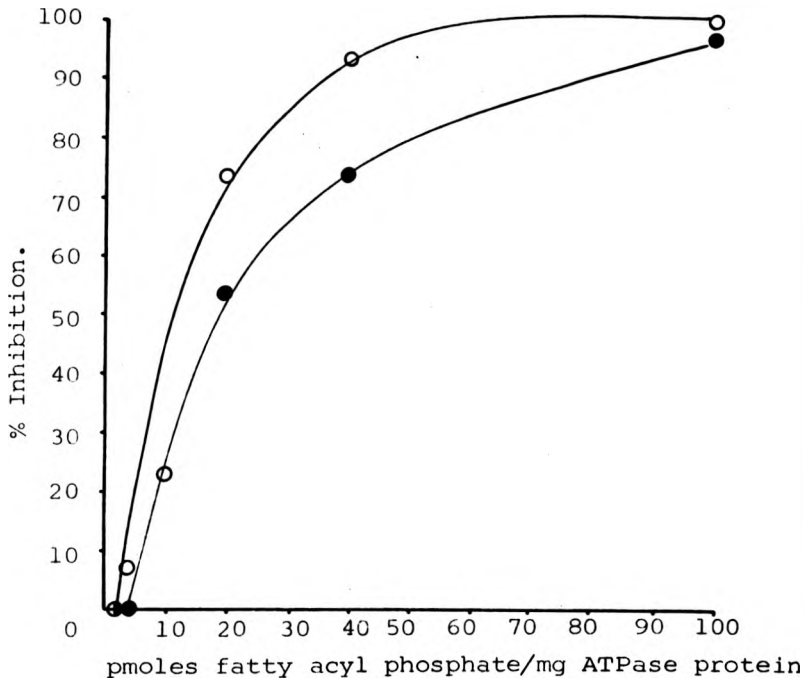


Fig 5.4. Inhibition of oleoyl phosphate driven ATP synthesis by fatty acyl phosphates.

●—● elaidoyl phosphate
○—○ erucoyl phosphate

Assays containing 100µg chloroform released ATPase protein from bovine heart SMP were preincubated with fatty acyl phosphates, added as solutions in dimethylformamide, for 5 minutes at 30°C prior to addition of 1µmole oleoyl phosphate. Assays were incubated for 20 minutes at 30°C, terminated and ATP determined as described in table 5.8.

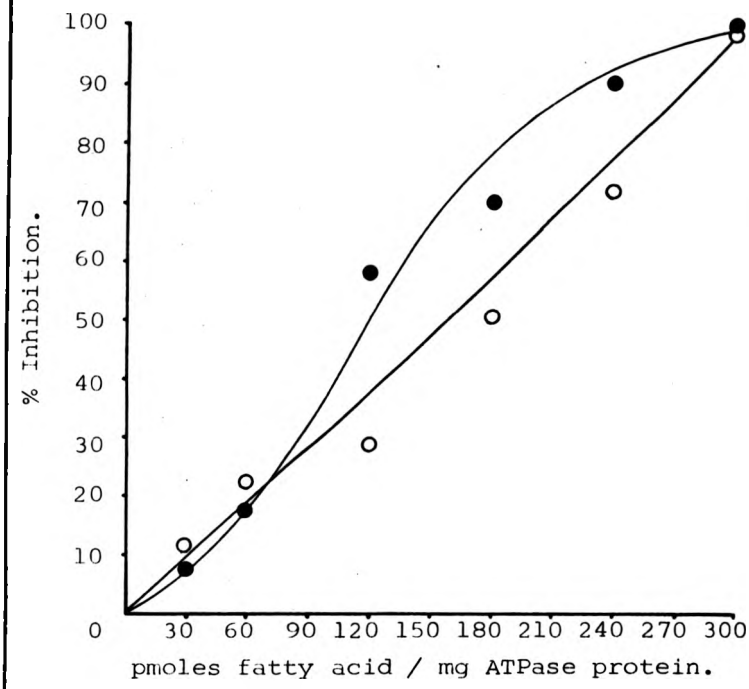


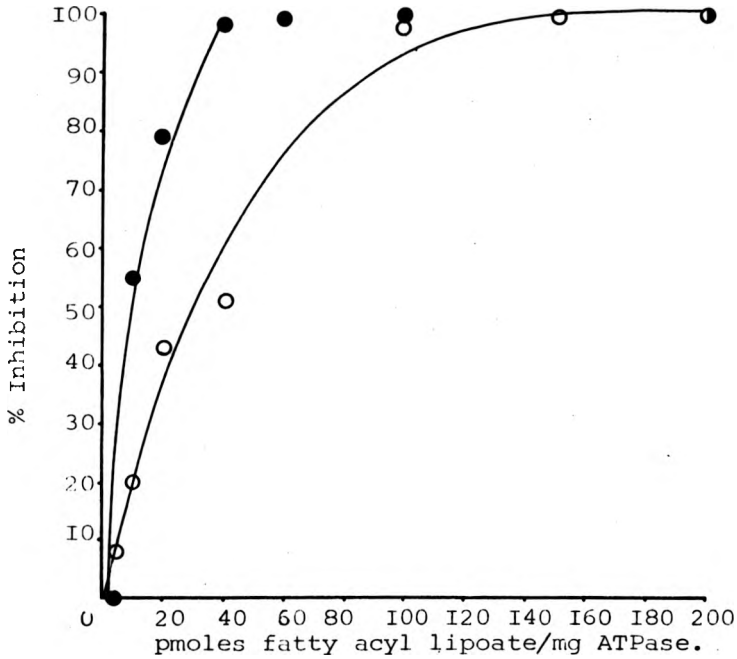
Fig 5.5. Inhibition of oleoyl phosphate stimulated ATP-^{32}Pi exchange by elaidic and erucic acid .

o—o elaidic acid.

●—● erucic acid.

Assays containing 100µg chloroform released ATPase protein from bovine heart SMP were preincubated with fatty acids, added as solutions in dimethylformamide, for 5 minutes at 30°C prior to addition of 10 µmoles ATP. ATP-^{32}Pi exchange was assayed as described in the methods section to chapter 3.

See also table 5.9 .



Fig,5.6.Inhibition of oleoyl phosphate stimulated ATP-^{32}Pi exchange by fatty acyl lipoates.

o—o elaidoyl lipoate

●—● erucoyl lipoate

Assays containing 100µg chloroform released ATPase from bovine heart submitochondrial particles were preincubated with fatty acyl lipoates added as dimethylformamide solutions for 5 minutes at 30°C prior to addition of 10µmoles ATP.ATP-^{32}Pi exchange was assayed as described in methods section chapter 3.

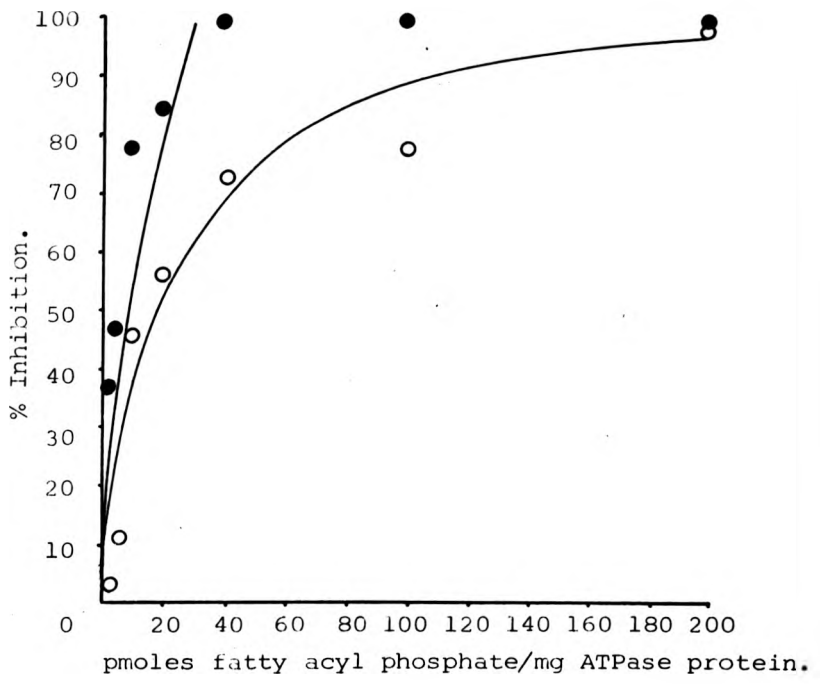


Fig 5.7. Inhibition of oleoyl phosphate stimulated ATP-^{32}Pi exchange by fatty acyl phosphates.

o—o elaidoyl phosphate.

●—● erucoyl phosphate.

Assays containing 100 µg chloroform released ATPase protein from bovine heart SMP were preincubated with fatty acyl phosphates, added as dimethylformamide solutions, for 5 minutes at 30°C prior to addition of 10 µmoles ATP. ATP-^{32}Pi exchange activity was assayed as described in the methods section to chapter 3.

See also table 5.9 .

Table 5.8 Oleoyl phosphate stimulated reactions in $CHCl_3$ released 'F_1' ATPase from bovine heart SMP: inhibition by fatty acyl derivatives, I_{50} values

Compound	ATP synthesis I_{50} value (pmoles/mg)	ATP-^{32}Pi exchange I_{50} value (pmoles/mg)
erucate	105	117
erucoyl lipoate	47	11
erucoyl phosphate	12	8
elaidate	155	160
eladioyl lipoate	72	30
elaidoyl phosphate	19	18
palmitoyl phosphate	> 1000	> 1000

Table 5.9 Oleoyl phosphate stimulated ATP-^{32}Pi exchange in F_1 ATPases from bovine heart mitochondria

Additions	ATP-^{32}Pi exchange nmoles/min/mg
$CHCl_3$ extracted 'F_1' ATPase	0
$CHCl_3$ extracted 'F_1' ATPase + oleoyl phosphate (1 µmole)	2653
$CHCl_3$ extracted 'F_1' ATPase + oleoyl phosphate (2 µmole)	4766
$CHCl_3$ extracted 'F_1' ATPase + oleoyl phosphate (1 µmole) + erucate (24 µmol)	30
$CHCl_3$ extracted 'F_1' ATPase + oleoyl phosphate (1 µmole) + elaidate (24 µmol)	796
$CHCl_3$ extracted 'F_1' ATPase + oleoyl phosphate (1 µmole) + erucoyl phosphate (20 µmol)	0
$CHCl_3$ extracted 'F_1' ATPase + oleoyl phosphate (1 µmole) + elaidoyl phosphate (20 µmol)	0
$CHCl_3$ extracted 'F_1' ATPase + oleoyl phosphate (1 µmole) +erucoyl lipoate (20 µmol)	0
$CHCL_3$ extracted 'F_1' ATPase + oleoyl phosphate (1 µmole) + elaidoyl lipoate (20 µmol)	0
F_1 ATPase (Knowles and Penefsky (262))	0
F_1 ATPase + oleoyl phosphate (1 µmole)	0

Assays containing 100 µg protein were incubated with fatty acid derivatives (not oleoyl phosphate) for 5 minutes at 30° C prior to initiation of reaction with oleoyl phosphate and ATP (10 µmoles). ATP-Pi exchange was determined as described in Chapter 3.

Table 5.10 Oleoyl phosphate-driven ATP synthesis in $CHCl_3$ released 'F_1' ATPases from bacterial vesicles

Additions	ATP synthesis µmoles/20 minutes		
	1	2	3
none	0	0	0
oleoyl phosphate (1 µmole)	0.71	0.66	0.61
oleoyl phosphate (2 µmole)	0.95	0.84	nt
oloeyl phosphate (1 µmole) + erucoyl phosphate (10 pmole)	0	0	0
oleoyl phosphate (1 µmole) + elaidoyl phosphate (10 pmole)	0	0	0
oleoyl phosphate (1 µmole) + erucate (25 pmole)	0	0	0
oleoyl phosphate (1 µmole) + elaidate (25 pmole)	0	0	0
oleoyl phosphate (1 µmole) + erucoyl lipoate (10 pmole)	0	0	0
oleoyl phosphate (1 µmole) + elaidoyl lipoate (10 pmole)	0	0	0
oleoyl phosphate (1 µmole) + FCCP (20 µg)	0	nt	nt
oleoyl phosphate (1 µmole) + DCCD (20 nmole)	0	nt	0.24

1 *Rhodospirillum rubrum* F_1 (50 µg/assay)

2 *Rhodopseudomonas capsulata* F_1 (50 µg/assay)

3 *Escherichia coli* F_1 (40 µg/assay)

Assays containing $CHCl_3$ released F_1 ATPase were preincubated with elaidoyl and erucoyl derivatives for 5 minutes at 30° C in a shaking water bath in a 1 ml glucose-hexokinase trap system, 0.2 mM in ADP. Assays were initiated by the addition of oleoyl phosphate (as a 0.2 M solution in dimethylformamide). Assays were terminated after 20 minutes with perchloric acid. ATP was estimated by the G-6-P method, as described in Chapter 3.

Table 5.11 Inhibition of bovine heart mitochondrial oxidative phosphorylation by fatty acyl phosphates

Additions	ATP synthesis nmoles/min/mg
none	0
1 mg mitochondria	0
1 mg mitochondria + 10 µmole pyruvate/1 µmole malate	218
1 mg mitochondria + 10 µmole pyruvate/1 µmole malate + 1 nmole oleoyl phosphate	91
1 mg mitochondria + 10 µmole pyruvate/1 µmole malate + 10 nmole oleoyl phosphate	69
1 mg mitochondria + 10 µmole pyruvate/1 µmole malate + 1 µmole oleoyl phosphate	0
1 mg mitochondria	0
1 mg mitochondria + 10 µmole pyruvate/1 µmole malate	150
1 mg mitochondria + 10 µmole pyruvate/1 µmole malate + 10 nmole erucoyl phosphate	57
1 mg mitochondria + 10 µmole pyruvate/1 µmole malate + 100 nmole erucoyl phosphate	0
1 mg mitochondria + 10 µmole pyruvate/1 µmole malate + 10 nmole elaidoyl phosphate	110
1 mg mitochondria + 10 µmole pyruvate/1 µmole malate + 100 nmole elaidoyl phosphate	80
1 mg mitochondria + 10 µmole pyruvate/1 µmole malate + 1 µmole elaidoyl phosphate	0

Assays containing 1 mg mitochondrial protein were preincubated, where indicated, with fatty acyl phosphates for 5 minutes at 30° C in a 1 ml glucose-hexokinase trap system, 0.2 mM in ADP in the presence of 5 units Sigma F-300 hexokinase. Assays were initiated by the addition of 10 µmoles sodium pyruvate + 1 µmole sodium malate, pH 7.3, and run for 20 minutes at 30° C in a shaking water bath. The assays were terminated by removal of an 0.2 ml aliquot into 0.2 ml 10% ice cold T.C.A. Coagulated protein was removed by bench centrifugation and an 0.2 ml aliquot from the supernatant was assayed for phosphate by the method of Fiske and Subbarow (see Chap. 3). Fatty acyl phosphates were added as solutions in dimethylformamide.

exchange activity was observed under these conditions (Table 5.9). This activity was inhibited by elaidoyl and erucoyl derivatives much as was the oleoyl phosphate stimulated ATP synthesis except that it was, if anything, slightly more sensitive (see Tables 5.8-9 and Figs. 5.5-7). Although oleoyl phosphate stimulated an ATP-Pi exchange activity in chloroform released F_1 ATPase, it failed to do so (or to drive ATP synthesis) in an F_1 ATPase of high specific activity (70-80 μmoles/min/mg) prepared by the method of Knowles and Penefsky (262) (Table 5.9).

Chloroform released F_1-like ATPases were also prepared from 'lysozyme' and 'French press' vesicles obtained from the bacterium Escherichia coli and from chromatophore preparations of the photosynthetic bacteria Rhodospirillum rubrum and Rhodopseudomonas capsulata. Oleoyl phosphate stimulated ATP synthesis in these enzymes. This synthesis was also sensitive to erucoyl and elaidoyl derivatives and DCCD (Table 5.10).

Pyruvate/malate driven oxidative phosphorylation in bovine heart mitochondria was inhibited by oleoyl, erucoyl and elaidoyl phosphates (Table 5.11).

Residual oleoyl phosphatase activity was observed in various ATPase preparations under conditions in which no oleoyl phosphate-driven ATP synthesis could be detected. Bovine heart mitochondria (Tables 5.12-13), ETP_H (Tables 5.14-15), chloroform released F_1 ATPase (Table 5.16) and an OS ATPase preparation from yeast mitochondria (Table 5.17) all appeared to catalyse this reaction at fairly low rates (0-75 nmoles/min/mg). This activity was inhibited by efrapeptin and aurovertin, (Tables 5.12-13, 15-18). Uncouplers occasionally inhibited the reaction (Table 5.13) but more usually had little effect (Table 5.14). Because of the difficulty with which some oleoyl phosphates disperse in aqueous buffer systems, various detergents were tested for their abilty to stimulate mitochondrial oleoyl phosphatase activity (Table

Table 5.12 Oleoyl phosphatase activity in bovine heart mitochondria

Additions	Oleoyl phosphatase nmoles Pi released/min/mg
none	0
0.2 µmole oleoyl phosphate	56
0.5 µmole oleoyl phosphate	21
1.0 µmole oleoyl phosphate	10
0.5 µmole oleoyl phosphate + 200 mg efrapeptin	0
0.5 µmole oleoyl phosphate + 10 µg aurovertin	5

Assays containing 115 µg enzyme were incubated with inhibitors, where indicated, for 5 minutes at 30° C in 1 ml 50 mM Tris-Cl, 5 mM $MgCl_2$, pH 7.4, 0.5 mM ADP prior to addition of oleoyl phosphate (as a 0.1 M methanolic solution). Assays were then incubated for 10 minutes @ 30° C before being terminated by the addition of 0.5 ml 10% T.C.A. Phosphate was determined by the method of Fiske/Subbarow, as described in Chapter 3.

Table 5.13 Oleoyl phosphatase activity in bovine heart mitochondria

Additions	Oleoyl phosphatase nmoles Pi released/min/mg
none	0
2.16 µmoles oleoyl phosphate	9.1
4.32 µmoles oleoyl phosphate	13.7
2.16 µmoles oleoyl phosphate + 5 µg efrapeptin	4.5
2.16 µmoles oleoyl phosphate + 5 µg DNP	1.4
2.16 µmoles oleoyl phosphate + 5 µg 1799	1.8
2.16 µmoles oleoyl phosphate + 5 µg S13	1.9

Assays containing 1.28 mg mitochondria were incubated at 30° C in 1 ml 50 mM Tris-Cl, pH 7.5; 5 mM $MgCl_2$ with uncouplers, where indicated, prior to addition of oleoyl phosphate as an 0.108 M solution in methanol. Assays were terminated by the addition of 0.5 ml 10% T.C.A. Phosphate was determined by the method of Fiske/Subbarow, as described in Chapter 3.

Table 5.14 Oleoyl phosphatase activity in bovine heart submitochondrial particles (ETP_H)

Additions	Oleoyl phosphatase nmoles Pi hydrolysed/min/mg
none	0
1.0 µmole oleoyl phosphate # 1	10
2.0 µmole oleoyl phosphate # 1	47
1.0 µmole oleoyl phosphate # 1 + '1799'	18
1.0 µmole oleoyl phosphate # 1 + DNP	18
1.0 µmole oleoyl phosphate # 2	14

Protocols. Assays containing 100 µg ETP_H in 1 ml 50 mM Tris-Cl, pH 7.4; 5 mM $MgCl_2$ and 2 µg uncouplers, added as methanolic solutions, where indicated, were incubated at 30° C for 5 minutes prior to the addition of oleoyl phosphate. After 15 minutes at 30° C, assays were terminated by addition of 0.5 ml 10% T.C.A. Phosphate was assayed by the method of Fiske/Subbarow, as described in Chapter 3. Values given above were corrected for any non-enzymic hydrolysis of oleoyl phosphate. Oleoyl phosphate was added as a 0.2 M solution in methanol.

Table 5.15 Oleoyl phosphatase activity in bovine heart submitochondrial particles (ETP_H): effect of pH

Additions	Oleoyl phosphatase nmoles Pi hydrolysed/min/mg	
	pH 7.4	pH 8.5
none	0	0
0.195 µmoles oleoyl phosphate	25.1	28.9
0.390 µmoles oleoyl phosphate	51.0	38.0
0.585 µmoles oleoyl phosphate	73.2	68.4
0.390 µmoles oleoyl phosphate + 1 µg efrapeptin	30.0	nt

Assays containing 224 µg ETP_H were assayed for oleoyl phosphatase activity as described in Table 5.14. Oleoyl phosphate was added as a 39 mM solution in dimethyl formamide.

Table 5.16 Oleoyl phosphatase activity in a chloroform released F_1 ATPase from bovine heart submitochondrial particles

Additions	Oleoyl phosphatase nmoles Pi released/min/mg
none	0
1 µmole oleoyl phosphate	47
1 µmole oleoyl phosphate + 10 µg efrapeptin	0
1 µmole oleoyl phosphate (- ADP)	0

Assays containing 100 µg $CHCl_3$ released ATPase were incubated with efrapeptin, where indicated in a buffer 50 mM in Tris-Cl, pH 8.5; 5 mM $MgCl_2$; 0.5 mM in ADP; 0.5% in potassium cholate prior to the addition of oleoyl phosphate (as a 0.1 M solution in methanol). Incubations were carried out at 30° C and were terminated by the addition of 0.5 ml 10% T.C.A. Phosphate was determined by the method of Fiske/Subbarow,as described in Chapter 3.

Table 5.17 Oleoyl phosphatase activity in an OS ATPase preparation from yeast mitochondria

Additions	Oleoyl phosphatase nmoles Pi released/min/mg
none	0
0.25 µmoles oleoyl phosphate	35
0.5 µmoles oleoyl phosphate	60
1.0 µmoles oleoyl phosphate	60
0.5 µmoles oleoyl phosphate + 1 µg efrapeptin	0

Assays containing 100 µg OS ATPase prepared by the method of Ryrie (429) were incubated in 1 ml 50 mM Tris-Cl, pH 8.5; 5 mM $MgCl_2$ at 30° C and were initiated by the addition of oleoyl phosphate (as an 0.05 M solution in the above buffer, 10% in potassium cholate). Assays were terminated by the addition of 0.5 ml 10% T.C.A. Phosphate was determined by the method of Fiske/Subbarow, as described in Chapter 3.

Table 5.18 Oleoyl phosphatase in bovine heart mitochondria: effect of detergents

Buffer system	Oleoyl phosphatase nmoles Pi/min/mg
50 mM Tris-Cl, pH 7.4; 5 mM $MgCl_2$; 0.5 mM ADP	0
50 mM Tris-Cl, pH 7.4; 5 mM $MgCl_2$; 0.5 mM ADP 0.5% in potassium cholate	21.4
50 mM Tris-Cl, pH 7.4; 5 mM $MgCl_2$; 0.5 mM ADP 0.5% in Triton X-114	8.9
50 mM Tris-Cl, pH 7.4; 5 mM $MgCl_2$; 0.5 mM ADP 0.5 % in Triton X-114 + 1 µg efrapeptin	0
50 mM Tris-Cl, pH 7.4; 5 mM $MgCl_2$; 0.5 mM ADP 0.5% in Tween 20	18.0
50 mM Tris-Cl, pH 7.4; 5 mM $MgCl_2$; 0.5 mM ADP 0.5% in Tween 20 + 1 µg efrapeptin	7.1
50 mM Tris-Cl, pH 7.4; 5 mM $MgCl_2$; 0.5 mM ADP 0.5% in Tween 80	18.0
50 mM Tris-Cl, pH 7.4; 5 mM $MgCl_2$; 0.5 mM ADP 0.5% in Tween 80 + 1 µg efrapeptin	1.8
50 mM Tris-Cl, Ph 7.4; 5 mM $MgCl_2$; 0.5 mM ADP 0.5% in potassium deoxycholate	0
50 mM Tris-Cl, pH 7.4; 5 mM $MgCl_2$; 0.5 mM ADP 0.5% in sodium dodecylsulphate	0

Assays containing 204 µg protein were assayed as described in Table 5.14

5.18), potassium cholate and the non-ionic detergents Tween 20 and 80 appeared to be the best. The various ATPase preparations usually required ADP to display oleoyl phosphatase activity (Table 5.16); this was not always the case, however, and oleoyl phosphatase rates of up to 80 nmoles/min/mg have been observed in the absence of ADP.

Discussion

Any discussion about the results presented in this chapter must be prefaced by an attempt to functionally classify the oleoyl phosphate preparations used. At best, oleoyl phosphate-driven ATP synthesis and oleoyl phosphate stimulated ATP-^{32}Pi exchange can be described as reactions which occur rarely and which are extremely difficult to reproduce. The reasons for this unreproducibility are not at all clear although some suggestions will be made in the course of this discussion.

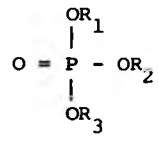
Oleoyl phosphate can be usefully classified into three functional groups: (a) those which drive ATP synthesis and ATP-Pi exchange, (b) those which can be partially phosphorylysed by various ATPases, i.e. ones which support a residual oleoyl phosphatase activity, (c) those which have no ability to drive any of the reactions reported driven by oleoyl phosphate (oleoyl phosphokinase, ATP-^{32}Pi exchange, oleoyl phosphatase).

Of the thirty or so preparations of oleoyl phosphate synthesised, only four could be classified in group (a), the rest being in (b) and (c), mainly in the latter. All four of the oleoyl phosphates in group (a) were synthesised by the method described previously and were in the region of 90-100% pure by acyl ester and hydrolysable phosphate content.

Some differences could be noticed in different oleoyl phosphate preparations. These were their physical state and their solubility in organic solvents. Some oleoyl phosphates appeared to become hard waxy solids at room temperature whereas some were dense, oily fluids only solidifying when cooled below 0° C. Preparations of oleoyl

phosphate differed in their solubility: some would dissolve easily in methanol up to at least 0.2 M; some, however, would hardly dissolve in methanol. Those that were soluble in methanol could be further classified by their ability to disperse when methanolic solutions were added to aqueous buffer systems. Some would, producing optically clear solutions, others would form emulsions on shaking, while others remained as large globules, waxy in texture and very difficult to disperse. Those methanolic solutions of oleoyl phosphate which would not disperse into aqueous buffer systems also precipitated on standing at 4° C or below. This precipitate was identical in acyl ester and hydroysable phosphate content to the original sample of oleoyl phosphate but would not redissolve in methanol when warmed above 4° C. All oleoyl phosphates tested were soluble in dimethylformamide, although here again not all dimethylformamide solutions would disperse into aqueous buffer systems.

The reasons for these differences are not clear. All the oleoyl phosphates were made by an identical procedure from similar starting materials (i.e. Sigma oleoyl chloride, grade 1, and Fisons '100% orthophosphoric acid'). There was some variation, however, in the nature of the orthophosphoric acid used; 100% orthophosphoric acid is extremely hydroscopic and some samples of the acid, even though stored when obtained under vacuum dessication, contained rather more hygroscopic water than others. The differences in solubility amd ability to disperse in aqueous buffers of the oleoyl phosphate preparations may be related to water retained by the phosphate grouping or differences in the nature of the phosphate group. Orthophosphates have the general structure:-

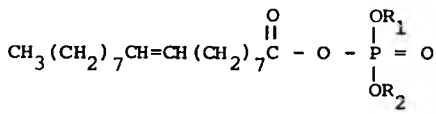


if $R_1R_2R_3$ = H orthophosphoric acid

R_1R_2 = H ; R_3 = Na sodium hydrogen phosphate

R_1 = H; R_2R_3 = Na disodium, hydrogen phosphate etc.

Oleoyl phosphate then could exist in a number of forms.

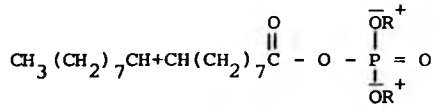


R_1R_2 = H oleoyl phosphoric acid

R_1 = Na; R_2 = H sodium hydrogen oleoyl phosphate

R_1R_2 = Na disodium oleoyl phosphate etc.

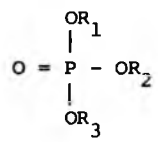
Any one, or a mixture,of these compounds could have been produced by the synthetic procedure used. Another complex salt of oleoyl phosphate, triethylammonium oleoyl phosphate, might have been produced as phosphate was solubilised by triethylamine in the synthetic procedure. Thus the following salt may have been produced:-



where one or both R^+'s is $(C_2H_5)_3\overset{+}{N}H$.

Wet orthophosphoric acid will dissolve in the acetonitrile used in the assay without the addition of triethylamine. This gives oleoyl phosphate that is indistinguishable chromatographically and functionally (with respect to groups (b) and (c)) from that prepared by the normal method. Triethylamine phosphate had no ability to act as substrate for any of the reactions attributed to oleoyl phosphate.

The existence of these different salts of oleoyl phosphate could account for both the solubility and physical differences observed in the samples. It might also account for the difference in ability to act as substrates for ATP synthesis with various ATPase enzymes which might require oleoyl phosphate (as substrate) as a specific salt, or in a specific hydration state. It is worth noting that all four preparations of oleoyl phosphate that routinely drove ATP synthesis

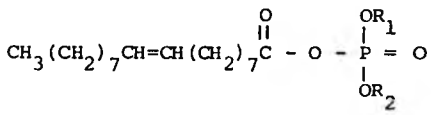


if $R_1R_2R_3 = H$ orthophosphoric acid

$R_1R_2 = H$; $R_3 = Na$ sodium hydrogen phosphate

$R_1 = H$; $R_2R_3 = Na$ disodium, hydrogen phosphate etc.

Oleoyl phosphate then could exist in a number of forms.



$R_1R_2 = H$ oleoyl phosphoric acid

$R_1 = Na$; $R_2 = H$ sodium hydrogen oleoyl phosphate

$R_1R_2 = Na$ disodium oleoyl phosphate etc.

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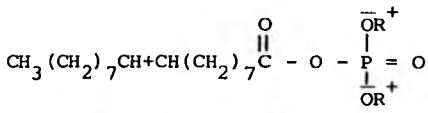

where one or both R^+'s is $(C_2H_5)_3\overset{+}{N}H$.

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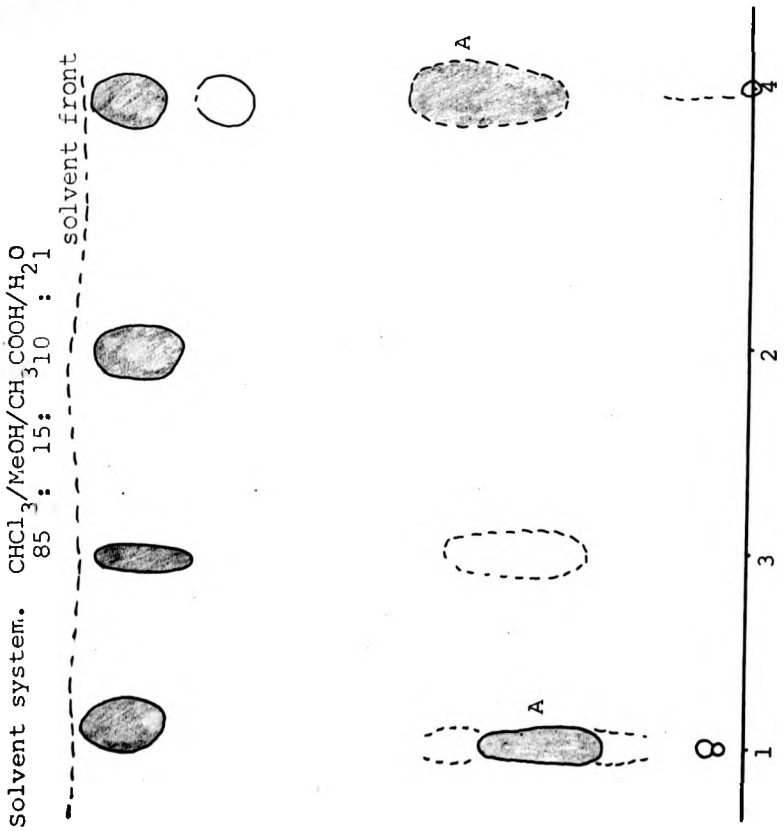
The existence of these different salts of oleoyl phosphate could account for both the solubility and physical differences observed in the samples. It might also account for the difference in ability to act as substrates for ATP synthesis with various ATPase enzymes which might require oleoyl phosphate (as substrate) as a specific salt, or in a specific hydration state. It is worth noting that all four preparations of oleoyl phosphate that routinely drove ATP synthesis

Fig 5.8.Thin layer chromatograms of oleoyl phosphate preparations.

1. oleoyl phosphate. 2. oleoyl phosphate
3. oleic acid. 4. oleoyl phosphate

u.v. visualised spot. Iodine vapour visualised spot.

Faint spot or smear.



were oils at room temperature solidifying below 4° C, dissolved in methanol and dimethylformamide, and these solutions when added to aqueous buffer systems gave optically clear solutions up to 2 μmoles oleoyl phosphate/ml buffer.

Further evidence for the view that functional oleoyl phosphate preparations are rather different in properties to non-functional ones may be obtained from their activity in ATPase assay systems. Both Johnson and Criddle (241) and Hyams et al. (246) report that oleoyl phosphates capable of driving ATP synthesis are fairly potent inhibitors of ATPase activity. Groups (b) and (c) oleoyl phosphates either have a slight stimulatory effect on ATPase activities or none (247).

Heavily loaded thin layer chromatograms of oleoyl phosphate (Fig. 5.8) show that a number of different species of oleoyl phosphate may be present although this must be a tentative conclusion as oleoyl phosphate decomposes on silica gel plates and the spots may be due to decomposition products. Only the spot, A, contains hydrolysable phosphate.

Because of the difficutly in observing oleoyl phosphate-driven ATP synthesis, the results presented in this chapter must be discussed in a manner which tries to define their 'true nature'. That is, is the observed ATP synthesis real or an artefact of the assay techniques used and, if real, is ATP synthesis catalysed by ATP synthase (ATPase) enzymes?

ATP synthesis was measured routinely in a glucose-hexokinase trap system (248). The buffer system used was 250 mM sucrose; 20 mM Tris-Cl, pH 7.3; 20 mM glucose; 5 mM $MgCl_2$; 0.5 mM EDTA; 0.2 mM ADP containing 5-30 units of hexokinase (Sigma types F-300 or C-300). This high level of hexokinase was used to minimise any possible ATP hydrolysis by the ATPase enzymes. The assay,usually containing 0.5-1.0 mg ATPase enzyme, was incubated in a shaking water bath at 30° C during the experiment, and was terminated by the addition of perchloric acid. After centrifugation to remove coagulated protein, addition of potassium hydroxide

to neutralise the perchloric acid and centrifugation to remove potassium perchlorate; aliquots were taken into a buffer containing 1-2 units of glucose-6-phosphate dehydrogenase and 0.5 µmoles NADP. Any ATP formed in the assay will be used to convert glucose to glucose-6-phosphate by hexokinase. This glucose-6-phosphate is then converted by glucose-6-phosphate dehydrogenase to D glucono-δ-lactone-6-phosphate with the concomitant reduction of NADP to NADPH. This NADPH can be assayed spectrophotometrically at 340 nm.

One obvious source of 'apparent' (i.e. non-real) ATP synthesis in this system would be a reaction in which oleoyl phosphate donates its phosphate directly to glucose, giving glucose-6-phosphate. This, however, is unlikely. Oleoyl phosphate has no non-enzymic effect when incubated in a glucose hexokinase trap system lacking an ATPase enzyme. The possibility remains, however, that there is an enzyme or a non-enzymic catalyst (e.g. protein surface or metal ion) present in the enzyme preparations used in the assays which catalyses this reaction. It is rather more difficult to definitely exclude this possibility, but there is evidence to suggest that it does not occur. This is that the observed ATP synthesis is sensitive to two specific inhibitors of the ATPase enzyme, efrapeptin and aurovertin. Also ATP synthesis has been observed with a number of ATPase preparations of varying degrees of purity from different tissue sources. The artefactual catalyst would have to copurify with the ATPases and be sensitive to the same (apparently specific) inhibitors of the ATPase. This is unlikely. The oleoyl phosphate-driven synthesis of 'ATP' (real or apparent) also requires ADP, which is strongly suggestive that ATP is being made. The sensitivity of the reaction to efrapeptin and aurovertin coupled with the substrate specificity of the reaction (other acyl phosphates do not appear to drive ATP synthesis) militates gainst the reaction being catalysed by a non-enzymic catalyst.

Other possible sources of 'non-real ATP synthesis' would be the production of substances which also absorb at 340 nm, thus appearing to be NADPH, or the production of substances which glucose-6-phosphate dehydrogenase (G6PDH) either converts to compounds absorbing at 340 nm or uses to produce NADPH. The first possibility is easily dismissed as control experiments show no production of substances absorbing at 340 nm prior to the addition of G6PDH and NADPH to the assay. The second possibility is also unlikely as G6PDH has a fairly strict specificity for glucose-6-phosphate. Also, control experiments show the 'ATP synthesis' requires an ATPase and ADP.

Another source of spurious 'ATP synthesis' could have been the production of turbidity which would cause light scattering, giving an absorption at 340 nm. Again this turbidity would require the presence of an ATPase, ADP, glucose-6-phosphate, hexokinase NADP and would be abolished or prevented by efrapeptin, aurovertin and DCCD. It should be pointed out at this point that the conditions of the assay routinely lead to measuring Δ $OD_{340's}$ of 0.2 - 0.4 OD units above the value of no substrate and no enzyme blanks (usually 0 - 0.15 OD units) and occasionally Δ $OD_{340's}$ of $\sim$ 1.0 OD units above the blank values, so small OD changes scaled up by large dilution factors can be excluded as a major error ($\pm$ 10% maximally) in estimating ATP made.

Is the observed ATP synthesis due to the action of the ATP synthase enzyme or is it due to other side reactions? Mitochondria and many commercially obtainable enzymes (e.g. hexokinase and gluocse-6-phosphate dehydrogenase) contain a (contaminating) 'myokinase' activity. This enzyme, or enzymic activity, as it is far from clear whether all 'myokinase' activity is due to a single enzyme, catalyses the dismutation of ADP to ATP and AMP, viz.

$$ADP + ADP \xrightleftharpoons[Mg^{2+}]{\text{myokinase}} ATP + AMP$$

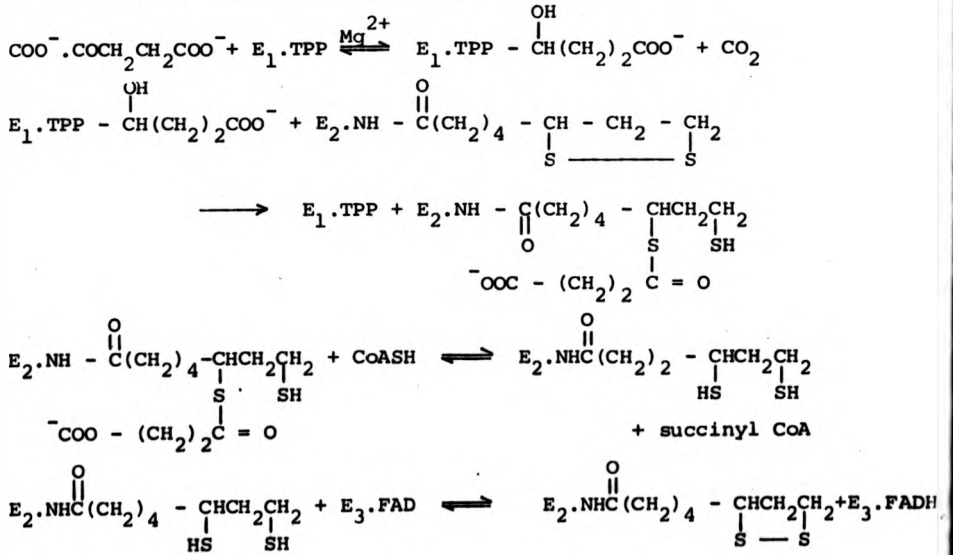
Thus, if mitochondria are incubated in a glucose hexokinase trap system containing ADP, but no other substrates, ATP synthesis due to myokinase activity can be observed. The first experiments carried out in which oleoyl phosphate-driven ATP synthesis was observed were done in a glucose-hexokinase trap system, 2 mM in ADP (Tables 5.3-6, Fig. 5.1). That is, submitochondrial particles were incubated with 2 μmoles of ADP. Myokinase activity could theoretically produce 2 μmoles ATP (measured as G-6-P equivalents) in this system. Apparent oleoyl phosphate-driven ATP synthesis could thus be due to myokinase or an oleoyl phosphate stimulated myokinase activity in this system. Certain considerations make this unlikely. All assays were measured against, or compared with, blanks containing substrate or enzyme, and hexokinase. The optical densities observed were substantially above these blank values (occasionally as much as 0.5 - 1.0 abs. 340 units). Synthesis was apparently sensitive to efrapeptin and DCCD and required the presence of oleoyl phosphate. Oleoyl phosphate may, however, specifically stimulate a mitochondrial myokinase activity. It is worth pointing out here that occasionally, in our hands, mitochondrial myokinase activity appeared to be partially inhibited by inhibitors of the ATPase enzyme such as oligomycin and efrapeptin. Thus it is very difficult to exclude the possibility that an oleoyl phosphate stimulated myokinase activity was responsible for the observed ATP synthesis. To remove or reduce this possible problem with contaminating myokinase activity, all subsequent glucose-hexokinase trap assay systems were made 0.2 mM in ADP. In this system, myokinase could maximally produce 0.2 μmoles in ATP (in fact levels of myokinase activity under the assay conditions used usually gave $\sim$ 0.1 moles ATP as a maximum). Oleoyl phosphate-driven ATP synthesis was observed under these conditions at levels substantially above this theroetical maximum (Tables 5.7-10,

Fig. 5.2-7). This shows that oleoyl phosphate-driven ATP synthesis is not due to a stimulation or induction of myokinase-like activities in the assay systems. This possibility cannot, however, be discounted in the case of the results presented by Johnson and Criddle (241).

Johnson and Criddle reported an F_1 ATPase catalysed synthesis of ATP from oleoyl phosphate and ADP. Although the controls they report make the action of an oleoyl phosphate stimulated myokinase activity unlikely as the source of this ATP synthesis, the levels of ATP produced could have been produced by myokinase activity. They do show, however, that ATP produced under their assay conditions is real ATP (that is, they use a direct chromatographic technique to detect ^{14}C -ATP) and that this synthesis is sensitive to compound '1799'.

The 'oleoyl' cycle hypothesis has many features in common with substrate level phosphorylation, so it would be useful to describe an example of this in some detail.

The conversion of α-ketoglutarate to succinate in the tricarboxylic acid cycle proceeds <u>via</u> succinyl∿SCoA and involves the phosphorylation of GDP to GTP. The reactions proceed as follows:-



where E_1 is α-ketoglutarate decarboxylase

E_2 is dihydrolipoate transsuccinylase

E_3 is dihydrolipoate(lipoamide)dehydrogenase.

In mammalian systems, succinyl thiokinase then catalyses.

$$\text{Succinyl}\sim\text{SCoA} + \text{MgGDP} + \text{Pi} \rightleftharpoons \text{succinate} + \text{MgGTP} + \text{CoASH}$$

The phosphate esterified in GTP can then be incorporated into ATP by the action of a nucleoside diphosphate kinase enzyme, viz.

$$\text{GTP(NTP)} + \text{ADP} \overset{Mg^{2+}}{\rightleftharpoons} \text{GDP(NDP)} + \text{ATP}$$

The situation is somewhat different in E. coli and higher plants; here succinyl thiokinase catalyses the following reactions:-

(i) $\text{Enz} + \text{succinyl}\sim\text{SCoA} + \text{Pi} \rightleftharpoons \text{Enz.succinyl}\sim\text{P} + \text{CoASH}$

(ii) $\text{Enz.succinyl}\sim\text{P} + \text{Pi} \rightleftharpoons \text{Enz}\sim\text{P} + \text{succinate}$

(iii) $\text{Enz}\sim\text{P} + \text{ADP} \rightleftharpoons \text{Enz} + \text{ATP}$

An unstable Enz∿CoA intermediate may also be formed in step 1. The enzyme catalysing these reactions has been isolated and purified. It has a molecular weight of 141,000 and two N-3 histidine residues are involved in its phosphorylation (249-253). A similar enzyme may be operational in mammalian systems, probably involving phospho-histidine residues as well (254).

The 'oleoyl' cycle postulated as a hypothetical framework for experimentation by Griffiths (235) requires the production of oleoyl lipoate, free or enzyme-bound, by the respiratory chain, analogous to the production of enzyme-bound succinyl lipoate. This is then acted on by CoASH or a similar acyl carrier protein-like grouping to produce an oleoyl∿S-CoA (or oleoyl∿S-ACP) moiety. This is then acted on by Pi to produce oleoyl phosphate which in turn donates its phosphate to ADP, giving ATP. All these reactions would be catalysed by the oligomycin sensitive ATP synthase (ATPase) complex of the inner membrane. The multisubunit enzyme would then catalyse a number of partial reactions. The terminal 'partial' reaction would be

an oleoylphosphokinase reaction in which oleoyl phosphate would act as the phosphate donor to ADP, converting it to ATP.

Oleoyl phosphate-driven ATP synthesis was observed in various ATPase preparations, ETP_H, 'complex V','proton translocating' ATPase and chloroform released F_1 ATPase, all from bovine heart mitochondria. This ATP synthesis was insensitive to various inhibitors of the ATPase enzyme, notably DBCT, TET and oligomycin. These inhibitors all act at the level of the F_O, membrane bound portions of the ATPase and are thought in chemiosmotic terms either to prevent (or block) proton pumping through the F_O portion of the ATPase, thus preventing the collapse of $\Delta\tilde{\mu}H$ and the concomitant synthesis of ATP (62) or, in terms of the chemical or conformational hypotheses,to prevent the conversion of a non-phosphorylated high energy state (X∿I or '∿') to a high energy phosphorylated state, X∿P or '∿P' (62). They would, therefore, not be expected to inhibit a reaction in which a putative X∿P or X∿P analogue drives the synthesis of ATP.

The inhibitors of ATP synthesis,efrapeptin and aurovertin, are thought to act at the level of the phosphorylated high energy state or intermediate, that is, they prevent the transphosphorylation between '∿P' and ADP giving ATP. Oleoyl phosphate-driven ATP synthesis is sensitive to these inhibitors and is also sensitive to DCCD in the chloroform released F_1 ATPase. Here DCCD also prevents ATPase activity (236) in a mechanism dissimilar to its inhibitory action in mitochondria, submitochondrial particles or purified F_1F_O ATPase preparations. Uncouplers, which discharge the high energy non-phosphorylated state of the membrane, have been reported to have varying effects on oleoyl phosphate-driven ATP synthesis (241, 246). Most (for example) FCCP, TTFB, 1799 have no effect on ATP synthesis in these systems, whereas 2,4-dinitrophenol strongly inhibits it. Compound '1799' was also reported to inhibit the synthesis in chloroform

released enzymes but not in vesicular preparations (241,246). These findings are generally confirmed here, though it must be pointed out that rather high levels of '1799' (1 mg/mg enzyme) were used to inhibit oleoyl phosphate-driven ATP synthesis in chloroform released enzymes.

Certain fatty acids have been shown to strongly inhibit dihydrolipoate-driven ATP synthesis in isolated F_1F_O ATPase preparations (see Chapter 3). Among the most interesting of these fatty acids were elaidic acid and erucic acid. Elaidic acid is the trans isomer of oleic acid, i.e. trans Δ 9.octadecenoic acid, and appeared to inhibit strongly reactions which involved oleic acid as a cofactor. Trans unsaturated C_{18} fatty acids, elaidate among them, have been reported to be ineffective in supporting the growth of unsaturated fatty acid auxotroph of Saccharomyces cerevisiae KD.115 (255) and to inhibit the growth of this strain supplied with oleate (256). Trans C_{18} fatty acids will also not support the growth of Escherichia coli mutants that could not synthesise fatty acids (256).

Rape seed oil, which can be as much as 45% erucic acid, has been observed to have severe cardiopathogenic effects when fed to laboratory animals (259). Fatty infiltration of rat cardiac muscle observed after short periods of higherucic acid rape seed oil feeding has been related to a decreased efficiency of mitochondrial ATP synthesis (257,258). Erucic acid is also almost completely unable to support the growth of the Saccharomyces cerevisiae fatty acid auxotroph KD.115 (255).

Work by Haslam et al. (260) has suggested that the inhibition of energy linked reactions produced by depletion of fatty acids from the mitochondrial membrane is not due to an effect on electron transport but is probably due to an effect on the respiratory control functions of these membranes, that is, an effect on the terminal steps involved

in oxidative phosphorylation. The results presented here on the inhibition of oleoyl phosphate-driven ATP synthesis and ATP-^{32}Pi exchange in chloroform released enzymes by elaidate, erucate and various derivatives of these fatty acids suggests a possible mechanism for the toxic effects of these (and other) fatty acids on oxidative phosphorylation; that is, they might prevent the terminal phosphate transfer of oxidative phosphorylation. The fact that the free fatty acids are less effective inhibitors than the fatty acyl lipoates,which in turn are less effective than the fatty acyl phosphates in inhibiting oleoyl phosphokinase and oleoyl phosphate stimulated ATP-^{32}Pi exchange , suggests that these compounds might act by competing for the same site as oleoyl phosphate, thus preventing or reducing the transfer of phosphate from oleoyl phosphate to ADP. Oleoyl phosphate was the only acyl phosphate tested that could support ATP synthesis; elaidoyl, erucoyl,palmitoyl would not.

Oxidative phosphorylation in bovine heart mitochondria,driven by respiratory chain substrates such as pyruvate/malate and succinate, is inhibited by oleoyl, elaidoyl and ercoyl phosphates. The mechanism is unclear, but a number of possibilities exist. The first is that if oleoyl phosphate is a true intermediate in ATP synthesis or a close analogue of an enzyme-bound acyl phosphate, it would only be present in the membrane in very low levels (repeated searches have failed to find a phosphorylated intermediate (261)). High levels of the intermediate might then cause a feedback inhibition of oxidative phosphorylation. Fatty acyl phosphates have a detergent-like structure, that is, they contain discrete hydrophobic and hydrophilic regions and may cause a disruption of the mitochondrial membrane, much as do other detergents such as Triton X-100, potassium cholate and potassium deoxycholate, thus preventing ATP synthesis. Fatty acids are known to uncouple oxidative phosphorylation in a manner that is assumed to be

related to their ability to collapse $\Delta\tilde{\mu}H$ across membranes (261). Fatty acyl phosphate could possibly act in a similar way.

As pointed out previously in this discussion, oleoyl phosphate-driven ATP synthesis is a reaction that is poorly reproducible. The reasons for this are not clear, but some of the data presented here may suggest why. Oleoyl phosphate preparations that drive ATP synthesis do not do so in all ATPase preparations, be they ETP_H or $CHCl_3$ released F_1 ATPases, even when these enzymes appear to exhibit their normal properties (oxidative phosphorylation and ATPase). This suggests that something is either periodically present or missing in the assay system or that the enzymes need to be in a particular state to catalyse oleoyl phosphate-driven reactions. The 'residual' oleoyl phosphatase activity displayed by certain enzymes with group (b) oleoyl phosphates may give us an indication about this 'state'. This reaction is often non-existent unless ADP is present which appears to induce the activity up to levels of 70-80 nmoles/min/mg. ADP might act by allowing the enzyme to adopt a configuration in which it can catalyse oleoyl phosphatase reactions. Various attempts have been made to stimulate oleoyl phosphate-driven ATP synthesis in those enzymes that normally display none. These include preincubation of the enzyme with oleoyl-S-CoA, coenzyme A, dihydrolipoate, oxidised lipoic acid, oleic acid and various metal ions such as Sn^{2+}, Ni^{2+}, Cr^{3+}, Cu^{2+}, Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}. None of these compounds induced oleoyl phospatase or ATP synthesis. The nature of a putative 'missing cofactor' then remains obscure. There is a possibility that there is normally in the assay system a component which inhibits oleoyl phosphate-driven reactions. All the chemicals used in the assay system were of the highest purity available, 'AristaR' grade where possible. The other main source for an inhibitory component in the assay is the substrate. As shown earlier, certain fatty acids and fatty acyl

derivatives are extremely potent inhibitors of oleoyl phosphate-driven ATP synthesis. Oleoyl phosphatepreparations could contain low levels of other fatty acids or fatty acyl phosphates which would inhibit these reactions. The level of contamination needed to inhibit these reactions would only need to be 1-10%. Thus 1 μmole oleoyl phosphate would only need to contain 10-100 nmoles fatty acyl-X to completely inhibit oleoyl phosphate-driven ATP synthesis (see Table 5.8). It may be that the group (a) oleoyl phosphates were fortuitously free from (or low in) these contaminants.

The evidence obtained with oleoyl phosphate suggests that an acyl phosphate (or similar enzyme-bound phosphate) may be involved in the terminal stages of ATP synthesis in oxidative phosphorylation. Oleoyl phosphate has appeared to give a stoicheiometric production of ATP in a reaction sensitive to several well known inhibitors of oxidative phosphorylation. Phosphate transfer from oleoyl phosphate to ATP could proceed by two pathways, one of which involved the formation of an enzyme phosphate intermediate, viz.

oleoyl phosphate + enzyme $\rightleftharpoons$ enzyme phosphate + oleoyl (carrier)

enzyme phosphate + ADP $\rightleftharpoons$ ATP + enzyme

The other possibility is that oleoyl phosphate may transfer its phosphate to ADP via a transition state; here an enzyme phosphate would have a transitory lifetime.

The involvement of an acyl phosphate intermediate in the terminal stages of oxidative phosphorylation could be incorporated into all the main hypothesis of oxidative phosphorylation without the sacrifice of their major principles. Because of the poor reproducibility of the reactions, however, much work needs to be done to confirm and define the role (if any) of acyl phosphates in oxidative phosphorylation.

CHAPTER 6

THE EFFECT OF DIHYDROLIPOIC ACID ON NICOTINAMIDE NUCLEOTIDE TRANSHYDROGENATION

Introduction

The nicotinamide nucleotide transhydrogenases (EC 1.6.1.1) are a group of enzymes that catalyse a direct and reversible transfer of hydrogen between NADH and $NADP^+$,

i.e. $NADH + NADP^+ \rightleftharpoons NAD^+ + NADPH$

The first example of this group was discovered by Colowick et al. in extracts from Pseudomonas fluorescens (263). The enzyme was later isolated from mammalian tissue extracts; the activity appeared to be associated with the mitochondrial inner membrane (264-4).

The first indication that this mitochondrial transhydrogenase activity was energy linked was found by Krebs (267) who found that NADPH dependent dehydrogenase reactions were sensitive to the uncoupler DNP. He suggested $NADP^+$ reduction was controlled by an ATP dependent flavoprotein. Analysis of the steady state reduction levels of NAD^+ and $NADP^+$ in mitochondria revealed that the $NADP^+$ pool was usually more reduced than the NAD^+ pool. This led Klingenberg and Slenczka to propose that mitochondria contain an asymmetric energy linked transhydrogenase controlled by oxidative energy (268). Much support was later forthcoming for this proposal (269). Danielson and Ernster demonstrated that in submitochondrial particles prepared by sonic disruption of mitochondria, ATP or respiration will provide energy which stimulates the reduction of $NADP^+$ by NADH (270). This energy input causes a shift in the equilibrium of the reaction $NADH + NADP^+ \rightleftharpoons NADPH + NAD^+$ from 1 to 500 in favour of NAD^+ and NADPH production (271). Later Lee et al demonstrated that the hydrogen transfer between NADH and NADPH is stereospecific for the 4A hydrogen of NADH and the 4B hydrogen of NADPH (272).

Respiring bacteria, such as Escherichia coli, and some photosynthetic bacteria, e.g. Rhodospirillum rubrum also possess transhydrogenases similar to the mitochondrial enzyme (269).

Mammalian submitochondrial particles prepared by sonic disruption of mitochondria carry out a rapid reduction of $NADP^+$ by NADH driven by ATP or respiration (270). Attempts to release this transhydrogenase activity from the inner membrane by detergent extraction cause an uncoupling of energy linked reactions probably due to disruption of the membrane, thus the released transhydrogenase only displays its non-energy linked activities, some of which can be restored if the enzyme is reincorporated into phospholipid vesicles. Kaplan et al. (264) used digitonin extraction of mitochondria followed by calcium phosphate gel adsorption chromatography to produce a transhydrogenase preparation 10 x purified with respect to digitionin particles. Further purification failed as the enzyme was very sensitive to delipidating agents such as acetone and bile salts. Other workers (273-4) produced preparations of transhydrogenase 14 x and 25 x purified with respect to digitonin particles, the latter by repeated sucrose density gradient centrifugation of digitonin particles. Again, further purification was prevented by the sensitivity of the preparation to inhibition by organic solvents, bile salts and phospholipases.

Rydstrom and co-workers (275) solubilised transhydrogenase selectivity from bovine heart submitochondrial particles by lysophosphotidyl choline. The preparation was 7 x purified and, unlike previous preparations of transhydrogenase, was largely free from cytochromes (except cytochrome c). Differential centrifugation of this enzyme produces a further purification to 12 x. The method was only useful for the preparation of small quantities of the enzyme. Cholate and ammonium sulphate fractionation of bovine heart SMP's followed by sucrose density gradient centrifugation in the presence of cholate and

phosphatidyl choline gives a 7 x purified transhydrogenase uncontaminated by cytochromes and ATPase activity (276). Both these last two preparations were reconstitutively active, that is, they are capable of catalysing some energy linked reactions when incorporated into liposomes (269).

Recently two methods producing enzymes purified to a high degree of homogeneity have been reported, those of Hojeberg and Rydstrom (277) and Anderson and Fisher (286).

Hojeberg and Rydstrom extended the method of Rydstrom et al. (276) by subjecting an ammonium sulphate fraction from this preparation to chromatography on a DEAE sepharose CL-6B column eluting with buffers containing triton X 100. Eluate fractions from this column were pooled and concentrated, and then subjected to hydroxylapatite column chromatography. Transhydrogenase activity was eluted from the column with a 100 mM potassium phosphate buffer. This final purification step gives an enzyme with a specific activity 40 x that of the original SMP preparation.

Table 6.1 Purification of bovine heart mitochondrial transhydrogenase (after 277)

	specific activity[1]	purification x fold
SMP	0.11	1.0
38-43p fraction	0.25	2.3
DEAE-sepharose pool	0.90	8.2
hydroxylapatite pool	4.40	40.0

[1] µmoles NADH formed/min/mg

The final preparation is devoid of respiratory chain activities such as NADH dehydrogenase and succinate dehydrogenase, ATPase and cytochromes. The absorption spectrum of the enzyme indicates that it is not a flavoprotein. Direct assay of flavin content indicates that the

preparation contains less than 0.1 mole flavin per mole enzyme. SDS PAGE indicates that the preparation is almost completely homogeneous with respect to a single 97,000 dalton component.

This purified transhydrogenase protein, when incorporated into lipid vesicles, catalyses the NAD^+, NADPH dependent uncoupler sensitive uptake of tetraphenylboron and also displays a rate of NAD^+ reduction by NADPH that can be stimulated 13 x by uncouplers or valinomycin and nigericin.

Anderson and Fisher have also purified bovine heart mitochondrial transhydrogenase to a high degree of homogeneity (286). Their method involves the treatment of SMP's with a chaotropic agent, sodium chlorate. This removes 20% of the NADH dehydrogenase from the inner membrane and leaves the remainder insoluble. The submitochondrial pellet is then treated with lysolecithin (final concentration 0.1%) which releases transhydrogenase activity. This is then removed from the insoluble SMP fraction by centrifugation. The transhydrogenase is then titrated with alumina gel (1 mg gel/mg protein) and eluted with a series of sodium phosphate buffers of varying concentrations, all 10 mM in mercaptoethanol and 0.05% in lubrol WX. The fractions containing transhydrogenase activity are then eluted and titrated with calcium phosphate gel (2 mg gel/mg protein). Activity is eluted from the gel with buffers similar to those used in the alumina gel system. The eluate from this is then passed down an NAD^+-sepharose affinity chromatography column twice. On its first passage, transhydrogenase activity is eluted without any binding to the affinity matrix, but is purified from several other proteins. On its second passage, transhydrogenase activity is immobilised on the column; this is eluted with a buffer system containing NADH. The eluate is then passed through a sephadex G-50 column. The eluate from this is the final purified 'transhydrogenase' fraction.

The purified product is fairly insoluble, losing 30-50% of its 3-acetylpyridine adenine dinucleotide reductase activity in 48 hours

when kept at 4° C. Freezing destroys its activity, even if done in the presence of substrates. The fluorescence spectum of the enzyme reveals that it contains tryptophan and no flavin. The enzyme is free of containinating cytochromes, NADPH dehydrogenase, NADH dehydrogenase, NADPH-ferricyanide reductase and NADPH-DCPIP reductase activities. Palmitoyl coenzyme A, Mg^{2+} and pH all have similar effects on the purified transhydrogenase as they do on SMP transhydrogenase activity. SDS PAGE reveals that the transhydrogenase preparation consists of essentially one protein of ∿ 120,000 molecular weight. Recent work from Fisher's laboratory has, however, suggested that this 120,000 M.W. protein may be composed of two subunits, one of which is ∿ 54,000 M.W. (304).

The relationship between transhydrogenase and the mitochondrial NAD(H) and NADP(H) dehydrogenases has been investigated, as both these types of activities are located in the same membrane and both sets of nucleotide binding sites are located on the same (M) side of the inner mitochondrial membrane. Little is known about the structure and topology of the transhydrogenase molecule and the only known link between the two systems is the functional one, that is the requirement for NAD(P)(H). Ubiquinone 9 has been reported to be a component of the transhydrogenase system on the basis of solvent extraction and reconstitution experiments. The effect of UQ_9 on this system is not defined, but is unlikely to be a 'general lipid activation' effect as the system requires UQ alone. Whether UQ_9 functions as a cofactor or has a more specific lipid activation effect is not clear (305-6).

A structure-function relationship between transhydrogenase and NADH dehydrogenase is possible (for the reasons outlined above) but is unlikely as the NAD(H) binding sites of the enzymes differ in their stereospecificity with respect to the 4H of NADH; the enzymes also differ in their sensitivity to various inhibitors. Hatefi and Hanstein (307)

and Ragan et al. (285, 157) have evidence showing that part of mtiochondrial transhydrogenase activity is recovered with complex I (NADH-UQ reductase) upon the detergent fractionation of the mitochondrial inner membrane. Activity is not recovered with the other complexes (307). Ragan has also shown that this activity can be removed from complex I by lysophosphatidyl choline treatment, suggesting that it is not an integral part of complex I activity. Rossi et al have reported that purified NADH dehydrogenase oxidises NADPH at a low rate (308), while Hatefi has shown that NADPH is oxidised under aerobic conditions in the absence of NAD^+ by the mitochondrial respiratory chain (307, 309) and proposed that this occurs via a separate pathway from that of NADH oxidation, NADPH entering on the oxygen side of FMN and the Fe-S centre 1 of NADH dehydrogenase, since NADPH only reduces Fe-S centres 2, 3, 4. NADPH oxidation exhibits a pH dependence similar to that of non-energy linked transhydrogenase activity. Hatefi proposed that these reactions were a reflections of transhydrogenase activity. This, however, is unlikely as none of these reactions are inhibited by palmitoyl CoA or trypsin. It was later shown that NADPH does reduce centre 1, but at a much lower rate than NADH (310). Thus there is little direct evidence to suggest structural or functional links between transhydrogenase and the nicotinamide nucleotide dehydrogenase.

Mitochondrial transhydrogenase is inhibited by a number of compounds. These can be divided into three main groups:-

(i) non-specific inhibitors. These include various SH reagents (154, 273, 278);

(ii) site directed inhibitors. These are compounds that appear to exhibit their inhibitory properties by competing for the $NAD^+(H)$ or $NADP^+(H)$ sites on the enzyme.

Table 6.2 Site specific inhibitors of mitochondrial transhydrogenase (after 269)

Inhibitor	Specificity	Ki μM	
		non-energy linked	energy-linked
Adenosine	NAD(H)	500	500
5'-AMP	NAD(H)	300	700
ADP	NAD(H)	300	400
Dephospho CoA	NAD(H)	9	40
Acetyl dephospho CoA	NAD(H)	11	40
2'-AMP	NADP(H)	700	1200
3'-AMP	NADP(H)	700	1200
CoA	NADP(H)	200	700
Acetyl CoA	NADP(H)	200	700
Palmitoyl CoA	NADP(H)	0.15	0.15
3':5'-AMP	NADP(H)	400	500

(iii) Energy transfer inhibitors: energy transfer inhibitors inhibit the energy linked transhydrogenase reactions by their ability to inhibit the generation of '∿' by the respiratory chain or the ATPase. These include oligmycin, trialkyltin halides, venturicidin, aurovertin, efrapeptin, DCCD and DBCT which inhibit the ATP-driven energy linked transhydrogenase. The uncouplers (e.g. FCCP, CCCP, '1799', TTFB, S13, DNP etc.) and the ionophores valinomycin + nigericin and gramicidin D, which dissipate the high energy state of the membrane, thus inhibiting all energy linked transhydrogenase reactions. Cyanide, antimycin A and other inhibitors of electron transport inhibit respiration-driven transhydrogenase but have no effect on the ATP-driven transhydrogenase activity.

There are several other compounds which inhibit transhydrogenase reactions. These include the anthraquinone derivative rhein, which was

thought to be a specific transhydrogenase inhibitor but was later shown to inhibit several other NAD^+ dependent reactions (281). Transhydrogenase activity is also inhibited by the sulphydryl reagent, 5,5'-dithiobis-(2-nitrobenzoic acid) (286).

Trypsin inhibits the enzyme irreversibly, suggesting that the transhydrogenase may contain an essential arginine residue (282). Confirmation of this suggestion came when Djavadi-Ohaniance and Hatefi found the arginine binding reagent, butanedione, selectivity inhibited the transhydrogenase of bovine heart mitochondria. NAD^+ and/or $NADP^+$ prevented this inhibition, suggesting an arginine residue might be involved at one of the binding sites (283).

Transhydrogenase activity is highly sensitive to delipidating agents such as detergents, organic solvents and phospholipases. The phospholipases have different effects, phospholipase A being more effective than phospholipase C, which is more effective than phospholipase D at inducing inhibition of the transhydrogenase (284).

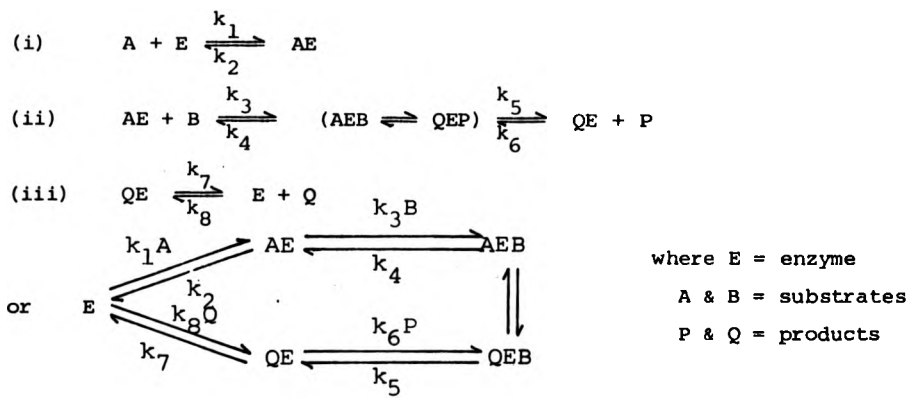
Rydstrom et al. (284) demonstrated that non-energy linked activity can be restored to an inactive delipidated lysophosphatidylcholine preparation of transhydrogenase by addition of phospholipids. Neutral lipids will not restore the activity. Cardiolipin was the most effective in restoring activity but inhibited it at high concentrations. Less effective, but still active, were phosphatidylcholine, phosphatidylethanolamine and lysolecithin. It is unlikely that this stimulation is due to a simple dispersion of the enzyme by phospholipids as detergents do not have a similar effect. It is possible that phospholipids act in stabilising an active configuration of the enzyme by occupying hydrophobic surfaces on the molecule. Ragan and Widger (285) have reported that a 4:1 phosphatidyl ethanolamine / phosphatidylcholine mixture was the most effective in reconstituting an energy linked transhydrogenase, but other workers have found that an energy generating

transhydrogenase (i.e. a reversal of the energy linked reaction generating ATP) functioned optimally with pure phosphotidylcholine as added phosphlipid (276), suggesting the 4:1 phosphatidyl ethanolamine, phosphatidyl choline system of Ragan and Widger probably reflects the phosphlipid dependence of the ATPase rather than the transhydrogenase (ATP-^{32}Pi exchange is optimally stimulated by the same mixture).

Kinetic properties of the transhydrogenase reaction

Steady state kinetic data indicate that the transhydrogenase reaction proceeds by way of a very short lifetime ternary complex, possibly by a 'Theorell-Chance' type mechanism (287-8).

Figure 6.1 'Theorell-Chance' type reaction mechanism



The Theorell-Chance mechanism is a special case of this general substrate mechanism where $k_5 >> k_7$, $k_4 >> k_2$, $(k_5 + k_7) >> k_6$ and $k_2 + k_4 >> k_3$. Under these conditions, the steady state concentrations of the ternary complexes are very low (transitory) and the first product appears to be formed from B by interaction with the A-E complex.

Kinetic analysis reveals competitive relationships between the oxidised and reduced forms of the nucleotides and non-competitive relationships between NAD^+ and $NADP^+$, and NADH and NADPH. Product inhibition patterns indicate that the transhydrogenase has separate sites for

$NAD^+(H)$ and $NADP^+(H)$. Studies with site specific inhibitors (see Table 6.2) indicate that NAD(H) binds before NADP(H) (289).

This mechanism is consistent with the observation of Fisher and Kaplan (290) that cleavage of the C-H bonds of the reduced nucleotides is not rate limiting.

At neutral pH the maximum initial velocities of NADPH and NADH formation differ by a factor of $\sim$ 5, the former being the slower. The reduction of $NADP^+$ by NADH is maximal at about pH 5.5, whereas NAD^+ reduction by NADPH is optimal at pH 7.0.When the reduction of $NADP^+$ by NADH reaches equilibrium, the rate constant of the reaction is increased, indicating that the activity of the transhydrogenase is related to the accumulation of NAD^+ and NADPH. Addition of energy to the system, from respiration or ATP hydrolysis, leads to a 5-10 x increase in the maximal initial velocities of $NADP^+$ reduction by NADH (272, 279, 288).

Table 6.3 Kinetic constants for the mitochondrial transhydrogenase reactions (adapted from 269)

Constant	Non-energy linked	Energy-linked
K_{NADH} (μM)	9.0	12.6
K_{NADP^+} (μM)	40.0	6.5
K_{NAD^+} (μM)	28.0	43.5
K_{NADH} (μM)	20.0	20.0
k_1 ($\mu M^{-1} min^{-1}$)	1.4	5.3
k_2 (min^{-1})	10.8	$\approx$ 0
k_3 ($\mu M^{-1} min^{-1}$)	0.3	10.2
k_4 ($\mu M^{-1} min^{-1}$)	3.0	2.1
k_5 (min^{-1})	20.6	166.0
k_6 ($\mu M^{-1} min^{-1}$)	2.1	1.0

where K's are the respective Michaelis constants and k_1, k_2, k_3, k_4, k_5, k_6 are rate constants for binding of NADH, release of NADH, binding of $NADP^+$, binding of NADPH, release of NAD^+ and binding of NAD^+ respectively.

The energy linked transhydrogenases are linked to the energy transfer systems in the membrane in which they are located. Mammalian and respiring bacterial transhydrogenases may be driven from any of their energy coupling sites, for example, in rat liver or beef heart mitochondria, energy linked activity can be stimulated by NADH, succinate, reduced cytochrome c or hydrolysis of ATP (272, 291). Similarly, energy linked transhydrogenase can be driven by ATP or respiration in the respiratory bacteria (292-3). In the photosynthetic bacteria, e.g. Rhodospirillum rubrum, energy linked transhydrogenation can be driven by light-induced electron transport, hydrolysis of ATP and GTP and the hydrolysis of inorganic pyrophosphate. In both bacterial and mammalian systems, half maximal stimulation of transhydrogenase by energy is attained at considerably lower energy levels than those required to give a half maximal rate of oxidative or light-driven phosphorylation.

The energy expenditure of the energy linked transhydrogenase reaction in mitochondria and SMP's has been estimated to be one high energy bond, or equivalent ('∿') per NADPH formed. This stoicheiometry was estimated in the initial phase of the reaction, that is, under non-equilibrium conditions (291, 294-5). The idea that energy expenditure is stoicheiometric with the net formation of NADP implies that the reduction of NAD^+ by NADPH should generate energy. This was shown to be the case by the work of Skulachev and co-workers (296-7) and van de Stadt et al. (298). The overall energy linked reaction can thus be written:-

$$NADH + NADP^+ + ATP \rightleftharpoons NAD^+ + NADPH + ADP + Pi$$

$$\text{or} \quad NADH + NADP^+ + \text{'}\sim\text{'} \rightleftharpoons NAD^+ + NADPH$$

It was assumed for a long time that the energy linked transhydrogenase was essentially irreversible, that is, could not be used to generate '∿'. However, Skulachev and co-workers found that the reduction of NAD^+ by NADPH in SMP's was linked to an uptake of lipophilic anions

similar to that observed during the oxidation of respiratory chain substrates or the hydrolysis of ATP. This uptake was abolished by uncouplers and was assumed to reflect a high energy state of the SMP membrane. The amount of lipophilic anion uptake may be used as a measure of the energy generated by the transhydrogenase reaction. Van de Stadt et al. went on to show that if the NAD^+ and NADPH concentrations were kept high and constant with regenerating systems, and if SMP's were incubated with ADP + Pi in a glucose-hexokinase trap system, then ATP was formed. This ATP synthesis was abolished by uncouplers. These reactions convincingly demonstrated that transhydrogenase is capable of generating energy provided that the nicotinamide nucleotide potential is sufficiently high. This has led to the suggestion that the transhydrogenase functions as a fourth coupling site, site O (299).

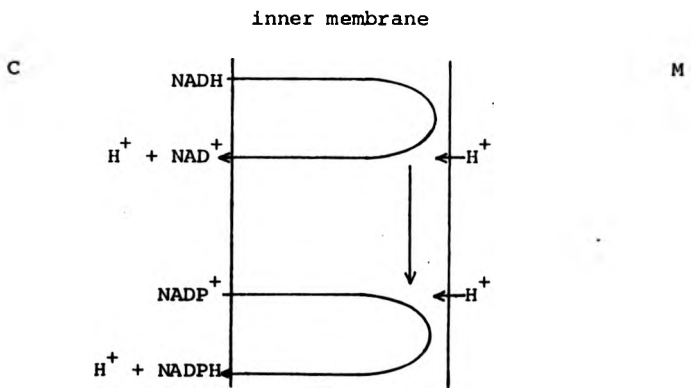
Mechanism of energy coupling

The mechanism by which energy is transferred from ATP hydrolysis or respiration to the transhydrogenase is unknown,as is the precise mechanism of energy coupling in general (see Chapter 2). Hypotheses have been proposed then, which come under the more general categories of the chemical, chemiosmotic and conformational hypothesis of energy coupling.

Early mechanisms of energy linked transhydrogenase involved either an alteration to or transport of one of the nicotinamide nucleotides involved. Danielson and Ernster (291) and Lee and Ernster (295) suggested that NADH (or $NADP^+$) may react as an NADH~ (or $NADP^+$~) form which is de-energised after the H transfer steps: here the reverse energy linked reaction was regarded as uncoupled. Klingenberg proposed a directional transhydrogenase where NAD^+ was constantly removed by an active ion transport mechanism (269). Papa et al. proposed a rather similar mechanism in which NADPH was removed from the transhydrogenase site in an energy linked manner (300).

Mitchell and Moyle proposed that the transhydrogenase acted in an electrogenic manner across the SMP membrane. Addition of NADH plus $NADP^+$, causing an acidification of the medium, and addition of NAD^+ plus NADPH, an alkalinization of the medium (301). They therefore proposed that the transhydrogenase acts as a proton pump carrying protonated species across the inner mitochondrial membrane (299). Later evidence suggested that the tranhydrogenase proton pumping driven by NAD^+ and NADPH exhibited an $H^+/2e^-$ ratio of close to 2, in agreement with the postulated chemiosmotic mechanism.

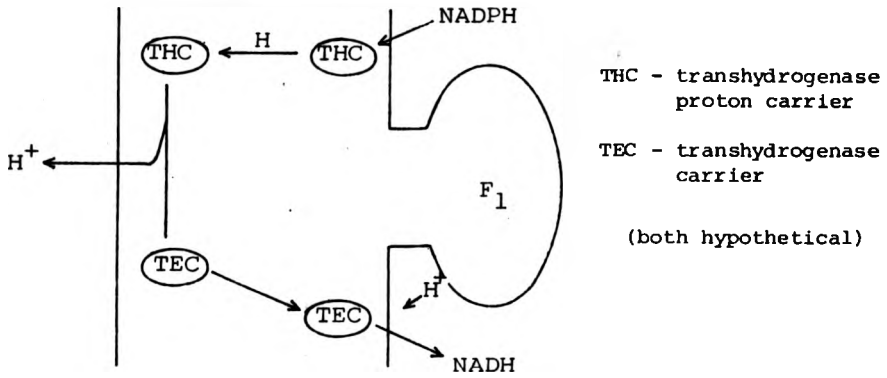
Fig. 6.2 Hypothetical chemiosmotic transhydrogenase mechanism (after 299)



A rather similar but subtly different mechanism has been proposed by Skulachev et al. (296-7), based on the distribution of lipophilic anions transported across submitochondrial particle membranes in response to transhydrogenase reactions. Here the transhydrogenase is in close relationship with the ATPase molecule and acts as a fourth chemiosmotic loop, loop O.

Both Mitchell's and Skulachev's hypotheses are unlikely as even in their protonated forms NAD^+ and $NADP^+$ are insoluble in the mitochondrial inner membrane.

Fig. 6.3 Transhydrogenase as coupling loop 'O' (after 297)



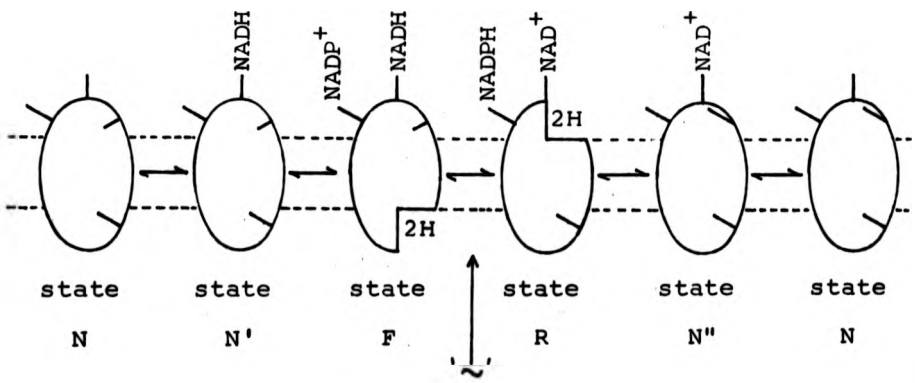
Several mechanisms based on conformational hypotheses have been proposed. As with most conformational proposals, these suffer from being rather vague about the 'excited' state of the transhydrogenase molecule. Rydstrom et al. (288, 302-3) proposed that energy, possibly derived from ΔpH, drove conformational changes in the transhydrogenase molecule, converting it from an inactive to an active form, viz.

$$\text{energy} + TH \longrightarrow TH^* \qquad TH = \text{transhydrogenase}$$

$$NADH + NADP^+ + TH^* \rightleftharpoons TH + NAD^+ + NADPH$$

This model was later expanded to a more complex form.

Fig. 6.4 Conformational model of transhydrogenase action



Conformational changes in the transhydrogenase molecule on addition of various nicotinamide nucleotides are well documented, but it is far from clear if these can be used to produce or convert redox energy in a form which can be used to drive energy linked transhydrogenation or whether they are merely a result of these energy linked processes.

The precise mechanism of the mitchondrial energy lined transhydrogenase is unclear and its elucidation will probably depend on a further understanding of the general mechanism of energy coupling.

The transhydrogenase reactions in some photosynthetic bacteria are rather interesting. Orlando (311) has reported a stimulation of the light-driven transhydrogenase of Rhodopseudomonas chromatophores by a soluble thiol containing protein factor and certain thiol and dithiol compounds. These also stimulate the ATP driven (dark) energy linked transhydrogenase. Of these compounds, only dihydrolipoic acid stimulated a dark energy linked transhydrogenase reaction in the absence of ATP. This reaction was as fast as the 'soluble protein factor' stimulated ATP-driven energy linked transhydrogenase. Similarly, Knoblock has reported that in crude cell free extracts from Rhodopseudomonas palustris, ATP can drive an energy linked $NADP^+$ transhydrogenation and reversed electron transport (from cytochrome c to NAD^+). ATP can be replaced by ADP or pyrophosphate as energy donor. Likewise, acetyl phosphate or acetyl CoA (+ Pi) can function as an energy source. Similarly, lipoic acid with thiamin pyrophosphate and pyruvate and/or acetate will function even more effectively in replacing ATP as the energy donating system (312). Oligomycin inhibited the ATP and acetylphosphate dependent reactions and arsenate inhibited the acetyl CoA and acetylphosphate driven reactions.

As dihydrolipoate apparently exhibited the ability to drive ATP synthesis in mitochondrial preparations, it was decided to see if it had

any effect on energy linked reactions such as transhydrogenation of nicotinamide nucleotides. The results of these investigations are presented in this chapter.

Methods

Mitochondria, submitochondrial particles, Escherichia coli vesicles and Rhodospirillum rubrum vesicles were all prepared as described in Chapter 3 or Chapter 5.

The ATP and respiration driven transhydrogenases were assayed as described by Sweetman and Griffiths (305) with slight modifications. Submitochondrial particles (0.5 - 2.0 mg) were suspended in 3 ml of a buffer, 0.25 M in sucrose; 50 mM in Tris-Cl, pH 8.0; 5 mM in $MgCl_2$. An NADH regenerating system, consisting of 40 nmoles NAD^+, 10 µl ethanol and 5-10 units alcohol dehydrogenase, was then added. The non-energy linked reaction was initiated by the addition of 0.5 µmoles $NADP^+$. The ATP-driven transhydrogenase activity was assayed in the presence of rotenone (1 µg) and antimycin A (1 µg) per mg submitochondrial particle protein or in the presence of rotenone (1 µg) and KCN, 1 mM. Assays were initiated by the addition of ATP and monitored at 340 nm in a Pye-Unicam SP1800 dual beam spectrophotometer connected to a Pye-Unicam chart recorder. Assays were monitored againt control cuvettes, containing all the above additions,with the exception of $NADP^+$ and ATP, at room temperature (18 - 20° C).

Respiration-driven transhydrogenase was monitored similarly except for the omission of antimycin A and cyanide. The non-energy linked reaction was initiated by the addition of 0.5 µmoles $NADP^+$. Energy linked activity was initiated by the addition of sodium succinate and monitored as described for ATP driven transhydrogenase.

Reverse electron transport activity, that is ATP-driven reduction of NAD^+ by succinate, was assayed essentially as described by Griffiths and Roberton (314). Submitochondrial particles (0.5 - 1 mg) were

incubated in a 3 ml buffer system containing 0.25 M sucrose; 5 mM $MgCl_2$; 50 mM Tris-Cl, pH 8.0; 1 mM NAD^+; 1 mM KCN or 1 µg/mg protein antimycin A; 5 mM succinate at 30° C. Assays were initiated by the addition of 2 µmoles ATP and monitored as described for transhyrdogenase activity.

Inhibitors were usually added as methanolic solutions (1 mg/ml). Dihydrolipoic acid solutions were prepared as described in Chapter 3. Oleoyl lipoate, elaidoyl lipoate and oleoyl phosphate were prepared as described in Chapters 3 and 5.

Materials

All chemicals used were of 'AnalaR' or equivalent grade. Organic solvents were redistilled prior to use. NAD^+, NADH, ATP and alcohol dehydrogenase were all obtained from the Boehringer Mannheim chemical corporation. Oligomycin, gramicidin D, valinomycin, quercetin and DCCD were obtained from Sigma Chemical Company. Efrapeptin and leucinostatin were kind gifts from Dr. R. Hammill, Eli Lilley and Co.

Results

Submitochondrial particles display non-energy linked and energy linked transhydrogenase activities. The energy linked activity can be stimulated by energy derived from respiration or from ATP hydrolysis (see Fig. 6.5). Previous work in this laboratory has suggested that lipoic acid or a derivative of it may be either involved in the mechanism of ATP synthesis or be able to supply energy to the mitochondrial coupling system in such a way that it can be used to drive energy linked reactions (see Chapter 3) (248, 235). It was therefore decided to investigate whether lipoic acid and derivatives had any effect on the transhydrogenase reactions. Dihydrolipoic acid has been reported to have the ability to reverse the DBCT induced inhibition of various energy linked reactions including ATP-driven transhydrogenase (315). Dihydrolipoic acid also appeared to have the ability under certain circumstances to reverse the inhibition of ATP-driven trans-

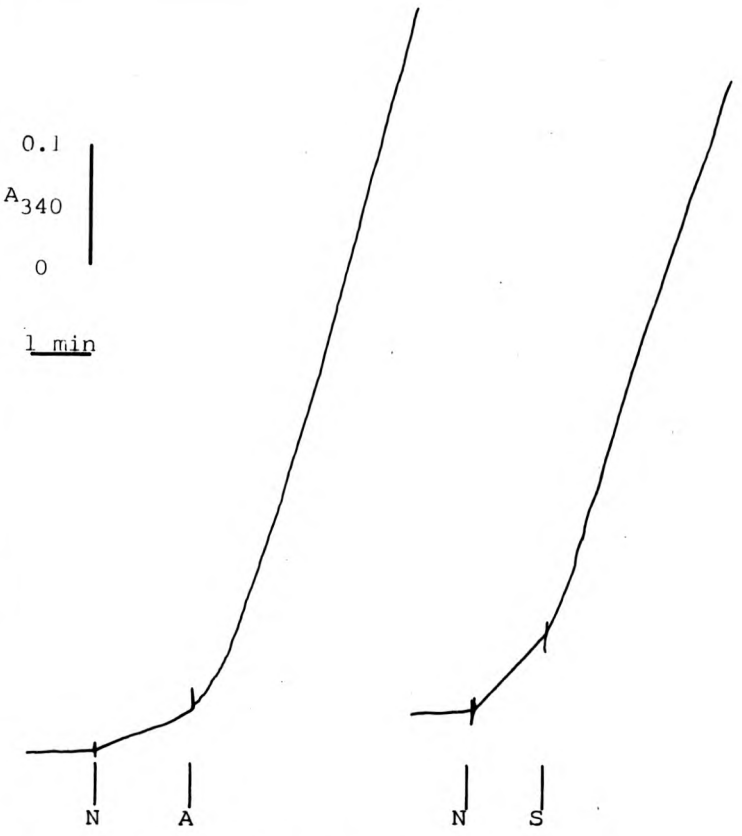


Fig 6.5. ATP driven and respiration driven transhydrogenase.

Assays were performed as described in the methods section to chapter 6.

N : 0.5 µmoles $NADP^+$. A: 5 µmoles ATP.
S : 10 µmoles sodium succinate.

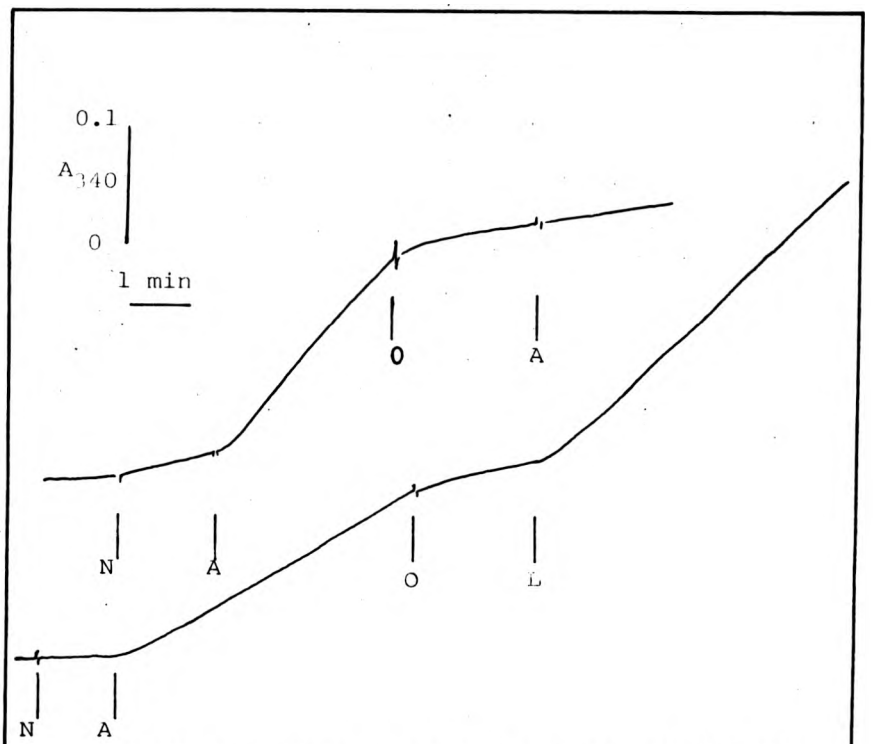


Fig 6.6.Apparent reversal of oligomycin inhibition of ATP driven transhydrogenase by 'dihydrolipoate'.

Assays were performed as described in chapter 6.
N : 0.5µmoles $NADP^+$. A : 5µmoles ATP.
O : 0.5µg oligomycin. L : 400nmoles 'dihydrolipoate'.

Assays contained 1mg bovine heart submitochondrial particles.

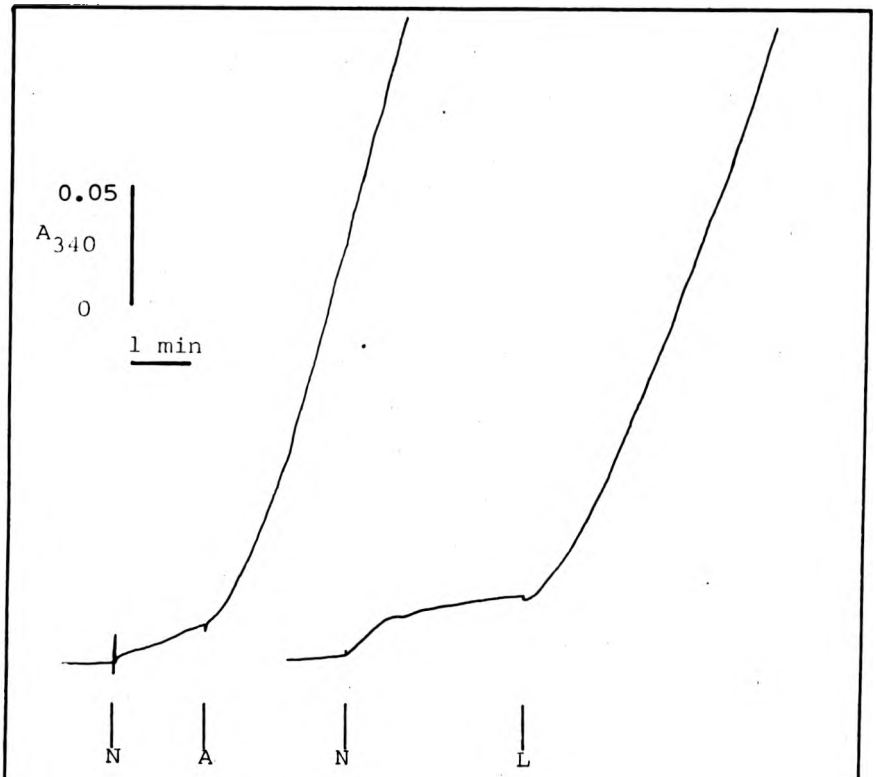


Fig 6.7.'Dihydrolipoate' driven transhydrogenase in bovine heart submitochondrial particles.

Assays containing 1mg SMP protein, 1µg rotenone, 1µg antimycin A, were performed as described in the methods section to chapter 6.

N : 0.5µmoles $NADP^+$. A : 5µmoles ATP.
L : 1 µmole 'dihydrolipoate.

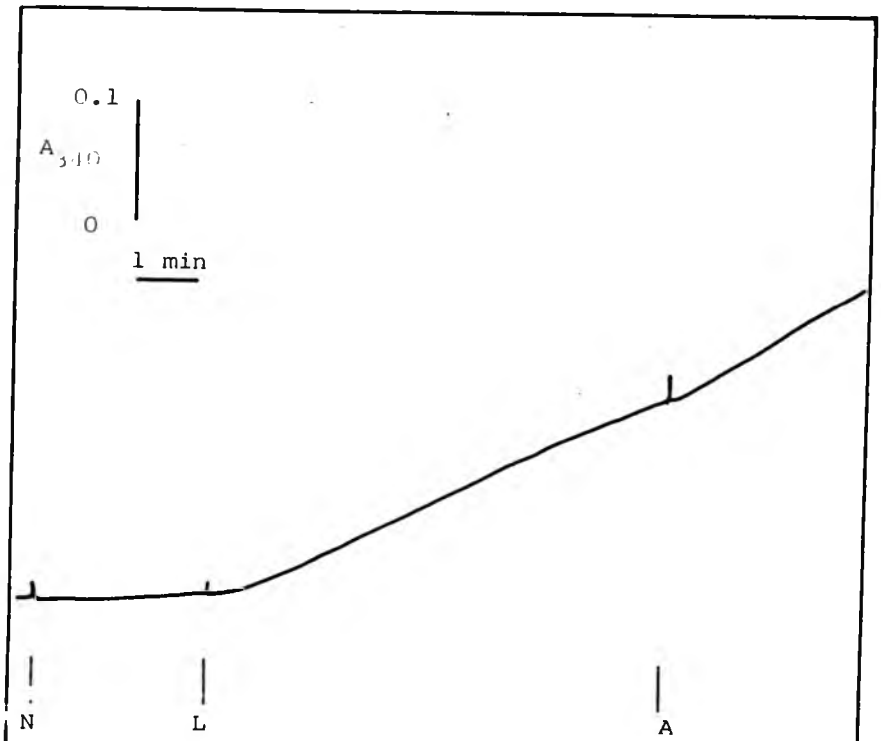


Fig 6.8.'Dihydrolipoate' driven transhydrogenase in bovine heart submitochondrial particles: effect of antimycin A.

Assays containing 1mg SMP protein, 1µg rotenone, were performed as described in the methods section to chapter 6.

N : 0.5µmoles. L : 1 µmole 'dihydrolipoate'.

A : 2 µg antimycin A.

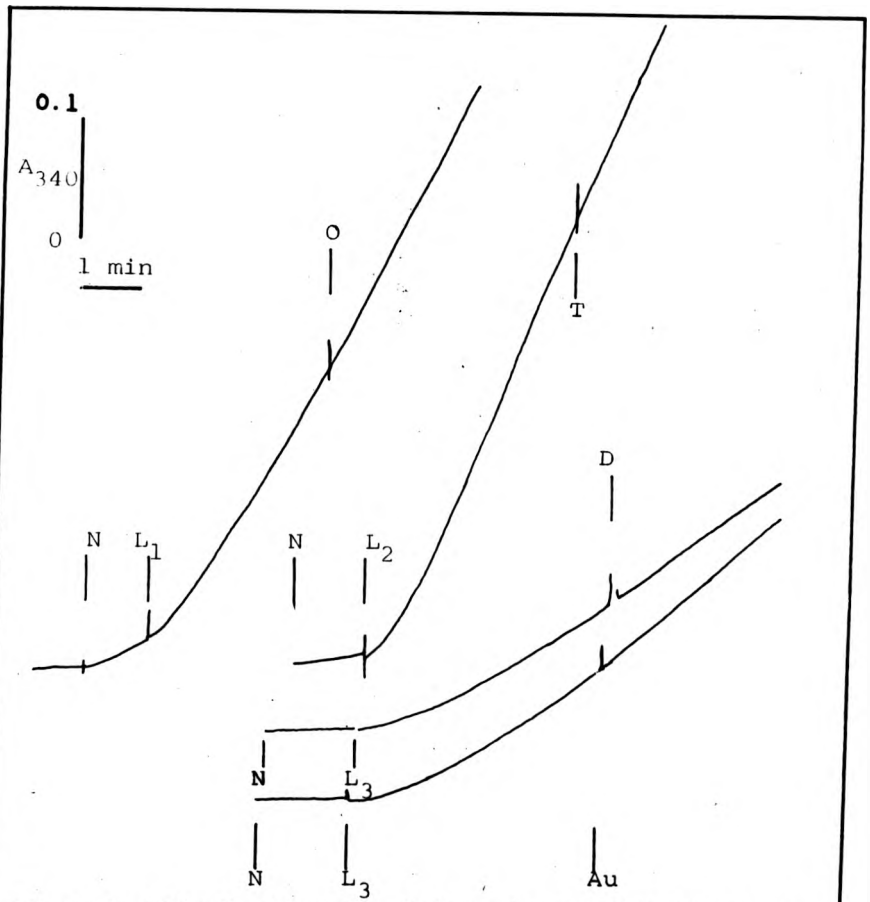


Fig 6.9.'Dihydrolipoate driven transhydrogenase in bovine heart SMP : effect of ATPase inhibitors.

Assays containing 1mg SMP protein, 1µg rotenone, 1µg antimycin A were performed as described in the methods section to chapter 6.

N : 0.5 µmole $NADP^+$. L_1 : 2 µmole 'dihydrolipoate'.
Au: 5µg aurovertin B. L_2 : 1 µmole " .
O : 2µg oligomycin. L_3 : 0.4 µmole " .
T : 2µg tri-ethyl tin sulphate. D : 5 µg dibutyltin dichloride

hydrogenase by oligomycin (Fig. 6.6). This restored activity was not sensitive to further additions of oligomycin. Oligomycin inhibition could not be reversed by the addition of further ATP. This reversal was not a true reversal of the inhibited ATP-driven activity as the dihydrolipoate preparation that caused this apparent reversal had the ability to stimulate an energy linked transhydrogenase activity at rates similar to those driven by ATP (Fig. 6.7). This dihydrolipoate-driven transhydrogenase reaction could only be obsered with certain preparations of dihydrolipoic acid. Of the 10 - 15 preparations of dihydrolipoate tested, only 4 had the ability to stimulate this energy linked reaction. Dihydrolipoate stimulated transhydrogenase activity was observed in the presence of rotenone and antimycin A, the latter appearing to somewhat stimulate the reaction (Fig. 6.8). The activity was not observed in SMP preparations inhibited with cyanide rather than antimycin A. Dihydrolipoate stimulated transhydrogenase was not inhibited by the F_1 ATPase inhibitor aurovertin, or the F_1F_O ATPase inhibitors oligomycin, DCCD, DBCT, TET and DBT (Fig. 6.9). Higher levels of TET do induce some inhibition of activity, possible because of an uncoupling effect due to its ability to mediate chloride-hydroxyl exchange. Uncoupling agents, such as '1799' TTFB and S13 have the ability to completely inhibit dihydrolipoate stimulated activity (Fig. 6.10). The ionophores gramicidin D and valinomycin (in the presence of K^+) also have the ability to inhibit the reaction (Fig. 6.11). The reaction is not inhibited by the iron chelating agent, bathophenanthroline, or the plant flavinoid, quercetin, which inhibits some ATP utilising reactions (Fig. 6.12). It is inhibited, however, by leucinostatin, a compound known to affect ATP utilising reactions (Fig. 6.12). Its mode of action here, though, is probably due to its ability to uncouple energy linked reactions in higher concentrations. The stoicheiometry of NADPH production from dihydrolipoate was

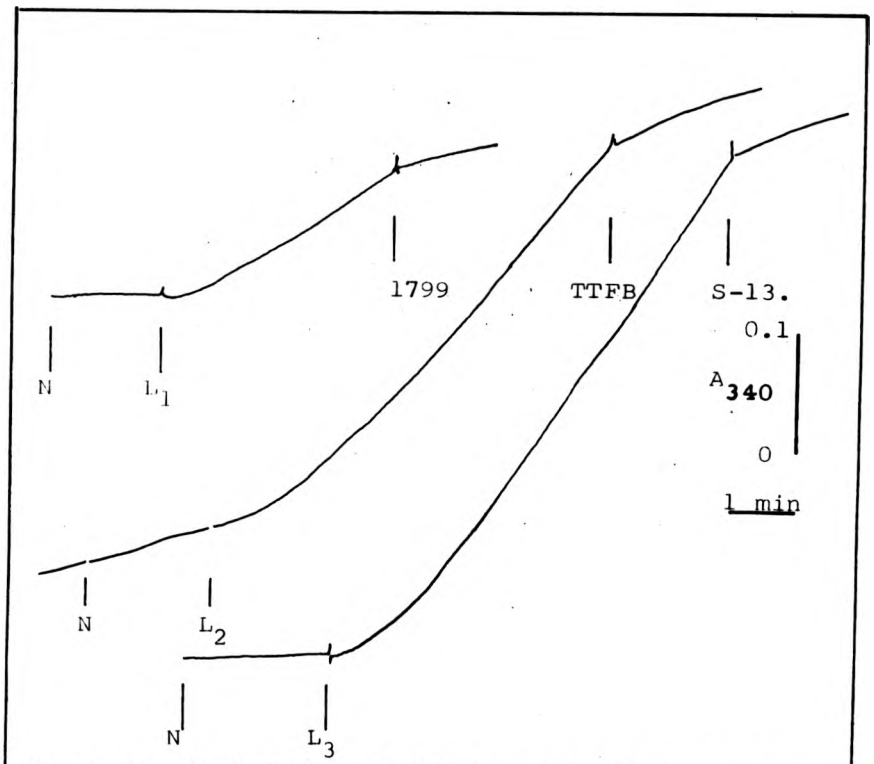


Fig 6.10. 'Dihydrolipoate' driven transhydrogenase in bovine heart SMP: effect of uncouplers.

Assays containing 1mg SMP protein, 1µg rotenone, 1µg antimycin A were performed as described in the methods section to chapter 6.

N : 0.5 µmcle $NADP^+$. L_1: 0.4 µmole 'dihydrolipoate'.
L_2: 1.0 µmole "
L_3: 2.0 µmole "

1799 : 2 µg compound '1799'. S-13 : 4µg S-13.

TTFB : 2 µg TTFB.

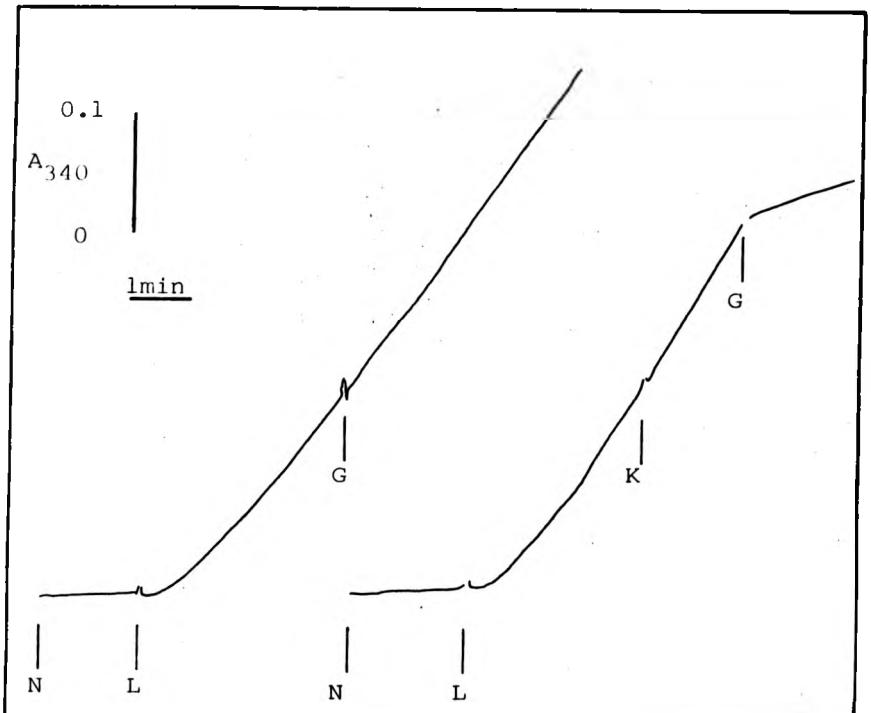


Fig 6.11. 'Dihydrolipoate' driven transhydrogenase in bovine heart SMP: effect of ionophores.

Assays containing 1mg SMP protein, 1µg rotenone, 1µg antimycin A were performed as described in the methods section to chapter 6.

N : 0.5 µmole $NADP^+$. L : 1 µmole 'dihydrolipoate'
G : 4µg gramicidin D. K : 0.75 µmole KCl.

Valinomycin also inhibits this reaction (317).

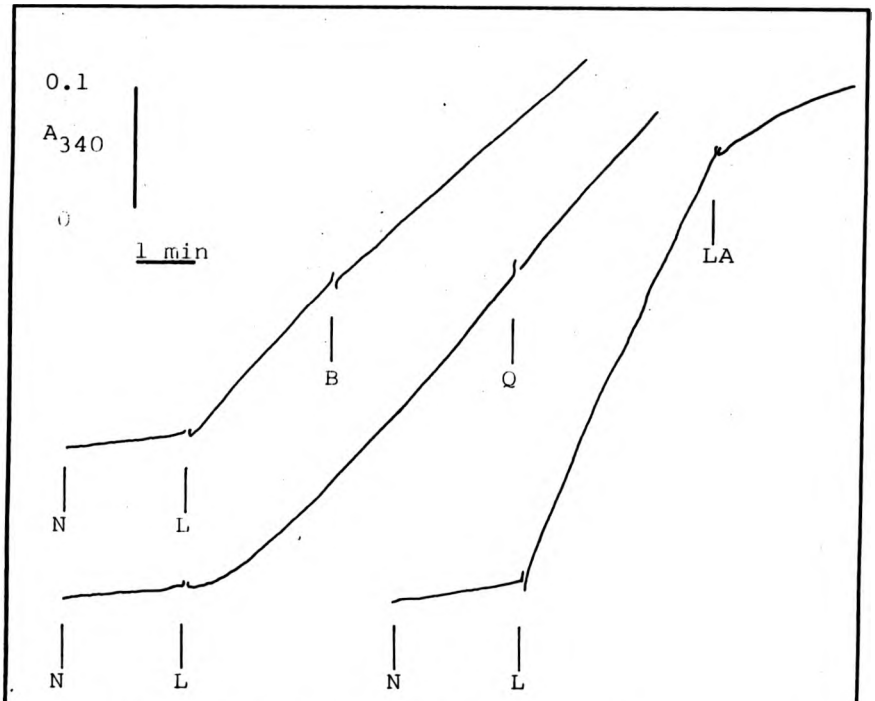


Fig 6.12.'Dihydrolipoate' driven transhydrogenase in bovine heart SMP : effect of quercetin, leucinostatin and bathophenanthroline.

Assays containing 1mg SMP protein, 1µg rotenone, 1µg antimycin A, were performed as described in the methods section to chapter 6.

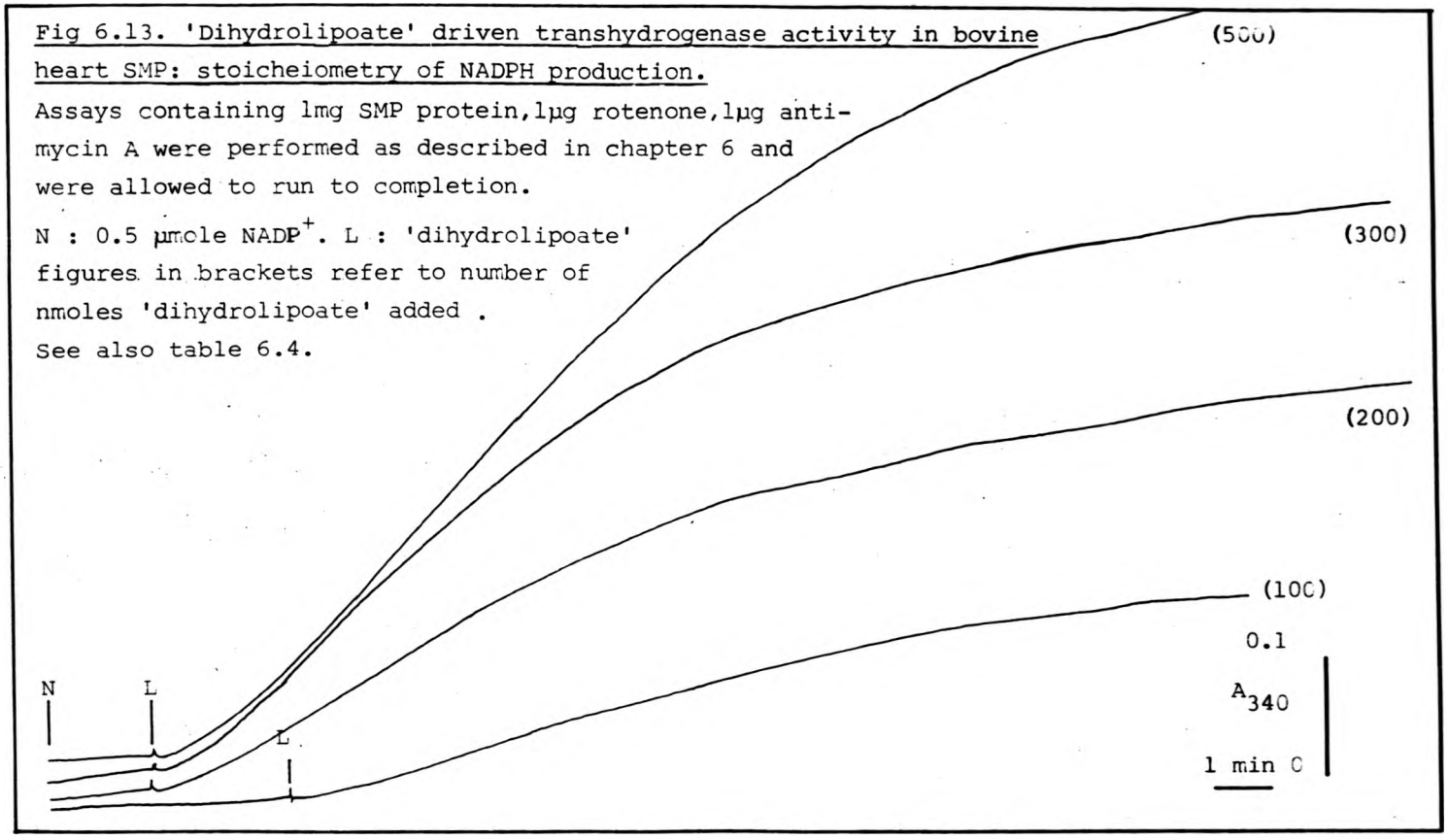
N : 0.5 µmole $NADP^+$. L : 1 µmole 'dihydrolipoate'. B : 10 µg bathophenanthroline. Q : 20 µg quercetin. LA : 4 µg leucinostatin.

Fig 6.13. 'Dihydrolipoate' driven transhydrogenase activity in bovine heart SMP: stoicheiometry of NADPH production.

Assays containing 1mg SMP protein, 1µg rotenone, 1µg antimycin A were performed as described in chapter 6 and were allowed to run to completion.

N : 0.5 µmole $NADP^+$. L : 'dihydrolipoate' figures in brackets refer to number of nmoles 'dihydrolipoate' added .

See also table 6.4.



investigated, the results of which are recorded in Table 6.4 and in Fig. 6.13.

Table 6.4 Dihydrolipoate driven transhydrogenase in bovine heart SMP: stoicheiometry of NADPH production

Dihydrolipoate added nmoles	NADPH produced nmoles	lipoate:NADPH ratio
100	90.73	1:0.93
200	162.36	1:0.82
300	237.00	1:0.79
500	307.90	1:0.62
1000	429.70	1:0.43
1000	570.00	1:0.57

Assays were performed as described in the methods section to Chapter 6. Assays were allowed to run to completion, that is until the 'dihydrolipoate' stimulated rate returns to the non-energy linked rate. See also Fig. 6.13.

A K_m value for dihydrolipoate was estimated (Fig. 6.14) and calculated to be 175 μM.

Various other compounds were tested for an ability to stimulate transhydrogenase activity; these included oxidised lipoic acid, dihydrolipoamide and the postulated 'oleoyl cycle' intermediates oleoyl-S-lipoate and oleoyl phosphate (235). None of these had any ability to stimulate transhydrogenase activity (Fig. 6.15). The oleolyl-S-lipoate analogue, eladioyl-S-lipoate, had the ability to inhibit the dihydrolipoate stimulated reaction (Fig. 6.16). Dihydrolipoate preparations that have no ability to stimulate transhydrogenase activity fall into three categories; those which have an inhibitory effect on dihydrolipoate stimulated transhydrogenase, those which have no effect on this reaction and those which have a stimulatory effect (Fig. 6.17). An analogous series of effects is observed with ATP-driven transhydrogenase (Figs. 6.18; 3.6).

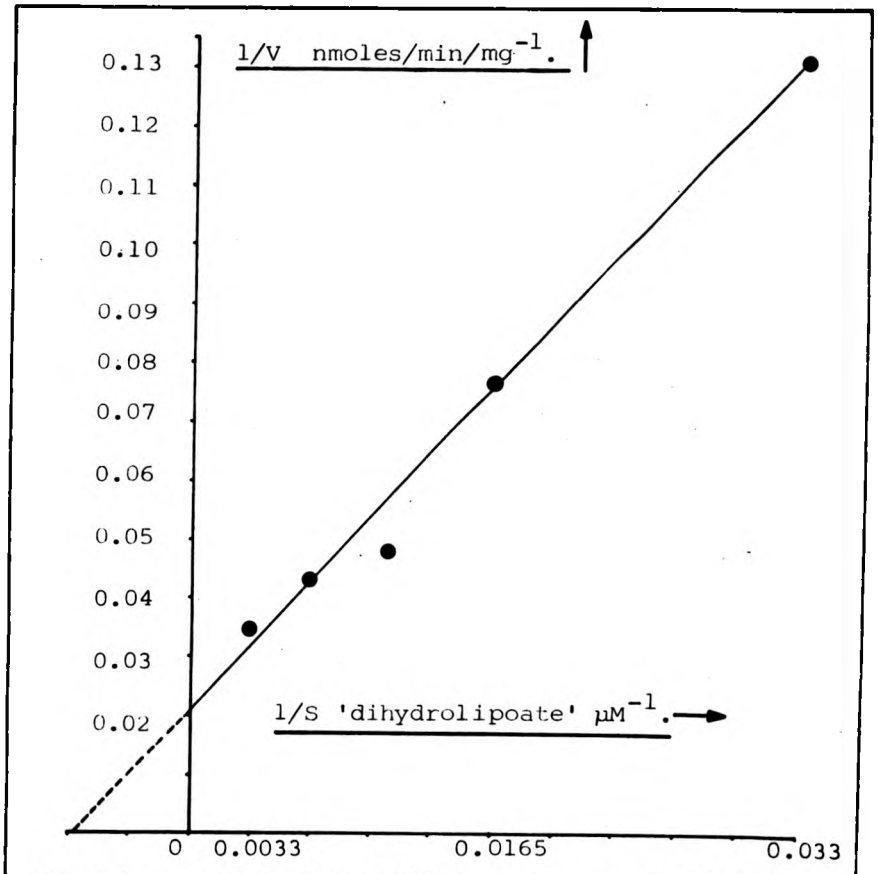


Fig 6.14. 'Dihydrolipoate'driven transhydrogenase in bovine heart SMP: Km (lipoate) determination.

Assays were performed as described in the methods section to chapter 6.

Km = 175 µM 'dihydrolipoate'.

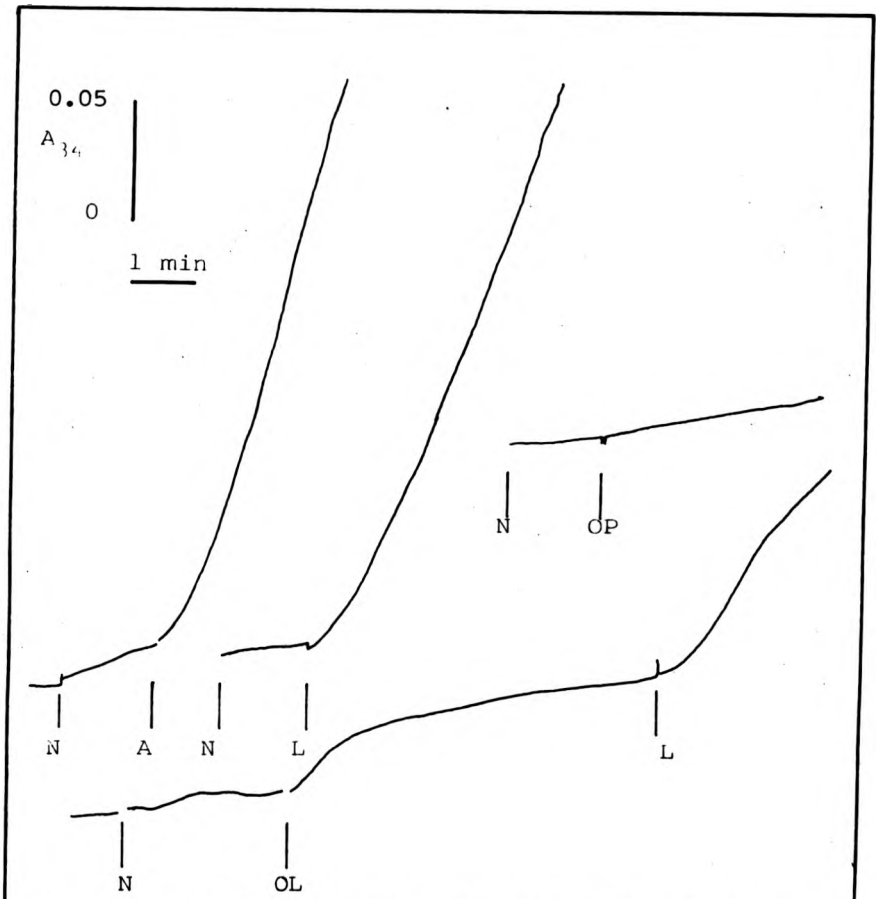


Fig 6.15 Effect of 'Oleoyl cycle intermediates' on nicotinamide nucleotide transhydrogenation in bovine heart SMP.

Assays containing 1mg SMP protein, 1µg rotenone, 1µg antimycin A were performed as described in methods section to chapter 6. L : 1 µmole 'dihydrolipoate'

N : 0.5 µmole $NADP^+$. A : 5 µmole ATP.

OL : 1 µmole oleoyl lipoate. OP : 1 µmole oleoyl-phosphate.

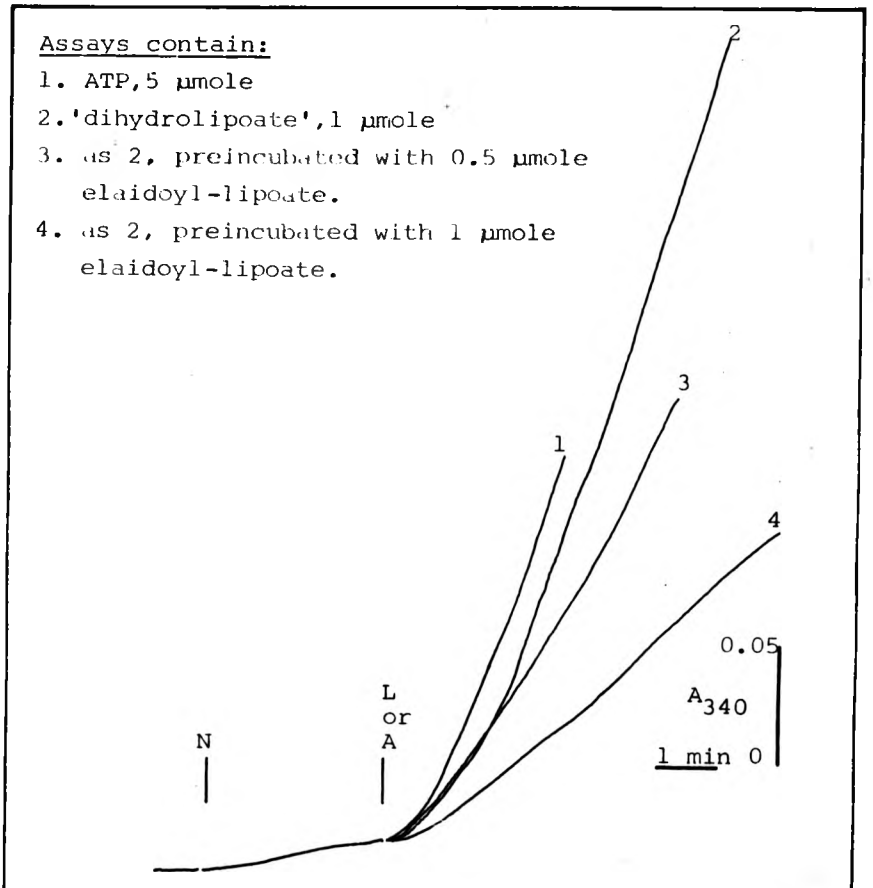


Fig 6.16 Inhibition of dihydrolipoate driven transhydrogenase by elaidoyl-lipoate.

Assays containing 1mg bovine heart SMP were preincubated with elaidoyl-lipoate, where indicated, for 10 minutes at R.T. Transhydrogenase activity was monitored as described in the methods section to chapter 6. N : 0.5 µmoles $NADP^+$. L : 1 µmole 'dihydrolipoate'. A : 5 µmoles ATP, assay 1 only.

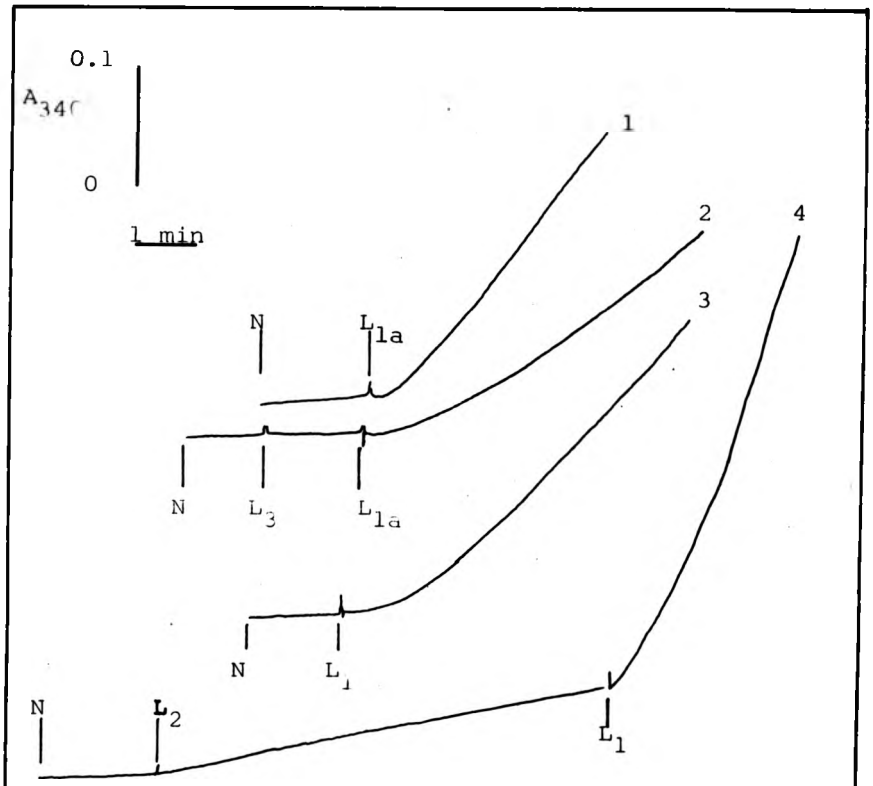


Fig 6.17 'Dihydrolipoate'driven transhydrogenase in bovine heart SMP:effect of different lipoates.

Assays containing 1mg SMP protein, 1µg rotenone, 1µg antimycin A were performed as described in chapter 6.

N : 0.5 µmole $NADP^+$. L_1: 1 µmole active dihydrolipoate
L_{1a}:0.4 µmole "

L_2 :1 µmole stimulatory dihydrolipoate.

L_3 :1 µmole inhibitory lipoate ; $plip(SH)_2$ see ch. 3.

Assays 1&2 : 3&4 are paired assays.

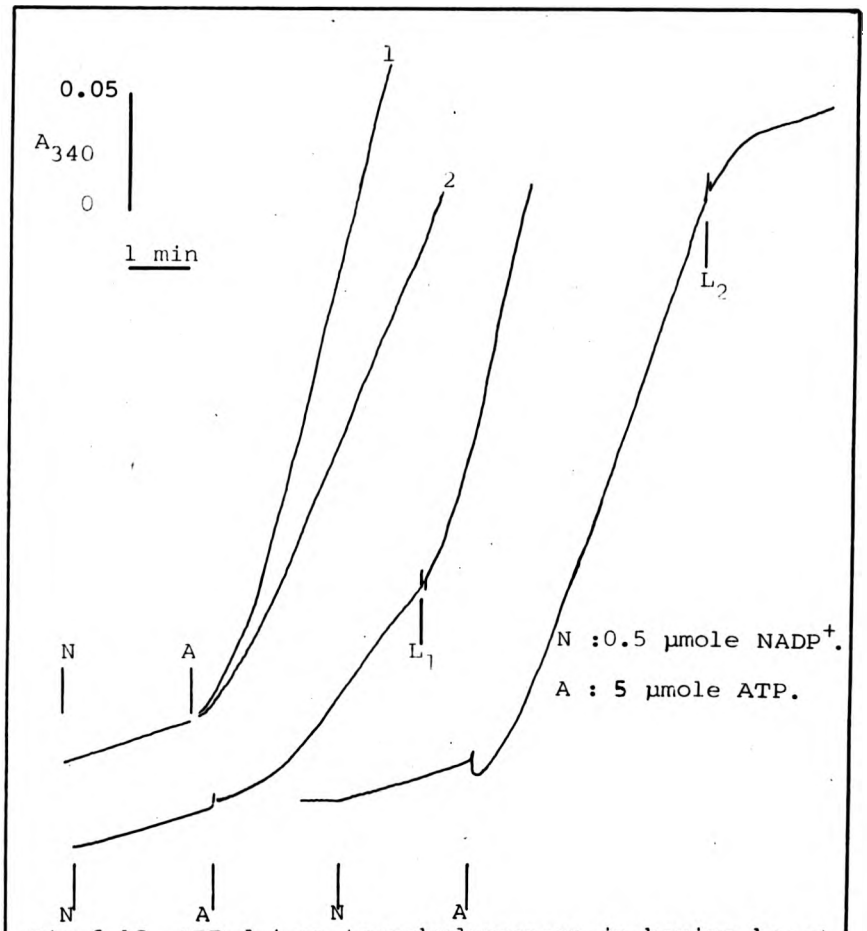


Fig **6.18.** ATP driven transhydrogenase in bovine heart SMP : effect of different lipoates.

L_1 : 1 µmole stimulatory dihydrolipoate

L_2 : 0.4 µmole inhibitory lipoate ; plip$(SH)_2$.

Assays 1&2 are paired, assay 2 is as assay 1 but preincubated with 1 µmole freshly prepared dihydrolipoic acid

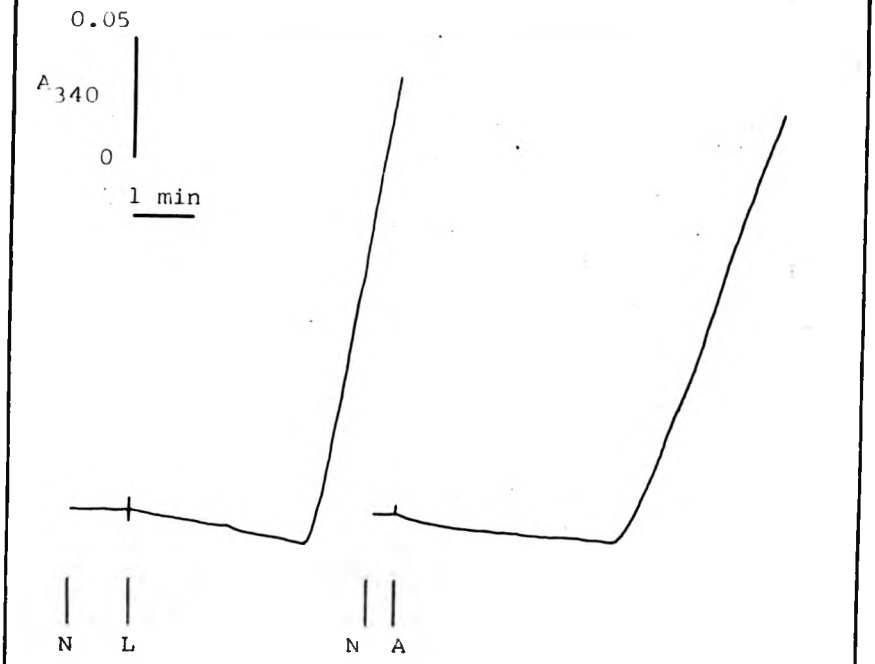


Fig 6.19.Energy linked reduction of NAD^+ by succinate in bovine heart SMP. effect of 'dihydrolipoate'.

Assays containing 1mg SMP protein were performed as described in the methods section to chapter 6.

N : 1 µmole NAD^+. A : 1 µmole ATP.
L : 1 µmole 'dihydrolipoate'.

Dihydrolipoate was also tested for the ability to stimulate the reduction of NAD^+ by succinate. This it consistently failed to do, even under conditions in which an energy linked transhydrogenase activity was stimulated except on one occasion (Fig. 6.19).

Discussion

Mitochondrial energy linked transhydrogenase reactions have been known for a number of years. These have been classified into two types; those driven by the oxidation of respiratory chain substrates, such as succinate, NADH and ascorbate/TMPD, and those driven by energy derived from the hydrolysis of ATP. Both these reactions are thought to generate a high energy state, '~', in the membrane which can be used by the transhydrogenase molecule to facilitate the transfer of hydrogen from NADH to NADPH.

Dihydrolipoic acid appeared to have the ability to reverse the oligomycin inhibition of the ATP-driven reaction, but this was not the case as later experiments showed that certain preparations of dihydrolipoate had the ability to stimulate a transhydrogenase reaction in the absence of either respiratory chain substrates or ATP. This dihydrolipoate stimulated activity can be classified as an energy linked activity as it is inhibited by uncoupling agents, gramicidin D and valinomycin in the presence of potassium ions. All these compounds have the ability to discharge the high energy state of the mitochondrial inner membrane. The dihydrolipoate stimulated reaction is unaffected by F_1 ATPase inhibitors, such as aurovertin, and inhibitors which have their locus of action in the F_O membrane sector of the ATPase, e.g. oligomycin, DBCT, DCCD, TET. It also is unaffected by the electron transfer inhibitor rotenone and antimycin A. This distinguishes the reaction from respiratory chain substrate-driven transhydrogenase which is affected by antimycin A. The dihydrolipoate-driven transhydrogenase appears to be unlike both the ATP-driven and the respiration-driven reactions.

ATP-driven transhydrogenase is inhibited by an analogue of lipoic acid, 8-methyl-lipoic acid (316), while the respiration-driven reaction is unaffected. The dihydrolipoate-driven reaction was also inhibited by the 8-methyl derivative (317). Thus, in its sensitivity to inhibition, the dihydrolipoate driven reaction can be considered as lying between or sharing characteristics of both the ATP and respiration stimulated transhydrogenase (see Table 6.5). The transhydrogenase has been estimated to consume one high energy bond, '~', per NADPH formed. The stoicheiometry of the dihydrolipoate stimulated reaction was investigated. The results from this are recorded in Table 6.4. At low levels (0 - 200 nmoles) of dihydrolipoate, the stoicheiometry of moles NADPH formed per mole dihydrolipoic acid is almost 1:1, while at higher levels (1 - 2 moles) this stoicheiometry fell to about 0.5:1.

Table 6.5 Effect of inhibitors on the energy linked transhydrogenase

Inhibitor	Respiration-driven (succinate)	ATP-driven	DHL-driven
Rotenone	-	-	-
Antimycin A	+	-	- (s)
Cyanide	+	-	+
Oligomycin	-	+	-
TET	-	+	-
DBT	-	+	-
Aurovertin	-	+	-
1799	+	+	+
TTFB	+	+	+
S13	+	+	+
Gramicidin	+	+	+
Valinomycin + K^+	+	+	+
8-methyl lipoic acid	-	+	+

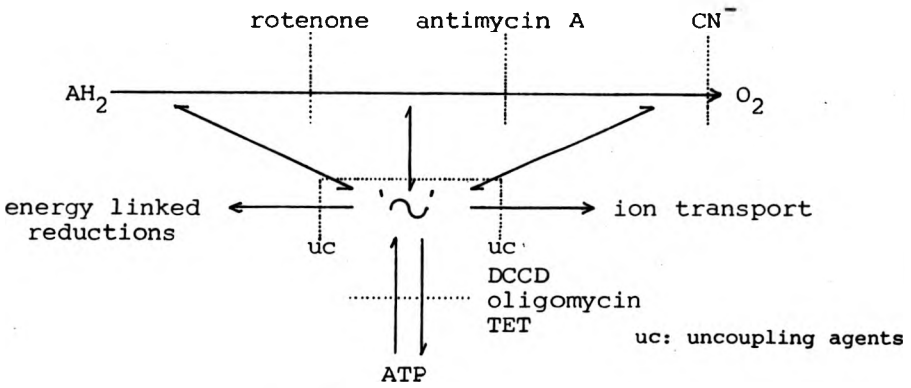
+ denotes inhibition; - denotes non-inhibition; s denotes stimulation

These values compared very well with experimentally determined stoicheiometries for ATP and extremely well with stoicheiometries obtained with respiration-driven systems (the latter are usually much lower than the theoretical 1:1 ratios). This high stoicheoimetry suggests a rather efficient conversion of energy, somehow bound up in dihydrolipoate to '$\sim$', and hence to the transhydrogenase. A K_m value for lipoic acid was deteremined and found to be $\sim$ 175 µM, which is of the same order of magnitude as K_m values for the nicotinamide nucleotides. The rate of the dihydrolipoate-driven reaction was comparable to that of the ATP-driven reaction in particles in which it was observed, the highest rate being $\sim$ 90 nmoles NADPH produced/min/mg submitochondrial particle protein.

Most dihydrolipoates tested exhibited no ability to stimulate a transhydrogenase reaction in bovine heart SMP's. Those that did not either had no effect on ATP and dihydrolipoate-driven activities or stimulated them or inhibited them. This latter class of reactions has been observed with many derivatives of both oxidised and reduced lipoic acid. This seems to be due to a general effect of these derivatives on energy-linked reactions as oxidative phosphorylation was also inhibited. The mechanism of this inhibition is discussed in Chapter 3. More interesting and possibly more informative was the ability of certain dihydrolipoate preparations to stimulate the ATP and dihydrolipoate-driven reactions. Incubation of aged submitochondrial particles with dihydrolipoate stimulates the ATP-driven reaction to a rate comparable to that of the rate in freshly prepared particles (Fig. 6.18), suggesting that dihydrolipoate can reduce a group (possibly a thiol) functionally involved in energy linked transhydrogenation.

The commonly shared view of the energy coupling system is outlined in Fig. 6.20.

Fig. 6.20 Mitochondrial energy conservation system



Here the nature of '~' is not defined

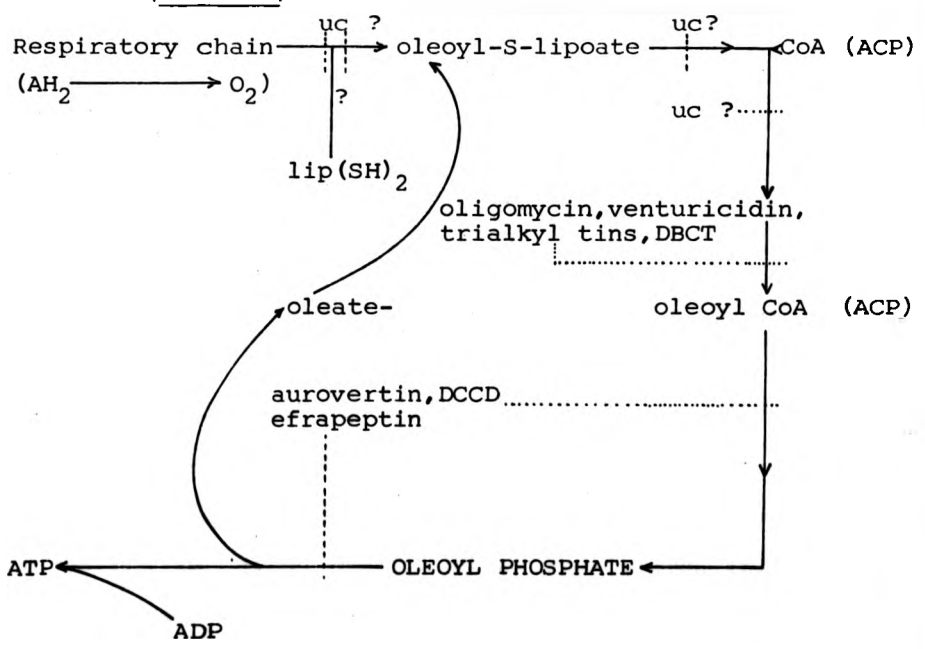
It can be seen that reactions driven by the '~' state should be unaffected by ATPase inhibitors such as oligomycin, DCCD, trialkyltins and aurovertin, and by respiratory chain inhibitors such as rotenone, antimycin A and cyanide. They should, however, be inhibited by compounds which abolish the '~' state, that is, uncoupling agents and certain ionophoric compounds.

A comparison of the inhibitor sensitivity of the dihydrolipoate-driven reaction to the sensitivity of '~'-driven reactions reveals a close similarity between the two. The only major difference in inhibitor sensitivity between the two reactions is that CN^- inhibits the dihydrolipoate-driven reaction. This, however, may be due to the formation of a thiocyanate adduct of lipoic acid which, like other lipoate derivatives, e.g. 8-methyl-lipoic acid, inhibits the dihydrolipoate-driven reaction. Cyanide has been reported to inhibit $lip(SH)_2$ driven ATP synthesis (248). It is tempting,then, to suggest that dihydrolipoate drives the reaction from around the level of '~'.

If this is the case, what is the nature of the interaction between lipoic acid and the energy coupling apparatus? Griffiths has suggested

that the terminal steps of oxidative phosphorylation may be analogous to those of substrate level phosphorylation and has proposed the hypothetical 'oleoyl cycle' as an experimental framework (Fig. 6.21). Here dihydrolipoate-driven ATP synthesis would be sensitive to oligomycin and aurovertin, but dihydrolipoate-driven transhydrogenase would not be sensitive to these inhibitors as it taps into the system between '∿' and the oleoyl cycle of reactions. Dihydrolipoate, then, would drive transhydrogenase because it was directly involved in the formation or subsequent utilisation of the energy stored as '∿'. It is worth noting here that other postulated intermediates of the oleoyl cycle, oleoyl S lipoate and oleoyl phosphate consistently failed to stimulate transhydrogenase activity, even under conditions in which the dihydrolipoate stimulated reaction was observed.

Fig. 6.21 The oleoyl cycle hypothesis of oxidative phosphorylation (after 248)

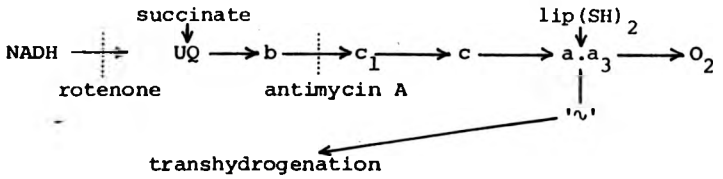


..... inhibition

uc: uncouplers

Two alternative explanations that do not need to invoke the existence of such a sequence of reactions can be proposed to explain dihydrolipoate transhydrogenase activity. These are that dihydrolipoate causes a respiration-driven reaction to occur or that dihydrolipoate somehow interacts with '∿' or the transhydrogenase molecule, donating the redox energy in such a way that it can be used to drive transhydrogenase.

Dihydrolipoate-driven transhydrogenase is inhibited or prohibited by the presence of CN^- in the assay system. Cyanide has its major locus of action in mitochondria at the level of cytochromes $a.a_3$. Dihydrolipoate-driven transhydrogenase activity was usually measured in a system containing rotenone and antimycin A. The possibility then remains that the reaction could be mediated <u>via</u> coupling site 3, <u>viz</u>.



Several artificial redox systems have been shown to generate '∿' at site 3; dihydrolipoate might possibly fulfil a similar function. It would be possible to eliminate this pathway if the reaction was performed under anaerobic conditions as oxygen would be consumed during this reaction. This experiment was not performed, so the possibility of a site 3-driven transhydrogenation remains open. There are two considerations, however, that make this rather unlikely. The first, and most important,is that NADPH production has been observed at levels in these experiments which are in excess of the molar concentration of oxygen present in the assay buffers. Oxygen would diffuse back into these buffers if it was being depleted, but this would happen at a low rate as the surface to volume ratio of the buffers in the cuvettes used to perform the assay was rather

low. It is possible (but unlikely) that O_2 was not being consumed in these reactions because the low O_2 tension under which the assays were performed may have inhibited oxidative electron flow. The second consideration making site 3-driven transhydrogenation unlikely is the stoicheiometry of the reaction. Site 3-driven transhydrogenation rarely exhibits a stoicheiometry of 1:1 (substrate added:NADPH produced), and is usually of the order $\sim$ 1:0.1. Dihydrolipoate-driven transhydrogenase usually exhibited stoicheiometries in the order of 1:0.5 - 0.9 (moles dihydrolipoate added:NADPH produced).

Thiol groups have been reported to be involved in the mechanism of both energy coupling and transhydrogenation (318, 269); the precise natures of these involvements are unclear. Dihydrolipoate may exert its effect by supplying energy at the right redox level to a series of membrane thiol groups directly involved in these reactions. This idea is , though, very vague and rather difficult to test. The ability of certain dihydrolipoate preparations to stimulate ATP-(and dihydrolipoate)-driven transhydrogenase does suggest that they may be acting on some membrane bound group in such a way, although much work remains to be done to clarify the issue.

Dihydrolipoate can act as a substrate for the diaphorase enzyme (NADH:lipoamide oxidoreductase EC 1.6.4.3) which is present in mitochondrial preparations. This catalyses the reactions.

$$\underset{\text{(lipoate)}}{\text{lipoamide}} + \text{NADH} + \text{H}^+ \xleftrightarrow{\text{diaphorase}} \underset{\text{(dihydrolipoate)}}{\text{dihydrolipoamide}} + \text{NAD}^+$$

Under the transhydrogenase assay conditions used, submitochondrial particles are incubated with 40 - 50 nmoles NAD^+ and up to 2 µmoles dihydrolipate. Diaphorase activity might then produce 40 - 50 nmoles NADH. This might act to push the equilibirum of the non-energy linked transhydrogenase reaction over to the right, i.e.

$$NADH + NADP^{+} \longrightarrow NADPH + NAD^{+}$$

The NAD^{+} produced could then be rereduced by the diaphorase enzyme and the process would continue. Thus dihydrolipoate would exert its action by effectively stimulating the non-energy linked reaction. This is, however, not an adequate explanation for dihydrolipoates' mode of action. This stimulation would be of the non-energy linked reaction, which is unaffected by uncoupling agents, and ionophoric compounds. The dihydrolipoate-driven process is, indicating that an energy linked reaction is being observed.

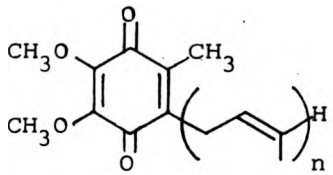
As with other dihydrolipoate stimulated reactions, the transhydrogenase was only observed rarely; the reasons for this are unknown (see Chapter 3). The reaction has been observed in other laboratories (319), although here it was interpreted to proceed *via* coupling site 3. The reaction has also been observed in preparations of *E. coli* vesicles in this laboratory (320). Orlando has reported a similar dihydrolipoate stimulated transhydrogenase in vesicles prepared from the photosynthetic bacteria, *Rhodopseudomonas spheroides* (311). This activity was also difficult to observe and intermittent in nature (321). The chemistry of dihydrolipoate in solution is extremely complex. It may be that one of its (photo-induced) breakdown products or lactone forms is of the correct redox potential for it to tap into the sequence of reactions between the coupling site and the transhydrogenase molecule (see Chapter 3 for further discussion). The intermittent nature of the observed reaction makes this a difficult problem to investigate.

CHAPTER 7

SOLVENT EXTRACTION STUDIES ON MITOCHONDRIAL ENERGY COUPLING: THE ROLE OF UBIQUINONE

Introduction

The ubiquinones are a series of 2,3-dimethoxy,5,methyl 6 polyisopentenyl (prenyl) benzoquinones, differing in the length of their prenyl side chains.



Ubiquinone-n (UQ_n)

The natural members of this group are those where n = 6-9 (although lower chain length homologues are known as breakdown or biosynthetic products of these ubiquinones). The commonest type in higher plants and animals is ubiquinone 10; ubiquinone 6 is common in yeast species, while ubiquinone 9 is the major quinone in rat liver mitochondria.

Ubiquinones were first discovered as nonsaponifiable lipids with absorption maxima around 272-275 nm in mammalian tissue extracts (331). It was only after their discovery in heart submitochondrial particles by Crane et al. (332) and the elucidation of their structure by Morton and co-workers (333-4) that a possible role in electron transport was postulated. Ubiquinone was later found in mitochondria and respiratory particles from many sources (76). There have been several reports that ubiquinone is also localised in other tissue fragments such as nuclei and microsomes, and that it may be involved in aldehyde oxidase activity; the evidence presented for this, however, does not absolutely exclude contamination by mitochondria or mitochondrial fragments (76).

Cytochrome aa_3 can be used as a standard against which the stoicheiometry of the other components of the respiratory chain can be

measured. Using this standard then, it can be shown that ubiquinone occurs in mitochondria in a several fold molar excess to cyt aa_3. In mammalian tissues, the UQ/cyt aa_3 ratio varies but is usually between 6 - 8.5, whereas in yeast and some bacterial preparations ratios as high as 36 have been observed.

Table 7.1 Content of quinones in the respiratory chain

Source	Preparation	UQ µmoles/g protein	cyt aa_3 µmoles/g protein	UQ/aa_3 ratio
rat heart	mitochondria	4.0	0.5	8.0
rat liver	mitochondria	1.8	0.25	7.2
rat skeletal muscle	mitochondria	3.0	0.35	8.6
rat kidney	mitochondria	1.6	0.27	5.9
bovine heart	mitochondria	3.5	1.3	2.7
pig heart	mitochondria	4.0	-	-
pig kidney	mitochondria	1.1	-	-
guinea pig kidney	mitochondria	1.6	-	-
pigeon breast muscle	mitochondria	3.9	0.47	8.3
flight muscle (locusta migratoria)	mitochondria	3.5	0.53	6.6
Saccharomyces cerevisiae	mitochondria	5.4	0.15	36
Mycobacterium phlei	particles	7.2	0.16	45
Azotobacter vinelandii	particles	8.9	0.67	13
Arum spadix	mitochondria	1.4	-	-
spinach	leaves	0.1[1]	0.0025[2]	40

[1] plastoquinone moles/mole chlorophyll

[2] cytochrome f mole/mole chlorophyll

Ubiquinone is present in the inner mitochondrial membrane at a much higher molar concentration than the other components of the respiratory chain.

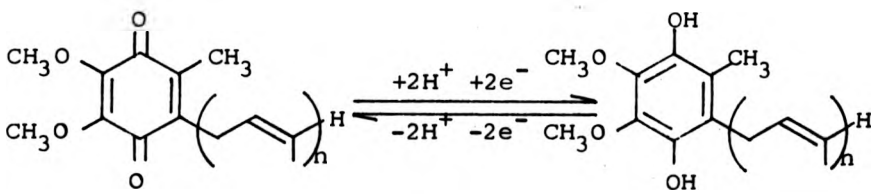
Table 7.2 Estimated stoicheiometry of respiratory enzymes in heart mitochondria

Component	Ratio[1]
malate dehydrogenase	0.50
glutamate dehydrogenase	0.01
succinate dehydrogenase	0.20
NADH dehydrogenase	0.15
ubiquinone	7
cytochrome b	1.10
cytochrome c	0.40
cytochrome c_1	1
cytochrome aa_3	1

[1] estimated per mole cyt aa_3

Table adapted after (76)

Various spectroscopic techniques have shown that ubiquinone undergoes redox changes during respiratory chain electron transport. These changes involve both the quinone and quinol forms of ubiquinone



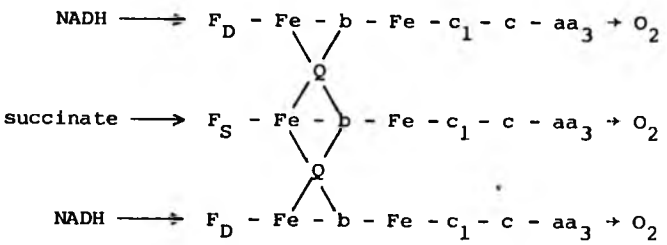
quinone quinol

The quinone absorption spectrum has a maximum at 275 nm with a molar extinction coefficient of 14,000 whereas the quinol has a maximum at 290 nm with a millimolar extinction coefficient of 12.2. The maximum rate of these redox changes (in vivo) as well as their kinetic characteristics are consistent with its role as an electron carrier.

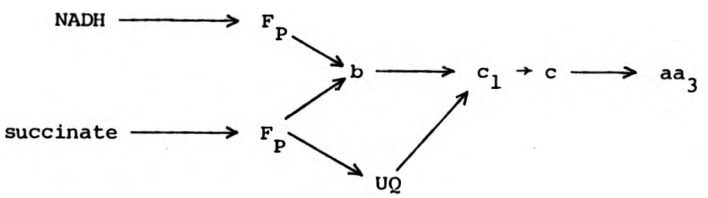
Spectrophotometrical techniques and 'crossover point' studies

show that ubiquinone acts at an early stage in electron transfer between the dehydrogenases and oxygen. The site(s) of ubiquinone's function as an electron (proton) carrier has been generally thought to lie between the flavin linked dehydrogenases, NADH and succinate dehydrogenase and the b cytochromes (b_{566}, b_{562}), or possible directly between the dehydrogenases and cytochrome c_1, the former being the most favoured position (76, 92, 24). The actual position(s) of ubiquinone in the respiratory chain is still far from clear. A pool function for ubiquinone as a mobile redox component between complexes I, II and complex III has been suggested by Ernster et al. (119) on the basis of solvent extraction studies of mitochondria. Various organic solvent systems have been used to selectively remove ubiquinone from the inner mitochondrial membrane. Ubiquinone can be reconstituted into these extracted systems with the concomitant return of certain enzymic activities. These techniques are treated more fully in the discussion section to this chapter. Solvent extraction studies have established beyond doubt that ubiquinone is an essential component of respiratory chain activity between NADH and oxygen and succinate and oxygen.

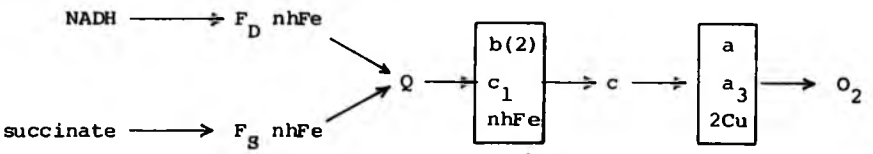
Early studies of the relationships between the kinetics of the redox reactions of ubiquinone and its function in electron transport gave rise to contradictory conclusions. Change and Redfearn (335) and Storey and Chance (336-7) concluded from spectrophotometric studies that ubiquinone was not involved in the main pathway of respiratory chain electron transfer but in a bypass or side reaction, possibly in equilibrium with the main pathway. Redfearn later pointed out that while his earlier kinetic data on ubiquinone could not be reconciled with a position in a single pathway, this did not necessarily imply that it is on a side pathway of electron transport (338). He suggested that it might act as a link between individual electron transport chains, e.g.



Storey and Chance suggested that electron transfer followed the following pathway in electron transport particles (336):-



Studies by Green and coworkers on complexes I - IV lead them to propose the following pathway for respiratory chain electron transport.



Perhaps the most important studies which finally defined the role and position of ubiquinone in respiratory chain electron transport were those of Kroger and Klingenberg (76, 121, 339). In a series of elegant kinetic investigations, they showed that the redox responses of ubiquinone were consistent with its function as a component of the respiratory chain, having a single position between the flavin linked dehydrogenases and the b or c_1 cytochromes. They went on to show that the mitochondrial ubiquinone pool consists of two parts, an active part (that is, involved in electron transport), UQ_a, and an inactive part, UQ_i (defined as that part of the ubiquinone pool non-reducible in the presence of substrates and KCN). UQ_i can be as much as 10-20% of total ubiquinone, UQ_t,

then $UQ_t = UQ_a + UQ_i$,

also $UQ_a = UQ_{oxidised} + UQ_{reduced}$.

They showed that the oxidation of UQ_{red} on aeration of anaerobic SMP followed first order kinetics and had a reaction constant = V_o/UQ_a, where V_o = oxidation rate of the SMP's. Further analysis showed that the rate of UQ reduction (steady state) was equal to the overall oxidation rate of the particles, i.e.

$$(\delta UQ_{ox}/\cdot \delta t)_{st.st} = V = (\delta UQ_{red}/\delta t)_{st.st}$$

Thus ubiquinone fulfils the kinetic requirements of an obligatory respiratory component.

Although electron transfer in the respiratory chain is a bimolecular process, transitions of respiratory carriers from the reduced to the oxidised state follow first order reaction kinetics. This is feasible for a chain of fixed carriers along which electrons are drained towards oxygen. It is, though, difficult to rationalise that a pool of mobile proton (electron) carriers such as ubiquinone also follows first order reaction kinetics. Instead, a mixed zero and first order reaction would be expected, since ubiquinone can be assumed to saturate both its donating and accepting components. Kroger and Klingenberg attempted to explain this by proposing that the oxidation reaction of UQ_{red} reflected the oxidation of a subsequent fixed carrier (B).

i.e. $SH_2 \rightarrow \rightarrow \rightarrow \rightarrow D \longrightarrow UQ \rightleftharpoons B \longrightarrow C - - \rightarrow O_2$

This would hold if B and UQ are in a close equilibrium which is adjusted faster than B is oxidised. The larger pool size of UQ in equilibrium with B as compared to that of C would cause C to reach its steady state level almost before the oxidation of B_{red} starts. As a result, the oxidation of B_{red}, and consequently UQ_{red}, would follow a pseudo first order reaction. The properties postulated for B and C are consistent with the observed properties of cyt b and cyt c respectively. The redox potentials of UQ and cyt b are almost equal under uncoupled conditions (340); this would enable close equilibrium to exist between them. The half time of oxidation of the b cytochromes

is of the same order of magnitude as that of ubiquinone, i.e. ∿ 0.1 s, which may indicate that the equilibrium is adjusted faster than b is oxidised (123). The half time of oxidation of the c cytochromes (∿ 5 ms), is considerably faster than those of UQ and the b cytochromes, consistent with the proposed scheme.

The redox reactions of plastoquinones involve plastoquinone semiquinone radicals (339). The first order reaction kinetics followed by the redox reactions of UQ_a exclude the possibility that radicals of UQ react with the donor or the acceptor of UQ. Radicals may be formed, though, as intermediary products of the reduction of UQ_{ox} and of the oxidation of UQ_{red}.

$$UQ_{red} + B_{ox} \longrightarrow \dot{UQ}H + B_{red} + H^+$$

The transfer of single electrons is probable since iron-sulphur proteins and a cytochrome are likely candidates for the donor and acceptor to UQ (D and B in Kroger and Klingenberg's scheme).

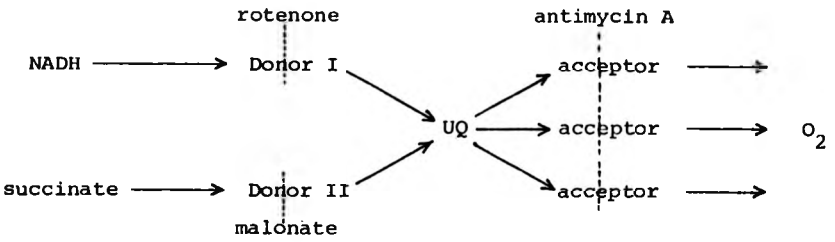
Electron spin resonance spectroscopy indicates that the amount of semiquinone radicals within the mitochondrial membrane under equilibrium conditions in the presence of substrate + KCN did not exceed 1-5% of UQ_{total} (341). The steady state concentration would not be expected to be high, however, since the disproportionation of the radical

$$2\dot{UQ}H \longrightarrow UQ_{ox} + UQ_{red}$$

is much faster than its formation by electron transport. Redox active ubiquinone, UQ_a, appears to be kinetically homogeneous. The relationship

$$UQ_{ox}/UQ_a = V_{ox}/(V_{red} + V_{ox})$$

derived by Kroger and Klingenberg is valid for UQ_a if either NADH or succinate is used as substrate, demonstrating that each ubiquinone molecule is accessible to reducing equivalents from both NADH and succinate dehydrogenases. This led Kroger and Klingenberg to propose a mobile pool function for ubiquinone, i.e.



This view contrasts to that proposed by Lenaz et al. (122) who suggested that there was a functional compartmentation of ubiquinone between NADH and succinate dehydrogenases based on observations of the reconstitution of these activities in pentane extracted mitochondria. They found NADH respiration has a far higher specificity for various ubiquinone homologues than succinate respiration, and requires far greater amounts of ubiquinone for reconstitution of pentane extracted mitochondiral activity than does succinate respiration. Lenaz' interpretation is based on the assumption that ubiquinone is relatively fixed in the membrane. His observations may be easily explained, however, if ubiquinone is mobile and the dehydrogenases display different enzyme specificities and different $Km_{(UQ)}$ values for ubiquinone.

Mitchell has proposed a rather different role for ubiquinone in the respiratory chain in his 'proton motive Q cycle' hypothesis, which was devised in order to allow the theoretical transport of $2H^+$ from the inside to the outside of the inner membrane by the transfer of only one electron from the dehydrogenase site of ubiquinone to cyt c_1. This cycle replaces loops2 and 3 of the chemiosmotic hypothesis (see Chapter 2) (342-3).

(a) $2UQ^{\cdot -} + 2H^{+} \rightleftharpoons UQ + UQH_2$

(b) $UQ^{\cdot -} + 2H^{+} = e^{-} \longrightarrow UQH_2$

(c) $UQH_2 \longrightarrow UQ + 2H^{+} + 2e^{-}$

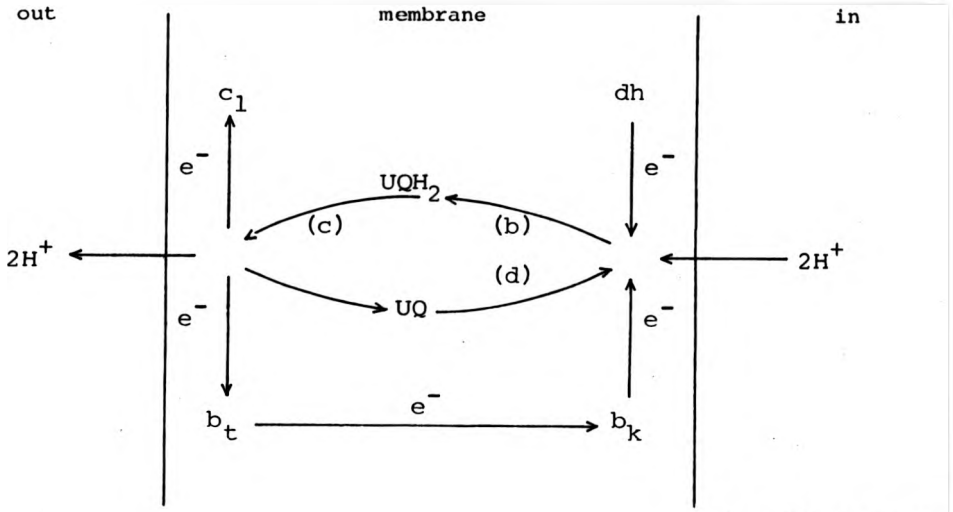
(d) $UQ + e^{-} \longrightarrow UQ^{\cdot -}$

This scheme of reactions has been criticised on theoretical grounds by Kroger (344), mainly on the basis of the stability of the radical anion of ubiquinone $UQ^{\cdot -}$ which dismutates to UQ and UQH_2 far faster than it can be produced by the electron transport chain.

The view of ubiquinone as a free mobile proton (electron) carrier in the membrane may need to be revised as King and co-workers have reported the presence of a number of specific ubiquinone binding proteins in the inner membrane. These are found in association with complex III (124), as the 'natural electron acceptor' of succinate dehydrogenase (123,345) and in association with complex I (124). They claim that it is these protein bound ubiquinones that are the redox active forms in the membrane. If this is so, then it raises many as yet unanswered questions. How is ubiquinone 'bound' to protein? What reactions does it undergo? What is its relationship to free ubiquinone? What then is the role of the large free ubiquinone pool?

There have been several suggestions in the past that ubiquinone might play a direct role in energy coupling in mitochondria. Clark postulated that a hydroquinone phosphate might act as a phosphate donor to ADP in oxidative phosphorylation (346). Other schemes involving quinone derivatives as possible 'high energy' intermediates have been proposed (see (25)). There is, however, little experimental evidence for any of these schemes. ATP synthesis driven by a quinol phosphate derivative has been reported (392) but at a very much slower rate than normal oxidative phosphorylation. Yamamoto *et al.* have reported that pentane extraction of ubiquinone from *Rhodospirillum rubrum* chromato-

Fig. 7.1 Proton motive ubiquinone cycle after Mitchell (343)



Two electrons and protons are transferred by the diffusion of UQH_2 from the inside to the outside of the membrane. Here UQH_2 is oxidised to UQ which diffuses back inside, only $1e^-$ is passed on to oxygen via c_1; the other is conducted back inside via the b cytochromes where it is transferred to UQ together with an electron donated from the dehydrogenase. In the oxidation reaction, the specific transfer of $2e^-$ may be achieved by the b-c_1 complex without the involvement of intermediates such as ubiquinone radicals. In contrast to this in the reduction of UQ to UQH_2 radicals have to be liberated in order to equilibrate with UQ and UQH_2 by the dismutation reaction:-

(a) $2UQ^{\cdot -} + 2H^+ \rightleftharpoons UQ + UQH_2$

This is necessary to explain the reduction of ubiquinone in the presence of antimycin A. This situation requires that cyt b reacts specifically with UQ and the dehydrogenase specifically with the radical. The ubiquinone cycle can then be described by the reactions a-d, i.e.

(a) $2UQ^{\cdot -} + 2H^{+} \rightleftharpoons UQ + UQH_2$

(b) $UQ^{\cdot -} + 2H^{+} = e^{-} \longrightarrow UQH_2$

(c) $UQH_2 \longrightarrow UQ + 2H^{+} + 2e^{-}$

(d) $UQ + e^{-} \longrightarrow UQ^{\cdot -}$

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phores results in a loss of ATP-Pi exchange activity, oligomycin sensitivity, coupled ATP hydrolysis and ATP synthesis. Readdition of ubiquinone 10 to this system results in a stimulation of these activities. They suggest that ubiquinone 10 may function as the link between the electron transport and energy conservation systems leading to ATP synthesis (347).

This hypothesis together with a report by Dr. E. Bertoli that pentane extraction of bovine heart mitochondria reduced sensitivity to oligomycin (326) lead to an investigation of possible functions of ubiquinone, other than those expected of a strict redox carrier in mitochondrial membranes.

Materials

All chemicals used were of 'AnalaR' or similar grade where available. Oligomycin was obtained from the Sigma Chemical Company. Yeast strains D273-10B, E3-24, E3-149 were kind gifts from Dr. Alex Tzagoloff. Ubiquinones were obtained from Hoffman La Roche Co. Ltd., Basel, Switzerland.

Methods

Bovine heart mitochondrial and submitochondrial particles were prepared as described in Chapter 3.

ATPase, ATP-^{33}Pi exchange and oxidative phosphorylation were assayed as described in Chapter 3.

Oxidase activities were assayed using a 'Clarke' type oxygen electrode connected to a Servoscribe chart recording device in 2 or 3 ml of a buffer, 0.25 M sucrose; 10 mM Tris-Cl, pH 7.5; 10 mM KCl; 5 mM $MgCl_2$; 5 mM potassium phosphate at 30° C. Values used for the oxygen content of buffer solutions were those of Eastabrook (399), i.e. 210 nmoles O_2/ml buffer at 30° C.

Yeast (strain D22, ad_2o^+,α) growth was carried out on a large scale in 10 L New Brunswick fermenter vessels under vigorous stirring (400 rpm)

and aeration (1OL/min). The growth media contained 0.5% (w/v) yeast extract; 0.1% (w/v) bacterial peptone; 0.01% (w/v) adenine sulphate, mineral salts (328); 1.0% v/v ethanol and tributyl citrate (0.01% v/v as an antifoaming agent. The culture was inoculated with a 1% (v/v suspension) of cells in late logarithmic stage and was harvested in late logarithmic phase (36-40 hr) by centrifugation in an MSE Mistral 6L centrifuge, 4 x 1.25 (rotor at 2000 rpm). Harvested cells were washed twice with cold distilled water and recentrifuged as before. Cells were then resuspended in an equal volume of 0.5 M sorbitol; 20 mM Tris-Cl, pH 7.5; 1 mM EDTA; 0.1% in bovine serum albumin and kept at 4^{o} C; all following operations were also carried out at 4^{o} C.

Yeast cells were disrupted by shaking cell suspensions with glass beads (0.45 - 0.5 mm diam.) for 30 seconds in a cooled Braun shaker (B. Braun Co., Melsungen) at 4000 rpm. The glass beads were washed with 10 ml media between shakes. The cell debris was pelleted by spinning the homogenate at 3000 rpm for 10 minutes in a Sorval RC-2B centrifuge G.S.A. rotor. The pellet was discarded and the supernatant recentrifuged until no more pellet was observed (usually 2-3 centrifugations). The mitochondria were sedimented by centrifugation at 14,000 rpm for 20 minutes in a Sorval RC-2B centrifuge SS34 rotor. The mitochondrial pellet was resuspended to $\sim$ 20 mg/ml protein concentration in isolation media. This suspension was layered on to a discontinuous sucrose gradient (14 ml each of 15%, 30%, 50% and 70% (w/v) sucrose solutions containing 20 mM Tris-Cl, pH 7.5; 1 mM EDTA) and centrifuged at 23,000 rpm for 3 hr in a Beckman L-2.50 centrifuge S.W. 25.2 rotor. The mitochondrial band at a density of 1.20 g/cm^3 was collected and diluted in mitochondrial isolation media (minus bovine serum albumin) and centrifuged as described above to pellet the mitochondria. This pellet was resuspended to 20 mg protein/ml and represents the purified mitochondrial preparation.

Submitochondrial particles were prepared by sonication of this suspension for 1 min (4 x 15 seconds with 1 minute intervals between each) at 4° C in an M.S.E. 60 W sonicator at maximum amplitude. The sonicated suspension was diluted with 2 volumes of buffer and centrifuged in a Sorval RC-2B centrifuge, SS34 rotor, 14,000 rpm for 20 minutes. The supernatant was then centrifuged at 100,000 g for 30 minutes in a Beckman L2-50 or L50 centrifuge. The submitochondrial pellet was then resuspended in 0.25M sucrose; 10 mM Tris-Cl, pH 7.5; 1 mM EDTA and washed by recentrifugation at 100,000 g for 30 minutes. The final pellet was resuspended in the same buffer to a concentration of 20 mg protein/ml.

Lyophilisation and pentane extraction procedures were based on methods originally described by Szarkowska (329) with substantial modifications which are described in the discussion section to this chapter.

Yeast strain E3-24 was grown under identical conditions to D22 except that growth media contained 2% glucose (w/v) and no ethanol. Cells were harvested after growth for 15 hr at 28-30° C.

Table 7.3 Effect of lyophilisation conditions on bovine heart mitochondrial activities

Mitochondrial preparation	Succinate oxidase[1]	Ascorbate/TMPD oxidase[1]	Respiratory control
Freshly prepared	197	98	Yes
Lyophilised			
Lyophilised in 5 mM $MgCl_2$, pH 7.5	193	95	No
Lyophilised in 5 mM $MgCl_2$; 2 mM ATP	170	67	No
Lyophilised in 5 mM $MgCl_2$; 2 mM ATP + 1 mM EDTA	182	63	No
Lyophilised in 5 mM $MgCl_2$; 2 mM ATP + 1 mM EDTA + 1 mM DTT	194	95	No
Lyophilised in 5 mM $MgCl_2$; 2 mM ATP + 1 mM EDTA + 1 mM DTT + 1 mg/ml BSA	186	64	No

[1] nmoles O_2 consumed/min/mg

(for legend to Table, see overleaf)

Legend to Table 7.3 (previous page)

Bovine heart mitochondria were suspended to 40 mg/ml protein in 5-10 ml buffer placed in a round bottomed quickfit flask and rapidly frozen as a thin film in liquid nitrogen. The contents were then lyophilised at R.T for 2-3 hr. Oxidase activities were assayed as described in the methods section to Chapter 7.

Table 7.4 Reconstitution of succinate oxidase activity in pentane extracted bovine heart mitochondria

Additions	Succinate oxidase activity nmoles O_2 consumed min/mg
None	0
Ubiquinone 9 (100 nmole)	189
Dihydrolipoate (1 µmole)	0
Cholesterol (100 nmole)	0
Oleic acid (100 nmole)	0
Phospholipids (asolectin) (1 mg)	0
Vitamin K_1 (100 nmole)	0
Ubiquinone 9 (20 nmole)	22

Assays containing 1 mg bovine heart mitochondria were preincubated with lipids for 15 minutes at 30° C in a shaking water bath in 1 ml 0.25 M sucrose; 10 mM Tris-Cl, pH 7.5; 5 mM $MgCl_2$; 5 mM potassium phosphate; 10 mM potassium chloride, and then transferred to an oxygen electrode assay chamber (final volume, 2 ml of above buffer). Succinate oxidase activity was assayed as described in methods section to this chapter.

Results

Several lyophilisation and pentane extraction procedures were used to facilitate ubiquinone extraction from mitochondrial membranes. Lyophilisation media which proved most useful in these investigations were ones designed to protect the ATPase from disruption particularly those containing magnesium chloride, ATP, DTT and EDTA (Table 7.3). It was important to ensure that mitochondria were thoroughly washed in these media prior to lyophilisation as the presence of sucrose and buffers such as 'Tris' produced preparations that required long periods of lyophilisation and which could only be poorly extracted by pentane. Lyophilisation was usually only necessary for 1-3 hours when small volumes of mitochondrial suspensions were used, and was carried out by freezing 5-10 ml of mitochondria (20 mg protein/ml) as a thin film in liquid nitrogen in a round bottomed 250 ml quickfit flask. This flask was then connected to a freeze dryer (Virtis freeze drier, Virtis Co. Gardiner, N.Y. 12525, U.S.A.) and removed after lyophilisation was complete. The lyophilised mitochondria were then resuspended in redistilled n-pentane (30 ml/100 mg protein). The suspension was then shaken for 3 hr at 4° C, after which time it was separated by centrifugation (5000 rpm for 10 minutes, RC-2 B centrifuge SS34 rotor). The supernatant was kept; the mitochondria were resuspended in pentane (30 ml/100 mg protein) and shaken for 1 hr at 4° C. This latter step was repeated two more times. The supernatants were pooled, evaporated to dryness and the residue dissolved in a minimum volume of ethanol. This complex solution was hereafter termed 'pentane extract'. Any remaining n-pentane was removed from the mitochondria by rotary evaporation. The dried mitochondria were either used immediately or stored at -20° C. Before use, lyophilised mitochondria were resuspended in lyophilisation medium to 1-5 mg protein/ml and washed in this buffer twice before being washed in 250 mM sucrose; 10 mM

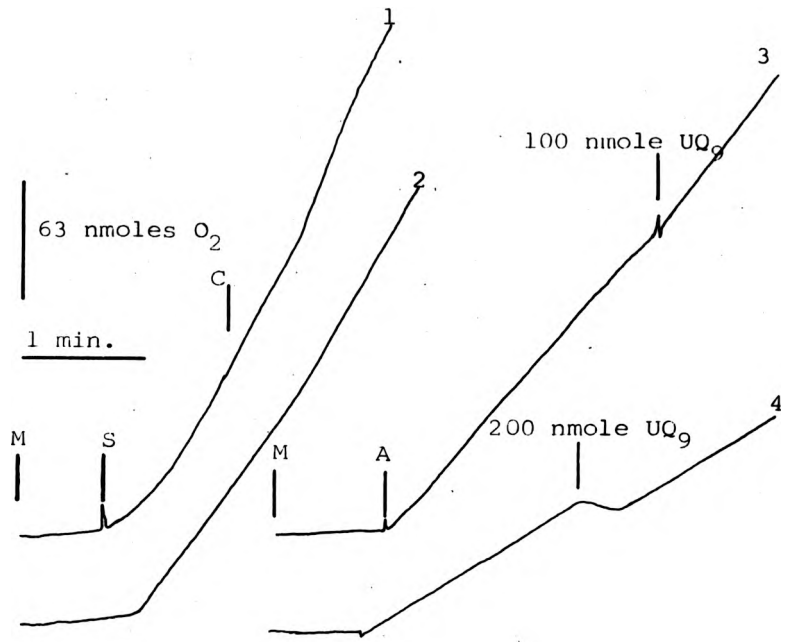


Fig 7.2 Oxidase activities in pentane extracted bovine heart mitochondria.

Assays containing 1mg of protein (except assay 4, ½ mg protein) were measured in a Clarke type O_2 electrode as described in the methods section ch.7. Assays 1&2 were preincubated with UQ_9 (100nmole/mg protein) for 15 min @30°C in a shaking water bath prior to assay.

S : 10 µmoles sodium succinate pH 7.5 .

A : 10 µmoles sodium D iso ascorbate/ 1 µmole TMPD.

C : 0.5 mg cytochrome C.

M : mitochondria.

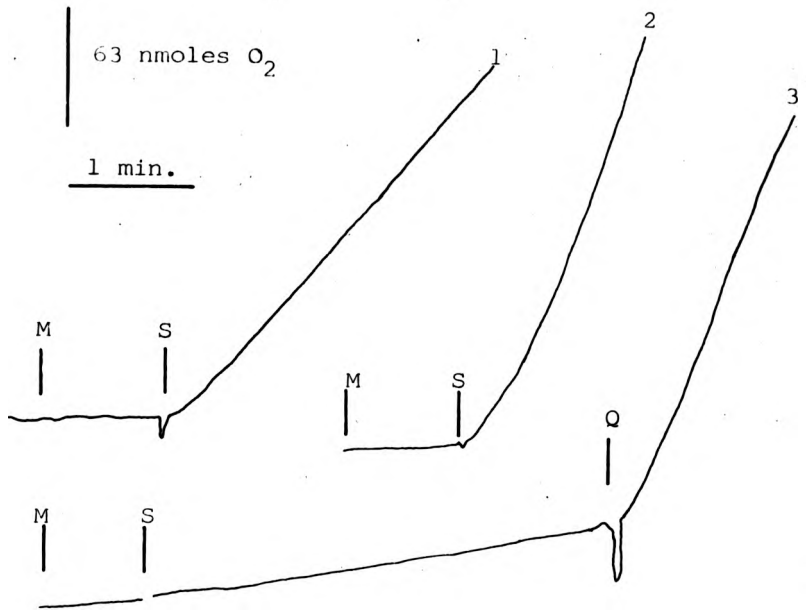


Fig 7.3 Succinate oxidase activity in pentane extracted bovine heart mitochondria:effect of ubiquinone by direct addition and preincubation.

Oxidase activities were monitored in a Clarke type O_2 electrode as described in the methods section to chapter 7.

S : 25 µmoles sodium succinate pH 7.5.

Q : 200 nmoles ubiquinone-9.

1. 1mg normal mitochondria

2. 1mg pentane extracted mitochondria,princubated with 200 nmoles ubiquinone-9,see fig 7.2.

3. 1mg pentane extracted mitochondria.

M : mitochondria.

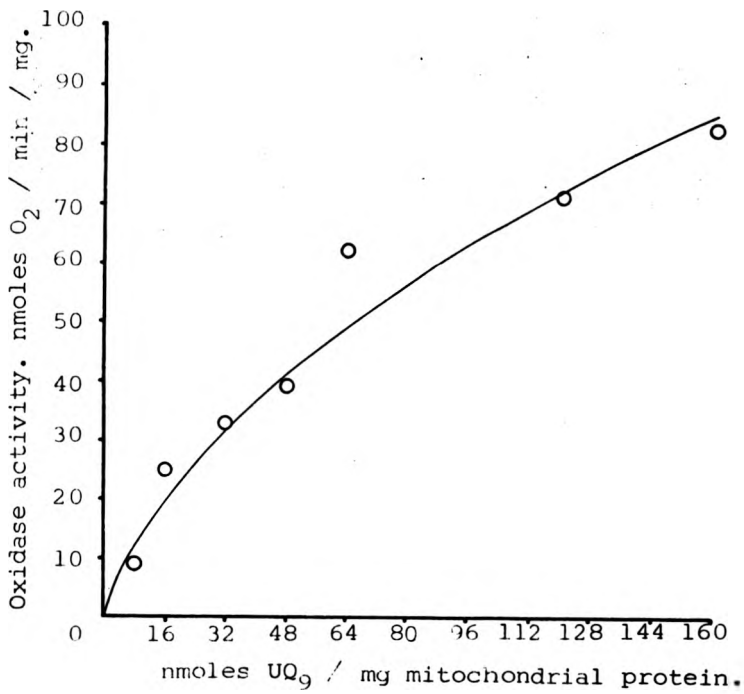


Fig7.4.Succinate oxidase activity in pentane extracted bovine heart mitochondria:effect of UQ_9.

Succinate oxidase activity was monitored as described in fig 7.3.

UQ_9 (added as a 20mM solution in ethanol) was preincubated with 2.5 mg mitochondrial protein in 4mls 0.25M sucrose:10mM Tris-Cl pH 7.5:1mM EDTA for 10 minutes at 30°C in a shaking water bath. 2ml samples were then taken into the reaction chamber of a Clarke type oxygen electrode and assayed for succinate oxidase activity.

Assays were initiated by the addition of 10 µmoles sodium succinate pH 7.5

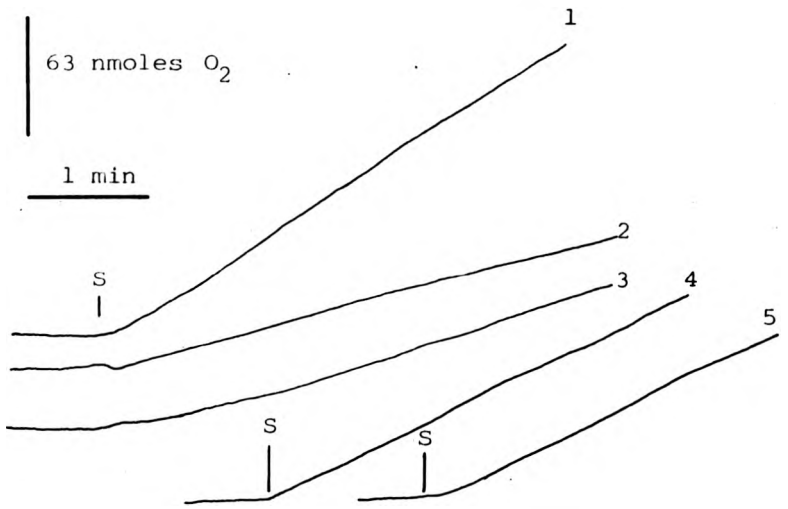


Fig 7.5. Stimulation of succinate oxidase in pentane extracted bovine heart mitochondria:effect of DBCT.

Assays containing 1mg mitochondrial protein were monitored in a Clarke type O_2 electrode as described in the methods section to chapter 7.

S : 10 µmoles sodium succinate pH 7.5.

1 . Pentane extracted mitochondria,preincubated with pentane extract (= to 2nmoles UQ.).

2 . As 1. but preincubated with 2 nmoles UQ.

3. As 1. but pentane extract preincubated with 3 nmoles DBCT prior to addition to assay.

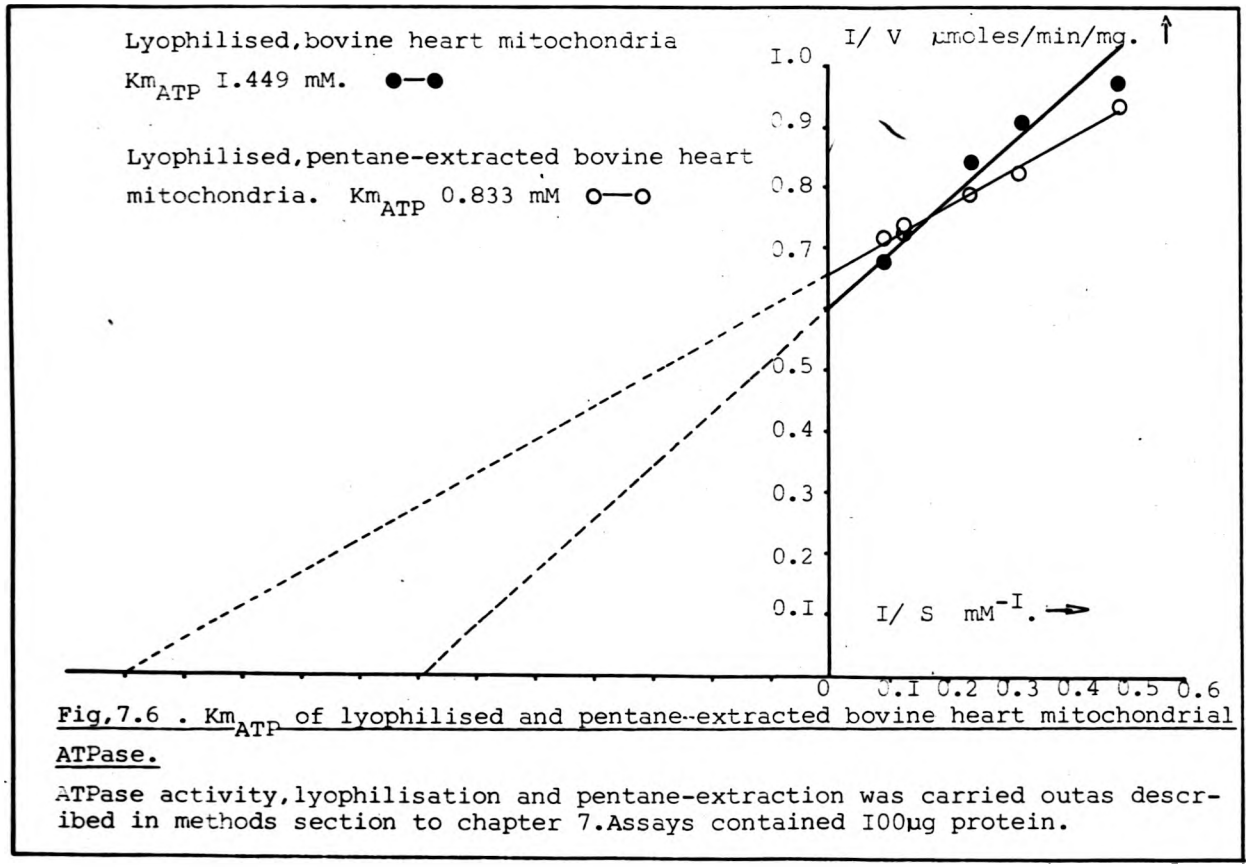
4. Pentane extracted mitochondria preincubated with 3 nmoles DBCT prior to addition of pentane extract as in 1.

5. As 1 then 3 nmoles DBCT added prior to sodium succinate.

Tris-HCl; 1 mM EDTA; 5 mM $MgCl_2$ twice, and resuspended in the same to a final concentration of 20 mg protein/ml. Pentane extract or ubiquinone was reincorporated into mitochondria by one of three methods. These were (i) direct addition back to the mitochondria in a simple preincubation step prior to initiation of assay, (ii) addition back in a preincubation carried out in a shaking water bath on a batch of mitochondria prior to start of an experiment, (iii) addition of 'pentane extract' to a suspension of mitochondria in n-pentane, shaking for 30 minutes at 4° C and then removing pentane by rotary evaporation.

Pentane extraction under these conditions produces a mitochondrial preparation that is minimally disrupted by the physical techniques used. Removal of ubiquinone in this way completely inhibits the succinoxidase activity of the mitochondria, whereas ascorbate/TMPD oxidase activity is left largely unaffected (Fig. 7.2). Cytochrome c has no stimulatory effect on succinoxidase activity in ubiquinone reconstituted, pentane extracted mitochondria, indicating that the inner membrane system has been minimally disrupted by the lyophilisation and extraction techniques used (Fig. 7.2). Succinoxidase activity can be reconstituted by addition back of pentane extract or ubiquinone to extracted particles, (Figs. 7.3-4); other lipid compounds do not restore this activity (Table 7.4). Ubiquinone 9 added back, either as a direct addition to the assay or by incorporation into a suspension of mitochondria in pentane, reconstitutes activity, the latter being slightly the more efficient method (Fig. 7.3).

Investigations showed that 'pentane extract' appeared to be more effective in reconstituting succinoxidase activity than would be expected from its ubiquinone content. Its efficacy in reconstituting succinoxidase could be reduced to that expected from its ubiquinone content it it was incubated with dibutylchloromethyltin chloride, an inhibitor of energy linked fuctions in bovine heart mitochondria, prior to addition to assay (Fig. 7.5).



Fig,7.6 . Km_{ATP} of lyophilised and pentane-extracted bovine heart mitochondrial ATPase.

ATPase activity,lyophilisation and pentane-extraction was carried outas described in methods section to chapter 7.Assays contained I00µg protein.

Table 7.5 Pentane extraction of bovine heart mitochondria: effect of inhibitors

Additions	ATPase specific activity[1]	% Activity
Lyophilised mitochondria	1.08	100
Lyophilised mitochondria + oligomycin	0.03	2.7
Lyophilised mitochondria + venturicidin	0.03	2.7
Lyophilised mitochondria + DCCD	0.03	2.7
Pentane extracted mitochondria	1.48	100
Pentane extracted mitochondria + oligomycin	1.27	85
Pentane extracted mitochondria + venturicidin	0.45	30
Pentane extracted mitochondria + DCCD	1.16	78
Pentane extracted, ubiquinone reconstituted mitochondria	1.46	100
Pentane extracted, ubiquinone reconstituted mitochondria + oligomycin	0.04	2.7
Pentane extracted, ubiquinone reconstituted mitochondria + venturicidin	0.10	6.8
Pentane extracted, ubiquinone reconstituted mitochondria + DCCD	0.23	15.7

[1] µmoles ATP hydrolysed/min/mg

Assays containing 80 µg lyophilised mitochondria or 125 µg pentane extracted and pentane extracted/ubiquinone reconstituted mitochondria in 1 ml 50 mM Tris-Cl, pH 8.5; 5 mM $MgCl_2$ were preincubated with inhibitors (1 µg/assay) for 5 minutes at 35° C prior to initiation of assay with 5 µmoles ATP. Assays were terminated after 5 minutes by addition of 0.5 ml 10% T.C.A. Phosphate released was assayed as

(continued on next page)

described in Chapter 3. Ubiquinone was reincorporated into mitochondria by addition of an ethanolic solution of ubiquinone to mitochondria suspended in a minimum volume of n-pentane which was shaken for 10 minutes at 4° C. Pentane and ethanol were removed by rotary evaporation. The mitochondria were washed and resuspended in 0.25 M sucrose; 10 mM Tris-Cl, pH 7.5; 1 mM EDTA; 5 mM $MgCl_2$ to 20 mg protein/ml. Ubiquinone was added back to a level of 100 nmoles/mg mitochondrial protein.

Table 7.6 Effect of pentane extraction on ATP-^{33}Pi exchange activity in bovine heart mitochondria

Preparation	Specific activity nmoles/min/mg
Lyophilised mitochondria	52.0
Pentane extracted mitochondria	8.6
Pentane extracted mitochondria + ubiquinone 9 (10 nmole)	23.9
Pentane extracted mitochondria + ubiquinone 9 (20 nmole)	36.0
Pentane extracted mitochondria + pentane extract (2 nmole[1])	28.7

[1] Pentane extract added contains 2 nmoles ubiquinone

Assays containing 1 mg mitochondrial protein were preincubated with ubiquinone where indicated for 10 minutes in a shaking water bath at 35° C, prior to initiation of assay with 12 µmoles ATP. Assays were terminated after 5 minutes incubation at 30° C with 200 µl 30% P.C.A. ATP-^{33}Pi exchange activity was determined as described in Chapter 3.

Table 7.7 Effect of inhibitors on ATP-^{33}Pi exchange activity in pentane extracted bovine heart mitochondria

Preparation	Specific activity nmoles/min/mg
Pentane extracted mitochondria	31.39
Pentane extracted mitochondria + ubiquinone 9 (10 nmole)	53.29
Pentane extracted mitochondria + ubiquinone 9 (20 nmole)	67.89
Pentane extracted mitochondria + pentane extract (10 nmole[1])	21.9
Pentane extracted mitochondria + ubiquinone 9 (10 nmole) + oligomycin	0
Pentane extracted mitochondria + ubiquinone 9 (10 nmole) + TTFB	0
Pentane extracted mitochondria + ubiquinone 9 (10 nmole) + DBCT	0

[1] Pentane extract added contains 10 nmoles ubiquinone

Lyophilised mitochondria sp. act. 61 nmoles/min/mg

Assays containing 1 mg mitochondrial protein were preincubated with ubiquinone where indicated for 10 minutes in a shaking water bath at 35° C. Inhibitors (5 µg/mg protein) were added and preincubation was continued for 5 minutes at 30° C. Assays were initiated with 12 µmoles ATP and were terminated by addition of 200 µl 30% P.C.A. after incubation for 5 minutes at 30° C. ATP-^{33}Pi exchange activity was determined as described in Chapter 3.

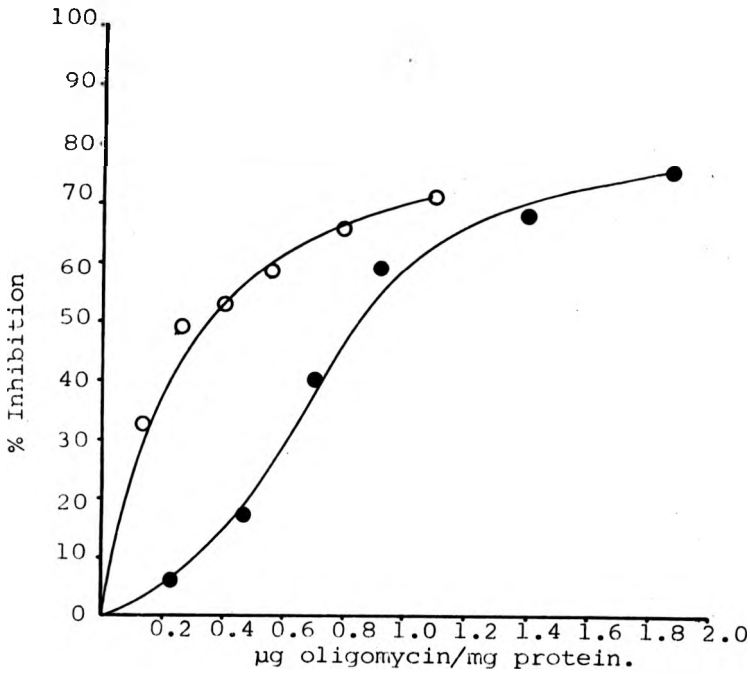


Fig 7.7.Effect of pentane extraction on oligomycin inhibition of bovine heart mitochondrial ATPase.

Assays were preincubated with oligomycin for 5 min at 30°C in 1ml 50mM Tris-Cl pH 8.5;5mM $MgCl_2$prior to addition of 5 µmoles ATP.Assays were terminated after incubation at 30°C for 5 minutes, by addition of 0.5 ml 10% TCA.Phosphate was determined as described in the methods section to chapter 3.

O—O lyophilised mitochondria.

●—● pentane extracted mitochondria.

Specific activity lyophilised mitochondrial ATPase = 1.24 µmoles / min / mg mitochondrial protein (uninhibited).

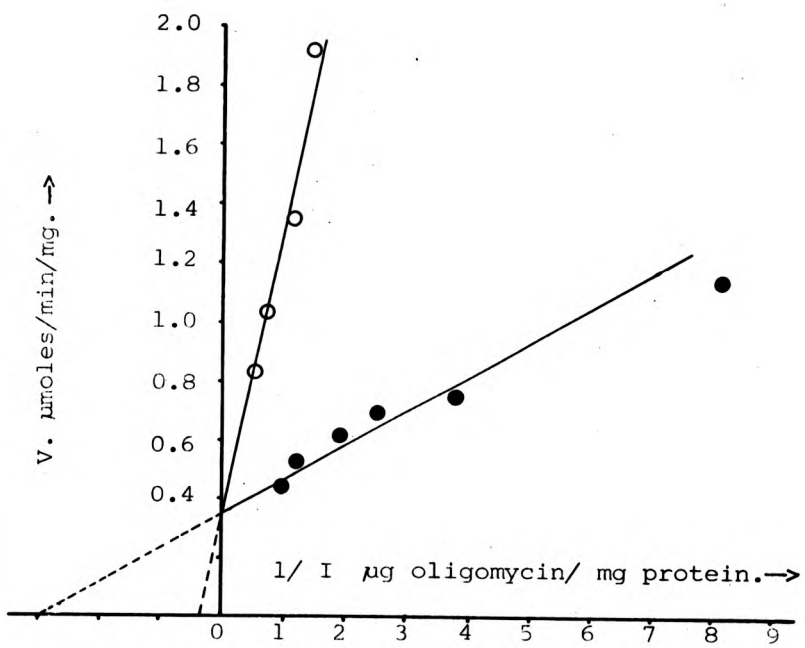


Fig 7.8. Dixon plots of oligomycin inhibition of bovine heart mitochondrial ATPases.

ATPase activity was determined as described in fig 7.7.

●—● lyophilised mitochondria.

○—○ pentane extracted mitochondria.

Ki = 0.325 µg oligomycin/mg lyophilised mitochondrial protein.

Ki = 3.22 µg oligomycin/mg pentane extracted mitochondrial protein.

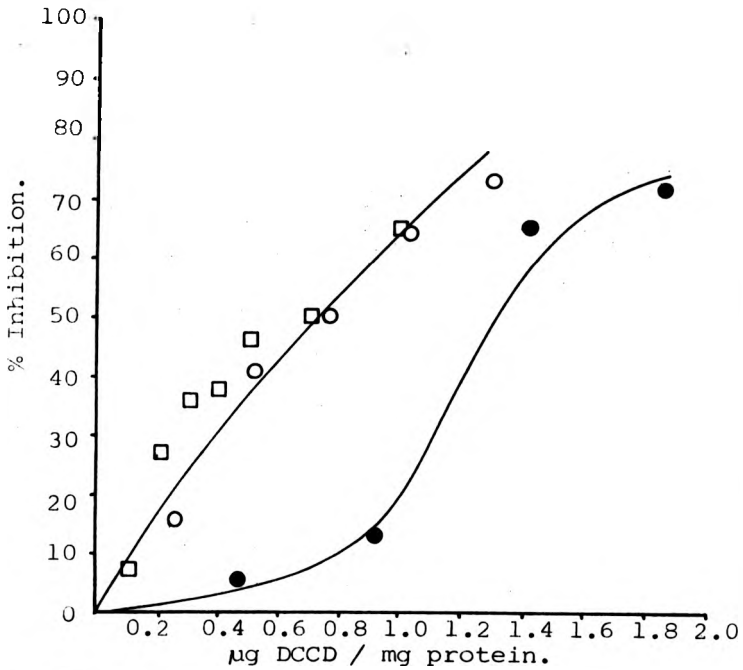


Fig 7.9 Effect of pentane extraction on the DCCD sensitivity of bovine heart mitochondrial ATPase.

Assays were preincubated with DCCD for 5 minutes at 30°C in 1ml 50 mM Tris-Cl pH 8.5; 5mM $MgCl_2$ prior to addition of 5 µmoles ATP. Assays were terminated after 5min @ 30°C by addition of 0.5 ml 10% TCA. Phosphate was determined as described in chapter 3. Specific Activity lyophilised mito ATPase= 0.89.

O—O lyophilised mitochondria.

●—● pentane extracted mitochondria.

□—□ ", reconstituted with ubiquinone-9 incorporated by preincubation (100nmole/mg protein) in a shaking water bath at 30°C for 15 min prior to addition of DCCD.

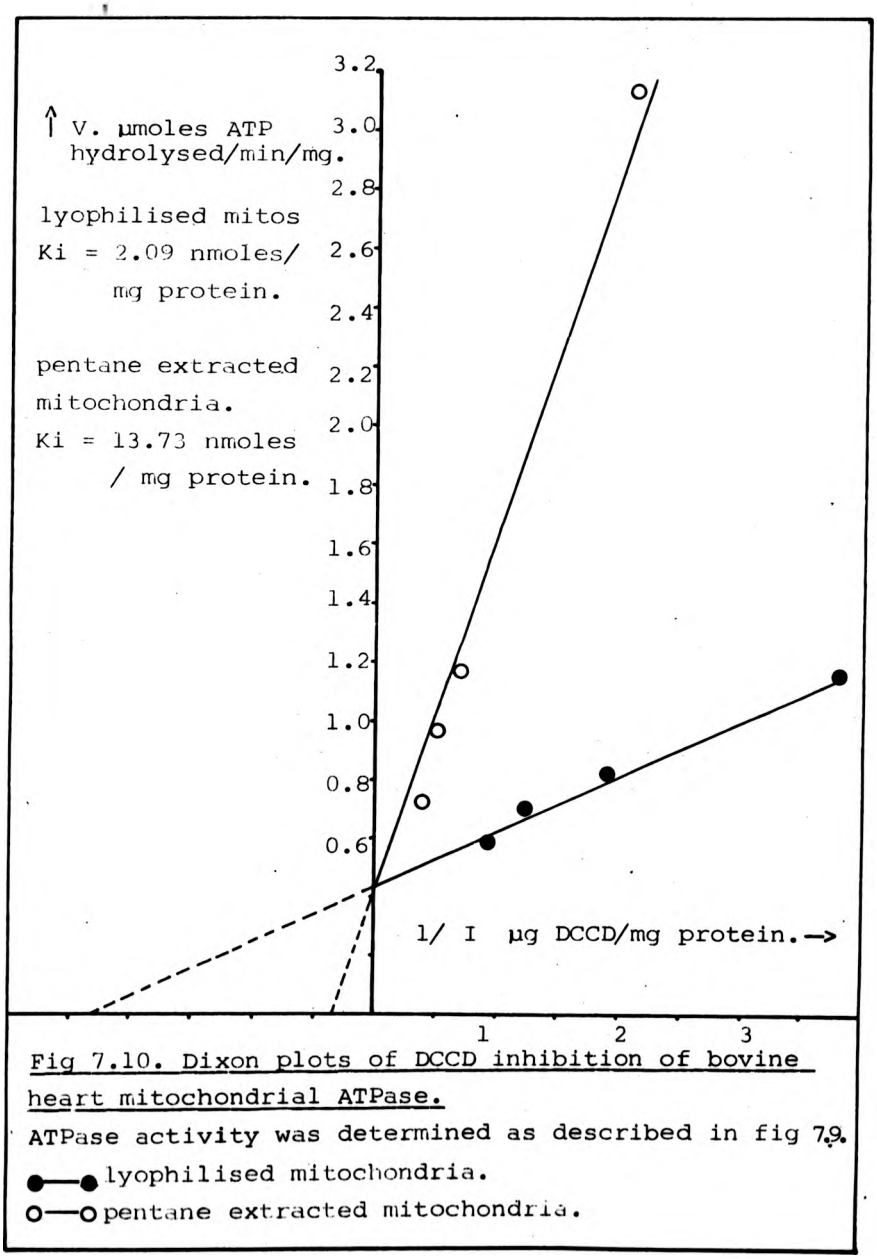


Fig 7.10. Dixon plots of DCCD inhibition of bovine heart mitochondrial ATPase.

ATPase activity was determined as described in fig 7.9.

●—● lyophilised mitochondria.

○—○ pentane extracted mitochondria.

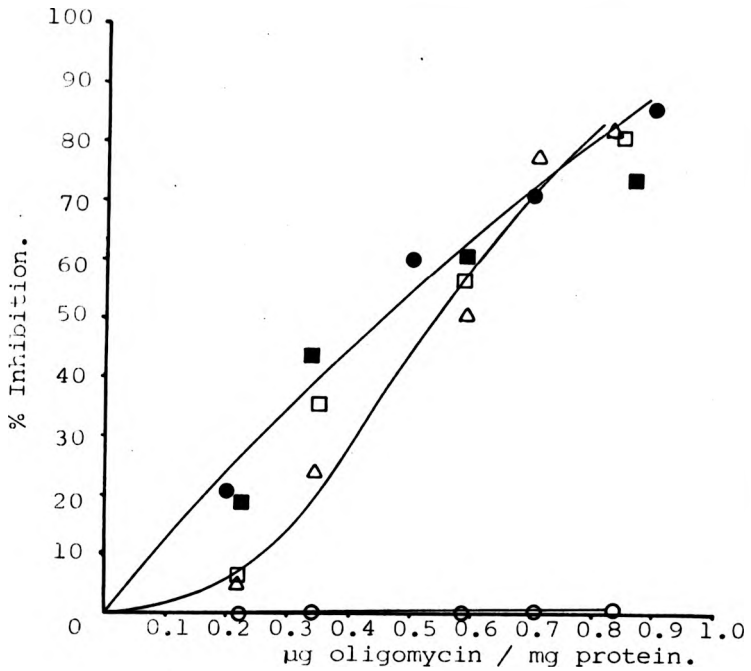


Fig 7.11. Reconstitution of oligomycin sensitivity of pentane extracted bovine heart mitochondrial ATPase.

ATPase activity was determined as described in fig 7.7.

●—● lyophilised mitochondria.

○—○ pentane extracted " assayed immediately after preparation.

△—△ as above assayed after standing for 6hr at 4°C.

■—■ pentane extracted mitochondria reconstituted ubiquinone-9 , assayed immediately. (100nmole/mg).

□—□ as above , but reconstituted with pentane extract (100 nmoles ubiquinone / mg protein.).

Specific activity lyophilised mitochondrial ATPase = 1.43 µmoles / min / mg protein (uninhibited).

Table 7.8 Effect of acetone extraction on bovine heart mitochondrial ATPase activity

Mitochondrial preparation	Specific activity[1]
Normal mitochondria	0.666
'Acetone extracted' mitochondria	0.894
'Acetone extracted, ubiquinone reconstituted mitochondria	0.866
'Acetone extracted, acetone extract reconstituted' mitochondria	1.075

[1] µmoles ATP hydrolysed/min/mg

Reconstitution conditions:-

Ubiquinone 9 reconstitution:- 1 ml acetone extracted mitochondria (11 mg) were incubated with 1 µmole ubiquinone 9 (20 µl of a 50 mM solution in ethanol) in a shaking water bath for 10 minutes at 30° C.

Acetone extract reconstitution:- Acetone extract was vacuumed down to remove acetone. The remaining water was taken to 66% ethanol with absolute ethanol. 1 ml acetone extracted mitochondria + 1 ml 0.25 M sucrose; 10 mM Tris-Cl, pH 7.5; 5 mM $MgCl_2$; 5 mM $MnCl_2$ + 50 µl acetone extract were incubated for 10 minutes at 30° C in a shaking water bath.

ATPase activity was determined as described in Table 7.5

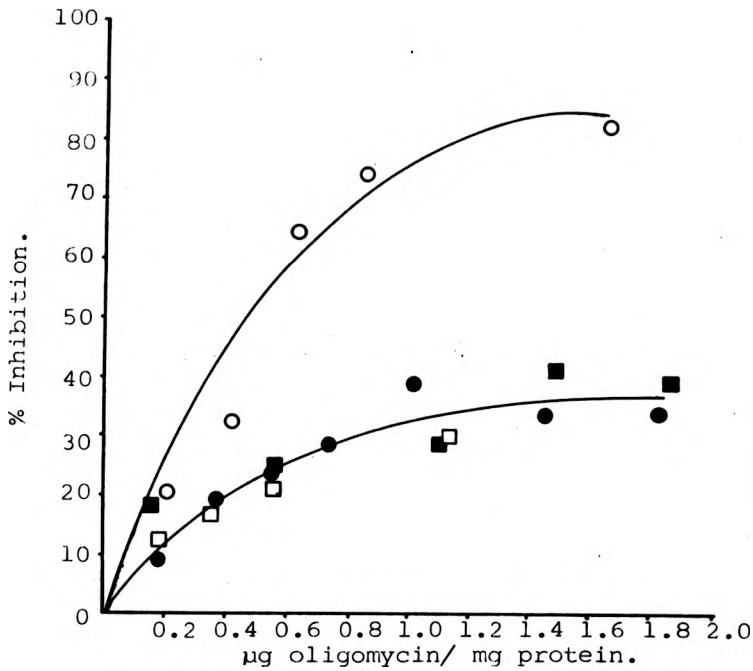


Fig 7.12. Effect of acetone extraction on the oligomycin sensitivity of bovine heart mitochondrial ATPase.

Assays were performed as described in fig 7.7. Acetone extraction procedures were those described in tab 7.8.

O—O normal mitochondria.

●—● acetone extracted mitochondria.

□—□ as above, UQ-1 reconstituted see table 7.8.

■—■ as above but pentane extract reconstituted see table 7.8.

Table 7.9 Lack of reconstitution of oxidative phosphorylation in acetone extracted bovine heart submitochondrial particles (ETP_H)

Additions	Oxidative phosphorylation
ETP_H + succinate	68.7
Acetone extracted ETP_H	0
Acetone extracted ETP_H + ubiquinone 9 (100 nmol)	0
Acetone extracted ETP_H + ubiquinone 9 (100 nmol) + dihydrolipoate (100 nmol) + oleate (30 nmol)	0
Acetone extracted, acetone extract reconstituted ETP_H	0

[1] nmoles/min/mg ATP synthesis measured as phosphate disappearance

Assays containing 0.5 mg submitochondrial protein and 1 µg rotenone were preincubated with lipids, where indicated, for 5 minutes at 30° C in a shaking water bath prior to initiation of assay with 10 µmoles sodium succinate, pH 7.5. Oxidative phosphorylation was assayed in a 1 ml glucose hexokinase trap system, 0.2 mM in ADP, as described in Chapter 3.

ETP_H were acetone extracted by addition of 0.5 ml ETP_H (10 mg/ml) to 1 ml 100% acetone, whirlimixing suspension and removing protein by centrifugation. Acetone extract reconstitution performed essentially as described in legend to Table 7.8.

The effect of pentane extraction on the activities of the ATPase enzyme was investigated. Pentane extraction produces a slight stimulation of ATPase activity in bovine heart mitochondria. This stimulated ATPase also loses its sensitivity to the inhibitors oligomycin and DCCD after extraction. If ubiquinone is reconstituted into the particles, sensitivity to ubiquinone and DCCD is regained (Table 7.5). Pentane extraction reduces the K_m value for ATP of the enzyme, indicating a higher affinity for ATP (Fig. 7.6). Extracted particles when reconstituted with ubiquinone 9 or 'pentane extract' regain their sensitivity to oligomycin and DCCD (Figs. 7.7-9). Dixon plots indicate that extraction reduces the affinity of the enzyme for these inhibitors (Figs. 7.8, 7.10). If pentane extraction was not complete, then the ATPase did not lose its sensitivity to oligomycin and DCCD. Extracted particles left to stand at 4^{o} C for 6 hrs regained full sensitivity to inhibition by oligomycin (Fig. 7.11).

ATP-^{33}Pi exchange activity in bovine heart mitochondria was reduced or abolished by pentane extraction. The activity could be reconstituted by either ubiquinone 9 or pentane extract (Table 7.6) and was fully sensitive to uncouplers and ATPase inhibitors (Table 7.7).

Acetone can also be used as a solvent system to extract ubiquinone from the mitochondrial membrane. Extraction stimulates ATPase activity and also reduces its sensitivity to oligomycin. This sensitivity is not restored by readdition of pentane extract' or ubiquinone 9 to the extracted particles (Table 7.8 and Fig. 7.12). Oxidative phosphorylation is abolished by acetone extraction and is not reconstituted by addition of ubiquinone 9 or 'pentane extract' (Table 7.9).

Pentane extraction of yeast mitochondria or submitochondrial particles has similar effects on them as on bovine heart mitochondria. ATPase activity is slightly stimulated; this stimulation is reduced by

Table 7.10 Effect of pentane extraction on yeast submitochondrial particle ATPase activity

Preparation	ATP hydrolysis[1]
Yeast submitochondrial particle lyophilised	2.85
Pentane extracted submitochondrial particles	3.68
Pentane extracted, ubiquinone reconstituted submitochondrial particles	2.99
Pentane extracted, pentane extract reconstituted submitochondrial particles	2.94

[1] µmoles ATP hydrolysed/min/mg

ATPase activity was in a buffer 50 mM in Tris-Cl, pH 9.5; 5 mM $MgCl_2$ essentially as described in Chapter 3.

Yeast SMP were washed in a buffer 10 mM in $MgCl_2$, 1 mM EDTA, 1 mM ATP twice before being resuspended to a concentration of 10 mg protein/ml in the same buffer. This suspension was then frozen as a thin film in liquid nitrogen and lyophilised for 3 hr at R.T. The SMP preparation was then homogenised in n-pentane (30 ml/100 mg protein) and shaken in the same for 1 hr at 4° C. This procedure was repeated two more times. Pentane extracts were pooled and vacuumed to dryness, the residue being dissolved in a minimum volume of absolute ethanol. Extracted SMP's were lyophilised to remove pentane and were washed twice in a buffer system 0.25 mM in sucrose; 10 mM Tris-Cl, pH 7.5; 1 mM EDTA; 5 mM $MgCl_2$ before being resuspended in the same buffer system to a protein concentration of 10 mg/ml. Ubiquinone and pentane extract were reconstituted with SMP's by incubating together in a shaking water bath for 15 minutes at 35° C.

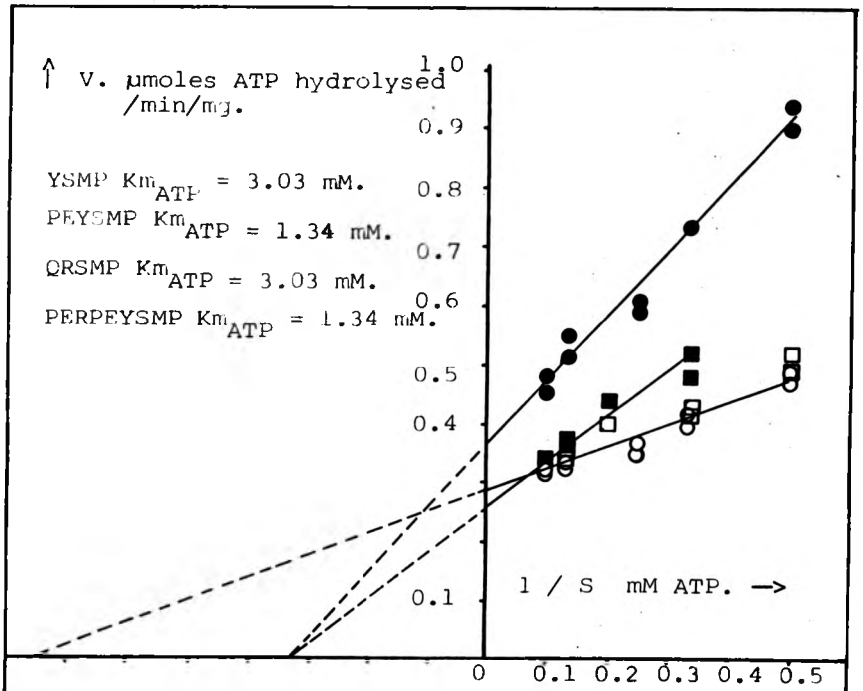


Fig 7.13. Km_{ATP} determinations for yeast SMP ATPase.
ATPase activity was determined as described in the methods section to ch.3.

●—● yeast submitochondrial particles, YSMP.

○—○ pentane extracted yeast SMP , PEYSMP.

■—■ as above , UQ-9 reconstituted 100 nmoles UQ/mg QRSMP.

□—□ as above but reconstituted with pentane extract (100 nmoles UQ / mg protein). PERPEYSMP.

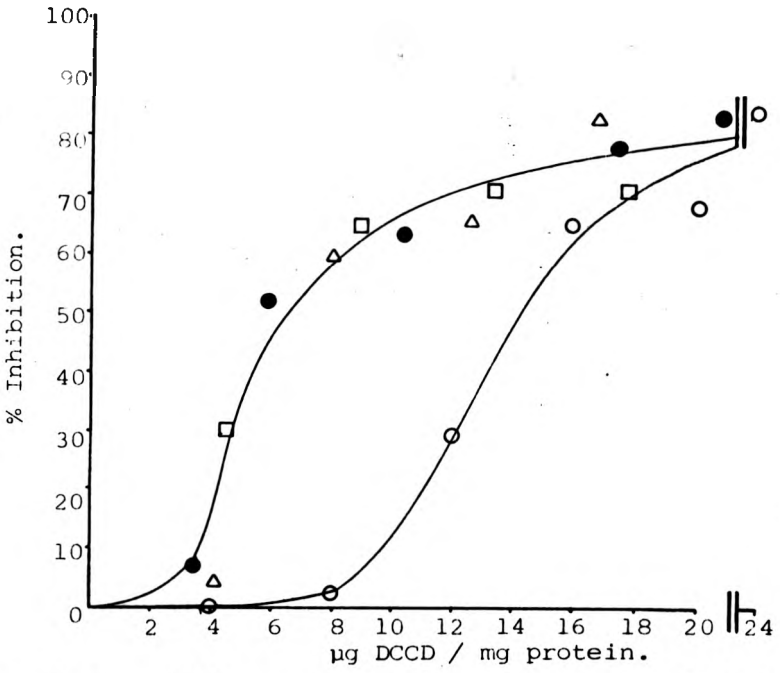


Fig 7.14. DCCD. inhibition of yeast SMP ATPase: effect of pentane extraction, ubiquinone and pentane extract reconstitution.

Assays were performed as described in fig 7.7 in a 50 mM Tris-Cl pH 9.0; 5 mM $MgCl_2$ buffer system. Reconstitution was performed as described in fig 7.13.

●—● YSMP.
○—○ PEYSMP.
□—□ QRYSMP.
△—△ PERPEYSMP.

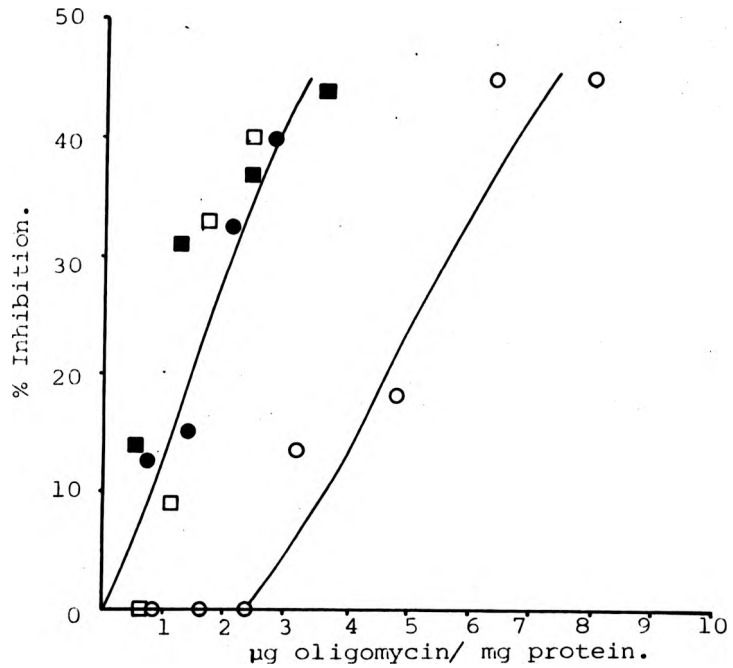


Fig 7.15 Oligomycin inhibition of yeast SMP ATPase: effect of pentane extraction , ubiquinone and pentane extract reconstitution.

Assays were performed essentialy as described in fig 7.9 in a 50 mM Tris-Cl pH 9.0;5 mM $MgCl_2$ buffer system.

●—● YSMP.

○—○ PEYSMP.

■—■ QRYSMP.

□—□ PERPEYSMP.

Table 7.11 Comparison of specific activities of yeast submitochondrial ATPase from two yeast strains

Yeast strain		Specific activity[1]
D273-10B	preparation 1	2.03
E2-34 (ubiquinone-less mutant)	preparation 1	1.16
D273-10B	preparation 2	2.04
E2-34	preparation 2	0.675

[1] μmoles ATP hydrolysed/min/mg

ATPase activity was assayed in a buffer 50 mM Tris-Cl, pH 8.5; 5 mM $MgCl_2$ essentially as described in Chapter 3.

Table 7.12 ATP-^{32}Pi exchange activities in yeast mitochondria from two yeast strains

Strain	Rate[1]	
	preparation 1	preparation 2
D273-10B	53.37	49.87
E2-34	0.69	0.70

[1] nmoles ^{32}Pi exchanged/min/mg

Assays containing 1 mg mitochondria were performed essentially as described in Chapter 3.

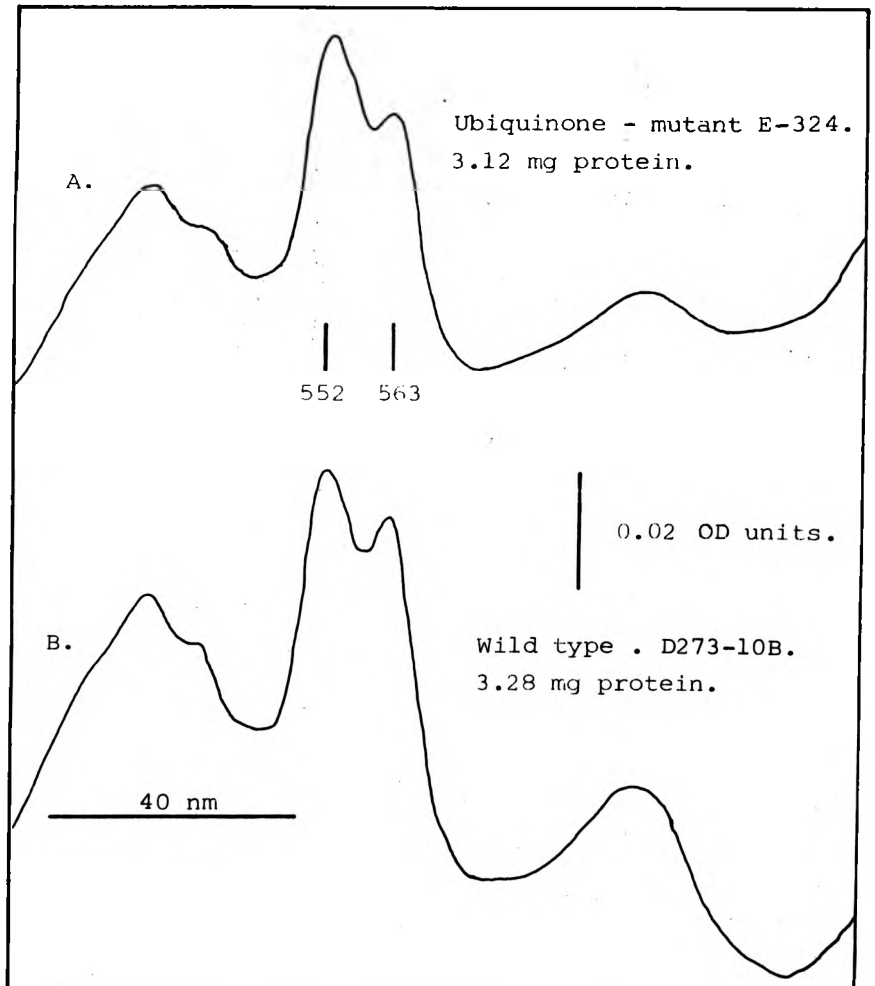


Fig 7.16. Yeast mitochondria cytochrome spectra.
Spectra were recorded in a 1 ml volume on a Pye-Unicam SP1800 uv-visible spectrophotometer. $Fe(CN)_6^{3-}$ oxidised reference cuvette vs. $Na_2S_2O_4$ reduced.

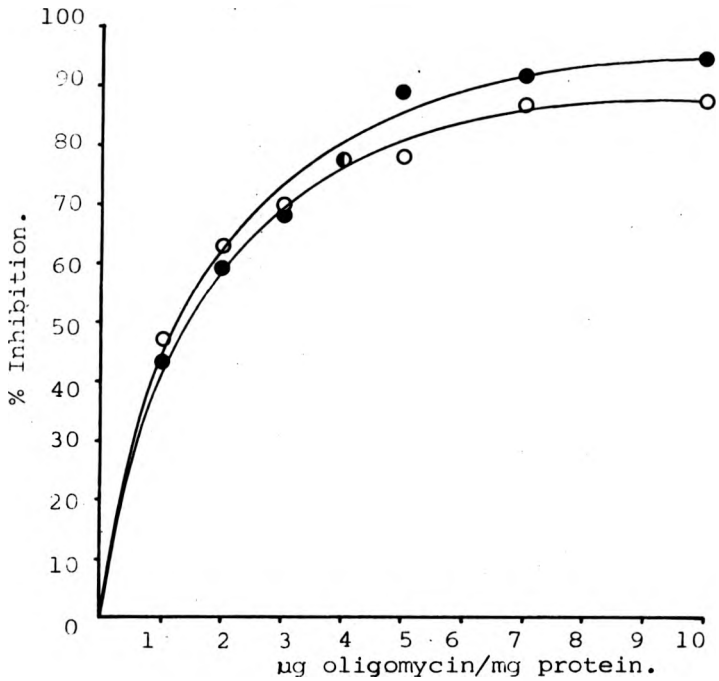


Fig 7.17 Oligomycin inhibition of yeast mitochondrial ATPase.

Assays were performed as described in fig 7.14.

O—O wild type yeast mitochondria I_{50} =1.27 µg / mg strain D273-10B.

●—● ubiquinone - mutant mitochondria:strain E-324. I_{50} = 1.20 µg oligomycin / mg protein.

For specific activities of uninhibited ATPases see table 7.11.

reincorporation of ubiquinone 9 or 'pentane extract' (Table 7.10). The K_m value for ATP in yeast SMP is reduced by pentane extraction and increased back to the 'normal' value by subsequent reincorporation of ubiquinone 9 or 'pentane extract' (Fig. 7.13). Sensitivity to oligomycin and DCCD in yeast SMP is reduced by pentane extraction and restored by addition of ubiquinone 9 or 'pentane extract' to extracted particles (Figs. 7.14-15).

The effects of pentane extraction suggested that ubiquinone might be involved in some way with the membrane bound portions of the ATPase enzyme (F_o). To further investigate this,various activities were assayed in a ubiquinone-less mutant of the yeast Saccharomyces cerevisiae wild type strain D273-10B, termed mutant 'E2-34'. This mutant has a full complement of cytochromes (Fig. 7.16). It also has a lowered ATPase activity and a very low rate of ATP-^{32}Pi exchange (Tables 7.11-12). It does not, however, display a lowered sensitivity to inhibition by oligomycin (Fig. 7.17).

Discussion

Several solvent systems have been used to extract ubiquinone from the mitochondrial membrane in a manner which will allow its subsequent readdition and (hopefully) reconstitution of enzymic activities. These include iso-octane (137), acetone (330) and n-pentane (329, 122). Iso-octane extraction is not a particularly useful technique as it not only does not remove all the ubiquinone but it is also retained by the particles and inhibits succinoxidase activity (92). Acetone extraction is more effective in removing ubiquinone but also removes many other lipids from the membrane, most notably phospholipids. Thus it is not possible to ascribe the effects of acetone extraction solely to the removal of ubiquinone. Reconstitution of oxidase activities requires not only ubiquinone but also cytochrome c (and occasionally phospholipids) (330). Extraction using n-pentane was introduced by

Szarkowska and taken up by other workers. It proved to be a far more efficient and specific method of removing ubiquinone from mitochondria than either iso-octane or acetone extraction (329, 122). Mitochondria have to be lyophilised before extraction with n-pentane. Szarkowska (329) and Lenaz et al. (122) both used a lyophilisation medium, 150 mM in KCl. This unfortunately results in a preparation that lacks respiratory control and oxidative phosphorylation. Several media were investigaged in order to discover if these activities could be protected in some way and thus be retained after lyophilisation of bovine heart mitochondria. These included systems containing respiratory chain substrates (succinate, pyruvate/malate and ascorbate) and ADP and ATP. None of these were able to prevent loss of respiratory control and oxidative phosphorylation on lyophilisation. The most effective lyophilisation media tested were those containing 5-10 mM $MgCl_2$. Mitochondria lyophilised under these conditions retained high succinoxidase and ascorbate/TMPD oxidase activity prior to pentane extraction (Table 7.3) and good ascorbate / TMPD oxidase activity after extraction. Mitochondria lyophilised in this system and then pentane extracted appeared to be less severely disrupted by solvent extraction as they did not require addition of cytochrome c or phospholipids as well as ubiquinone to reconstitute full succinate oxidase activity (Fig. 7.2).

Succinoxidase activity can be restored in pentane extracted mitochondria by reincorporation of ubiquinone or 'pentane extract'. The lyophilisation methods used allow reincorporation either to be done directly, that is by addition of ubiquinone immediately prior to assay, or by preincubation steps,both of roughly equal efficacy. A range of other compounds was tested for the ability to reconstitute succinate oxidase activity in pentane extracted mitochondria; only ubiquinone was able to do so, showing that reconstitution was not due to a 'general

lipid activation' effect on the extracted membrane. During the course of experiments on the reconstitution of succinoxidase activity in extracted mitochondria, it was noticed that some 'pentane extracts' (notably those prepared from mitochondria lyophilised in the presence of ATP and dithiothreitol) were more effective in restoring activity than would be expected from their ubiquinone content. If these pentane extracts were preincubated with an equimolar or slight molar excess (compared to ubiquinone content) of DBCT prior to addition to the assay, then they lost this ability and would only stimulate succinate oxidase activity to a level expected from their ubiquinone content. Pre-incubation of lyophilised and lyophilised, extracted mitochondria with DBCT prior to initiation of the assay with sodium succinate had no effect on succinoxidase activity (Fig. 7.5). These results suggest that 'pentane extract' contains either a form of ubiquinone or another component that is sensitive to a known energy transfer inhibitor which effects succinate oxidase activity. If DBCT is titrating a component that it reacts with in the membrane, then these results provide evidence, although of a very tentative nature, of a link between electron transfer and energy transfer systems.

Attention was then focussed on the effect that pentane extraction of mitochondria had on ATPase and ATPase linked activities. Pentane extraction slightly stimulated the specific activity of bovine heart mitochondrial ATPase, in ubiquinone reconstituted particles. ATPase activity was approximately the same as in extracted particles. The main difference in ATPase activity lay in its sensitivity to oligomycin, venturicidin and DCCD. Pentane extraction substantially reduces ATPase sensitivity to inhibition by oligomycin and DCCD and slightly reduces sensitivity to venturicidin. Reincorporation of ubiquinone reconstitutes sensitivity to oligomycin, venturicidin and DCCD (other lipids do not do this) (Table 7.5). Pentane extraction also reduces

the K_m value for ATP of bovine heart mitochondrial ATPase (Fig. 7.6). Similar results were observed in yeast submitochondrial particles (Table 7.10, Figs. 7.13-15). Here the K_m ATP was also reduced. Reincorporation of ubiquinone or 'pentane extract' increased the K_m value to that of the unextracted preparation. All these results suggest that ubiquinone has a kinetic effect on the ATPase enzyme. If ubiquinone was involved in the coupling process, then its removal might be expected to stimulate the reverse ATPase reaction and also decrease the K_m for ATP of the enzyme. The effect that ubiquinone extraction and reincorporation has on oligomycin and DCCD inhibition suggests that ubqiuinone acts in the F_o portion of the ATPase, that is, at the level at which '$\sim$' is thought to be transduced. Thisview is reinforced by the effect that ubiquinone extraction has on ATP-Pi exchange activity, which is thought to be a reflection of ATP synthase activity in the enzyme. Ubiquinone extraction reduces ATP-Pi exchange to low levels in bovine heart mitochondria. This activity can be restimulated by the reincorporation of ubiquinone. This restimulated activity is fully sensitive to energy transfer inhibitors and uncouplers.

As lyophilised mitochondria exhibit no respiratory control or oxidative phosphorylation, lyophilised, pentane extracted mitochondria have limited use as a system to study the possible roles of ubiquinoe in ATP synthesis. Acetone can be used as a solvent system for extracting ubiquinone from aqueous suspensions of mitochondria. Acetone extracted mitochondria and SMP's lose sensitivity to oligomycin and ATP synthase activity. They do not, however, regain these activities on reconstitution with 'acetone extract' or ubiquinone. It is probable that acetone treatment causes a dislocation between the F_1 and F_o portions of mitochondrial ATPase activity, producing an F_1-like ATPase activity.

The effect of pentane extraction on ATPase oligomycin and DCCD sensitivity is only apparent if complete (or as complete as possible)

extraction of the ubiquinone pool is achieved. Incomplete extraction results in no change in oligomycin sensitivity. If extracted particles are left to stand at 4^{o} C for long periods (6-24 hrs) this reduction in oligomycin sensitivity is reversed (see Fig. 7.11). This may be due to residual ubiquinone redistributing itself in the membrane as it is not possible to remove all the ubiquinone by solvent extraction. Even if 95% of ubiquinone is removed, this would still leave $\sim$ 0.5-1 nmole UQ/mg mitochondrial protein in the membrane which is sufficient to saturate any possible site that may exist in association with the ATPase (0.05-0.10 nmoles/mg mitochondrial protein). The major difficulty in interpreting these results lies in differentiating between specific and non-specific (general lipid) effects of ubiquinone on the one hand and effects due to disruption by pentane extraction on the other. It is probable that ubiquinone is having a more direct effect than just that of an integrative lipid in the inner mitochondrial membrane as other lipids (phospholipids, cholesterol, vitamin K_1) do not mimic its effects in these solvent extracted systems. The recovery of oligomycin sensitivity on standing may be due to a reintegration of the membrane after disruption by pentane rather than be due to a dispersal of residual ubiquinone through the system.

To further investigate ubiquinone's role in energy coupling and to remove any possible spurious effects induced by solvent extraction, a mutant strain of Saccharomyces cerevisiae lacking ubiquinone was investigated. This mutant,designated E3-24, was derived from a wild type yeast stain constructed by Tzagaloff and co-workers, D273-10B (for a further description of these yeast strains, see (391)). Strain E3-24 lacked NADH and succinate oxidase activities and oxidative phosphorylation but did possess a full complement of the other respiratory chain components as addition of ubiquinone alone fully

reconstituted NADH and succinate oxidases. Cytochrome difference spectra of D273-10B and E3-24 are almost identical (Fig. 7.16). Strain E3-24 had a lowered mitochondrial ATPase activity compared with D273-10B, but this activity was fully sensitive to inhibition by oligomycin, suggesting that ubiquinone plays no direct role in the mechanism of oligomycin inhibition in yeast mitochondria (Fig. 7.17). Strain E3-24 did, however, display a very low ATP-Pi exchange activity (Table 7.12). Attempts were made to reconstitute or stimulate this activity with ubiquinone, but these have so far proved unsuccessful.

Site 3 mediated oxidative phosphorylation has been reported in a similar ubiquinone-less mutant of yeast, strain E3-149, albeit at a very low rate. The reconstitution of site 2 mediated oxidative phosphorylation after addition of ubiquinone to carefully prepared E3-149 derived mitochondria has also been observed (326). Currently, further attempts to reconstitute ATP-Pi exchange and to investigate the kinetics of ATP requiring reactions in these yeast mutants are under way.

Recently a model for oxidative phosphorylation has been proposed by Santiago and López-Moratolla (348), based on the presence of redox active iron in ATPase preparations. They propose that ubiquinone and/or a flavin act as ligands to this iron. They have adduced the stimulation of F_1 ATPase preparations by FAD and ubiquinone as evidence to support this hypothesis (349).

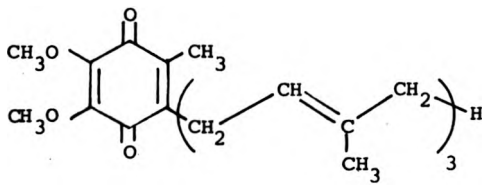
It would seem then that ubiquinone plays no direct role in the mechanism of ATP synthesis. It is possible though that it exhibits a 'kinetic control' on the energy coupling apparatus, possibly contributing in some way to the mechanism by which respiratory control is expressed.

CHAPTER 8

THE EFFECT OF UBIQUINONE ON ENERGY LINKED REACTIONS

Introduction

Solvent extraction studies on bovine heart mitochondria have indicated that ubiquinone may have a more complex function in the inner membrane than would be supposed if it merely fulfilled its well-known role as a mobile proton (electron) carrier in the electron transport chain. To further investigate these possible roles, attention was focussed on a lower homologue of the natural ubiquinones 9-10, ubiquinone 3.



Ubiquinone 3

This has been reported to possess the ability to uncouple oxidative phosphorylation at site II (322). The uncoupling was not a straightforward reaction as higher homologues, e.g. ubiquinone 7, had the ability to reverse it (see Table 8.1). Data presented in the previous chapter has suggested that ubiquinone may play some role in or close to energy coupling reactions in mitochondria. Ubiquinone 3 was tested for its effect on various mitochondrial reactions, both energy linked and non-energy linked.

Table 8.1 Effect of UQ_3 on oxidative phosphorylation of rat heart mitochondria, after (322)

Addition	Oxidative phosphorylation µmoles/min/mg protein	P:O ratios
None	0.125	1.37
UQ_3 (67 µM)	0.140	0.04
UQ_7 (67 µM)	0.100	1.46
UQ_3 + UQ_7 (67 µM each)	0.120	0.51

Materials

All chemical used were of 'AnalaR' or equivalent grade. All organic solvents were redistilled before use. Ubiquinones were obtained from Hoffman-LaRoche, Basel, Switzerland. Ubiquinone 3 was the kind gift of Dr. E. Bertoli. Oligomycin was obtained from the Sigma Chemical Company.

Methods

Bovine heart mitochondria and submitochondrial particles were prepared as described in Chapter 3. Oxidative phosphorylation was measured in a glucose-hexokinase trap system, 0.25 M in sucrose; 20 mM glucose; 20 mM Tris-Cl, pH 7.3; 5 mM potassium phosphate; 5 mM $MgCl_2$; 0.5 mM EDTA; 0.2 mM ADP, containing 5 units hexokinase (Sigma type C-300 or F-300). ATPase activity was assayed as described in Chapter 3. Transhydrogenase activity was assayed as described in Chapter 6.

All preincubations with ubiquinones were carried out in a shaking water bath, usually at 30° C. The conditions (time, temperature) of preincubation were important as increased preincubation times often improved inhibition by UQ_3, e.g. see Figs. 8.4-5. Ubiquinones were used as solutions in absolute ethanol.

Mitochondrial oxidase activities were monitored in a Clarke type electrode system attached to a Servoscribe chart recorder, as described in Chapter 7.

Purified F_1F_O ATPase was prepared by the method of Berden and Voorn-Brouwer (323). A suspension of bovine heart submitochondrial particles, 6 mg/ml in 200 mM sucrose; 10 mM Tris-Cl, pH 7.5; 0.2 mM EDTA; 1 mM dithiothreitol; 1 mM ATP; 2% methanol; 1% Triton X-100 was made 0.5 M in Na_2SO_4 by the addition of the solid. After stirring for 5 minutes, the homogenate was centrifuged at 150,000 g for 30 minutes. The pellet was discarded while the supernatant was dialysed overnight against 50 volumes of the same medium but without Na_2SO_4. The dialysate was then centrifuged at 50,000 g for 30 minutes. Virtually

all the oligomycin sensitive ATPase activity was concentrated in the precipitate. This was suspended in 250 mM sucrose; 50 mM Tris-Cl, pH 7.5; 1 mM dithiothreitol; 0.2 mM EDTA and 2% methanol to a final concentration of 3 mg/ml and taken to 0.4% in potassium cholate prior to the addition of saturated $(NH_4)_2SO_4$ to a final concentration of 30%. After centrifugation at 50,000 g for 15 minutes, the pellet was discarded and the supernatant brought to 50% saturation with saturated ammonium sulphate and recentrifuged. The pellet which contained the ATPase activity was resuspended in 250 mM sucrose; 50 mM Tris-Cl, pH 7.5; 1 mM dithiothreitol; 0.2 mM EDTA. ATPase of specific activity around 10 µmoles ATP hydrolysed/min/mg was prepared by this method.

Results and Discussion

Ubiquinone 3 was found to inhibit NADH oxidation in agreement with the results reported by Lenaz et al (324). It did not have such an effect on succinate or ascorbate/TMPD oxidation, however, as it appeared to stimulate succinate oxidation by as much as 95%, while it had very little effect on ascorbate/TMPD oxidation (Table 8.2). These stimulated (and inhibited) activities were true electron transfer chain activities, that is they were not due to bypass mechanisms from UQ 3 to oxygen, as they were fully sensitive to rotenone, antimycin and cyanide respectively (results not shown). Ubiquinone 9 had very little effect on any of these oxidase activities at similar concentrations (0-300 nmoles/mg mitochondrial protein).

Ubiquinone 3 had little effect on SMP ATPase activity (Table 8.3), producing only 16% inhibition at concentrations of 4 µmoles UQ_3/mg protein. Ubiquinone 9 produced 11% inhibition at similar concentrations. Ubiquinone 3 did, however, affect the oligomycin inhibition of bovine heart SMP ATPase activity (Fig. 8.1) reducing the efficacy of oligomycin as an inhibitor. Ubiquinone 9 has no effect at similar concentrations. A rather similar effect was observed in the purified F_1F_O ATPase

Table 8.2 Effect of UQ_3 on bovine heart mitochondrial activities

Additions	Rate nmoles O_2 consumed/ min/mg	% Activity	% Inhibition
NADH	258	100	0
NADH + 100 nmoles UQ_3	176	68.4	31.6
NADH + 150 nmoles UQ_3	164	63.5	36.5
NADH + 200 nmoles UQ_3	151	58.5	41.5
NADH + 300 nmoles UQ_3	126	48.8	51.2
			% Stimulation
Succinate	76	100	0
Succinate + 100 nmoles UQ_3	145	190.7	90.7
Succinate + 150 nmoles UQ_3	148	194.7	94.7
Succinate + 200 nmoles UQ_3	132	173.6	73.6
Succinate + 300 nmoles UQ_3	151	198.6	98.6
			% Inhibition
Ascorbate/TMPD	173	100	0
Ascorbate/TMPD + 100 nmoles UQ_3	170	98.2	1.8
Ascorbate/TMPD + 150 nmoles UQ_3	104	94.8	5.2
Ascorbate/TMPD + 200 nmoles UQ_3	170	98.2	1.8
Ascorbate/TMPD + 300 nmoles UQ_3	177	102	-2

Oxidase activities were determined as described in Chapter 7. Assays containing 1 mg bovine heart mitochondrial protein were initiated with either 4 µmoles NADH, 10 µmoles sodium succinate or 10 µmoles sodium-D-isoascorbate/1 µmole TMPD. Mitochondria were preincubated with ethanolic solutions of UQ_3 (20 mM) for 15 mins at 30° C prior to assay. Figures quoted above are corrected for any effect of the ethanol on oxidase activity.

Table 8.3 Effect of UQ_3 and UQ_9 on bovine heart submitochondrial particle ATPase

Additions	ATPase	
	% Activity	% Inhibition
None	100 [1]	
100 nmoles UQ_3	100	0
200 nmoles UQ_3	88	12
300 nmoles UQ_3	82	18
400 nmoles UQ_3	84	16
200 nmoles UQ_9	100	0
400 nmoles UQ_9	89	11

ATPase assays were performed as described in Chapter 3. 98 µg protein was suspended in 1 ml 50 mM Tris-Cl, pH 8.5, and preincubated with ubiquinone for 15 minutes at 30° C prior to initiation of the assay with 5 µmoles ATP. Assays were terminated by the addition of 0.5 ml 10% T.C.A. Phosphate was determined as described in the methods section to Chapter 3. Ubiquinones were added as 20 mM solutions in ethanol. Assays were compared with ones in which ATPase activity was determined in the presence of ethanol (i.e. 5, 10, 15, 20 µl).

[1] Specific activity 1.53 µmoles Pi released min^{-1} mg^{-1}

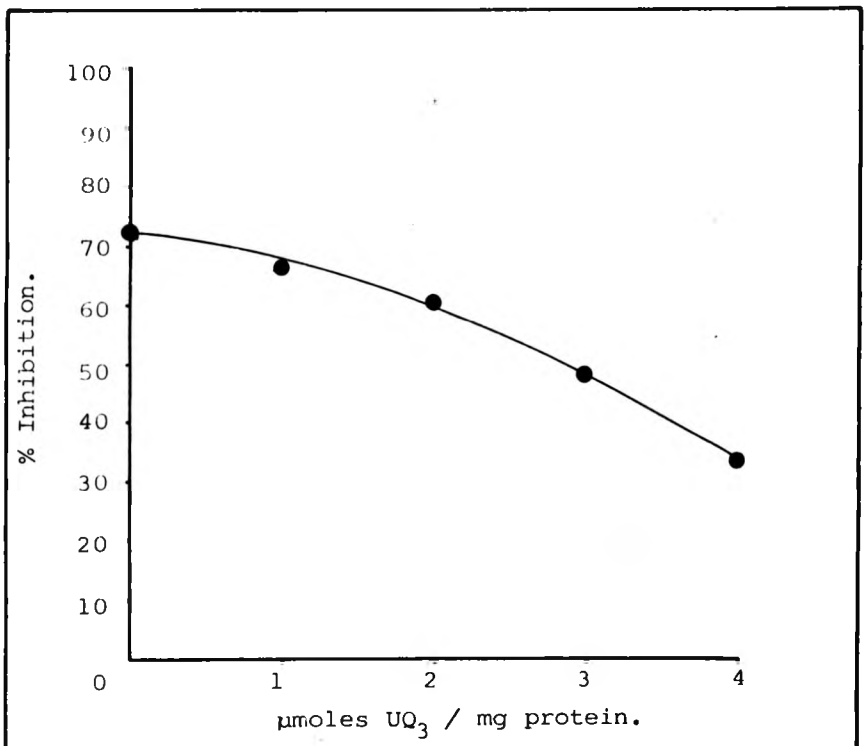


Fig 8.1. Reversal of oligomycin inhibition of bovine heart mitochondrial ATPase:inhibition by UQ_3.

Assay conditions as in table 8.3.

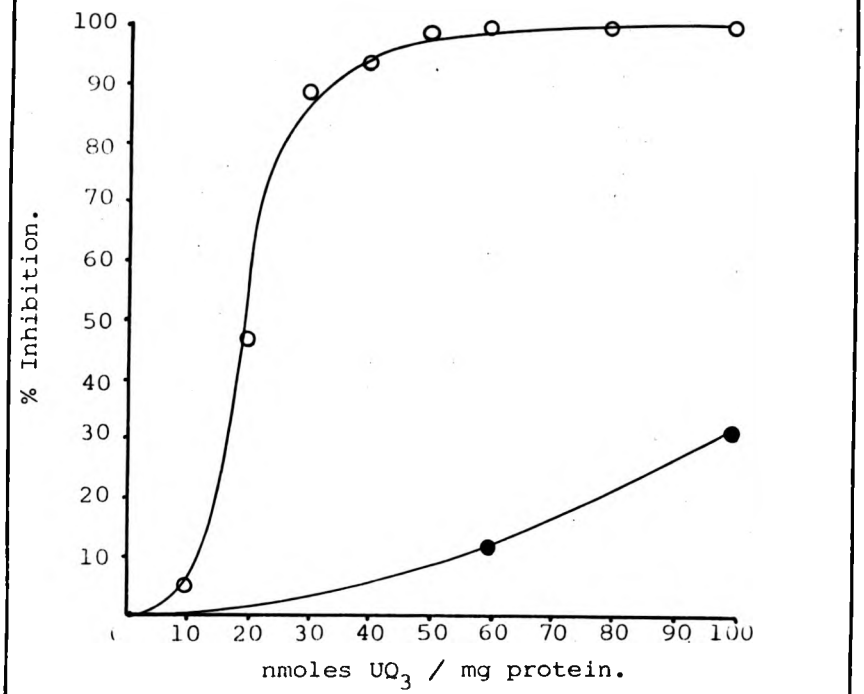


Fig 8.. Effect of UQ_3 on site 1 mediated activities in bovine heart mitochondria.

o—o pyruvate/malate driven oxidative phosphorylation[a].

●—● NADH oxidase[b].

Assays containing 1mg protein were preincubated with UQ_3 for 15 minutes at 30°C prior to initiation with either pyruvate / malate (10µmoles/1µmole), or 4 umoles NADH. Oxidative phosphorylation and NADH oxidase activities were assayed as described in the methods section to chapter 3.

[a]specific activity. 137 nmoles ATP formed/min/mg.

[b]specific activity. 250 nmoles O_2 consumed/min/mg.

preparation of Berden and Voorn-Brouwer (Table 8.4). Here ubiquinone 3 also reduces the inhibition induced by oligomycin. This effect is reduced or prevented by coincubating the enzyme with UQ_9 in the presence of UQ_3. Neither of these ubiquinones had any effect on F_1F_O ATPase activity itself.

Ubiquinone 3 inhibited oxidative phosphorylation driven by a pyruvate/malate (10:1) couple in bovine heart mitochondria (Fig. 8.2), but at much lower levels than those required to inhibit NADH oxidase activity. Succinate-driven and ascorbate/TMPD-driven-oxidative phosphorylation in bovine heart mitochondria and submitochondrial particles was also inhibited by ubiquinone 3 at similar levels to those that inhibit pyruvate/malate driven activity (Figs. 8.3-5). Ubiquinone 9 had little effect at similar concentrations; it could, however, reverse the inhibition induced by UQ_3 (Table 8.5). Similar effects, inhibition by ubiquinone 3 and its reversal by ubiquinone 9, were observed on the ATP-^{32}Pi exchange activity of bovine heart mitochondria (Fig. 8.6; Table 8.5).

ATP-driven transhydrogenase activity was also inhibited by ubiquinone 3; this inhibition was reversed by ubiquinone 9, which was without effect on the system (Figs. 8.7-8).

Ubiquinone 3 has been shown to affect a number of mitochondrial activities. The inhibition of oxidative phosphorylation driven by pyruvate/malate could bethought to be due to an inhibition of NADH oxidase activity by UQ_3. Analysis of the inhibition characteristics of these reactions makes this unlikely, however, as oxidative phosphorylation is far more sensitive than NADH oxidase to inhibition by UQ_3 (Table 8.6).

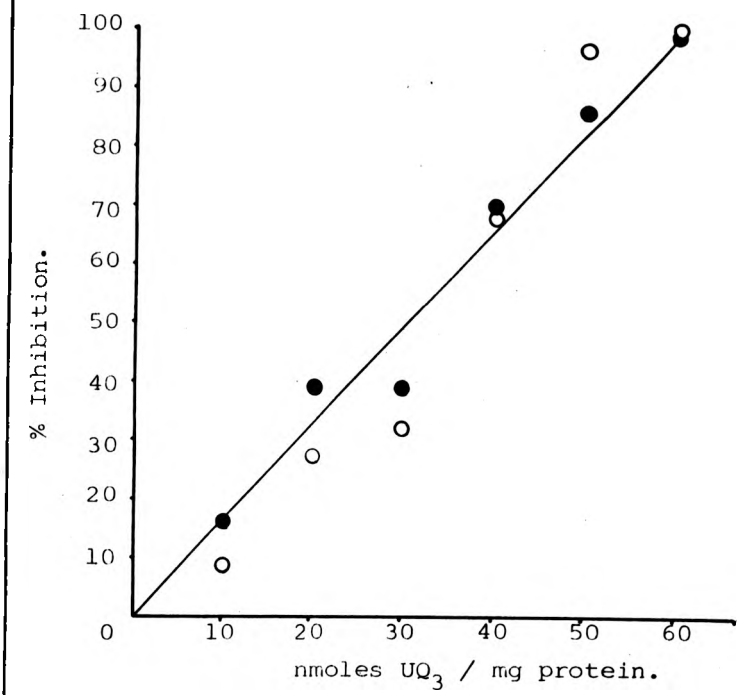


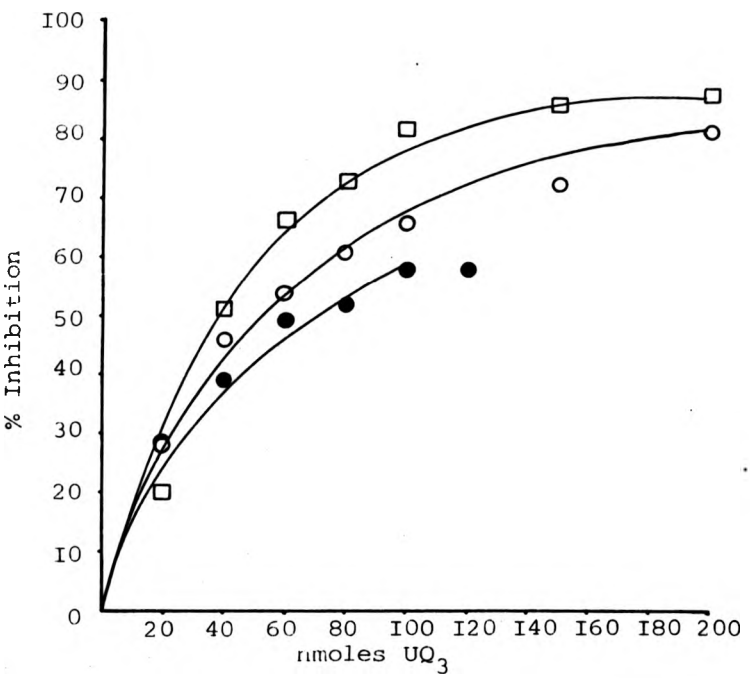
Fig 8.3. Inhibition of succinate driven ATP synthesis in bovine heart mitochondria by UQ_3.

Assays containing 1mg mitochondrial protein were preincubated with UQ_3 for 15 minutes at 30°C. Assays were initiated by addition of 10 µmoles sodium succinate and terminated after 20 minutes at 30°C with 10% TCA. Oxidative phosphorylation was measured by the dissapearence of phosphate as described in the methods section to chapter 3.

O—O assay 1.

●—● assay 2.

Specific activity: 121 nmoles ATP formed / min / mg.



Fig,8.4. Inhibition of succinate driven ATP synthesis in bovine heart SMP by UQ_3.

Assays containing Img SMP were preincubated, as indicated, with UQ_3. Assays were initiated by addition of I0µmoles sodium succinate and terminated after indicated times with I0% TCA. Oxidative phosphorylation was measured by the disappearence of phosphate as described in the methods section, chapter 3.

●—● expt.I. preincubated with UQ_3 I5'@R.T. inc I0'.
○—○ expt.2. " " inc I5'.
□—□ expt.3. " I2hr.@4°C; I5'@30°C inc 30'.

Assay incubations were carried out @ 30°C.
specific activity expt.1&2 =113nmoles ATP formed/min /mg. expt 3. 89 nmoles ATP formed /min/mg.

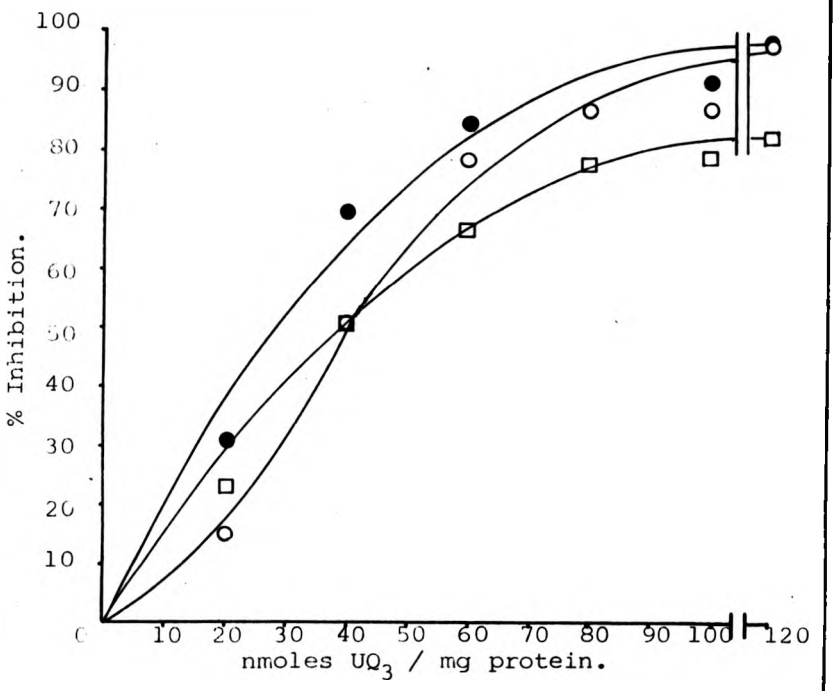


Fig 8.5. Inhibition of ascorbate / TMPD driven **ATP synthesis** in bovine heart mitochondria by UQ_3.

o—o expt.1.preincubated with UQ_3 10 minutes at 30°C.
●—● expt.2. " " 15 " "
□—□ expt.3. " " 10 " "

Assays containing 1mg mitochondria were preincubated with UQ_3 as indicated,then initiated by the addition of 10 µmoles ascorbate/0.5 µmoles TMPD and incubated at 30°C.Assays were terminated with 1M PCA,after 30'. Oxidative phosphorylation was measured by the appearance of glucose-6-phosphate as described in the methods section to chapter 3.

Specific activity = 79 nmoles ATP formed /min/mg.

Table 8.4 Effect of UQ_3 and UQ_9 on the oligomycin inhibition of a purified F_1F_O ATPase preparation

Addition	ATPase % Activity	% Inhibition	Δ %
None	100 [1]		
Oligomycin (5 µg)	32	68	
Oligomycin + UQ_3 10 nmoles	64	36	32
Oligomycin + UQ_3 30 nmoles	141	-41	(109)
Oligomycin + UQ_3 50 nmoles	136	-36	(104)
Oligomycin + UQ_3 70 nmoles	138	-38	(106)
Oligomycin + UQ_3 100 nmoles	138	-38	(106)
Oligomycin + UQ_9 100 nmoles	64	36	32
Oligomycin + UQ_3 50 nmoles UQ_9 50 nmoles	41	59	9

Assays were performed essentially as described in Table 8.3. Assays containing 5 µg F_1F_O ATPase, prepared by the method of Berden and Voorn-Brouwer (323), and asolectin were preincubated with ubiquinones for 5 minutes at 30° C

[1] Specific activity = 10.09 µmoles Pi released min^{-1} mg^{-1}

Table 8.5 Effect of UQ_3 and UQ_9 on bovine heart mitochondrial oxidative phosphorylation and ATP-^{32}Pi exchange activity

Additions	Oxidative phosphorylation	
	% Activity	% Inhibition
Pyruvate/malate [1]	100	0
Pyruvate/malate + 20 nmole UQ_3	53	47
Pyruvate/malate + 50 nmole UQ_3	1	9
Pyruvate/malate + 100 nmole UQ_9	100	0
Succinate [2]	100	0
Succinate + 60 nmole UQ_3	31	69
Succinate + 300 nmole UQ_3	0	100
Succinate + 60 nmole UQ_9	100	0
Succinate + 300 nmole UQ_9	60	40
Ascorbate/TMPD [3]	100	0
Ascorbate/TMPD + 40 nmole UQ_3	41	59
Ascorbate/TMPD + 100 nmole UQ_3	7	93
Ascorbate/TMPD + 100 nmole UQ_9	100	0
Ascorbate/TMPD + 100 nmole UQ_3 + 100 nmoles UQ_9	88	12
	ATP-^{32}Pi exchange	
ATP + ^{32}Pi [4]	100	0
ATP + ^{32}Pi 80 nmoles UQ_3	39	61
ATP + ^{32}Pi 80 nmoles UQ_9	100	0
ATP + ^{32}Pi 80 nmoles UQ_3 80 nmoles UQ_9	100	0

[1] Specific activity: 114 nmoles ATP/min/mg

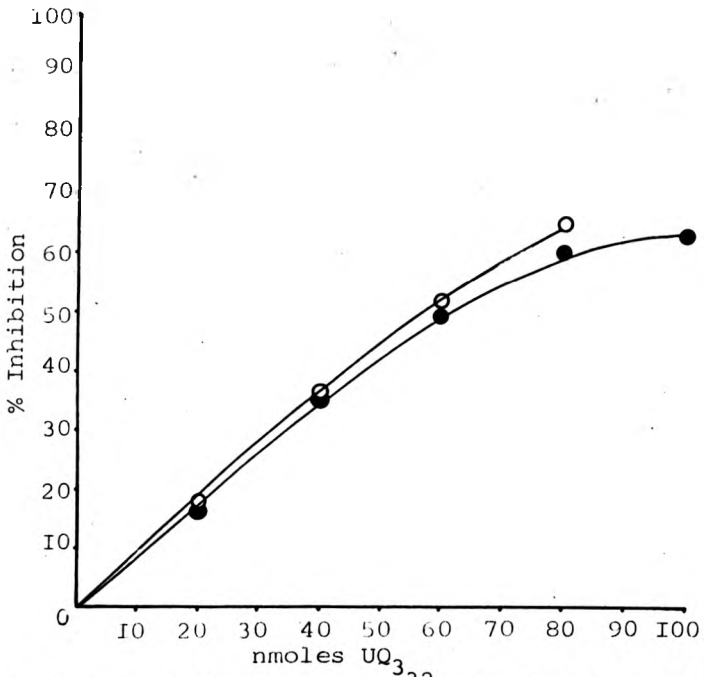
[2] Specific activity: 120 nmoles ATP formed/min/mg

[3] Specific activity: 68 nmoles ATP/min/mg

[4] Specific activity: 49.5 nmoles $^{32}P\ min^{-1}\ mg^{-1}$

Oxidative phosphorylation was assayed in a glucose-hexokinase trap system, 0.2 mM in ADP as described in the methods section to this chapter.

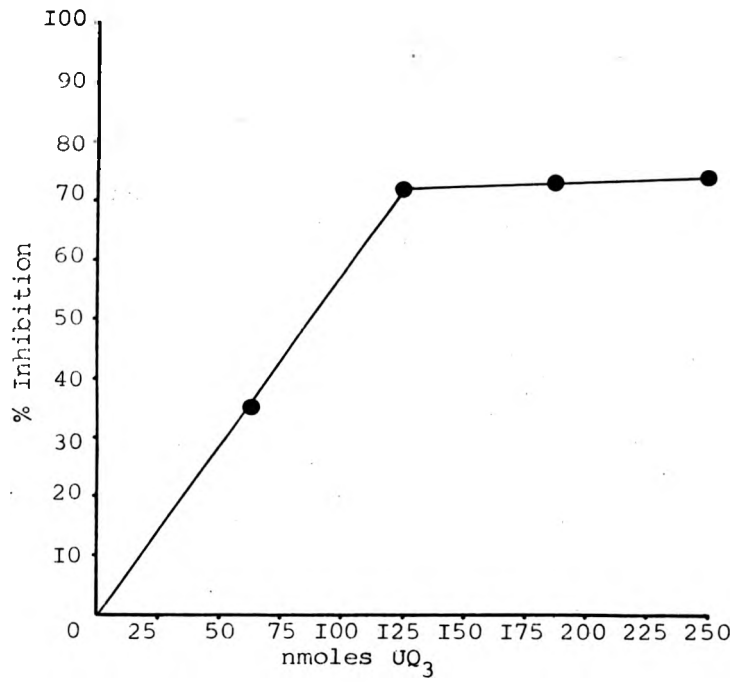
ATP-^{32}Pi exchange was assayed as described in Chapter 3.



Fig,8.6.Inhibition of ATP - ^{32}Pi exchange in bovine heart SMP by UQ_3.

O—O expt.I. specific activity 49.5nmoles/min/mg.
●—● expt.2. specific activity 48.3nmoles/min/mg.

Assays containing Img protein were preincubated with UQ_3 for I5 minutes at 30°C in a shaking water bath prior to initiation by addition of I0µmoles ATP.Assays were terminated after 5 minutes by addition of I0% TCA.ATP - ^{32}Pi exchange was estimated as described in chapter three.



Fig,8.7. Inhibition of ATP driven transhydrogenase in bovine heart SMP by UQ_3.

Assays containing Img protein were preincubated with UQ_3 for I5 minutes at 30°C prior to assay. Transhydrogenase activity was estimated as described in chapter 6.

Specific activity uninhibited transhydrogenase = 72 nmoles $NADP^+$ reduced /min / mg SMP protein.

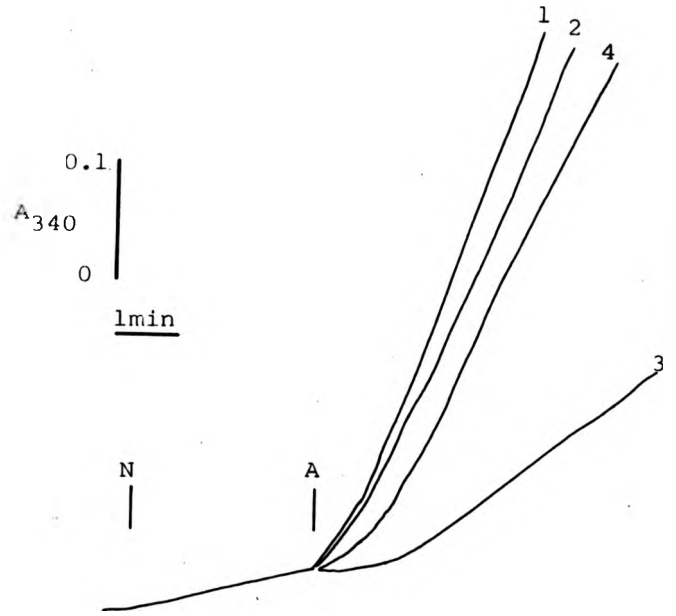


Fig 8.8. Effect of UQ_3 and UQ_9 on ATP driven transhydrogenase activity in bovine heart SMP.

N : 0.5 µmoles $NADP^+$. A : 5 µmoles ATP.

1. no additions. 2. + 80 nmoles UQ_9.

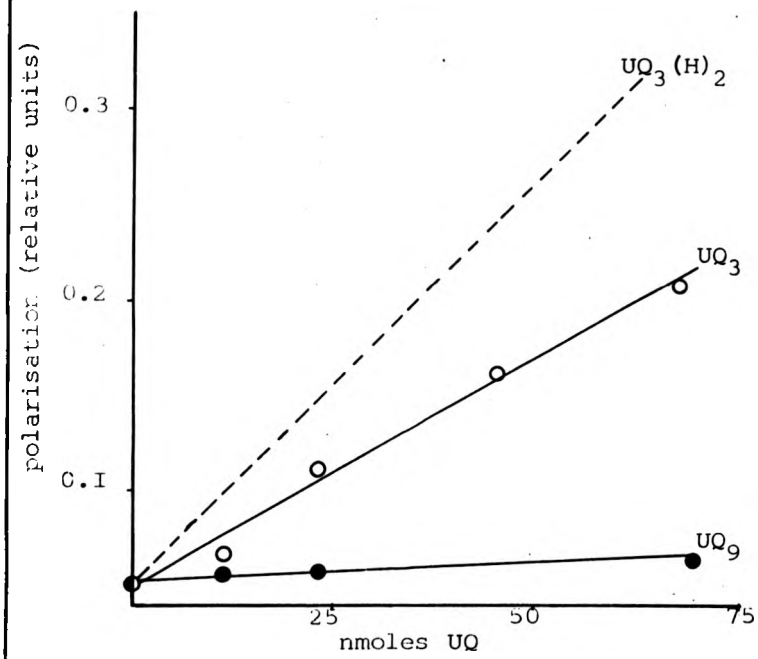
3. + 80 nmoles UQ_3. 4. + 80 nmoles UQ_3 + 80 nmoles UQ_9.

Assays containing 1mg protein were preincubated with ethanolic solutions of ubiquinones, where indicated, for 15 minutes at 30°C prior to initiation. Transhydrogenase activity was assayed as described in the methods section to chapter 6. Activities were corrected for any effect of ethanol.

Table 8.6 Inhibition of bovine heart mitochondrial activities by UQ_3: I_{50} values

Activity	I_{50} nmoles mg^{-1} protein
NADH oxidase	∿ 300
Pyruvate/malate oxidative phosphorylation	18
Succinate oxidative phosphorylation	∿ 30
Succinate oxidative phosphorylation (SMP)	40-70
Ascorbate/TMPD oxidative phosphorylation	27-40
ATP-^{32}Pi exchange	55-60
ATP-driven transhydrogenase (SMP)	∿ 80

Oxidative phosphorylation driven from sites 2 and 3 is also inhibited by UQ_3 while the respective oxidase activities are either stimulated or unaffected. This strongly suggests that UQ_3 is not acting by dislocating or inhibiting electron transport, but is having a more direct action on the coupling mechanism. This view is reinforced by the inhibition of other energy linked reactions, ATP-^{32}Pi exchange and ATP-driven transhydrogenase, which have no (known) requirement for electron transport in order to exhibit their activity. The locus of inhibition may be at the level of the ATPase molecule itself, as all these energy linked reactions involve its action in some way. Ubiquinone 3 has very little effect on ATPase activity; this is probably because of the exact location of ATPase activity in the membrane The active site of ATPase is located on the F_1 portion of the molecule which is commonly thought to protrude out of the membrane into the aqueous matrix space, i.e. away from the disruptive effects of UQ_3. While ATPase activity is unaffected, UQ_3 will reverse the oligomycin induced inhibition of ATPase activity. Oligomycin has its site of action in the F_O membrane bound portion of the ATPase. This suggests that UQ_3 is having a disruptive effect on the ATPase molecule, dislocating the site of oligomycin inhibition away



Fig,8.9.Fluorescence polarisation of perylene in rabbit heart mitochondria:effect of ubiquinones.

Presented by kind permission of Dr. E.Bertoli.

Fluorescence polarisation of perylene probe was measured in an Hitachi-Perkin Elmer MPF-32 recording spectrofluorimeter (at 440nm with excitation at 4I0nm) as described in [327] .

from the ATPase active site, thus producing a functional disruption of the ATPase molecule and an 'uncoupling' of energy linked reactions.

What is the mode of UQ_3's disruptive action? Spisni et al. have reported that short chain quinones are not freely diffusible across bilayers and that they become stacked within the phospholipids (325), thus immobilising the membrane and disrupting many activities which take place within it. Support for this hypothesis has come from studies on the fluorescence polarisation of perylene incorporated into rabbit heart mitochondria (326). The fluorescence polarisation of these mitochondria labelled with perylene was found to increase markedly with increase of (added) UQ_3 concentration. This indicates that the rotational mobility of perylene decreases with the increase of UQ_3 into the membrane. Longer chain quinones such as UQ_7 and UQ_9 have little effect on the system (see Fig. 8.9 reproduced here by kind permission of Dr. E. Bertoli). While this may be an adequate description of the mode of UQ_3's inhibition of NADH oxidase activity, it is not at all clear whether it adequately explains the inhibition of oxidative phosphorylation which occurs at much lower quinone levels. It does not explain either the ability of the longer chain quinones to reverse this inhibition. The coupling process is notoriously labile, so low levels of UQ_3 may produce a sufficiently large disruptive effect to uncouple it. The longer chain quinones may reverse this effect by increasing the fluidity of the membrane, allowing these processes to take place again.

APPENXIX A

Compounds that inhibit energy linked reactions

1. (a) F_1 ATPase inhibitors

   (b) F_1F_O ATPase inhibitors

2. Uncouplers

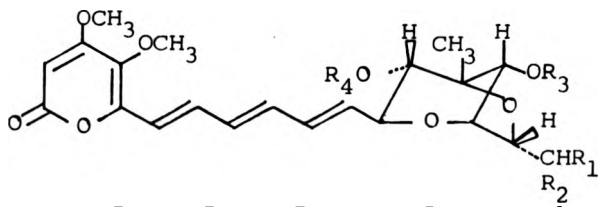
3. Ionophores

4. Electron transport inhibitors

1. ATPase inhibitors

(a) F_1ATPase inhibitors

Aurovertins.- The aurovertins are a class of compounds having the general formula:-



	R_1	R_2	R_3	R_4	molecular weight
Aurovertin A	H	Me	COMe	COMe	501
B	H	Me	COMe	H	445
C	H	H	COMe	H	451
D	OH	Me	COMe	H	475
E	H	Me	H	H	417

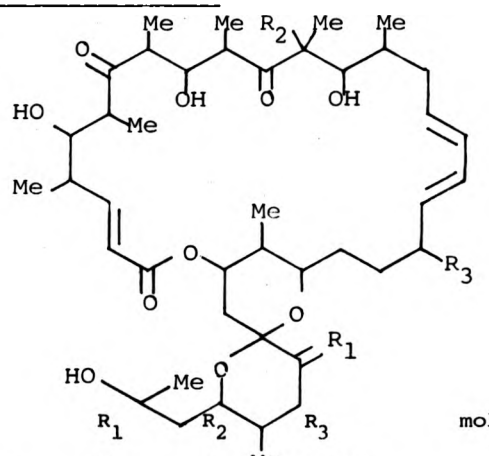
They are obtained from cultures of the fungus Calcorisporium arbuscula and are potent inhibitors of mitochondrial ATP synthesis; ATPase; $H_2{}^{18}O$-Pi exchange; ATP-^{32}Pi exchange; ATP-driven transhydrogenase; ATP-driven reversed electron transport; uncoupler induced swelling. D and B are the most potent forms.

The locus of action appears to be at the F_1 ATPase.

Efrapeptins.- Efrapeptins are a group of lipophilic polypeptides obtained from the hyphomycete Tolypocladium inflatum. The structures are unclear, but hydrolysis yields glycine, leucine and alanine in a ratio of 6:7:1 and an unknown amino acid. The polypeptides do not appear to possess N-terminal amino acids but do contain a free carboxyl group. They have a molecular weight somewhere in excess of 1500. They have a mode of action similar to that of the aurovertins but, although they act on the F_1 ATPase, they appear to have a binding site distinct from that of the aurovertins.

(b) F_1F_O ATPase inhibitors

Oligomycin



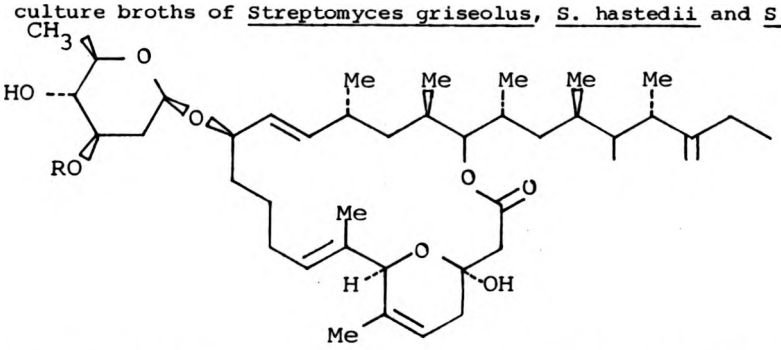
					molecular weight
Oligomycin	A	H_2	OH	CH_3CH_2	760
	B	O	OH	CH_3CH_2	784
	C	H_2	H	CH_3CH_2	744
	D	O	OH	CH_3	760

The oligomycins are a group of complex antibiotics obtained from cultures of Streptomyces diasto chromogenes (oligomycins A-C) and Streptomyces rutgersensis (NRRL. B-1256 (oligomycin D, also known as rutamycin)).

The oligomycins are the 'classical inhibitors' of the F_1F_O ATPase in mitochondria, and are among the most potent known: 1 molecule is thought to be sufficient to completely inhibit 1 molecule of ATPase.

It inhibits oxidative phosphorylation, ATP-Pi exchange, ATP-ADP exchange, F_1F_O ATPase and all energy linked activities consuming or producing ATP. It has no effect on the isolated F_1 ATPase. Its precise mode of action is unclear; it is thought either to block the formation of the '~P' state or to prevent proton pumping through the F_O portion of the ATPase. It probably interacts with 'subunit 9' of the ATPase. Sulphydryl groups have been implicated in its binding site.

Venturicidin The venturicidins are a group of compounds obtained from culture broths of Streptomyces griseolus, S. hastedii and S. xanthophaeus.



Venturicidin A	R = NH_2CO	molecular weight	750
B	R = H	molecular weight	707

Venturicidin has a mode of action similar to that of the oligomycins although it is slightly less potent. It acts on the F_O portion of the ATPase, inhibiting all ATP utilising and producing reactions. It appears to bind to subunit 6 of the ATPase.

Leucinostatin This is a lipophilic polypeptide isolated from the fungus Penicillium lilacinum A-267. Hydrolysis yields leucine and 4 unknown amino acids. It has a complex mode of action, being an uncoupler in high concentration but appearing to act like oligomycin or venturicidin at lower concentrations. It has no effect on isolated F_1 ATPase.

Dicyclohexylcarbodiimide (DCCD) DCCD is a potent inhibitor of energy linked reactions. It appears to exert its inhibitory characteristics by covalently binding to subunit 9 of the F_O ATPase in mitochondria and

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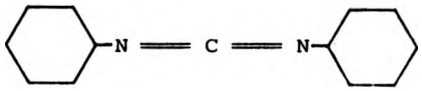


DCCD

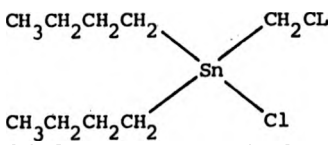
Trialkyltins These are compounds having the general formula

$R_1R_2R_3SnX$ where $R_1R_2R_3$ are hydrocarbon groupings

X = halogens or -OH (usually).

They are potent inhibitors of ATP utilising or producing energy linked reactions (the compounds where $R_1R_2R_3$ are: n-ethyl or n-butyl,n-propyl or phenyl appear to be the most effective). They do not inhibit F_1 ATPase activity and appear to bind to the subunit 9 component of the membrane bound (F_O) portion of the ATPase. They generally act like oligomycin but may also facilitate a $Cl^- \longleftrightarrow OH^-$ exchange reaction across the mitochondrial inner membrane.

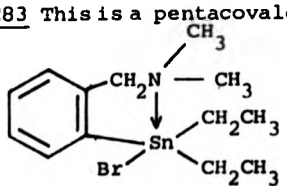
Dibutyl chloromethyl tin chloride This is a trialkyl tin, having the structure:-



molecular weight 318

It appears to bind to a nonprotein lipophilic component of the inner mitochondrial membrane. It has a similar efficacy, and general properties like, the other trialkyl tin compounds.

Compound VE2283 This is a pentacovalent organotin compound having the structure



molecular weight 390

It appears to act like oligomycin and is at least as potent an inhibitor (on a molar basis). Its precise mode of action is at present under investigation.

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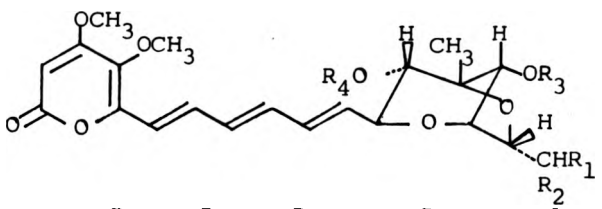

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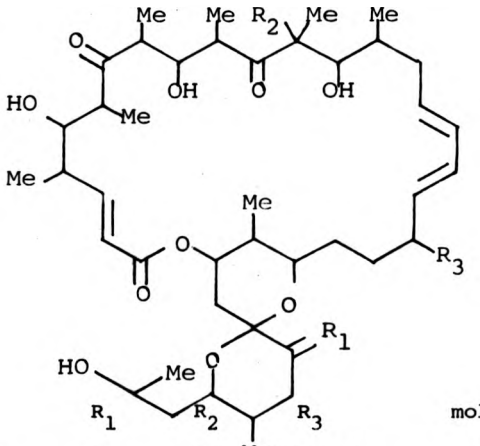

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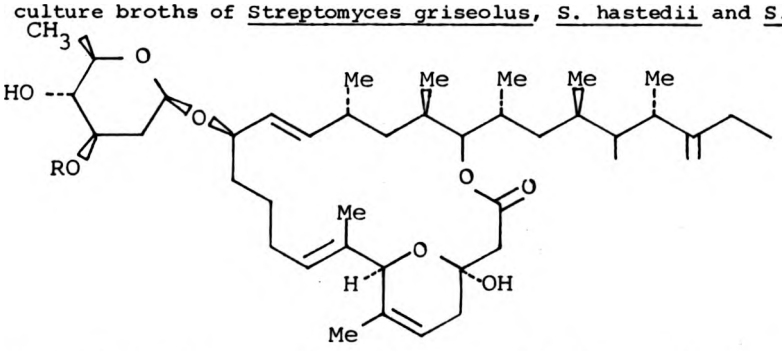

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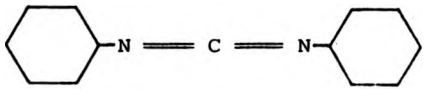

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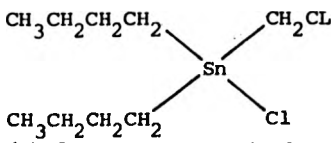

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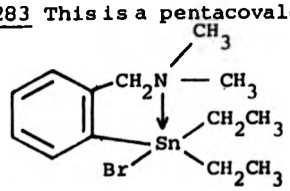
It appears to bind to a nonprotein lipophilic component of the inner mitochondrial membrane. It has a similar efficacy, and general properties like, the other trialkyl tin compounds.

Compound VE2283 This is a pentacovalent organotin compound having the structure

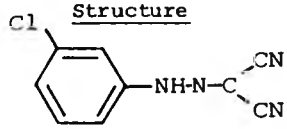
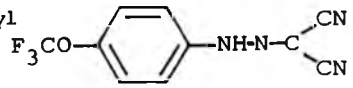
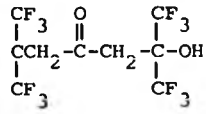
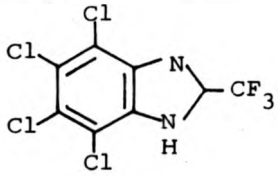
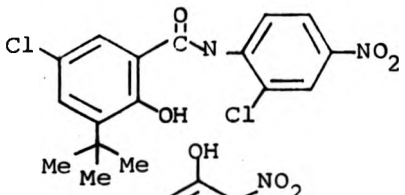
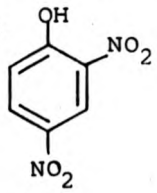
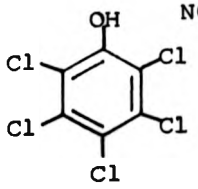


molecular weight 390

It appears to act like oligomycin and is at least as potent an inhibitor (on a molar basis). Its precise mode of action is at present under investigation.

2. Uncouplers

These are compounds that dissipate the high energy state,'∿', of the inner membrane; whether they act by dissipating a proton gradient or in a more specific manner (e.g. binding to a component of the energy coupling apparatus) is not completely clear, although most are lipophilic weak acids or bases, capable of conducting protons across artificial lipid bilayers. For a more detailed discussion of the mechanism of uncoupling, see Chapter 2.

Name	Structure	Acronym
m-chloro carbonyl cyanide phenyl hydrazone		CCCP
trifluoromethoxy carbonyl cyanide phenylhydrazone		FCCP
bis hexafluoro acetonyl acetone		'1799'
4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole		TTFB
5-chloro-3-tert butyl-2'-chloro-4'nitrosalicylanilide		S-13
2,4-dinitrophenol		DNP
Pentachlorophenol		PCP

3. Ionophores

These are a group of compounds that have the ability to transport cations across lipid membranes. They uncouple oxidative phosphorylation either by collapsing the $\Delta\phi$ component of the proton motive force or by facilitating energy linked, futile cycles of ions across bioenergetic membranes, thus dissipating '$\sim$'.

Gramicidin D Isolated from cultures of Bacillus brevis. It is a lipophilic polypeptide consisting of 15 amino acids of alternating L and D forms, i.e.

HCO-val-gly-ala-leu-ala-val-val(tryp-leu)-tryp-$NHCH_2CH_2OH$

L L D L D L D

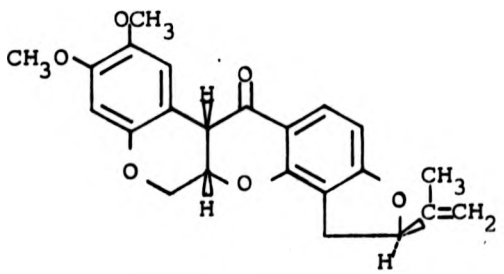
It is thought to adopt a helical form in the membrane, effectively poking a hydrophilic hole through it, through which ions can pass. It transports K^+ and H^+ with similar efficiencies (K^+ slightly > H^+).

Valinomycin This is isolated from Streptomyces fulvissimus. It is one of the 'cage' form ionophores which enclose cations with a hydrophobic shell. It contains 3 moles of L-valine, D-α-hydroxy isovaleric acid, D-valine, L-lactic acid, linked alternately to form a 36 membered ring. It principally transports potassium across membranes, but will also transport other monovalent cations with lowered effectivity.

4. Electron transport inhibitors

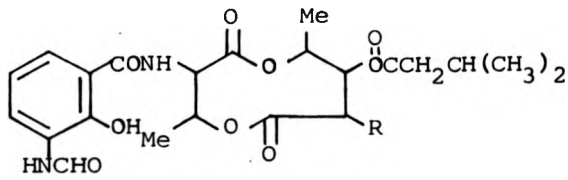
These are compounds that act by blocking electron flow between respiratory substrates and oxygen.

Rotenone Obtained from derris root. It appears to block electron transport between NADH dehydrogenase and ubiquinone.



molecular weight 393

Antimycin A Blocks electron transport between cyt b and cyt c; it binds to a protein subunit of complex III.



Antimycin A_1	R = n-hexyl	molecular weight 558
Antimycin A_3	R = n-butyl	molecular weight 530

Cyanide, azide, sulphide, carbon monoxide All block electron transport at the level of cytochrome oxidase. They act by irreversibly binding to the 'free ligand' of the iron in cytochrome a_3's haem grouping.

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