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Generation Of Oxygen-derived Free Radicals Stimulated By The Fumigant Insecticide Phosphine: In Vivo And In Vitro Studies

Caroline Jane Bolter

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GENERATION OF OXYGEN-DERIVED FREE RADICALS STIMULATED BY THE FUMIGANT INSECTICIDE PHOSPHINE: *IN VIVO* AND *IN VITRO* STUDIES

by

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ABSTRACT

Previous studies have shown that phosphine (PH_3) inhibits cytochrome oxidase and that a direct relationship exists between oxygen concentration during fumigation and insect mortality. Insects take several days to die suggesting that a toxic product, accumulating over time, may be responsible for death. This study was undertaken to test the hypothesis that mortality is due to cumulative damage of cellular components by free radicals derived from superoxide ($O_2^{\cdot-}$) generated by the inhibited respiratory chain.

Hydrogen peroxide (H_2O_2), a product of $O_2^{\cdot-}$ dismutation, was measured spectrophotometrically using yeast cytochrome c peroxidase as an indicator. The respiratory inhibitors; antimycin, myxothiazol and PH_3 stimulated H_2O_2 release from mitochondria isolated from granary weevil (Sitophilus granarius) and mouse liver. Peroxide release increased with the addition of α-glycerophosphate. It was concluded that glycerophosphate dehydrogenase was a source of H_2O_2. The concentration of quinone, known to be a major site of $O_2^{\cdot-}$ generation, was measured spectrophotometrically. Quinone levels were low in granary weevils compared to other species and were unaltered after in vivo treatment with PH_3 (LD_{50}). Difference spectra, obtained after insect mitochondria were treated with respiratory inhibitors, provided information concerning sites of $O_2^{\cdot-}$ generation.

The effect of PH_3 on the oxygen defence system of PH_3-sensitive (S) and -resistant (R) insects was observed. No glutathione peroxidase activity was found in this species however it did contain glutathione, the concentration of which was unaffected by PH_3 treatment (LD_{70}). Peroxidase activity, observed using p-phenylenediamine as an indicator, was the same in S and R insects. Activity was reduced by 65% in S and 45% in R insects three days after fumigation (LD_{30}). Catalase activity was significantly higher (62%) in S insects than R. This activity was inhibited by 34% in S insects three days after treatment (LD_{30}), but was unaffected in R insects. A pyrogallol assay was used to measure superoxide dismutase. Two isozymes were present, a cyanide (CN)-insensitive form in the mitochondria and a CN-sensitive in the cytosol. Activity of the latter enzyme increased two-fold after in vivo PH_3 treatment (LD_{30}) in S insects, no change was observed in R insects.

Damage to cellular components resulting from attack by oxygen-derived radicals was measured after S insects were exposed to PH_3 in vivo and after isolated membranes were exposed to free radicals generating systems in vitro. Polyunsaturated fatty acids (PUFA's) are susceptible to free radical attack and a decrease of 12.3% and 32.7% in PUFA concentration relative to saturated fatty acids was observed in microsomes after in vivo exposure to PH_3 (LD_{60} and LD_{80} respectively). No changes were observed in vitro. A 31.0%

decrease in sulphydryl group content was seen after *in vivo* exposure to PH_3 (LD_{65}). No change was observed *in vitro*. H^+-ATPase activity increased by 11% after *in vivo* treatment, probably due to inhibition of the regulatory protein. A 30% decrease in activity was recorded after *in vitro* treatment.

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GLOSSARY

ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
3-AT	3-amino 1,2,4-triazole
BSA	bovine serum albumen
Ca^{2+}	calcium ion
CCP	cytochrome c peroxidase
CuZnSOD	cuprozinc-superoxide dismutase
DTPA	diethylenetriamine pentaacetic acid
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-bis-(-amino-ethyl ether)N,N'-tetraacetic acid
FAD	flavin adenine dinucleotide, oxidised form
FADH	flavin adenine dinucleotide, reduced form
FAME	fatty acid methyl ester
H_2O_2	hydrogen peroxide
$HO^{\cdot}$	hydroxyl radical
MnSOD	mangano-superoxide dismutase
NAD+	nicotine adenine dinucleotide, oxidised form
NADH	nicotine adenine dinucleotide, reduced form
NADPH	nicotine adenine dinucleotide phosphate, reduced form
$O_2^{\cdot-}$	superoxide radical
PH_3	phosphine
PUFA	polyunsaturated fatty acid
Sigma	Sigma Chemical Co. (St. Louis, Mo., U.S.A.)
SMP	sub-mitochondrial particle
SOD	superoxide dismutase

INTRODUCTION

1.1 Properties of Phosphine

Phosphine (PH_3) is a colourless gas (MW=34) with a low boiling point (-87.4°C). It diffuses rapidly and penetrates deeply into materials such as large bulks of grain. Phosphine is one of the most toxic fumigants used for the control of stored product insects and is effective at very low concentrations if the exposure time is long enough. Toxicity declines as the temperature falls and it is not recommended for use below 5°C (Bond 1984).

Under physiological conditions, PH_3 is a weak nucleophile, a sluggish but powerful reducing agent and can act as a metal ligand (Bond *et al*. 1984). The known reactivity of PH_3 provides many opportunities for the toxic inhibition of biological pathways (Banks 1975). The gas is toxic to several species of mites, nematodes, insects and to all vertebrates including mammals (Singh *et al*. 1967, Sinha *et al*. 1967). It does not appear to have any adverse effects on the germination of most plant seeds under normal conditions (Strong and Lindgren 1960, Fam *et al*. 1974).

Recently, reports that many different species of insects have become resistant to PH_3 (Champ and Dyte 1976, Bell *et al*. 1977, Borah and Chalal 1979, Hole 1981, Tyler *et al*. 1983) have resulted in renewed interest in the mode of action of this fumigant. Since resistance does not appear to be due to increased metabolism of PH_3 by mixed function microsomal

oxidases (Rajak and Hewlett 1971), identification of the site of toxic action could help in establishing the mechanism of resistance. For example, the altered molecular structure of an enzyme, a decrease in its accessibility, or an increase in its concentration could result in resistance. Such investigations could prove to be very important in the design of a fumigation regime which includes factors such as variable atmospheric temperature, oxygen and carbon dioxide concentrations as well as the use of PH_3 with fumigants which may act synergistically.

Results from early studies on the dosage/mortality response on insects (Quereshi et al. 1965, Lindgren and Vincent 1966, Reynolds et al. 1967) lacked consistency. The standard relationship between concentration (c) and time (t) of $c \times t = k$ for use with PH_3 was questioned (Bond et al. 1969, Howe 1974). Many of the apparent anomalies probably resulted from the use of PH_3 concentrations above the range of a normal toxic response (Winks 1984). Exposure of several different species to concentrations of above 0.5mg/l resulted in a pronounced and systematic deviation from linearity in the ct relationship and a significant increase in tolerance (Bang and Telford 1966, Lindgren and Vincent 1966, Bond et al. 1969, Monro et al. 1972, Bell 1979, Winks 1984).

PH_3 inhibited oxygen uptake in adult Sitophilus zeamais (Nakakita et al. 1974) and by isolated mitochondria from a variety of insect and mammalian sources in a concentration dependent manner (Nakakita et al. 1971, Chefurka et al. 1976).

There was almost no toxic action when insects were exposed to PH_3 under anaerobic conditions (in an atmosphere of 100% N) even when the dosage (c x t) was increased more than 1000 fold above that used in air (Bond and Monro 1967, Bond *et al*. 1967). It was concluded that the presence of oxygen during fumigation was essential for PH_3 toxicity (Bond *et al*. 1969) and that the concentration of oxygen during the period after fumigation was related to toxicity (Bond *et al*.1967).

The observation that PH_3 toxicity was dependent on oxygen concentration led Kashi (1974) to look at its effect on the electron transport chain. *In vitro* studies using insect and mammalian mitochondria suggested that the primary biochemical lesion of PH_3 was the inhibition of cytochrome *c* oxidase, specifically cytochrome *a* (Chefurka *et al*. 1976, Kashi 1974, Nakakita 1976). Kashi and Chefurka (1976) concluded that PH_3 reacts with the oxidised form of cytochrome *a* which may explain why the gas had no toxic action in the absence of oxygen. Spectral and circular dichroism studies revealed that interactions occurred between the heme moiety of cytochrome *a* and PH_3 involving a conformational change (Kashi and Chefurka 1976). Inhibition of cytochrome *c* oxidase by PH_3 was noncompetitive and only slowly reversible, if at all (Kashi 1974). Nakakita (1987) detected a 40-50% inhibition of cytochrome *c* oxidase in insect mitochondria after exposure to PH_3 *in vivo*.

Bond (1963) found that PH_3 also inhibited catalase (hydrogen peroxide:hydrogen peroxide oxidoreductase E.C. 1.11.1.6) activity after *in vivo* exposure. Catalase catalyses the reduction of hydrogen peroxide (H_2O_2) to water (See Introduction Section 1.4). Price and Walter (1987) observed that weevils of the species *Rhyzopertha dominica* fed the catalase inhibitor 3-amino-1,2,4-triazole (3-AT) survived as long as controls and showed no increased susceptibility to PH_3, suggesting that catalase inhibition (and consequent accumulation of H_2O_2) was not the cause of insect mortality.

1.2 Mitochondrial Electron Transport Chain

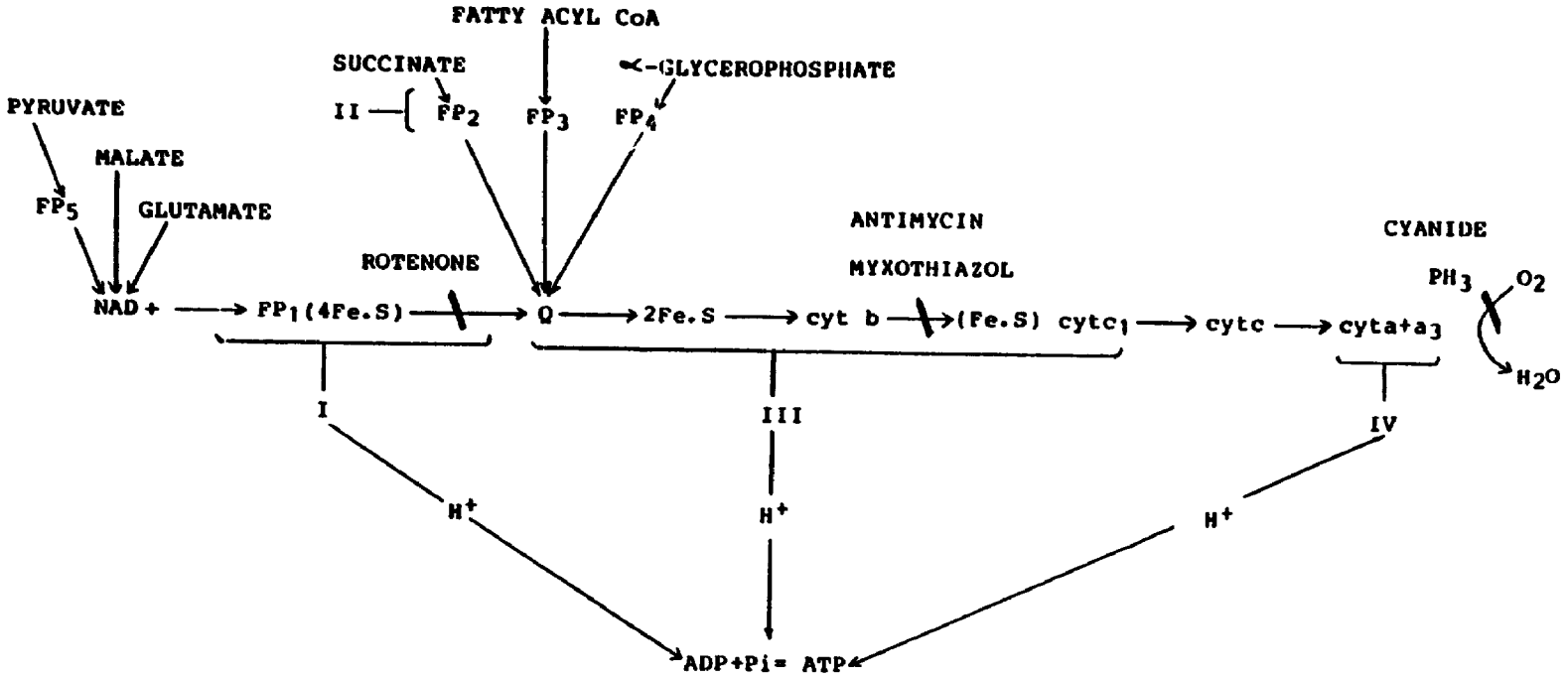
A proton gradient is generated across the inner mitochondrial membrane by the flow of electrons through three energy-conserving sites in the respiratory chain (Fig. 1). The three complexes involved in coupling are asymmetrically oriented and span the membrane so that protons can be pumped from the mitochondrial matrix to the intermembrane space. Reducing equivalents are fed into the respiratory chain from various substrates via nicotinamide adenine dinucleotide (NAD^+) or flavin adenine dinucleotide (FAD). Electrons are transferred through a series of electron carriers: flavins, iron-sulphur complexes, quinones and hemes. The electron-carriers, with the exception of quinones, are prosthetic groups of proteins.

Figure 1

Simplified Diagram of the Mammalian Respiratory Chain

The sites where NAD-linked substrates, pyruvate, malate and glutamate and FAD-linked substrates, succinate, fatty acyl CoA and α-glycerophosphate feed into the chain are shown. The regions of the chain blocked by certain electron transport inhibitors, rotenone, antimycin, myxothiazol, cyanide and PH_3 are also shown. Complexes I, III and IV contain energy coupling sites responsible for movement of protons across the membrane creating a proton gradient. Protons flow back into the matrix via H^+-ATPase resulting in ATP generation. Abbreviations: **Complex I**, NADH-ubiquinone reductase; **Complex II**, succinate-ubiquinone reductase or NADH dehydrogenase; **Complex III**, ubiquinol-cytochrome c reductase; **Complex IV**, cytochrome c oxidase; **FP**, flavoprotein; FP_1, NADH dehydrogenase; FP_2, succinate dehydrogenase; FP_3, fatty acyl CoA dehydrogenase; FP_4, α-glycerophosphate dehydrogenase; **Fe-S**, iron-sulphur centre; **Q**, Ubiquinone; **cyt**, cytochrome.

Modified from Lehninger 1975.


FATTY ACYL CoA
SUCCINATE
α-GLYCEROPHOSPHATE
PYRUVATE
II
FP2
FP3
FP4
MALATE
FP5
GLUTAMATE
ANTIMYCIN
ROTENONE
MYXOTHIAZOL
CYANIDE
PH3
O2
NAD+
FP1(4Fe.S)
Q
2Fe.S
cyt b
(Fe.S)
cytc1
cytc
cyta+a3
H2O
I
III
IV
H+
H+
H+
ADP+Pi= ATP

1.2.1 Substrates

Pyruvate is an important substrate for insect respiration, in contrast to intermediates of the tricarboxylic acid cycle such as citrate, isocitrate, α-ketoglutarate, succinate, fumarate and malate, the amino acids glutamate and aspartate which when added to isolated mitochondria are not very effective respiratory chain substrates (Chance and Sacktor 1958, Sacktor and Childress 1967).

Intact mitochondria are impermeable to NADH formed as a result of the oxidation of glyceraldehyde 3-phosphate in glycolysis. Two shuttle systems allow the passage of electrons across the membrane to the respiratory chain. Firstly, the malate-aspartate shuttle is utilized in mammalian heart and liver (Kornacker and Ball 1965) and both radioisotope and enzyme studies suggest that this pathway also exists in insects to some extent (Walker and Bailey 1970). Electrons are transferred from NADH to oxaloacetate producing malate in the cytosol in a reaction catalysed by malate dehydrogenase. Malate transverses the inner membrane and is reoxidised resulting in the formation of NADH in the matrix. Malate is oxidised to oxaloacetate which cannot cross the membrane and a transaminase reaction occurs resulting in the formation of aspartate which moves freely out of the matrix. This series of reactions can only occur if the NADH/NAD^+ ratio is higher in the cytosol than the mitochondrial matrix.

The second and most important system in insects is the α-glycerophosphate shuttle. Chance and Sacktor (1958) pro-

posed that α-glycerophosphate is a very important substrate in insects since it is the only known substrate that can sustain the respiratory activity of muscle at levels comparable to those of active flight. It can be metabolised by insect mitochondria 10 to 100 times faster than citric acid cycle intermediates such as citrate, succinate and malate. Cytosolically generated NADH reacts with dihydroxyacetone phosphate reducing it to α-glycerophosphate in a reaction catalysed by NAD-linked glycerophosphate dehydrogenase (1.1.1.8) of the cytosol (Lehninger 1975). α-Glycerophosphate passes through the outer mitochondrial membrane and is oxidised by an FAD-linked glycerophosphate dehydrogenase (1.1.99.5) located on the inner membrane. The position of the enzyme is a matter of discussion, an outer surface location has been suggested (Donnellan *et al*. 1970) while Slack and Bursell (1977) favour a position on the inner surface. Electrons from the reduced enzyme are then transferred to ubiquinone (Salach and Bednarz 1973) (Fig 1). The exact site of entry into the electron transport chain is still not known but Wu (1958) hypothesised that it is a different pathway from succinate since polyurethane, thought to be an inhibitor of cytochrome *b*, had no effect on reduction of the dye 2,6-dichlorophenolindophenol by α-glycerophosphate while it inhibited choline-dye or succinate-dye reactions. When glycerophosphate dehydrogenase-linked $FADH_2$ transfers reducing equivalents to ubiquinone only two energy conserving sites are activated and consequently only two molecules of ATP are

formed. This is a wasteful process since three molecules of ATP are produced when NADH transfers electrons to the respiratory chain via NADH dehydrogenase, a third coupling site. However, it allows the passage of reducing equivalents into mitochondria against an NADH concentration gradient unlike the malate/aspartate shuttle.

Glycerophosphate dehydrogenase is inhibited by EDTA (Eastabrook and Sacktor 1958b); a concentration as low as 0.1mM EDTA reduced activity by 25%. Addition of Ca^{2+} restored enzyme activity and it was hypothesised that an endogenous chelator capable of functioning like EDTA could act as a control mechanism for the enzyme *in vivo*. When the insect is at rest, the enzyme is inhibited and a low level of respiration is achieved by other substrates, in flight the inhibition is reversed and the high respiratory rate characteristic of α-glycerophosphate is observed. During coupled state 3 respiration protons are pumped out of the mitochondria and a high potential exists across the inner mitochondrial membrane. Wohlrab (1977) postulated that calcium vacates the active site of the enzyme in response to the ionic gradient and moves toward the matrix leaving the enzyme inactivated. This effect is overcome by addition of excess substrate. In situations where Ca^{2+} leaks from the mitochondria, for example when the ATP level is reduced due to high activity or inhibition of electron transport, the enzyme would be reactivated and oxidise α-glycerophosphate efficiently.

The degree to which the α-glycerophosphate shuttle would be utilized at rest, pertinent to this situation since the insect species under investigation has a comparatively sedentary existence, has not been studied in detail. Kashi (1974) reported that the granary weevil contained 50μM α-glycerophosphate per gramme whole insect wet weight which is a high concentration considering that locust, a potentially highly active insect was found to have only 0.45μM/gm whole insect. There is a dearth of literature on substrates utilized to drive oxidative phosphorylation in other insect systems and flight muscle at rest. Chefurka (1981a) reported that free fatty acids were the major endogenous substrate in mouse liver mitochondria, and the possibility that this is the case in insect species was investigated in this study.

Fatty acids are linked to Coenzyme A in a reaction catalysed by acyl CoA synthetase. Long chain fatty acids activated in this way are transported across the inner mitochondrial membrane as carnitine derivatives in mammals. Medium chain fatty acids do not require carnitine for transport. It was found that carnitine was not necessary for fatty acid oxidation in two species of moths tested (Stephenson 1968). Requirements for this molecule have not been established in the granary weevil. In the mitochondria the acyl CoA undergoes a recurring sequence of four reactions: oxidation linked to FAD, hydration, oxidation linked to NAD^+, and thiolysis by CoA. The fatty acyl chain is shortened by two carbonatoms and $FADH_2$, NADH and CoA are generated. Consequently, reducing

equivalents enter the respiratory chain at two levels, NADH dehydrogenase and ubiquinone when fatty acids undergo β-oxidation (Fig. 1). There are several different inhibitors of fatty acid oxidation, 4-pentenoic acid inhibits 3-Ketoacyl-CoA thiolase, one of the enzymes involved in the β-oxidation cycle (Schultz 1987). This inhibitor will be used in this study to ascertain the identity of the endogenous substrate in the granary weevil.

1.2.2 The Q-cycle Hypothesis

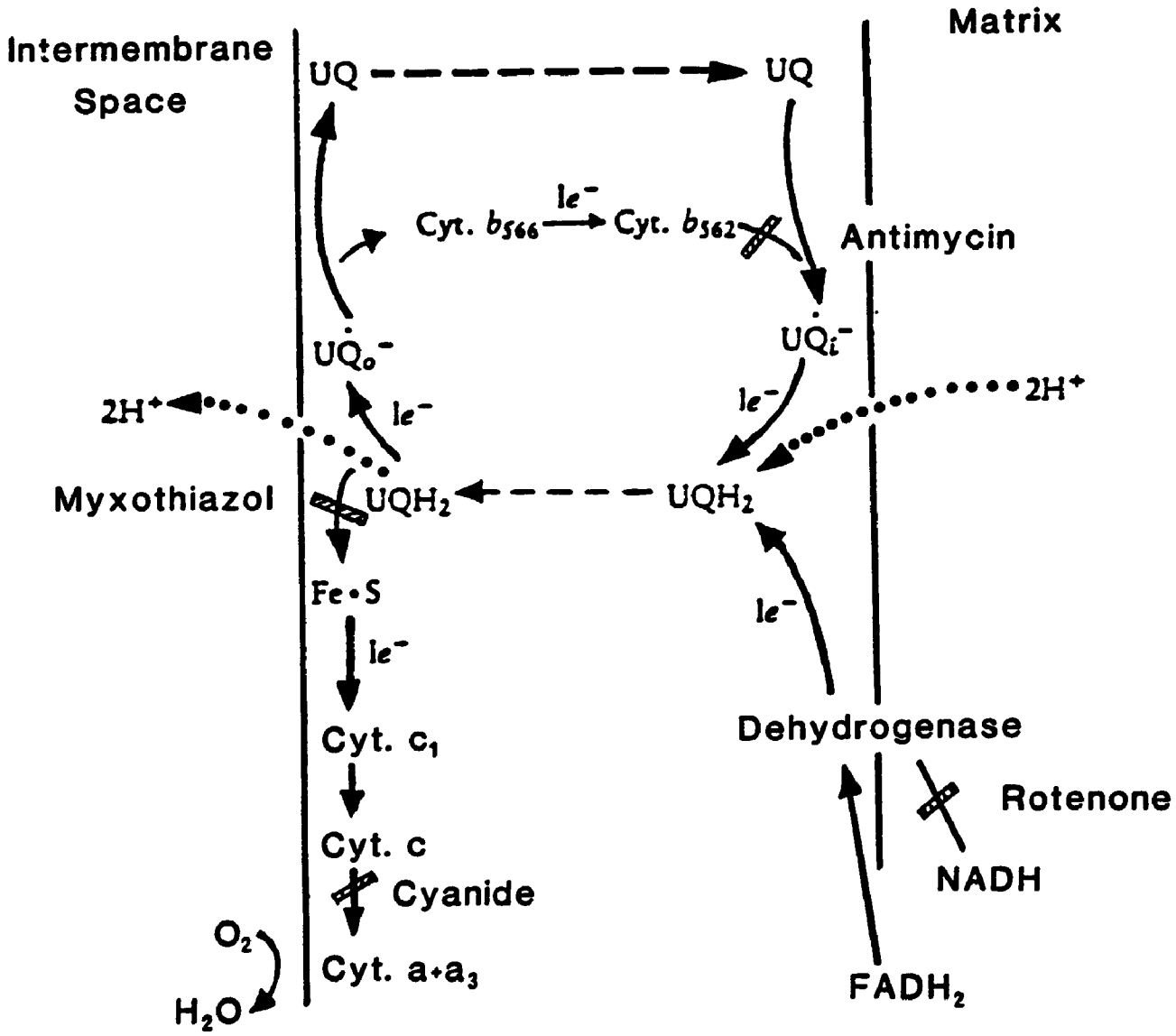
Fig. 1 shows the simplified version of the sequence of electron carriers in the respiratory assembly. It has been found that ubiqninol-cytochrome c reductase is much more complex than originally proposed (Mitchell 1976). More recently the use of inhibitors of this region of the respiratory chain has provided convincing evidence for the existance of the Q-cycle model (Von Jagow and Link 1984). The Q-cycle hypothesis can be simplified as follows (Fig. 2). Initially an electron donated from cytochrome b_{562} reduces ubiquinone (UQ) to ubisemiquinone ($UQ_i^{\cdot}-$). Ubisemiquinone is further reduced to ubiquinol (UQH_2) accepting an electron from a substrate dehydrogenase (or complex I), combined with the uptake of two protons from the matrix. UQH_2 formed on the matrix side diffuses to the cytosolic side where it discharges two protons. One electron is accepted by the Rieske iron-sulphur centre and then passed via cytochrome c to oxygen. UQ_o donates the second electron to cytochrome b566. The

Figure 2

Diagram of the Proposed Mitochondrial Q-cycle

Events occurring in one turn of the cycle. The dehydrogenase shown could be NADH-, α-glycerophosphate-, succinate- or fatty acyl CoA-dehydrogenase. An electron from cyt b_{562} reduces ubiquinone to ubisemiquinone. This is followed by further reduction from an electron donated by dehydrogenase and the addition of two protons from the matrix to form ubiquinol. This diffuses across the membrane to the intermembrane space side. Here, the protons are released, one electron is donated to the Rieske-iron sulphur centre and the other electron reduces cyt b_{566}. The b-cytochromes span the membrane and the electron is carried to the matrix-side where it can continue in the cycle. Sites of inhibition of this region are also shown. Antimycin blocks passage of electrons from cyt b_{562} to ubiquinone and myxothiazol blocks electron movement from ubiquinol to the Rieske iron-sulphur centre. Abbreviations: cyt, cytochrome; UQH_2, ubiquinol; UQ, ubiquinone; UQ^-, ubisemiquinone anion bound at the inner (i) and outer (o) centres. **Dashed lines**, diffusion or translocation; **dotted lines**, proton flow; **solid lines**, electron flow.

Modified From Harold (1986)


Intermembrane Space
Matrix
UQ
UQ
Cyt. b_{566}
le^-
Cyt. b_{562}
Antimycin
UQ_o^-
UQ_i^-
$2H^+$
le^-
le^-
$2H^+$
Myxothiazol
UQH_2
UQH_2
Fe•S
le^-
le^-
Cyt. c_1
Dehydrogenase
Rotenone
Cyt. c
Cyanide
NADH
O_2
Cyt. a+a_3
H_2O
$FADH_2$

two b-cytochromes span the membrane and the electron passed to cytochrome b_{562} is donated to UQ to complete the cycle. Thus, in each turn of the Q-cycle one electron is transferred to cytochrome c and two protons translocate across the membrane (Harold 1986, Rich 1986).

1.2.3 Inhibitors

Inhibitors of the respiratory chain are available which can be used to investigate the complexes in detail. Rotenone, a plant product used as an insecticide, inhibits electron transfer within the NADH-UQ reductase complex (NADH dehydrogenase or complex I) and prevents the generation of a proton gradient at site 1 (Tyler 1966). Binding of rotenone is slow but virtually irreversible (Wainio 1970) and the exact site of inhibition within the complex remains a matter of controversy. Rotenone has no effect on the generation of a proton gradient at site 2 when FAD-linked substrates are utilized, however it does prevent ATP-energized reverse electron transfer from succinate to NAD^+.

Antimycin, an antibiotic and a natural product of various species of Streptomyces, blocks the flow of electrons from the heme b_h centre of cytochrome b_{562} to oxidised ubiquinone (UQ) (Fig. 2) (Von Jagow and Link 1986). Both b centres are reduced in the presence of antimycin when electrons are fed into the respiratory chain.

Myxothiazol, an antibiotic produced by the myxobacterium Myxococcus fulvus structurally resembles part of ubiquinone

(UQ). It blocks reduction of the Rieske iron-sulphur centre and of cytochrome b via the UQ_o site, but allows reduction of cytochrome b_{562} by reversed electron transfer (Fig. 2). The extent of reduction of the two b centres depends on their potential and the redox equilibrium of the system under investigation. In mitochondria and sub-mitochondrial particles (SMP) it is common for half the heme b to be reduced, which is attributed to the b_h centre of cytochrome b_{562} (Von Jagow and Engel 1981).

Cyanide inhibits cytochrome a_3 of the cytochrome oxidase complex, however although fully reduced cytochrome aa_3 binds cyanide, molecular oxygen readily oxidises the resulting complex (Keilin and Hartree 1939). The partially oxidised cytochrome oxidase ($a^{2+}a_3^{3+}Cu_B^+$; where Cu_B^+ represents the copper atom associated with cytochrome a_3) inhibited by cyanide is the most stable complex (See Nicholls 1983 for discussion). PH_3 also inhibits cytochrome c oxidase, but reacts with the oxidised form of cytochrome a (Kashi and Chefurka 1976).

1.3 Products of Reduced Oxygen

Broadly defined free radicals are molecules or molecular fragments with unpaired electrons (Slater 1984). This definition includes most transition metals, the hydrogen atom and even the oxygen molecule, since O_2 has two unpaired electrons in different antibonding orbitals (Halliwell and Gutteridge 1984). This electron configuration imposes restrictions on its reactivity because oxygen cannot accept a

pair of electrons from another atom or molecule unless both new electrons have parallel spins enabling them to fit into the vacant antibonding orbital spaces. Because of this, O_2 reacts slowly with non-radical species, accepting electrons individually. If O_2 obtains a single electron it enters one of the antibonding orbitals producing the superoxide radical, $O_2^{\cdot-}$. This reactive radical forms naturally in most aerobic cells and forms more quickly when the oxygen tension is above atmospheric (Fridovich 1975, 1978). In aqueous solutions, and especially at acidic pH, $O_2^{\cdot-}$ spontaneously dismutates to form hydrogen peroxide (H_2O_2) and water. The peroxide ion O_2^{2-} formed as a result of the addition of an electron to $O_2^{\cdot-}$ is protonated rapidly at physiological pH.

$$2O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + O_2 \qquad [1]$$

Two hydroxyl radicals ($HO^{\cdot}$) are formed when the O-O bond of H_2O_2 is broken. It has been proposed that an iron-catalysed Haber-Weiss reaction occurs in which the homolytic fission of H_2O_2 is catalysed by ferrous iron (McCord and Day 1978, Winterbourn 1981).

$$Fe^{3+} + O_2^{\cdot-} \longrightarrow Fe^{2+} + O_2 \qquad [2]$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO^{\cdot} + OH^- \qquad [3]$$

$$\text{Net } O_2^{\cdot-} + H_2O_2 \longrightarrow O_2 + HO^{\cdot} + OH^- \qquad [4]$$

Almost any oxidant can oxidise ferric iron to ferrous rendering it available to react with H_2O_2 (Winterbourne 1979).

It seems likely that $HO^{\cdot}$ is produced *in vivo* (Nohl and Hegner 1978, Freeman *et al*. 1982), however it is difficult to measure because it is extremely unstable and reactive, and therein lies its danger. The hydroxyl radical reacts with virtually all cellular components, phospholipids, sugars, amino acids, DNA bases and organic acids.

1.4 Antioxidant Defence

As described above, the successive univalent reduction of dioxygen results in the production of the intermediates $O_2^{\cdot-}$, H_2O_2 and $HO^{\cdot}$, all of which are highly reactive and threaten the integrity of living cells. As a requirement for an aerobic existance, a complex defence system has evolved to counter this threat.

Superoxide dismutase (SOD)(E.C.1.15.1.1) is the name given to several different metalloenzymes that catalyse the following reaction.

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + O_2 \qquad [5]$$

This reaction occurs spontaneously but the rate is increased by 10^6 fold by SOD at pH=7.4 (Fridovich 1975). The cytosol of eukaryotic cells contain a SOD with a molecular weight of 32,000 made up of two identical subunits each containing one Cu^{2+} and one Zn^{2+} ion. Bacteria have a totally

unrelated SOD isozyme which has a molecular weight of 40,000 and is made up of two subunits with one atom of manganese per subunit. At pH=7.0 it is as active as CuZnSOD, as the pH is raised it becomes less active while CuZnSOD is unaffected by pH in the range 5.5-10.0 (Fridovich 1975). Eukaryote mitochondria have an equivalent MnSOD but it contains four subunits instead of two. MnSOD is insensitive to cyanide, while CuZnSOD is reversibly inhibited by it and because of this it is possible to differentiate between these two isozymes in homogenates. A third form of SOD containing iron is found in some bacteria.

Mitochondrially produced H_2O_2, the majority of which results from $O_2^{\cdot-}$ dismutation (Boveris and Cadenas 1975, Dionisi et al. 1975) is removed by catalase or peroxidases. Catalase, (E.C.1.11.1.6) a heme protein, dismutes H_2O_2 to water and molecular oxygen by catalysing the reaction:

$$H_2O_2 + H_2O_2 \longrightarrow 2H_2O + O_2 \qquad [6]$$

In mammals catalase is a homotetrameric enzyme with a molecular weight of 240,000. It is found in the peroxisomes where it actively removes H_2O_2 produced therein. Compound I, formed when Fe(V) heme is divalently oxidised by the first H_2O_2, is a powerful oxidant and can be reduced by small molecules other than H_2O_2 such as ethanol and nitrite (Aebi 1974). In this role it is acting as a peroxidase.

Peroxidases catalyse the reduction of H_2O_2 by a variety of electron donors. This group of enzymes is particularly important in removing low concentrations of H_2O_2. In mammals the most important peroxidase is the seleno-enzyme glutathione peroxidase (E.C.1.11.1.9) which uses glutathione specifically as its co-substrate.

$$2GSH + H_2O_2 \longrightarrow G\text{-}S\text{-}S\text{-}G + 2H_2O \qquad [7]$$

Glutathione reductase (E.C.1.6.4.2) prevents the depletion of cellular glutathione (GSH) by using NADPH to reduce the disulphide (G-S-S-G). However, no glutathione peroxidase activity could be measured in pupae of chinese silk moths or flesh flies (Smith and Shrift 1979), at any stage of the housefly, Musca domestica (Allen et al. 1983), or in the larvae of the Southern armyworm Spodoptera eridania (Ahmed et al. 1988). Despite the lack of peroxidase, glutathione was found in the housefly and Allen et al. (1983) proposed that glutathione itself was an important antioxidant in insects. It can react with free radicals spontaneously and also reactivate enzymes by reducing oxidised-SH groups (Jocelyn 1962). Ascorbic acid plays a similar antioxidant role.

The antioxidant α-tocopherol reacts with the chain propagating fatty acid radical yielding the α-tocopherol radical (Burton and Ingold 1981). It is a non polar molecule and is effective at removing peroxide radicals formed deep within the membrane (DiLuzio 1973, Corongiu et al. 1985).

Ascorbate can reduce α-tocopherol radicals, on the membrane surface back to α-tocopherol (Fridovich and Freeman 1986).

Davies (1986) describes a secondary antioxidant defense system comprising of proteolytic systems, DNA repair systems and lipolytic enzymes. He stresses the importance of removing the oxidatively damaged proteins which would otherwise accumulate as "useless cellular debris", particularly when the primary antioxidants are unable to cope with increased oxidative stress.

1.5 Extramitochondrial Hydrogen Peroxide Production

It has been observed that H_2O_2 is released from intact mitochondria utilizing NADH-linked substrates when cytochrome c oxidase is inhibited by cyanide (Boveris and Turrens 1980). Electrons, unable to move along the electron transport chain are donated from NADH dehydrogenase (Fig. 1) to oxygen which is reduced to superoxide ($O_2^{\cdot-}$), a reactive cytotoxic radical. $O_2^{\cdot-}$ is highly unstable and rapidly decomposes to H_2O_2 in a spontaneous reaction the rate of which if further increased by the enzyme superoxide dismutase (SOD)(See Introduction section 1.4). Mitochondria contain a barrage of non-enzymatic and enzymatic mechanisms to defend the tissue against the products of reduced oxygen. However, if the production of H_2O_2 ($O_2^{\cdot-}$) increases sufficiently the system is overwhelmed and H_2O_2, which can pass through membranes as easily as water (Freeman et al. 1982), can be measured extramitochondrially.

Since PH_3 also inhibits cytochrome c oxidase it was considered possible that H_2O_2 production could occur with this inhibitor when mitochondria are supplied with NADH-linked substrates.

NADH dehydrogenase is generally not the major site of $O_2^{\cdot-}$ generation in mitochondria. Turrens et al. (1985) established that ubisemiquinone (ubiquinone reduced by one reducing equivalent) is the most important source of $O_2^{\cdot-}$ in beef heart mitochondria. However, the proportion generated at each site depends on the concentration of ubiquinone which varies considerably between species (Turrens et al. 1982). Production of $O_2^{\cdot-}$ from the ubiquinone-cytochrome b region of the respiratory chain occurs only when cytochrome c is in the oxidised state i.e. production from this site is inhibited when cyanide is added to mitochondria (Cadenas and Boveris 1980). This observation agrees with the Q-cycle theory of electron transport proposed by Mitchell (1976) with certain modifications (Bowyer and Trumpower 1981) (Introduction section 1.2.2). In summary, ubiquinone is reduced to ubiquinol by two electrons, one from cytochrome b, the other from a respiratory chain substrate in a reaction catalysed by a dehydrogenase. One electron is transferred to cytochrome c, via the Rieske iron-sulphur centre and then to oxygen to form water. The other electron from ubisemiquinone normally reduces the b-cytochromes and cycles back to ubiquinone (Fig. 2). The system is not perfect, however, and electrons can "leak" from ubisemiquinone when FAD-linked substrates specifically succinate are used to supplement mitochondria. The electrons

react with oxygen to form superoxide (Introduction section 1.3). If the movement of electrons through the b cytochromes to ubiquinone is blocked by antimycin, ubisemiquinone autoxidises more readily and there is a concomittant increase in extramitochondrial H_2O_2. In order for this electron to react univalently with oxygen, the other electron must be free to move to cytochrome c_1. Consequently, any inhibitor that blocks the respiratory chain after ubiquinone should inhibit H_2O_2 production resulting from ubiquinone autoxidation. Examples of this are cyanide, as mentioned and myxothiazol, which inhibits between ubiquinone and the Rieske centre, correspondingly, PH_3 should also inhibit H_2O_2 production from this source.

The primary substrate for insect flight muscle is α-glycerophosphate. It is the only known substrate that can sustain the respiratory activity of flight muscle (Chance and Sacktor 1958) (Introduction section 1.2.3). Because of the potentially high activity of α-glycerophosphate dehydrogenase in insects, α-glycerophosphate was utilized as the exogenous substrate during this study. As previously mentioned, it is an FAD-linked substrate providing reducing equivalents directly to ubiquinone in the same way as succinate. For this reason it is expected that $O_2^{\cdot-}$ production would occur via ubisemiquinone autoxidation if the respiratory chain was inhibited by antimycin and that PH_3 would prevent $O_2^{\cdot-}$ release.

By inhibiting cytochrome c oxidase, cyanide and presumably PH_3, will allow the flow of electrons back along the chain to

NADH dehydrogenase by a process known as reverse electron flow. This movement is toward NAD^+, a more electronegative component and requires energy in the form of ATP. Mitochondria may contain sufficient ATP to fuel reverse electron flow, but sub-mitochondrial particles must be supplemented with ATP. This mechanism also results in the production of $O_2^{\cdot-}$ from the autoxidation of NADH dehydrogenase and is driven specifically by FAD-linked substrates. Although reverse electron transfer is generally thought to be an *in vitro* phenomenon, under suitable conditions it could happen *in vivo*.

1.6 Damage From Free Radical Attack

As mentioned above (Introduction section 1.3) the hydroxyl radical ($HO^{\cdot}$) formed in a metal-catalysed Haber-Weiss reaction is highly reactive and cytotoxic. It is a strong oxidant and reacts with almost every type of molecule found in living cells, consequently membrane lipids, proteins and nucleic acids as well as other cytosolic molecules are at risk from free radical attack.

Lipid peroxidation, the result of $HO^{\cdot}$ attack on membrane lipids is one of the most commonly measured forms of damage, probably because of its far-reaching consequences. Membrane damage can eventually result in the destruction of an entire organelle. A schematic representation of polyunsaturated fatty acid peroxidation is shown in Fig. 3 (Buege and Aust 1977). Unsaturated bonds of lipid fatty acids are most susceptible to peroxidation and the process becomes

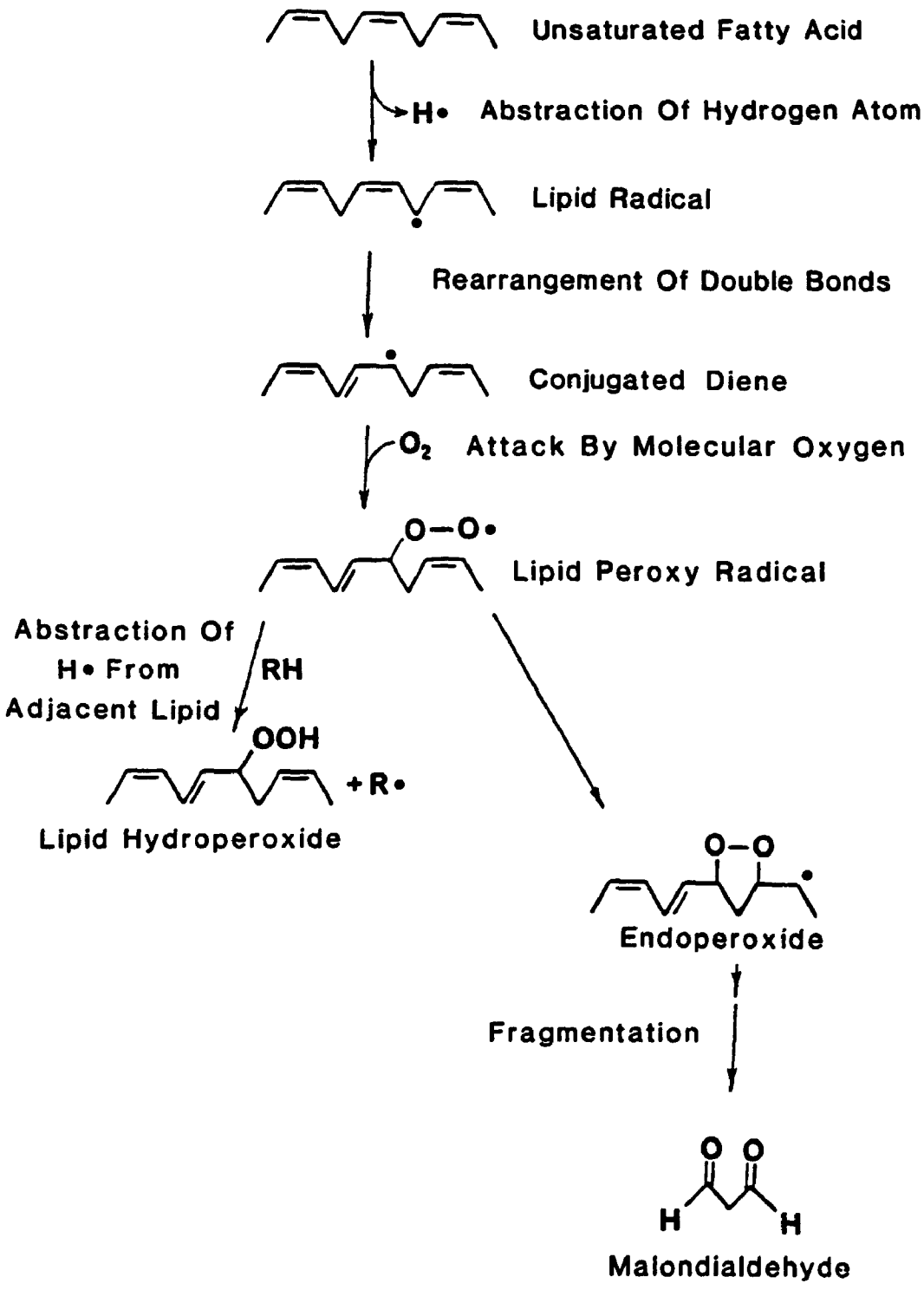
Figure 3

Peroxidation of Membrane Lipids

A simplified scheme of polyunsaturated fatty acid peroxidation is shown. Peroxidation can result in the initiation of another chain reaction and thus propagation of peroxidation throughout the membrane or it can lead to formation of endoperoxides. If the endoperoxide contains three or more double bonds the fragmentation products include the highly toxic malondialdehyde molecule.

See Introduction section 1.6 for details.

From Buege and Aust 1977.


Unsaturated Fatty Acid
H•
Abstraction Of Hydrogen Atom
Lipid Radical
Rearrangement Of Double Bonds
Conjugated Diene
O2
Attack By Molecular Oxygen
O—O•
Lipid Peroxy Radical
Abstraction Of
H• From
Adjacent Lipid
RH
OOH
+R•
Lipid Hydroperoxide
O—O
Endoperoxide
Fragmentation
O O
H H
Malondialdehyde

autocatalytic after initiation. Peroxidation begins with the abstraction of a hydrogen atom from an unsaturated fatty acid and the formation of lipid radical. A conjugated diene is formed as a result of the rearrangement of double bonds. These dienes have an absorbance peak at 233nm and can be measured spectrophotometrically (Corongiu and Milia 1983). A lipid peroxy radical is produced after attack by molecular oxygen and this can form an endoperoxide or abstract another hydrogen atom from the adjacent lipid initiating another sequence. The formation of endoperoxides can lead to the production of malondialdehyde in unsaturated fatty acids containing at least three methylene interrupted double bonds (Buege and Aust 1977). The formation of this oxidation by-product can be measured with thiobarbituric acid (TBA) (Asakawa and Matsushita 1979ab, Bird and Draper 1984). Malondialdehyde is not, however, a good indicator of lipid peroxidation since it is metabolised *in vivo* and reacts with cell lipids and proteins (Donato 1981). Its reaction with primary amines yields fluorescent conjugated Schiff's bases detectable at 470nm following excitation at 365nm (Dillard and Tappel 1984). Malondialdehyde can also cause cross-linking and polymerization of membrane components (Hochstein and Jain 1981) and reacts with the nitrogenous bases of DNA. Slater (1984) recommends that lipid peroxidation is most accurately measured by the decrease in polyunsaturated fatty acids since all the breakdown products are susceptible to further metabolism.

From this description of lipid peroxidation it can be seen that it is a very damaging event. Intrinsic membrane properties such as fluidity and permeability can be affected. These properties are of fundamental importance in maintaining the membrane in a state that allows efficient functioning of intramembrane proteins including enzymatically active proteins and those involved in ion transport.

Proteins are also susceptible to free radical attack resulting in denaturation. Damage is caused by hydrogen abstraction by $HO^{\cdot}$ from α-carbon atoms of tryptophan, tyrosine, phenylalanine and histidine followed by a reaction with O_2 to produce the peroxyl radical (Davies and Delsignore 1987). Covalently bound protein aggregations, extensive changes in electrical charge and the loss of tryptophan were common occurrances when a variety of proteins were exposed to $HO^{\cdot}$ (Davies 1987).

Free radicals can also react with the sulphur-containing amino acid residues of proteins, methionine and cysteine. Enzymes, such as many dehydrogenases, are dependent on these amino acids for activity and will be inactivated by free radical attack (Haugaard 1968). $HO^{\cdot}$ removes hydrogens from thiol groups which can then form aggregates as a result of cross-linking mediated by interprotein dissulphide bonds. Protein susceptibility depends on their amino acid composition as well as their intracellular location.

The effect of free radical attack on oligomycin-sensitive Mg^{2+}-dependent H^{+}-ATPase was investigated in the present study.

This enzyme is a complex of proteins with a hydrophobic membrane bound (F_o) that acts as a proton channel and an enzymatically active headpiece (F_1)(Harold 1986). Since Fo-ATPase spans the inner mitochondrial membrane, any changes in the fluidity of the microenvironment surrounding this module, as a result of lipid peroxidation, may affect its ability to transport protons and would result in a deceased enzyme activity. Residue analysis of F_1-ATPase demonstrated that it contains many aromatic- and sulphydryl-amino acids (Walker et al. 1985) which are susceptible to attack by free radicals. H^+-ATPase is, therefore, a suitable subject for investigation into the effects of free adical damage.

1.7 Objectives of This Study

1. To establish whether or not PH_3 stimulates release of H_2O_2 from mitochondria, isolated from insects and mouse liver, utilizing various substrates.
2. To look at the effect of respiratory chain inhibitors, rotenone, antimycin and myxothiazol an extramitochondrial release of H_2O_2 with and without PH_3.
3. To establish the relationship between the respiratory inhibitors, antimycin, myxothiazol and PH_3 by recording the difference spectra of mitochondrial cytochromes.
4. To look at the effect of PH_3 on bovine ubiquinone in vitro and insect ubiquinone after in vivo treatment with PH_3.

5. To look at the effect of PH_3 on components of the oxygen defence system, catalase, peroxidase, superoxide dismutase and glutathione both *in vitro* and after insects are treated with PH_3 *in vivo*.
6. To establish whether or not there is any measurable damage to cellular components, such as lipids, sulphydryl groups and protein (using the example of the transmembrane H^+-ATPase) of the type associated with free radical attack.

MATERIALS AND METHODS

2.1 Materials

All reagents were obtained from Sigma Chemical Co. (St.Louis, Mo., U.S.A.) and Aldrich Chemical Co. (Milwaukee, Wi., U.S.A.). The organic solvents used in the lipid extraction and lipid peroxidation assay were of spectroscopic grade.

2.2 Insects

The granary weevil, Sitophilus granarius (L) was the insect species used in this study. Insects were reared at 25±1°C and 70±5% RH on whole wheat kernels. The variety of wheat varied with availability. Insects showing resistance to PH_3 were selected from the normal wild-type, PH_3-sensitive population after exposing adults to 0.5mg PH_3/l for 24-72 hrs. Survivors were bred for the next generation. This selection procedure was repeated 2-3 times per year.

2.3 Preparation of Phosphine

The gas was generated from commercial formulations of aluminium phosphide (Gastoxin® Casa Bernardo Ltda, Sao Paulo Brazil).

$$AlP + 3H_2O \longrightarrow PH_3 + Al(OH)_3 \qquad [1]$$

In the laboratory phosphine (PH_3) was prepared daily by immersing pellets of aluminium phosphide in a graduated cylinder filled with distilled water, an inverted funnel direc-

directed the gas into a gas burette (Kashi & Bond 1975, Hobbs 1984). PH_3 collected in the headspace of the burette, was removed through a rubber septum at the top of the burette. It was free of contaminating ammonia and carbon dioxide since these dissolve in water. Alternatively, PH_3 was used directly from a cylinder acquired from Matheson (Whitby, Ontario).

The effect of PH_3 on enzyme activity *in vitro* was carried out by bubbling the gas into a cuvette equipped with a septum or into the chamber of an oxygen electrode. However, because of its instability in aqueous medium, there was no reliable method of quantitatively assaying the PH_3 concentration in solution. Consequently, the concentration of PH_3 in solution is denoted as the volume of gas bubbled into a specified volume of reaction medium.

The effect of PH_3 on enzyme activity after *in vivo* exposure was observed. Insects were treated with a specific dose of PH_3, reared on wheat for a certain duration and homogenated preparations made from the insects were assayed for enzyme activity.

2.4 Treatment of Insects with Phosphine

Two to four week-old adults were used in all experiments. They were separated from wheat using graduated sieves, weighed into batches of one gramme (1g = about 300 insects) and transferred into plastic screened cages (53mm length X 20mm diameter). These were suspended in 12.6 l rubber stoppered flasks which had been equilibrated to 25±1°C and

70±5% RH. The required volume of PH_3 was injected into the flask via a septum in the rubber stopper. Initial concentrations of PH_3 in the flask were measured by withdrawing a sample after 30 minutes and analysing it with a model 10A10 gas chromatograph (Photovac Inc., Thornhill, Ontario) equipped with a photoionization detector and a Teflon column 1m X 32mm ID packed with Tenax® (Chromatographic Specialites Inc. Support Canada Toronto)(60/80 mesh). At 36°C and a flow rate of 30ml.min^{-1} of high purity air carrier gas, the retention time of PH_3 was 0.35 mins. Peak areas were quantified using a model 3390A integrator (Hewlett-Packard, Avondale, PA) calibrated using standards of known amounts of PH_3.

A measure of mortality was made after every exposure by retaining 0.5g - 1gm (150-300 insects) and rearing the insects in a petri dish with wheat for at least 14 days before a mortality count was made.

2.5 Preparation of Cellular Fractions

Two methods of preparation were employed depending on the cell fraction to be studied. Crude extracts were prepared as follows. Insects 2-7gms were homogenized in a cold room at 4°C using a Polytron homogenizer (Brinkman Instruments, Westbury, N.J.) with a PT-10 generator (1cm diameter) at a speed setting of 0.7 (approx. 13,000 rpm). Ice cold 40mM HEPES buffer pH=7.4 containing 0.25M sucrose and 0.1mM EDTA was used. The homogenate was centrifuged at 300xg for 5 mins in a Sorval, Model RC-2 centrifuge. The supernatant was

carefully decanted and recentrifuged at 100,000g for 45 mins at 4°C in a Beckman Ultracentrifuge L8-80M. The supernatant was decanted and filtered through glass wool to remove fatty material. When intra-organelle enzymes were to be assayed, the organelles were fragmented in an MSE ultra sonicator set at 1.5 amps. Samples were kept on ice throughout the sonication procedure and were sonicated for 15 secs followed by 45 seconds of cooling. This was repeated five times. The suspension was centrifuged at 10,000xg for 10 minutes to remove unbroken mitochondria and other organelles and then at 100,000xg for 45 mins. to precipitate the sub-mitocondrial particles and other fragments. The supernatant contained soluble enzymes, the activity of which was measured.

The second method of preparation was used when intact mitochondria or microsomes were required (Kashi 1974). Insects, 5-7gms, were gently crushed in an ice cold pestle and mortar containing 20ml 0.25M sucrose and 0.1mM EDTA (pH=7.4) for approximately three minutes. The homogenate was filtered through moistened cotton cloth under vacuum using a Buchner funnel the residue was washed with 10ml buffer and refiltered. The filtrate was centrifuged at 300xg for 10 mins and the supernatant centrifuged at 8700xg for 10 mins. The resulting supernatant was removed and centrifuged at 10,000xg for 10 mins. to precipitate remaining lysosomes and peroxisomes (Boveris *et al*. 1972). The supernatant of this spin was centrifuged again at 100,000xg for 45 mins. to precipitate microsomes. The precipitate of the 8700xg spin was rinsed

carefully with 0.25M sucrose and 0.1mM EDTA to remove the fluffy layer consisting of broken mitochondria leaving intact mitochondria as a pellet. This tightly packed mitochondrial pellet was homogenized in a hand-held glass homogenizer with a teflon plunger, maintained on ice, and dispersed in the appropriate volume of isolation medium to the desired protein content. An aliquot of suspension was removed for protein determination (Methods Section 2.6) while fat-free bovine serum albumen was added to the remainder (1mg BSA/ml suspension). If sub-mitochondrial particles were required, the mitochondria were fragmented by sonication as described above.

Assay Procedures

2.6 Protein Determination

Protein concentrations were determined using the method of Bradford (1976) with fat-free bovine serum albumen as a standard.

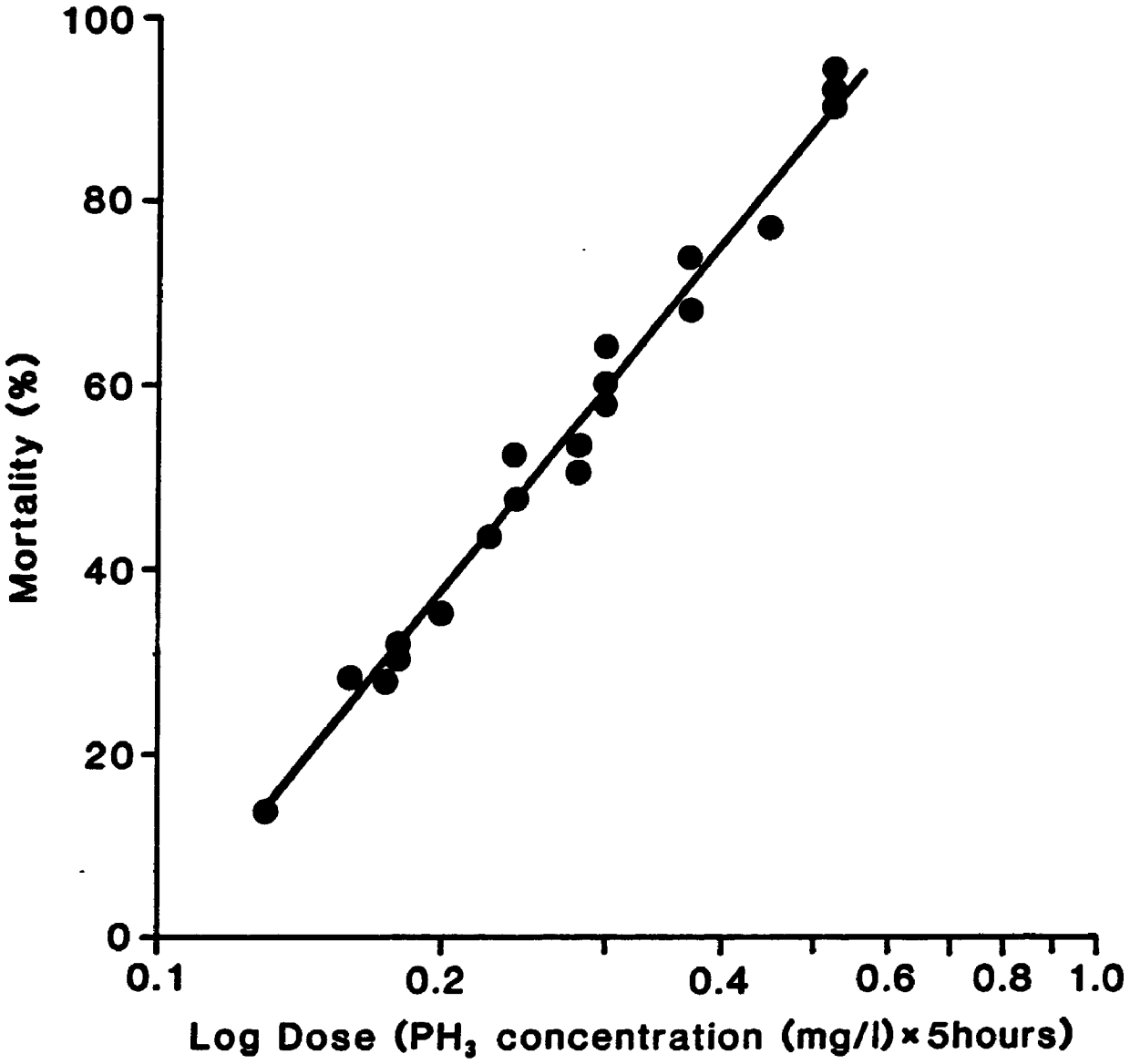
2.7 Relationship Between Mortality and Phosphine Concentration

Insects, 6-10gms, were exposed to known concentrations of PH_3 for variable durations and a log (concentration ($mg.l^{-1}$) X time) *vs* mortality curve was constructed. From this curve it was possible to estimate the dose of PH_3 that should be applied to insects to give a certain level of mortality. The graph shown (Fig. 4) contains data obtained after exposure to

Figure 4

Dose-Response Curve

Mortality is expressed as % of insects dead 14 days after exposure. Dose of PH_3 is expressed as log (concentration x time product). Concentration is in mg $PH_3.l^{-1}$. Time of exposure was 5 hours. The graph represents the line of best fit as determined by regression analysis.


100
80
60
40
20
0
Mortality (%)
0.1
0.2
0.4
0.6
0.8
1.0
Log Dose (PH_3 concentration (mg/l) × 5hours)

a specific concentration of PH_3 for 5 hours. In some experiments, insects were exposed to PH_3 for 12 or 18hrs, however, these results have not been included since it was found that mortality was lower than expected with longer durations. A possible explanation for this is that PH_3 breaks down with time into a product that is not toxic to insects. This observation meant that it was essential to make a mortality count after every exposure.

2.8 Measurement of Extramitochondrial-Hydrogen Peroxide Release

Cytochrome c peroxidase (CCP), with an absorbance peak at 407nm forms an enzyme-substrate complex with H_2O_2 which has a peak at 419nm (Boveris et al. 1972). The complex was stable as long as no reduced cytochrome c was present. Cytochrome c peroxidase cannot pass through the outer membrane of intact mitochondria and consequently will not come in contact with mitochondrial cytochrome c.

$$\underset{(407nm)}{CCP} + H_2O_2 \longrightarrow \underset{(419nm)}{CCP\text{-}H_2O_2\ (ES\ complex)} \qquad [2]$$

$$CCP\text{-}H_2O_2 + 2cyt.c^{2+} \longrightarrow CCP + 2cyt.c^{3+} + 2OH^- \qquad [3]$$

On addition of H_2O_2, E_{407} decreases and E_{419} increases with the formation of cytochrome c peroxidase $-H_2O_2$ complex. This forms the basis of a very sensitive assay for H_2O_2 ($E_{419-407}$ =

$50mM^{-1}.cm^{-1}$). In this present study horseradish peroxidase was initially used in place of yeast cytochrome c peroxidase, ($E_{417-402}$ = $50mM^{-1}.cm^{-1}$) however, the rate of complex formation was very slow because of significant interference from catalase and other hydrogen donors (Andreae 1955). Assays were performed using an Aminco-Chance DW-2a dual wave-length spectrophotometer (Technical Marketing Ass. Ltd., Mississauga, Ont.). In this study the sample wavelength was set at 424nm with an active reference at 404nm (Autor & Stevens 1980). This combination was chosen because it gave consistently good results. Extinction co-efficient with this wavelength pair was $55mM^{-1}.cm^{-1}$. Extramitochondrial H_2O_2 release was measured using various substrates and inhibitors of the electron transport chain. One problem encountered with peroxide assays is that catalase, often contaminating mitochondrial preparations, removes H_2O_2 and could result in an underestimation of production. Azide (10-30mM) is normally used to inhibit catalase (Boveris et al. 1972), however, it could not be utilized in these experiments since it inhibits cytochrome c oxidase, the major target site for PH_3 (Kashi and Chefurka 1976). Catalase in free solution at a concentration of (0.05-0.1μM) does not compete successfully with 1-2μM cytochrome c peroxidase for H_2O_2 (Boveris et al 1972) and it was observed that the catalase concentration was less than this in the mitochondrial preparation used.

Yeast cytochrome c peroxidase (CCP) is not available commercially. The original batch used in this study was

supplied by Dr. Ann English (Concordia University, Montreal, Que.) and additional quantities were prepared by the method of English et al. (1986) with certain modifications (See Methods section 2.8.1).

Intact mitochondria, either insect or mouse liver (0.3-0.6mg protein) were introduced into a cuvette containing 50mM phosphate buffer pH=7.4 with 0.25M sucrose, 0.1mM EDTA, 3.75mM $MgSO_4$ and 2.0μM CCP having a final volume of 3ml. BSA (1mg.ml^{-1}) was present in the buffer on certain specified occasions. Different substrates, 5mM pyruvate + 1mM malate, 4mM succinate and variable concentrations of α-glycerophosphate were added to the reaction medium and CCP-H_2O_2 complex formation observed. The effect of varying concentrations of respiratory inhibitors, PH_3, antimycin, myxothiazol and rotenone, (see Introduction section 1.2.4) on extramitochondrial H_2O_2 release was also tested. Substrates and inhibitors were added after the H_2O_2 release stimulated by endogenus substrate had ceased. In an attempt to establish the identity of the endogenous substrate, 4-pentenoic acid, an inhibitor of fatty acid oxidation was incubated with mitochondria for three mins prior to addition of CCP.

2.8.1 Isolation and Purification of Cytochrome c Peroxidase

Yeast cytochrome c peroxidase was prepared using the method of English et al. (1986) with certain modifications. Five kg. Fleischmans baker's yeast were dried on trays for 36 hours in which time they lost 30% of their original weight.

Yeast was divided into three 4000ml beakers on ice in the fumehood and 300ml cold (4°C) ethyl acetate were added to each beaker. The mixture was stirred to a sticky paste and left overnight to lyse by which time it had reached room temperature. The beaker was surrounded with fresh ice and 550ml of extraction buffer composed of 0.05M sodium acetate pH=5.0 and 1.25mM EDTA were added to each beaker. The extract was thoroughly stirred and centrifuged in 500ml polypropylene centrifuge tubes in a Beckman J2-21 M/E centrifuge (#10 rotor) at 4800 rpm (rav = 2500xg rmax = 3800xg) for 20 mins at 4°C. All the following steps were carried out at 4°C. The brown supernatant was vacuum filtered through Watman #1 filter paper using a Buchner funnel and gently stirred for 2 hrs with 300ml of suspended DEAE-agarose (DEAE sepharose CL-6B Pharmacia) equilibrated with extraction buffer (English personal communication 1986). The bulk adsorption technique described by English was further modified using a bulk extraction method as follows: DEAE-agarose enzyme complex was vacuum filtered using Whatman #52 filter paper and the filtrate tested for CCP activity (See Methods section 2.8.2). The residue containing CCP was washed with 500ml extraction buffer, then resuspended in 500ml of 0.5M sodium acetate buffer pH = 7.5 and stirred for 20 mins removing the enzyme by bulk extraction. The filtrate after vacuum filtration through Whatman #52 filter paper contained CCP activity which was assayed (Table 1) (See Method section 2.8.2).

The filtrate was dialysed against deionised distilled

water for 24 hrs with three changes of water and lyophilized for 24 hours until it crystalized (Freezemobile 3 Virtis Co.Ltd. Gardiner, N.Y.). The lyophilized protein was resuspended in 3 ml 0.5M sodium acetate buffer pH=7.5 and applied to a 100X2.6cm column of degassed Sephadex G-75 (Pharmacia) equilibrated with 0.1M sodium acetate buffer pH=5.0. The pressure was kept at 60cm at all times during gel filtration by maintaining the flask of buffer at this distance from the bottom of the column. The enzyme was eluted with the same buffer at a rate of approx. 1 ml/min. CCP was observed as a brown band on the column. The first 100ml of eluant were discarded and the next 40 fractions (each fraction being 4.3ml) were collected. The purity index (E_{408}/E_{280}) was measured for each fraction (Yonetani 1967). Fractions 11 to 29 had purity indices of 0.98-1.38 and were pooled. The activity of pooled enzyme extract was measured (approx. 60ml). The extract was dialyzed against four changes of deionized distilled water lyophilized for 4 hours to a crystaline powder which was resuspended in 5ml of 10mM phosphate buffer pH=7.0 and the activity and purity index measured (See Methods section 2.8.2). Results of periodic tests for CCP activity are shown on Table 1. The enzyme was divided into aliquots of 0.5ml, sealed in tubes and frozen at -20°C until required for H_2O_2 assay.

2.8.2 Measurement of Cytochrome c Peroxidase Concentration

Two methods were employed to establish the concentration

TABLE 1

Extraction, Purification and Quantification of Yeast Cytochrome c Peroxidase

	Volume (ml)	Activity* (units)	Purity**	% Recovery	Total*** mgCCP
Crude extract (filtered)	4780	0.08	0.04	100	380
Bulk DEAE extract	470	0.72	0.08	88	336
Total G75 eluant	82	4.80	1.10	68	260
After dialysis, lyophilization and resuspension	5	96.00	1.02	63	240

Extraction and purification procedure was carried out according to English et al. (1986) with modifications. The table shows the results of the most successful procedure performed during this study.

* 1 unit= ΔA_{550}.10sec^{-1}.10μl preparation. (Yonetani and Ray 1965).

** purity index = $\frac{A_{408}}{A_{280}}$ (Yonetani 1967)

*** 1 unit=20μM enzyme hematin. MW=50,000g (Yonetani and Ray 1965).

of cytochrome c peroxidase. CCP has an absorbance maximum at 408nm and the concentration was found using the extinction coefficient $e=93.0mM^{-1}cm^{-1}$ (Yonetani 1967). The second method measured the initial rate of peroxidatic oxidation of ferricytochrome c catalyzed by CCP. The reaction was initiated by addition of H_2O_2 (180μM) to 0.1M sodium acetate buffer pH=6.0 containing 90% reduced ferrocytochrome c (1mM) and CCP (0.1-1μM corresponding to $A_{408}=0.01-0,1cm^{-1}$). Absorbance at 550nm decreased as cytochrome c was oxidised and the initial decrease in 10 secs per 10μl of enzyme solution were used as an arbitrary unit of enzyme activity. One unit corresponded to 10μM enzyme hematin (Yonetani and Ray 1965). The purity index is the ratio of absorbance at 408 and 280 nm (Nelson et al. 1977).

2.8.3 Calibration of the Cytochrome c Peroxidase Assay

The ability of CCP to detect H_2O_2 released into solution was examined using the oxidation of glucose by glucose oxidase which results in oxygen utilization and H_2O_2 production:

$$C_6H_{12}O_6 + O_2 + H_2O \longrightarrow C_6H_{12}O_7 + H_2O_2. \qquad [4]$$

The rate of oxygen consumption was measured polarographically using a Clark-type oxygen electrode maintained at 25°C. Glucose oxidase (0.3-6.0 units) was added to the chamber of the oxygen electrode containing air-equilibrated 30mM HEPES buffer pH=7.4, with 145mM KCl, 3mM $MgCl_2$, 5mM potassium phosphate, 0.1mM EGTA and 10mM glucose (3ml final

volume). Oxygen consumption was compared to H_2O_2 generation by measuring the rate of formation of the CCP-H_2O_2 complex using DW-2a dual wavelength spectrophotometer with sample wavelength at 424nm and reference at 404nm (See Methods section 2.8). Glucose oxidase (0.004-0.01 units/ml) was added to a cuvette containing 2μM CCP in the 10mM glucose medium described above. Since oxygen consumed was stoichiometrically related to H_2O_2 generated the result of the assays allows the proportion of H_2O_2 detected by CCP to be calculated.

Oxygen consumption and formation of the cytochrome c peroxidase-H_2O_2 complex were linearly related to concentration of glucose oxidase (Fig. 5). Interpolation from the graph reveals that two units of glucose oxidase resulted in the consumption of 0.29μmoles O_2. min^{-1} and $2X10^{-2}$ units generated 2.78 nanomoles $H_2O_2.min^{-1}$. This suggests that the cytochrome c peroxidase used to indicate H_2O_2 generation in this study was highly sensitive and detected almost 100% of H_2O_2 produced.

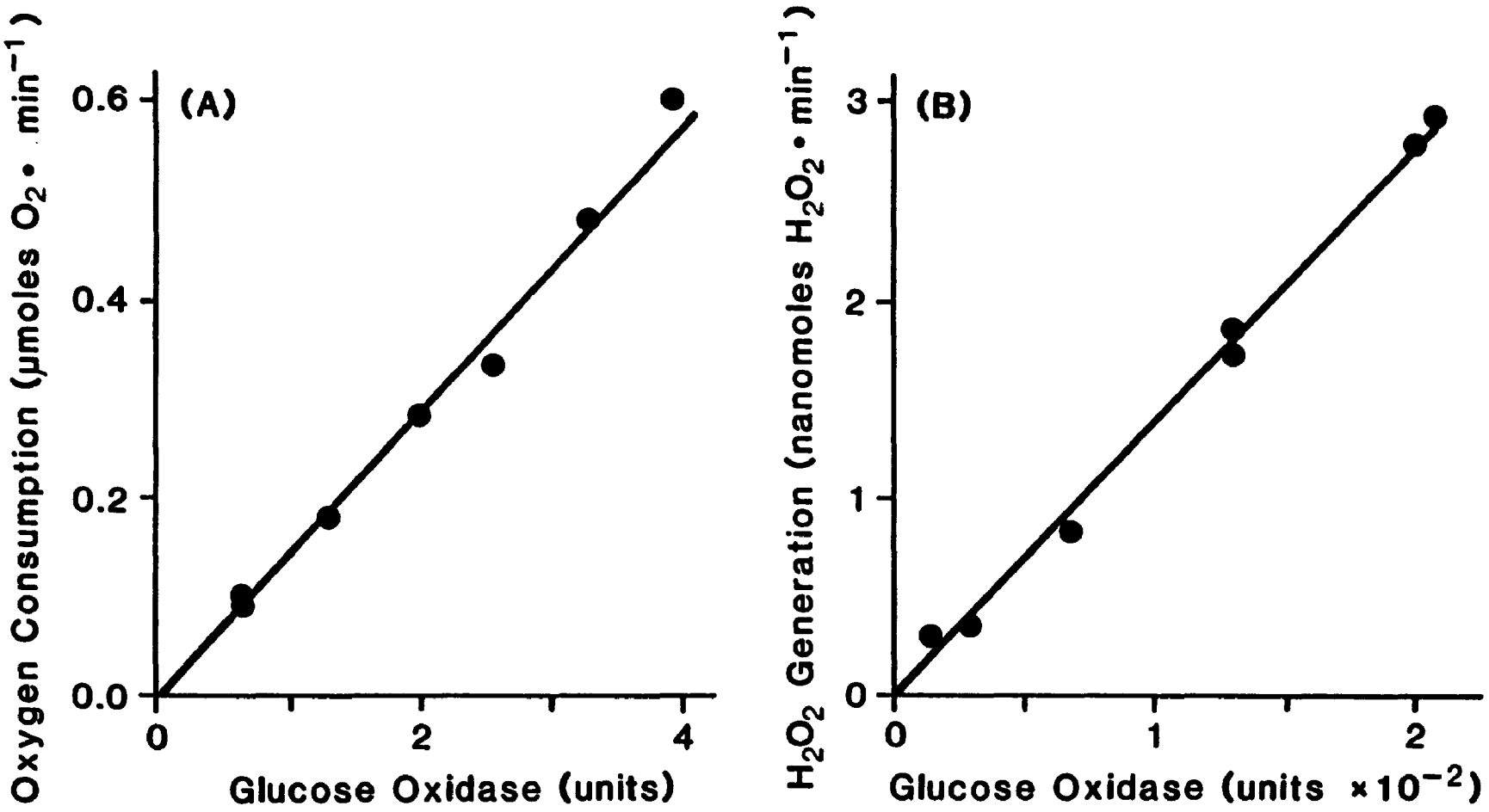
2.9 Spectral Studies on Mitochondrial Cytochromes

Spectral studies were carried out using an Aminco-Chance DW-2a dual wavelength spectrophotometer in split-beam scanning mode with wavelength scale 375-675nm. Insect or mouse liver mitochondria (2.5-3mg protein) were added to aerated 50mM HEPES buffer pH=7.0 containing 0.9% NaCl (3ml final volume) in two cuvettes. Baseline was zeroed with reference and sample cuvettes in the spectrophotometer. Spectral scans were made at medium speed with the absorbance adjusted as required

Figure 5

Calibration of the Cytochrome c Peroxidase Assay

Figures A and B show the rates of oxygen consumption and H_2O_2 generation respectively. The concentration of glucose oxidase is given in units in a final volume of 3ml of 30mM HEPES buffer pH=7.4 containing 5mM phosphate, 145mM KCl, 3mM $MgSO_4$, 0.1mM EGTA and 1mM glucose. 2μM CCP were used in the spectrophotometric assay for H_2O_2. For experimental detail see Methods Section 2.2.1.


(A)
Oxygen Consumption (μmoles O_2 • min^{-1})
0.6
0.4
0.2
0.0
0
2
4
Glucose Oxidase (units)
(B)
H_2O_2 Generation (nanomoles H_2O_2 • min^{-1})
3
2
1
0
0
1
2
Glucose Oxidase (units $\times 10^{-2}$)

using a higher absorbance range, 0.1 or 0.2 between 375-480nm and reducing the range to 0.02 or 0.05, between 480-675nm. Complete reduction of cytochromes was acheived using a few crystals of sodium dithionite in the sample cuvette, resulting in the production of a difference spectrum.

PH_3 (300µl) added to the sample cuvette resulted in reduction of cytochromes and the difference spectrum was recorded. No reduced peak representing cytochrome b was observed which was unexpected and it was thought that it could have been masked by cytochrome c, consequently, mitochondria from 20 gms of insects were depleted of cytochrome c using the technique described by Jacobs and Sanadi (1960).The mitochondrial pellet was suspended in 20ml 0.015M KCl, a hypotonic solution which breaks open the membranes, left for 10 mins at 0°C, centrifuged at 6000Xg for 10 mins and the pellet resuspended in 20 ml of 0.15M KCl to extract the cytochrome c. After 10 mins at 0°C it was centrifuged at 5000xg for a further 10 mins. and the residue washed twice more with 20ml of 0.15M KCl. The supernatants from the first two 0.15M KCl extractions were combined and analysed spectrally for cytochrome c content. Extracted mitochondrial residue was suspended in 3ml of 0.3M sucrose, and the difference spectrum of reduced cytochromes observed after PH_3 (300µl) addition.

The absolute spectrum from the supernatant containing cytochrome c was observed with concentrated supernatant in the sample cuvette and 0.15M KCl in the reference cuvette.

Whole mitochondria were used to test the effect of in-

hibitors on cytochrome spectra. The contents of the sample cuvette was reduced using antymycin (1.6μM), myxothiazol (0.9μg), PH_3 (300μl) and combinations of the three respiratory chain inhibitors and the difference spectra recorded.

2.10 Ubiquinone

Recordings were made of the absolute spectra of an ethanolic solution of oxidised bovine ubiquinone (Q_{10}) (Sigma), as well as ubiquinone reduced by the addition of a few crystals of sodium borohydride, (scanning from 230-320nm) (Redfearn 1967). These spectra were compared with those obtained after bubbling PH_3 (100μl) into a cuvette containing oxidised or reduced ubiquinone.

Ubiquinone was extracted from insects exposed to PH_3 (LD_{50}) and compared to controls. Extraction procedures were described by Redfearn (1967) using mitochondria containing 20-40mg protein.ml^{-1}. One ml of freshly prepared mitochondria was placed in a 15ml glass stoppered centrifuge tube and denatured by rapid addition of 4ml cold (-20°C) methanol containing pyrogallol (1mg/ml) to prevent ubiquinol oxidation. Petroleum ether (5ml) was added immediately, the tube stoppered and vortexed (Vortex mixer, Fisher) for 1 min and centrifuged at 1000g for 10 mins in a Sorvall GLC-2 centrifuge to separate the phases. The upper ether layer was transferred to another 15ml glass-stoppered tube and denatured residue further extracted twice with petroleum ether. Combined ether extracts were treated with 2ml of 95% (v/v) methanol, the

mixture shaken gently by inversion for 30 secs. and the phases separated again by centrifugation. The ether layer was removed by aspiration, placed in a 100ml round-bottomed flask and reduced to dryness using a rotary evaporator. The resulting residual lipid was dissolved in 3ml distilled ethanol and the flask placed in a water bath (60°C) for 30 secs to ensure complete solution. The spectrum of the ethanolic solution was recorded in the range of 220-320nm. The presence of ubiquinone was indicated by an absorption maximum at 275nm, ubiquinol had a peak at 291nm (Redfearn 1967). Ubiquinone was reduced by the addition of a few crystals (approx. 0.2mg) of sodium borohydride followed by thorough mixing. After two minutes the spectrum was redetermined and the difference in absorbance at 275nm was used to calculated ubiquinone concentration (molecular extinction coefficient $(e_{ox}-e_{red})_{275}=12,250$).

$$[\text{ubiquinone}].\text{mg protein}^{-1} = \frac{3 \times E_{275}}{12.25 \times \text{mg protein/ml}} \qquad [5]$$

2.11 Effect of Phosphine on the Oxygen Defence System

2.11.1 Superoxide Dismutase

Superoxide dismutase (SOD) activity was measured using the technique described by Marklund (1985) based on inhibition of pyrogallol (1,2,3-benzenetriol) autoxidation (See Introduction section 1.4 for SOD function). Pyrogallol autoxidizes rapidly in alkaline solution with the formation of a product

that absorbs at 420nm and $O_2^{\cdot-}$ which then participates as a chain propagating species in the process. Autoxidation is highly dependent on $O_2^{\cdot-}$ and is inhibited 97.5% by SOD.

Pyrogallol autoxidation was initiated by addition of 25µl of 24mM pyrogallol in 10mM HCl (0.2mM) to a cuvette containing 50mM tris-HCl buffer pH=8.2 with 0.1µM catalase and 1mM DTPA. This was used as a chelator since EDTA is unable to prevent acceleration of pyrogallol autoxidation by iron (Fe^{3+}) (Marklund & Marklund 1974). The buffer was equilibrated with air by rapid stirring for 20 mins. Maximum repeatability was obtained when pyrogallol autoxidation resulted in a change in absorbance A_{420}=0.02 min^{-1}. Inhibition of autoxidation was linearly related to concentration of purified bovine CuZnSOD up to about one unit of activity. A unit is defined as the activity that inhibits the reaction by 50% and is equivalent to about 400µg bovine CuZnSOD (Marklund 1985). The total protein concentration was maintained below 60µg.ml^{-1} since higher concentrations affected the assay (Del Maestro & McDonald 1987). Activities of two isozymes of SOD were distinguished in the basis of their sensitivity to cyanide. SOD located in the cytosol (CuZnSOD) is inhibited 95% by 1mM cyanide at pH=8.2, while mitochondrial SOD (MnSOD) is resistant to cyanide. Cyanide reduced the rate of pyrogallol autoxidation by 20-30% and this was accounted for when calculating SOD activity.

$$\text{CuZnSOD (units)} = \text{"Total SOD"} - \frac{(\%\text{reduction by CN} \times \text{CN-resistant})}{0.95}$$

MnSOD = "Total SOD" - CuZnSOD

(units)

Activity was expressed as units SOD.mg^{-1} instead of concentration of SOD since no conversion exists for insect species. The pyrogallol assay for SOD activity was used in most of the experiments performed in this study. It has many advantages, it is convenient and reliable, the reagents are stable and inexpensive and the reaction is linear. However, since pyrogallol is a good substrate for peroxidase, catalase (0.1μM) was used in the assay to remove H_2O_2 which could be utilized in the peroxidase reaction (Marklund 1985). Without catalase, at protein concentrations of greater than 60$\mu g.ml^{-1}$, SOD activity was underestimated with control groups in this study, since these groups had high peroxidase activity.

This method was used to assay cyanide sensitive and insensitive SOD activity in susceptible insects after exposure to PH_3 (LD_{30})(See Methods section 2.4) at various durations after exposure. Homogenates from susceptible and resistant insects exposed to PH_3 (LD_{50} for susceptible insects) were tested for SOD activity three days after exposure.

It was observed that pyrogallol autoxidation was inhibited by PH_3 and a method described by Beauchamp and Fridovich (1971) was utilized in experiments investigating the direct effect of PH_3 on SOD. Xanthine oxidase catalyses the release of O_2- from xanthine.Nitrobluetetrazolium (NBT) is reduced by

superoxide to formazan the formation of which can be followed spectrophotometrically since it absorbs at 560nm. The reaction was initiated by the addition of xanthine oxidase ($3.3X10^{-9}M$) to air-saturated 0.05M sodium carbonate buffer pH=10.2 containing $2.5X10^{-5}M$ NBT, 0.1mM xanthine and 0.1mM EDTA. Addition of SOD to the reaction medium decreases the rate of formazan formation. The effect of PH_3 (100µl) on one unit of bovine CuZnSOD (Sigma), MnSOD extracted from rat liver mitochrondria and SOD present in whole insect homogenates was tested using this assay, in a final volume of 3 ml.

MnSOD was extracted according to the method of Geller and Winge (1982). The liver was removed from a single adult male rat and homogenized in three volumes (w/v) of 0.25M sucrose, 20mM Tris-HCl, 1mM EDTA pH=7.4 using a Potter Elvehjem tissue grinder. After dilution with another six volumes of buffer the homogenate was centrifuged at 300xg for 15 mins. The supernatant was centrifuged at 10,000xg for 15 mins. and the pellet resuspended to a final concentration of about 50mg protein/ml with buffer. This was incubated with 5mg recrystallized digitonin for 10 mins at 4°C. Digitonin was recrystallized by boiling 1gm commercial digitonin with 50ml ethanol, cooling to -20°C for 2 hours, filtering through filter paper and dehydrating the digitonin precipitate. After incubation with digitonin, three volumes of homogenization buffer were added and the homogenate centrifuged at 10,000xg for 10 mins. The pellet containing mitochrondria and MnSOD was resuspended in 2ml buffer, sonicated for 5 mins at 55W

(MSE ultrasonicator) and further centrifuged for 15 mins at 48,000xg. The supernatant contained partially purified MnSOD.

Bovine CuZnSOD (Sigma) and rat liver MnSOD were incubated with PH_3 (100µl) in cuvettes sealed with rubber septa for 30mins at 25°C. Activity was measured before and after exposure to PH_3.

2.11.2 Catalase

Catalase is an enzyme that removes hydrogen peroxide from the cell (see Introdution section 1.4). Catalase activity was determined spectrophotometrically by following the disappearance of hydrogen peroxide (H_2O_2) at 240nm (Aebi 1974). Absorbance was measured at 25°C using a model UV-260 recording spectrophotometer (Shimadzu Corp. Kyoto, Japan) with a slit width of 2nm.

Aliquots of suspension were added to a cuvette containing 0.05M potassium phosphate buffer pH=7.0. The reaction was started by the addition of 0.02M H_2O_2 prepared from 30% (v/v) H_2O_2 solution (Analytical grade Fisher Scientific). The concentration of H_2O_2 used gave an absorbance of approximately A=0.5 before the suspension was added. Decrease in absorbance was measured for the first 30-40 seconds when the rate was linear. One Sigma unit of activity was defined as the amount of catalase required to decompose one µmole of H_2O_2 per min at 25°C. Activity was expressed as units per mg protein.

The effect of PH_3 on catalase in insect homogenates was investigated. Crude homogenates were sonicated and centrifug-

ed at 100,000xg for 45 minutes (See Methods section 2.5). An aliquot of supernatant corresponding to a final concentration of 0.04mg protein.ml^{-1} as exposed to PH_3 (100μl) in a cuvette which was shaken thoroughly and the catalase activity measured.

Homogenates from insects exposed to PH_3 (LD_{15-20}), (0.04mg PH_3.l^{-1}X5 hrs, as described in Methods Section 2.4) were assayed for catalase activity for 15 days after exposure.

Catalase activity in homogenates extracted from resistant and susceptible insects was assayed 3 days after exposure to PH_3 (LD_{50}).

2.11.3 Peroxidase

Peroxidase enzymes also remove H_2O_2 using a variety of electron donors (see Introduction section 1.4). Glutathione peroxidase activity was measured following the method of Flohé and Gunzler (1984), using mitochondrial preparations or supernatant from sonicated homogenates (Methods section 2.5). The assay was based on the coupled oxidation of reduced glutathione by glutathione peroxidase [6] to the disappearance of NADPH by glutathione reductase [7].

$$2\ GSH + t\text{-}BOOH \longrightarrow GSSG + H_2O + t\text{-}BOH \qquad [6]$$

$$GSSG + NADPH + H^+ \xrightarrow[(340nm)]{} 2GSH + NADP^+ \qquad [7]$$

The decrease in absorbance at 340nm [E_{340}=6.22mM^{-1} cm^{-1}], representing the removal of NADPH, was measured using 260 split beam spectrophotometer. t-Butyl hydroperoxide was used since this is not a substrate for catalase. An aliquot of homogenate was incubated with 3ml of 50mM phosphate buffer pH=7.0 containing 0.5mM DTPA, 0.16mM NADPH, 2mM reduced glutathione and 1.0 unit of glutathione reductase for 1 min at 25°C and the baseline rate of oxidation recorded at 340nm. The reaction was initiated by addition of 20µl 0.09M t-butyl hydroperoxide. The specific activity was expressed as units /mg protein. One unit was defined as the amount of enzyme that converted one µmole NADPH to $NADP^+$ per min at 25°C.

Activity of an unidentified peroxidase was measured spectrophotometrically according to the method of Armstrong et al. (1978), using p-phenylenediamine as a hydrogen donor co-substrate which is oxidised to a compound that absorbs at 485nm. An aliquot of mitochondria or of supernatant from 100,000g spin was added to a cuvette containing 3ml of 0.15M phosphate buffer pH=7.5 with 20mM H_2O_2 and 18mM p-phenylenediamine. Initial experiments also included 2.5mM sodium azide to ensure that catalase was not involved. Absorbance was followed for two mins and enzyme activity was expressed as change in absorbance per min per mg protein.

The effect of PH_3 on peroxidase activity was tested. PH_3 (100µl) was bubbled through reaction medium containing 0.04mg/ml supernatant and peroxidase activity was measured. Homogenates from insects exposed to PH_3 (LD_{15-20}) were assayed

for peroxidase activity over a period of 15 days. Resistant and susceptible insects were exposed to PH_3 (LD_{50} for susceptible insects) and the supernatant tested for enzyme activity three days after then end of fumigation.

2.11.4 Glutathione

Glutathione is the specific electron donor for glutathione peroxidase activity (see Introduction section 1.4). Glutathione concentrations were determined spectrofluorimetrically according to the method of Hissin and Hilf (1976) using an Aminco-Bowman fluorescence spectrophotometer (American Instrument Co. Silver Springs Ma). Reduced glutathione (GSH) and oxidised glutathione (GSSG) standards (2µg/100ml) were prepared and standard curves constructed daily. GSH was prepared in 0.1M sodium phosphate buffer pH=8.0 containing 5mM EDTA while GSSG was prepared in 0.1N NaOH.

Insects (2g) were homogenized on ice with the Polytron in 5ml phosphate-EDTA buffer containing 5% phosphoric acid to precipitate proteins and vacuum filtered through cotton to remove large particulate matter. The homogenate was centrifuged at 100,000xg for 30 mins at 4°C and the pellet kept for protein analysis (Allen personal communication). GSH concentration was measured as follows: phosphate-EDTA buffer (4.5ml) described above was added to 0.5ml 100,000g supernatant. The final assay mixture (3ml) contained 100µl diluted supernatant, 2.8ml phosphate-EDTA buffer and 100µl containing 150µg OPT in distilled methanol. The reaction medium was

incubated for 15 mins at room temperature. GSH reacts specifically with OPT at pH=8.0 yielding a highly fluorescent product that is activated at 350nm with an emission peak at 420nm (Hissin & Hilf 1976).

GSSG concentration was assayed after incubating 0.5ml supernatant with 200µl freshly prepared 0.04M N-ethylmaleimide (NEM) in ethanol for 30 mins at room temperature and diluting with 4.3ml 0.1N NaOH. Assay procedures were carried out as described above except that 0.1N NaOH was used in the incubation medium instead of phosphate-EDTA buffer since GSSG reacts with OPT at pH=12.

The protein concentration of the sample was determined using the precipitate of the 100,000g spin (Allen personal communication 1987). Precipitated proteins were resuspended to original total volume, obtained after vacuum filtration, in 0.1N NaOH to neutralize the acid. The suspension was diluted 10X and proteins assayed using the method of Bradford (1976) with BSA standards made up in 0.01N NaOH. Glutathione levels were measured 24 hrs after susceptible insects were exposed to PH_3 (LD_{65}, LD_{90}) and after resistant insects were exposed to PH_3 (LD_{65}).

2.12 Damage Resulting from Free Radical Attack

Superoxide ($O_2^{\cdot-}$) can react with H_2O_2 in an iron catalysed reaction to give the highly reactive cytotoxic oxidant, hydroxyl radical $HO^{\cdot}$ (see Introduction section 1.3)(McCord & Day 1978). Free radicals can damage; proteins, because of the

reactivity of aromatic and sulphydryl-containing molecules with free radicals (Pryor 1976); nucleic acids and DNA, causing chromosomal aberrations and membrane lipids, because unsaturated bonds of membrane fatty acids readily undergo free radical initiated peroxidation, (see Introduction section 1.6).

In this study damage caused by H_2O_2 and free radicals was observed by measuring lipid peroxidation, sulphydryl group content and the activity of the inner mitochondrial membrane enzyme, oligomycin sensitive ATPase. This enzyme was chosen because it is a trans-membrane protein, susceptible to radical attack and whose inactivation would have serious consequences to the organism (See Introduction section 1.6).

2.12.1 Lipid Peroxidation Determination

The extent of lipid peroxidation on mitochondrial and microsomal membranes from control and PH_3-treated insects ($0.065 mg.l^{-1}$ for 6 hrs, LD_{70}) was determined by measuring the concentration of lipid soluble fluorescent products and polyunsaturate to saturated fatty acid ratios. Other methods were attempted, such as measuring thiobarbituric acid (TBA) reactive materials eg. malondialdehyde (Ohkawa et al. 1979) with no success. Malondialdehyde was found to be consumed by mitochondrial enzymes (Recknagal and Ghoshal 1966) and microsomal peroxidation was not studied using this technique.

Mitochondrial and other membranes, isolated from whole insects were exposed to free radical generating systems in

vitro in order to assess membrane susceptibility to peroxidation.

(i) *Lipid Peroxidation In Vitro*

Three different free-radical generating systems were utilized in an attempt to peroxidize insect-membrane lipids.Mitochondrial and microsomal membranes were exposed to two non-enzymatic systems. The first system described by Rehncrona *et al*. (1980), used freshly prepared 0.3mM $Fe(NH_4)_2SO_4$, 0.1mM ADP and 0.02mM ascorbate in the incubation medium. In the second system, membranes were incubated with freshly prepared 0.1mM $Fe(NH_4)SO_4$, 0.5mM AMP and 0.05mM H_2O_2 (Aust personal communication 1988). Membrane homogenates (5-10mg proteins.ml^{-1}) were divided into two aliquots of 3 ml in 10 ml beakers, one of which contained the free radical generating system was incubated at 25°C for 1 hour.

Another *in vitro* system relied on free radical production from the mitochondrial electron transport chain and consequently only mitochondria or sub-mitochondrial particles (SMP) were used.Membranes were incubated for 1.5 hrs with 0.1mM $Fe(NH_4)_2SO_4$, 0.1mM EDTA, 4mM α-glycerophosphate with or without 500μl PH_3. The beaker with PH_3 was sealed with parafilm to prevent escape of the gas during its injection into the medium and neoprene stoppers were used to seal all the beakers for the duration of incubation. The $Fe(NH_4)_2SO_4$ solution was prepared immediatley prior to use.

(ii) Extraction of Mitochondrial & Microsomal Lipids

Lipids were extracted immediately following isolation of the membrane under investigation, either mitochondria or microsomes from control or exposed insects in vivo and membranes that had been exposed to free-radical generating systems. The following procedure was used with volumes of suspension up to 1ml containing up to 50mg cell fraction dry weight (with insect mitochrondria 1mg protein corresponded to 7.5mg dry weight and the maximum protein content for this procedure was 6.7mg/ml). After in vitro experiments the volume ranged between 3-5ml with 15 to 50mg protein and the volumes of extraction solvents were increased accordingly. The technique for lipid extraction was described by Kates (1986). The suspension of cell fraction was placed in a 15ml glass stoppered centrifuge tube. To this 5ml methanol: chloroform (1:1 v/v) were added, the mixture was vortexed for one hour and the solid material precipitated by centrifugation in a IEC tabletop centrifuge 950xg (2360rpm) for 10 mins. The supernatant extract was transferred using a Pasteur pipet to another glass stoppered centrifuge tube into which 2.5ml chloroform and 2.5ml distilled water were added.The mixture was shaken and centrifuged as before to separate the phases. The upper aqueous layer including any emulsion remaining at the interface was carefully removed using a Pasteur pipet and discarded while the lower chloroform phase was withdrawn, placed in a 100ml round-bottomed flask and reduced to dryness in a rotary evaporator. The lipid residue was dissolved in

3ml absolute methanol and 30µl removed for phosphorus analysis (See Method section 2.12.1(v)). If fluorescence products were to be assayed 2ml of extract was removed and analysed (See Methods Section 2.12.1(iii)). The remainder was brought to dryness on the rotary evaporator and fatty acids were methylated in preparation for gas chromatographic analysis (See Methods Section 2.12.1(iv)).

(iii) Measurement of Lipid Soluble Fluorescent Products

Fluorescent products, formed in the reaction of malondialdehyde with amines (see Introduction section 1.6)(Freeman et al. 1982), were measured using the method of Dillard and Tappel (1984). The methanol extract (See Methods section 2.12.1(ii)) was placed in a 25ml conical flask, reduced to dryness on a rotary evaporator and resolubized in 2ml chloroform and 0.1ml methanol. The relative fluorescence was measured using an Aminco Bowman spectrofluorimeter (American Instrument Co Inc., Silver Spring MD) fitted with a ratio potentiometer, at an excitation wavelength of 340nm and an emission wavelength of 430nm. Quinine sulphate (1µg/ml) in $0.1NH_2SO_4$ was used for fluorescence intensity calibration. The relative fluorescence intensity of the sample was expressed in terms of inorganic phosphorus content of the lipids.

(iv) Measurement of Polyunsaturated to Saturated Fatty Acid Ratio

Fatty acids were methylated before gas chromatographic

analysis (Morrison and Smith 1964). The methanol lipid extract (See Methods section 2.12.1(ii)) was brought to dryness using a rotary evaporator, the flask was then flushed with nitrogen and sealed with a glass stopper. Boron trifluoride methanol reagent (140g BF_3 per liter methanol) was added in a ratio of 1ml reagent per 4-16mg lipid. In this study 2ml was generally sufficient. The mixture was swirled around the flask and quickly transferred using a Pasteur pipette to a 10ml tube with a Teflon-lined screw cap that was flushed with nitrogen before and after addition of the mixture. The sealed tube was then placed in a boiling water bath for 15 mins, allowed to cool completely and the methyl esters were extracted using two volumes of hexane (4ml) and one volume of water (2ml). The phases were mixed by inversion and centrifuged in the IEC tabletop centrifuge at 950xg (2360rpm). The upper hexane layer contained 97-99% of the methyl esters. An aliquot, 1-2μl, of the sample was applied to a Hewlett-Packard 5890A gas chromatograph equipped with an ionization detector set at 250°C and a Chrompack capillary column 25MX0.22mM (I.D.) (wall coated open tubular column with fused silica CP=Wax52CB). Chromatograph parameters were set as follows: splitter vent = 107.1ml min^{-1}; purge = 5ml min^{-1}; column flow = 0.89ml min^{-1}; column head pressure = 25psi; injector temperature = 270°C. It was programmed such that the oven temperature was 150°C for the first minute and then increased by 4°C min^{-1} to 225°C. The carrier gas was Helium (98.4ml min^{-1}). Data was collected and analysed on a Hewlett

Packard 3390A integrator. Standards of the following fatty acid methyl esters (FAME) in hexane were used to establish retention times; myristic acid ($C_{14}:_0$), palmitic acid ($C_{16}:_0$), palmitoleic acid($C_{16}:_1$), steric acid ($C_{18}:_0$); oleic acid ($C_{18}:_1$), linoleic acid ($C_{18}:_2$), linolenic acid ($C_{18}:_3$) and arachidonic acid ($C_{20}:_4$). Then ratio of polyunsaturated fatty acid to saturated fatty acid methyl esters was used to compare extracts.

(v) Assay of Phosphorus Content

Phosphorus was assayed according to Bartlett (1959) with some modifications. An aliquot of the lipid extract in methanol containing 0.5-10μg P was pipetted into a 10ml acid-washed Pyrex Kjeldahl flask and the solvent evaporated to dryness under a stream of nitrogen. Perchloric acid (400μl) was added to the lipid extract and the sample digested by heating at 240°C in a Kjeldahl digestion rack for 30 min. The digest was allowed to cool and 5ml distilled water, 0.2ml 5% ammonium molybdate and 0.2ml Fiske-Subba Row reagent were added. Fiske-Subba Row reagent was made purifying 1-amino-2-naphthol-4-sulfonic acid with acid according to the method of Chanley et al. (1952) and combining 0.025gms of the purified product with 10ml freshly prepared 15% sodium metabisulphite followed by addition of 0.05gms sodium sulfite (Bartlett 1959). The mixture in the Kjeldahl flask was vortexted for 10 secs, heated in a boiling waterbath for 7 mins using an acid-washed marble to cover the opening of the

flask. After 15 mins cooling the absorbance of the stable blue colour was measured against a reagent blank at 830nm in a Schimadzu dual beam UV260 spectrophotometer. Standard solutions of potassium phosphate 0.5-10μg P final concentration were used to calibrate a standard curve from which sample concentrations could be read. Beer's law was valid up to 10μg P.

2.12.2 Measurement of Sulphydryl Group Content

The sulphydryl group content of mitochondria isolated from both control and PH_3-exposed insects 24 hours after exposure (LD_{70}) and from preparations exposed *in vitro* to a free radical generating system (Fe^{2+}-ADP/ascorbate, see Methods Section 2.12.1 (i)) were determined using the method described by Ellman (1959) as modified by Khare *et al*. (1982). An aliquot of the preparation, exactly 750μg protein, was incubated with 2ml 0.1M potassium phosphate buffer pH=8.0 containing 0.67mM DTNB [5,5^1-dithiobis(2-nitrobenzoic acid)] for 10 mins. The mixture was centrifuged at 10,000Xg for 10 mins in a Sorvall RC2-B centrifuge, the supernatant carefully removed and the absorbance measured at 412 nm. The coloured nitrophenol anion, released during the reaction of DTNB with sulphydryl groups, had an extinction coefficient E_{412}=13.6mM^{-1}.cm^{-1}. The sulphydryl group content was expressed as nmole thionitrobenzoic acid.mg protein^{-1}.

2.12.3 Oligomycin-Sensitive ATP-ase

The activity of oligomycin-sensitive ATPase complex was assayed using the method of Tzagoloff (1979). Mitochondria isolated from control and PH_3-exposed insects 24 hours after exposure (LD_{70}, 0.074mg $PH_3.l^{-1}$ for 5hrs) as well as after *in vitro* exposure to a free radical generating system (Fe^{2+}-ADP/ascorbate; see Methods Section 2.12.1(i)) were examined for ATP-ase activity. Activity was assayed by measuring the concentration of inorganic phosphorus released after mitochondria had been challenged with ATP for exactly 20 mins. Different buffers were used to isolate mitochondria after *in vivo* exposure to PH_3 and *in vitro* exposure to a free radical generating system. A medium consisting of 0.25M sucrose, 5mM EDTA and 1mg BSA.ml^{-1} pH=7.4 was used to homogenize insects that had been exposed to PH_3 *in vivo* while mitochondria prepared for *in vitro* exposure were isolated in 50mM HEPES buffer pH=7.4 containing 0.25M sucrose and 1.0mM EDTA.Mitochondrial suspension (exactly 200μg protein) was added to 1.0ml of 50mM Tris buffer pH=8.5 containing 4mM magnesium sulphate and the reaction started by addition of ATP (10mM) pH=7.0. Three controls were prepared one with suspension and oligomycin (10μg.ml^{-1}), and two reagent blanks, with and without oligomycin. When mitochondria were treated with the free radical generating system another reagent blank was used containing ADP (0.1mM). After 20 mins incubation at 25°C the reaction was terminated by addition of 0.2ml 50% (w/v) trichloroacetic acid (TCA).

Samples were centrifuged at 10,000xg for 10 mins to remove denatured proteins and 0.4ml of the supernatant was tested for inorganic phosphorus content using a modified form of the method described by Sumner (1944). Ammonium molybdate (10gms) was dissolved in 32ml concentrated sulfuric acid and made up to 1 litre with water. This stock was kept indefinitely. A solution of 25ml stock and 1gm Fe_2SO_4 was used within four hours of its preparation. The sample (protein range 0.1-0.4mg) was incubated with 1.9ml water and 2ml Fe_2SO_4 solution for 5 mins and the absorbance read against a reagent blank at 660nm. Inorganic phosphorus concentration was established using a potassium phosphate standard (0-1mgP).

2.13 Statistical Analysis of Data

All data presented in this study are expressed as mean values $\pm$ standard error of the mean (SEM). Statistical analysis was performed according to the type of data obtained. All comparisons were made using formulae taken from Zar (1974). Differences at the 95% confidence level (ie. $p \leq 0.05$) were considered statistically significant.

RESULTS

3.1 Extramitochondrial Release of Hydrogen Peroxide

The concentration of H_2O_2 released from mitochondria was assayed as a measure of $O_2^{\cdot-}$ generation by the electron transport chain. This indirect measurement was used because superoxide dismutase (SOD), present in the matrix, rapidly catalysis the reduction of $O_2^{\cdot-}$ making it difficult to measure $O_2^{\cdot-}$ directly. In this study yeast cytochrome c peroxidase (CCP) was used as an indicator of H_2O_2 released from mitochondria isolated from PH_3-sensitive insects and mouse liver.

3.1.1 Insect Mitochondria

Uninhibited insect mitochondria utilizing only endogenous substrate released H_2O_2, the amount of which decreased with duration after mitochondrial preparation. When freshly prepared organelles were used there was a release of H_2O_2 during the first minute of the assay which decreased to zero after 2-3 mins. No additions of substrates or inhibitors were made until H_2O_2 release driven by endogenous substrate had ceased.

PH_3 added to insect mitochondria (0.15mg protein.ml^{-1}) resulted in a significant release of H_2O_2 that was directly related to PH_3 concentration from 25μl to 100μl (Fig. 6a). When PH_3 was added to mitochondria already treated with 4-pentenoic acid (3mM), an inhibitor of fatty acid oxidation within the mitochondria, a slow release of H_2O_2 was observed

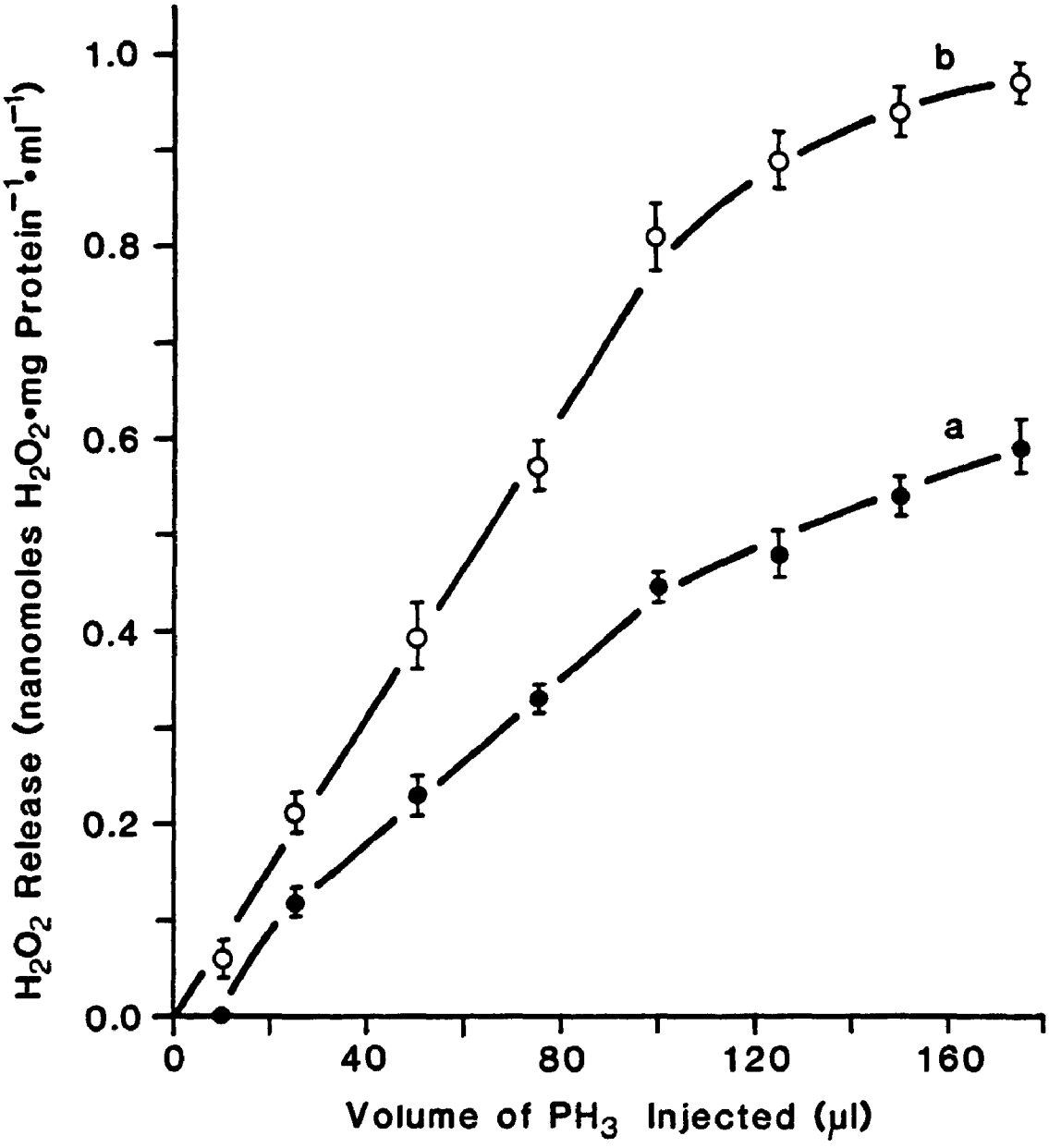
Figure 6

Effect of PH_3 on Release of H_2O_2 from Insect Mitochondria

Extramitochondrial H_2O_2 generation was followed by measuring the change in absorbance $\Delta A_{(404-424)}$ resulting from the formation of the CCP-H_2O_2 complex (extinction coefficient = 55-$mM^{-1}.cm^{-1}$). H_2O_2 release stimulated by endogenous substrate alone was allowed to cease before the addition of α-glycerophosphate or PH_3. A volume of PH_3 (10-175μl) was injected into a cuvette containing 3ml of 10mM phosphate buffer pH=7.4 with 0.25M sucrose, 0.1mM EDTA, 3.75mM $MgSO_4$, 2μM CCP and 0.3mg mitochondrial protein.ml^{-1}, with or without 1.5mM α-glycerophosphate. (See Methods Section 2.8 for experimental detail) Data points represent mean ± SEM n=5. All points on a are significantly different from b, $p \leq 0.05$ (Scheffé's test)

Graph: a= mitochondria utilizing endogenous substrate

b= mitochondria supplemented with 1.5mM α-glycerophosphate


1.0
0.8
0.6
0.4
0.2
0.0
0
40
80
120
160
b
a
H_2O_2 Release (nanomoles H_2O_2•mg Protein^{-1}•ml^{-1})
Volume of PH_3 Injected (µl)

which ceased within three minutes (Fig. 7, compare with result shown in Fig. 10) suggesting that fatty acid was the endogenous substrate.

There was no increase in H_2O_2 when mitochondria were supplemented with α-glycerophosphate (See Introduction section 1.2.3 for details on respiratory chain substrates) until PH_3 was added. The rate of H_2O_2 release was significantly higher with this substrate than endcgenous (Fig. 6a and b, Table 2 and 3) and was proportional to α-glycerophosphate concentrations (Fig. 8) reaching a peak at 0.75mM α-glycerophosphate with 0.3mg protein.ml^{-1}. When PH_3 (100μl) and α-glycerophosphate (0.75mM) were used peroxide release increased with protein concentration to 0.31mg protein.ml^{-1} (Fig. 9). The addition of succinate (4mM) to PH_3-inhibited mitochondria had no effect on H_2O_2 release (Table 3). No effect was observed when the NADH-linked substrates, pyruvate (5mM) and malate (1mM) were added to mitochondria with or without inhibitors.

Inhibition of the insect mitochondrial electron transport chain utilizing endogenous substrate with antimycin, which blocks the cytochrome $\underline{b}$-$\underline{c}_1$ segment, resulted in a faster rate of extramitochondrial H_2O_2 production than with PH_3 (Table 2)(See Introduction 1.2.4 for detailed explanation of sites of inhibitor action). With α-glycerophosphate or succinate, release was further stimulated by 23% (Table 3).

With mitochondria using endogenous substrate, myxothiazol a respiratory chain inhibitor that blocks electron transport between ubiquinone and the Rieske iron-sulphur centre,

Figure 7

Spectrophotometric Tracing of CCP-H_2O_2 Complex Formation by Insect Mitochondria Using Inhibitors and an Exogenous Substrate

The rate of formation of the CCP-H_2O_2 complex was measured by following $\Delta A_{(404-424)}$. Mitochondrial protein was used at a concentration of 0.15mg ml^{-1}. 4-Pentenoic acid, an inhibitor of fatty acid oxidation was incubated with mitochondria for three mins. prior to addition of 2μM CCP (cytochrome c peroxidase). H_2O_2 release was initiated when PH_3 (300μl) was bubbled into the cuvette and further stimulated with addition of the substrate α-glycerophosphate. (See Methods Section 2.8 for experimental details) The numbers on the trace represent nanomoles H_2O_2 released per mg protein per minute, and is the average of three repeats.

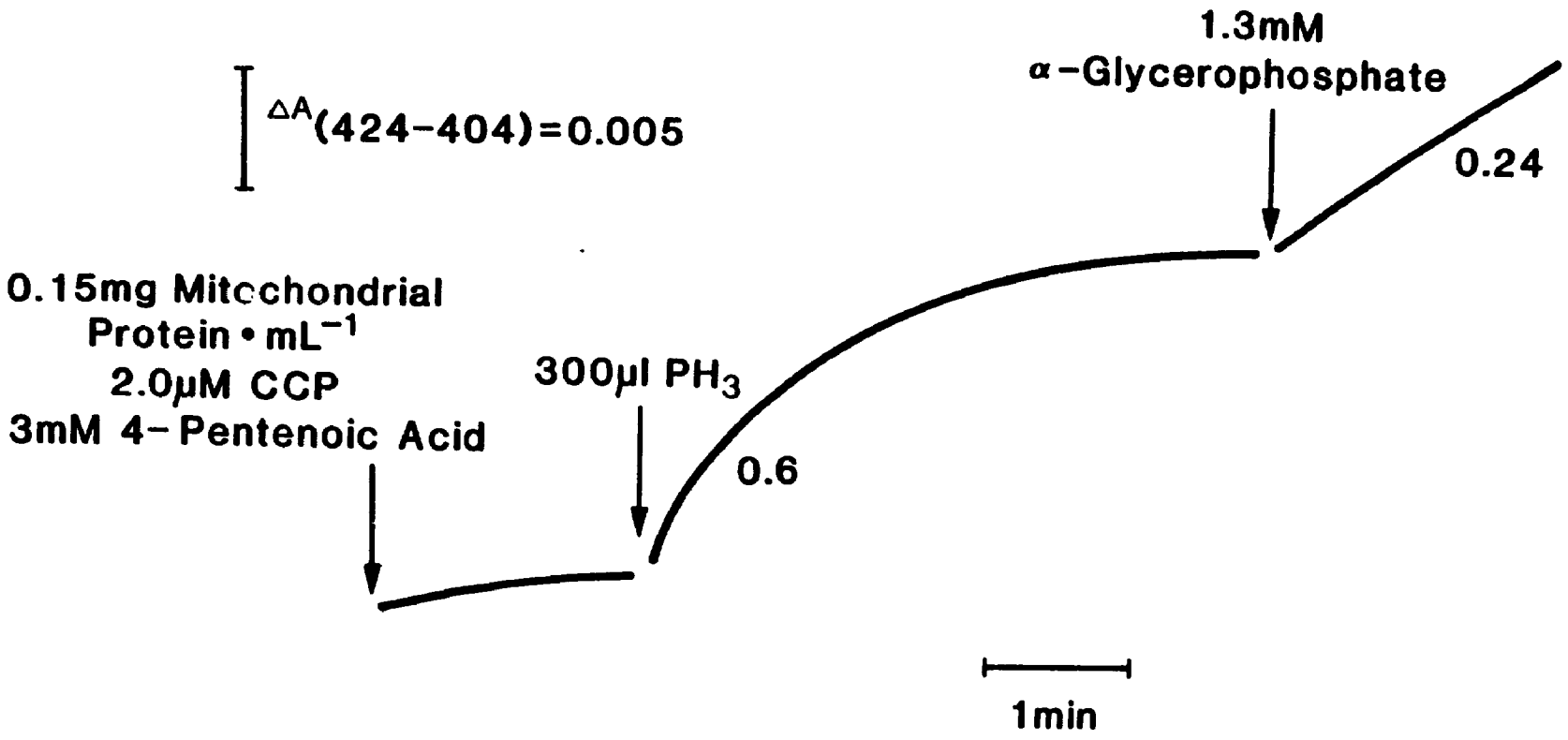

1.3mM
α-Glycerophosphate
0.24
ΔA(424-404)=0.005
0.15mg Mitochondrial
Protein • mL⁻¹
2.0μM CCP
3mM 4-Pentenoic Acid
300μl PH3
0.6
1min

TABLE 2

Hydrogen Peroxide Release from Insect Mitochondria

Inhibitor	n	Extramitochondrial H_2O_2 Release (nmole H_2O_2.mg protein^{-1}.min^{-1})
PH_3	9	0.71 ± 0.01^a
PH_3 + AA	3	0.91 ± 0.04^b
PH_3 + mx	3	0.94 ± 0.05^b
PH_3 + rot (0.15×10^{-2} nmoles/mg)	3	0.91 ± 0.06^b
AA	4	1.20 ± 0.06^c
AA + PH_3	3	0.88 ± 0.05^b
AA + mx	3	0.90 ± 0.04^b
AA (0.04μM)	3	0.27 ± 0.05^d
AA (0.04μM) + PH_3	3	0.90 ± 0.04^b
AA (0.04μM) + mx	3	0.88 ± 0.04^b
mx	5	0.89 ± 0.03^b
mx (0.45μg)	3	0.48 ± 0.04^e
mx (0.45μg) + AA	3	0.91 ± 0.06^b
mx (0.45μg) + PH_3	3	0.92 ± 0.08^b
rot	3	0.019 ± 0^f
rot + AA	3	0.15 ± 0.08^g
rot + PH_3	3	0.09 ± 0.05^g

H_2O_2 release from insect mitochondria utilizing endogenous substrate. Respiratory chain inhibitors, antimycin (AA) (1.6μM), myxothiazol (mx) (0.9μg) and PH_3 (300μl) at concentrations shown unless otherwise stated, were used in the order shown. Protein concentration was 0.15-0.3mg.ml^{-1}. Values represent mean ± SEM, n = number of replications. Values followed by different letters are significantly different $p \leq 0.05$ (Scheffé's test).

TABLE 3

Hydrogen Peroxide Release from Insect Mitochondria Supplemented with Substrate

Addition	n	Extramitochondrial H_2O_2 Release (nmole H_2O_2.mg protein^{-1}.min^{-1})
succ + PH_3	3	0.74 ± 0.05[a]
mal, pyr + PH_3	3	0.70 ± 0.04[c]
α-gp + PH_3	6	1.06 ± 0.06[b]
α-gp, PH_3 + AA	3	1.03 ± 0.12[b]
α-gp, PH_3 + rot	4	1.07 ± 0.10[b]
succ + AA	3	1.51 ± 0.09[c]
α-gp + AA	4	1.56 ± 0.05[c]
α-gp, AA + PH_3	3	1.14 ± 0.05[b]
α-gp, AA + mx	3	1.12 ± 0.03[b]
α-gp + AA (0.032μM)	3	0.35 ± 0.04[d]
α-gp, AA (0.032μM) + mx	3	1.19 ± 0.08[b]
α-gp + mx	3	1.08 ± 0.11[b]
α-gp + mx (0.18μg)	3	0.75 ± 0.07[a]
α-gp, mx (0.18μg) + PH_3	3	1.62 ± 0.17[c]

H_2O_2 release from insect mitochondria with substrate added before inhibitors; the substrates were 1.3mM α-glycerophosphate (α-gp), 4mM succinate (succ) or 1mM malate (mal) plus 5mM pyruvate (pyr) unless otherwise stated. Respiratory chain inhibitors were antimycin (AA) (1.6μM), myxothiazol (mx) (0.9μg), rotenone (rot) (5.6 nanomoles.mg protein^{-1}) and PH_3 (300μl) at the concentrations shown unless otherwise stated, used in the order shown. Protein concentration was 0.15-0.3 mg.ml^{-1}. Values represent means ± SEM, n=number of repeats (Total volume = 3ml). Values with different letters are statistically different according to the Scheffé's test ($p \leq 0.01$).

Figure 8

Relationship Between Substrate Concentration and Extramitochondrial H_2O_2 Release from PH_3-Inhibited Insect Mitochondria

Extramitochondrial H_2O_2 generation followed by measuring the change in absorbance ΔA_{404-42}, resulting from the formation of the CCP-H_2O_2 complex (extinction coefficient=55.mM^{-1}.cm^{-1}). Protein concentration used was 0.3mg protein.ml^{-1}. H_2O_2 release stimulated by endogenous substrate was allowed to cease before addition of variable concentrations of the FAD-linked substrate α-glycerophosphate (0.15-0.75mM) to the cuvette containing 3ml of 10mM phosphate buffer pH=7.4 with 0.25M sucrose, 0.1mM EDTA, 3.75mM $MgSO_4$ and 2μM CCP. After 2 mins. PH_3 (100μl) was injected into the medium to inhibit the respiratory chain. (See Methods Section 2.8 for experimental details). Data points represent mean ± SEM, n=5. There were statistically significant differences (Scheffé's test; $p \leq 0.01$) between rates of H_2O_2 release at all intervals below 0.25mM α-glycerophosphate.

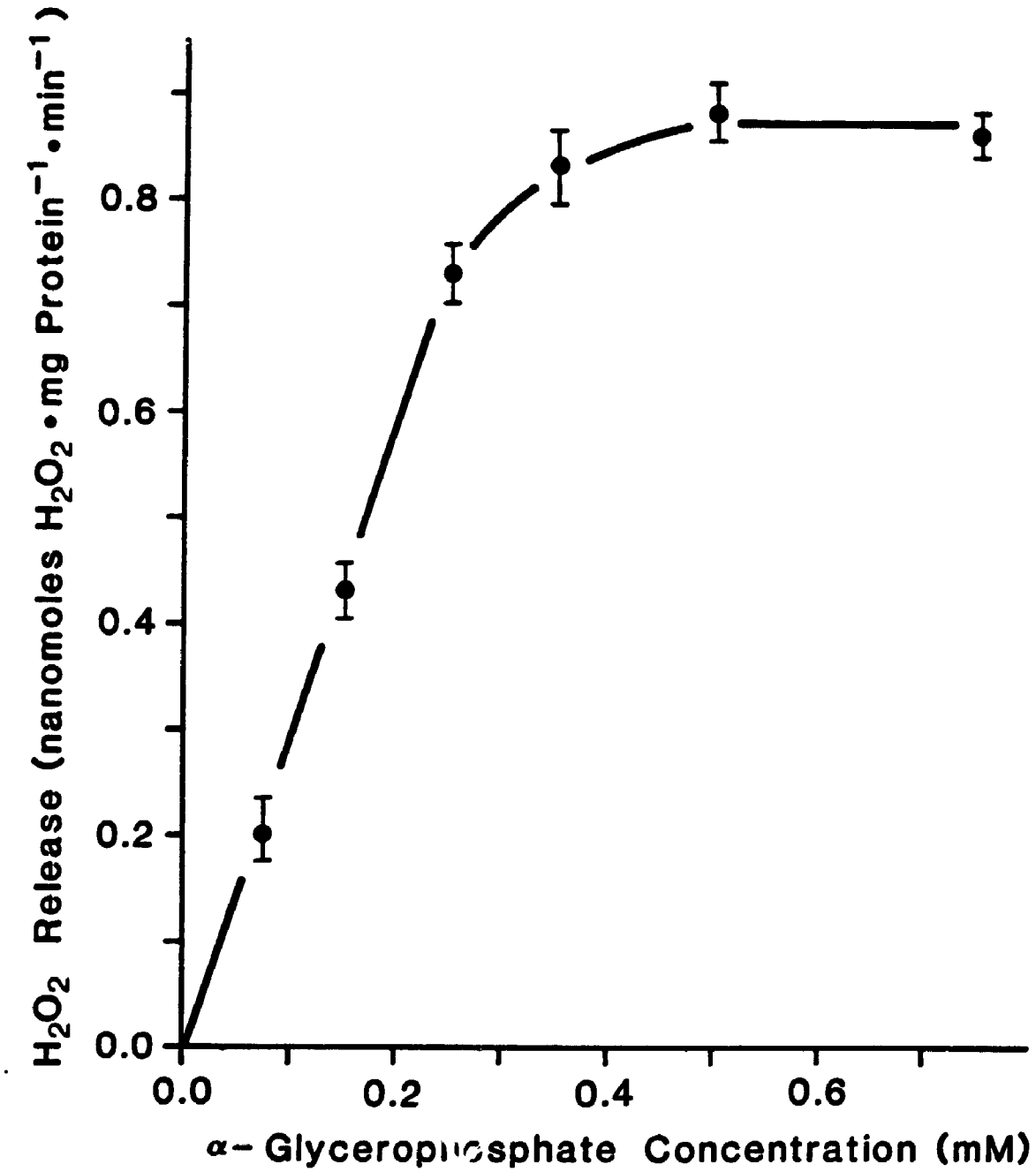
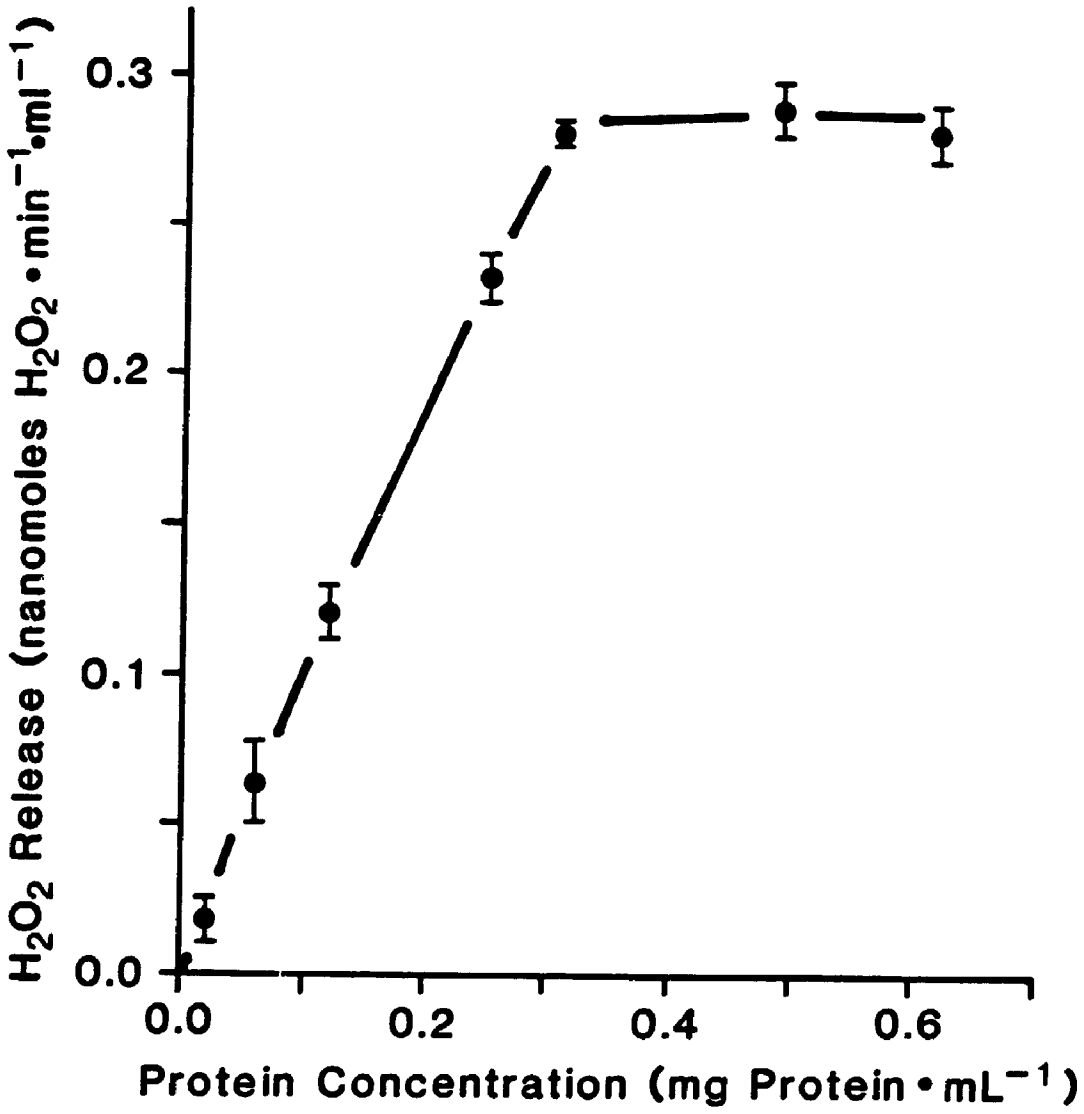

H_2O_2 Release (nanomoles H_2O_2 • mg Protein^{-1} • min^{-1})
0.0
0.2
0.4
0.6
0.8
0.0
0.2
0.4
0.6
α– Glyceropnosphate Concentration (mM)

Figure 9

Relationship Between Protein Concentration and Extramitochondrial H_2O_2 Release from PH_3-Inhibited Substrate-Supplemented Insect Mitochondria

Extramitochondrial H_2O_2 release was followed by measuring the change in absorbance $\Delta A_{(404-424)}$ resulting from the formation of the CCP-H_2O_2 complex (extinction coefficient=55.$mM^{-1}.cm^{-1}$). Protein concentration was varied between 0.02-0.6mg.ml^{-1}. Mitochondria were supplemented with 1.5mM α-glycerophosphate after H_2O_2 release stimulated by endogenous substrate had ceased. PH_3 (100μl) was used to inhibit the respiratory chain. Final volume=3ml of 10mM phosphate buffer pH=7.4 with 0.25M sucrose, 0.1mM EDTA, 3.75mM $MgSO_4$ and 2μM CCP. See Methods Section 2.8 for experimental details. Data points represent mean ± SEM, n=3. There were statistically significant differences (Scheffé's test; $p \leq 0.05$) between rates of H_2O_2 release at all intervals below 0.31mg protein.ml^{-1}.


H_2O_2 Release (nanomoles $H_2O_2 \cdot min^{-1} \cdot ml^{-1}$)
0.3
0.2
0.1
0.0
0.0
0.2
0.4
0.6
Protein Concentration (mg Protein $\cdot mL^{-1}$)

resulted in a rate of H_2O_2 release that was higher than PH_3 but lower than antimycin (Table 2). With α-glycerophosphate as substrate, myxothiazol resulted in a production of H_2O_2 at the same rate as PH_3 with substrate (Table 3).

Addition of rotenone (3.1 nmoles.mg^{-1}), which inhibits between NADH dehydrogenase and ubiquinone, led to a very slight increase in CCP.H_2O_2 complex formation from mitochondria utilizing endogenous substrate (Table 2). No change in the release of H_2O_2 was observed when α-glycerophosphate was added to rotenone-inhibited mitochondria.

The effect of combining respiratory inhibitors was observed with both endogenous substrate and added α-glycerophosphate (Tables 2 & 3). With endogenous substrate, addition of antimycin, myxothiazol and rotenone ($0.15X10^{-2}$nmoles.mg^{-1}) to PH_3-inhibited insect mitochondria resulted in an increase in H_2O_2 production from 0.71±0.01 nanomoles H_2O_2.mg^{-1}.min^{-1} to a maximal rate with an average value of 0.9±0.03 nanomoles H_2O_2.mg^{-1}.min^{-1} (Fig. 10, Table 2). The rates were not significantly different from each other or from those obtained with myxothiazol alone (0.89±0.03 nanomoles H_2O_2.mg^{-1}.min^{-1}), although they were significantly less than with antimycin alone (1.20±0.06).

When a low concentration of antimycin was used giving a rate of production 4-fold less than saturating concentrations, the addition of myxothiazol or PH_3 resulted in a maximal increase in H_2O_2 release that was essentially the same as myxothiazol alone (Fig. 11, Table 2).

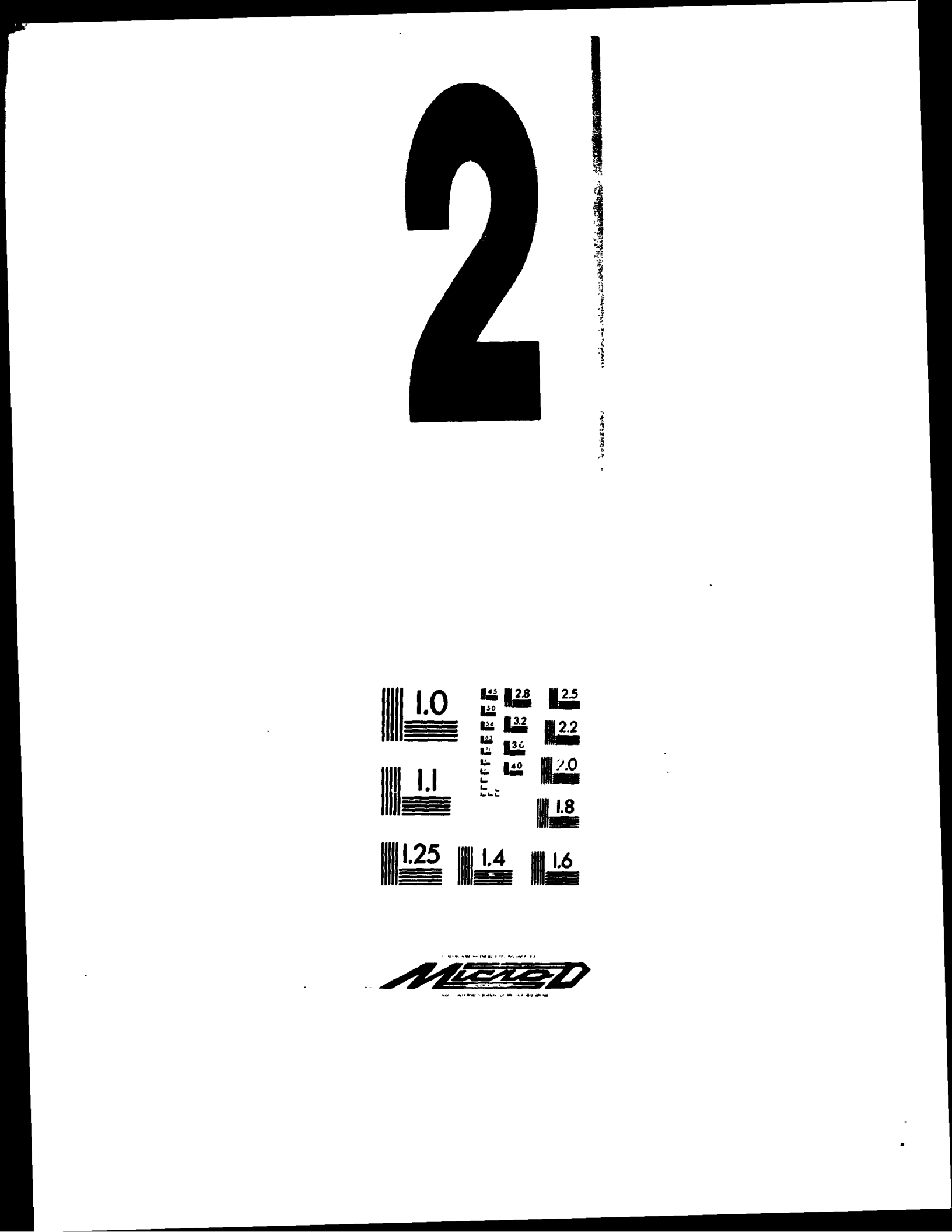

2
1.0
1.1
1.25
1.4
1.6
1.8
2.0
2.2
2.5
2.8
3.2
3.6
4.0
4.5
5.0
5.6
6.3
7.1
MicroD

Figure 10

Spectrophotometric Tracing of CCP-H_2O_2 Complex Formation by Insect Mitochondria Using Inhibitors.

The rate of formation of the CCP-H_2O_2 complex was measured by following $\Delta A_{(404-424)}$. Mitochondrial protein was used at a concentration of 0.15mg protein.ml^{-1}. PH_3 (300μl) was added after the release of H_2O_2 stimulated by endogenous substrate had ceased. Antimycin (1.6μM) an inhibitor of the respiratory chain was added after 1.5 mins. Tabulated results are shown on Table 2. The numbers on the trace represent nanomoles H_2O_2 released per mg protein per minute.

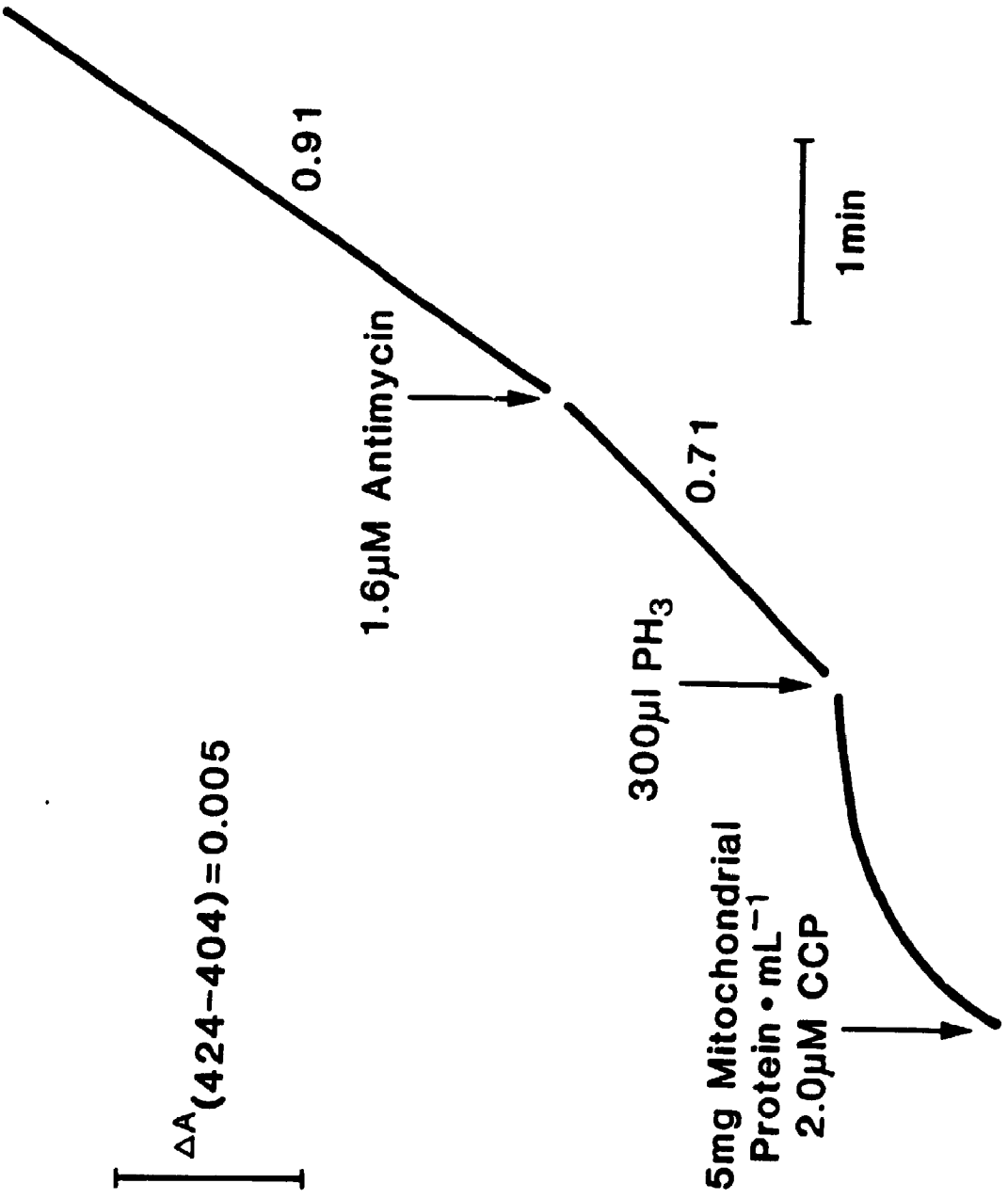
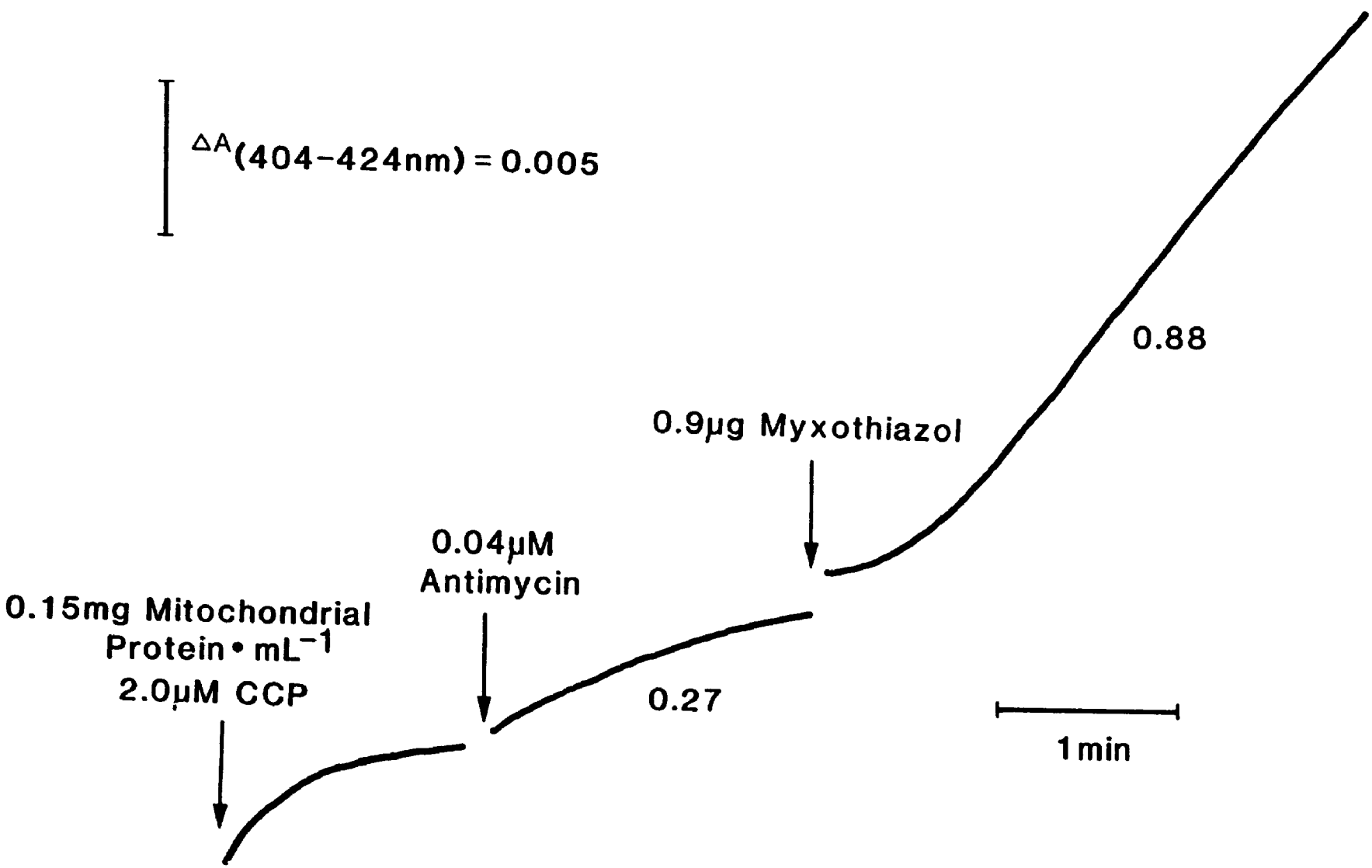

0.91
1.6μM Antimycin
0.71
300μl PH_3
0.15mg Mitochondrial Protein • mL^{-1}
2.0μM CCP
1min
$\Delta A_{(424-404)} = 0.005$

Figure 11

Spectrophotometric Tracing of CCP-H_2O_2 Complex Formation by Insect Mitochondria Using Inhibitors

The rate of formation of the CCP-H_2O_2 complex was measured by following $\Delta A_{(404-424)}$. Mitochondrial protein was used at a concentration of 0.15mg protein.ml^{-1}. Antimycin (1.6μM) was added after the release of H_2O_2 stimulated by endogenous substrate had ceased. Myxothiazol, also an inhibitor of the respiratory chain (See Introduction 1.1.4) was added after two mins. See Methods Section 2.8 for experimental details. Tabulated results are shown in Table 2. The numbers on the trace represent nanomoles H_2O_2 released per mg protein per minute.


ΔA(404–424nm) = 0.005
0.88
0.9µg Myxothiazol
0.04µM
Antimycin
0.15mg Mitochondrial
Protein • mL⁻¹
2.0µM CCP
0.27
1 min

A similar rate of release was observed when antimycin or PH_3 were added to mitochondria half maximally inhibited by myxothiazol (Fig. 12, Table 2), again demonstrating that under these conditions there is a maximum rate of H_2O_2 production.

When PH_3 or antimycin were added to rotenone (3.1nmoles.mg^{-1}) inhibited mitochondria a small but significant increase was observed which gradually levelled off to zero after four mins (Table 2).

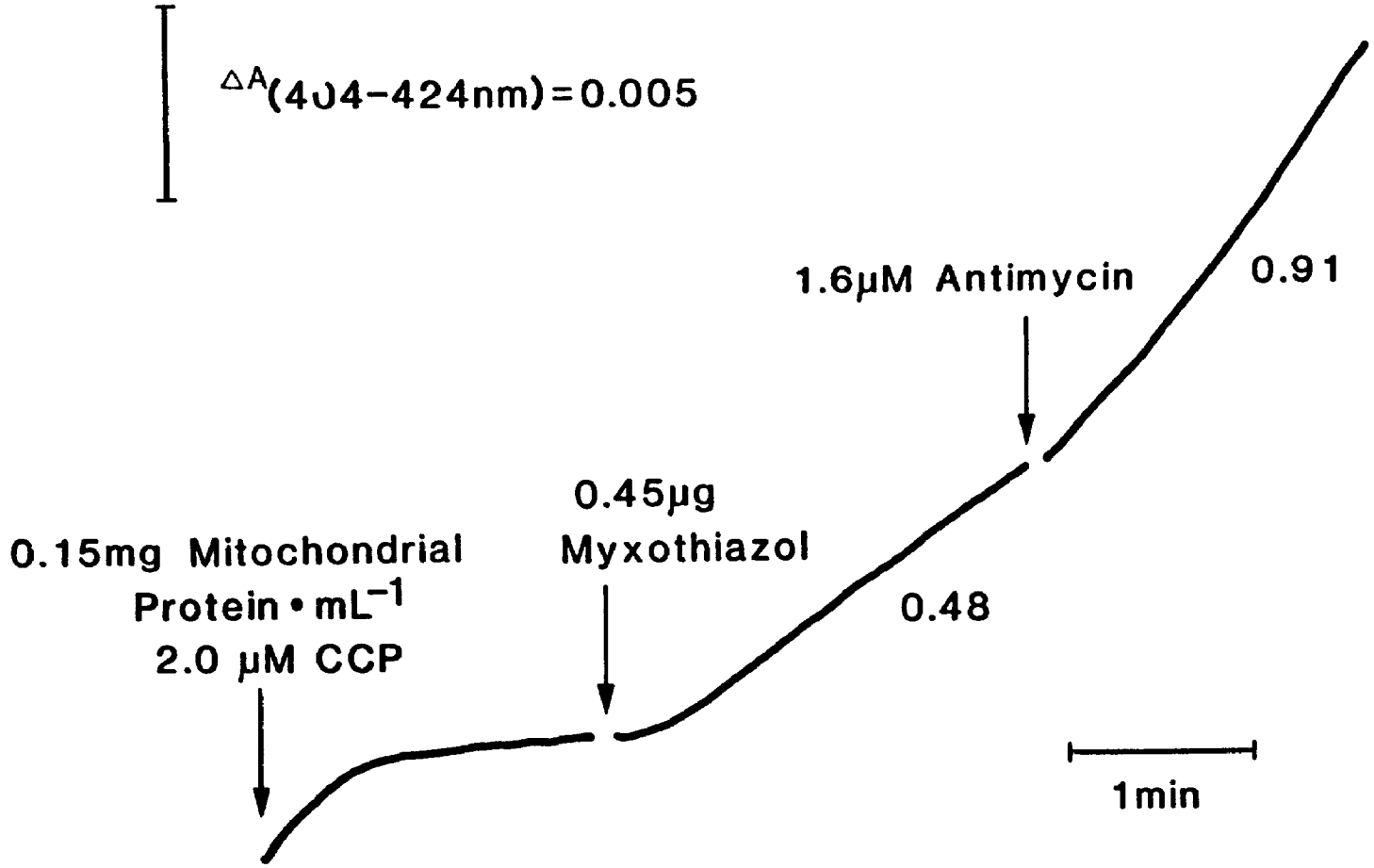
With α-glycerophosphate (0.75mM) as substrate and PH_3 inhibiting cytochrome c oxidase, addition of antimycin produced no significant increase in H_2O_2 (Table 3). A decrease of 28% was observed when myxothiazol was added to antimycin inhibited mitochondria. With antimycin inhibiting at sub-maximal concentrations (0.35±0.04 nanomoles H_2O_2. $mg^{-1}.min^{-1}$), myxothiazol increased the rate to a level that was not significantly different from myxothiazol alone. PH_3 following low concentrations of myxothiazol (0.75±0.07 nanomoles $H_2O_2.mg^{-1}.min^{-1}$) had the unexpected effect of increasing the rate to values as high as antimycin alone (Table 3).

The addition of ADP ($3.8X10^{-4}M$) to insect mitochondria inhibited by PH_3 utilizing endogenous substrate had the effect of increasing the rate of H_2O_2 production to 0.93±0.08 (n=3), while the addition of ATP ($2.5X10^{-4}M$) to similarly inhibited mitochondria inhibited extramitochondrially released H_2O_2 completely.

Figure 12

Spectrophotometric Tracing of CCP-H_2O_2 Complex Formation by Insect Mitochondria Using Inhibitors

The rate of formation of the CCP-H_2O_2 complex was measured by following $\Delta A_{(404-424)}$. Mitochondrial protein was used at a concentration of 0.15mg protein.ml^{-1}. Myxothiazol (0.45μg) was added after the release of H_2O_2, stimulated by endogenous substrate had ceased. Antimycin (1.6μM) was added after 2.5 mins. See Methods Section 2.8 for experimental details. Tabulated results are shown in Table 2. The numbers on the trace represent nanomoles H_2O_2 released per mg per minute.


ΔA(404-424nm)=0.005
1.6μM Antimycin
0.91
0.45μg
Myxothiazol
0.15mg Mitochondrial
Protein • mL^{-1}
2.0 μM CCP
0.48
1min

3.1.2 Mouse Mitochondria

The release of H_2O_2 from mouse mitochondria utilizing endogenous substrate was directly related to PH_3 concentration (Fig. 13) with rates that were significantly less than those obtained with insect mitochondria (Fig 6a). H_2O_2 release and protein concentration were directly related with mitochondria inhibited by PH_3 (75μl) up to approx. 0.5mg protein.ml^{-1} (Fig. 14).

The addition of the oxidative phosphorylation uncoupler 2,4-dinitrophenol (2,4-DNP) to mouse mitochondria with no added substrate did not result in any extramitochondrial H_2O_2 release, however, on addition of PH_3 the rate increased dramatically to a level that was nearly 2-fold higher than the rate observed with PH_3 alone, from 0.24$\pm$0.03 (n=3) to 0.44$\pm$0.07 nanomoles H_2O_2 mg protein^{-1}.min^{-1} (n=3).

3.2 Spectral Studies on Mitochondrial Cytochromes

Oxidised and reduced cytochromes have distinct spectra which can be utilized to determine their redox state using mitochondrial tissue *in vitro* (Chance and Williams 1958)(See Introduction section 1.2 for description of the respiratory chain). This is a useful technique when observing effects of respiratory inhibitors on mitochondria, the "cross-over point" providing information on the site of inhibition. In the

Figure 13

Effect of PH_3 on Extramitochondrial H_2O_2 Release by Mouse Liver Mitochondria

Extramitochondrial H_2O_2 generation was followed by measuring the change in absorbance $\Delta A_{(404-424)}$ resulting from the formation of the CCP-H_2O_2 complex (extinction coefficient=55.mM^{-1}.cm^{-1}). After H_2O_2 release stimulated by endogenous substrate had ceased (1-2mins.), a volume of PH_3 (25-200μl) was injected into a cuvette containing 3ml of 10mM phosphate buffer pH=7.4 with 0.25M sucrose, 0.1mM EDTA 3.75mM $MgSO_4$, 2μM CCP and 0.2mg protein.ml^{-1}. See Methods Section 2.8 for experimental details. Data points represent mean $\pm$ SEM, n=4. There were statistically significant differences (Scheffé's test; $p \leq 0.05$) between the rates of H_2O_2 release at all intervals below 150μl PH_3.

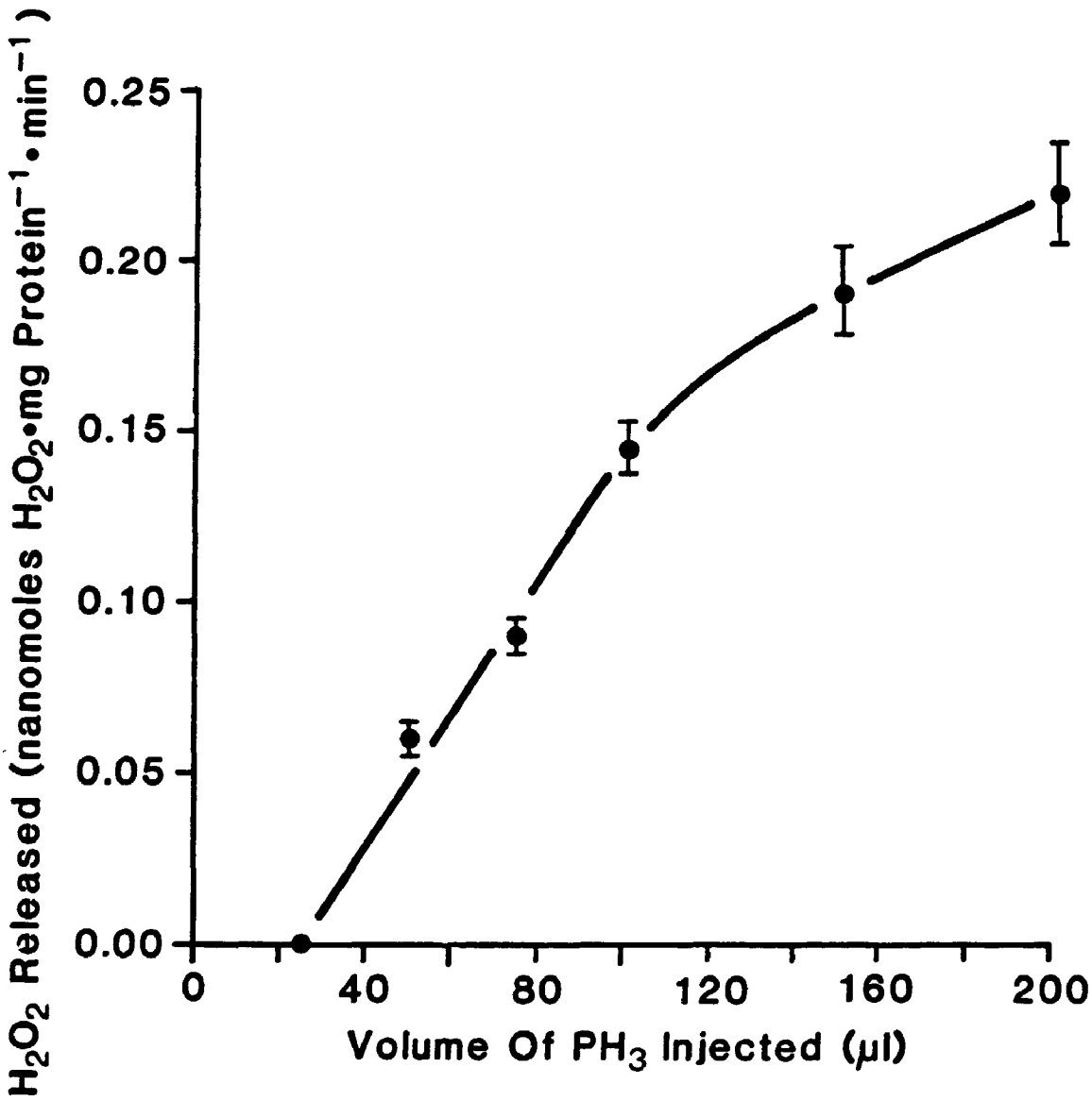
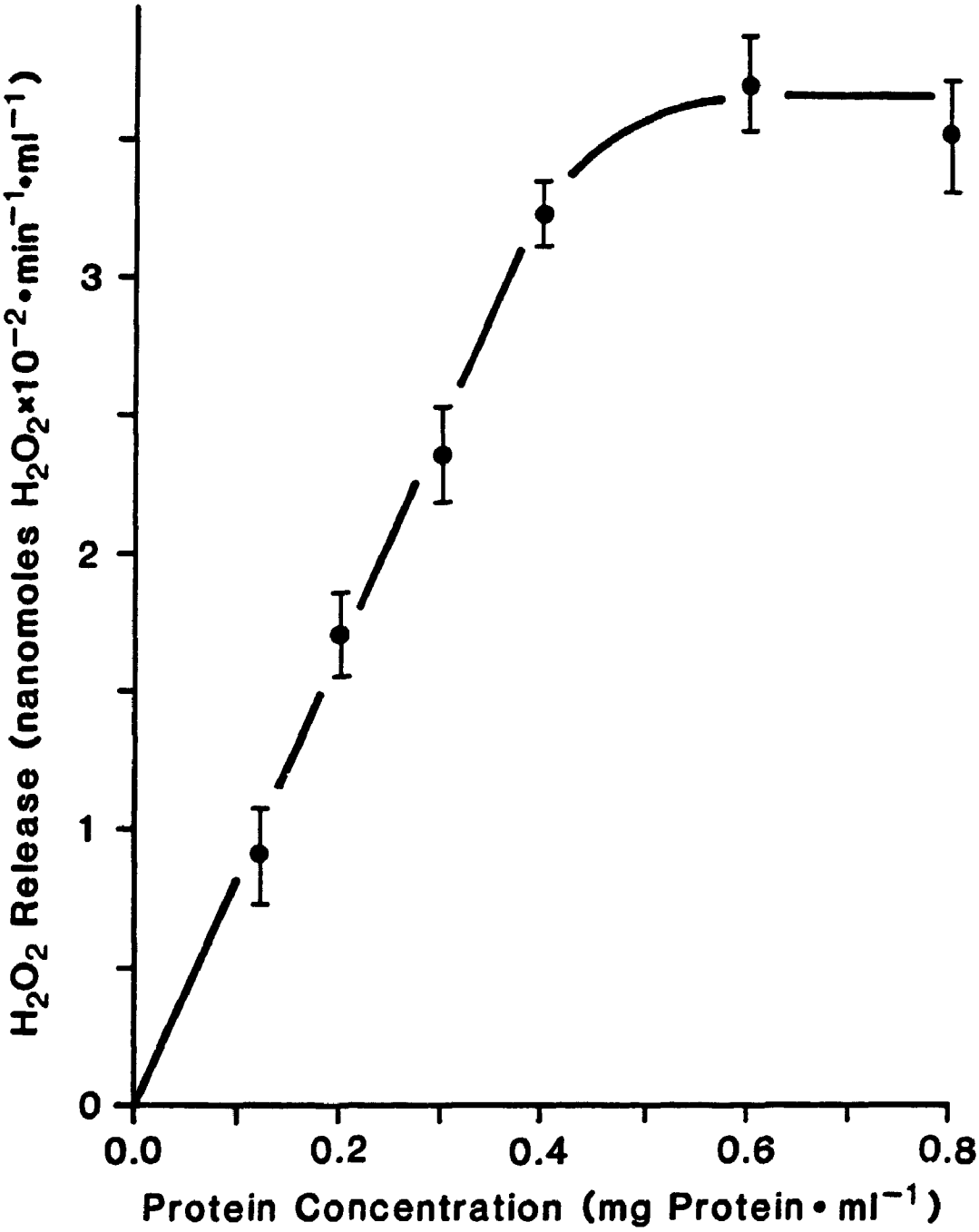

H_2O_2 Released (nanomoles H_2O_2•mg Protein^{-1}•min^{-1})
0.25
0.20
0.15
0.10
0.05
0.00
0
40
80
120
160
200
Volume Of PH_3 Injected (µl)

Figure 14

Relationship Between Protein Concentration and Extramitochondrial H_2O_2 Release from PH_3-Inhibited Mouse Liver Mitochondria

Extramitochondrial H_2O_2 release was followed by measuring the change in absorbance $\Delta A_{(404-424)}$ resulting from the formation of the CCP-H_2O_2 complex (extinction coefficient=55.$mM^{-1}.cm^{-1}$). Protein concentration was varied between 0.12-0.8mg protein.ml^{-1}. Mitochondria were supplemented with 1.5mM α-glycerophosphate after H_2O_2 release stimulated by endogenous substrate had ceased. PH_3 (75μl) was used to inhibit the respiratory chain. Final volume = 3ml of 10mM phosphate buffer pH=7.4 with 0.25M sucrose, 0.1mM EDTA and 2μM CCP. See Methods Section 2.8 for experimental details. Data points represent mean ± SEM, n=3. There were statistically significant differences (Scheffé's test; $p \leq 0.05$) between H_2O_2 release at all intervals below 0.4mg protein.ml^{-1}.


H_2O_2 Release (nanomoles $H_2O_2 \times 10^{-2} \cdot min^{-1} \cdot ml^{-1}$)
0
1
2
3
0.0
0.2
0.4
0.6
0.8
Protein Concentration (mg Protein • ml^{-1})

following experiments, mitochondria were not supplemented with exogenous substrate in an attempt to emulate *in vivo* conditions.

3.2.1 Insect

The difference spectrum was recorded after complete reduction of the cytochromes with sodium dithionite. It revealed peaks at 431.5 and 445nm, probably the Soret bands of *b* and $a+a_3$ respectively, 552.1nm representing $c+c_1$, a broad peak at 560-563.2nm attributed to reduced cytochrome *b*, and one at 601nm representing reduced $a+a_3$ (Fig. 15).

The spectra of cytochromes from aerobic mitochondria challenged with PH_3 was observed (Fig. 16). Peaks were recorded at 550.5nm corresponding to cytochrome *c* ($+c_1$) that was about 66% reduced compared to full reduction by dithionite, and at 601.2nm which was 100% of dithionite reduced $a+a_3$. This result suggested that the *b*-cytochromes (peak at 563nm) were not reduced after PH_3 inhibition. It was considered possible that a small reduced peak could be masked by the peak at 550.5nm and for this reason cytochrome *c*-depleted mitochondria were utilized. Complete reduction of cytochrome *c*-depleted mitochondria by dithionite resulted in a spectrum (Fig. 17A) that was almost identical to unwashed mitochondria except that a new small peak became evident at 552.1nm possibly representing cytochrome c_1 or contamination from cytochrome *c*. The supernatant containing cytochrome *c* reduced with dithionite had peaks at 416.7 and 549nm (Fig. 17B).

Figure 15

Difference Spectrum of Insect Mitochondrial Cytochromes Reduced by Dithionite

Reference cuvette contained 0.8mg protein.ml^{-1}. Sample cuvette contained the same protein concentration but cytochromes were reduced using sodium dithionite crystals. Total volume used was 3ml of aerated 50mM HEPES buffer pH=7.0 containing 0.9% NaCl. Cytochromes were scanned from 375 to 675nm. Using OD range = 0-0.2 from 375 to 500nm and OD range = 0-0.05 from 500 to 675nm. See Methods Section 2.9 for experimental details.

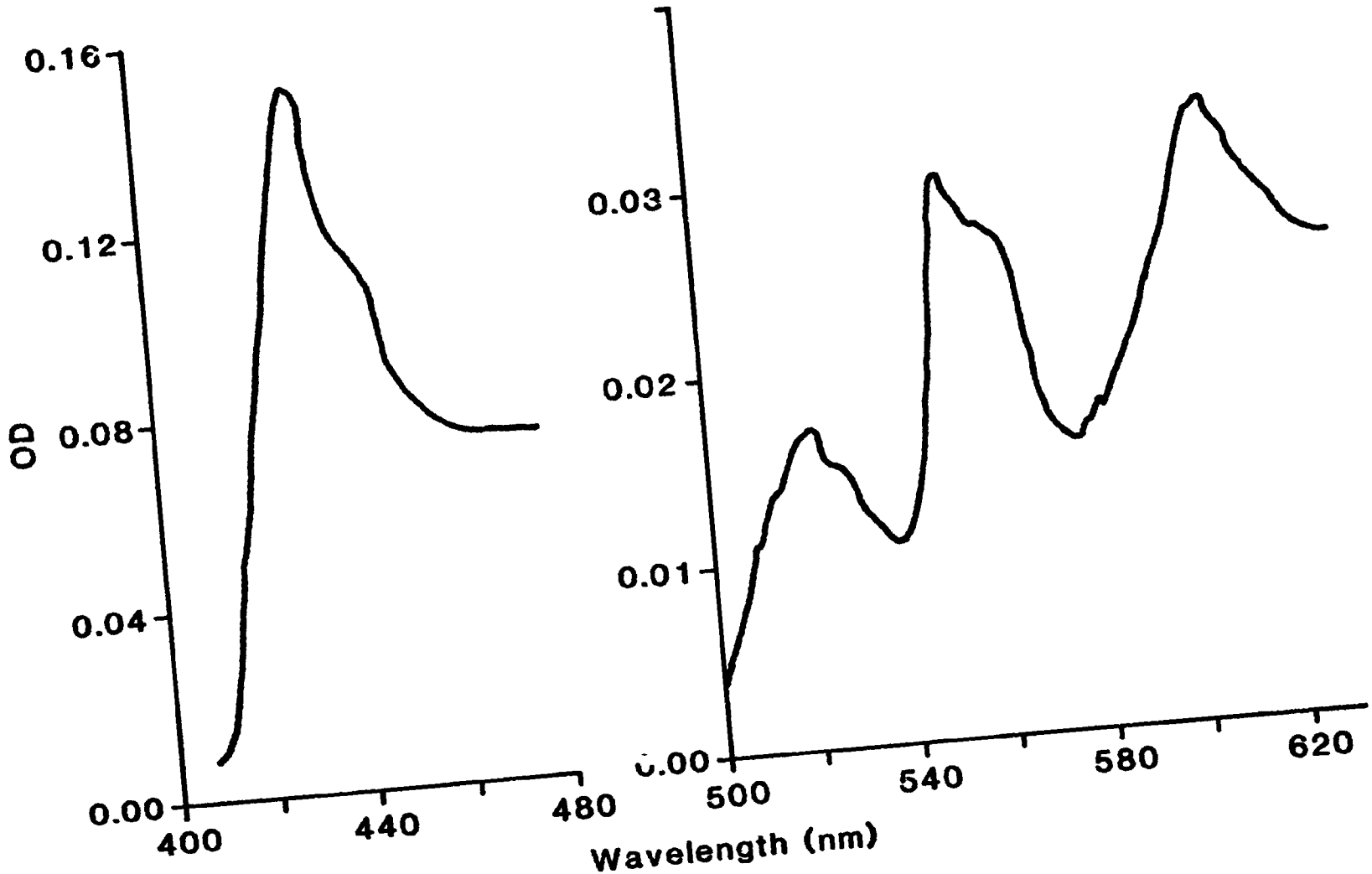

0.16
0.12
0.08
0.04
0.00
OD
400
440
480
0.03
0.02
0.01
0.00
500
540
580
620
Wavelength (nm)

Figure 16

Difference Spectrum of Insect Mitochondrial Cytochromes Reduced by PH_3

Reference cuvette contained 0.8mg protein.ml^{-1}. Sample cuvette contained the same protein concentration but cytochromes were reduced by bubbling PH_3 (300μl) into the cuvette. Total volume was 3ml of aerated. Cytochromes were scanned from 375 to 675nm using OD range = 0-0.2 from 375 to 500nm and OD range = 0-0.05 from 500 to 675nm. See Methods Section 2.9 for experimental details.

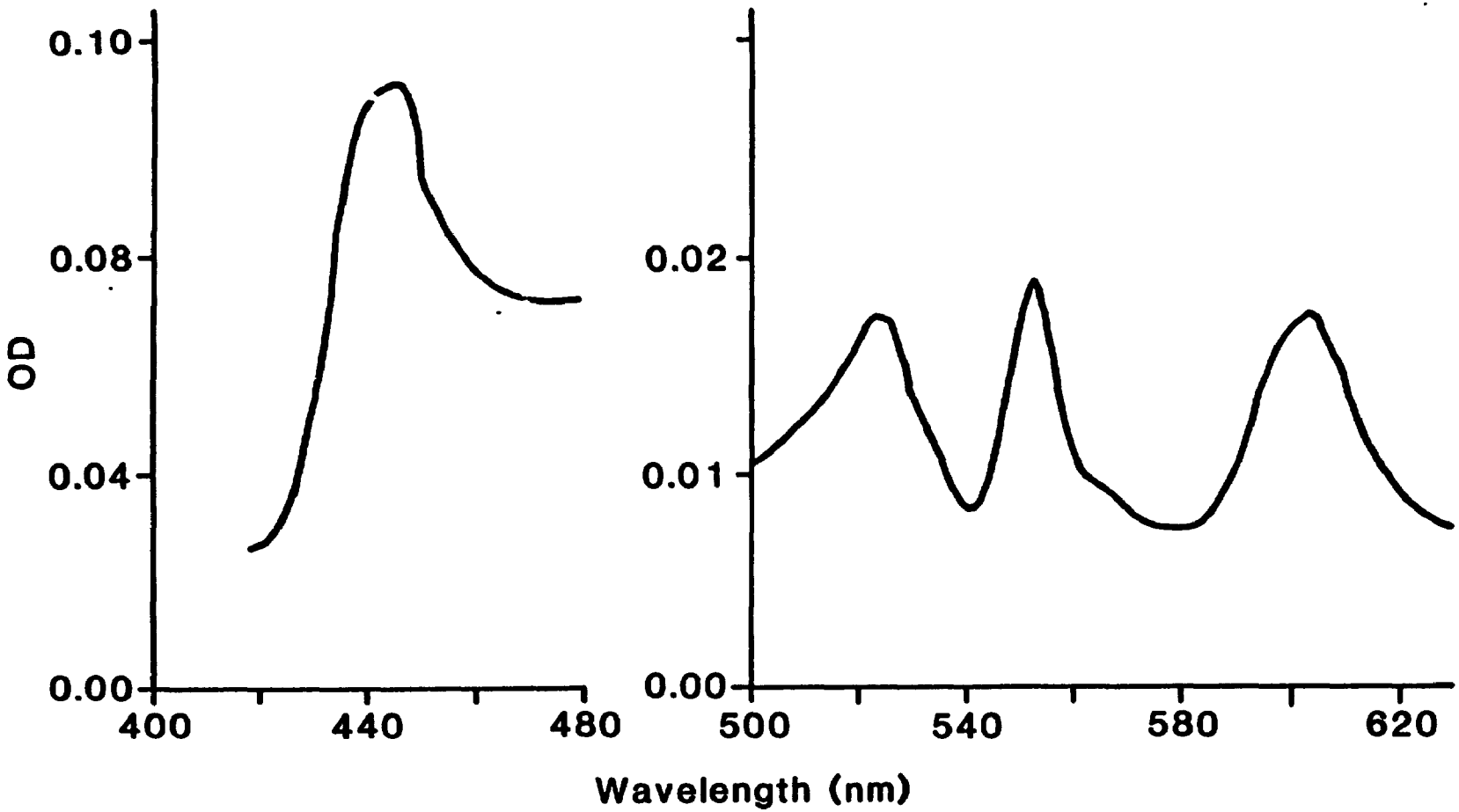

0.10
0.08
0.04
0.00
OD
400
440
480
0.02
0.01
0.00
500
540
580
620
Wavelength (nm)

Figure 17A

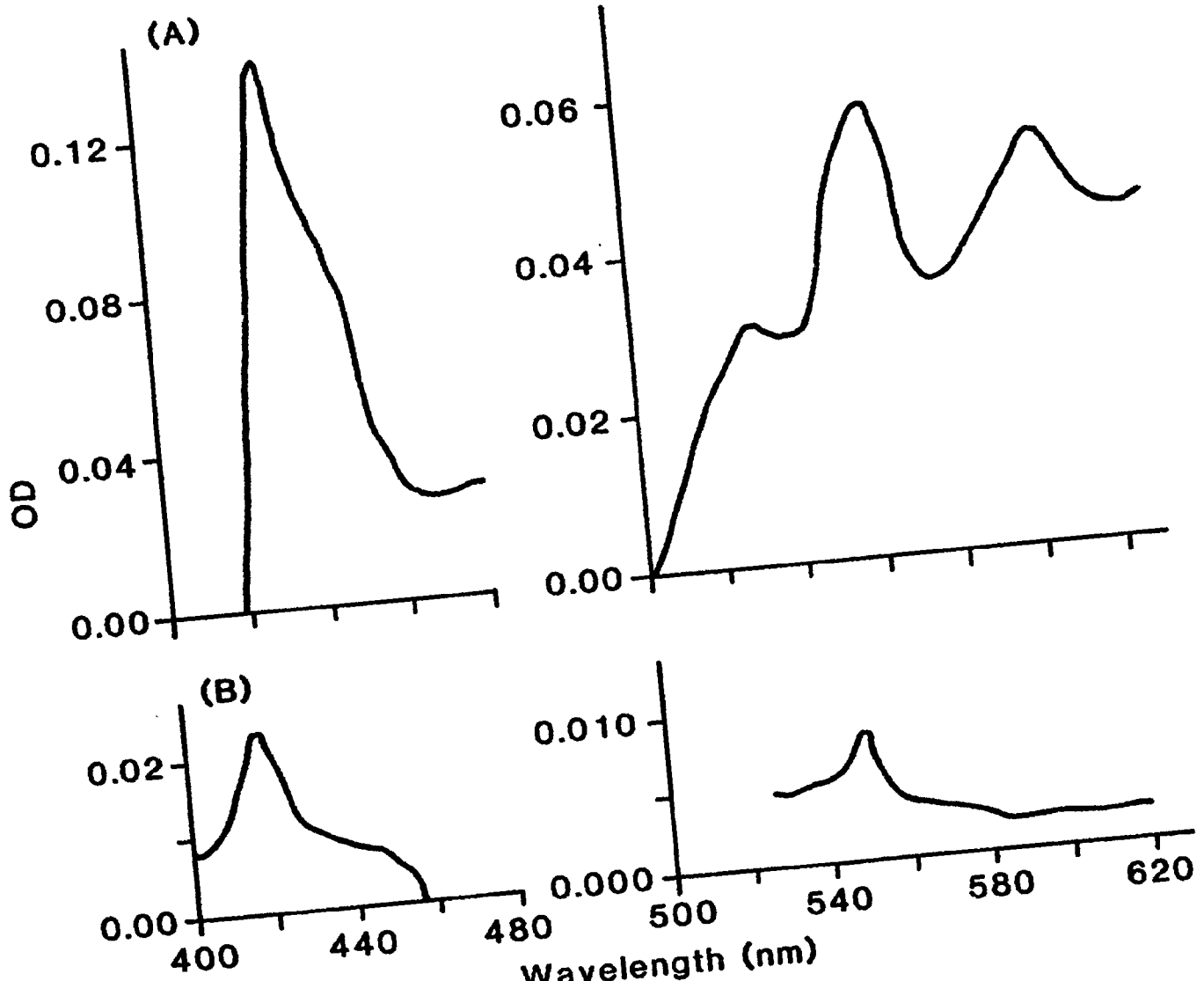
Difference Spectrum of Cytochrome c-Depleted Insect Mitochondria Reduced by Dithionite.

Reference cuvette contained 1.5mg protein.ml^{-1}. Sample cuvette contained the same protein concentration with cytochromes reduced by sodium dithionite crystals. Total volume was 3ml of aerated 50mM HEPES buffer pH=7.0 containing 0.9% NaCl. Cytochromes were scanned from 375nm to 675nm using OD range = 0-0.2 from 375 to 500nm and OD range = 0-0.1 from 500 to 675nm. See Methods Section 2.9 for experimental details.

Figure 17B

Absolute Spectrum of Cytochrome c Wash

Reference cuvette contained 0.15MKCl. Sample cuvette con tained combined supernatant of initial two 10ml 0.15MKCl washes undiluted reduced by sodium dithionite crystals. Cytochromes were scanned from 375 to 675nm using OD range = 0-0.05 from 375 to 500nm and OD range = 0-0.01 from 500 to 750nm. See Methods Section 2.9 for experimental details.


(A)
0.12
0.08
0.04
0.00
OD
0.06
0.04
0.02
0.00
(B)
0.02
0.00
400
440
480
0.010
0.000
500
540
580
620
Wavelength (nm)

Mitochondria depleted of cytochrome c and exposed to PH_3 had a small peak at 551nm which increased slightly with time and a fully reduced peak at 600.2-602.5nm (Fig. 18) indicating that cytochrome b was not reduced in this case.

A series of difference spectra were recorded using whole insect mitochondria and combinations of respiratory inhibitors, myxothiazol, antimycin and PH_3. When whole insect mitochondria were inhibited by PH_3 followed by the use of antimycin, a peak appeared at 561nm (54% reduced when compared to full reduction by dithionite) suggesting reduction of cytochrome b. The peaks remained at 551nm and 603nm (Fig. 19A).

Using the inhibitor myxothiazol a peak was observed at 560nm (cytochrome b) that was about 50% of the antimycin peak (27% reduced). There was no change in the spectrum with the addition of antimycin (Fig. 19B).

Myxothiazol followed by the addition of PH_3 resulted in the appearance of a small peak at 551nm (cytochrome c) (22% reduced) and complete reduction of $a+a_3$ (Fig. 20A). Since myxothiazol inhibits the reduction of cytochromes after the Rieske-iron centre, this peak, representing cytochrome c, probably suggests incomplete inhibition by myxothiazol.

When antimycin was used to inhibit the ubiquinone-cytochrome b region of mitochondria a single large peak was observed at 563nm (cytochrome b)(54% reduced). Addition of PH_3 to these mitochondria resulted in large peaks at 551nm, cytochrome c (63% reduced) and 601nm, cytochromes $a+a_3$ (fully reduced), while the peak at 563nm remained (Fig. 20B). This

Figure 18

Difference of Cytochrome c-Depleted Insect Mitochondria Reduced by PH_3

Reference cuvette contained 1.5mg protein.ml^{-1}. Sample cuvette contained the same protein concentration with cytochromes reduced by PH_3 (300μl). Spectrum recorded at time (t=mins) after PH_3 addition. Total volume was 3ml of aerated 50mM HEPES buffer pH=7.0 containing 0.9% NaCl. Cytochromes were scanned from 500 to 675nm using OD range = 0-0.05. See Methods Section 2.9 for experimental details.

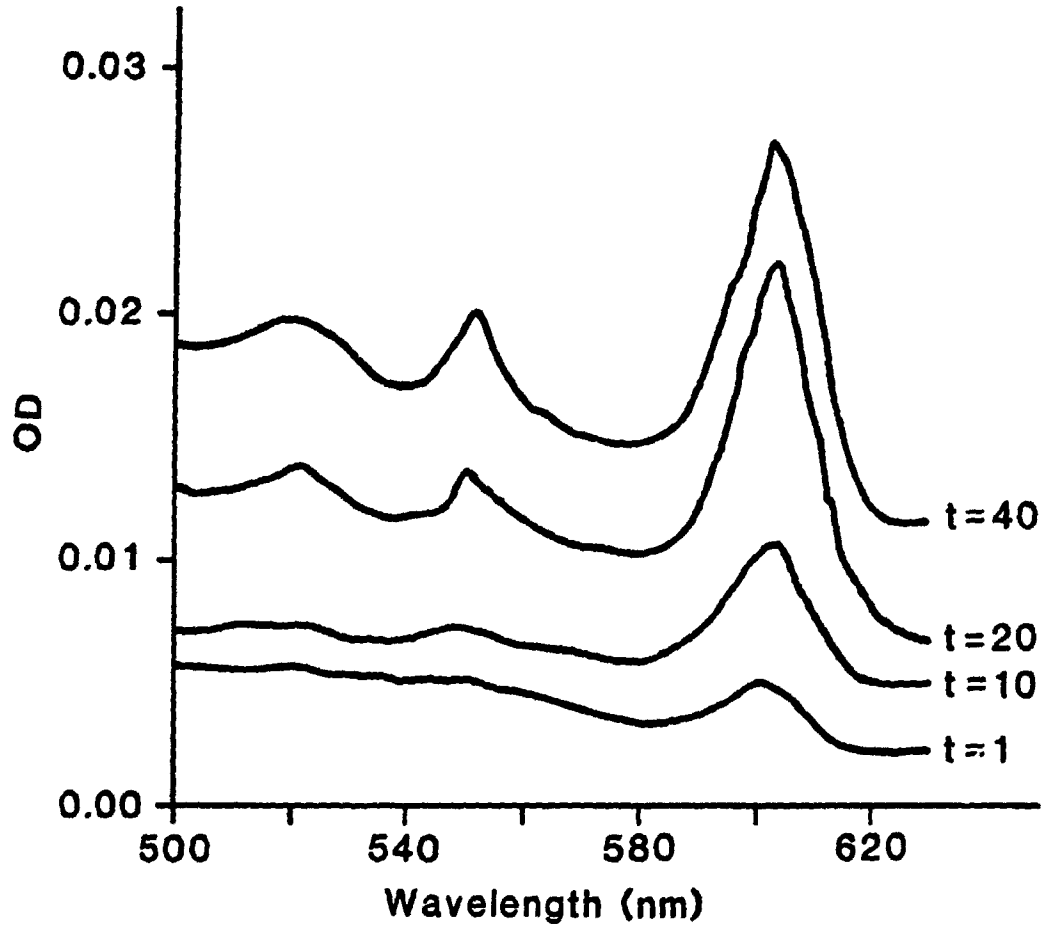

0.03
0.02
0.01
0.00
OD
500
540
580
620
Wavelength (nm)
t=40
t=20
t=10
t=1

Figure 19

Difference Spectra of Insect Mitochondrial Cytochomes Exposed to Inhibitors of Electron Transport

Reference cuvette contained 0.8mg protein.ml^{-1}. Sample cuvette contained the same amount of protein but the respiratory chain was blocked by inhibitors. Total volume was 3ml of aerated 50mM HEPES buffer pH=7.0 containing 0.9% NaCl. Cytochromes were scanned from 375 to 675nm using OD range = 0-0.2 from 375 to 500nm and OD range = 0-0.05 from 500 to 675nm.

A. Trace (i) mitochondria inhibited by PH_3 (300μl)

(ii) mitochondria inhibited by PH_3 (300μl) followed by antimycin (1.6μM).

B. Trace (i) mitochondria inhibited by myxothiazol (1.8μg)

(ii) mitochondria inhibited by myxothiazol (1.8μg) followed by antimycin (1.6μM).

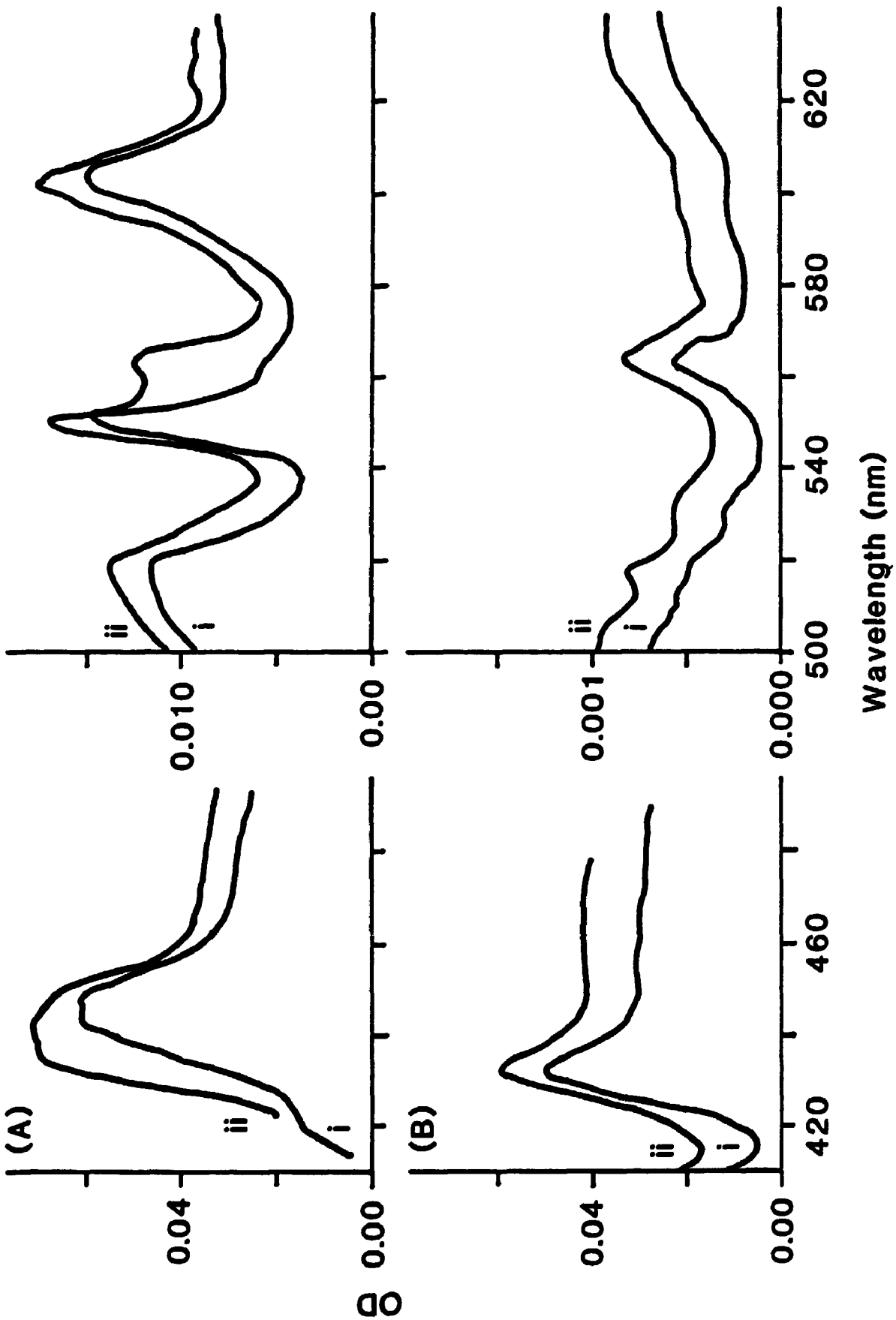

(A)
(B)
i
ii
OD
0.04
0.00
0.010
0.001
0.000
420
460
500
540
580
620
Wavelength (nm)

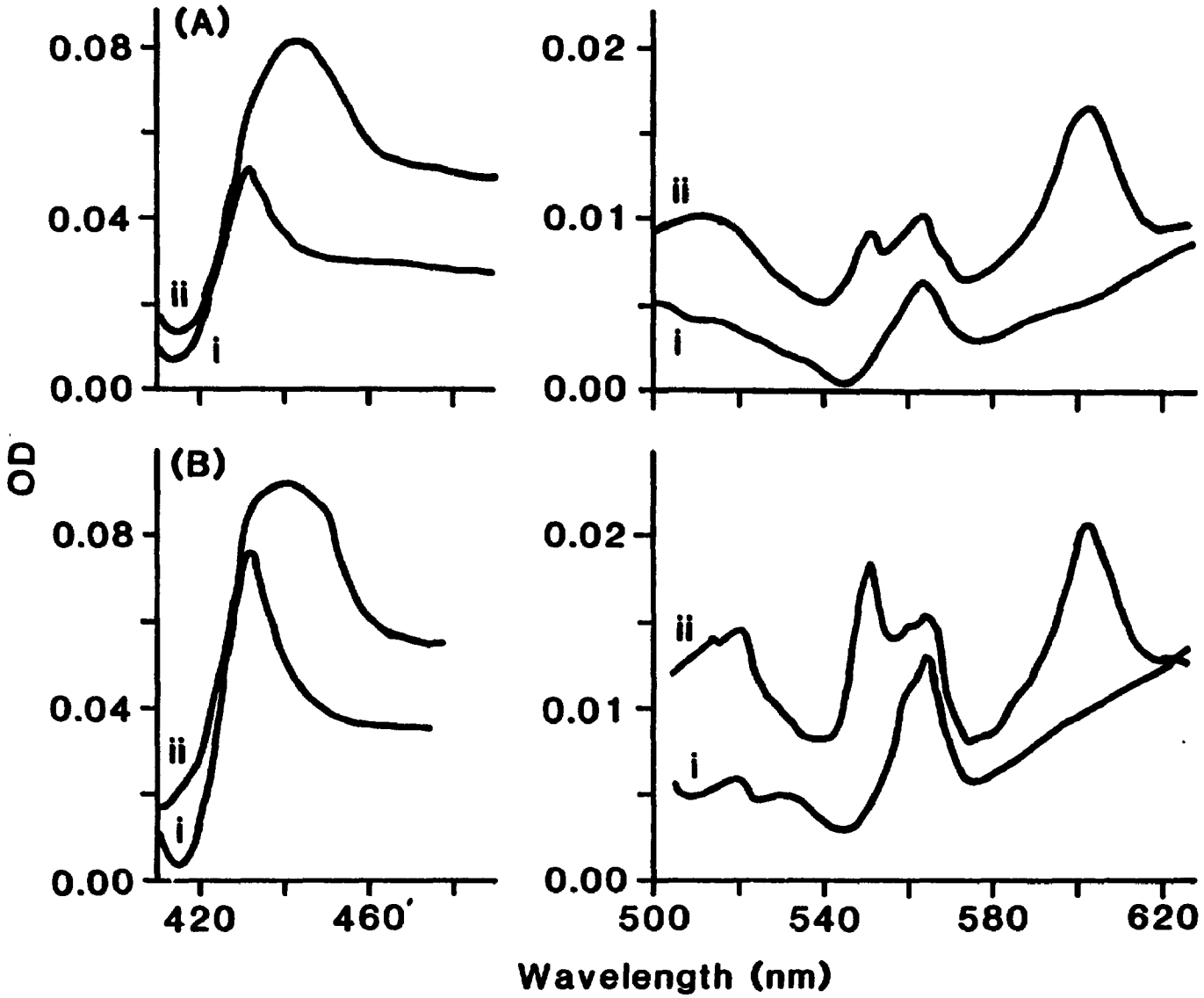
Figure 20

Difference Spectra of Insect Mitochondrial Cytochromes Exposed to Inhibitors of Electron Transport

Reference cuvette contained 0.8mg protein.ml^{-1}. Sample cuvette contained the same amount of protein but the respiratory chain was blocked by inhibitors. Total volume was 3ml of aerated 50mM HEPES pH=7.0 containing 0.9% NaCl. Cytochromes were scanned from 375 to 675nm using OD range = 0-0.2 from 375 to 500nm and OD range = 0-0.05 from 500 to 675nm

A. Trace i: mitochondria inhibited by myxothiazol (1.8μg)
ii: mitochondria inhibited by myxothiazol (1.8μg) followed by PH_3 (300μl)

B. Trace i: mitochondria inhibited by antimycin (1.6μM)
ii: mitochondria inhibited by antimycin (1.6μM) followed by PH_3 (300μl)


(A)
(B)
0.08
0.04
0.00
0.02
0.01
0.00
ii
i
OD
420
460
500
540
580
620
Wavelength (nm)

observation suggested that reducing equivalents were able to move along the respiratory chain to cytochrome c even when the mitochondria were inhibited with antimycin.

3.2.2 Mouse Liver

After complete reduction of mouse liver mitochondrial cytochromes with dithionite the spectrum was very similar to that recorded with insect mitochondria. Peaks were observed at 428nm (probably cytochrome b) and 444nm (cytochromes $a+a_3$) in the Soret band and at 519nm, 552nm (cytochrome c) 562nm (cytochrome b) and 603nm (cytochromes $a+a_3$) in the γ-region. In mouse mitochondria the peaks at 552 and 562nm were barely distinguishable (Fig.21A).

The addition of PH_3 to mouse liver mitochondria resulted in a spectrum with peaks at 552nm (cytochrome c)(51% reduced as compared to full reduction by dithionite) and 603nm ($a+a_3$)(fully reduced) as observed with insect mitochondria (Fig. 21B). However, there was a distinct peak at 562nm representing reduced cytochrome b (24% reduced) which was not present with insect tissue after PH_3 inhibition (Fig. 19A). Addition of myxothiazol resulted in a 30% decrease in height of the peak at 552nm while peaks at 562nm and 603nm remained constant.

When mitochondria were inhibited by myxothiazol a small reduced peak was observed at 562nm representing cytochrome b(21% reduced). This peak r ained and a second peak at 603nm representing cytochromes $a+a_3$ (72% reduced) was observed with

Figure 21A

Difference Spectrum of Mouse Liver Mitochondrial Cytochromes Reduced by Dithionite.

Reference cuvette contained 1mg protein.ml^{-1}. Sample cuvette contained the same concentration of protein but cytochromes were reduced with sodium dithionite. Total volume was 3ml of aerated 50mM HEPES buffer pH=7.0 containing 0.9% NaCl. Cytochromes were scanned from 375 to 675nm using OD range = 0-0.2 from 375 to 500nm and OD range = 0-0.05 from 500 to 675nm.

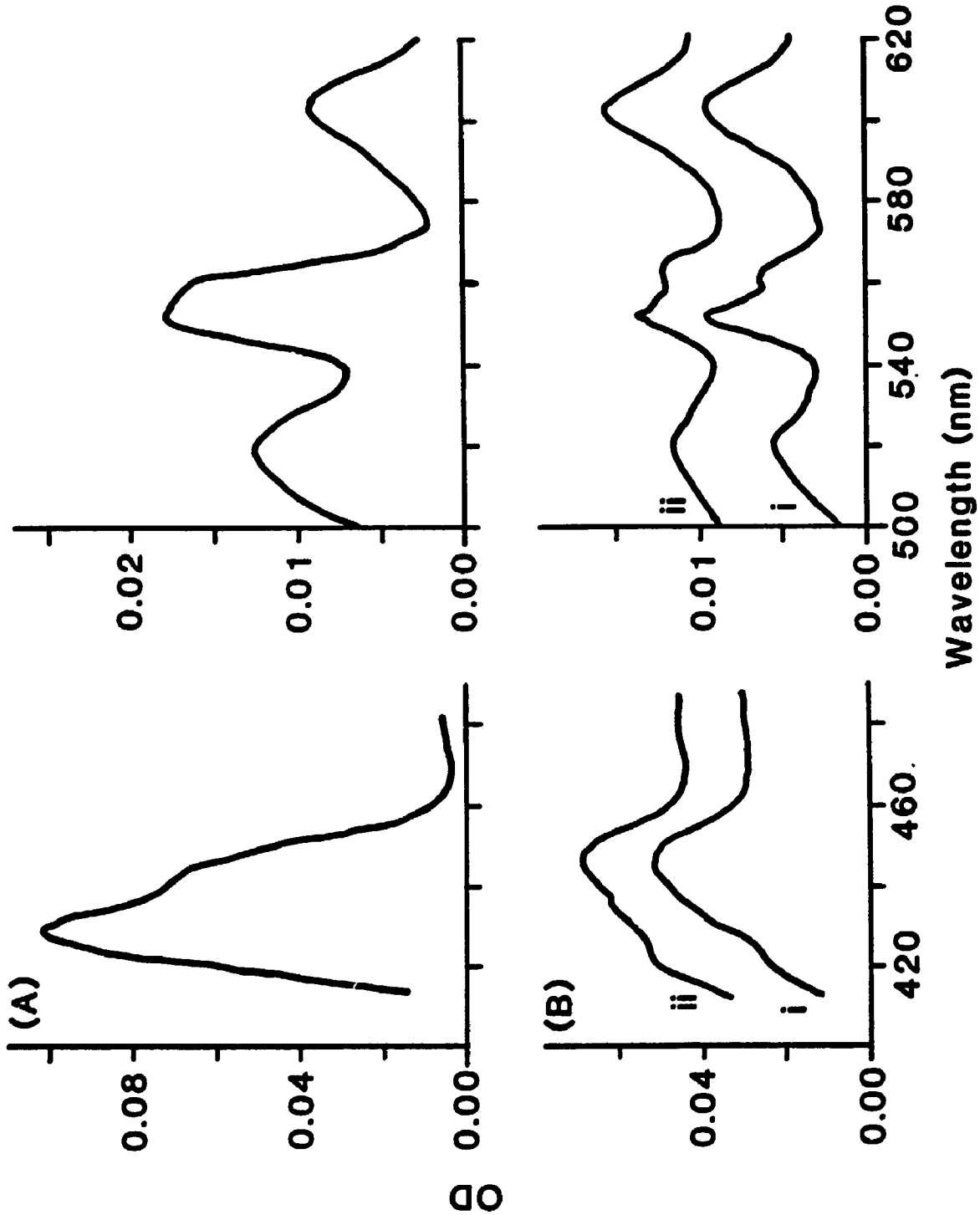
Figure 21B

Difference Spectrum of Mouse Liver Mitochondrial Cytochromes Exposed to Inhibitors of Electron Transport

As above but not reduced with dithionite.

Trace i: mitochondria inhibited by PH_3 (300μl)

ii: mitochondria inhibited by PH_3 (300μl) followed by myxothiazol (1.8μg).


(A)
(B)
OD
0.08
0.04
0.00
0.02
0.01
ii
i
420
460
500
540
580
620
Wavelength (nm)

the addition of PH_3 (Fig 22A). Cytochrome c was not reduced under these conditions, indicating that in this case, myxothiazol had fully inhibited the respiratory chain.

Inhibition of the electron transport chain by antimycin resulted in a peak at 562nm (38% reduced) which remained after addition of PH_3 (Fig. 22B). A small peak at 552nm (32% reduced) and a large peak at 603nm (86% reduced) was also observed with PH_3. In insects this combination had resulted in a greater reduction of cytochrome c (550nm)(66%) (Fig. 20B).

3.3 Ubiquinone

PH_3 bubbled into a cuvette containing 0.04mg ubiquinone (Sigma) per ml ethanol had no effect on the spectrum after 60 mins incubation and a peak was observed at 275nm. On addition of sodium borohydride ubiquinone was reduced to ubiquinol which had an absorbance peak at 291nm. PH_3 had no effect on this peak when added before or after borohydride. When ubiquinone was suspended in 0.25M sucrose, 0.1mM EDTA pH=7.2 it was reduced with a shifted peak at 285nm. PH_3 had no effect on this peak.

Material extracted from insect mitochondria according to the method of Redfearn had a spectrum that was very different from that described by Pumphrey and Redfearn (1960) from pig heart mitochondria. A series of peaks were observed in this

Figure 22

Difference Spectrum of Mouse Liver Mitochondrial Cytochromes Exposed to Inhibitors of Electron Transport

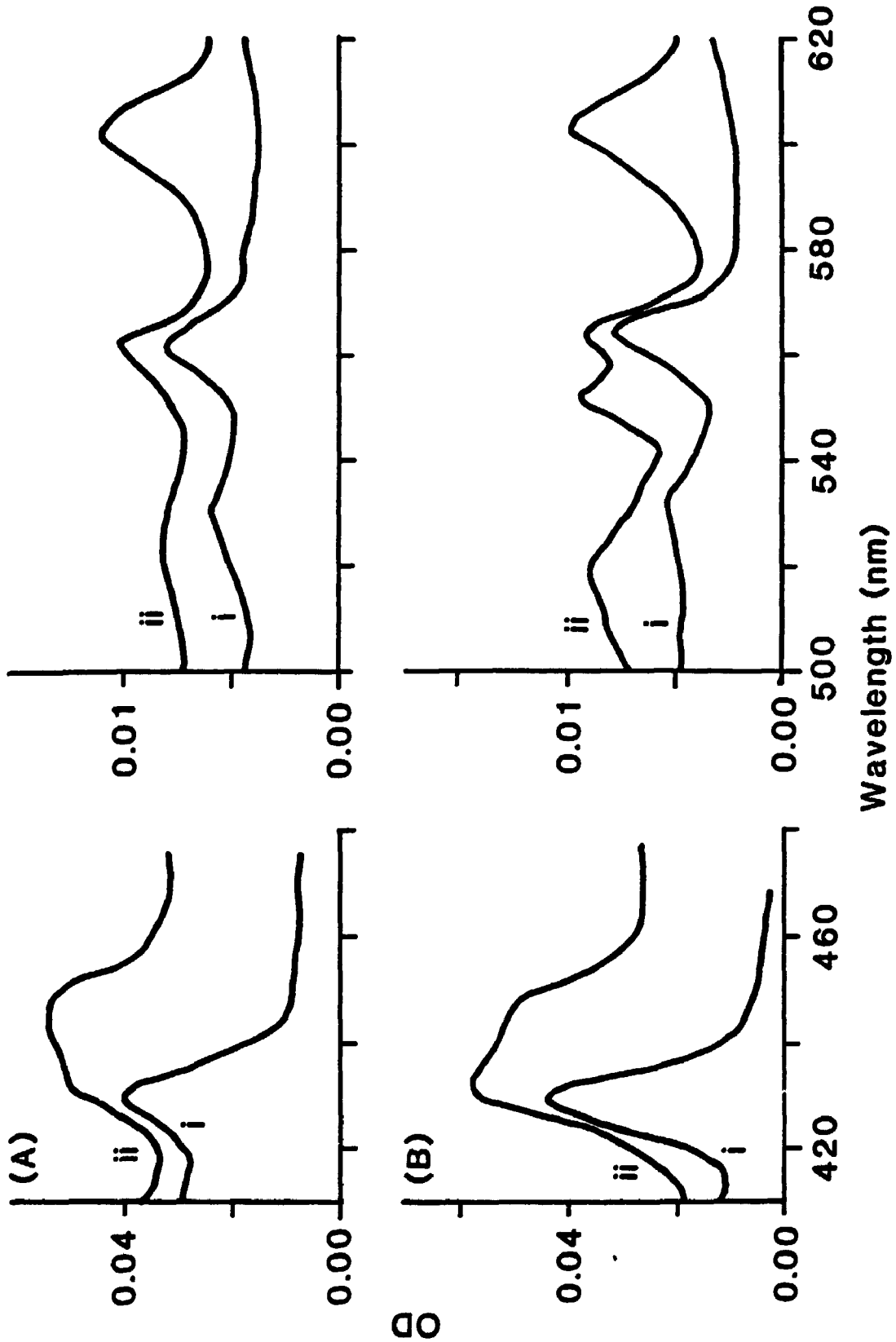
Reference cuvette contained 1mg protein.ml^{-1}. Sample cuvette contained the same concentration of protein but the respiratory chain was blocked with inhibitors. Total volume was 3ml of aerated 50mM HEPES buffer pH=7.0 containing 0.9% NaCl. Cytochromes were scanned from 375 to 675nm using OD range = 0-0.2 from 375 to 500nm and OD range = 0-0.05 from 500 to 675nm.

A. Trace i: mitochondria inhibited by myxothiazol (1.8μg)

ii: mitochondria inhibited by myxothiazol (1.8μg) followed by PH_3 (300μl)

B. Trace i: mitochondria inhibited by antimycin (1.6μM).

ii: mitochondria inhibited by antimycin (1.6μM) followed by PH_3 (300μl).


(A)
(B)
ii
i
0.04
0.00
0.01
0.00
420
460
500
540
580
620
Wavelength (nm)
OD

study from 240-270nm. The quinone peak appeared to be at 280nm compared to 275nm in pig heart while the absorbance peak of the reduced form was at about 291nm (Fig. 23).

Quinone concentrations were not statistically different in mitochondria from control insects and those exposed to PH_3 (LD_{50})(Table 4) when PH_3-sensitive insects were used.

3.4 Effect of Phosphine on the Oxygen Defence System

3.4.1 Superoxide Dismutase (SOD)

The activities of bovine CuZnSOD (1 unit), MnSOD extracted from rat liver mitochondria (1 unit) and SOD present in whole insect homogenates were unaffected after 30 minutes incubation with PH_3 (100μl).

Insects (PH_3-sensitive) exposed to PH_3 (LD_{30}) had an increased total SOD activity (Table 5). It was established, using cyanide, that this increase was due to cyanide-sensitive SOD (CuZnSOD) which was two-fold higher in treated insects. No difference was observed within the first 6 hours after exposure, however by 12 hours the difference was maximal and remained unaltered for 6 days, measurements were not made after this time. Activity of the cyanide-insensitive isozyme (MnSOD) was not changed after fumigation.

SOD levels in resistant insects were not significantly different from those measured in PH_3-sensitive insects. No increase in cyanide sensitive SOD activity was observed three days after exposure to PH_3 (LD_{30}) (Table 5). This is an

Figure 23

Absolute Spectrum of Ubiquinone from Insect Mitochondria

Ubiquinone was extracted from 1ml insect mitochondrial homogenate containing 17.6mg protein ml^{-1}. Pyrogallol added during extraction to prevent oxidation of reduced ubiquinone. Total extract was resuspended in 3ml ethanol and used directly for spectral analysis. Extraction was performed four times, of which this is one example. See Methods section 2.10 for experimental details.

Trace a: direct measurement after extraction

Trace b: after reduction with sodium borohydride.

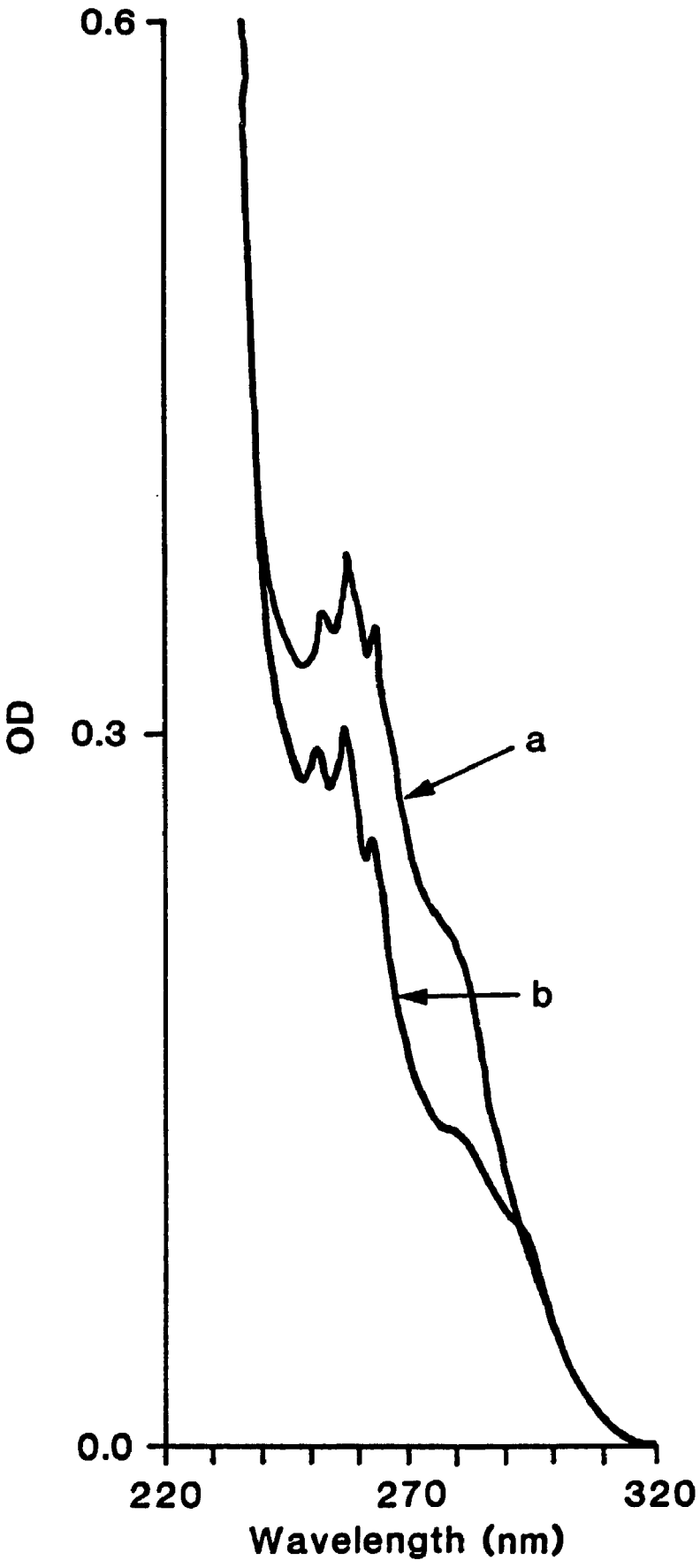

0.6
0.3
0.0
OD
a
b
220
270
320
Wavelength (nm)

TABLE 4

Comparison of Oxidised Quinone Concentration in Control and Treated Insect Mitochondria

Preparation	n	Quinone concentration (nanomoles quinone . mg^{-1})
Control	4	1.04±0.13
Treated*	4	0.89±0.20

Insects used in this experiment were PH_3-sensitive. The spectrum of ubiquinone in ethanol was determined in the range of 220-320nm. Oxidised ubiquinone concentration was calculated by subtracting the peak height value, after reduction by dithionite, from the peak height observed at 280nm before reduction. The molecular extinction coefficient $(e_{ox}-e_{red})=12.25 mM^{-1}cm^{-1}$ (Redfearn 1967). See Methods Section 2.10 for detail of procedure. *Treatment: Insects were exposed to 0.055mg $PH_3.l^{-1}$ for 5 hours (LD_{55}). Quinone was extracted from 1ml mitochondrial homogenate containing 20-40mg protein and the total extract resuspended in 3ml ethanol. No statistically significant difference was observed between control and treated preparations.

TABLE 5

Comparison of Superoxide Dismutase Activity in Susceptible and Resistant Insects

Preparation	n	Superoxide Dismutase (c) (units SOD.mg protein^{-1}) CN-sensitive(d)	CN-insensitive	SOD Total
a. Control				
PH_3-sensitive	10	2.8 ± 0.24	5.6 ± 0.52	8.4 ± 0.61
Resistant	10	2.9 ± 0.56	4.2 ± 0.43	7.1 ± 0.60
b. Treated				
PH_3-sensitive	10	5.7 ± 0.42#	5.9 ± 0.44	11.6 ± 0.78#
Resistant	10	3.1 ± 0.19*	4.3 ± 0.31	7.4 ± 0.51*

Enzyme activities were measured as described in Methods Section 2.11.1 using sonicated whole insect homogenates. (b) Treatment: insects were exposed to PH_3 ($0.085 mg.l^{-1}$ X 5hrs, LD_{30}) and activities determined after 3 days. (c) One unit SOD is amount required to inhibit pyrogallol antoxidation by 50%. (d) Cyanide (CN)-sensitive SOD and CN-insensitive. Values represent mean ± SEM, n represents number of replications. Statistically significant differences between resistant and PH_3-sensitive strains are designated *($p \leq 0.05$) and between treated insects and controls, #($p \leq 0.05$) (Students t-test).

important observation since it suggests that resistant insects were not challenged in the same way as susceptible and could provide a key to the mechanism of resistance (See Discussion section 4.2).

3.4.2 Catalase

Catalase activity in supernatants from PH_3-sensitive insects exposed to PH_3 ($0.04mg.l^{-1}$ for 5 hrs, LD_{30}) was inhibited by nearly 60% as compared to controls in the first 36 hours after fumigation from 20.65 ± 1.96 to 8.33 ± 1.50 units.mg $protein^{-1}$ (Mean$\pm$ SEM,n=4). Activity increased with time until there was no significant difference between control and exposed insects after 11 days (Fig. 24) Table 6 shows the catalase activity three days after fumigation in PH_3-sensitive and resistant insects.

Resistant insects had a significantly lower catalase activity (62%) than PH_3-sensitive (Table 6), which was unchanged three days after insects were exposed to PH_3 (LD_{30}). The catalase activity in resistant insects was not significantly different from the activity remaining in susceptible insects after fumigation.

3.4.3 Peroxidase

Adults of *S. granarius* had no glutathione peroxidase activity. There was no increase in absorbance at 340nm with any of the homogenate concentrations used.

Figure 24

Effect of PH_3 on Catalase Activity from Insects Exposed In Vivo

Insects (PH_3-sensitive), exposed to PH_3 were homogenized and catalase activity measured at specified time intervals after exposure. Protein concentration was 0.03-0.05 mg protein.ml^{-1}. One unit of catalase decomposes one umole H_2O_2 per min at 25°C. See Methods Section 2.11.2. Data points represent mean ± SEM n=4. Statistically significant differences between control and treated preparations are denoted by *($p \leq 0.01$) and x($p \leq 0.02$).

Trace a: Control insects

Trace b: PH_3 treated (0.035mgPH_3.l^{-1} for 5hrs, LD_{30})

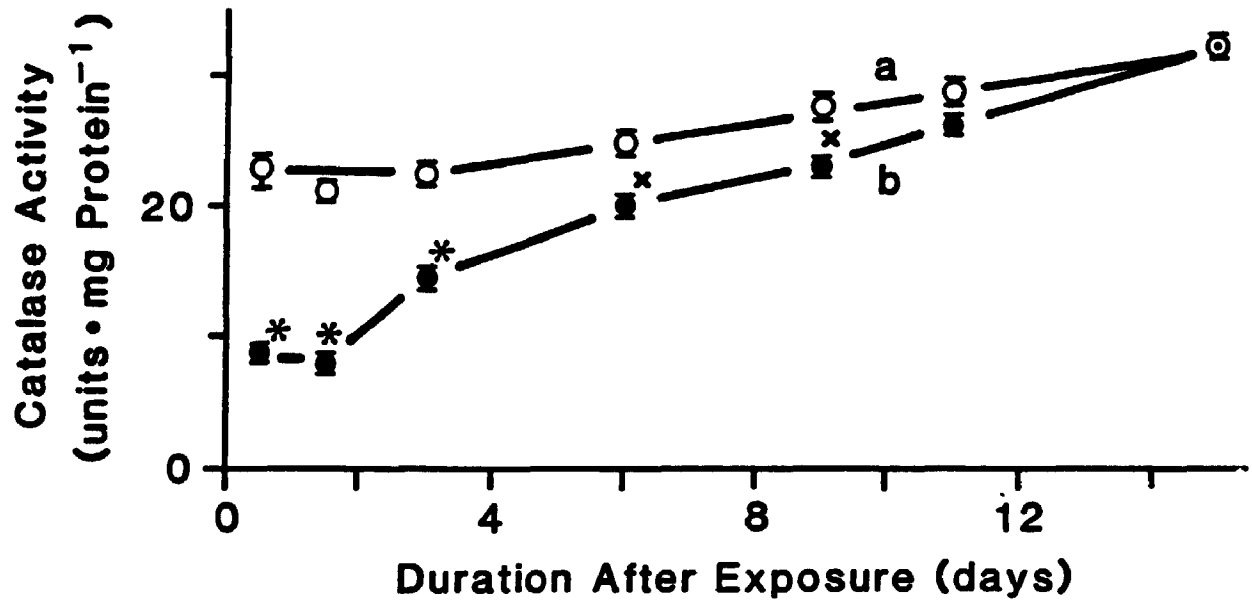
Figure 25

Effect of PH_3 on Peroxidase Activity from Insects Exposed In Vivo

Insects (PH_3-sensitive), exposed to PH_3 were homogenized and peroxidase activity measured at specified time intervals after exposure. Protein concentration 0.03-0.06mg protein.ml^{-1}. See Methods Section 2.11.3 for experimental details. Data points represent mean ± S.E.M. n=4. Statistically significant differences between control and treated preparations are denoted by *($p \leq 0.01$) and x($p \leq 0.05$).

Trace a: Control insects

Trace b: PH_3 treated (0.035mgPH_3.l^{-1} for 5hrs LD_{30}).


Catalase Activity
(units • mg Protein^{-1})
20
0
0
4
8
12
Duration After Exposure (days)
a
b

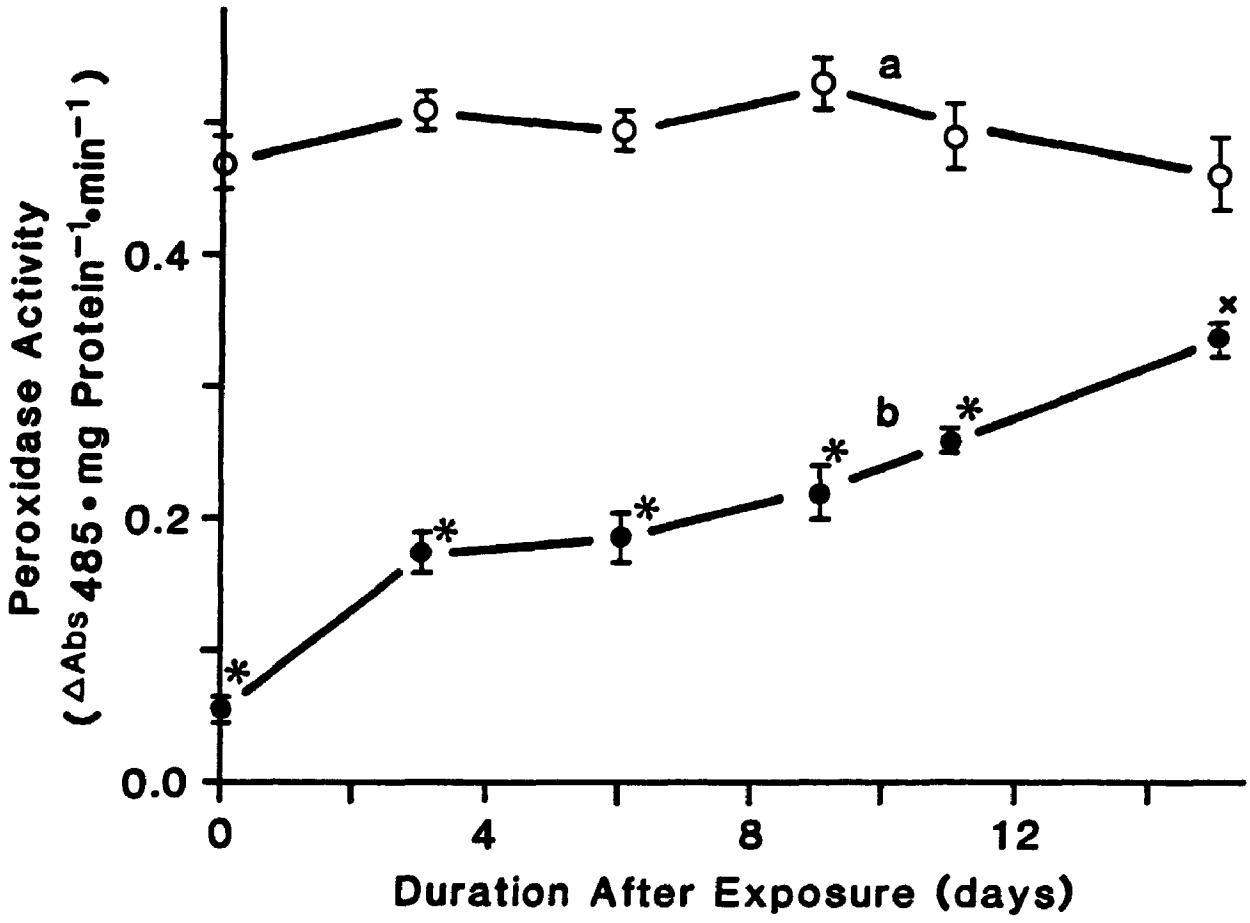

Peroxidase Activity
(ΔAbs 485 • mg Protein^{-1}•min^{-1})
0.4
0.2
0.0
0
4
8
12
Duration After Exposure (days)
a
b

TABLE 6

Comparison of Catalase and Peroxidase Activity in Susceptible and Resistant Insects

Preparation	n	Catalase(c) (units.mg protein^{-1})	Peroxidase(d) ($\Delta OD_{485}.mg^{-1}.min^{-1}$)
a. Control			
PH_3-sensitive	4	23.2 ± 1.45	0.51 ± 0.02
Resistant	4	8.9 ± 1.30*	0.54 ± 0.03
b. Treated			
PH_3-sensitive	4	8.5 ± 0.95#	0.18 ± 0.02#
Resistant	4	7.9 ± 0.50	0.28 ± 0.02*#

Enzyme activities were measured as described in Methods Section 2.11.2-2.11.3 using sonicated whole insect homogenates. (b) Treatment: insects were exposed to PH_3 ($0.085mg.l^{-1}$ X 5hrs, LD_{30}) and activities determined after 3 days. (c) One unit catalase is amount that decomposes $1\mu MH_2O_2.min^{-1}$. (d) Peroxidase activity measured in terms of reactivity with p-phenylenediamine. Values represent mean ± SEM, n represents number of replications with same preparation used for each enzyme. Statistically significant differences between resistant and PH_3-sensitive strains are designated *($p \leq 0.05$) and between treated insects and controls, #($p \leq 0.05$) (Students t-test).

General peroxidase activity, observed using p-phenylenediamine as an indicator was high in both PH_3-sensitive and resistant insects (Table 6). Immediately after PH_3-sensitive insects were exposed <u>in vivo</u> to PH_3 ($0.04mg.l^{-1}X5hrs$, LD_{30}), peroxidase activity had dropped to 12% of the control value (Fig. 25). After three days peroxidase activity was 34% of the control and increased to 53% of the control 11 days after fumigation. By 15 days the peroxidase activity was up to 73% of control. Three days after resistant insects were exposed to PH_3 (LD_{30}), peroxidase activity was 52% of control significantly less inhibited than in PH_3-sensitive insects under the same conditions (Table 6).

A comparison of peroxidase concentration in mitochondria and cytosolic supernatant three days after exposure to PH_3 ($0.043mg.l^{-1}$, LD_{35} and $0.065mg.l^{-1}$, LD_{55}) showed that approximately one third of the peroxidase activity was located in the mitochondria. After the LD_{35} exposure, the mitochondrial fraction was only inhibited by 36% while to the cytosolic peroxidase was inhibited by 68%. After the LD_{55} exposure the mitochondrial fraction was inhibited by 71% while cytosolic peroxidase was also inhibited by 68% (Table 7).

3.4.4 <u>Glutathione</u>

There are several non-enzymatic antioxidants that are important members of the oxygen defence system (See Introduction section 1.5). Glutathione, a tripeptide, found in all

TABLE 7

Peroxidase Activity in Mitochondrial and Cytosolic Fractions

Preparation	n	Peroxidase ($\Delta OD_{485}.min^{-1}.mg\ protein^{-1}$) Mitochondria	Cytosol
Control	3	0.14±0.00	0.34±0.01
Treatment (a)	3	0.09±0.01*	0.11±0.01*
Treatment (b)	3	0.04±0.00*	0.11±0.00*

Insects used in this experiment were PH_3-sensitive. Peroxidase activity measured according to the method of Armstrong *et al*. (1978)(see Methods section 2.11.3 for details) three days after exposure to treatment (a) 0.043mg $PH_3\ l^{-1}$ for 5hrs (LD_{35}) and (b) 0.065mg $PH_3.l^{-1}$ for 5hrs (LD_{55}). Mitochondria were isolated as a pellet after a 10,000xg centrifugation and the supernatant of this spin represented the cytosol, (see Methods section 2.5 for details). n = the number of replications of a single experiment. Statistically significant differences between control and treated preparations are denoted by an asterisk (*) ($p \leq 0.05$) (Student's t-test).

eukaryotes is one of the most important of these. It can be utilized in conjunction with glutathione peroxidase or, as in insects where this enzyme is not found, it can function independently. The reduced form (GSH) is oxidised to the disulphide (GSSG) by free radicals. A linear relationship was observed between the standard reduced glutathione (GSH) or oxidized (GSSG)(0-2.0μg/3ml) concentration and arbitrary fluorescence units.

After exposure to PH_3 (0.04mg.ml^{-1}X5hrs LD_{30}) there was no significant difference in glutathione (GSH or GSSG) between control and exposed PH_3-sensitive insects (Table 8).

3.5 Damage Resulting from Free Radical Attack

In this study three types of damage, resulting from free radical attack on cellular components, were investigated (see Introduction section 1.6). Firstly, free radicals oxidise membrane polyunsaturated fatty acids resulting in their fragmentation and the production of lipid soluble fluorescent material which was measured. Another consequence of the breakdown of PUFA's is that their concentration will be decreased with respect to saturated fatty acids, the ratio of the two types of fatty acid was used as another measure of membrane lipid peroxidation. Secondly, free radicals oxidise the sulphydryl groups of non-protein molecules such as glutathione as well as proteins, whose activity may be adversly effected by this damage. Thiol content was measured

TABLE 8

Comparison of Glutathione Concentration in Susceptible and Resistant Insects

Preparation	GSH[d] (μg.mg protein^{-1})[f]	GSSG[e] (μg.mg protein^{-1})
a. **Control**		
PH_3-sensitive	101.2±3.2	53.6±3.1
Resistant	109.6±4.4	59.0±4.3
b. **Treated (LD_{65})**		
PH_3-sensitive	110.4±3.1	56.3±2.1
Resistant	103.4±3.8	58.4±1.6
c. **Treated (LD_{90})**		
PH_3-sensitive	109.1±3.6	59.2±3.4

Spectrofluorimetric determination of glutathione in insect mitochondria. λ_{ex}=350nm λ_{em}=420nm. Treatments: (b) insects exposed to 0.065mgPH_3/1x5hrs; (c) exposed to 0.16mgPH_3/1x5hrs, glutathione levels determined 3 days after exposure. (d) Reduced glutathione (GSH) and (e) oxidised glutathione (GSSG) levels interpolated from standard curves. (f) Protein concentrations determined by resuspending the denatured protein pellet. See Methods Section 2.11.4 for details. Values represent means ± SEM (n=4). No significant differences observed relating to treatments.

as an indicator of attack. Lastly, the activity of a specific enzyme, H^+-ATPase was measured. Free radicals can attack aromatic residues as well as sulphur-containing residues resulting in enzyme inactivity.

3.5.1 Lipid Peroxidation

Lipid peroxidation was assessed by measuring the level of lipid soluble fluorescent material and the ratio of unsaturated to saturated fatty acid methyl esters (FAME's). Quinine sulphate solution was used to make a standard curve from which the concentration of fluorescent material (lipofuscin) could be interpolated. Standard FAME's were used to identify the sample peaks using gas chromatography.

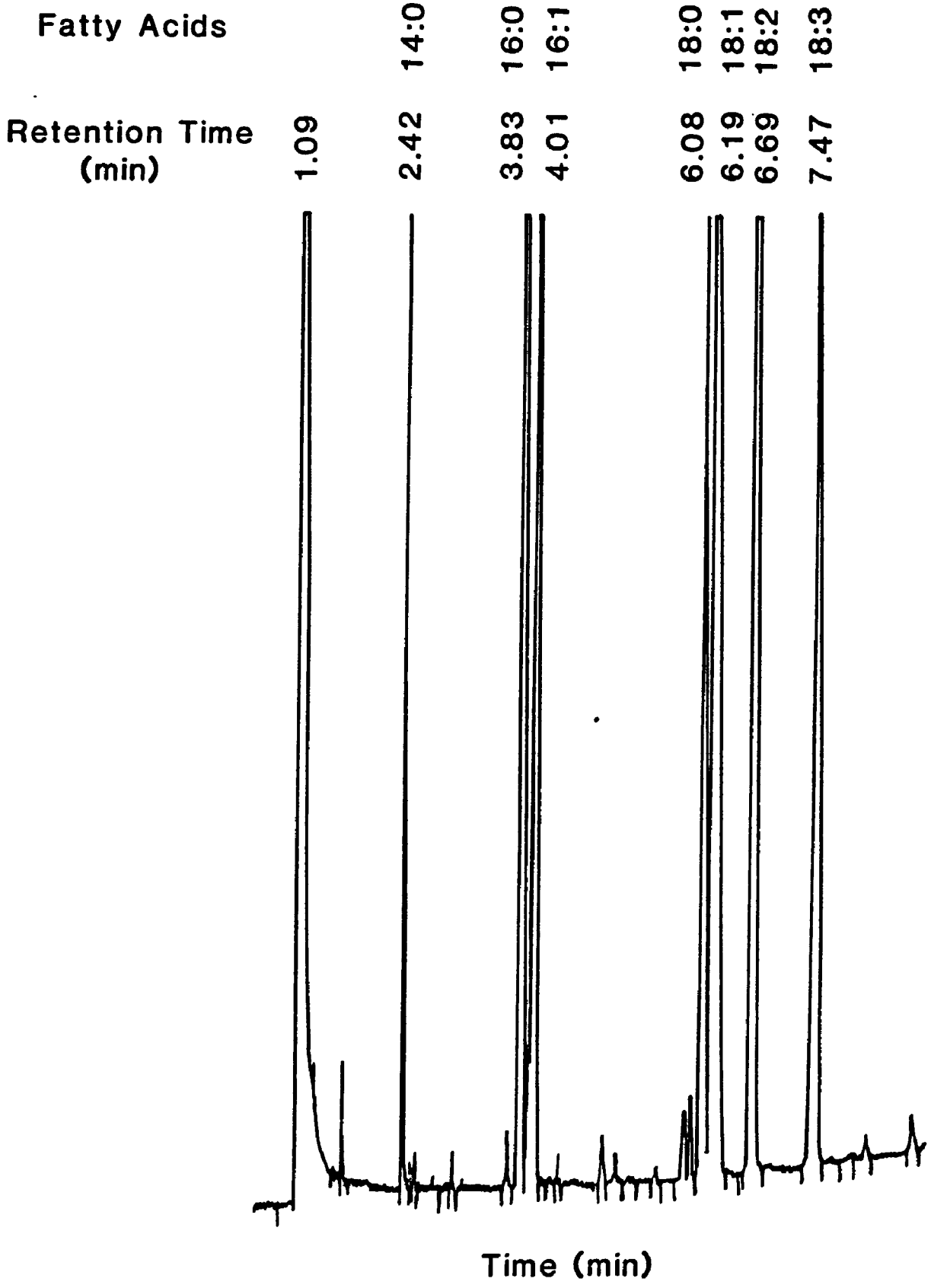
The ratio of unsaturated to saturated FAME's was calculated using peak area values for $C_{18:2}$ and $C_{18:3}$ and comparing them to those of $C_{14:0}$ and $C_{16:0}$. These FAME's were chosen because the peaks were consistantly integrated and well defined, (See Fig. 26 for an example chromatogram).

Following *in vitro* exposure to free radical generating systems, Fe^{2+}/ADP ascorbate, Fe^{2+}/AMP H_2O_2 there was no significant difference in FAME composition or fluorescent product accumulation between control and exposed mitochondria or microsomes (Table 9). However, it was observed that PUFA concentration in control and treated preparations of both mitochondria and microsomes were significantly decreased

Figure 26

Gas Chromatogram Identifying Fatty Acids

This is a sample chromatogram showing the separation obtained with fatty acids isolated from insect membranes. Three saturated fatty acids were recorded; $C_{14:0}$, myristic acid; $C_{16:0}$, palmitic acid, and $C_{18:0}$, stearic acid. Four unsaturated fatty acids were also recorded; $C_{16:1}$, palmitoleic acid; $C_{18:1}$, oleic acid; $C_{18:2}$, linoleic acid and $C_{18:3}$, linoleic acid. The identity of the fatty acids was found using standards. For experimental detail see Methods Section 2.12.1 (iv). A ratio of $C_{18:2}$ + $C_{18:3}$ to $C_{14:0}$ + $C_{16:0}$ was used when comparing polyunsaturated fatty acid content before and after treatment.


Fatty Acids
14:0
16:0
16:1
18:0
18:1
18:2
18:3
Retention Time (min)
1.09
2.42
3.83
4.01
6.08
6.19
6.69
7.47
Time (min)

compared to the concentrations in membranes taken directly from insects. If the ratio of PUFA: saturated fatty acids is considered and the proportion of PUFA's is taken as having a value of one, then the saturated fatty acids; in insect microsomes have a value of 0.66 while after incubation they average 0.77; in insect mitochondria there is an average of 0.75 and after incubation the proportion is increased to 0.86. The corresponding decrease in PUFA content suggests that a one hour incubation at 25°C is sufficient to peroxidase some of the lipids.

When the Fe^{2+}/EDTA + α-glycerophosphate + PH_3 system was used with submitochondrial particles (SMP), no differences were observed in PUFA content between control and treated preparations. There was a significantly higher accumulation of fluorescent material containing Fe^{2+}/EDTA + α-glycerophosphate both with and without PH_3 (Table 9).

After <u>in vivo</u> exposure to PH_3 (LD_{80}) the microsomal fraction only had significantly different ratios of unsaturated:saturated fatty acids. Table 10a presents the results on five different sets of insects. The results were highly variable but did show a statistically significant decrease in PUFA concentration (an average of 23.7%) after exposure. Mitochondria isolated from the same insects showed no difference in FAME levels. Insects exposed to a lower level of PH_3 (LD_{60}) had a 12.3% decrease in microsomal PUFA's, which was

TABLE 9

Lipid Peroxidation Levels in Insect Mitochondria after Exposure to a Free Radical Generating System

Preparation	n	Lipid Soluble Fluorescence (RFU: λ_{ex}=340, λ_{em}=430nm)	Ratio of FAME ($C_{18:2}$+$C_{18:3}$) : ($C_{14:0}$+$C_{16:0}$)
a) **Mitochondria**			
Control	5	N.D.	1:0.86±0.010
Fe^{2+}-ADP/ascorbate	5	N.D.	1:0.88±0.019
Fe^{2+}-AMP/H_2O_2	5	N.D.	1:0.83±0.022
b) **SMP's**			
Control	5	20.5±1.2	1:0.91±0.023
Fe^{2+}-EDTA	5	38.5±3.1*	1:0.92±0.009
Fe^{2+}-EDTA/PH_3	5	43.5±2.4*	1:1.01±0.021
c) **Microsomes**			
Control	4	N.D.	1:0.77±0.029
Fe^{2+}-AMP H_2O_2	4	N.D.	1:0.78±0.027

Insects used in this experiment were PH_3-sensitive. Lipid peroxidation in terms of relative fluorescence units (RFU) see Methods Section 2.12.1(iii), and change in polyunsaturated to saturated fatty acid methyl ester (FAME) ratio, see Methods section 2.12.1(iv). Three different preparations, a, b and c were exposed to radical generating systems described in Methods Section 2.12.1(i) SMP's are sub-mitochondrial particles. Data represents mean values of n, number of replications ± SEM. No data is indicated as N.D. Statistically significant differences between treated and control preparations are denoted by a asterisk (*) $p \leq 0.05$.

TABLE 10

Analysis of Fatty Acid Methyl Ester Content of Insect Microsomes

Experiment		Peak Areas from G.C. (c) $C_{18:3}$+$C_{18:3}$	(d) $C_{14:0}$+$C_{16:0}$	Ratio (c):(d)	% Decrease in PUFA's
a) LD_{80}					
1	Control	71201	47238	1:0.66	
	Treated	97797	92907	1:0.95	30.1
2	Control	83454	55998	1:0.67	
	Treated	49239	38481	1:0.78	14.1
3	Control	73154	48582	1:0.66	
	Treated	31279	25143	1:0.80	17.3
	Treated	61209	47942	1:0.78	15.2
4	Control	24268	16114	1:0.66	
	Treated	17143	20127	1:1.17	43.6
5	Control	237899	154158	1:0.65	
	Treated	199259	165783	1:0.83	21.9
b) LD_{60}					
1	Control	237899	154648	1:0.65	
	Treated	294215	223865	1:0.73	14.5
2	Control	24268	16113	1:0.66	
	Treated	15214	11304	1:0.74	10.6
3	Control	83454	55831	1:0.67	
	Treated	85511	46401	1:0.75	10.6
4	Control	71201	47238	1:0.66	
	Treated	82352	62752	1:0.76	13.3

Insects used in this experiment were PH_3-sensitive. Fatty acid methyl ester (FAME) analysis using gas chromatography (GC). FAME's made according to Methods Section 2.12.1 (iv) from fatty acids extracted from insect microsomes 3 days after in vivo exposure to a) 0.06mg $PH_3.l^{-1}$x5hrs, and b) 0.09mg PH_3 l^{-1}x5hrs. Volume of sample in hexane was 1-2µl containing 0.04-18µg inorganic phosphorous, see Methods Section 2.12.1 (v). Raw data is shown in terms of peak area as integrated by the G.C. Results from individual experiments are shown because of the variability. % Decrease in polyunsaturated fatty acid (PUFA) content was calculated from the ratio.

less variable than at higher concentrations (Table 10b), again no differences were observed in mitochondrial fatty acid ratio's from the same insects.

3.5.2 Sulphydryl Group Content

The sulphydryl group content of mitochondria was measured because free radicals such as $HO^{\cdot}$ are powerful oxidising agents which react with thiols, rendering them undetectable by this technique. Consequently, a decrease in sulphydryl group content suggests attack by free radicals. Thiol content was measured 24 hours after the end of fumigation (LD_{60}). There was a 25% decrease in sulphydryl group content in exposed insects when compared to controls (Table 11).

There was no difference between control and treated mitochondria after *in vitro* exposure of mitochondria to a free radical generating system, Fe^{2+}-ADP/ascorbate.

3.5.3 Mitochondrial Oligomycin Sensitive ATPase Activity

The activity of membrane bound oligomycin Mg^{2+}-dependent, H^{+}-ATPase was observed after insects were exposed *in vivo* to PH_3 (LD_{60}) and after insect mitochondria were exposed *in vitro* to two free-radical generating system, Fe^{2+}-ADP/ascorbate.

The activity of this enzyme was observed for two reasons. Firstly, if mitochondrial membrane lipids are peroxidised after $HO^{\cdot}$ attack the concommitant decrease in fluidity (See Introduction 1.6) could affect membrane bound proteins such

TABLE 11

Comparison of Sulphydryl Group Content in Control and Treated Insect Mitochondria

	Preparation	n	Sulphydryl group content (nmole thionitrobenzoic acid.mg protein^{-1})
a)	In vivo control	7	39.7±3.1
	PH_3-treated	7	29.6±3.2*
b)	In vitro Control	4	23.2±1.2
	(Fe^{2+} ADP)/ascorbate	4	22.6±2.5

Insects used were PH_3-sensitive. Sulphydryl group content measured a)24 hours after insects exposed to 0.06mg PH_3.l^{-1}x5hrs and b)after insect mitochondria were exposed to a free radical generating system (5-10mg protein.ml^{-1}). See Methods Section 2.13 for details. Values are means ± SEM. The asterisk (*) indicates a statistically significant difference between sulphydryl content of mitochondria rom control and treated insects. ($p \leq 0.05$, Student's t-test).

as ATPase. Secondly HO· can react directly with sulphur-containing and aromatic amino acids which may affect enzyme function.

ATPase from PH_3-sensitive insects exposed to PH_3 *in vivo* had an 11% increase in activity compared to controls (suggesting a disinhibition of the enzyme which is usually controlled by an inhibitory protein) while ATPase activity after exposure to free-radical generating system was 30% less than controls indicating inhibition of the enzyme (Table 12). Phosphorus production was 5-fold higher after *in vitro* experiments than *in vivo*, which also suggested disinhibition.

TABLE 12

Comparison of Oligomycin-Sensitive H+-ATPase Activity in Control and Treated Insect Mitochondria

Preparation	n	ATPase Activity[(c)] (nmoles Pi released.mg protein^{-1}.20 mins^{-1})
a) In vivo		
control	5	1.41±0.03
PH_3	5	1.59±0.06*
b) In vitro		
Control	4	7.13±0.31
Control + ADP	4	7.24±0.28
(Fe^{2+} ADP)/ascorbate	4	4.96±0.35*

Insects used were PH_3-sensitive. H^+-ATPase activity measured in terms of inorganic phosphorus production during 20 mins incubation with mitochondria (See Methods Section 2.14). Mitochondria used were a)extracted from insects 24-hours after exposure to 0.06mg $PH_3.l^{-1}$x5hrs and b)extracted from insects and exposed to a free radical generating system (see Methods Section 2.1.12(i)). Values represent means ± SEM. The asterisk (*) indicates a statistically significant difference between control and treated preparations where $p \leq 0.05$ for <u>in vivo</u> and $p \leq 0.01$ for <u>in vitro</u> studies (Student's t-test).

DISCUSSION

4.1 Effect of Inhibitors on the Respiratory Chain.

The effect of respiratory chain inhibitors, including PH_3 on the extramitochondrial release of H_2O_2 was observed using various substrates. The effect of inhibitors on individual cytochromes was recorded spectrophotometrically in order to ascertain the relationship between H_2O_2 release and site of inhibitor attack. Finally, the concentration of ubiquinone in insect mitochondria was measured and the effect of PH_3 on this member of the respiratory chain observed. Ubiquinone is thought to be the major site for H_2O_2 production in mitochondria (Boveris and Turrens 1980).

4.1.1 Extramitochondrial Release of Hydrogen Peroxide

It has been observed that mitochondria from a variety of different sources produce hydrogen peroxide (See Foreman and Boveris 1982 for examples) as a result of $O_2^{\cdot-}$ dismutation (Boveris and Cadenas 1975). The rate of H_2O_2 formation varies considerably between organs and species, as well as with experimental conditions. In most eukaryotes a system of enzymatic and non-enzymatic free radical scavengers are present in cytosolic and mitochondrial fractions. These defence mechanisms are essential since both $O_2^{\cdot-}$ and H_2O_2 are cytotoxic albiet indirectly. However, a significant increase in H_2O_2 production can overwhelm H_2O_2 scavenging enzymes resulting in the appearance of extramitochondrial peroxide.

a. Insect Mitochondria

This study shows that extramitochondrial H_2O_2 release was sustained for no more than 1-2 mins in the absence of exogenous substrate. Even after the addition of succinate, α-glycerophosphate or NADH-linked substrates to insect mitochondria, no increase in H_2O_2 release was observed. It is probable that there was an increased production of H_2O_2 at the respiratory chain level, however the oxygen defence system located in the mitochondrial matrix was apparently able to remove the H_2O_2 as it was produced, thereby preventing release of H_2O_2 extramitochondrially.

It was observed that inhibition of cytochrome c oxidase *in vitro* by PH_3 stimulated in the production of H_2O_2 from mitochondria utilizing endogenous substrate. The rate of release was approximately 2-fold higher in insects than mouse liver (see 4.1(b)). Since fatty acids comprise a significant proportion of endogenous substrate in mouse (Chefurka 1981a) and rat (Bryła *et al*. 1967) liver mitochondria, it was thought possible that fatty acids were also the endogenous substrates for the insect respiratory chain. Fatty acids supply electrons to two sites in the electron transport chain, NADH dehydrogenase and ubiquinone (Lehninger 1975). Experiments were performed on insect mitochondria with an inhibitor of fatty acid oxidation. 4-Pentenoic acid inhibits 3-Ketoacyl-CoA thiolase thereby preventing fatty acid oxidation in the mitochondrial matrix (Schultz 1987). This

inhibitor resulted in significant reduction of H_2O_2 release from PH_3-treated mitochondria, suggesting that the endogenous substrates were fatty acids. However, the addition of α-glycerophosphate to 4-pentenoic acid-inhibited, PH_3 treated mitochondria resulted in an increase in H_2O_2 production which was significantly less than before inhibition by 4-pentenoic acid. Since this inhibitor should only prevent H_2O_2 formation stimulated by reducing equivalents from fatty acids, the decrease observed when α-glycerophosphate was used indicates that 4-pentenoic acid may have damaged respiratory chain components as well as the thiolase although this has not been reported in the literature.

The addition of antimycin, an antibiotic that inhibits the ubiquinone-cytochrome b region of the respiratory chain, (see Introduction section 1.4) to mitochondria utilizing endogenous substrate also resulted in a significant increase in the release of H_2O_2. This was 69% higher than that observed after inhibition by PH_3.

These data suggests a connection between the Q-cycle and the generation of $O_2^{\cdot-}$ (Mitchell 1975, Bowyer and Trumpower 1981). It has been observed that H_2O_2 (from $O_2^{\cdot-}$) is produced at two major sites in mitochondria, NADH dehydrogenase (Rich and Bonner 1978, Boveris and Turrens 1980), and ubiquinone (Boveris *et al*. 1972, Loschen *et al*. 1973). Generation from the ubiquinone-cytochrome b region is rapid when it is highly reduced, as a result of inhibition by antimycin and supplemented with succinate (Nohl and Jordan 1986,

Konstantinov et al. 1987). Electrons, prevented from passage to the b cytochromes by antimycin, are donated by ubisemiquinone to intramitochondrial oxygen forming $O_2^{\cdot-}$ (Trumpower and Simmons 1979)(see Introduction section 1.2.2). Consequently, production of $O_2^{\cdot-}$ increases with increasing oxygen tension (Boveris and Chance 1973). Nohl and Werner (1986) suggested that $O_2^{\cdot-}$ generation from this region of the respiratory chain is the result of oxidation of cytochrome b_{566} and not ubisemiquinone. The actual source of $O_2^{\cdot-}$ remains a point of debate.

Addition of cyanide to antimycin-inhibited, succinate-supplemented mitochondria results in cessation of $O_2^{\cdot-}$ release (Cadenas and Boveris 1980)(See Introduction section 1.5). Myxothiazol, which inhibits electron flow through the Rieske iron-sulfur centre to cytochrome c, (Becker et al. 1981), has a similar effect (Turrens et al. 1986, Nohl and Jordan 1986). It was hypothesised that cytochrome c has to be in the oxidised state and able to accept the second electron carried by ubiquinol, for $O_2^{\cdot-}$ to be formed. These requirements are not met by cyanide, myxothiazol or PH_3. When cyanide is used in the presence of NADH, however, there was a significant generation of $O_2^{\cdot-}$ from bovine heart SMP's (Turrens and Boveris 1980), and it was concluded that autoxidation of NADH dehydrogenase was responsible for $O_2^{\cdot-}$ production with this inhibitor when NADH was used as substrate. NADH dehydrogenase autoxidises without the complication of electron cycling and there is no requirement

for passage of electrons to cytochrome c as there is with ubiquinone. Production is lower at this site, fully reduced NADH dehydrogenase of beef heart SMP's yields 0.9±0.07 (SEM) nmol $O_2^{\cdot-}$ min^{-1}.mg protein $^{-1}$ while the ubiquinone-cytochrome b site generates 1.85 ±0.20 (SEM) nmol $O_2^{\cdot-}$ $min^{-1}.mg^{-1}$. With antimycin and NADH-supplemented systems, $O_2^{\cdot-}$ will be generated at both sites (Turrens et al. 1982). Thus the observation that H_2O_2 was released from insect mitochondria at a higher rate with antimycin than PH_3 suggests that with PH_3, $O_2^{\cdot-}$ (H_2O_2) was generated exclusively by autoxidation of NADH dehydrogenase while it was generated from the ubiquinone-cytochrome b region as well as NADH dehydrogenase when antimycin was used. Turrens et al. (1982) observed a similar increase with antimycin compared to that with rotenone using porcine lung mitochondria utilizing endogenous substrate. Turrens and Boveris (1980) reported a 50% increase in $O_2^{\cdot-}$ production with antimycin over rotenone using mitochondria supplied with NADH-linked substrates, because of ubiquinone autoxidation. In this study the difference was significantly higher and could indicate that electrons were also supplied directly to ubiquinone via FAD-linked dehydrogenase, as predicted if the endogenous substrates were fatty acids.

Observations using the respiratory chain inhibitor rotenone are less easy to explain in terms of the Q-cycle. Other studies have hinted at the unusual behaviour of this inhibitor of NADH dehydrogenase (Boveris and Chance 1973, Boveris 1977, Boveris and Turrens 1980, Turrens and Boveris

1980). Rotenone did not stimulate H_2O_2 release in the same way as antimycin, myxothiazol or PH_3 when it was used to inhibit insect mitochondria utilizing endogenous substrate and with 3.1nmol rotenone per mg protein only a very small release of H_2O_2 was measured. This is unexpected if NADH dehydrogenase is the site of $O_2^{\cdot-}$ generation. Turrens et al. (1982b) observed that 5nmol rotenone per mg protein resulted in significant release of H_2O_2 NADH-linked substrates and porcine lung mitochondria. However, Boveris and Turrens (1980), using otherwise uninhibited beef heart SMP's and NADH, found that H_2O_2 production increased with rotenone concentration up to 0.36 nmol rotenone per mg protein after which no further increase was observed. Cadenas et al. (1977) reported a concentration-dependent inhibition of NADH supplemented H_2O_2 production from isolated beef heart complex I by rotenone up to 10nmol rotenone per mg protein. These authors apparently used 2.5nmol per mg as their lowest concentration. No explanation has been proposed for this lack of consistency.

Inhibition of H_2O_2 release by rotenone was reported when mitochondria or SMP's supplemented with NADH-linked substrates were initially inhibited by antimycin (Boveris 1977, Turrens and Boveris 1980). Boveris and Chance (1973) observed a slight increase in H_2O_2 with concentration up to 2nmol rotenone per mg using pigeon heart mitochondria which were uncoupled with carbonyl cyanide p-trifluoromethoxy-phenylhydrazone and inhibited with antimycin, but above this concentration the

release of H_2O_2 was decreased. Low concentrations up to approximately 0.36nmol per mg increased release of H_2O_2 in antimycin-inhibited bovine heart concentration of rotenone above this level resulted in a sudden decrease in H_2O_2 (Turrens and Boveris 1980). It was hypothesised that at these high concentrations rotenone might have an effect on the ubiquinone-cytochrome b site of $O_2^{\cdot-}$ generation. In the present study, it was observed that H_2O_2 production from PH_3-inhibited insect mitochondria utilizing endogenous substrate was increased when a very low concentration of rotenone ($0.15X10^{-1}$nmol per mg) was used. However, when PH_3 or antimycin were added to mitochondria inhibited by higher concentrations of rotenone (3.1nmol per mg) H_2O_2 release was significantly less than when PH_3 or antimycin were used alone. This decrease caused by rotenone can not be explained by its inhibitory effect on ubiquinone at high concentrations, since H_2O_2 production following PH_3 addition is not via the ubiquinone-cytochrome b region. From these observations it is apparent that rotenone is an unusual inhibitor and that the results are difficult to interpret. It seems clear that a relatively low concentration, 0.5nmol rotenone/mg, inhibits NADH dehydrogenase (Hatefi 1967) probably resulting in maximal H_2O_2 production and that there is a certain concentration of rotenone, specific to the organism, tissue or state of mitochondria above which H_2O_2 release decreases. Consequently, the observation that an unexpectedly small release of H_2O_2 accompanied rotenone (3.1nmol per mg protein) inhibition of

insect mitochondria utilizing endogenous substrate, probably has little significance in terms of determining the source of endogenous electrons.

The addition of ADP to uninhibited mitochondria utilizing endogenous substrate, had no effect on the rate of extramitochondrial H_2O_2 release, however subsequent inhibition by PH_3 resulted in a rate of H_2O_2 production that was 30% higher than in the presence of PH_3 alone. It was also observed that PH_3 added to 2,4-DNP uncoupled mouse liver mitochondria resulted in a 2-fold increase in H_2O_2 release compared to the addition of PH_3 to state 4 mitochondria. The addition of ADP and uncouplers to PH_3-inhibited mitochondria appear to have the same effect, that of releasing the respiratory chain from the constraints resulting from controlled proton movement. Cadenas and Boveris (1980) suggested that antimycin-insensitive oxygen consumption, which results in $O_2^{\cdot-}$ production, supports proton extrusion which can generate a proton motive force across the membrane (Mitchell and Moyle 1967). Protophores, inophores and Ca^{2+} all of which result in increased extramitochondrial H_2O_2 release from antymycin inhibited mitochondria, collapse membrane potential meaning that electrons can move rapidly through the system. With rat liver mitochondria in state 3u the addition of antimycin resulted in a rate of production five-fold higher than with antimycin alone (Cadenas and Boveris 1980), however, the rate was 24% less with antimycin in uncoupled pigeon lung (Boveris et al. 1972) and 28% less

in rat heart mitochondria (Loschen et al. 1973), demonstrating again in the complex nature of respiratory chain inhibition. ATP had the opposite effect, restricting H_2O_2 release from PH_3-inhibited mitochondria utilizing endogenous substrate. The effect of ATP on antimycin-inhibited pigeon heart mitochondria using endogenous substrate was very similar until respiration was released by uncouplers (Boveris and Chance 1973, Cadenas and Boveris 1980). Presumably ATP decreased the rate of electron passage in these examples. Protons are pumped out of the matrix as electrons move through the chain and then return along a concentration gradient via ATPase. If the matrix ATP concentration is high the ATPase channels are effectively 'closed' meaning that electrons can no longer flow along the respiratory chain. However, addition of ATP to cyanide-inhibited SMP's supplemented with succinate resulted in a significant production of $O_2^{\cdot-}$ via reverse electron transfer (Boveris and Turrens 1980) which was inhibited by rotenone, uncoupling and oligomycin, an inhibitor of ATPase. It appears, then that the effect of ATP depends upon the substrate used, with NADH-linked substrates electron flow is decreased, thereby reducing H_2O_2, while in succinate supplemented SMP's electrons were moved by reverse electron transfer to NAD+ in an energy dependent reaction resulting in increased $O_2^{\cdot-}$ generation from NADH dehydrogenase. Nohl and Hegnar (1978) found that intact tightly coupled rat heart mitochondria presumably utilizing endogenous substrate, had a measurable rate of extramitochondrial $O_2^{\cdot-}$ release when

supplemented with 200μM ATP. Since almost 80% of $O_2^{\cdot-}$ produced by the respiratory chain was dismutated to H_2O_2 and they were only measuring the 20% that escaped, their results suggest that very high concentrations of H_2O_2 would have been measured. A large release of H_2O_2 with ATP probably indicates the effect of reverse electron flow and suggests that the endogenous substrate was FAD-linked or that succinate was used in this experiment. Boveris and Chance (1973) reported a slight release of extramitochondrial H_2O_2 on addition of ATP to uninhibited pigeon heart mitochondria utilizing endogenous substrate which they attributed to increased fatty acid oxidation.

Thus, both ADP and ATP can result in an increase in $O_2^{\cdot-}$ generation depending on the circumstances. When PH_3 is utilized as a fumigant it might be expected that the electron transport chain would be in a reduced state. It is assumed that inhibition of cytochrome c oxidase by PH_3 would not be complete *in vivo* and that insects do not die from a lack of ATP, because unlike the situation with cyanide poisoning, they take several days to die. It is therefore necessary to hypothesis another reason for insect mortality. After fumigation there would be an accumulation of ADP in the mitochondria and the oxygen tension in the tissues would increase since it is not reduced by cytochrome oxidase to water (Turrens 1988 in press). ADP has been shown to stimulate $O_2^{\cdot-}$ production in PH_3-inhibited mitochondria utilizing endogenous substrate and the increased partial

pressure of oxygen, would further increase generation of $O_2^{\cdot-}$ as observed by Turrens et al. (1982b). ADP also forms a complex with ferrous iron which can catalyse hydroxyl radical formation from H_2O_2 (Rehncrona et al. 1980). Damage caused by free radical attack on membranes initiates a self perpetuating chain reaction. Mortality resulting from the gradual alteration of organelle membranes and proteins might be a relatively slow process explaining the extended duration between fumigation and death.

When insect mitochondria were supplemented with an exogenous FAD-linked substrate, α-glycerophosphate and inhibited by antimycin, myxothiazol or PH_3 there was an increase in H_2O_2 production which was not expected according to the Q-cycle theory of $O_2^{\cdot-}$ formation from the ubiquinone-cytochrome b region. Antimycin is the only inhibitor that results in $O_2^{\cdot-}$ generation from this site when FAD-linked substrate is used (Turrens et al. 1985). Superoxide generated from succinate-supplemented antimycin-inhibited SMP's decreased on addition of cyanide (Cadenas and Boveris 1980) or myxothiazol (Nohl and Jordan 1986), as previously explained. Extrapolation from these data suggests that PH_3 addition to antimycin-inhibited mitochondria utilizing FAD-linked substrates should result in virtually complete inhibition of H_2O_2 by reducing cytochrome c. However, when mitochondria supplemented with α-glycerophosphate and inhibited by antimycin were further inhibited by PH_3 there was only a slight decrease in extramitochondrial release of H_2O_2.

When mitochondria were partially inhibited by antimycin and then supplied with PH_3 the rate of H_2O_2 release actually increased to a rate that was the same as PH_3 alone. Both these observations appear to contradict one of the basic premises of the Q-cycle hypothesis; that cytochrome c must be in the oxidised state for $O_2^{\cdot-}$ generation to occur. It was also observed that the addition of myxothiazol to antimycin-inhibited mitochondria resulted in a similarly small decrease in peroxide release, and that myxothiazol added to mitochondria that were partially inhibited by antimycin also increased the rate of H_2O_2 release to a level equivalent to that attained by myxothiazol alone. The maximal rate with α-glycerophosphate was the same whether myxothiazol or PH_3 were utilized singly or in combination. It is apparent that H_2O_2 production could not have been from the ubiquinone-cytochrome b region when PH_3 and myxothiazol were used, if the Q-cycle hypothesis is accepted.

In an attempt to explain these observations, it was initially hypothesised that H_2O_2 production was due to reverse electron transfer fuelled by ATP present in the intact mitochondria. However, when rotenone (0.5nmoles.mg protein^{-1}) was used to block NADH dehydrogenase it had no effect on H_2O_2 release from PH_3-inhibited, α-glycerphosphate-supplemented mitochondria, indicating that reverse electron transfer (Turrens and Boveris 1980) was not the process involved. Another alternative that might account for the data is that α-glycerophosphate dehydrogenase was itself the source of

peroxide. One observation that supports this conclusion is that mitochondria, inhibited by PH_3 and utilizing succinate as a substrate instead of α-glycerophosphate have a rate of H_2O_2 release that is not significantly different from PH_3 with endogenous substrate. α-Glycerophosphate is an important substrate in insect flight muscle mitochondria (see Introduction section 1.2.1). When α-glycerophosphate supplemented mitochondria are inhibited with antimycin the rate of H_2O_2 production is higher than with either myxothiazol or PH_3. This can be explained in the same way as the observation that a higher rate of H_2O_2 release was observed with mitochondria using endogenous substrate inhibited by antimycin than by PH_3; that is that there is a release of peroxide from the autoxidation of ubiquinone with this inhibitor and not with the others. When antimycin-inhibited mitochondria utilizing α-glycerophosphate were further inhibited with PH_3 or myxothiazol the rate of H_2O_2 release was reduced presumably because these inhibitors prevented ubiquinone autoxidation. These data suggest that α-glycerophosphate could be an endogenous substrate, as well as fatty acids. Kashi (1974) reported that untreated *S. granarius* contained approximately 50μM α-glycerophosphate per gramme insect tissue which is significantly higher than that found in *Locusta migratoria*, a potentially very active insect (Bücher *et al.* 1958), indicating that this substrate is probably very important in *S. granarius*.

The production of superoxide anions in the reactions of several different reduced flavins and flavoproteins with molecular oxygen had been reported (Massey et al. 1969). They observed that flavoprotein oxidases and hydroxylases did not produce significant amounts of $O_2^{\cdot-}$ whereas flavoprotein dehydrogenases did. Singer and Edmondson (1974) tested a variety of flavoprotein enzymes for their behaviour toward a series of acceptors including oxygen. They concluded that only two of the enzymes tested mimicked xanthine oxidase in eliciting superoxide production. They obtained negative results with both α-glycerophosphate and NADH dehydrogenase. However, the latter enzyme is known to autoxidise, generating $O_2^{\cdot-}$ when the electron transport chain is inhibited. The leakage of electrons from both enzymes may only occur in situations where the chain is reduced as a result of inhibition.

The location of α-glycerophosphate dehydrogenase has been a matter of some controversy. Donnellan et al. (1970) hypothesised that it was situated on the outer surface of the inner mitochondrial membrane while Slack and Bursell (1971) placed this enzyme on the inner surface of the inner membrane. The location is important to this study since an outer surface position indicates that H_2O_2 (or $O_2^{\cdot-}$) could diffuse away from mitochondria without contacting the mitochondrial defence system.

b. Mouse Liver Mitochondria

Mouse liver mitochondria showed no extramitochondrial H_2O_2 production with endogenous substrate or succinate and only a small increase with α-glycerophosphate which ceased within one minute. When challenged with PH_3 fresh mitochondria utilising endogenous substrate released H_2O_2 but at a much slower rate than insect mitochondria. If mouse mitochondria were aged for 1-2 hours after preparation, inhibition by PH_3, myxothiazol or antimycin had no effect on H_2O_2 production until α-glycerophosphate was added and then it increased to a rate similar to that observed with PH_3 and endogenous substrate in freshly prepared mitochondria. Rotenone (0.5 nanomoles.mg protein^{-1}) did not inhibit this H_2O_2 production suggesting that it was not due to reverse electron flow and that glycerophosphate dehydrogenase present in mammalian mitochondria is also autoxidisable. There was a slower rate of H_2O_2 release than with insect mitochondria probably because glycerophosphate dehydrogenase is present at a lower concentration in mammals. This enzyme is not very active in mammalian tissue in contrast to insect mitochondria where metabolism of α-glycerophosphate is 10-100 times greater than citric acid cycle intermediates (Eastabrook and Sacktor 1958b).

4.1.2 Spectral Studies on Mitochondrial Cytochromes

Spectral studies were performed on insect and mouse mitochondria utilizing endogenous substrate since this more closely represents the *in vivo* situation. The effect of

combining the three respiratory inhibitors PH_3, antimycin and myxothiazol on cytochrome spectra was recorded as a means of understanding their interaction, and the relationship between inhibition and hydrogen peroxide production. All spectral studies were carried out at 25°C which does not allow differentiation between cytochrome c and c_1 of the electron transport chain. Eastabrook and Sacktor (1958a) found no evidence of cytochrome c_1 in housefly mitochondria using low temperature spectra, no such observations have been made using the granary weevil.

Addition of PH_3 to insect mitochondria results in peaks in the γ-region at 551nm and 603nm due to the reduction of cytochromes c and c and $a+a_3$. In contrast, PH_3-inhibited mouse liver mitochondria had a significantly reduced cytochrome b peak at 561nm as well as cytochrome c and cytochrome oxidase. It is therefore possible that the large peak due to reduced cytochrome c masked a peak at 561nm in insects. To investigate this, insect mitochondria depleted of cytochrome c were subjected to PH_3 inhibition, but no reduction of cytochrome b occurred. Chance (1952) made a similar observation when he used cyanide to inhibit succinate-supplemented mammalian heart mitochondria. With cyanide and a high substrate concentration (3.2mM), cytochrome oxidase and cytochrome c were highly reduced while cytochrome b was 97% less reduced than with succinate alone. When only 32μM of succinate were used with cyanide, cytochrome b was not reduced at all. This was one of the earliest hints of the complexities of this part of the

electron transport chain which later developed into the Q-cycle hypothesis. The dependence of cytochrome b reduction on substrate concentration could be the key to spectral differences observed between insect and mouse liver mitochondria.

PH_3 reacts directly with cytochrome c oxidase resulting in the formation of a reduced peak at 603nm (Kashi and Chefurka 1976). These authors also detected a weak interaction between PH_3 and cytochrome c. In an attempt to establish whether the peak at 552nm (cytochrome c), observed when mitochondria were inhibited with PH_3 in the present study, was due to reduction of cytochrome $c(+c_1)$ by electrons or to direct interaction of cytochrome c with PH_3, the following experiment was performed: Mitochondria were first inhibited by myxothiazol which blocks electron transport between ubiquinone and the Rieske iron-sulphur centre and prevents the passage of electrons to cytochrome c and were then challenged with PH_3. If PH_3 reacted directly with cytochrome c, a reduced peak would have been recorded at 551nm despite the presence of myxothiazol, however no such peak was observed. This demonstrates that most if not all of the reduced cytochrome c was due to the passage of reducing equivalents from ubiquinone.

The site of electron transport chain inhibition by antimycin and myxothiazol has been discussed (Introduction section 1.2.3). Beckar et al. (1980, 1981) found that both these inhibitors block electron transfer between cytochromes

b and c_1 reducing b and not c but that myxothiazol reduced 50% less cytochrome b than antimycin. Corresponding differences were observed in the present study using both mouse liver and insect mitochondria. Furthermore, Beckar et al. (1980) using beef heart SMP's reported that myxothiazol induced a red shift of ferrocytochromes b which was different from that induced by antimycin and by dithionite, from a peak at 562nm with dithionite to 568nm with myxothiazol and 561nm with antimycin. The dramatic shift in peak position of cytochrome b was not observed in insects or mouse liver. With insect mitochondria inhibited by myxothiazol, the b centre was at 560nm and with antimycin it was 563nm compared the dithionite-reduced peak between 560-563nm.

In summary then, these spectral data agree with Kashi and Chefurka (1976) that PH_3 reacts directly with cytochrome c oxidase, reducing it and thereby preventing the reduction of oxygen to water. PH_3 does not react in this way with other cytochromes of the electron transport chain, cytochrome c reduction resulting from the passage of electron from ubiquinol. Cytochrome b is not reduced in insects when mitochondria are inhibited by PH_3, from which it can be concluded that there is no movement of electrons from ubisemiquinone to cytochrome b. According to the Q-cycle hypothesis, cytochrome c reduction must have occurred only during the first turn of the cycle. As expected, both antimycin and myxothiazol result in cytochrome b reduction, antimycin by forward movement of electrons, myxothiazol by reverse movement as discussed in

Introduction section 1.2.3. Superoxide will not be generated at this segment of the respiratory chain unless ubisemiquinone is first formed by the partial oxidation of ubiquinol. An electron must be donated from ubiquinol to cytochrome c which can only occur if cytochrome c is in the oxidised state (Cadenas and Boveris 1980). This is the case after myxothiazol inhibition, however, cytochrome c is not reducible since myxothiazol blocks electron flow between ubiquinol and the Rieske iron-sulphur centre. Consequently, these spectral studies further support the conclusion drawn in the previous experiments concerned with release of H_2O_2; that when mitochondria are inhibited with myxothiazol or PH_3, the observed H_2O_2 is not formed from superoxide generated at ubiquinone.

4.1.3 Ubiquinone Concentration

Mitochondria from S. granarius contained 1.04±0.14nmol ubiquinone per mg protein which is a relatively low concentration when compared to other organisms. Turrens et al. (1982) concluded that ubisemiquinone autoxidation was responsible for H_2O_2 production in uninhibited porcine lung mitochondria supplemented with succinate. They correlated the low rate of extramitochondrial peroxide release, 0.045nmol H_2O_2 per mg protein with the low concentration of ubiquinone, 1.6±0.2 nmol per mg protein. Insect mitochondria contained even less ubiquinone than porcine lung mitochondria suggesting that it is unlikely to be a major site of $O_2^{\cdot-}$ generation in this species. Indeed, when uninhibited mitochondria were used, it

was observed that FAD-linked substrates, succinate and α-glycerophosphate resulted in a very low release of H_2O_2 which ceased within two minutes.

4.2 Effect of Phosphine on the Oxygen Defence System

Partially reduced oxygen species such as H_2O_2, $O_2^{\cdot-}$ and $HO^{\cdot}$ are produced *in vivo* as a byproduct of normal aerobic metabolism. They are reactive and cytotoxic and the cell contains a variety of enzymes and quenching molecules whose primary role is one of oxygen defence, These enzymes, including superoxide dismutases, catalase, glutathione peroxidases and other peroxidases as well as quenching molecules such as vitamin E, glutathione, ascorbic acid and β-carotene prevent these toxic species from damaging membranes, proteins, nucleic acids, DNA and etc. However, if the rate of production of superoxide common source of H_2O_2 and ultimately of $HO^{\cdot}$ is increased by ionizing radiation, by xenobiotics generating free radicals *in vivo* or by drugs capable of redox cycling, the defence system can be overwhelmed allowing the toxic species to attack and damage cellular constituents. Consequently, since it is hypothesised that inhibition of the electron transport chain by PH_3 *in vivo* results in increased release of H_2O_2 (presumably from $O_2^{\cdot-}$) any effect on the activity of the oxygen defence system after exposure is important.

Cyanide-(CN-)insensitive SOD activity was relatively high in this insect, approximately six units per mg protein in total tissue homogenate, and was located within the mitochond-

ria. Cyanide-sensitive SOD was located in the cytosol and had an activity of less than three units per mg protein. Experiments were not performed to establish the identity if the metal group(s) associated with these two isozymes, however, it is likely that the CN-sensitive form is CuZnSOD while the CN-insensitive form is probably MnSOD.

Three days after insects (PH_3-sensitive) were exposed to PH_3, CN-sensitive SOD activity increased two-fold to a level that was not significantly different from CN-insensitive SOD while CN-insensitive SOD activity remained constant. This increase in CN-sensitive SOD could have been due to increased synthesis, decreased degradation, enzyme activation or a combination of these processes. Further investigations could be made to establish the cause of the increased activity, however it seems likely that an induction of enzyme synthesis occurred. Increased rate of synthesis can be stimulated by the presence of an inducer which interacts with the gene resulting in an increased rate of transcription. A variety of different molecules can act as inducers, such as proteins, lipid peroxides and in this case, something that might be produced as a result of an increased levels of $O_2^{\cdot-}$, H_2O_2 or $OH^{\cdot}$. The inducer of CN-sensitive SOD was apparently unable to induce CN-insensitive SOD, a situation that has been described before by Steven and Autor (1977) and Hass and Massaro (1979b). The concentration of CN-sensitive SOD measured in this insect species may suggest that, under normal metabolic conditions there is little release of $O_2^{\cdot-}$ into the cytosol

from any of its biological sources; eg mitochondria, endoplasmic reticulum, plasma membrane, nuclear membrane and etc.. Superoxide, formed as a result of autoxidation of ubisemiquinone, that was released into the matrix of the mitochondria would come in immediate contact with CN-insensitive SOD (Boveris and Chance 1973, Turrens et al. 1982).

Nohl and Hegner (1978) suggested that as much as 20% of the $O_2^{\cdot-}$ produced by the electron transport chain escaped into the cytosol, presumably overwhelming mitochondrial CN-insensitive SOD. If this did occur then extramitochondrial release of $O_2^{\cdot-}$ might increase with increasing $O_2^{\cdot-}$ generation. However, release of $O_2^{\cdot-}$ from mitochondria is still controversial (Freeman and Crapo 1982). Fridovich (1974, 1975) stated that $O_2^{\cdot-}$ has a significant "lifetime" enabling it to diffuse from the site of formation. In the absence of protons (at physiological pH), $O_2^{\cdot-}$ may dissolve as the extremely stable potassium salt (Halliwell 1981) which can cross some biological membranes. Rumyantseva et al. (1979) reported the movement of $KO_2^{\cdot-}$ through an artificial liposome membrane. Certainly, if the extramitochondrial release of $O_2^{\cdot-}$ did increase it would be easier to explain the increase in CN-sensitive, cytosolic SOD, since, although it is unlikely to be an inducer, without such an increase in $O_2^{\cdot-}$ there is no 'need' for increased activity of this isozyme. It is not necessary to hypothesis an increase in cytosolic $O_2^{\cdot-}$, however. An increase in H_2O_2 combined with a decrease in catalase and peroxidase activity in PH_3-inhibited cells in vivo would probably result in a

significant increase in H_2O_2 reaching the cytosol. Hydroxyl radicals formed from the reaction of H_2O_2 and reduced transition metals, even in the absence of $O_2^{\cdot-}$ (Winterbourn 1979) could attack and damage cytosolic components. Since most, if not all extramitochondrial H_2O_2 is derived from the dismutation of $O_2^{\cdot-}$ (Dionisi et al. 1975, Boveris and Cadenas 1975), any products released from damaged membranes etc. would indicate a severe increase in $O_2^{\cdot-}$ the removal of which would be enhanced by increased SOD activity. Another effect of increased H_2O_2 is the inhibition of CuZnSOD. Hydrogen peroxide reduces enzyme-bound Cu^{2+} to Cu^{1+} and then reacts with Cu^{1+} to give $HO^{\cdot}$ which attacks adjacent active site histidine residues required for catalytic activity (Hodgson and Fridovich 1975, McMahon and Stern 1979).

In the present study there was an increase of about 38% in the total SOD activity after fumigation while CN-sensitive SOD activity was almost 100% higher. Fridovich (1979) indicated that even a small increase in SOD activity could be significant. A 50% increase in SOD activity occurring in rat lungs after in vivo exposure to 85% oxygen for seven days was found to increase their tolerance to 100% oxygen (Crapo and Tierney 1974). Induction of CN-sensitive SOD has been observed during aging in plant tissue (Boveris et al. 1978) and due to increased oxygen tension in rat liver cells (Hass and Massaro 1987ab) and rat lung (Crapo and McCord 1976). Increased CN-insensitive, MnSOD activity has been observed in bacteria (Gregory and Fridovich 1973, Hassan and Fridovich

1977), neonatal rat lung (Stevens and Autor 1977) and mouse heart (Oberley et al. 1987) in response to increased free radical production. There was no apparent connection between the site of radical generation and the identity of the induced isozyme.

One of the most interesting observations made in the present study was that insects selected for resistance to PH_3 showed no increase in CN-sensitive SOD activity after in vivo treatment. If it is assumed that an increase in SOD is due to induction at the gene level, it is possible that there is no production of an inducer in resistant insects. Since the inducer is likely to be a molecule produced as a byproduct of free radical attack it can be inferred that damage to cellular components did not occur to the same extent in resistant insects as it does in PH_3-sensitive insects. It was observed that peroxidase activity was significantly inhibited in both resistant and PH_3-sensitive insects while catalase was inhibited in sensitive insects and was already very low in resistant ones. This suggests that if extramitochondrial release of H_2O_2 was increased there would be a limited defence system available to remove it in both populations. Consequently, it is perhaps necessary to consider the actual production of H_2O_2. Is there less H_2O_2 released from mitochondria isolated from resistant insects than from sensitive ones? Unfortunately, no attempt was made to answer this question, but this presents a fascinating area for future research.

Glutathione peroxidase activity could not be measured in

this species, which is apparently true of other insect species (Smith and Shrift 1979), however, activity of another peroxidase enzyme was observed, using PH_3-sensitive insects, in both mitochondria (30%) and cytosol (70%). The identity of this peroxidase was not established, however it did not utilize glutathione as a co-substrate and was highly active in control preparations. Catalase has a relatively high Vmax, breaking down high concentrations of H_2O_2 rapidly (Chance et al. 1979) but is almost ineffective at decomposing low concentrations because of its low affinity (Halliwell 1981). Catalase is thought to be present in peroxisomes in vivo where it breaks down high concentrations of H_2O_2 produced there. Glutathione peroxidase has a lower Km (and a higher affinity) for H_2O_2 than catalase (Cohen and Hochstein 1963) and is important in removing low levels of peroxide. It is probable that the peroxidase present in insect cells has similar properties and would take on the role of detoxifying low levels of H_2O_2, such as those observed after PH_3-inhibition.

It should be noted that peroxidase could interfere with the catalase assay used in this study by consuming H_2O_2 if the co-substrate necessary for peroxidase activity was present in the homogenate. Other methods might be more suitable, such as a polarographic technique which follows the removal of oxygen from the medium as a measure of catalase activity (Del Rio et al. 1977).

It was observed that the peroxidase was unaffected by PH_3 in vitro. However its activity was strongly inhibited (88%)

36-hours after PH_3-sensitive insects were exposed to a low dose of PH_3 *in vivo*. Catalase was inhibited by 60%, after the same dose. Hypophosphite, the major breakdown product of PH_3 (Robinson and Bond 1972) is known to inhibit catalase (Nicholls 1961) and could be responsible for the decrease in activity. Hobbs (1984) reported the time dependent inhibition of insect catalase by a PH_3-saturated buffer. Alternatively, O_2^- itself is also capable of inhibiting this enzyme as well as glutathione peroxidase (Kono and Fridovich 1982). A drastic decrease in H_2O_2 scavenging enzymes could result in the mitochondrial release of damaging concentrations of H_2O_2, however, tissue damage was obviously not excessive in this experiment since only 30% of the insects died. Price *et al.* (1982) and Price and Dance 1983) suggested that a dramatic inhibition of catalase alone was responsible for insect toxicity. They observed that PH_3-resistant strains of the lesser grain borer (*Rhyzopertha dominica*) had higher catalase activity than susceptible. In a more recent article, however, they found that insects fed with 3-amino 1,2,4 triazole (3-AT), which reduced catalase activity by more than 50%, showed no increased mortality before or after exposure to PH_3, suggesting that decreased catalase activity was not important in toxicity after all (Price and Walter 1987). Peroxidase activity, which was not measured, was probably not affected by 3-AT, since this inhibitor reacts with the protein and not the heme of catalase (Nicholls 1961). Peroxidase may have compensated for the decreased catalase levels if it had been

induced over the two-week period that insects were fed with 3-AT-treated wheat.

In this study, after PH_3-sensitive insects were exposed to PH_3 (LD_{30}), only 34% of the intramitochondrial peroxidase was inhibited compared to 68% of the cytosolic peroxidase and this may have left enough peroxidase activity to remove the H_2O_2 produced in the majority of insects. At higher concentrations of PH_3 generation of $O_2^{\cdot-}$ could be such that H_2O_2 levels in the mitochondria overwhelm the remaining peroxidase resulting in a significant release of H_2O_2. It is not known why the intramitochondrial form of peroxidase should be less susceptible to PH_3.

It was observed that insects selected for resistance to PH_3 had the same peroxidase activity and significantly less catalase activity than PH_3-sensitive insects. Peroxidase activity was decreased by 48%, three-days after resistant insects were tr· ated with PH_3 (LD_{30}). Inhibition of peroxidase was, therefore, significantly less in this population than in PH_3-sensitive insects which showed a decrease of 65% after the same treatment. This difference could be very important when considering the adaptations of resistant insects to PH_3. The cellular distribution of peroxidase was not investigated in the present study using resistant insects, however, if a high proportion of peroxidase was located in the mitochondria in resistant insects, this might explain the observed difference in inhibition.

Catalase activity was significantly higher in PH_3-

sensitive insects than resistant ones. After fumigation (LD_{30}) catalase activity was inhibited in PH_3-sensitive insects to a level that was not significantly different from the level observed in uninhibited resistant insects, while activity in resistant insects remained unchanged treatment. This is difficult to explain since it is expected that PH_3 would inhibit catalase activity equally in both populations. The finding that catalase activity was the same in inhibited, PH_3-sensitive insects as it is in resistant insects may be important. It seems to suggest that there is a certain proportion of enzyme that is insensitive to attack by PH_3. Jones and Masters (1972) and Masters (1982) argue convincingly that there are at least two forms of catalase, a peroxisomal form (cat-1) that migrates to the anode during electrophoresis and a cytosolic form (cat-2,3,4,5) which migrates toward the cathode and is an epigenetic modification of cat-1. They suggest that catalase is synthesised in the cytosol and is rapidly incorporated into the peroxisomal membrane. It then moves into the peroxisome as an active form that is highly soluble. Catalase is later released from the peroxisomes into the cytosol (the extraparticulate cytoplasmic or EPC fraction), where it also functions as an active enzyme albeit with a modified structure, until it is degraded. Structural modifications are such that, in mouse liver, the peroxisomal form is depressed by 80% by the inhibitor, 3-AT while the activity of the EPC-form is only reduced by 49% (Jones and Masters 1972). These authors suggest that, since the soluble

catalase activity is shared equally between peroxisomes and cytosol in this species, the peroxisomal catalase must have been altered to a form that is less sensitive to 3-AT on release into the cytoplasm. It is known that PH_3 inhibits by reacting with the heme moiety of catalase (Nicholls 1961) and that 3-AT binds covalently with histidine-74 of catalase (Margoliash et al. 1960). However, despite these different modes of inhibition, it seems likely that a change in conformation of the type alluded to by Masters (1982) could also alter the binding potential of PH_3 making it more or less able to react with catalase. Resistant insects have less catalase activity than PH_3-sensitive ones and if it is hypothesised that there is a lower proportion of the EPC-form, possibly by virtue of an increased rate of degradation, and that it is this form that is most susceptible to attack by PH, observed differences in catalase activity could be explained. The differences observed in the present study may appear unexpectedly large, however, Masters (1982) stressed that there are distinct variations in localization, turnover rate and number of forms of catalase between homologous tissues in different species, suggesting that some of the biological characteristics of this enzyme are not highly conserved. This ability to change according to environmental pressures may account for the differences observed between PH_3-sensitive and resistant insects, although at this stage it is impossible to say how such a decrease in resistant insects could be a beneficial adaptation to PH_3 fumigation. If peroxidase is more important

than catalase in removing a small increase in H_2O_2 such as that resulting from PH_3-treatment, then the higher activity of peroxidase remaining in resistant insects, described previously, could be sufficient to remove excess H_2O_2 before it is oxidised to HO·.

Catalase and peroxidase levels in PH_3-sensitive insects increased gradually with time after PH_3 exposure. Eleven days after treatment, catalase activity had increased from 60% inhibition to a level that was equivalent to controls after 15 days. Peroxidase activity increased from 88% less than controls 11 days after fumigation to 27% less after 15 days. This increase is probably too slow to be accounted for by resynthesis, which in mammals only takes 3-4 days for catalase (Price et al. 1961), and may indicate that there has been damage to the genetic material required for resynthesis. It would be interesting to repeat this experiment using resistant insects.

Glutathione was the only non-enzymatic quenching molecule investigated in this study. Reduced glutathione (GSH) interacts with $O_2^{\cdot-}$, HO· and singlet oxygen, as well as inorganic and organic peroxides non-enzymatically, producing oxidised glutathione (GSSG) which is then reduced by glutathione reductase (Forman and Fisher 1981, Meister 1983). The enzyme glutathione peroxidase increases the rate of these reactions dramatically, but is not present in any of the insects so far studied (Smith and Shrift 1969). Increased levels of GSH were observed after paraquat, a radical forming

herbicide; diamide, an oxidant of glutathione or diethyldithiocarbamate, a copper chelator were administered to houseflies (Allen et al. 1984ab, Sohal et al. 1984). These authors suggested that glutathione has a major role in radical defence, even in the absence of glutathione peroxidase. Gutathione confers protection to proteins because it is more accessible to free radicals than enzyme -SH groups (Halliwell 1981). In this study it was found that whole insect homogenates from PH_3-sensitive insects contained an average of 0.1mg GSH per mg protein and 0.54mg GSSG per mg protein which was not significantly different from concentrations measured after fumigation. This indicated that, glutathione was not induced as a response to oxidative stress.

In summary then, the inhibition of cytochrome c oxidase by PH_3 and the resultant release of H_2O_2 combined with decreased catalase and peroxidase activity in PH_3-sensitive insects could result in the accumulation of H_2O_2 and the formation of $HO^{\cdot}$. Attack by the highly reactive hydroxyl radical could result in chemical modifications of proteins, lipids, carbohydrates and nucleotides which could lead to serious metabolic and cellular damage (Slater 1984). The increased SOD activity observed in PH_3-sensitive insects after fumigation reaffirms the hypothesis that there is an increase in superoxide generation following treatment. It has been suggested by other researchers that an increase in SOD activity without an accompanying increase in H_2O_2 scavenging enzymes, could be actually harmful to the cell (Koppenol and

Butler 1977, Mavelli et al. 1981).

Insects selected for resistance to PH_3 were found to have a number of modifications: They have significantly less catalase activity than sensitive insects which is apparently unaffected by PH_3-treatment; there is 17% more peroxidase activity remaining in resistant than PH_3-sensitive insects after fumigation; and there is no observed increase in CN-sensitive SOD activity following treatment. It is difficult to create a model for resistance from these data, suffice it to say that oxygen-derived free radicals do appear to play a part in the scheme. The levels of peroxidase may prove to be the key to resistance in this selected population.

4.3 Effect of Free Radicals

If reactive free radicals are produced in vivo after PH_3 fumigation, in concentrations sufficient to overcome the oxygen defence system it is possible that metabolic and cellular damage will occur. It has been hypothesised that an iron-catalysed Haber-Weiss reaction occurs when $O_2^{\cdot-}$ reduces Fe^{3+}-complexes to Fe^{2+}-complexes which then react with H_2O_2 to give the highly reactive hydroxyl radical, $HO^{\cdot}$ (McCord and Day 1978). Reductants such as ascorbate and probably hypophosphite, a major breakdown product of phosphine, can reduce Fe^{3+} suggesting that production of $OH^{\cdot}$ can occur in the absence of $O_2^{\cdot-}$ (Winterbourn 1979). Free radicals are very unstable and cannot be measured directly in vivo. However it is possible to assess tissue damage resulting from free radical attack,

giving an indirect measure of free radical concentration. In this study, damage to insect tissue was observed by measuring the extent of lipid peroxidation, changes in the activity of membrane-bound, oligomycin-sensitive, Mg^{2+}-dependent H+-ATPase and changes in sulphydryl group content.

4.3.1 Unsaturated to Saturated Fatty Acid Ratio

Unsaturated bonds of membrane fatty acids react with unstable free radicals and undergo peroxidation. Once initiated this process becomes an autocatalytic chain reaction ultimately destroying membrane integrity. Polyunsaturated fatty acids (PUFA's) are essential for maintaining membrane fluidity and the activity of membrane-bound proteins (Fourcans and Jain 1974). Lipid peroxidation is only the beginning of a series of damaging reactions which yield lipid peroxides, lipid alcohols and aldehydic by-products resulting from PUFA breakdown (Mead *et al.* 1979). Slater (1984) stressed the importance of following the disappearance of PUFA's from membranes as a technique for establishing the extent of tissue damage. Other products utilized to measure lipid peroxidation are readily metabolised and give an erroneous negative result. In this study the ratio of PUFA's to saturated fatty acids was measured as an index of structural change (Nohl and Hegner 1978).

After *in vivo* exposure to PH_3, mitochondria isolated from insect tissue had no significant decrease in PUFA content compared to controls. Alternatively analysis of microsomal

membranes revealed that exposed insects had a significantly lower PUFA to saturated fatty acid ratio. It should be noted that microsomes are artifactual debris formed upon homogenization by the disruption of the cell plasma membrane and the endoplasmic reticulum (Gutteridge 1987). With PH_3 (LD_{60}) the PUFA concentration in microsomes was on average 12.3% lower than in control membranes. When an LD_{80} treatment was used the results were not repeatable varying from a 13.8-43.0% (with an average of 24%) reduction in PUFA's. These inconsistencies observed with the LD_{80} dose might have been because of mitochondrial contamination brought about by slight changes in the isolation procedure. The initial homogenization with a pestle and mortar is one of extreme importance, if the grinding was performed too vigorously, mitochondria if broken into small vesicles, could precipitate with microsomes. Thus mitochondria, which are apparently unaffected by PH_3-treatment, could mask changes in microsomal PUFA content to varying degrees. However, this variability was only observed in insects treated with an LD_{80} dose of PH_3 while all the control preparations, and those from insects exposed to LD_{60} levels had fairly consistent PUFA to saturated fatty acid ratios.

It is also possible that the variability in PUFA concentration in treated insects (LD_{80}) was due to other factors brought on by approaching death even though the insects used were still alive. Slater (1984) stated that in <u>in vivo</u> situations it is impossible to be certain if lipid peroxidation is a primary cause of injury or a secondary consequence

of damage that has already occurred, but either way he felt that it was still important in the injury process.

Comparison of fatty acids extracted from mitochondrial membranes of three- and 23-month-old rats demonstrated a decrease in PUFA content of almost 30% in the older animals which correlated with increased steady state concentrations of H_2O_2 and $O_2^{\cdot-}$ from intact mitochondria (Nohl and Hegner 1978). It is difficult to explain the lack of mitochondrial peroxidation after <u>in vivo</u> exposure to PH_3 when it has been hypothesised that H_2O_2 ($O_2^{\cdot-}$) is produced from this organelle after the respiratory chain has been inhibited by PH_3. Microsomal membranes were found to have 10.5% more PUFA's ($C_{18:2}$ + $C_{18:3}$) with respect to the saturated fatty acid ($C_{14:0}$ + $C_{16:0}$) content than mitochondria and this would render them more susceptible to radical attack. Studies on the inner mitochondrial membrane of mammalian liver revealed that they contain a very high proportion of proteins, approximately 80%, compared to only 20% of lipid (Sjostrand and Barajas 1970). A relatively high protein content could account for the observed lack of lipid peroxidation in mitochondria, since by occupying so much of the membrane, the proteins would be more accessible to oxidation than the lipids.

It is possible that the mechanisms responsible for repair of membrane damage are more efficient in the mitochondria. The concentrations of α-tocopherol and ascorbate were not investigated but these antioxidants do remove phospholipid peroxides from membranes preventing free radical generation

from the lipid hydroperoxides. Glutathione levels were measured however, and no differences were seen between concentrations of reduced and oxidised forms before and after PH_3 fumigation. It may be significant that glutathione levels were measured in whole insect extract and not in individual organelles which may have shown some differences. Glutathione peroxidase catalyses the removal of damaged lipids (Ursini and Bindoli 1987) and it is possible that the peroxidase present in insect mitochondria has a similar activity. It was found that 68% of the cytosolically-located peroxidase was inhibited by PH_3, compared to only 34% of the mitochondrial enzyme, which may have provided sufficient protection against H_2O_2 in the mitochondria, as mentioned previously.

Another reason why microsomal and not mitochondrial lipids were peroxidised, which has not been examined in this study, is the possibility that in vivo free radicals may be generated from components of the non-phosphorylating electron transport chain of the endoplasmic reticulum after exposure to PH_3. Phosphine inhibits a variety of heme-containing enzymes (Kashi 1974) and one of the microsomal electron transport chains contain cytochrome P_{450} which has a heme moiety, as well as the flavoprotein NADPH-cytochrome P_{450} reductase, both of which are thought to be sources of H_2O_2 (Chance et al. 1979). Production of $O_2^{\cdot-}$ may occur through autoxidation of the partially reduced flavin co-factor or because electrons are donated to molecular oxygen from the cytochrome P_{450}-substrate complex. Porcine lung microsomes

exposed to 100% oxygen produced 19.7nmol H_2O_2 per gm tissue when supplied with suitable substrate, a six-fold higher rate than mitochondria under the same conditions (Turrens et al. 1982).

When in vitro free radical-generating systems were used there were no significant differences observed between the fatty acid composition of control and exposed membranes (mitochondrial or microsomal). A decrease in PUFA content was noticed in both mitochondria and microsomes after in vitro exposure in control and treated samples which was probably due to peroxidation of PUFA's as a result of exposure to air for 1.5 hours. The lack of any differences between control and treated membranes could be due to catalase contamination of mitochondria and microsomes which might have removed exogenously added H_2O_2. The presence of catalase in the system could also explain the lack of diene conjugation (Gutteridge et al. 1983). The choice of a free radical-generating system is complex and an incorrect balance of components could be responsible for the lack of success. Halliwell and Gutteridge (1981) demonstrated that concentrations of EDTA as low as 0.1-0.2mM inhibited radical formation. Autoxidation of the Fe^{2+}-EDTA complex at pH=7.4 is much faster than Fe^{2+} alone resulting in the formation of Fe^{3+}-EDTA, a complex that is an ineffective generator of HO·. Too much Fe^{2+} (10μM) inhibits HO· production (Schneider et al. 1964), while the absolute ratio of Fe^{3+} to Fe^{2+} was considered to be the most important factor by Braughler et al. (1986). Minotti and Aust (1986, 1987) also

hypothesised that both ferrous and ferric iron were required for peroxidation and that all factors affecting redox activity must be considered including, the nature of iron chelators, pH and the concentration of oxidants and reductants used. A variety of reductants could be used with ferric iron, providing that an effective concentration of ferrous-ferric complex was maintained. Catalase can actually stimulate lipid peroxidation in situations where H_2O_2 concentration is sufficient to oxidise iron before initiation occurs.

An enzymatic free radical-generating system could have been used to demonstrate that these membranes are vulnerable to lipid peroxidation and may have been more successful than the non-enzymatic techniques used in this study. One example of such a system, described by Kellogg and Fridovich (1975), uses Fe^{2+}-(hypoxanthine/xanthine). The oxidation of hypoxanthine to uric acid generates both $O_2^{\cdot-}$ and H_2O_2 which can react together in the presence of Fe^{2+} to form $HO^{\cdot}$.

Another result of membrane damage which has not been considered and which probably causes the most devastating effects on cell integrity is an increased permeability to Ca^{2+} that occurs after peroxidation. Permeability changes and a decrease in ATP levels resulting from inhibition of the electron transport chain both effect the mitochondrial regulation of cytosolic Ca^{2+} leading to a sustained increase in Ca^{2+} in the cytosol (Smith et al. 1984). The Calcium ion is highly biologically active and is usually maintained at low levels in the cytosol by specific translocases in the plasma

membrane, endoplasmic reticulum and mitochondrial membrane. The thiol groups of endoplasmic reticular Ca^{2+}-ATPase, which sequesters Ca^{2+} from the cytosol are highly susceptible to oxidative damage and if this enzyme, or others like it were inhibited by $HO^{\cdot}$, the process that maintains Ca^{2+} homeostasis would be further disrupted. One of the major roles of Ca^{2+} is to regulate the exoskeletal structure. A rise in Ca^{2+} concentration in the cytosol causes plasma membrane blebbing by altering the structure of the microfilament system, resulting in changes in cellular function and eventually cell death (Smith et al. 1984).

4.3.2 Sulphydryl Group Content

It was observed that mitochondria from PH_3-sensitive insects exposed to PH_3 in vivo contained 25.4% fewer sulphydryl groups than control mitochondria. This includes protein thiol groups, both membrane-bound and soluble, and non-protein thiols such as glutathione. Protein thiol groups are readily oxidised by free radicals, including lipid peroxy radicals, leading to profound changes in enzyme activity (Finley and Lundin 1980, Slater 1984). Certain enzymes, for example papain and glyceraldehyde 3-phosphate dehydrogenase, depend on the thiol containing amino acid cysteine for activity, consequently, any event that results in oxidation of this residue will inhibit the enzyme. A major cause of enzyme inhibition is the formation of aggregations resulting from the cross-linking of sulphydryl groups.

Although there is no glutathione peroxidase activity in this insect species, they do possess the thiol-containing tripeptide glutathione (GSH), which reduces free radicals, lipid peroxides and radical-oxidised proteins non-enzymatically. Glutathione is also used by thiol transferases that maintain protein sulphydryl groups in the reduced state (Guarnieri et al. 1980). The possibility that the observed large decrease in sulphydryl group content was due to glutathione oxidation was investigated by quantifying the concentrations of glutathione in insect tissue. Vladimirov et al. (1980) reported a 25% decrease in -SH content following UV irradiation of rat erythrocyte membranes which correlated with an accumulation of the lipid peroxidation product malondialdehyde. They apparently attributed this decrease to glutathione oxidation. In the present study no difference in glutathione concentration was observed between control and exposed insects suggesting that this was not responsible for the measured decrease in sulphydryl content. Guarnieri et al. (1980) studying the effect of hypoxia followed by reoxygenation on perfused rat heart observed that the cellular content of acid soluble thiol groups, insoluble thiol groups and reduced glutathione were all decreased, while oxidised glutathione remained unchanged. They measured total thiol concentration following the same procedure used in the present study and non-protein thiols by denaturing homogenates with 50% trichloroacetic acid (TCA) before the assay. Guarnieri et al. (1980) observed that protein thiol content decreased

by approximately 25% compared to control values. This demonstrates that the large changes in sulphydryl group content observed in the present study could have been due to protein thiol oxidation.

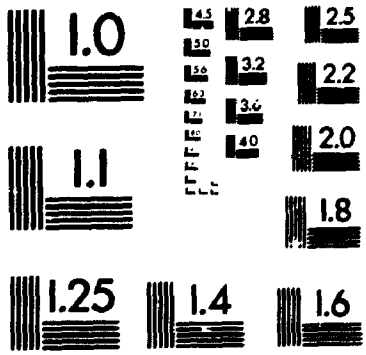
4.3.3 Oligomycin-Sensitive H+-ATPase

The effect of PH_3 fumigation on oligomycin-sensitive, Mg^{2+}-dependent H+-ATPase was investigated by measuring enzyme activity in terms of phosphorous production after incubation with ATP for 20 minutes. ATPase is a complex of proteins making up two modules, the transmembrane proton pore (F_0) and the enzymatically active headpiece (F_1). ATPase activity could be affected by peroxidation of lipids surrounding the F_0-module and by free radical attack on aromatic and sulphur-containing residues of both modules.

ATPase activity was significantly higher (11%) in insects exposed to PH_3 *in vivo*. Hostman and Racker (1970) reported a five- to 10-fold increase in ATPase activity from bovine heart after trypsin digestion and an endogenous regulatory protein that inhibits the enzyme in the presence of Mg^{2+} was isolated. The dependence of the regulatory protein on Mg^{2+} was investigated by Chefurka (1981b) using house fly mitochondria supplemented with various concentrations of EDTA. At low concentrations EDTA sequesters Mg^{2+}, thereby inhibiting the regulatory protein. A peak of enzyme activity occurred at 2mM EDTA. At higher concentrations of EDTA, ATPase activity was decreased presumably because EDTA sequesters most of the

available Mg^{2+}, some of which is essential for the functioning of ATPase itself. The observed increase in ATPase activity after *in vivo* exposure could result from direct free radical damage to the inhibitor protein. This protein contains five histidine, one tyrosine and two phenylalanine residues (Frangione *et al.* 1981), all of which are targets of free radical attack. In the bacterium *E. coli* the inhibitor is thought to be located between F_1 and F_0 (Pedersen *et al.* 1980), its location in insects has not been established.

Senior (1973) observed that a change in F_1-ATPase conformation led to increased ATPase activity. This could also be an explanation for the increase in enzyme activity observed in this study. The F_1-ATPase complex is made up of five individual subunits, α_3 β_3 γ δ and ϵ, all of which contain methionine residues (10,10,6,2 and 1 respectively) (Walker *et al.* 1985). Senior (1973) reported that there are also 12 half cystine residues per molecular weight of 360,000 of which eight are cysteines and four are involved in intersubunit disulphide bonds between γ and ϵ, and an intrachain disulphide bond in the α-subunit. Walker *et al.* (1985) suggested that Senior may have overestimated the number of half cystines, however both methionine and cysteine are sulphur-containing amino acids which are prone to oxidation by free radicals. If the cysteine residues were oxidised new disulphide bridges could be formed resulting in changes in conformation that could lead to either an increase or a decrease in enzyme activity.



Disinhibition by alteration of the regulatory protein or a change in the conformation of ATPase itself may also explain the five-fold increase in activity seen after *in vitro* experiments. It is possible that incubation of the diluted enzyme for 1.5 hours at 25°C damaged the regulatory protein or its binding site. These mitochondria were isolated and incubated in a medium containing 1mM EDTA which would probably sequester sufficient Mg^{2+} to prevent the protein binding to the enzyme, however the assay was performed in a buffer containing Mg^{2+} which should reactivate the inhibitor.

Conversely, there was a significant decrease in ATPase activity from mitochondria exposed to a free radical-generating system compared to controls. Assuming that the generator did not actually protect the inhibitor, it seems likely that the decrease is due to the effect of free radical attack. Several different reasons for a decrease in enzyme activity have been postulated (Vladimirov *et al*. 1980). If lipid peroxidation occurred there would be a concomitant decrease in PUFA's in the membrane leading to a decrease in membrane fluidity. Such a modification of the physical state of the bilayer results in increased membrane permeability to calcium ions, allowing calcium to leave the matrix and protons to enter, thereby dissipating the proton gradient required for ATPase activity. Measurement of changes in membrane fluidity using a probe such as the fluorescene of 1-anilino-8-naphthalene sulfonate (ANS) would provide useful information. However, in the present study, the observed decrease in ATPase

activity was not correlated with a decrease in PUFA content. It is possible that peroxidation of lipids was highly localised and of insufficient magnitude to register as a difference in unsaturated to saturated fatty acid ratio.

Other possible explanations for decreased enzyme activity are a change in the conformation of the enzyme resulting from cysteine oxidation, as mentioned above and the peroxidation of aromatic residues. The F_1-complex contains many aromatic amino acids; 37 tyrosine, 45 phenylalanine and 22 histidine residues (Walker et al. 1985). It was observed that the reaction of 4-chloro-7-nitrobenzofuran with a single tyrosine residue of the β-subunit abolishes ATPase activity (Ferguson et al. 1975). This indicates the importance of this amino acid in enzyme function, and attack by free radicals could have a similar devastating effect.

4.4 Suggested Strategy for Application of Phosphine

A working hypothesis for the mode of action of phosphine, based on the data obtained in this study, is that oxygen-derived free radicals generated in vivo from the inhibited respiratory chain attack cellular components and that the cumulative effect of this damage could be responsible, at least in part, for insect mortality.

This suggests a new strategy for the use of PH_3. It consists of two steps: first, by exposing insects to a low partial pressure of oxygen, the activity of the free radical

scavenging systems would be reduced; second, fumigation with PH_3 at a partial pressure of oxygen higher than atmospheric should result in the accumulation of toxic products derived from superoxide radicals. The duration required at each step would have to be investigated , however it appears that this technique might reduce the concentration of PH_3 required thereby decreasing its contribution to environmental pollution.

4.5 Future Research

This research has not provided a final answer to the question of how phosphine kills insects, but it has opened the door to a new way of looking at this old problem. The effect that PH_3 has on the enzymes involved in oxygen defence indicates that oxygen-derived free radicals may mediate cellular damage after treatment. Results obtained using insects selected for resistance are not as easy to interpret however. It appears that these insects do not have the same free radical challenge as PH_3-sensitive insects; catalase activity is significantly lower in resistant insects and there is no induction of cyanide-sensitive SOD after fumigation. In the light of these results there are several experiments that could be performed to clarify the situation:

1. To establish whether or not mitochondria isolated from resistant insects release H_2O_2 when inhibited by PH_3.

2. To find out if induction at the gene level is responsible for the observed increase in CN-sensitive SOD activity in PH_3-sensitive insects after fumigation. A radioactively-labelled nucleic acid probe is available for mammalian CuZnSOD RNA which might have a sufficiently conserved structure to enable it to interact with messenger-RNA coding for the insect SOD isozyme.

3. The effect of PH_3 fumigation over time on catalase, peroxidase and superoxide dismutase could be measured using PH_3-resistant insects. In the present study, measurements were only made three-days after exposure.

4. Significantly more peroxidase activity was inhibited in PH_3-sensitive insects after fumigation that in resistant insects. It would be interesting to investigate the sub-cellular distribution of peroxidase in resistant insects since it was observed that mitochondrial peroxidase is less susceptible to inhibition than the cytosolically-located form.

5. To isolate and characterize the peroxidase detected in these insects.

6. To observe whether or not fumigation of resistant insects results in free radical-mediated damage of cellular

components, for example if there is a decrease in the sulphydryl group content or peroxidation of microsomal lipids.

7. To identify other enzymes that are affected by free radical attack. When PH_3-sensitive insects were fumigated there was no change in glutathione levels while the sulphydryl group content of mitochondria was significantly decreased suggesting that cysteine residues of proteins, either in the matrix or the membrane itself, were oxidised. It is unlikely that the effect on H^+-ATPase or its regulatory protein accounts for all the observed decrease and by testing the activity of other enzymes it might be possible to distinguish other targets for attack.

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