DOKUZ EYLÜL UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES

THE EFFECT OF BROMOPROPYLATE ORGANOPHOSPHATE PESTICIDE ON THE SOME METABOLITES AND MITOCHONDRIAL ELECTRON TRANSPORT ENZYMES IN TRICHODERMA HARZIANUM

by
Zehra TAVŞAN

July, 2011
İZMİR

THE EFFECT OF BROMOPROPYLATE ORGANOPHOSPHATE PESTICIDE ON THE SOME METABOLITES AND MITOCHONDRIAL ELECTRON TRANSPORT ENZYMES IN TRICHODERMA HARZIANUM

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by

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M.Sc THESIS EXAMINATION RESULT FORM

We have read the thesis entitled **"THE EFFECT OF BROMOPROPYLATE ORGANOPHOSPHATE PESTICIDE ON THE SOME METABOLITES AND MITOCHONDRIAL ELECTRON TRANSPORT ENZYMES IN *TRICHODERMA HARZIANUM*"** completed by **ZEHRA TAVSAN** under supervision of **ASSOC. PROF. DR. HULYA AYAR KAYALI** and we certify that in our opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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ABSTRACT

In order to identify the aspects of organophosphate pesticide bromopropylate concentrations effect on electron transport system (ETS) enzymes; succinate dehydrogenase (SDH) and cytochrome c oxidase (COX), tricarboxylic acid (TCA) cycle metabolites; citrate, α-ketoglutarate and fumarate and glycolysis last metabolite pyruvate level as well as lipid peroxidation (LPO) levels of eukaryote model, *Trichoderma harzianum* were investigated with respect to incubation period. The mitochondrial respiratory system is one of the most important systems in the central metabolism and includes glycolysis, TCA and ETS.

In the present study, the SDH activities of *T. harzianum* increased with the rises in the bromopropylate concentration up to 2.5 mg/L. This may be inhibition effect of bromopropylate on the SDH activity. In contrast to SDH, COX activities increased respect to increases in the bromopropylate concentration by showing induction effect of the pesticides on COX enzyme. As an end product of glycolysis, pyruvate levels increased markedly up to 2.5 mg/L of bromopropylate. The decreases in pyruvate levels higher than 2.5 ppm bromopropylate may suggest that *T. harzianum* metabolism towards to pentose phosphate pathway to generate NADPH. Although the intermediate of the TCA cycle, the intracellular citrate levels rose with the increases in the bromopropylate concentration up to 7.5 mg/L, the other TCA cycle intermediates, and intracellular α-ketoglutarate and fumarate levels increased up to 5.0 mg/L of bromopropylate. However, the variations of ATP, ADP and AMP levels showed positive correlation with the α-ketoglutarate, fumarate levels. The results may indicate that the pesticides have been reported to influence intracellular nucleotide phosphate concentration with respect to TCA cycle and oxygen uptake. As an indicator of membrane damage, LPO levels enchanced with respect to

increases in bromopropylate concentrations and incubation periods which is probably due to leakage of electrons from ETS.

Keyword: Bromopropylate, *Trichoderma harzianum*, ETS enzymes, TCA cycle, Glycolysis, LPO.

TRİCHODERMA HARZİANUM DA ORGANOFOSFATLI PESTİSİTLERDEN BROMOPROPİLAT IN BAZI METABOLİTLER VE MİTOKONDRİYEL ELEKTRON TRANSPORT ENZİMLERİ ÜZERİNE ETKİSİ

ÖZ

Gerçekleştirilen çalışmada; organofosfatlı pestisitlerden bromopropilatın farklı derişimlerinin ökaryotik model olan *Trichoderma harzinum* suşunda elektron transport sistemi (ETS) enzimlerinden süksinat dehidrogenaz (SDH) ve sitokrom c oksidaz (COX) aktiviteleri; glikolizis son metaboliti pürivat; sitrat çevrimi (TCA) ara ürünlerinden sitrat, α-ketoglutarat ve fumarat; ve membran hasarının göstergesi olan lipid peroksidasyon (LPO) seviyeleri üzerine etkileri inkübasyon periyodu süresince incelenmiştir. Metabolizmada temel sistemlerden biri olan mitokondriyel solunum sistemi: glikolizis, TCA ve ETS olmak üzere 3 ana çevrim üzerinden yürümektedir.

Sunulan tez çalışmasının sonuçları: *T. harzianum* da, SDH aktiviteleri besi ortamındaki bromopropilatın 2,5 mg/L e kadar artışıyla hızlı artışlar göstermiştir. Bu bromopropilatın SDH aktivitesi üzerindeki inhibisyon etkisinden kaynaklanabilir. SDH ın aksine, pestisitlerin COX enzimini indükleme etkisi nedeniyle, COX aktivitesi bromopropilatın artan derişimiyle artış göstermiştir. Glikolizisin son ürünü olan pruvat düzeyleri ise 2,5 mg/L bromopropilata kadar artış göstermiştir. 2,5 mg/L bromopropilat derişimi üzerinde pruvat düzeylerinin düşüşünün sebebi, *T. harzianum* metabolizmasının NADPH üretimini tetiklemek amacıyla pentoz fosfat yoluna yönelmesinden kaynaklanabilir. TCA çevrimi ara ürünü olan hücre içi sitrat düzeyi 7,5 mg/L a kadar artan bromopropilat derişimi ile artarken diğer TCA metabolitleri olan hücre içi α-ketoglutarat ve fumarat düzeyleri 5,0 mg/L bromopropilata kadar artış göstermiştir. Bununla birlikte; ATP, ADP ve AMP düzeyleri, α-ketoglutarat ve fumarat düzeyleri ile pozitif korelasyon göstermektedir. Bu sonuçlar, çalışılan pestisitin TCA çevrimi ve oksijen alımına bağlı olarak hücre içi nükleotid fosfat derişimini etkilemesiyle açıklanabilir. Membran hasarının göstergesi olan LPO

düzeyleri, bromopropilat derişimine ve inkübasyon süresine bağımlı ETS deki elektron kaçaklarının artmış olabileceğini göstermektedir.

Anahtar Sözcükler: Bromopropilat, *Trichoderma harzianum*, ETS enzimleri, TCA Çevrimi, Glikolizis, LPO.

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CHAPTER ONE

INTRODUCTION

Every day, people are exposed simultaneously to various xenobiotics through the different ways. Xenobiotics are low molecular weight (below ~1000 Da) chemical compounds foreign to the body. These are drugs, food constituens, food additives such as flavors and coloring agents, cosmotics, doping agents, hallucinogens such as ecstasy, LSD and cocaine, social stimulants such as nicotine and alcohol, industrial and synthetic compounds such as fertilizers, pesticides and heavy metals. Nowadays, pesticides, one of these xenobiotics, have contributed to the increase of product yields and quality in agriculture by controlling pests and diseases (Bhatnagar, 2001; Rekha, & Prasad, 2006). Because of growing population, to increase world food necessity was needed. A way to increase crop productivity is effective pest management because more than 45% of annual food production is lost to pest infestation. Such substances are applied directly to soil or sprayed over crop fields and hence are released directly to the environment. In our country, 1483 formulations which contain 346 different pesticides, have been using. But, less than 1% of pesticides reaches to target organism, because they are not specific chemicals. In addition of this, uncontrolled use of pesticides brought risks as a consequence of their long residual life-time and accumulation in food chains**.** Therefore, the residues of pesticides left after treatment may penetrate plant tissues and appear in the pulp and juice of fruits and vegetables, although their concentrations are, in general, lower than those observed in whole fruit (Council Directive 91/414/EEC, 1991; Albero, Sa´ nchez-Brunete & Tadeo, 2005). Pesticides can also remain as residue in foodstuffs after their application and can spread in the environment (soils, surface and underground waters) and have been contaminated non-target organisms such as human and animals. Unfortunately, the presence of pesticides which found in environmental media and food is one important concern for human, due to their possible long-term adverse health effects (Camino-Sa´nchez et al., 2011) and non-target organisms are exposed by different routes such as inhalation, ingestion and dermal contact (Rekha et al., 2006). According to the Food and Agriculture

Organization (FAO) inventory (FAO, 2001), more than 500000 tons of unused and obsolete pesticides are threatening the environment and public health in many countries. Increased interest in environmental pollutant-induced oxidative stress, and knowledge of the interactions between free radicals, related oxidants, and cellular systems, has increased the profile of reactive oxygen species (ROS) in reproductive toxicology (Oakes et al., 2003). In recent years, several studies have indicated that increase of acute and chronic health problems are associated with exposure to pesticides. Cancer, chronic kidney diseases, suppression of the immune system, sterility among males and females, endocrine disorders, neurological and behavioral disorders have been attributed to chronic pesticide poisoning (Agnihotri, 1999). Government agencies and international organizations limit the amount of pesticides in food establishing maximum residue limits (MRLs), with the aim of protecting consumers' health. Several European Union (EU) directives have set different MRLs for pesticide residues in vegetables and fruits at the low microgram per kilo level (Camino-Sa´nchez et al., 2011). Many scientists have estimated the pesticide residues (PRs) in various fruits including banana, mango, apple, peach, watermelon, melon, grape, orange, lemon, pear, pineapple, strawberry, raspberry, kiwi fruit, beet, papaya and litchi, etc. (Boon, 2008; Chen, 2009; Cunha, 2009; Ferrer, 2007; Gonzalez-Rodriguez, 2009; Hernandez-Borges, 2009; Huskova, 2008; Knezevic, 2009; Kruve, 2008; Ortelli, 2004; Rial-Otero, 2002) vegetables like tomato, cabbage, green beans, pepper, cucumber, pea, eggplant, spinach, marrow, onion, potato, carrot, cauliflower, lettuce, aubergine, chard, sprout, leek, sweet pepper, green salad, brinjal, okra, green chilly, mint, radish, ginger and smooth gourd (Amoah, 2006; Gonzalez, 1998; Hajslova, 1998; Hernandez, 2006; Rial-Otero, 2005; Salvador, 2006; Sawaya, 2000; Szymczyk, 1998; Xiao-Zhou, 2006; Walorczyk, 2006; Wennrich, 2001) and reported the occurrence of pesticide residues to be even more than maximum residue level (MRL) values recommended by european union (EU), world health organization (WHO) and food and agricultural organization (FAO) (Sharma, Nagpal, Pakade & Katnoria, 2010).

1.1 Pesticide

The “pesticide” name is derived from the Latin words, pestis (pestilence, plague) and caedere (to kill).

Food and Agriculture Organization (FAO) has defined the term of pesticide as: “any substance or mixture of substances intended for preventing, destroying or controlling any pest, including vectors of human or animal disease, unwanted species of plants or animals causing harm during or otherwise interfering with the production, processing, storage, transport or marketing of food, agricultural commodities, wood and wood products or animal feedstuffs, or substances which may be administered to animals for the control of insects, arachnids or other pests in or on their bodies” (FAO, 2007).

1.1.1 Classification

Pesticides can be classified based on chemical structure (biopesticides and synthetic) or their target species (e.g. insecticides, fungicides, herbicides, acaricides, etc.) (Hajslova, 1999; Van der Hoff, 1999).

1.1.1.1 According to Chemical Structure

Based on chemical structure, pesticides are classified three classes, inorganic, botanical and synthetic pesticides.

1.1.1.1.1 Inorganic Pesticides. There are at least 18 elements that characterize in inorganic pesticides. Ten of these elements, such as chromium, copper, zinc, phosphorus, sulfur, tin, arsenic, selenium, fluorine, and chlorine, have been shown to be essential for normal growth. In any event, experience has shown that toxicity is not an argument against essentiality. Some highly toxic elements such as iron, selenium, arsenic, and fluorine certainly are essential to normal development (Clarkson, 2001).

1.1.1.1.2 Botanical Pesticides. Botanical pesticides are extracted from various plant species' parts such as stems, seeds, roots, flower heads. Natural pyrethrins extracted from dried flower heads of *Chrysanthemum cinerariaefolium*, they had been known for centuries as a potent insecticide, but they were rapidly inactivated, when exposed to sunlight. They are biodegradable (Devlin & Zettel, 1999) and their use in crop protection is a practical sustainable alternative. They have effect on biological diversity of predators and reducing environmental contamination and human health hazards. Examples of botanical pesticides are pyrethrins, sabidilla, rotenone, nicotine, ryania, neem and limonene.

1.1.1.1.3 Synthetic Pesticides. They are synthesized by human, do not naturally occur in the environment. Six sub-classes of synthetic pesticides are organochlorins, organophosphates, carbamates, pyrethroids, insect growth regulators, and microbial pesticides.

1.1.1.1.3.1 Organochlorins. Organochlorine pesticides constitute a major environmental problem, because of their high toxicity, persistence in the environment and ability to bioaccumulate in the food chain (Ntow, 2005; Xue et al., 2006). Although most developed countries established bans and restrictions on the use of several organochlorins during the 1970s and 1980s, they are still being used in certain countries for agricultural and public health purposes because of their low cost and versatility as pest control (Itawa et al., 1993; Xue et al., 2006). The use of most organochlorin pesticides such as DDT, BHC, dieldrin, chlordane, aldrin, endrin, heptachlor and methoxychlor has been prohibited in the most country all over world. Despite of being prohibited several years ago, they are also detecting in food.

1.1.1.1.3.2 Organophosphates. Organophosphorus pesticide (OPs) group is the most widely used class of agricultural pesticides (Bai, 2006; Chen, 2009; Lyton, 1996; Pope, 2005; Subhani, 2001; Toan, 2007; Wang, 2008). OPs are either more toxic or less toxic. These group pesticides are not persistent and can break down in 30 days. But they are exerted pharmological and toxicological effect through inhibition of acetylcholinesterase (AChE), required for the transmission of impulse

across the cholinergic synapse. In recent years, many studies have proved OPs to be mutagenic, carcinogenic (Chen, 2009; Huskova, 2008; Pope, 2005; Sanghi, 2001; Sarabia, 2009; Tao, 2009), cytotoxic (Giordano, 2007; Wagner, 2005), genotoxic (Cakir, 2005; Garry, 1989; Rahman, 2002), teratogenic (Kang et al.,2004) and immunotoxic (Crittenden, 1998; Yeh, 2005). Common organophosphate pesticides are malathion, chlorpyrifos, diazinon, dichlorvos.

1.1.1.1.3.3 Carbamates. These group pesticides are less persistent in the environment than the OPs and have neurotoxic effect as OPs. The difference between carbamates and OPs is known; OPs irreversibly link the AChE by the phosphate group (Sultatos, 1994), while carbamates compete with the substrate acetylcholine (Mineau, 1991). Carbamates only leave small inorganic molecules such as carbon dioxide. Common carbamate insecticides are aldicarb, carbaryl, propoxur, oxamyl and terbucarb.

1.1.1.1.3.4 Pyrethroids. These group pesticides synthesized from botanical pesticides, pyrethrins. The stability of pyrethrins in the environment were increased by modification. These synthetic pyrethroids are more toxic nervous system through affecting Ca^{+2} pump in neuron cells. Common pyrethroids are acetamiprid, bifenthrin, cypermethrin, deltamethrin.

1.1.1.1.4 Other Synthetic Organic Pesticides. These group have chemicals such as methoprene, hydroprene, fenoxycarb and hexaflumuron that affect the ability of insects to growth and mature normally. They affect mostly by adversely affecting chitin synthesis so the insect fails to complete a moult from one larval stage to the next. Another novel insecticide, tebufenozide, causes larvae to form precocious adults; that is, they attempt to moult into an adult before sufficient larval development has taken place (Matthews, 2006). Some microorganisms such as a bacteria *Bacillus thuringiensis*, a fungi *Metarhyzium*, a nematode *Steinernema feltiae* that are grown in manufacturing plants can use as a biopesticide. Neonicotinoids and nicotine, juvenile hormones, pheromones and phenylpyrazoles are other using synthetic organic pesticides.

1.1.1.2 According to Target Organism

According to target organism, pesticides are classified as (Grene & Pohanish, 2005);

- Insecticides are a pesticide used against insects. They include ovicides and larvicides used against the eggs and larvae of insects respectively.
- Acaricides are pesticides that kill members of the Acari group, which includes ticks and mites
- Algicides a substance used for killing and preventing the growth of algae in ponds, lakes, canals, swimming pools and industrial air conditioners.
- Fungicides are chemical compounds or biological organisms used to kill or inhibit fungi or fungal spores. Fungi can cause serious damage in agriculture, resulting in critical losses of yield, quality and profit. Fungicides are used both in agriculture and to fight fungal infections in animals (Haverkate, Tempel & Den Held, 1969).
- Herbicides commonly known as a weedkiller, is a type of pesticide used to kill unwanted plants. Selective herbicides kill specific targets while leaving the desired crop relatively unharmed. Some of these act by interfering with the growth of the weed and are often synthetic "imitations" of plant hormones. Herbicides used to clear waste ground, industrial sites, railways and railway embankments are non-selective and kill all plant material with which they come into contact (Kellogg, Nehring, Grube, Goss & Plotkin, 2000).
- Molluscicides also known as snail baits and snail pellets, are pesticides against molluscs, which are usually used in agriculture or gardening to control gastropod pests like slugs and snails that can damage crops by feeding on them.
- Nematicides are a type of chemical pesticide used to kill parasitic, microscopic, worm-like organisms, nematodes that feed on plant roots.

- Biocides are a chemical substance or microorganism which can deter, render harmless, or exert a controlling effect on any harmful organism by chemical or biological means.
- Ovicides are a type of chemical pesticide used to kill eggs of mites and insects.
- Rodenticides are a category of pest control chemicals intended to kill rodents.

1.2 Electron Transport Chain

Mitochondria are the primary source of cellular ATP. Electrons are transferred from NADH to O_2 through a chain of four large protein complexes called NADH: ubiquinone oxidoreductase, succinate dehydrogenase, ubiquinol: cytochrome c oxidoreductase, and cytochrome c oxidase. The final electron acceptor is molecular oxygen. Electron flow within these transmembrane complexes leads to the transport of protons across the inner mitochondrial membrane. ATP is generated via an ATP synthase that utilizes an inner mitochondrial membrane and pH gradient uses as the driving force (Brown & Yamamoto, 2003).

1.2.1 The Mitochondria

Mitochondria are intracellular organelles, varying in both shape and size. They may be spherical or elongated, or even branched, and the number may vary from 6–12 small discrete organelles per rat thymus lymphocyte. Despite the wide variability in number and morphology, all mitochondria share several fundamental properties regardless of the cell type. Mitochondria has two lipid bilayer membranes. The outer membrane is permeable to ions (M_R <5.000), solutes up to 14 kDa and move freely through transmembrane channels formed by a family of integral membrane proteins called porins. It is rich in cholesterol and contains enzymes that interface the mitochondria with the rest of the cellular metabolic network. The inner membrane encloses a water containing compartment called matrix, where mitochondrial DNA and various soluble enzymes, such as the pyruvate dehydrogenase complex, the citric acid cycle, the tricarboxylic acid cycle, the β-oxidation pathway and the pathways of

amino acid oxidation, are located. This membrane is not freely permeable to ions including protons (H^+) and metabolites, but contains special membrane proteins that transport selected metabolites across the membrane. However, specific transporters carry pyruvate, fatty acids, and amino acids or their α-keto derivatives into the matrix for access to the machinery of the citric acid cycle. ADP and P_i are specifically transported into the matrix as newly synthesized ATP is transported out. This feature, the protein-regulated permeability of the inner membrane, is of vital importance for the morphological and functional integrity of the mitochondria, therefore it is also the most common target for mitochondrial toxicants. Many foreign chemicals damage mitochondria either by increasing the permeability of the inner membrane or by inhibiting transport proteins within it. The lipid composition of the inner membrane is unique and it also contains large amounts of cardiolipin and virtually no cholesterol. The inner membrane also contains many different proteins that participate in various metabolic activities, including the production of energy. It also contains a mobile electron carrier, ubiquinone, dissolved in the lipid phase of the membrane (Wallace & Starkov, 2000). In 1948, Eugene Kennedy and Albert Lehninger discovered that mitochondria are the site of oxidative phosphorylation in eukaryotes.

1.2.2 Electron Transport

1.2.2.1 Electron Carriers

The mitochondrial respiratory chain consists of a series of electron carriers, most of which are integral proteins with prosthetic groups capable of accepting and donating either one or two electrons.

These are;

- NADH
- Flavoproteins (FAD and FMN)
- Ubiquinone or coenzyme Q
- Cytochromes (cytochrome a, b and c)
- Iron-sulfur clusters.

NADH; Nicotinamide adenine dinucleotide, abbreviated NAD^+, is a coenzyme found in all living cells. As seen in Figure 1.1, the compound is a dinucleotide, since it consists of two nucleotides joined through their phosphate groups, with one nucleotide containing an adenine base and the other containing nicotinamide.

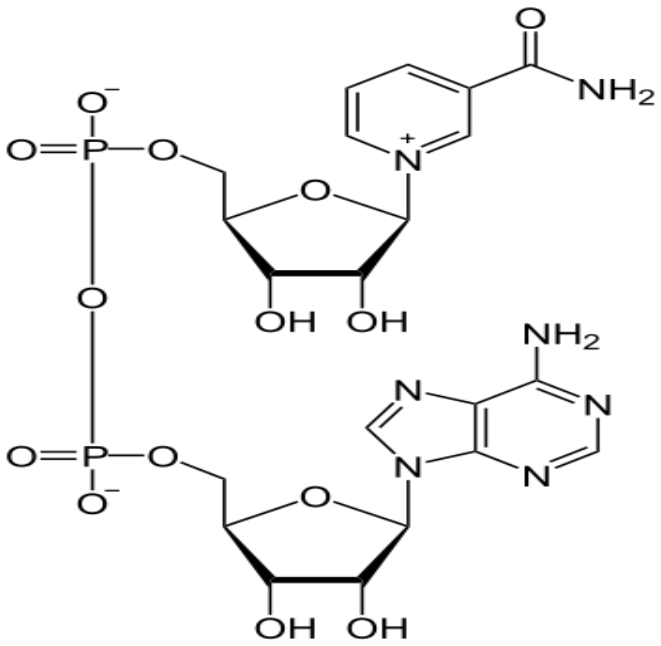


Figure 1.1. The structure of NADH.

In metabolism, NAD^+ is involved in redox reactions (Figure 1.2), carrying electrons from one reaction to another. The coenzyme is, therefore, found in two forms in cells: NAD^+ is an oxidizing agent, it accepts electrons from other molecules and becomes reduced. This reaction forms NADH, which can then be used as a reducing agent to donate electrons. These electron transfer reactions are the main function of NAD^+. However, it is also used in other cellular processes, the most notable one being a substrate of enzymes that add or remove chemical groups from proteins, in posttranslational modifications.

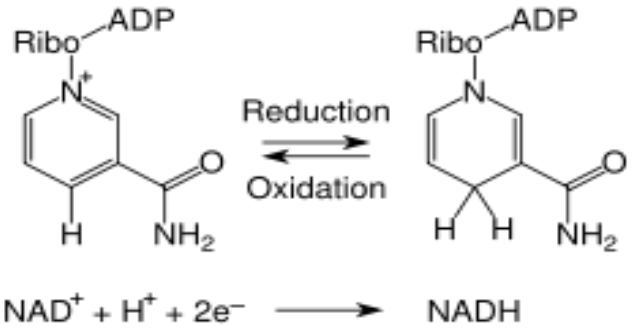


Figure 1.2. Redox equilibrium between NAD^+ and NADH.

Flavoproteins (FAD and FMN); Flavoproteins are proteins that contain a nucleic acid derivative of riboflavin: the flavin adenine dinucleotide (FAD) or flavin mononucleotide (FMN). FAD and FMN can exist in two different redox states, which it converts between by accepting or donating electrons (Figure 1.3 and 1.4). FAD molecule consists of a riboflavin moiety (vitamin B_2) bound to the phosphate group of an ADP molecule. The flavin group is bound to ribitol, a sugar alcohol, by a carbon-nitrogen bond, not a glycosidic bond (EA1) (Metzler, 2001). FAD can be reduced to $FADH_2$, whereby it accepts two hydrogen atoms (a net gain of two electrons):

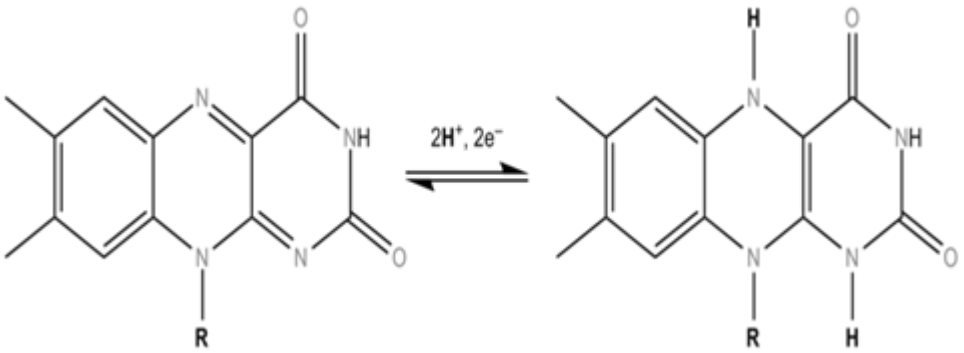


Figure 1.3. Redox equilibrium between FAD and $FADH_2$.

During catalytic cycle, the reversible interconversion of oxidized (FMN), semiquinone ($FMNH^{\bullet}$) and reduced ($FMNH_2$) forms occurs in the various oxidoreductases (Figure 1.4).

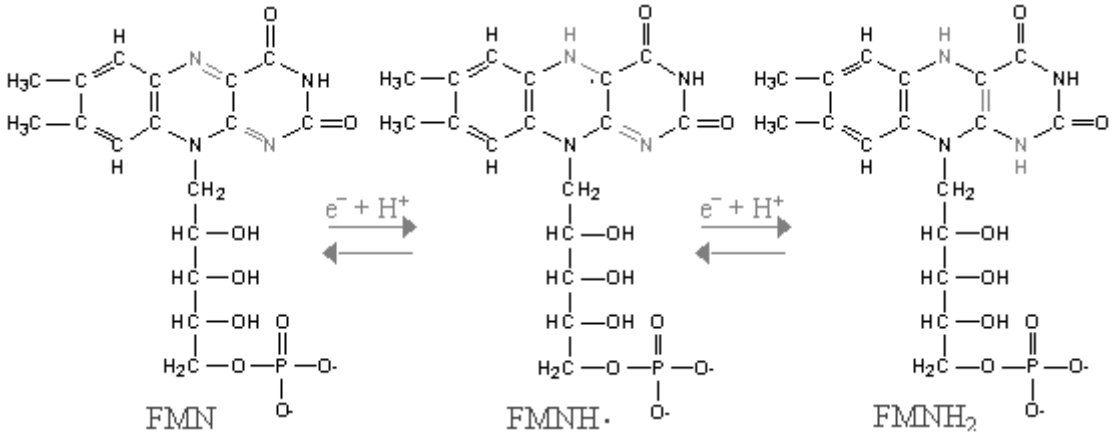


Figure 1.4. Redox equilibrium between FMN, $FMNH^{\bullet}$ and $FMNH_2$.

Ubiquinone or coenzyme Q; Within the inner mitochondrial membrane, the lipid-soluble electron carrier coenzyme Q_{10} (Q) carries both electrons and protons by a redox cycle (Crane, 2001). This small benzoquinone molecule is very hydrophobic, so it diffuses freely within the membrane. When Q accepts two electrons and two protons, it becomes reduced to the ubiquinol form (QH_2); when QH_2 releases two electrons and two protons, it becomes oxidized back to the ubiquinone (Q) form (Figure 1.5). As a result, if two enzymes are arranged so that Q is reduced on one side of the membrane and QH_2 oxidized on the other, ubiquinone will couple these reactions and shuttle protons across the membrane (Mitchell, 1979). Some bacterial electron transport chains use different quinones, such as menaquinone, in addition to ubiquinone (Søballe & Poole, 1999).

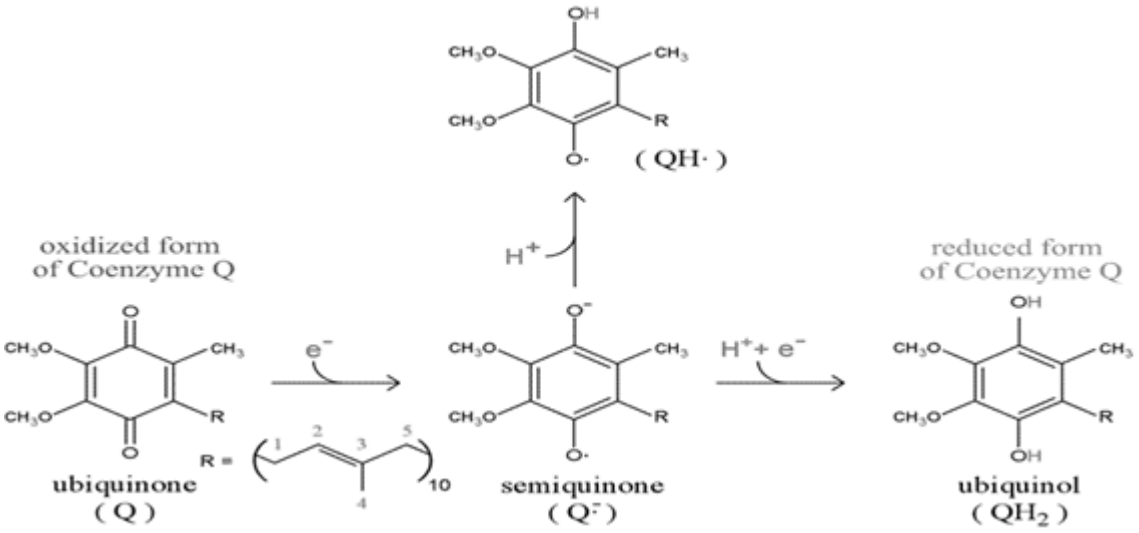
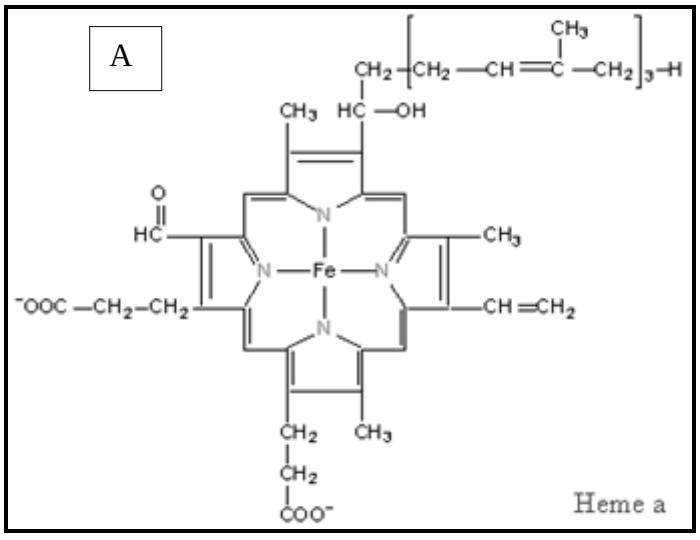


Figure 1.5. Redox equilibrium between ubiquinone, semiquinone and ubiquinol.

Cytochromes; are, in general, water-soluble, mitochondrial inner membrane-bound hemoproteins that contain heme groups and transfer electrons within the intermembrane space by reduction and oxidation of an metal atom. The heme group is a highly-conjugated ring system (which allows its very mobile electrons) surrounding a metal ion, which readily interconverts between the oxidation states. For many cytochromes, the metal ion present is that of iron, which interconverts between Fe^{2+} (reduced) and Fe^{3+} (oxidised) states (electron-transfer processes) or between Fe^{2+} (reduced) and Fe^{3+} (formal, oxidized) states (Mathews, 1975). In mitochondria, these cytochromes are often combined in lots of metabolic pathways (Table 1.1). Especially cytochrome a, b and c forms are in electron transport chain (Figure 1.6).

Table 1.1. The cytochromes and their place in metabolism.

Cytochromes	**Combination**
a and a_3	Cytochrome c oxidase (Complex IV) with electrons delivered to complex by soluble cytochrome c (hence the name)
b and c_1	Coenzyme Q - cytochrome c reductase (Complex III)
b_6 and f	Plastoquinol—plastocyanin reductase



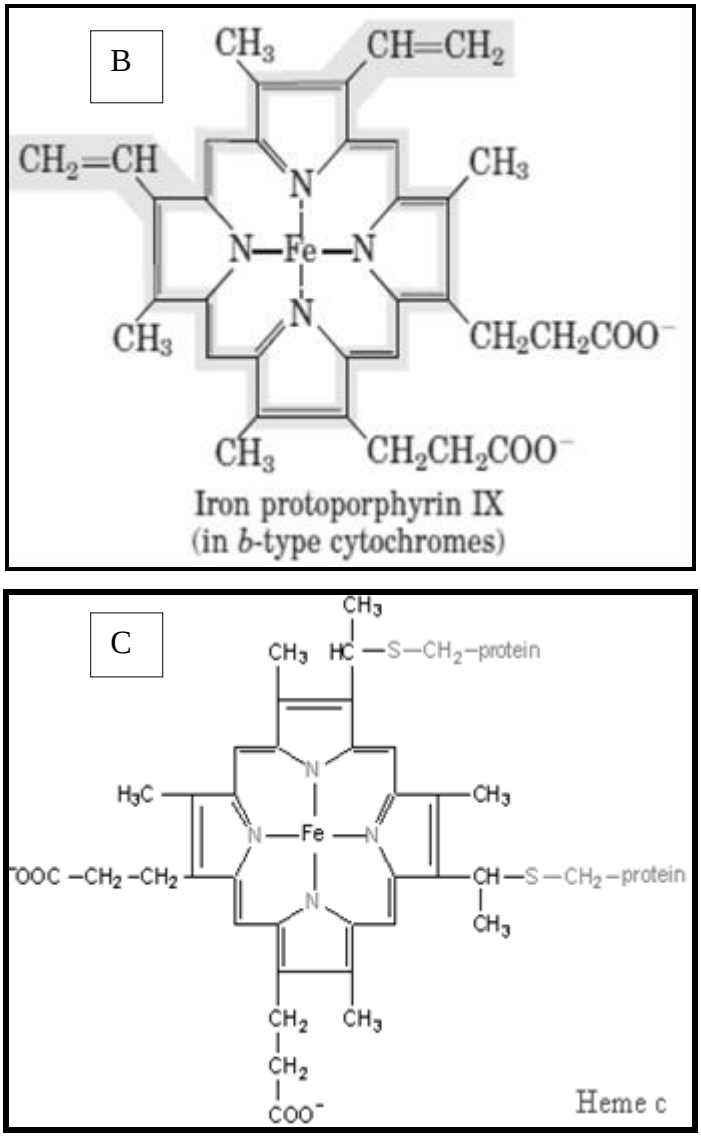


Figure 1.6. The cytochrome types, A) cytochrome a, also called heme a; B) cytochrome b also called heme b; C) cytochrome c also called heme c.

Iron-sulfur clusters; There are several types of iron–sulfur cluster (Figure 1.7). The simplest kind found in the electron transfer chain consists of two iron atoms joined by two atoms of inorganic sulfur; these are called [2Fe–2S] clusters. The second kind, called [4Fe–4S], contains a cube of four iron atoms and four sulfur atoms. Each iron atom in these clusters is coordinated by an additional amino acid, usually by the sulfur atom of cysteine. Metal ion cofactors undergo redox reactions without binding or releasing protons, so in the electron transport chain they serve

solely to transport electrons through proteins. Electrons move quite long distances through proteins by hopping along chains of these cofactors (Page, Moser, Chen & Dutton, 1999). This occurs by quantum tunnelling, which is rapid over distances of less than 1.4×10−9 M (Leys & Scrutton, 2004).

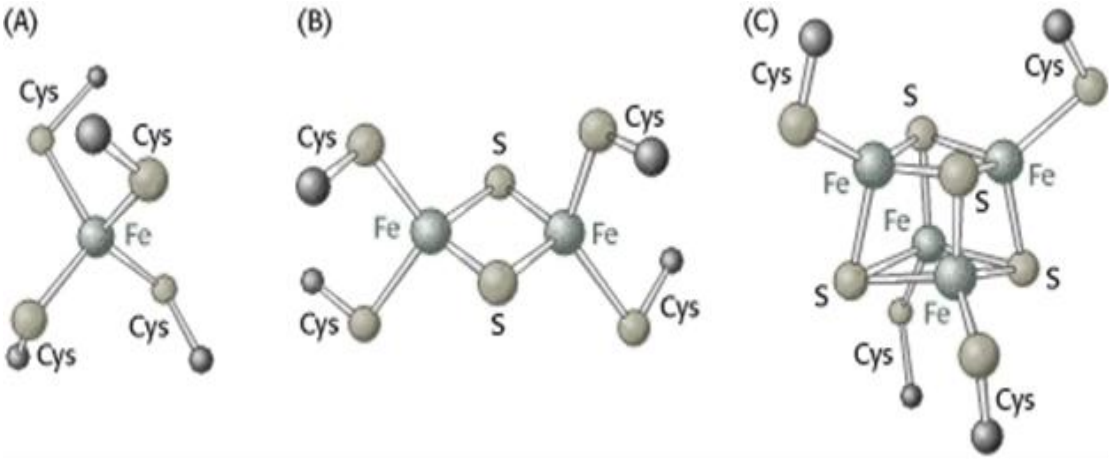


Figure 1.7. The types of Fe-S clusters A) Fe-S B) [2Fe–2S] C) [4Fe–4S].

The mitochondrial respiratory chain has 4 enzyme within the inner mitochondrial membrane. Their some properties were given in Table 1.2 (DePierre & Ernster, 1977; Hatefi, 1985; Walker, 1992).

Table 1.2. Some properties of mitochondrial respiratory chain enzymes.

					Oxidant or reductant	
Enzyme complex	**Mass (kDa)**	**Subunits**	**Prosthetic group**	**Matrix side**	**Membrane core**	**Cytosolic side**
NADH: ubiquinone oxidoreductase	880	> 34	FMN Fe-S	NADH	Q	
Succinate dehydrogenase	140	4	FAD Fe-S	Succinate	Q	
Ubiquinol-cytochrome c oxidoreductase	250	10	Heme b_H Heme b_L Heme c_1 Fe-S		Q	Cytochrome c
Cytochrome c oxidase	160	10	Heme a Heme a_3 Cu_A and Cu_B			Cytochrome c

1.2.2.2 Complex I (NADH: Ubiquinone Oxidoreductase)

Complex I (EC 1.6.5.3) is the first complex of the oxidative phosphorylation system. It is the entry point for electrons into the respiratory chain by oxidation of NADH and transport of electrons to coenzyme-Q_{10} also known as ubiquinone. Ubiquinone is a hydrophobic quinone that diffuses rapidly within the inner mitochondrial membrane. With a relative molecular mass of 880 kDa, it is the largest complex of the respiratory chain. Complex I consists of 42 different polypeptide chains, including an FMN-containing flavoprotein and at least six iron-sulfur centers, forming a characteristic L-shaped configuration (Carroll et al., 2006). The hydrophilic peripheral arm stretches out into the mitochondrial matrix and catalyzes the NADH oxidation and electron transport. The hydrophobic membrane arm is embedded in the inner mitochondrial membrane and contains the proton-transport activity (Janssen et al., 2007). At this step, Complex I catalyzes transfer of two high-potential electrons from NADH to to the flavin mononucleotide (FMN) prosthetic group of this complex and the reduced $FMNH_2$ forms. But FMN can also accept one electron. Therefore, ubiquinone accepts the other electron and forms a semiquinone radical intermediate (Figure 1.8).

$$NADH + H^+ + Q \longrightarrow NAD^+ + QH_2$$

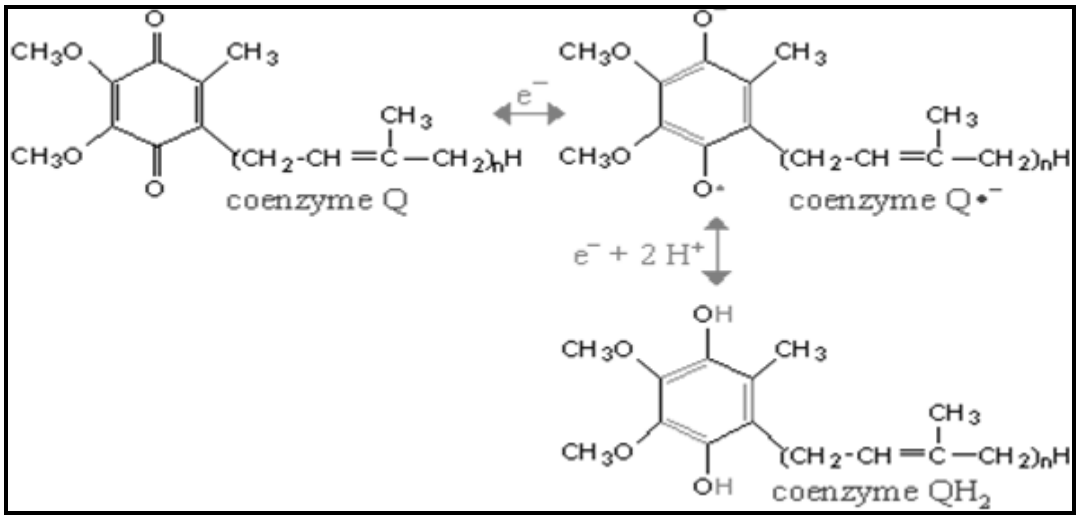


Figure 1.8. An electron influx from ubiquinone to ubiquinol.

Then, the electron of $FMNH_2$ transfer to second prosthetic group, Fe-S proteins. NADH-Q oxidoreductase contains both 2Fe-2S and 4Fe-4S clusters. At the end, Fe-S proteins transfer an electron to semiquinol and semiquinone forms. With electron transfer, four protons transfer from the matrix to the intermembrane species. So, the matrix becomes negative charged and the intermembrane species becomes positive charged (Figure 1.9).

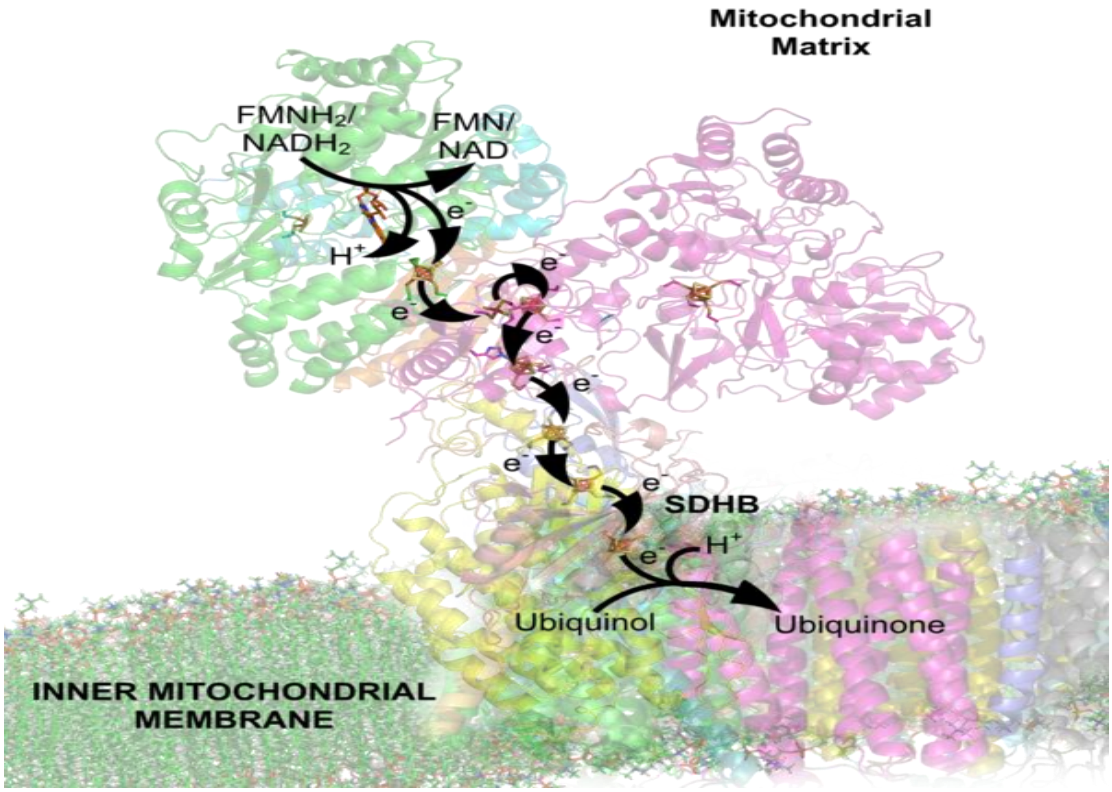


Figure 1.9. The mechanism of Complex I.

1.2.2.3 Complex II (Succinate Dehydrogenase)

Complex II (EC 1.3.5.1) is the only membrane-bound enzyme of the citric acid cycle. It contains five prosthetic groups of two types and four different subunits called A, B, C and D. Its moleculer mass is relatively 140 kDa. Subunits C and D are integral proteins. These contain heme b and a binding of ubiquinone. Subunits A nd B are extend into the matrix. These contains three 2Fe-2S centers, bound FAD, and a binding site for the substrate, succinate. The electron flow is from succinate to FAD, then through Fe-S centers to ubiquinone. It oxidizes succinate to fumarate and

reduces ubiquinone. As this reaction releases less energy than the oxidation of NADH, complex II does not transport protons across the membrane and does not contribute to the proton gradient (Figure 1.10).

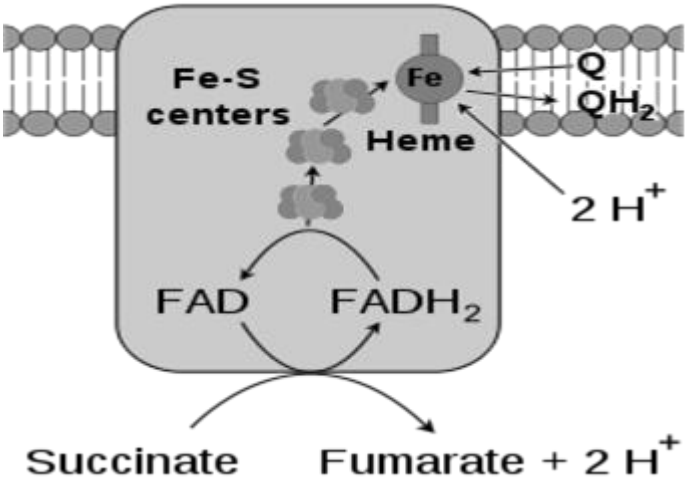


Figure 1.10. The mechanism of Complex II.

Not through Complex II, but there are entry points to the electron transport chain that transfer electrons to ubiquinone into the respiratory chain. Second entry point is ETF: ubiquinone oxidoreductase. It transfers electrons from acyl-CoA to FAD. Then, through electron-transferring flavoprotein (ETF), the electrons reach ubiquinone. It is an enzyme in the mitochondrial matrix contains a flavin and a [4Fe–4S] cluster, but, unlike the other respiratory complexes, it attaches to the surface of the membrane and does not cross the lipid bilayer (Zhang, Frerman & Kim, 2006). In mammals, this metabolic pathway is important in beta oxidation of fatty acids and catabolism of amino acids and choline, as it accepts electrons from multiple acetyl-CoA dehydrogenases (Ikeda, 1983; Ruzicka, 1977). Another enzyme is cytocolic glycerol–3- phosphate dehydrogenase that is located on the outer surface of inner membrane of mitochondria. Glycerol 3-phosphate, formed either from glycerol released by triacylglycerol breakdown or by the reduction of dihydroxyacetone phosphate from glycolysis. This enzyme transfers electrons from cytocolic NADH to ubiquinone (Figure 1.11).

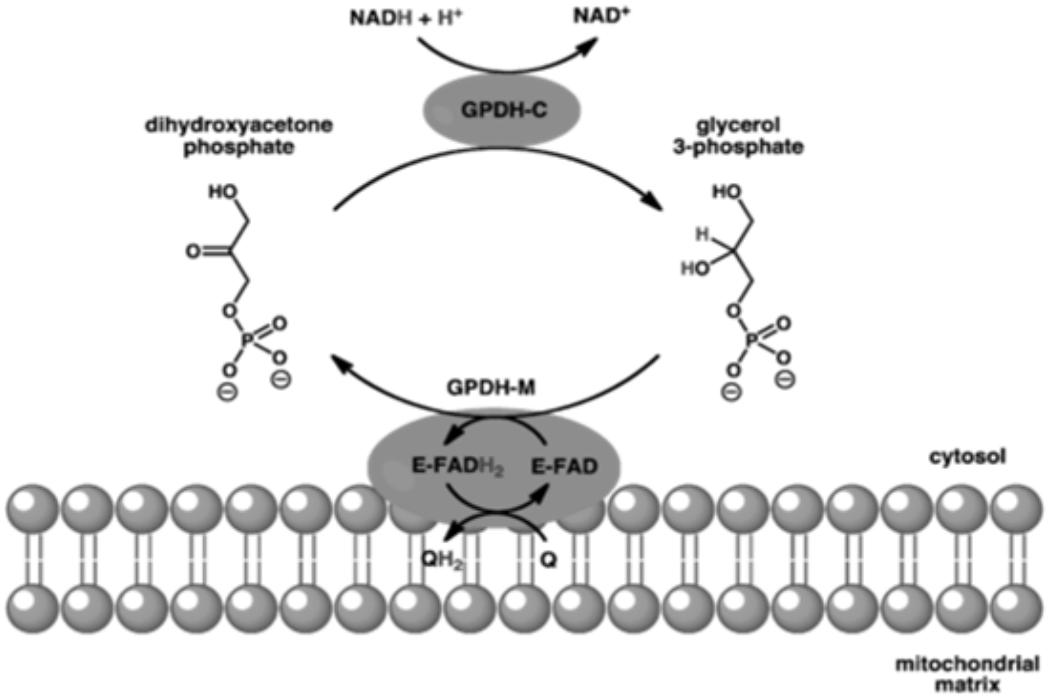


Figure 1.11. The mechanism of gliserol 3- phosphate dehydrogenase.

At the end of these four reactions, ubiquinone reduced to ubiquinol (Figure 1.8).

1.2.2.4 Complex III (Ubiquinol: Cytochrome c Oxidoreductase)

Complex III (EC 1.10.2.2), is a dimer, with each subunit complex containing 11 protein subunits, an [2Fe-2S] iron–sulfur cluster and three cytochromes: one cytochrome c_1 and two b cytochromes such as b_L, b_H in mammals (Iwata, Lee & Okada, 1998). A cytochrome is a kind of electron-transferring protein that contains at least one heme group. The iron atoms inside complex III's heme groups alternate between a reduced ferrous (+2) and oxidized ferric (+3) state as the electrons are transferred through the protein. For the reactions in this step, a general model has been proposed called Q-cycle.

The first QH_2 oxidizes to Q and two electrons are formed. Two protons pump to intermembrane space. An electron transfers to Fe-S center and the other transfers to cytochrome b_L. Cytochrome b_L transfers electron through b_H to other quinone and was formed semiquinone. Fe-S center transfers electron through cytochrome c_1 to

cytochrome c. The second QH_2 oxides to Q and other reaction as the oxidation of first QH_2. only difference is that with two protons tansfer from matrix and an electron from cytochrome b_H, semiqinone reduces to ubiquinol (Figure 1.12).

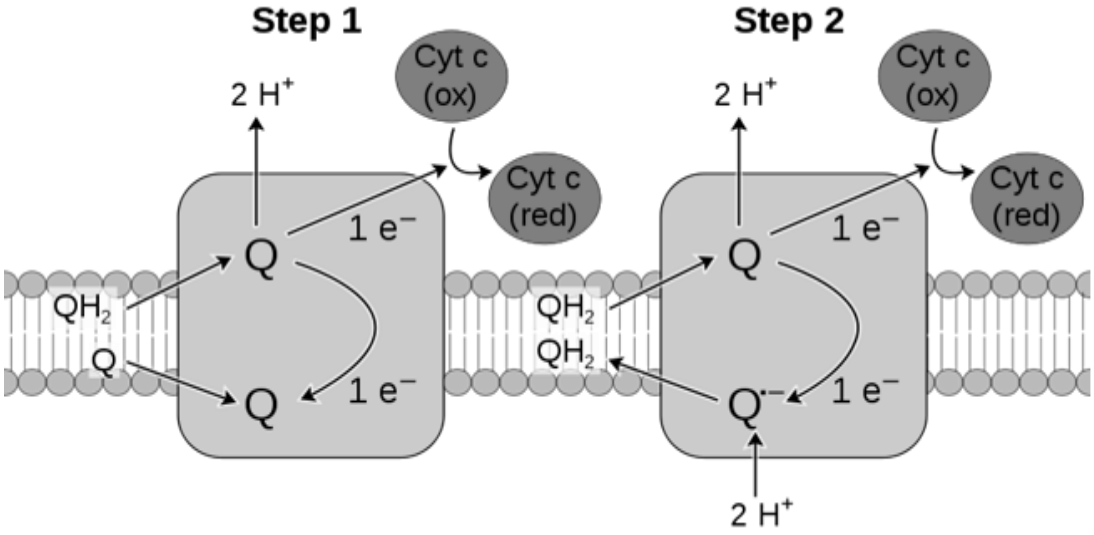


Figure 1.12. The mechanism of Complex III.

$$QH_2 + \text{cyt } c_1 \text{ (oxidized)} \longrightarrow Q^{\bullet -} + 2H^+_{(P)} + \text{cyt } c_1 \text{ (reduced)}$$

$$QH_2 + \text{cyt } c_1 \text{ (oxidized)} + Q^{\bullet -} + 2H^+_{(N)} \longrightarrow QH_2 + 2H^+_{(P)} + Q + \text{cyt } c_1 \text{ (reduced)}$$

The net equation of Q-cycle is:

$$QH_2 + 2 \text{ cyt } c_1 \text{ (oxidized)} + 2H^+_{(N)} \longrightarrow Q + 2 \text{ cyt } c_1 \text{ (reduced)} + 4H^+_{(P)}$$

1.2.2.5 Complex IV (Cytochrome c Oxidase)

Complex IV (EC 1.9.3.1) has an extremely complicated structure and contains 13 subunits, two heme groups, as well as multiple metal ion cofactors – in all three atoms of copper, one of magnesium and one of zinc (Tsukihara et al., 1996). It catalyzes oxidation of reduced cytochrome c and the reduction of moleculer O_2 to H_2O. Complex IV contains thirteen protein chains. Three of them are important and

other ten proteins encycle these three proteins. It has four prosthetic groups as Cu_A, Cu_B, cytochrome a and a_3. Cytochrome a_3-Cu_B complex is called Fe-Cu center.

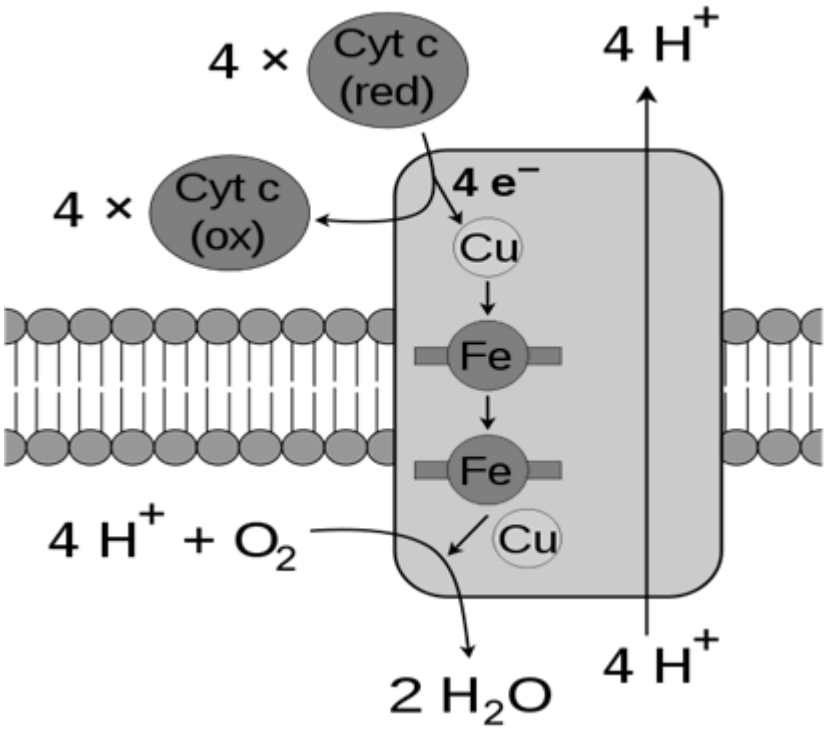


Figure 1.13. The mechanism of Complex IV.

Firstly, every cytochrome c transfer one electron to Cu_A and these two electrons move through cytochrome a to Fe-Cu center. Cu^{+2} reduces Cu^{+} and Fe^{+3} to Fe^{+2}. This reduced Fe^{+2} form can bind O_2 like hemoglobin. Then, two electrons transfer to O_2 and is formed O_2^{-2}. The last two electrons transfer to Cu_A and the other reactions are as same. With four protons from matrix, H_2O is formed from O_2^{-2}. Cytochrome c oxidase evolved to pump four additional protons from the matrix to the cytoplasmic side of the membrane (Figure 1.13).

1.2.2.6 ATP Synthase or F_0F_1 ATPase

ATP synthase, also known F_0F_1 ATPase, is a multisubunit transmembrane enzyme. Total moleculer mass is 450 kDa. It consists of two functional units, F_0 and F_1. F_0 is a water-soluble unit, composed of a, b, and c subunits. F_1 is a a water-soluble peripheral unit and composed of three α-, three β-, one each of γ-, α- and ε-

subunits. F_1 cannot synthesize ATP, hydrolyzes it and so called ATPase. F_o component is membrane-embedded and F_1 is connected to F_0 by a protein (Figure 1.14).

This enzyme is found in all forms of life and functions in the same way in both prokaryotes and eukaryotes (Boyer, 1997). The enzyme uses the energy stored in a proton gradient across a membrane to drive the synthesis of ATP from ADP and phosphate (P_i). Estimates of the number of protons required to synthesize one ATP have ranged from three to four (Van Walraven, 1996; Yoshida Muneyuki, 2001) with some suggesting cells can vary this ratio, to suit different conditions (Schemidt, Qu, Williams & Brusilow, 1998).

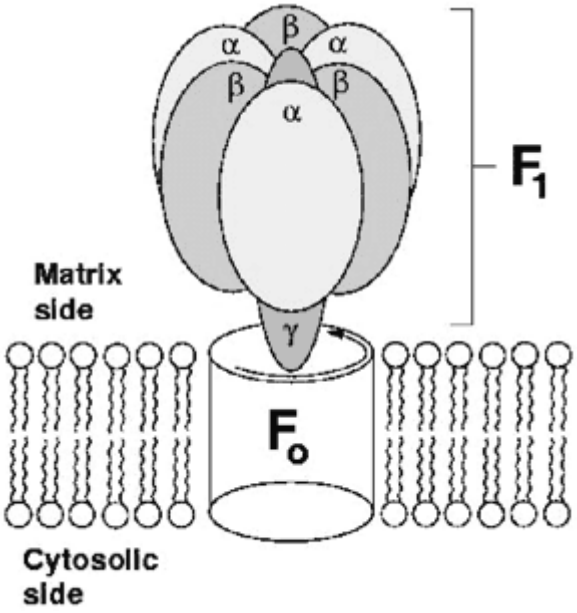


Figure 1.14. The structure of F_oF_1 ATPase.

1.2.3 Oxidative Phosphorylation

The chemiosmotic model, proposed by Peter Mitchell, is for this mechanism. According to the model, the free enegy of electron transport is conserved by pumping H^+ from the mitochondrial matrix to the intermembrane space and this creates an electrochemical H^+ gradient across the iner mitochondrial membrane and this potential uses for production of ATP. The formation of the proton gradient is

endegenous process, but discharge of the gradient is exogenous process. This free is harnessed by ATP synthase to drive the phosphorylation of ADP. Both the electron transport chain and the ATP synthase are embedded in a membrane, and energy is transferred from electron transport chain to the ATP synthase by movements of protons across this membrane, in a process called chemiosmosis (Mitchell & Moyle, 1967).

$$\mathrm{ADP} + \mathrm{P_i} + 4\ \mathrm{H^+_{intermembrane}} \rightleftharpoons \mathrm{ATP} + \mathrm{H_2O} + 4\ \mathrm{H^+_{matrix}}$$

This phosphorylation reaction is an equilibrium, which can be shifted by altering the proton-motive force (Figure 1.15). In the absence of a proton-motive force, the ATP synthase reaction will run from right to left, hydrolyzing ATP and pumping protons out of the matrix across the membrane. However, when the proton-motive force is high, the reaction is forced to run in the opposite direction; it proceeds from left to right, allowing protons to flow down their concentration gradient and turning ADP into ATP (Boyer, 1997). Indeed, in the closely related vacuolar type H+-ATPases, the same reaction is used to acidify cellular compartments, by pumping protons and hydrolysing ATP (Nelson et al., 2000).

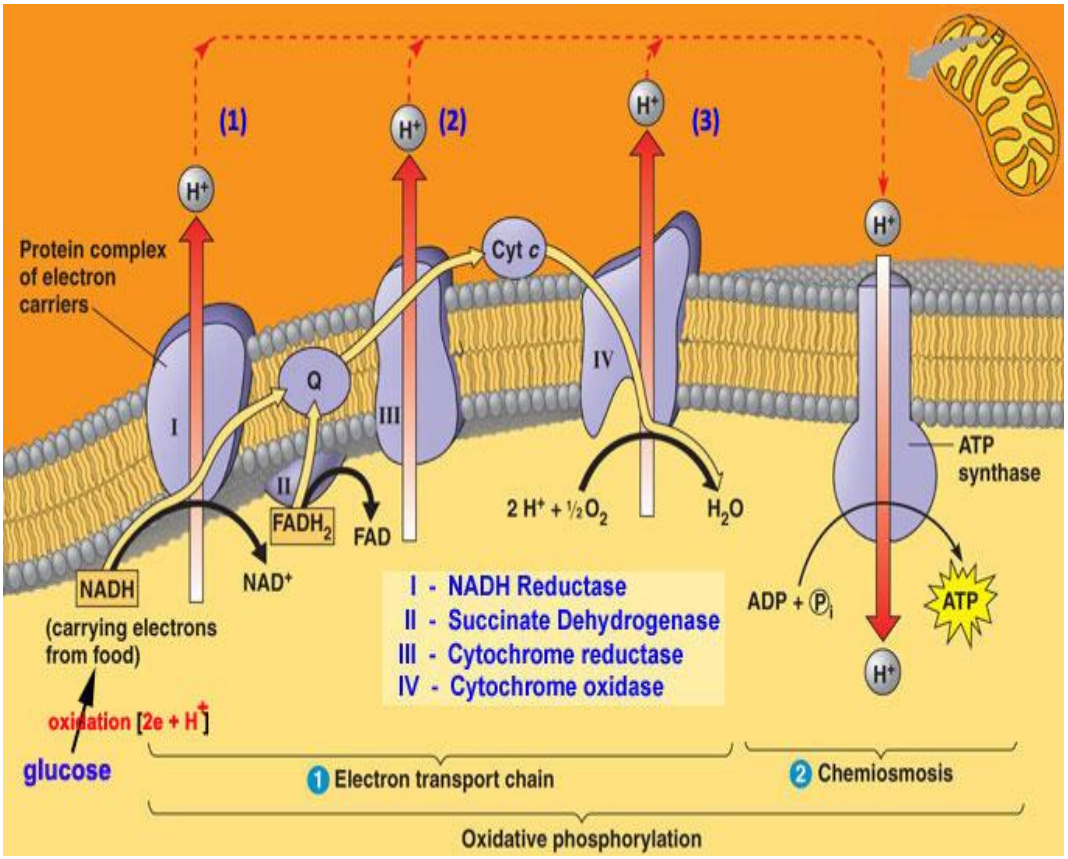


Figure 1.15. The general scheme of oxidative phosphorylation in the mitochondria.

1.2.4 Control of Oxidative Metabolism

The first electron acceptor of this system is NADH. But the inner membrane is not permeable to NADH. The generated NADH by glycolysis in the cytosol must be reoxidized to NAD^+ to continue the metabolism. Therefore, some special shuttle systems such as the malate-aspartate shuttle and the glycerol 3-phosphate shuttle carry reducing equivalents from cytosolic NADH into mitochondria by an indirect route.

The reducing equivalents of cytosolic NADH are first transferred to cytosolic oxaloacetate to yield malate, catalyzed by cytosolic malate dehydrogenase. The formed malate passes through the inner membrane via the malate-α-ketoglutarate transporter. The reducing equivalents are passed to NAD^+ by the action of matrix malate dehydrogenase, forming NADH. This NADH can pass electrons directly to

the respiratory chain. Cytosolic oxaloacetate must be regenerated by transamination reactions and regenerated in the cytosol (Figure 1.16).

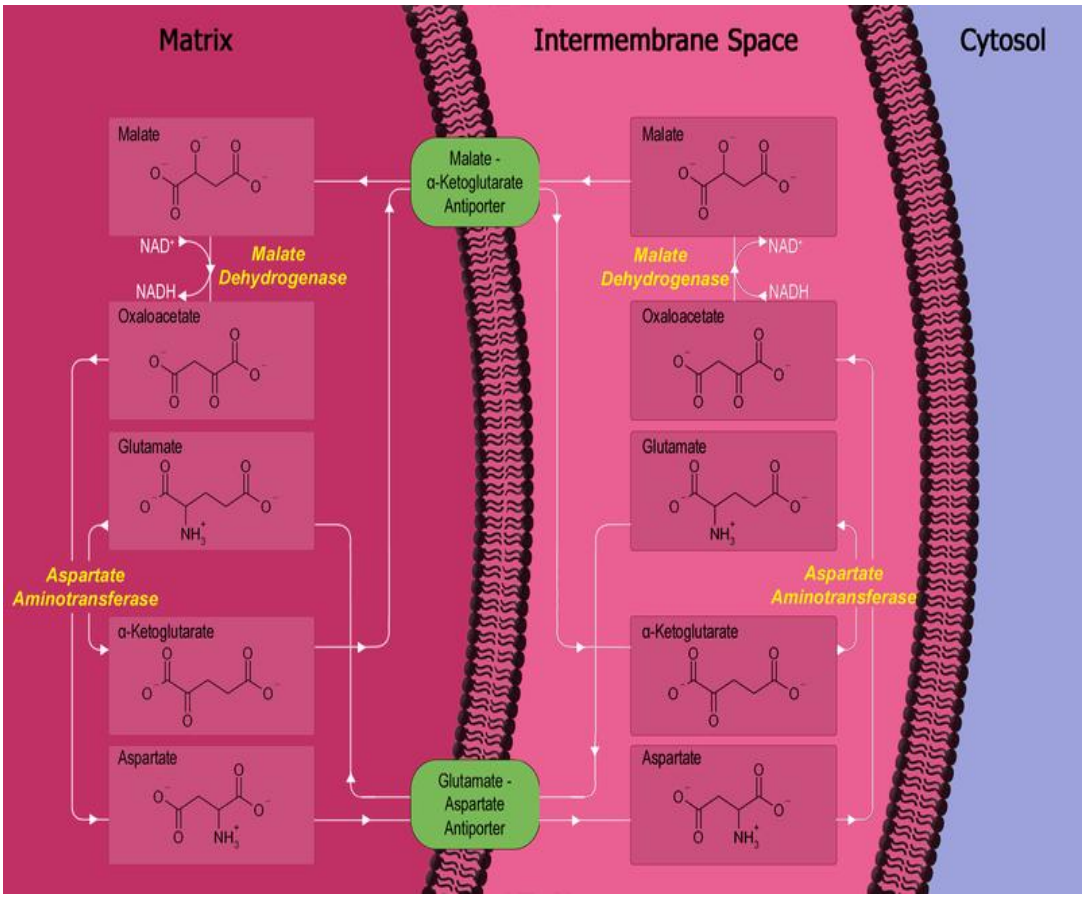


Figure 1.16. The malate-aspartate shuttle.

The glycerol 3-phosphate shuttle differs from the malate-aspartate shuttle, it transfers the reducing equivalents from NADH to ubiquinone and thus into Complex III, not Complex I (Figure 1.17).

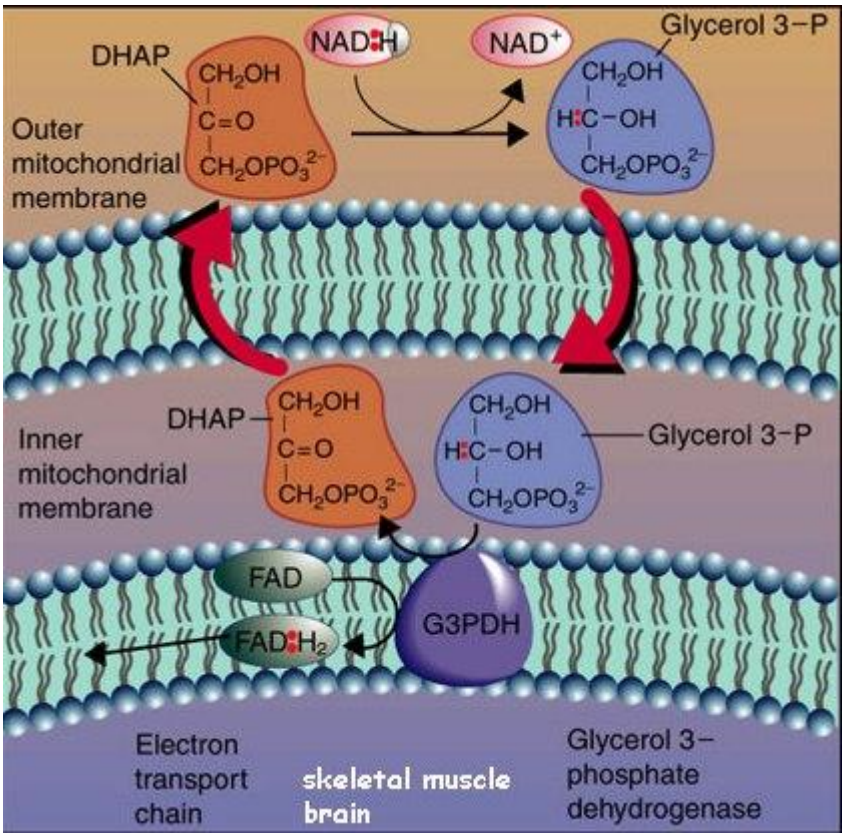


Figure 1.17. Glycerol 3-phosphate shuttle.

The other control mechanism is cellular energy need. O_2 consumption in mitochondria is generally limited by the availability of ADP as a substrate for phosphorylation. Dependence of the rate of O_2 consumption on the availability of the P_i acceptor ADP, the acceptor control of respiration, can be remarkable. In some animal tissues, the acceptor control ratio, the ratio of the maximal rate of ADP-induced O_2 consumption to the basal rate in the absence of ADP, is at least ten.

The intracellular concentration of ADP is one measure of the energy status of cells. Another, related measure is the mass-action ratio of the ATP-ADP system, [ATP]/([ADP][Pi]). Normally this ratio is very high, so the ATP-ADP system is almost fully phosphorylated. When the rate of some energy-requiring process increases, breakdown of ATP to ADP and Pi increases, as well as lowering the mass-action ratio. With more available ADP for oxidative phosphorylation, the rate of respiration increases, that causes regeneration of ATP. This continues until the mass-action ratio returns to its normal high level.

The relative concentrations of ATP and ADP control not only the rates of electron transfer and oxidative phosphorylation but also the rates of the citric acid cycle, pyruvate oxidation, and glycolysis. Whenever ATP consumption increases, the rate of electron transfer and oxidative phosphorylation increases. Simultaneously, the rate of pyruvate oxidation via the citric acid cycle increases, increasing the flow of electrons into the respiratory chain. These events can in turn evoke an increase in the rate of glycolysis, increasing the rate of pyruvate formation. When conversion of ADP to ATP lowers the ADP concentration, acceptor control slows electron transfer and thus oxidative phosphorylation.

ATP is an allosteric inhibitor of the glycolytic enzyme phosphofructokinase–1 and of pyruvate dehydrogenase. Therefore, glycolysis and the citric acid cycle are also slowed. When the citric acid cycle is slowed, citrate accumulates within mitochondria, then crosses into the cytosol. When the concentrations of both ATP and citrate rise, they produce an allosteric inhibition of phosphofructokinase–1 and this effects to slow glycolysis.

1.2.5 The Role of Mitochondria in Apoptosis and Oxidative Stres

Molecular oxygen is an ideal terminal electron acceptor because it is a strong oxidizing agent. The reduction of oxygen involves potentially harmful intermediates (Davies, 1995). The summary of producing ROS in mitochondria can be seen in Figure 1.18. Although the transfer of four electrons and four protons reduces oxygen to water, which is harmless, transfer of one or two electrons produces superoxide or peroxide anions, which are dangerously reactive. The mitochondrial respiratory chain is the major site of reactive oxygen species (ROS) production within the cell. The cytochrome c oxidase complex is highly efficient at reducing oxygen to water and it releases very few partly reduced intermediates; however small amounts of superoxide anion and peroxide are produced by the electron transport chain (Raha & Robinson, 2000). Particularly important is the reduction of coenzyme Q in complex III, as a highly reactive ubisemiquinone free radical is formed as an intermediate in the Q cycle. This unstable species can lead to electron "leakage" when electrons

transfer directly to oxygen, forming superoxide (Finkel & Holbrook, 2000). As the production of ROS by these proton-pumping complexes is greatest at high membrane potentials, it has been proposed that mitochondria regulate their activity to maintain the membrane potential within a narrow range that balances ATP production against oxidant generation (Kadenbach, Ramzan, Wen & Vogt, 2009). For instance, oxidants can activate uncoupling proteins that reduce membrane potential (Echtay, Roussel & St-Pierre, 2002). Superoxide is thought to be produced continually as a byproduct of normal respiration through the one electron reduction of molecular oxygen (Chance et al., 1979; Raha et al., 2000). Superoxide itself damages iron sulfur center-containing enzymes such as aconitase (Vasquez-Vivar, Kalyanaraman & Kennedy, 2000) and can also react with nitric oxide to form the damaging oxidant peroxynitrite, which is more reactive than either precursor (Beckman J. S., Beckman T.W., Chen, Marshall & Freeman, 1990). Nitric oxide diffuses easily into mitochondria and may also be produced there (Murphy, 1999) The mitochondrial enzyme manganese superoxide dismutase (MnSOD) converts superoxide to hydrogen peroxide, which, in the presence of ferrous or cuprous ions, forms the highly reactive hydroxyl radical, which damages all classes of biomolecules. The availability of free iron and copper within mitochondria is uncertain, although the reaction of superoxide with the iron sulfur center in aconitase releases ferrous iron (Vasquez-Vivar et al., 1990). Consequently, mitochondrial superoxide production initiates a range of damaging reactions through the production of superoxide, hydrogen peroxide, ferrous iron, hydroxyl radical, and peroxynitrite, which can damage lipids, proteins, and nucleic acids (James & Murphy, 2002). Mitochondrial function is particularly susceptible to oxidative damage, leading to decreased mitochondrial ATP synthesis, cellular calcium dyshomeostasis, and induction of the mitochondrial permeability transition, all of which predispose cells to necrosis or apoptosis (James et al., 2002).

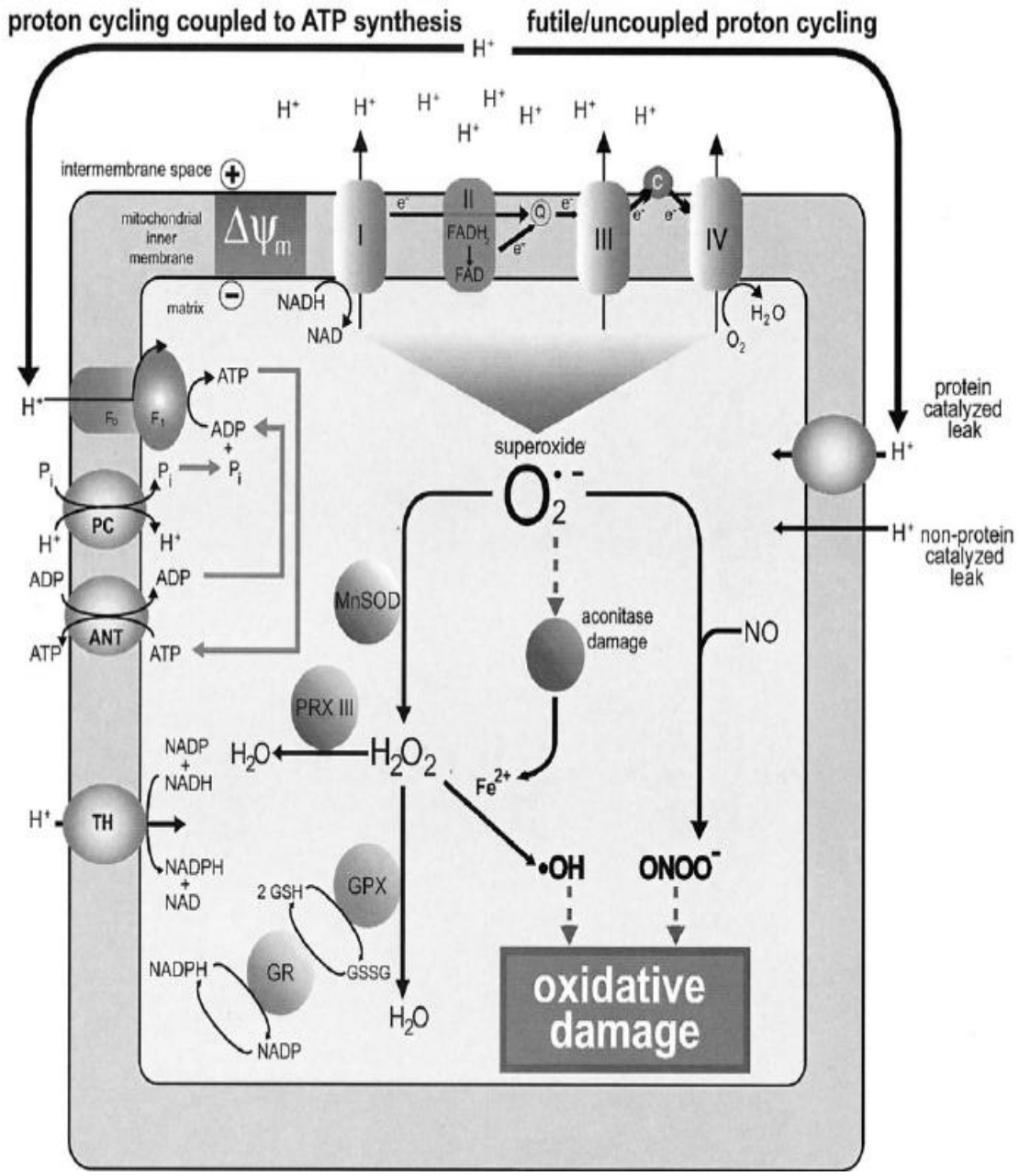


Figure 1.18. The production of ROS in mitochondria.

1.3 Citric Acid Cycle

The citric acid cycle is also known as the tricarboxylic acid cycle (TCA cycle) or the Krebs cycle. In eukaryotic cells, the citric acid cycle occurs in the matrix of the mitochondrion. In aerobic organisms, the citric acid cycle is part of a metabolic pathway involved in the chemical conversion of carbohydrates, fats and proteins into carbon dioxide and water to generate a form of usable energy. Other relevant

reactions in the pathway include those in glycolysis and pyruvate oxidation before the citric acid cycle and oxidative phosphorylation after it. In addition, it provides precursors for many compounds including some amino acids.

As seen in Figure 1.19, the first reaction of the cycle is condensation of acetyl-CoA with oxaloacetate (OAA). When the cellular energy charge increases the rate of flux through the TCA cycle will decline. Excess citrate is used to transport acetyl-CoA from the mitochondrion to the cytoplasm where they can be used for fatty acid and cholesterol biosynthesis. Additionally, the increased levels of citrate in the cytoplasm activate the key regulatory enzyme of fatty acid biosynthesis, acetyl-CoA carboxylase (ACC) and inhibit PFK-1. In non-hepatic tissues citrate is also required for ketone body synthesis. The isomerization of citrate to isocitrate by aconitase is stereospecific, with the migration of the –OH from the citrate being always to the adjacent carbon which is derived from the methylene ($–CH_2–$) of OAA. Isocitrate is oxidatively decarboxylated to α-ketoglutarate by isocitrate dehydrogenase (IDH). This is the rate-limiting step. The IDH of the TCA cycle uses NAD^+ as a cofactor. The CO_2, produced by the IDH reaction is the original C-1 carbon of the oxaloacetate used in the citrate synthase reaction. The control of carbon flow through the cycle is regulated at IDH by the negative allosteric effectors NADH and ATP and by the positive effectors; isocitrate, ADP and AMP. α-ketoglutarate is oxidatively decarboxylated to succinyl-CoA by the α-ketoglutarate dehydrogenase (α-KGDH) complex. This reaction generates the second TCA cycle equivalent of CO_2 and NADH. The conversion of succinyl-CoA to succinate by succinyl CoA synthetase involves use of the high-energy thioester of succinyl-CoA to drive synthesis of a high-energy nucleotide phosphate, by a process known as substrate-level phosphorylation. In this process a high energy enzyme--phosphate intermediate is formed, with the phosphate subsequently being transferred to GDP. Mitochondrial GTP is used in a trans-phosphorylation reaction catalyzed by the mitochondrial enzyme nucleoside diphospho kinase to phosphorylate ADP, producing ATP and regenerating GDP for the continued operation of succinyl CoA synthetase. Succinate dehydrogenase catalyzes the oxidation of succinate to fumarate with the sequential reduction of enzyme-bound FAD and non-heme-iron. The fumarase-catalyzed

reactions specific for the transform of fumarate to L-malate. L-malate is the specific substrate for MDH, the final enzyme of the TCA cycle. The forward reaction of the cycle, the oxidation of malate yields oxaloacetate (OAA).

The stoichiometry of the TCA cycle is:

$$\text{Acetyl-CoA} + 3\text{NAD}^+ + \text{FAD} + \text{GDP} + \text{P}_i + 2\text{H}_2\text{O} \rightarrow 2\text{CO}_2 + 3\text{NADH} + \text{FADH}_2 + \text{GTP} + 2\text{H}^+ + \text{HSCoA}$$

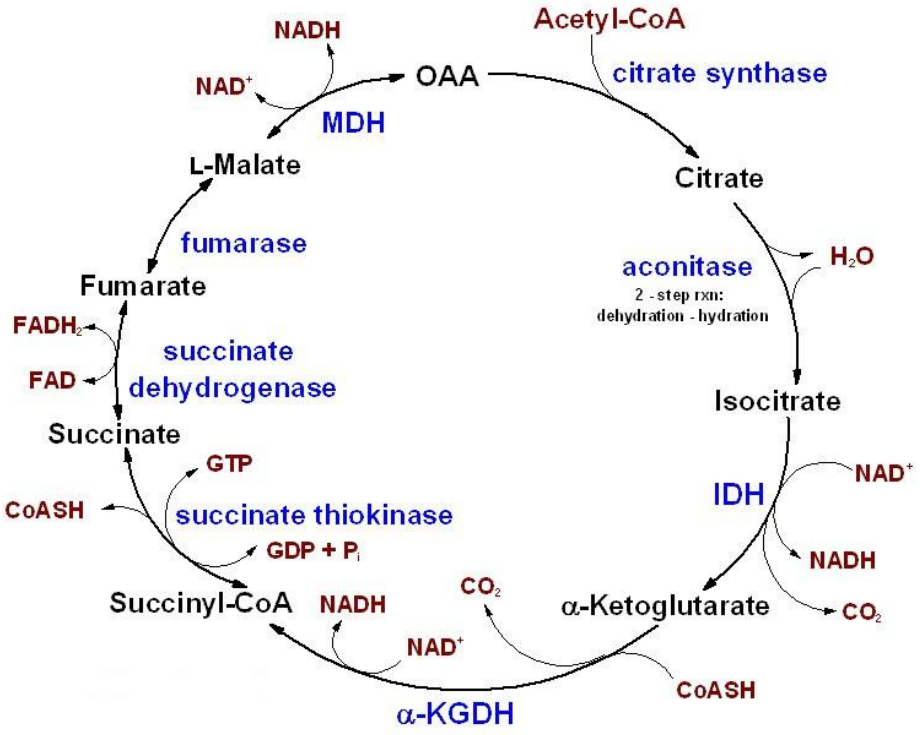


Figure 1.19. The citric acid cycle.

1.4 Lipid Peroxidation

Oxidative damage to DNA, proteins and lipids can ultimately lead to outcomes such as disorganisation, dysfunction and destruction of membranes, enzymes and proteins (Halliwell, 1994; Halliwell, 1997; Slater, 1984). Specifically, peroxidation of membrane lipids may cause impairment of membrane function, decreased fluidity,

inactivation of membrane-bound receptors and enzymes, increased permeability to ions and possibly eventually membrane rupture (Gutteridge, 1990, Gutteridge, 1995). If the oxidative stress is particularly severe, it can produce cell death (Dypbukt, 1994; Halliwell, 1997). Death can occur by necrosis, but in a number of cell types, such as neuronal cells, a mild oxidative stress can trigger the process of apoptosis, activating the intrinsic suicide pathway present within all cells (Hampton, 1997; Stoian, 1996). When lipids are oxidised without release of energy, unsaturated lipids go rancid due to oxidative deterioration when they react directly with molecular oxygen (De Zwart, 1999; Gutteridge, 1990; Gutteridge, 1995; Halliwell, 1990; Halliwell, 1993; Halliwell, 1999; Moore, 1998). This process is called lipid peroxidation and the insertion of an oxygen molecule is catalysed by free radicals (non-enzymatic lipid peroxidation) or enzymes (enzymatic lipid peroxidation) (Gutteridge, 1995; Halliwell, 1990; Halliwell, 1993). Lipid peroxidation is a well-established mechanism of cellular injury in both plants and animals, and is used as an indicator of oxidative stress in cells and tissues. Lipid peroxides are unstable and decompose to form a complex series of compounds including reactive carbonyl compounds.

1.4.1 The Lipid Peroxidation Chain Reaction

Initiation of lipid peroxidation is caused by attack of any species that has sufficient reactivity to abstract a hydrogen atom from a methylene group upon a PUFA (De Zwart, 1999; Gutteridge, 1990; Gutteridge, 1995; Halliwell, 1990; Halliwell, 1993; Halliwell, 1999; Moore, 1998;). As seen in Figure 1.20, since a hydrogen atom in principle is a free radical with a single unpaired electron, its removal leaves behind an unpaired electron on the carbon atom to which it was originally attached. The carbon-centred radical is stabilised by a molecular rearrangement to form a conjugated diene, followed by reaction with oxygen to give a peroxyl radical. Peroxyl radicals are capable of abstracting a hydrogen atom from another adjacent fatty acid side-chain to form a lipid hydroperoxide, but can also combine with each other or attack membrane proteins. When the peroxyl radical abstracts a hydrogen atom from a fatty acid, the new carbon-centred radical can react

with oxygen to form another peroxyl radical, and so the propagation of the chain reaction of lipid peroxidation can continue. Hence, a single substrate radical may result in conversion of multiple fatty acid side chains into lipid hydroperoxides.

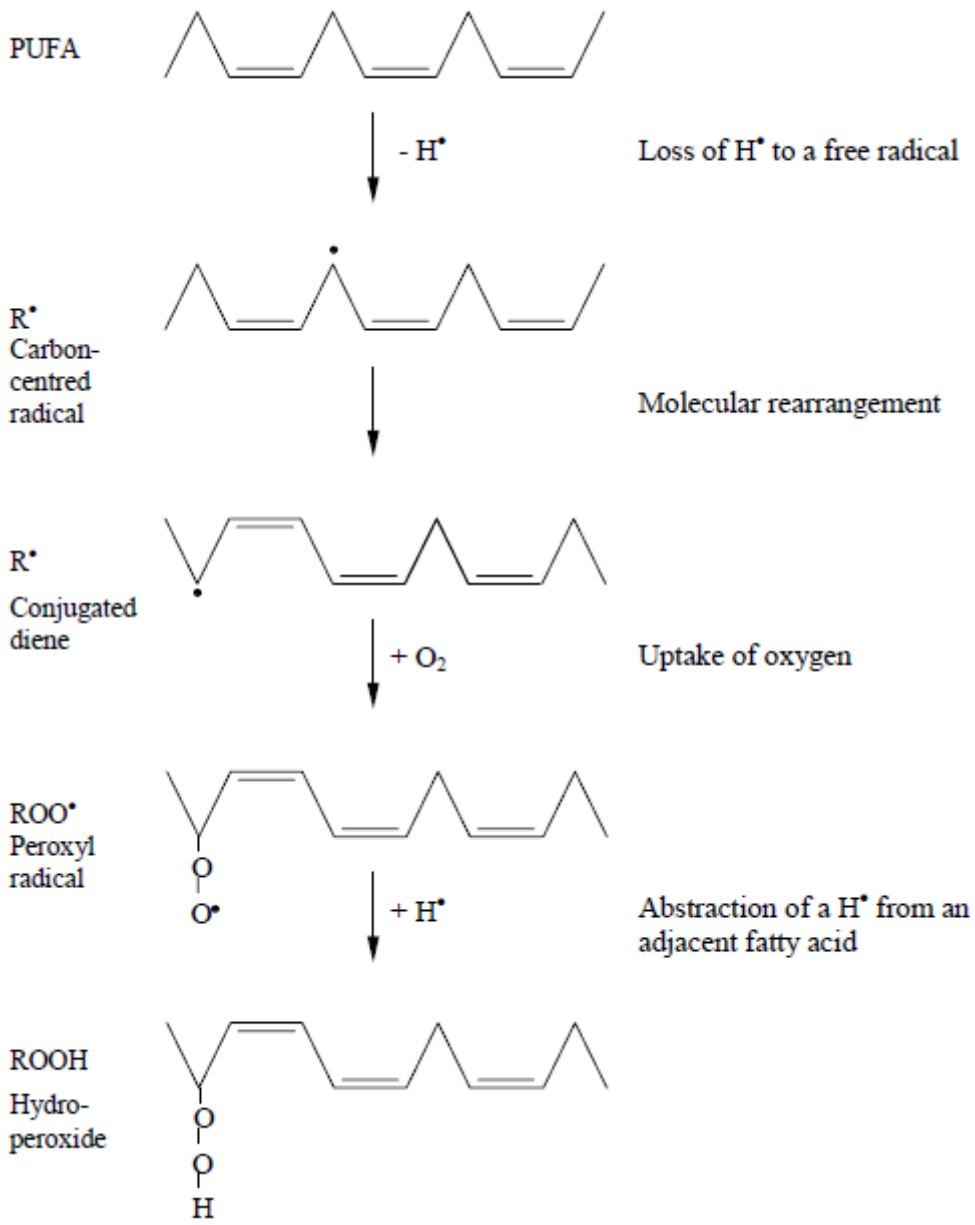


Figure 1.20. The Lipid Peroxidation Chain Reaction.

1.5 Protein Oxidation

Protein oxidation is defined as the covalent modification of a protein induced either directly by ROS or indirectly by reaction with secondary by-products of

oxidative stress. Agents that lead to protein oxidation include reagents such as H_2O_2 (Garrison, Jayko & Bennett, 1962) and HOCl (Heinecke, Li, Daehnke & Goldstein 1993; Handelman, Nightingale, Dolnikowski & Blumberg, 1998; Schraufstatter et al., 1990; Yan et al., 1996; Yang et al., 1997), xenobiotics such as paraquat (Korbashi, Kohen, Katzhendler & Chevion, 1986), CCl_4 (Hartley, Kroll & Petersen, 1997), and acetaminophen (Tirmenstein & Nelson, 1990), cigarette smoke (Eiserich, Van der Vliet, Handelman, Halliwell & Cross, 1995), reduced transition metals such as Fe^{2+} (Berlett & Stadtman, 1997) or Cu^{+} (Steinbrecher, Witztum, Parthasarathy & Steinberg, 1987; Yan, Lodge, Traber, Matsugo & Packer, 1997), γ-irradiation in the presence of O_2 (Davies, 1987; Davies, Delsignore & Lin, 1987; Fu & Dean, 1997; Garrison et al., 1962), activated neutrophils (Oliver, 1987), ultraviolet (UV) light (Balasubramanian, Du & Zigler, 1990; Hu & Tappel, 1992; Shen, Spikes, Kopecekova & Kopecek, 1996), ozone (Berlett, Levine & Stadtman, 1996; Cross et al., 1992), oxidoreductase enzymes (Fucci, Oliver, Coon & Stadtman, 1983), and by-products of lipid and free amino acid oxidation (Haberland, Fong & Cheng, 1988; Kim et al., 1997; Requena et al., 1997). Oxidative changes to proteins can lead to diverse functional consequences, such as inhibition of enzymatic and binding activities, increased susceptibility to aggregation and proteolysis, increased or decreased uptake by cells, and altered immunogenicity. The oxidative modification of the amino acid side chains, the conversion of one amino acid into a different one, the fragmentation of the peptide backbone and the formation of intra- and inter-molecular cross-links are common consequences of ROS-mediated protein oxidation (Stadtman & Levine, 2000). Carbonylation is an irreversible and non-enzymatic modification of proteins that involves the formation of carbonyl moieties induced by oxidative stress and other mechanisms (Berlett et al., 1997). Carbonyls (aldehydes and ketones) can be formed in proteins through four different pathways, namely, i) direct oxidation of the side chains from lysine, threonine, arginine and proline (Requena, Chao, Levine, & Stadtman, 2001), ii) non-enzymatic glycation in the presence of reducing sugars (Akagawa, Sasaki, Kurota, & Suyama, 2005); iii) oxidative cleavage of the peptide backbone via the α-amidation pathway or via oxidation of glutamyl side chains (Berlett et al., 1997; Garrison, 1987) and iv) covalent binding to non-protein carbonyl compounds such as 4-hydroxy–2-nonenal

(HNE) or malondialdehyde (MDA) (Feeney, Blankenhorn, & Dixon, 1975) (Figure 1.21). Among the four pathways, the direct oxidation of susceptible amino acid side chains has been highlighted as the main route for protein carbonylation and the most potent and major source of direct oxidative attack to proteins (Shacter, 2000; Stadtman, 1990; Stadtman, 2000). One of the greatest challenges in oxidation research today is the determination of oxidative stress in vivo. Because proteins are ubiquitous in all cells and tissues and are susceptible to oxidative modification, they can serve as useful markers of oxidative stres. Compared to measuring products of lipid peroxidation (Morrow et al., 1999) and DNA oxidative base modifications (Shigenaga, Aboujaoude, Chen & Ames, 1994), proteins offer some advantages as markers of oxidative stress. Proteins have unique biological functions, so there are unique functional consequences resulting from their modification (e.g., loss of clotting from oxidation of fibrinogen (Shacter, Williams & Levine, 1995), impaired ATP synthesis by oxidation of G3PDH (Levine & Ciolino, 1997). Products of protein oxidative modification are relatively stable and there are sensitive assays available for their detection; thus, from a purely technical perspective, they serve as suitable markers for oxidative stress. Importantly, the nature of the protein modification can give significant information as to the type of oxidant involved in the oxidation process.

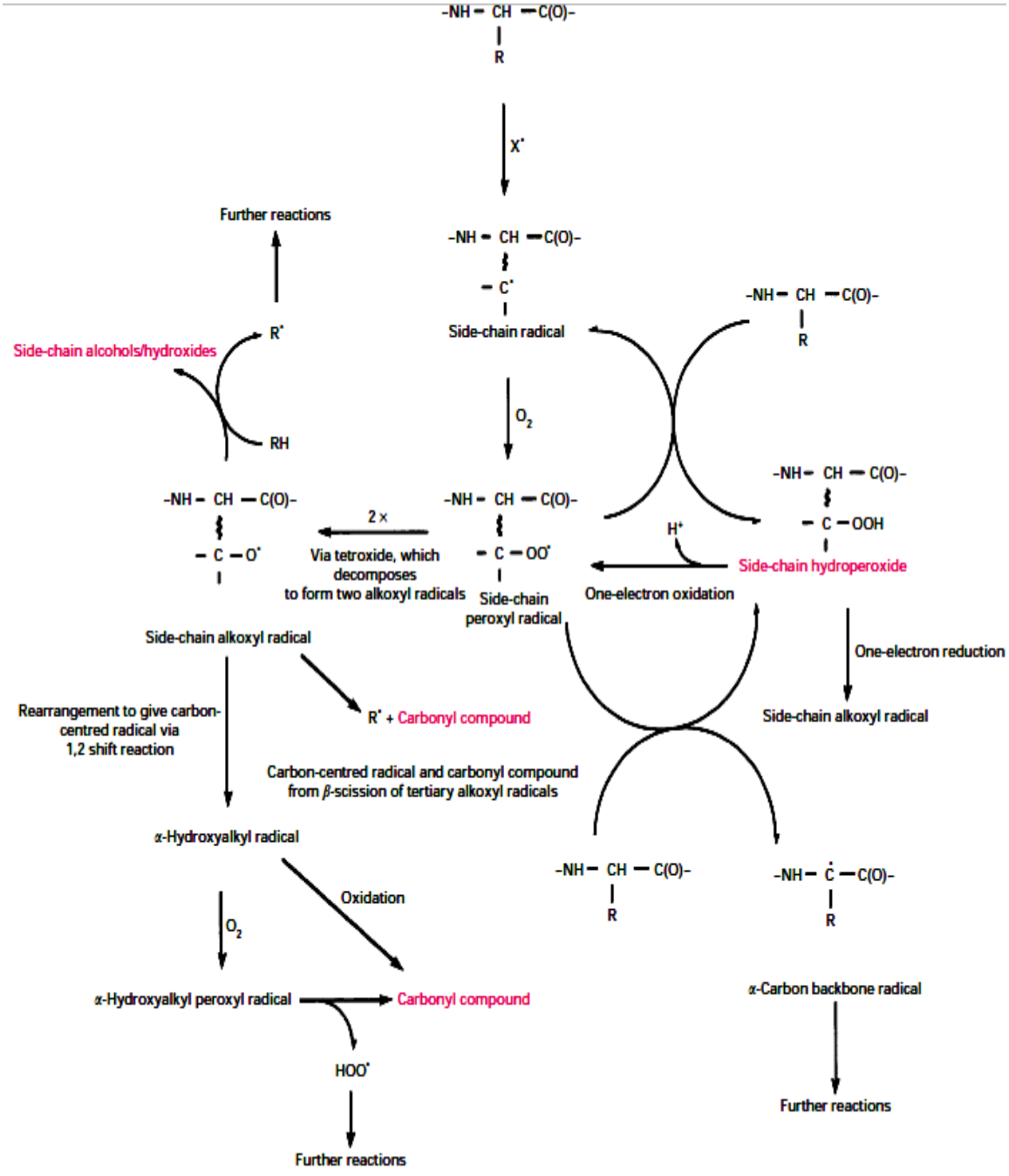


Figure 1.21. Some examples of protein oxidation reaction

1.6 Toxicity of Pesticides to Human

O_2 is a part of aerobic life, so it is not only essensial for enegy metabolism but also it has implicated most disease. It has two impair electrons and the stepwise reduction of O_2 is to form superoxide ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), hydroxyl

radical ($OH^{\bullet}$) and water. These reactive oxygen species (ROS) cause oxidative damage in a cell, tissue or organ. The targets of xenobiotics or metabolites are unsaturated fatty acyl chains in membranes, thiol groups in proteins, nucleic acid bases in DNA and enzymes.

1.6.1 Absorption

The ways of absorption are especially inhalation, ingestion, dermal. For showing the systemic effect of the pesticides that is absorbed from one of these ways, they must pass the biological membranes and reach the target location. Therefore, their effects can be different respect to the target and adsorption way.

Especially, the farmers that spray the pesticides onto their crops, can be influenced by dermal and inhalation absorption of the pesticides. Although the epidermis, nose and lungs are barrier for them, the high level absorption is toxic for human. Because the membran of epidermis cells has two lipid bilayers that bind covalently to protein. So, these pesticides are lipophilic substances. This property allows them to penetrate easily into the different cell compartments through the hydrophobic membrane barriers.

Because of various diseases, growers use the pesticides to protect the crops. However, the pesticides are penetrated through the outer surface of vegetables and fruits. If human eat these foods, the pesticides can be adsorbed from stomach and intestinal. Digestion system is covered by single layer of epithelial cells and intestinal cells are closely wrapped up lots of capillary vein. Therefore, adsorpsion of the pesticides from gastrointestion system and the passing to cardiovascular system are possible.

1.6.2 Biotransformation

Biotransformation reactions are essential chemical processes, mainly mediated by enzymes, which lead to the formation of metabolites that are excreted from the body.

Historically, this metabolism was known as a detoxication mechanism. Due to the large variety of chemical structures, biotransformation involves numerous and sophisticated reaction mechanisms and metabolic pathways that are catalysed by a large number of enzyme families. The general principle of biotransformation metabolism is to convert the lipophilic substances via several chemical steps, into hydrophilic, water-soluble derivatives which can easily be eliminated into urine and minimized the damage of xenobiotics. However, very highly lipophilic substances, polyhalogenated xenobiotics, such as some insecticides that can concentrate in the membrane compartment are sterically shielded from metabolic attack (Magdalou, Fournel-Gigleux, Testa & Ouzzine, 2003). But sometimes, the metabolites of these reactions can be toxic, too.

The metabolism of xenobiotics involves two sequential steps known as phase I and phase II reactions. During phase I, the xenobiotic is achieved by some reactions such as oxidation, reduction or hydrolysis. Phase I metabolites may be excreted prior to conjugation. During phase II, known as conjugation reactions with endogenous substrates such as glucuronic acid and sulphate, link to phase I metabolite. But some xenobiotics can be directly conjugated. The major function of xenobiotic metabolism is the production of physiologically useless compounds, some of which may be harmful. But especially pro-drugs can turn their active metabolite at the end of this reaction. Other metabolites, such as electrophiles, may be highly reactive entities able to bind covalently to circulating or intracellular proteins (formation of adducts), to enzymes (mechanism-based, irreversible inactivation), or to DNA (mutagenic and carcinogenic compounds). Enzyme systems contribute to xenobiotic metabolism are localised in the endoplasmic reticulum and cytocolic fraction of the cell. In the mammals, they are encountered in every tissue, but especially in the liver.

1.6.2.1 Phase I

Phase I reactions comprise oxidations (electron removal, dehydrogenation and hydroxylation), reductions (electron donation, hydrogenation and removal of oxygen), and hydrolytic reactions.

The oxidation reactions catalysed by particularly monooxygenases and other oxygenases (alcohol dehydrogenase, aldehyde dehydrogenase, monoamine oxidase). The main reaction is known as a monooxygenation and is supported by two main groups of monooxygenases: cytochromes P450 and flavine monooxygenases.

1.6.2.1.1 Oxidation Reactions

1.6.2.1.1.1 Cytochromes P450s. The cytochromes P450 constitute a superfamily of haem-thiolate enzymes (Lewis, 1996; Lewis, 2001). These are the major group of enzymes involved in the oxidation of xenobiotics. The cytochrome P450s play importantroles in the many fiels (Figure 1.22).

$$R—H + O_2 + NADPH + H^+ \longrightarrow R—OH + NADP^+ + H_2O$$

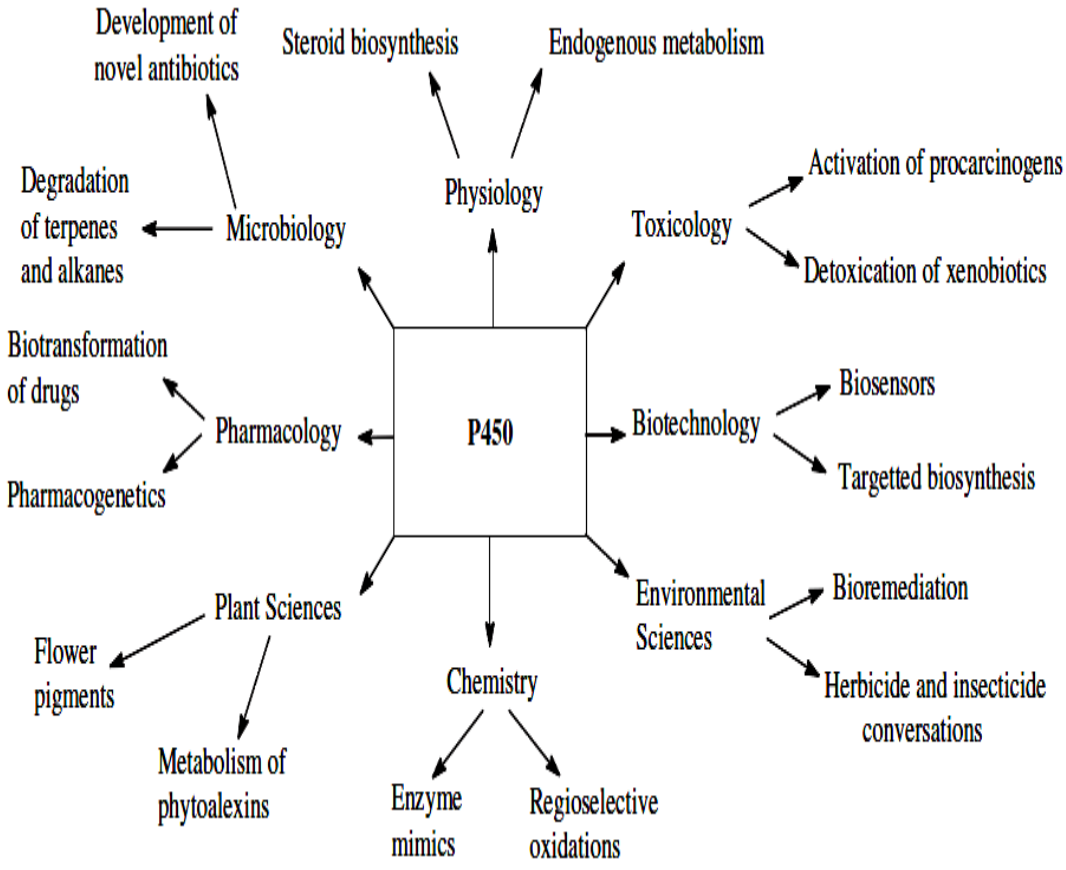


Figure 1.22. The many fields in which P450s play important roles.

In eukaryotic P450 systems, a membrane phospholipid bilayer such as that present in the microsomes of smooth endoplasmic reticulum is also able to bind. They represent up to 25% of the total microsomal proteins. Cytochromes P450 contain a molecule of haem, protoporphyrin IX, and a variable protein of MW~50 kDa. Due to the haem iron fifth ligand is a thiolate group, generally a cysteine residue, such protein exhibits a Soret absorption band at 450 nm in the CO-difference spectrum of a dithionite-reduced form.

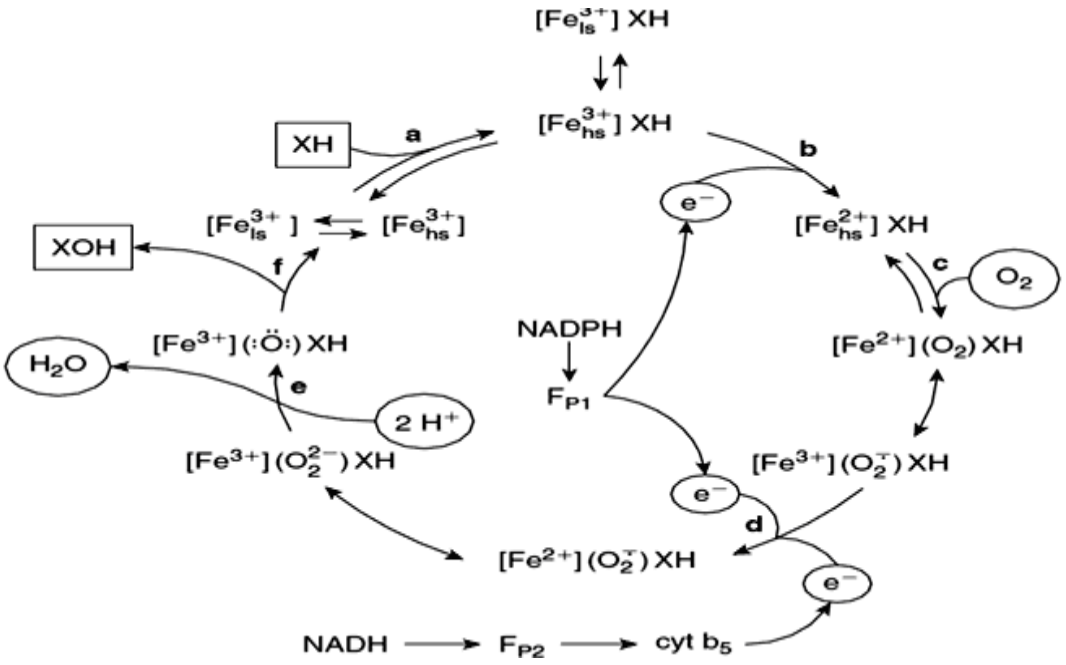


Figure 1.23. Catalytic cycle of cytochrome P450 associated with monooxygenase reactions. [Fe^{3}] = ferricytochrome P450; hs = high spin; ls =low spin; [Fe^{2}] = ferrocytochrome P450; F_{P1} = flavoprotein1 = NADPH-cytochrome P450 reductase; F_{P2} =NADH cytochrome b_5 reductase; cyt b_5 = cytochrome b_5; XH = substrate.

In Figure 1.23, cytochrome P450 requires a presentation of its catalytic cycle. The enzyme in its ferric (oxidized) form exists in equilibrium between two spin states, a hexacoordinated low-spin form whose reduction requires a high-energy level, and a pentacoordinated high-spin form. Binding of the substrate to enzyme induces a shift to the reducible high-spin form (reaction **a**). The first electron then enters the enzyme–substrate complex (reaction **b**), reducing the enzyme to its ferrous form, which has a high affinity for diatomic gases such as CO (a strong inhibitor of cytochrome P450) and dioxygen (reaction **c**). This allows molecular oxygen to bind as a third partner. The cycle continues with a second electron entering via either F_{P1}

or F_{P2} and reducing the ternary complex (reaction **d**). Electron transfer within the ternary complex generates bound peroxide anion (O_2^{2-}). The bound peroxide anion is split by the addition of two protons, liberating H_2O (reaction **e**). The remaining oxygen atom is equivalent to an oxene species, a neutral electrophilic species having only six electrons in its outer shell, stabilized by electron density from the iron. This is the reactive form of oxygen that will attack the substrate. The binary enzyme–product complex dissociates, thereby regenerating the initial state of cytochrome P450 (reaction **f**) (Testa, 2008).

1.6.2.1.1.2 Flavin Monooxygenases. Flavine monooxygenases (FMO) are a family of microsomal flavoproteins that catalyse the oxidation of numerous organic or inorganic compounds, including various structurally unrelated xenobiotics, in the presence of NADPH and oxygen. As for cytochrome P450, FMO are involved in detoxication and toxication reactions. FMO are 58 kDa proteins that possess a NADPH and flavine binding sites.

In Figure 1.24, the catalytic cycle of the reaction involves four steps:

(1) rapid reduction of FAD by NADPH;
(2) rapid introduction of molecular oxygen into FAD leading to the formation of the oxidating reagent, 4α-hydroxyperoxyflavine (E-FAD-OOH);
(3) attack of the oxygen on the nucleophilic group of the xenobiotic (X) with the concomitant formation of 4 α -hydroxyflavine;
(4) generation of the starting enzyme (E-FAD) upon release of a water molecule and $NADP^+$.

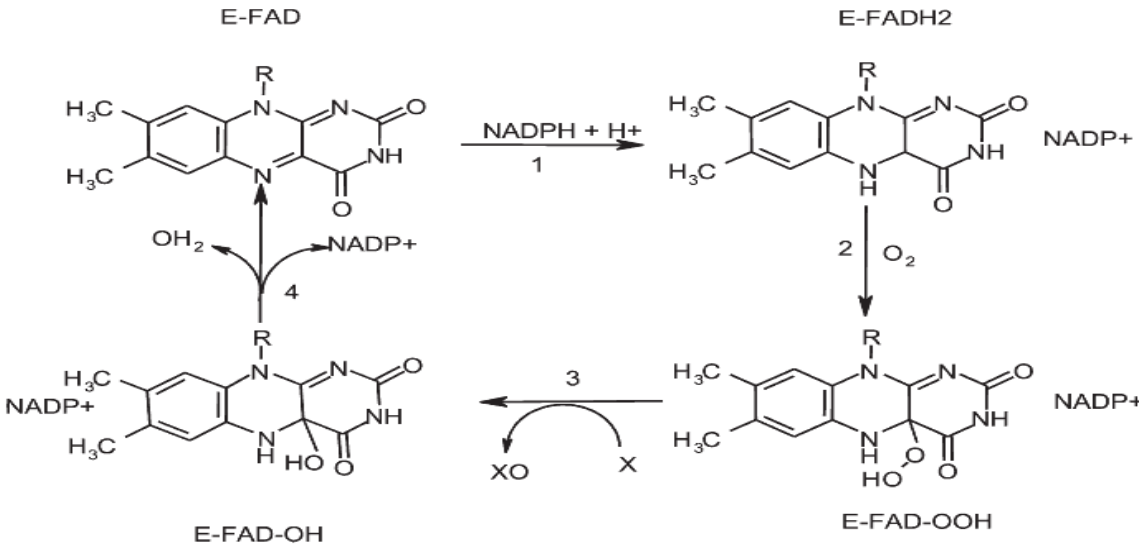


Figure 1.24. The catalytic cycle of flavin monooxygenases

1.6.2.1.1.3 Other Oxygenases. Alcohol dehydrogenases catalyse the oxidation of primary and secondary alcohols to aldehydes and ketones, respectively. Typical primary alcohols acting as substrate are ethanol, benzylic alcohol, phenylethanol, geraniol and retinol.

Alcohol dehydrogenases are zinc enzymes that use NAD^+ as coenzyme according to the reaction:

$$CH_3—CH_2—OH + NAD^+ \longrightarrow CH_3—CHO + NADH + H^+$$

The reaction involves the transfer of a hydride ion to the nicotinamide part of NAD^+ and is stereospecific. Aldehyde dehydrogenases transform aldehydes into carboxylic acids (e.g. succinaldehyde dehydrogenase). As with alcohol dehydrogenases, the key step of the reaction is the cleavage of the α-C—H bond, with a hydride transfer to NAD^+.

Monoamine oxidases (MAO) are mitochondrial enzymes existing in two forms, MAO-A and MAO-B. Their physiological function is to deaminate endogenous amines, in particular catecholamines. Although they are involved in xenobiotic metabolism, the products of reaction, ammonia, hydrogen peroxide and aldehyde are

themselves potentially toxic. The aldehydes formed may be further metabolized by aldehyde dehydrogenases or aldehyde oxidases corresponding to the carboxylic acids or by aldehyde reductases to the alcohols (Figure 1.25).

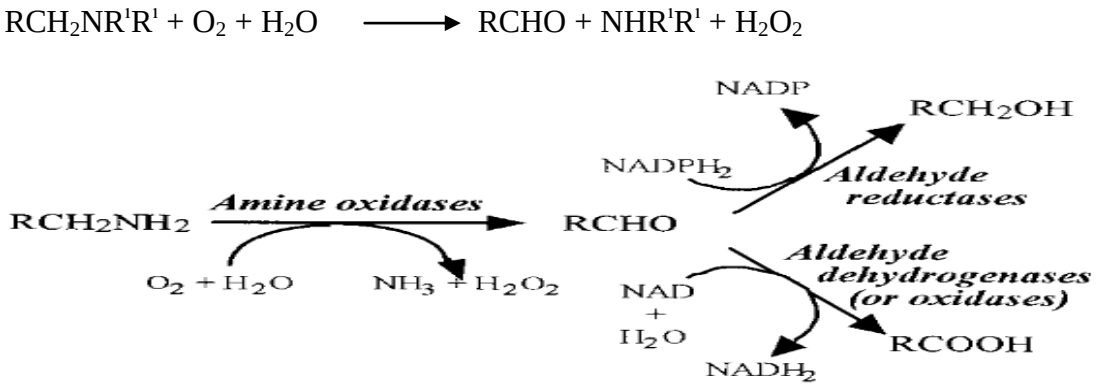


Figure 1.25. The basic reactions of amine oxidases.

1.6.2.1.2 Reduction Reactions

As seen in Figure 1.26, Aldehydes and ketones are readily reduced to primary and secondary alcohols, respectively. Quinones can be reduced to dihydrodiols either by a two electron mechanism (carbonyl reductase and quinone reductase) or by two single electron steps (cytochromes P450 and some flavoproteins). Dehalogenation reactions can also proceed reductively. Reductive dehalogenations involve replacement of a halogen by a hydrogen, or vic-bisdehalogenation.

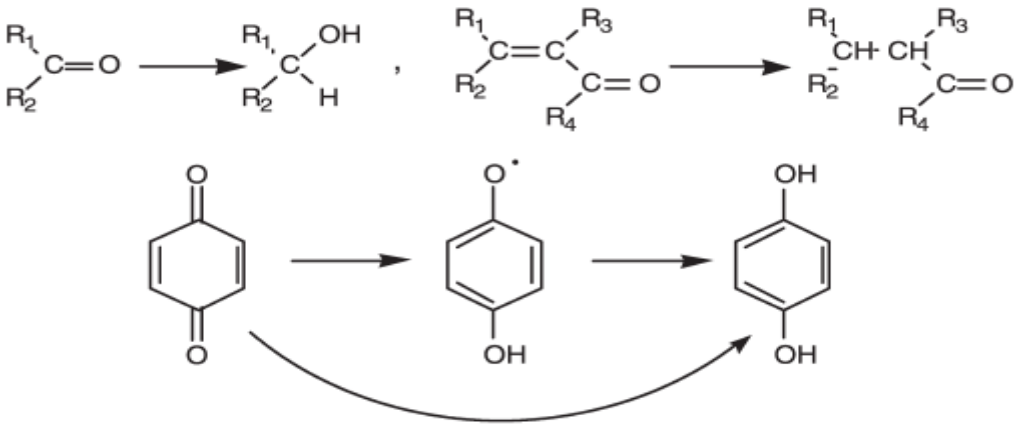


Figure 1.26. Some examples of reduction reactions.

1.6.2.1.3 Hydrolase Reactions

Hydrolases constitute a very complex ensemble of enzymes many of which are known or suspected to be involved in xenobiotic metabolism. Relevant enzymes among the serine hydrolases include carboxylesterases, arylesterases, cholinesterases and a number of serine endopeptidases. Other hydrolases are arylsulfatases, aryldialkylphosphatases, β-glucuronidases, epoxide hydrolases, cysteine endopeptidases, aspartic endopeptidases and metallo-endopeptidases. Arylsulfatases and β-glucuronidases can cleave the conjugates formed by the sulfo- and glucuronosyltransferases, leading back to the parent compound. The overall reaction catalyzed by epoxide hydrolases is the addition of a water molecule to an epoxide. Together with GSH conjugation, hydration is a major pathway in the inactivation and detoxification of arene oxides. Two main groups of epoxide hydrolases are located in the microsomal (mEH) and cytosolic (soluble, sEH) fractions. They present marked differences in substrate specificity. mEH catalyses the hydrolysis of the procarcinogenic polycyclic aromatic compounds, epoxide derivatives of 1,3-butadiene and aflatoxin B1 or drug epoxide metabolites of the anticonvulsant drugs, phenytoin or carbamazepine. Trans-substituted epoxides and aliphatic epoxides from fatty acid metabolism are substrates of sEH.

1.6.2.2 Phase II

Conjugation reactions link either to the original drug (if it is polar) or to the phase I metabolite. Conjugates are often more polar and water-soluble and readily excreted via the renal route. Conjugation reactions also occur for endogenous compounds, such as the toxic bilirubin formed from the metabolism of haemoproteins or steroid hormones and retinoic acid which can compete with drugs toward the same binding site. By abolishing their physiological properties and modulating their concentration, conjugation reactions play a major role in the regulation of the biological activity of numerous compounds involved in cell growth and differentiation, and are believed to participate in the evolution of major diseases, such as cancer (Neberti, 1994) Reactions of conjugation are catalysed by families of enzymes known as

transferases. The transferases are located in microsomes and in the cytosolic fraction (soluble forms).

The reactions of Phase II are classified as methylation, glucuronidation, sulfonation, acetylation and conjugation with coenzyme A and glutathione.

Metylation reactions involve the transfer of a methyl group from the cofactor S - adenosyl- L -methionine (SAM) and are catalyzed by methyl transferase. Sulfonation reactions consist in the transfer of a sulfate being transferred from the cofactor 3^{1} - phosphoadenosine 5^{1} -phosphosulfate (PAPS) by sulfotransferase. Sulfotransferases, which catalyze a variety of physiological reactions, are soluble enzymes (Banoglu, 2000; Kauffman, 2004; Nagata & Yamazoe, 2000; Testa, 2007; Testa & Krämer, 2008). The sulfate moiety in PAPS is linked to a phosphate group by an anhydride bridge whose cleavage is exothermic and supplies enthalpy to the reaction. The nucleophilic — OH or — NH — site in the substrate will react with the leaving SO_3^- moiety, forming an ester sulfate or a sulfamate. Some of these conjugates are unstable under biological conditions and will form electrophilic intermediates of considerable toxicological significance. Glucuronidation reactions involve the transfer to the substrate of a molecule of glucuronic acid from the cofactor uridine-5^{1} -diphospho- α - D -glucuronic acid (UDPGA). These reactions are catalyzed by UDP-glucuronosyltransferases which are microsomal enzymes. In acetylation reactions, there is a major enzyme system as arylamine N –acetyltransferase and their cofactor is acetylcoenzyme A. Mainly amines are the substrates of acetylation. Glutathione, the major intracellular thiol, and the associated enzymes have been widely studied for their implication in the protection against free radicals as well as in detoxification. Cytosolic glutathione S-transferases catalyze the conjugation of electrophilic compounds to reduced glutathione (GSH).

1.7 Bromopropylate

Chemical name: isopropyl–4,4'-dibromobenzilate

Formula:

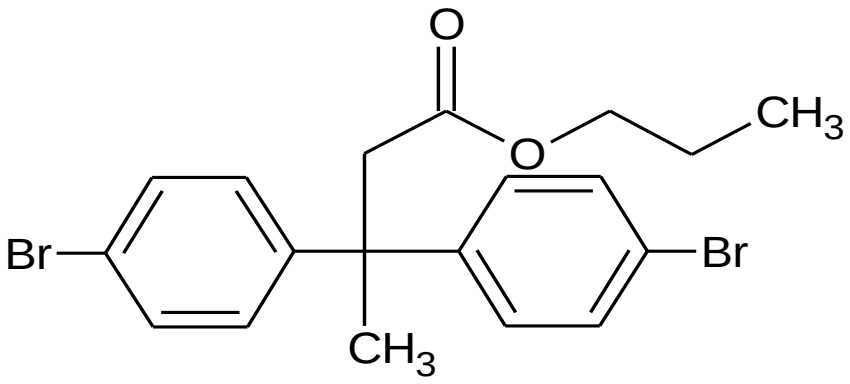


Figure 1.27. The chemical structure of bromopropylate.

1.7.1 General Properties

The general properties of bromoropylate were given in Table 1.3.

Table 1.3. The general properties of bromopropylate.

Physical state	White crystalline powder
Molecule formule	$C_{17}H_{16}Br_2O_3$
Moleculer weight	428.13 g/mol
Melting point	77°C
Vapour pressure	5.5×10^{-7} mm Hg at 20°C 5.25×10^{-3} mm Hg at 100°C
Density	1.59 g/cm^3 at 20°C
Solubility	<0.5 ppm in water at 20°C, soluble in organic solvents
Stability	Fairly stable in neutral or slightly acid media; half-life periods in aqueous 10% methanol at pH 0 (in HCI), 50 days;

	at pH 6–7 (water), >3 years; at pH 9 (0.05 M borax buffer), 15 days;

1.7.2 Uses of Bromopropylate

Bromopropylate is a contact miticide effective against all stages of mites such as *Eriophyidae* (eriphyid mites), *Tenuipalpidae* (false spider mites) and *Tetranychidae* (spider mites). Although the chemical does not have a pronounced ovicidal action, it displays some activity on the early egg development stages, Newly hatched larvae are killed by contact with the araricidal deposit on the foliage.

It has uses in various vagetables and fruits, especially cotton, grapes, soya beans, orange.

1.7.3 Toxicological Effects

The toxicological effects of bromopropylate were given in Table 1.4.

Table 1.4. The acute toxicity of bromopropylate.

Species	**Sex**	**Route**	**Purity**	**LD_{50} (mg/kg)**	**Reference**
Mouse	Male	Oral	Technic	8 000	(JMPR, 1993)
Rat	Male+Female	Oral	Technic	>5 000	(JMPR, 1993)
Rat	Male+Female	Oral	Formulation	6 000	(JMPR, 1993)
Rat		Oral	Formulation	>23 100	(JMPR, 1993)

Short-term studies were summarized as;

Paterson studied that groups including 10 male and 10 female rats were fed 0, 40, 200, 1000 and 5000 mg bromopropylate/kg bw by gavage as a suspension in 0.5% aqueous on 6^{th} days each week for four weeks. The highest dosage level produced polyuria throughout the 21 days before the animals were killed. The rate of body weight gain and food intake were reduced in this and the 1000 and 200 mg/kg groups. The absolute and relative liver weights were increased in the three highest dosage groups and cytoplasmic swelling and periportal infiltration were seen. Hepatic necrosis occurred at the highest dosage level (JMPR, 1993).

Paterson et al. studied that groups of 12 male and 12 female rats were fed for 90 days on diets containing 0, 100, 300 and 1000 ppm bromopropylate. Another group received diet containing 3000 ppm for 55 days and 4000 ppm for a further 35 days. In the highest dosage level group the food intake and rate of weight gain were below normal and at autopsy the liver, kidney and testes weights were heavier than in controls relative to body weight, and loss of basophil material, pigmentation and fatty infiltration were present in hepatocytes. At the 1000 ppm level food intake was slightly less than in controls although weight gain was similar. At the two lower levels smaller numbers of rats showed loss of basophil material and cytoplasmic swelling with focal vacuolation (JMPR, 1993).

Long-term studies were summarized as;

Coulston et al. studied that groups of 50 male and 50 female rats were fed for two years on diets containing 0, 15, 30 and 100 ppm bromopropylate. Five male and five female rats from each group were killed after six months and one year and autopsies performed. Food consumption and weight gain were similar in test and control animals throughout the test and survival rates were similar for the first 18 months. Approximately half of these control female animals alive at 18 months survived to 24 months while only a third of test animals on 30 and 100 ppm levels survived over this period. EM studies on the livers of animals of the 100 ppm group surviving two

years showed slightly less, focal enlargement of intracrestal space in the mitochondria, focal dilatation of SER and more prominent lipid accumulations than livers of control animals. These differences were not considered to be significant pathological alterations in cell ultrastructure. The number of tumours and their location were similar in control and test groups (JMPR, 1993).

1.8 Eukaryotes

Biological classification is a method that categorizes organisms by biological type, such as genus or species. Biological classification is a form of scientific taxonomy. According to biological classification, organisms firstly divide two domains as prokaryotes and eukaryotes. Difference between prokaryote and eukaryote is whether there are nuclear envelope which encloses the genetic material and membrane-bound organelles. Examples of prokaryotes are bacteria and blue-green algs. However, eukaryotes have six kingdoms as bacteria, protozoa, chromista, plantae, fungi and animalia. The some species of eukaryotes are large complex organisms including animals, plants and fungi, although some species of eukaryotic protists are microorganisms.

1.8.1 Fungi

Fungi are a diverse group of organisms belonging to kingdom Eukaryotes (Hageskal et al., 2009). They are non-motile, their species range from simple, single-celled organisms to very complex, multicellular organisms. Fungi are chemo-organotrophic eukaryotes and exhibit dynamic interactions with their nutritional environment that may be exemplified by certain morphological changes depending on nutrient availability. Most fungi are free living, can be classified into groups such as filamentous fungi (also called moulds or mycelia), yeasts, and mushrooms (Hageskal et al., 2009; Osiewacz, 2002).

Filamentous fungi such as *Trichoderma harzianum* are typically saprophytic microorganisms which secrete a wide array of enzymes involved in the

decomposition and recycling of complex biopolymers from both plant and animal tissues (Osiewacz, 2002). The majority of these enzymes are hydrolytic and play an important role in fungal nutrition, releasing carbon and nitrogen locked in insoluble macromolecules obtained from the metabolic activities of other organisms. For more than a century, fungi have been known to produce and secrete different types of enzymes in large quantities, which has resulted in an increasing interest in studying and using filamentous fungi in industrial processes. Filamentous fungi are known to produce many organic acids, polysaccharides, enzymes, plant growth regulators, alkaloids, pigments, mycotoxins, and antibiotics (El-Enshasy, 2007). The growth mechanism of the filamentous fungi in liquid culture ranges from dispersed mycelia to compact pellets (More, Yan, Tyagi & Surampalli, 2010).

Heavy metals can accumulate in soil and plants when sludge is applied as fertilizer and eventually can produce harmful effects in animals and humans. These metals are threats to the environment because of their non-biodegradability and consequent persistence (Datta, 2002; Lasheen, Ashmaway & Ibrahim, 2000). Fungi are known to have metal absorbing capability in their cell wall, or by extracellular polysaccharide slime. Presence of high concentration of some heavy metals is fatal for fungi, however, fungi are able to utilize them at low concentrations as their micronutrient source (Roy, Matthias, Mandal, Wendt-Potthoff & Bhattacharya, 2009). The cell walls of fungi contain high content of functional groups like amino, amide, hydroxyl, carboxyl, sulphydryl and phosphate. The metal binding capacity in the fungi is due to presence of these functional groups, which are implicated in metal binding (27) (Akhtar & Mohan, 1995).

In many industrial processes, certain toxic substances are generated which are found in the sludge together with normal pathogens and microorganisms. Industries such as olive oil, starch, distillery, cotton bleaching, pulp and paper processing produce several billion litres of coloured, toxic and harmful sludge solids all over the world. The presence of pesticides, polycyclic aromatic hydrocarbons (PAHs), Polychlorinated biphenyls (PCBs) in sludge may pose risk and can restrict the land applications of the sludge. The repeated application of highly concentrated and

readily degradable chemicals can also eventually increase its concentration in the soil (Harrison, Oakes, Hysell & Hay, 2006).

Lower eukaryotes, like filamentous fungi, are attractive organisms to study fundamental processes of the eukaryotic cell. Also, compared to bacteria, the fungi have advantage of being able to grow on a medium of low pH, low nitrogen content and low temperature (Sladka, 1966). Relative to higher eukaryotes, fungi have the advantage of easy cultivation on simple defined media with short generation times and easy accessibility towards molecular and classical genetics. The entire genome sequence of a growing number of fungal species is available, which enables to extensively apply modern system biology approaches. The fact that fungi are more related to animals, emphasizes the value of these organisms as favourable models for human cells (van der Klei & Veenhuis, 2006).

1.8.2 Trichoderma harzianum

The filamentous fungus *Trichoderma harzianum* Rifai is a common soil species used in biological control against a range of important airborne and soilborne phytopathogens such as *Botrytis cinerea, Fusarium* spp., *Pythium* spp., *Rhizoctonia solani* and *Sclerotium rolfsii* (Chet 1987; Papavizas 1985). In addition to the biocontrol of plant diseases, this species has potential for the enhancement of plant growth and resistance (Bailey & Lumsden, 1998). The biocontrol mechanisms of *Trichoderma* spp. are myriad and varied; they include mycoparasitism, antibiosis, competition for resources and space, and promotion of plant growth (Ghisalberti & Sivasithamparam, 1991). It is clear that the abilities of *Trichoderma* spp. to inhibit the growth of other fungi are probably due to the combined action of cell-wall degrading enzymes (CWDEs) (Lorito, Woo, Donzelli & Scala, 1996). Cellulases (Benkoet al., 2007), chitinases (Carsolio, Gutierrez, Jimenez, Montagu & Herrera-Estrella, 1994; El-Katatny, Gudelj, Elnaghy & Gubitz, 2001; Mach et al., 1999; Nguyen, Kim, Oh, Jung & Park, 2008), β -1,3-glucanases (El-Katatny et al., 2001) β-1,6-glucanases (Montero, Sanz, Rey, Monte & Llobell, 2005) and proteases (Geremia et al., 1993; Grinyer, Hunt, McKay, Herbert & Nevalainen, 2005; Pozo,

Baek, Garcia & Kenerley, 2004) have been described as important components of the multi-enzymatic system and the antifungal ability of *Trichoderma* spp.

CHAPTER TWO

MATERIAL AND METHOD

2.1 Media and Growth Conditions

Trichoderma harzianum was obtained from DSMZ. Malt peptone agar, malt yeast glucose agar and potato dextrose agar were studied at 25^{o}C for 7 days. Their contents were showed in Table 2.1. The best grown of *Trichoderma harzianum* was obtained on solid medium MYGA (Deane, Whipps, Lynch & Peberdy, 1998). The five different liquid cultures shown in Table 2.2 were investigated.

Table 2.1. Studied solid mediums.

Malt peptone agar (MPA)	**Malt yeast glucose agar (MYGA)**	**Potato dextrose agar (PDA)**
30 g malt extract, 30 g soya peptone, 15 g agar in 1 liter distilled water	5 g malt extract, 2.5 g yeast extract, 10 g glucose, 20 g agar in 1 liter distilled water	4 g potato infusion, 20 g glucose, 15 g agar in 1 liter distilled water

Table 2.2. Studied liquid mediums.

Liquid 1 (Sua´rez et al., 2005)	**Liquid 2** (Yang et al., 2009)	**Liquid 3** (Sarhy-Bagnon, Lozano, Saucedo-Castan˜eda, & Roussos, 2000)	**Liquid 4** (Liu et al., 2007)	**Liquid 5** (Deane et al., 1998
0.68 mg KH_2PO_4, 0.87 mg K_2HPO4, 1.7 g $(NH_4)_2SO_4$, 200 mg KCl, 200 mg $CaCl_2$, 200 mg $MgSO_4.7H_2O$, 2 mg $FeSO_4$, 2 mg $ZnSO_4$ in 1 liter distilled water	1.4 g $(NH_4)_2SO_4$, 2 g KH_2PO_4, 6.9 g Na_2HPO_4, 0.3 g $MgSO_4.7H_2O$, 1 g peptone, 0.3 g urea, 1% (w/v) glucose in 1 liter distilled water	7 g KH_2PO_4, 2 g Na_2HPO_4, 0.94 g $(NH_4)_2SO_4$, 1.5 g $MgSO_4.7H_2O$, 0.1 g $CaCl_2.7H_2O$, 8 mg $FeCl_3.6H_2O$, 0.1 mg $ZnSO_4$, 30 g glucose in 1 liter distilled water	7 g KH_2PO_4, 2 g Na_2HPO_4, 1.5 g $MgSO_4.7H_2O$, 2 mg $FeCl_3$, 0.5 g $(NH_4)_2SO_4$, 0.1 g $CaCl_2$, 1 mg $ZnSO_4.7$ H_2O, 30 g glucose in 1 liter distilled water	2 g KH_2PO_4, 1.4 g $(NH_4)_2SO_4$, 0.3 g $MgSO_4.7H_2O$, 0.3 g $CaCl_2.7H_2O$, 5 mg $FeSO_4.7H_2O$, 2 mg $ZnSO_4$, 2 mg MnSO4, 2 mg $CoCl_2$, 1 g peptone, 0.3 g urea, 0.3% (w/v) glucose in 1 liter distilled water

The pH was adjusted to 5.6 before autoclaving. The cultures were inoculated with 1 mL spore suspensions (OD_{620} = 2.000) in 250 ml shaking flasks containing 100 ml of culture and incubated with agitation at 180 rpm at 28°C. After the cultivation process, the cells were collected and followed by washing with distilled water and kept at -20°C.

2.2 Isolation of Mitochondria

100, 200 and 300 mM sucrose concentrations; homogenization and glassbead breakdown methods; 4–12 times isolation buffer volumes; 5, 10, 15, 2x15, 30 and 2x30 seconds homogenization periods; 1000, 1500 and 2000 rpm centrifugation rates were studied. The best isolation was determined such as; The thawed samples were resuspended in 10mM Trizma-HCl buffer, pH 7.5, containing 1 mM EDTA, 300 mM sucrose and 0.1% BSA in a volume equal to 8 times its weight. Sample cells were homogenized at 8500 rpm for 15 seconds in the ice. Cell suspension was ground in 1.5 ml plastic vials and centrifuged at 2000 rpm for 15 minutes and cell debris was removed. The supernatant was centrifuged at 15000 rpm for 15 minutes. Final pellet

contains mitochondria. Before assaying, the pellet was resuspended in isolation buffer without BSA.

2.3 Enzyme Activity Assay

2.3.1 Succinate Dehydrogenase Activity Assay (Complex II)

10, 20 and 30 mM of sodium succinate were investigated. The optimum concentration was determinated 20 mM sodium succinate. Succinate dehydrogenase was assayed by measuring the initial rate of decrease in DCIP absorbance at 600 nm. The reaction mixture contained 50 mM potassium phosphate buffer, pH 7.0, 1.0 mM EDTA, 20 mM sodium succinate, 3 mM sodium azid, enzyme solution and 32 µM DCIP.

2.3.2 Cytochrome c Oxidase Activity Assay (Complex IV)

20, 30 and 50 µM reduced cytochrome c were investigated. The optimum concentration was determinated 30 µM reduced cytochrome c. Cytochrome c oxidase was assayed by measuring the initial rate of decrease in cytochrome c absorbance which was reduced by $NaBH_4$ at 550 nm. The reaction mixture contained 50 mM potassium phosphate buffer, pH 7.0, 30 µM reduced cytochrome c. Cytochrome c in 10 mM potassium phosphate buffer, pH 7.5 was reduced by adding sodium borohydride and monitoring the absorbance at 550 nm and 565 nm. Enough sodium borohydride was added to produce an absorbance ratio at 550/565 nm greater than 10 (Anderson & Friedberg, 1980).

2.4 Bromopropylate Determination

2.4.1 The extraction

Extracellular bromopropylate in liquid culture was recovered by solid phase extractions using Envocarb C18 SPE cartridge. The method described by Gomis et

al. (1996) was modified as: the SPE cartridge (100 mg) was activated with 400 µL methanol, then 400 µL distillated water and finally with 1 mL of methanol/water (1:1) mixture. The extracellular liquid of growth medium was loaded up on the SPE cartridge, washed with 1.8 mL methanol: water (1:1) and dried for under vacuum. The elution of bromopropylate was performed with 1 mL hexane (Gomis, Mangas, Castano & Gutierrez, 1996).

2.4.2 GC/MS Condition

The pesticide concentration was measured by GC/MS. Sample extracts were injected into the GC/MS system under the following conditions: split injection mode; injection volume, 5 µL; injector temperature, 250°C; detector temperature, 280°C; and carrier gas, helium. The gas chromatograph was operated in constant flow mode (electronic pressure control) at 36.2 mL/min. GC temperature program: hold 0.75 min at 50°C, 50° to 150°C at 25°C/min, 150° to 200°C at 3°C/min, 200° to 280°C at 8°C/min, hold 15 min at 280°C. A 0.25mm x 30m x 0.25µm HP-5MS column was used.

2.5 Adenine Nücleotids and Organic Acids Content Assay

2.5.1 Sample preparation

The different extraction procedures were studied as; (i) boiling ethanol, (ii) 50% (v/v) boiling ethanol, (iii) boiling water, (iv) 30% cold perchloric acid (PCA), (v) 15% cold PCA, (vi) cold 6% PCA.

The samples were prepared by a modification of the procedure of Ganzera et al. (2006). (Ganzera, Vrabl, Wörle, Burgstaller & Stuppner, 2006). Intracellular metabolites from the cell pellet was extracted in a solution of boiling water for 15 minutes. Boiling water (w/v) was directly transferred into chilled sample. This solution was shaken for 15 min at 90 ^{o}C in a water bath, cooled in the ice, and then centrifuged at 12000 rpm for 10 min. The supernatant was quickly frozen and

lyophilised. The lyophilyzate was resolved in 200 µL of ultra pure water and then filtered through a 0.45 µm filter before injecting into the HPLC.

2.5.2 HPLC Conditions for Adenine Nücleotids

The HP 1100 HPLC system used was equipped with a photodiode detector and Thermo ODS-2 Hypersil column(250x4.6 mm). 50 mM aqueous triethylamin (TEA) buffer (adjusted with phosphoric acid to pH 6.5; A) and acetonitrile (B). Gradient elution was performed from 99 A/1B in 10 min to 95A/5B and changed in another 10 min to 92.5A/ 7.5B. Each run was followed by a 5-min wash with 70B/30 parts 0.1% phosphoric acid. Detection wavelength, flow rate, column temperature were set to 254 nm, 1 mL/min, 20 ^{o}C (Ganzera et al., 2006).

2.5.3 HPLC Conditions for Metabolites of Glycolysis and Tricarboxylacid Cycle

The HP 1100 HPLC system used was equipped with Alltech IOA–1000 column, a UV detector. 0.4 mL/min mobile phase using 9.0 mM H_2SO_4 solution was applied to the column. The column was operated at 42 ^{o}C. Standards were prepared for pyruvate, citrate, α-ketoglutarate and fumarate for UV detector (210 nm), and calibration curves were created (Ayar Kayalı, 2005).

2.6 Lipid Peroxidation

Cells homogenized in isolation buffer, (1:2 w/v), pH 7.5. 500µL homogenate was transferred into 2.5 mL 10% TCA, incubated 90ºC for 15 minute, cooled and then centrifuged at 3900 rpm for 10 minute. 2 ml supernatant was added into 1 mL 0.675% TBA solution. The mixture was incubated 90 ºC for 15 minute. After cooling, the absorbance was measured 532 nm. Malondialdehyde (MDA), an end product of fatty acid peroxidation, reacts with TBA and forms a coloured complex. This complex has maximum absorbance at 532 nm. MDA values in nanomoles were calculated from the absorbance coefficient of MDA-TBA complex at 532 nm, 1.56×10^{5} mol^{-1} x cm^{-1} (Buege & Aust, 1978).

2.7 Protein Determination

The protein content was determined by the method of Bradford et al. (1976) (Bradford, 1976). Bovine serum albumin as standard was used.

CHAPTER THREE

RESULTS AND DISCUSSION

3.1 Growth Medium Variations

3.1.1 Growth Curve Variations of T. harzianum Depending on Incubation Period

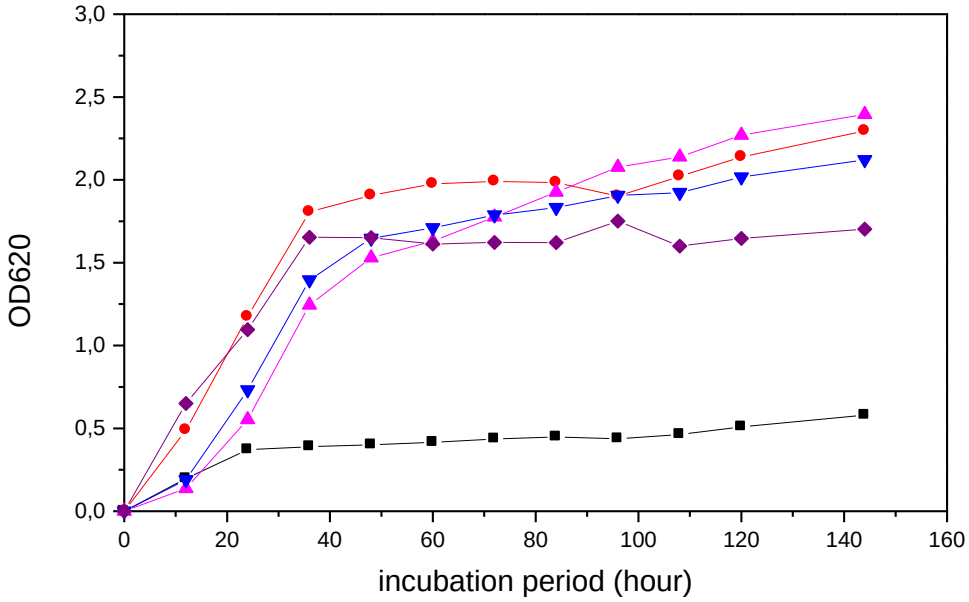


Figure 3.1 Variations in optic density (OD) level of *T. harzianum* in studied growth mediums depending on incubation period; liquid medium 1 (—■—), liquid medium 2 (—●—), liquid medium 3 (—▲—), liquid medium 4 (—▼—), liquid medium 5 (—♦—).

As shown in Figure 3.1, *T. harzianum* displayed logarithmic growth during the first 48 h for liquid medium 2. Then, after 48th h, it entered into a stationary phase. In liquid medium 1, biomass levels were significantly lower compared with the other used mediums. In liquid medium 5, logarithmic growth wasn't displayed during first

36 h. Liquid medium 2 was chosen for the following experiments (Grinyer et al., 2005).

3.1.2 pH Level Variations of Growth Mediums in T. harzianum Depending on Incubation Period

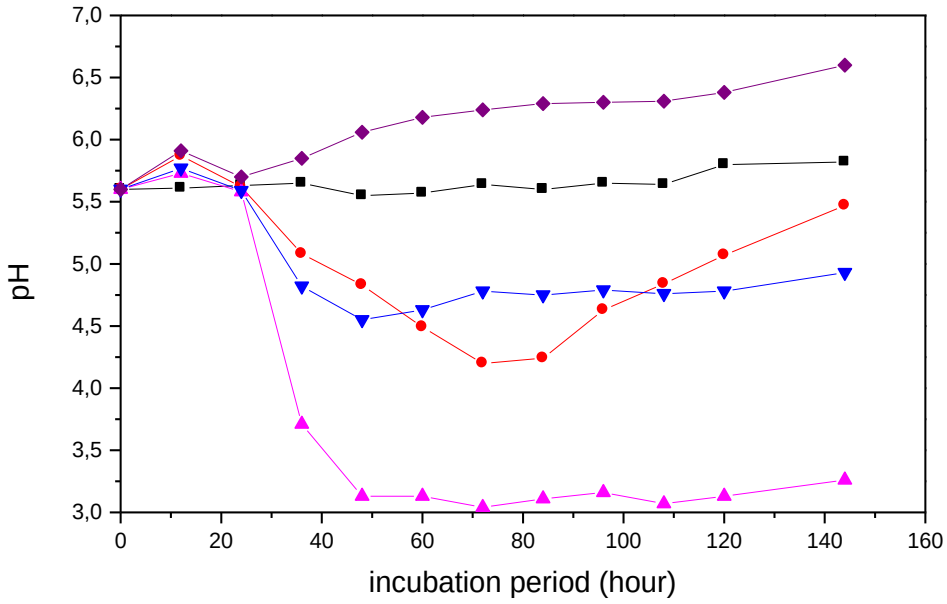


Figure 3.2 Variations in pH level in *T. harzianum* in studied liquid mediums depending on incubation period; liquid medium 1 (—■—), liquid medium 2 (—●—), liquid medium 3 (—▲—), liquid medium 4 (—▼—), liquid medium 5 (—♦—).

According to the results as seen in Figure 3.2, pH levels of *T. harzianum* rose up to 12th hour for all studied liquid mediums and then decreased continuously for liquid mediums 2, 3 and 4 until 48th h. For liquid medium 2, the decrease continued up to 72nd hour and pH rose up after 84th h. The pH levels in liquid medium 3 and 4 did not change significantly after 48th h. In liquid medium 1, the pH levels did not change markedly until the end of incubation period.

3.2 Variations in Succinate Dehydrogenase and Cytochrome c Oxidase Activities of *T. harzianum* Depending on Bromopropylate Concentration and Incubation Period

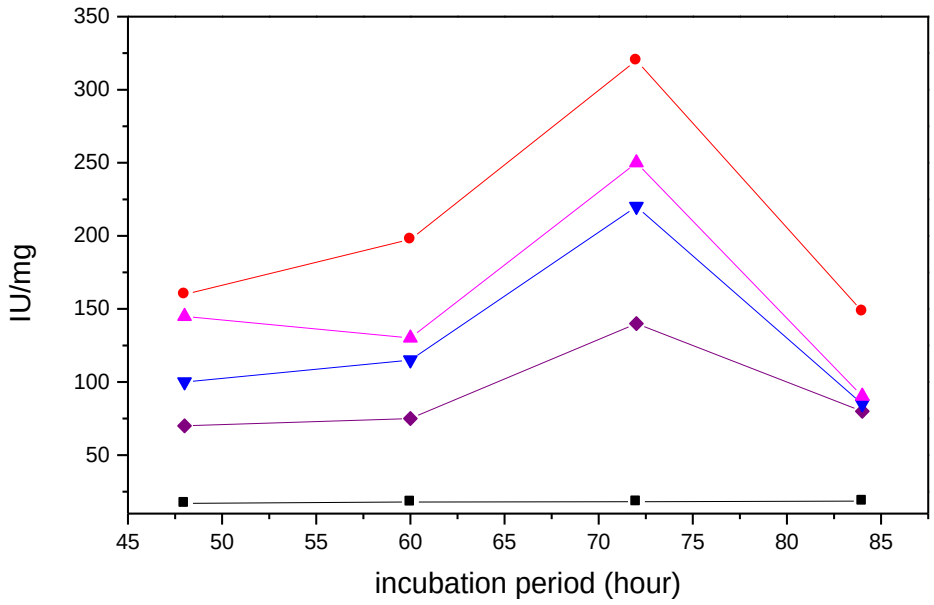


Figure 3.3 Variations in SDH activity in *T. harzianum* depending on the incubation period in medium containing; control (—■—), 2.5 mg/L (—●—), 5.0 mg/L (—▲—), 7.5 mg/L (—▼—), 10.0 mg/L (—♦—) bromopropylate concentrations.

For centuries, pesticides have been used in agriculture to enhance food production by eradicating unwanted insects and controlling disease vectors. Among common pesticides: organophosphorus compounds (OP) such as bromopropylate are widely used in agriculture, medicine and industry. According the results, SDH activities increased slowly with increasing in bromopropylate concentration in the range of 0.0 and 2.5 mg/L up to 60th h and then rose up significantly until 72nd h (Figure 3.3). The SDH activities decreased for following incubation period for all studied bromopropylate concentrations. The insignificant activity change was measured at control during all incubation period **(**$p < 0.01$**)**. The maximum SDH activity was measured as 320.1 IU/mg for 2.5 mg/L of bromopropylate on the 72nd h. The increases in SDH activity with the rises in bromopropylate concentration up to 2.5

mg/L may support the xenobiotic induction on the ETS enzyme system (Delgado, Streck, Quevedo and Dal-Pizzol, 2006). It seems that it is a compensatory effect of xenobiotics. On the other hand, the decreases in the SDH activity at the bromopropylate concentration higher than 2.5 mg/L may suggest an overall perturbation of the electron transfer pattern leading to absolute mitochondrial dysfunction as a result of bromopropylate toxicity. Since the mitochondrial respiration was found to be altered, this may further disrupt the supply of oxygen to various cells and generate a state of hypoxia. In contrast, Sıngh et al., and Gupta et al. also reported an increase in the activity of SDH in permethrin and malathion exposed rats (Gupta & Kapoor, 1975; Sıngh & Srıvastava, 1999). It appears that the stress may produce by an adverse effect on energy metabolism causes the SDH activity to rise.

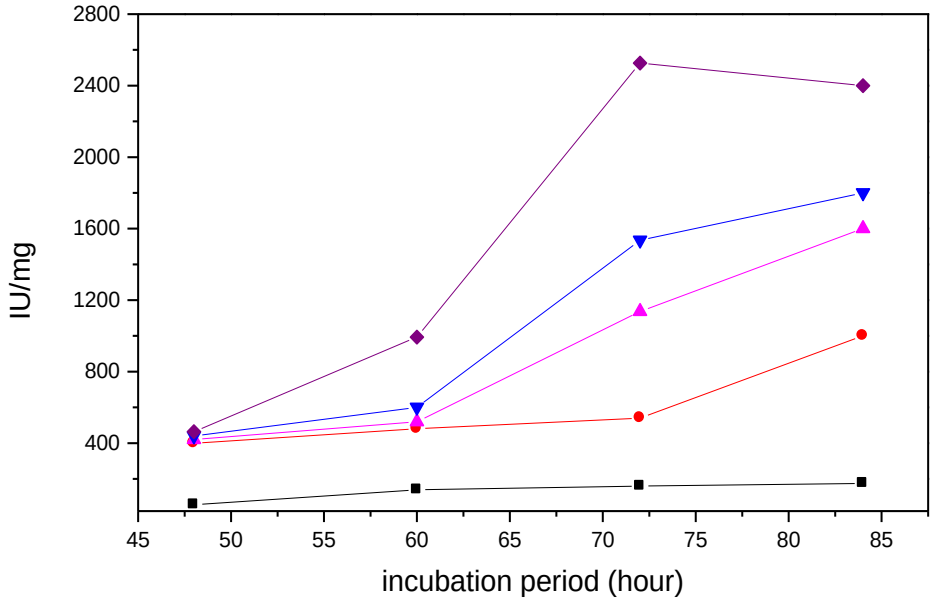


Figure 3.4 Variations in COX activity in *T. harzianum* depending on the incubation period in medium containing; control (—■—), 2.5 mg/L (—●—), 5.0 mg/L (—▲—), 7.5 mg/L (—▼—), 10.0 mg/L (—♦—) bromopropylate concentrations.

In contrast to SDH enzyme, COX activities showed positive correlation with increasing in bromopropylate concentration during all incubation period. The observation further confirms that the activities of complex IV of ETS system were induced with the pestiside. Therefore, bromopropylate specifically interacts at the level of succinate dehydrogenase (Complex II) and the translocation system of the metabolite. As seen from Figure 3.4, they increased slowly in the initial hours of incubation period and also similar activities were obtained for in the range of 0.0-10.0 mg/L bromopropylate. Then, COX activities rose significantly up to 72nd h while they did not change markedly for following incubation period ($p < 0.01$). The maximum COX activity was measured as 2400.056 IU/mg for 10.0 mg/L of bromopropylate on the 72nd h. The significant correlation between SDH and COX enzymes activity of ETS with respect to concentration of bromopropylate may also suggest the xenobiotic induction on the ETS enzyme system (Delgado et al., 2006).

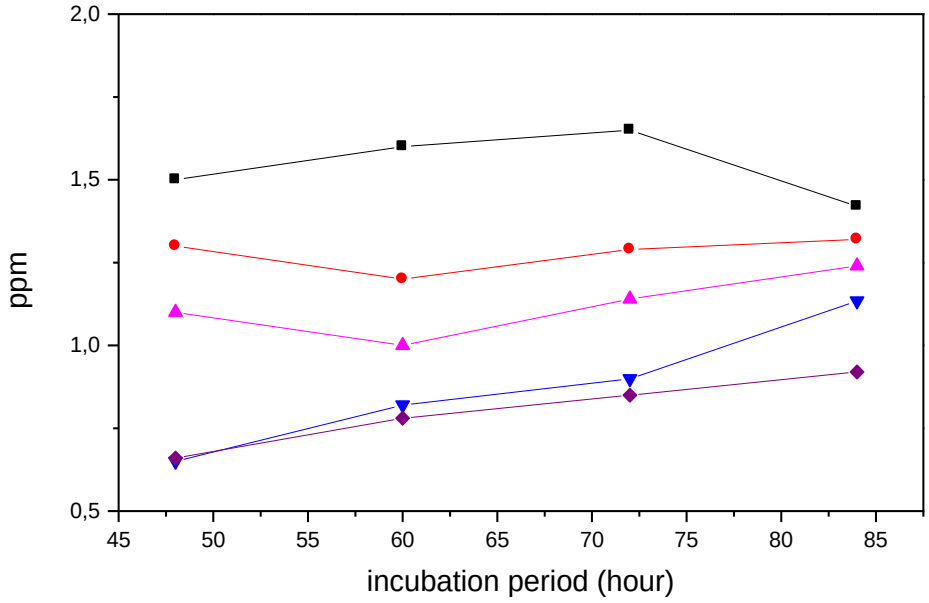


Figure 3.5 Variations in protein level in *T. harzianum* depending on the incubation period in medium containing; control (—■—), 2.5 mg/L (—●—), 5.0 mg/L (—▲—), 7.5 mg/L (—▼—), 10.0 mg/L (—♦—) bromopropylate concentrations.

Protein levels of *T. harzianum* decreased with increases in bromopropylate concentrations. Protein levels increase insignificantly up to 72nd h for control while it decreased at 84th h (Figure 3.5). On the other hand, protein levels decreased markedly up to 72nd h for 2.5 and 5.0 mg/L of bromopropylate and increased for following incubation period. In addition, they increased continuously for 7.5 and 10.0 mg/L of bromopropylate, respectively. The maximum protein level was measured as 1.65 ppm for control on 72nd h.

3.3 Variations in TCA Cycle Intermediate and Glycolysis Last Metabolite Levels of *T. harzianum* Depending on Bromopropylate Concentration and Incubation Period

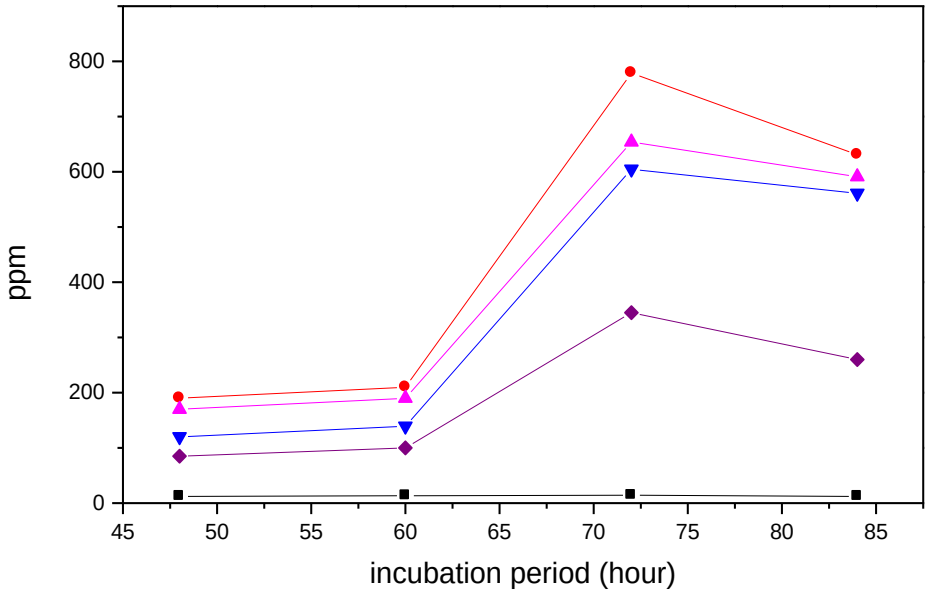


Figure 3.6 Variations of intracellular pyruvate level in *T. harzianum* depending on the incubation period in medium containing; control (—■—), 2.5 mg/L (—●—), 5.0 mg/L (—▲—), 7.5 mg/L (—▼—), 10.0 mg/L (—♦—) bromopropylate concentrations.

The intracellular pyruvate levels of *T. harzianum* did not change significantly for all studied bromopropylate concentrations up to 60th h (Figure 3.6). They increased markedly up to 2.5 mg/L of bromopropylate until 72nd h except control. For following incubation period, the intracellular pyruvate levels of *T. harzianum* decreased slowly. The intracellular pyruvate level of control did not change significantly during all studied incubation period. The maximum level was measured as 778.696 ppm for 2.5 mg/L of bromopropylate on the 72nd h.

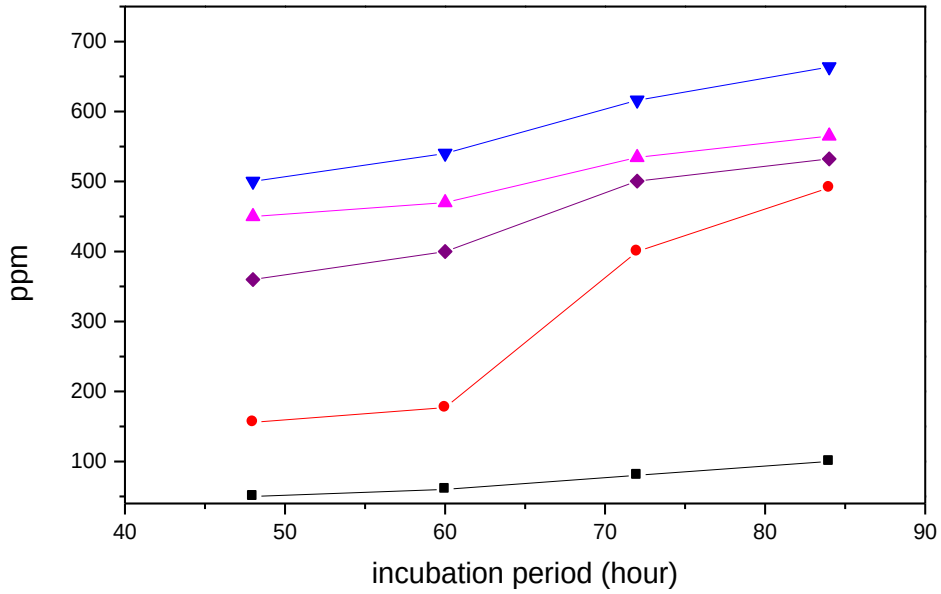


Figure 3.7 Variations of intracellular citrate level in *T. harzianum* depending on the incubation period in medium containing; control (—■—), 2.5 mg/L (—●—), 5.0 mg/L (—▲—), 7.5 mg/L (—▼—), 10.0 mg/L (—♦—) bromopropylate concentrations.

Intermediates of the tricarboxylic acid (TCA) cycle play an important role in metabolic reactions under aerobic conditions, forming many of the metabolites. According to the results, the intracellular citrate levels of *T. harzianum* did not change significantly in the range of 5.0-10.0 mg/L copper containing up to 60th h, increased slowly until 72nd h and then did not change markedly for following

incubation period (Figure 3.7). For 2.5 mg/L of copper, the intracellular citrate levels did not change markedly until 60th h while after 60th h, the significant increase was obtained until 72nd h. For the following incubation period, it rose up slowly. During all studied incubation period, the intracellular citrate levels of *T. harzianum* in the control medium did not change significantly ($p<0.01$). The maximum intracellular citrate level of *T. harzianum* was measured as 663.635 ppm for 7.5 mg/L of bromopropylate on the 84th h.

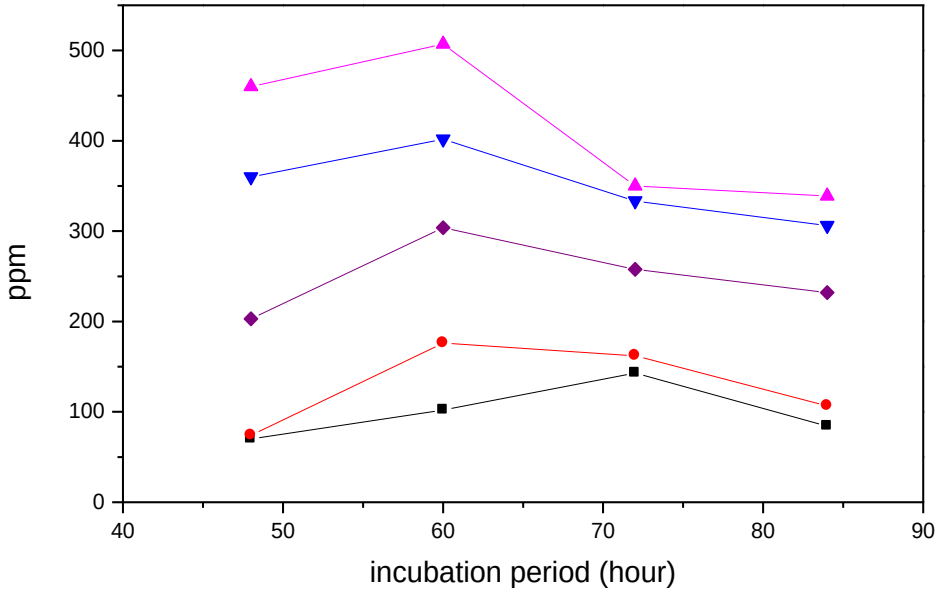


Figure 3.8 Variations of intracellular α-ketoglutarate level in *T. harzianum* depending on the incubation period in medium containing; control (—■—), 2.5 mg/L (—●—), 5.0 mg/L (—▲—), 7.5 mg/L (—▼—), 10.0 mg/L (—♦—) bromopropylate concentrations.

As can seen in Figure 3.8, the intracellular α-ketoglutarate levels of *T.harzianum* increased slowly 2.5 and 10.0 mg/L of bromopropylate and significantly for other studied bromopropylate concentrations until 60th h. Then, they decreased markedly up to 72nd h except control and 2.5 mg/L of bromopropylate. For the following incubation period, the intracellular α-ketoglutarate levels did not change significantly

for all studied bromopropylate concentrations. The maximum intracellular α-ketoglutarate level was measured as 506.977 ppm for 5.0 mg/L of bromopropylate on the 60th h.

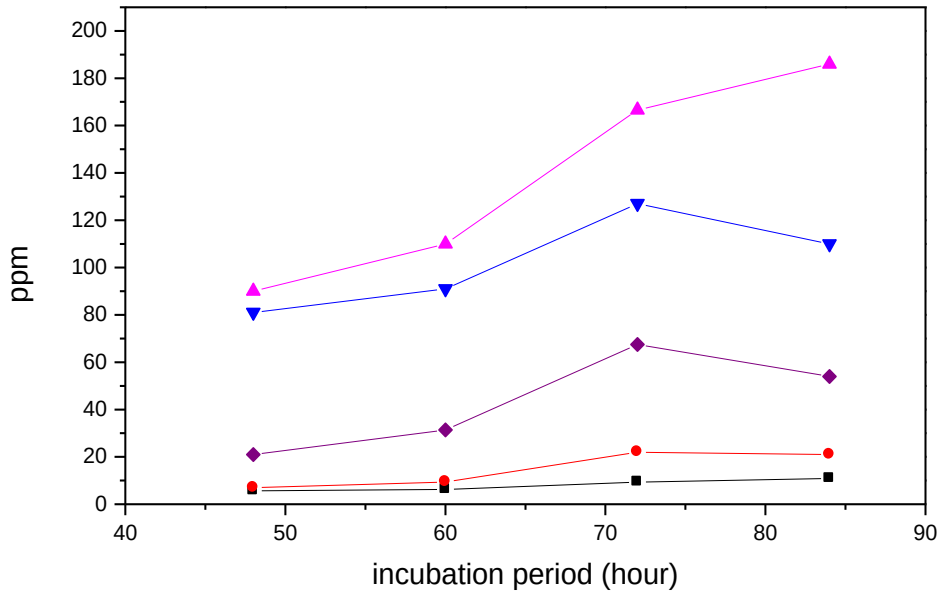


Figure 3.9 Variations of intracellular fumarate level in *T. harzianum* depending on the incubation period in medium containing; control (—■—), 2.5 mg/L (—●—), 5.0 mg/L (—▲—), 7.5 mg/L (—▼—), 10.0 mg/L (—♦—) bromopropylate concentrations.

As can seen in Figure 3.9, the intracellular fumarate levels in *T. harzianum* increased slowly except control in initial hours of the studied incubation period. They rose significantly in the range of 2.5–10.0 mg/L bromopropylate containing medium up to 72nd h while it did not change markedly for control medium. For the following incubation period, the intracellular fumarate levels decreased significantly for 5.0, 7.5 and 10.0 mg/L of bromopropylate while they did not change markedly for control and 2.5 mg/L of bromopropylate. The maximum intracellular fumarate level of *T. harzianum* was measured as 166.553 ppm for 5.0 mg/L of bromopropylate on the 72nd h.

3.4 Variations in Adenine Nucleotide Levels of *T. harzianum* Depending On Bromopropylate Concentration and Incubation Period

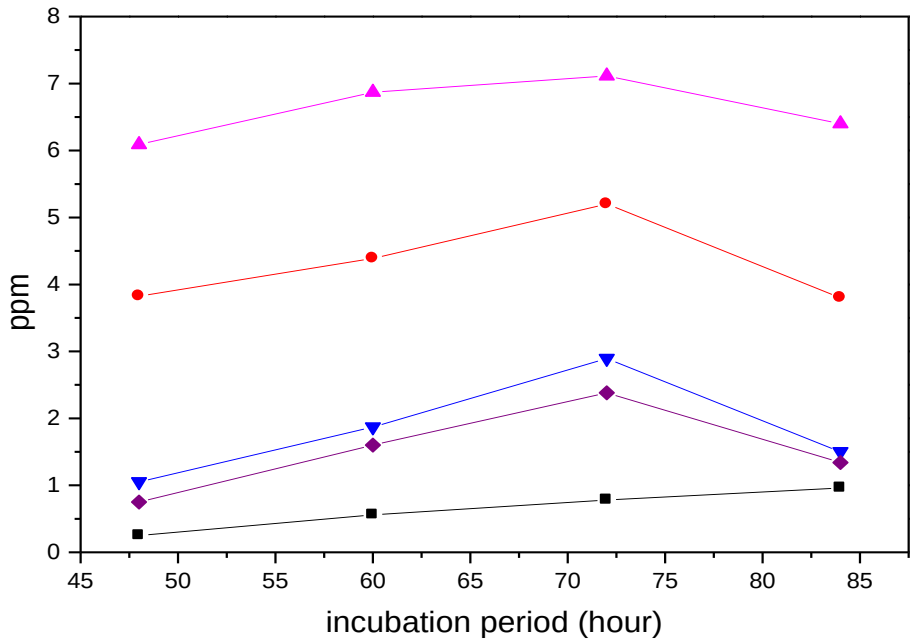


Figure 3.10 Variations in ATP level in *T. harzianum* depending on the incubation period in medium containing; control (—■—), 2.5 mg/L (—●—), 5.0 mg/L (—▲—), 7.5 mg/L (—▼—), 10.0 mg/L (—♦—) bromopropylate concentrations.

According the results, ATP levels of *T. harzianum* increased in the range of 2.5-10.0 mg/L of bromopropylate up 48th h (Figure 3.10). The increases continued until 60th hand then ATP levels of *T. harzianum* decreased significantly for the following incubation period. ATP levels of control medium did not change significantly during all studied incubation period. The maximum ATP level was measured as 7.113 ppm for 5.0 mg/L of bromopropylate on 72nd h. The results may indicate that the pesticides have been reported to influence oxygen uptake, intracellular ATP concentration (Lee and Park, 1979), mitochondrial energetics (Moreno and Madeira, 1991), and enzymes of the electron transport chain (Pardini, 1971). Thus, the energy metabolism was affected owing to pesticides exposure.

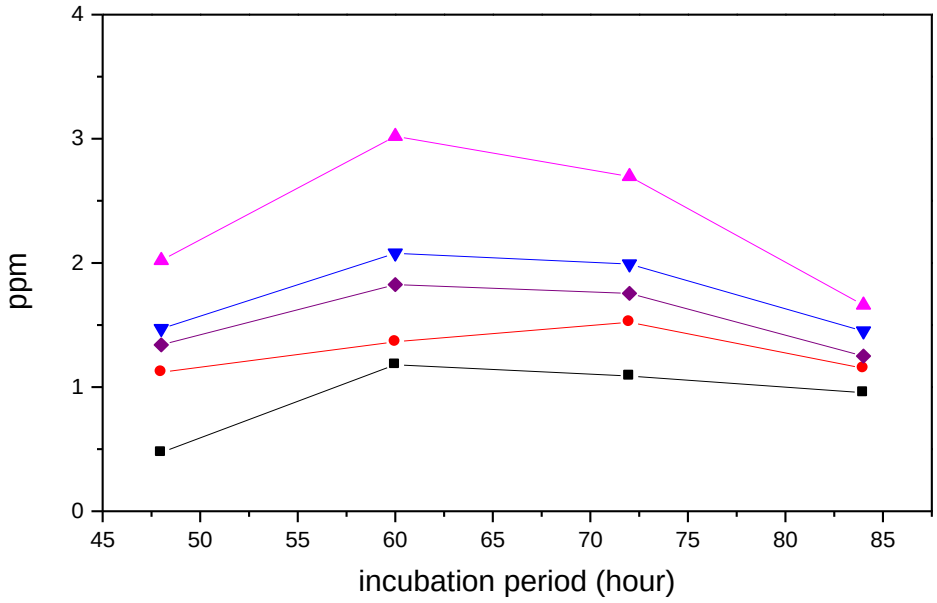


Figure 3.11 Variations in ADP level in *T. harzianum* depending on the incubation period in medium containing; control (—■—), 2.5 mg/L (—●—), 5.0 mg/L (—▲—), 7.5 mg/L (—▼—), 10.0 mg/L (—♦—) bromopropylate concentrations.

As seen in Figure 3.11, ADP levels of *T. harzianum* increased significantly up to 5.0 mg/L of bromopropylate until 60th h. The increases continued for control and 2.5 mg/L bromopropylate containing mediums while the decreases observed in the range of 5.0-10.0 mg/L of bromopropylate until 72nd hour. They decreased for all studied bromopropylate concentrations for the following incubation period. The maximum ADP level was measured as 3.02 ppm for 5.0 mg/L of bromopropylate on 60th h.

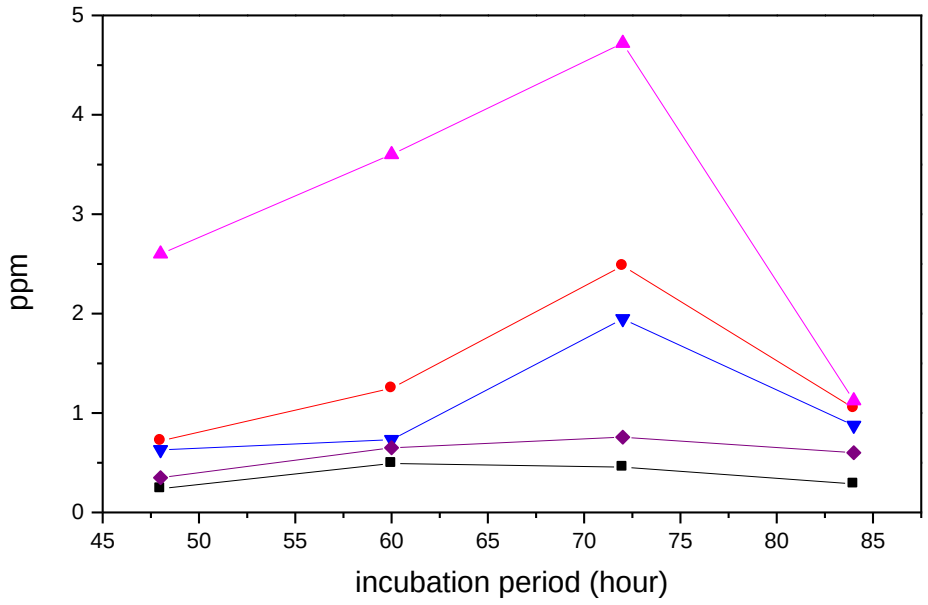


Figure 3.12 Variations in AMP level in *T. harzianum* depending on the incubation period in medium containing; control (—■—), 2.5 mg/L (—●—), 5.0 mg/L (—▲—), 7.5 mg/L (—▼—), 10.0 mg/L (—♦—) bromopropylate concentrations.

According the results, AMP levels increased significantly up to 5.0 mg/L of bromopropylate until 72nd h (Figure 3.12). The increases were slowly for control and 25.0 mg/L of bromopropylate. Then AMP levels decreased markedly in the range of 2.5-7.5 mg/L of bromopropylate while they increased slowly for control and 25.0 mg/L bromopropylate containing mediums for the following incubation period. The maximum AMP level of *T. harzainum* was measured as 4.72 ppm for 5.0 mg/L of bromopropylate on 72nd h.

3.5 Variations in LPO Levels of *T. harzianum* Depending on Bromopropylate Concentration and Incubation Period

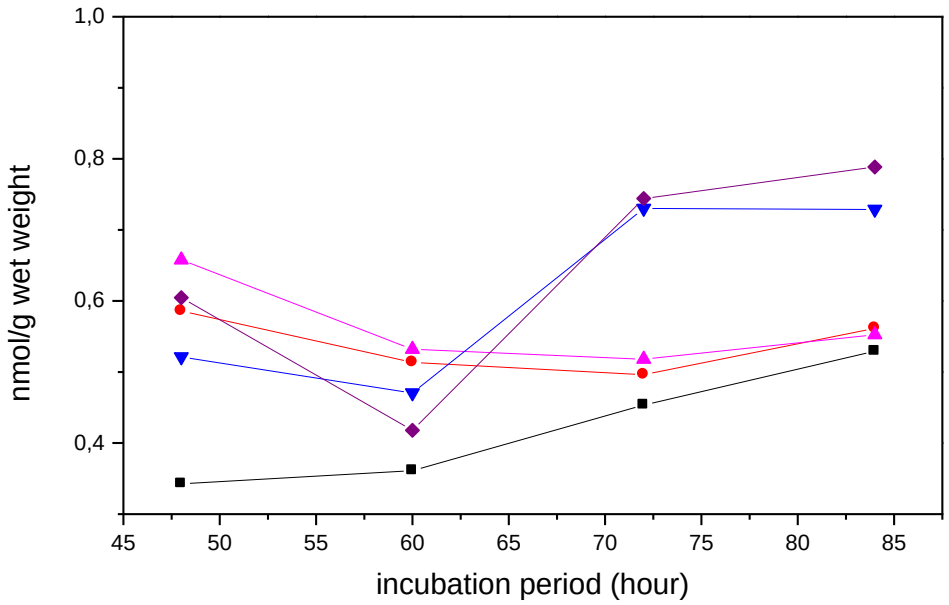


Figure 3.13 Variations of LPO level in *T. harzianum* depending on the incubation period in medium containing; control (—■—), 2.5 mg/L (—●—), 5.0 mg/L (—▲—), 7.5 mg/L (—▼—), 10.0 mg/L (—♦—) bromopropylate concentrations.

Recent findings indicate that toxic manifestations induced by OPs may be associated with an enchanced production of reactive oxygen species (Altuntas, Delibas and Sutcu, 2002). The LPO levels increased significantly with respect to increases in bromopropylate concentration (Figure 3.13). The results indicate that the chronic bromopropylate exposure caused to decreases in SDH enzyme activity and induced oxidative stres which cause cell damage by increasing the production of ROS. The results can also be explained with primary role of mitochondria in the pathogenesis of peroxidative damage, because mitochondria consume about 90% of inhaled oxygen, and reactive oxygen species such as superoxide, hydrogen peroxide, and the hydroxyl radical are formed in the mitochondria as physiologic metabolites of the respiratory chain. The results were supported by the enhanced lipid

peroxidation in the hepatic and cerebral tissues of rats threated with cypermethrin (Giray, Gürbay & Hincal, 2000).

3.6 Variations in Bromopropylate Levels of *T. harzianum* Depending on Bromopropylate Concentration and Incubation Period

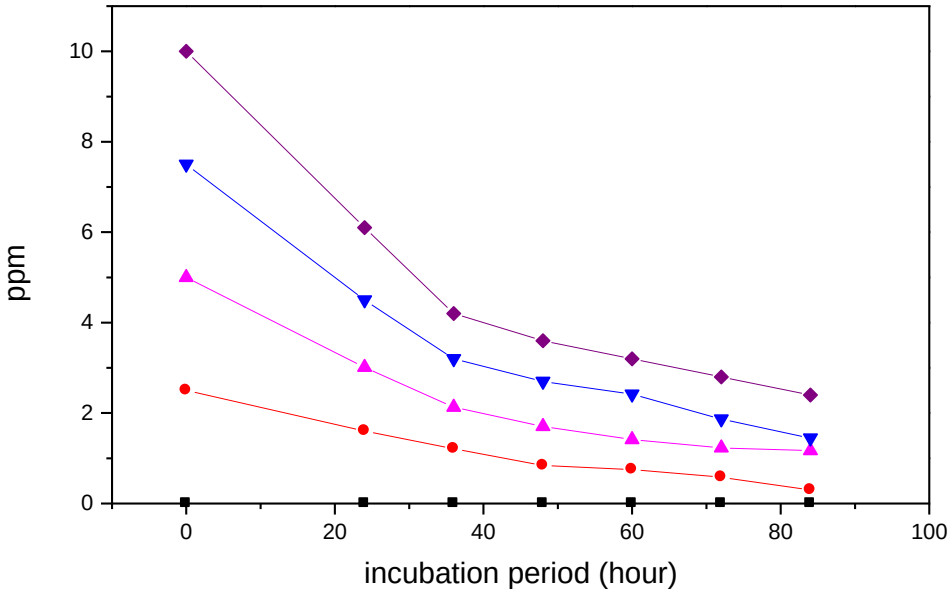


Figure 3.14 Variations of extracelluler bromopropylate level in *T. harzianum* depending on the incubation period in medium containing; control (—■—), 2.5 mg/L (—●—), 5.0 mg/L (—▲—), 7.5 mg/L (—▼—), 10.0 mg/L (—♦—) bromopropylate concentrations.

As can be seen from Figure 3.14, bromopropylate levels in *T. harzianum* decreased significantly with respect to incubation period ($p<0.01$). The highest increased was determined in the first 24^{th} h of incubation for all tested concentrations of bromopropylate and these rates were decreased for the following period of incubation. These results may suggest that the transport of the pesticide was highest rate in the beginning period. In addition, this uptake rate was markedly affected by the concentration of bromopropylate.

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