CHAPTER I
INTRODUCTION

Ketoconazole is an imidazole antifungal agent [1]. It has been effectively used to treat fungal infection, but not severe or acute cases. The pharmacology of ketoconazole has been studied extensively. Unlike others imidazole derivatives, which have side effects in man, ketoconazole has a very low side effect profile [2]. In this chapter the general pharmacological and toxicological informations of ketoconazole as well as a brief account on the mitochondrial respiratory chain and oxidative phosphorylation are presented.

Chemical structure and properties

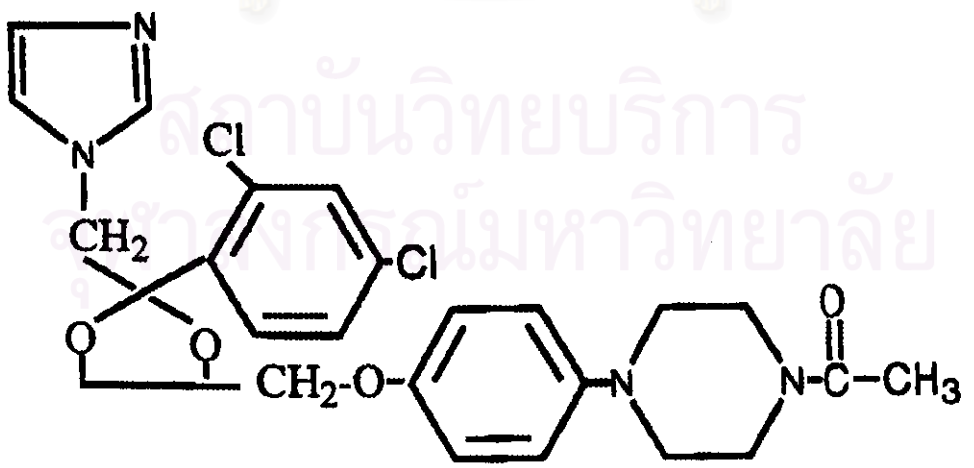

Fig 1. The chemical structure of ketoconazole

Ketoconazole , cis-1-Acetyl - 4 - 4 - [2 - (2 , 4 - dichlorophenyl) - 2 - (- 1H-1- imidazolyl-methyl) -1 , 3 - dioxolan -4 yl} - methoxy] piperazine , C_{26} H_{28} Cl_2 N_4 O_4 [3].

The compound has a mild basicdic property and a molecular weight of 531.44. It is a white odorless crystalline powder, insoluble in water and easily soluble in dimethyl sulfoxide (DMSO) [4].

Pharmacological and Toxicological Actions of ketoconazole

There are a great number of human diseases which can be attributed in some way to the effect of fungi. Their influence may be indirect, for instance by their contribution to the destruction of food stuffs, or may depend on their toxic or antifungal properties. Alternatively fungi may cause disease by invasion of tissue. Such infections caused by fungi, mycoses, have long been recognized as important, but common problems in all spheres of medicine.

As illustrated in table I and II, ketoconazole is active in the usual in vitro tests against a wide variety of dermatophytes, yeast and other fungi [5].

Table I In vitro activity of Ketoconazole against dermatophytes and yeasts.

Organism	No. of strains tested	Concentration (μg/ml)
dermatophytes		
Microsporum canis	24	0.1-64
Microsporum audouini	4	2-64
Microsporum gypseum	9	0.1-64
Microsporum cookei	1	1
Trichophyto mentagro	2	0.1-20
Trichophyto rubrum	75	10^{-5}-128
Trichophyto ajelloi	1	1
Trichophyto schoenleini	1	1
Trichophyto tonsurans	35	0.25-16
Epidermophyton floccosum	23	0.1-8
Yeasts		
Candida albicans	472	0.02-80

Candida tropicalis	45	0.1-64
Candida pseudotropicalis	2	25-50
Candida guilliermondii	4	0.4-50
Candida krusei	14	0.2-3.1
Candida parapsilosis	18	0.2-64
Candida stellatoidea	1	0.8
Cryptococcus neoformans	39	0.1-32
Torulopsis glabrata	124	0.8-64
Rhodotorula mucilanginosa	1	0.1
Trichospoon cutaneum	1	0.1

Table II In vitro activity of Ketoconazole against dimorphic and other fungi.

Organism	No. of stains tested	Concentration (μg/ml)
Dimorphic fungi		
Blastomyces	26	0.1-2

dermatitidis		
Coccodioudes immitis	30	0.1-0.8
Histoplasma capsulatum	26	0.1-0.5
Paracoccidioides brasiliensis	5	0.002-0.1
Eumycetes		
Acremonium falciforme	1	10
Madurella grisea	1	0.1
Madurella mycetomi	1	0.1
Petriellidium boydii	23	0.1-4
Actinomycetales		
Actinomudura madurae	2	10-25
Nacardia asterroides	1	1
Nacardia brasiliensis	2	32-10
Nacardia cavea	1	1
Streptomyces sp.	1	10
Phycomycetes		

Absidia corymbifera	1	1
Rizopus nigricans	1	100
Saprolegnia sp.	1	1
Various fungi		
Aspergillus flavus	2	1
Aspergillus flomigatus	55	1-100
Aspergillus glaucus	1	1
Aspergillus nidulams	1	1
Aspergillus niger	6	1-16
Aspergillus terreus	3	1
Aspergillus spp.	3	5.5-106
Ceotrichum candidum	1	1
Picdraia hortai	1	0.1
Sporothrix schenekii	23	0.1-16
Dematiacious fungi	29	0.1-6

Like other imidazole antifungus, ketoconazole shows some activity in vitro against certain gram-positive bacteria

such as Staphylococcus aureus, Staphylococcus epidermidis or Enterococcol streptococci, but it is less active in this regard than micronazole.

Mechanism of action

After fungi exposure to low dose of ketoconazole or other imidazole derivatives, 10-100 ng/ml, peculiar changes take place at the plasma membrance and the cell wall. The alterations consist of the formation of high osmiophilic vesicles deposited in the cell wall and in the central vacuole. At the same time a large increase in cell volume take place and abnormalities of cell division has occurred. The otherwise smooth cell surface becomes wrinkled and shows randomly distributed bud scars. The enlarge cells do not separate after normal budding and division. Exposure to higher doses, 0.5-50 μg/ml, induce deterioration of all subcellular organelles and the cells become angular in appearance due to loss of osmotic resistance [7]. Fatty overload is one of characteristic features seen with the high dosage regimen (fig.2 and 3) [5]

Pharmacokinetics

Absorption in man

A single oral dose of ketoconazole 200 mg as a tablet containing the free base produced peak plasma concentration(depend on dose) of 3 μg/ml. Higher and more

consistent plasma levels are reached when ketoconazole is taken with a meal. Its bioavailability is higher and more consistent when it is administered with or just before meal. Since it is a dibasic compound the acidity of the stomach plays an important role: ketoconazole requires sufficient gastric secretion for dissolution and subsequent absorption.

In healthy volunteers given increasing doses in tablet form, as well as a solution of ketoconazole as a reference, just before breakfast, the areas under the concentration-time curves correlate closely with peak plasma concentrations (figs. 4 and 5). Figure 4 illustrates the similar absorption pattern for the 200 mg tablet as compared with the solution, although the relative bioavailability of the tablet was slightly lower (about 75%). As suggested by figure 5, ketoconazole may undergo ' first pass' metabolism during the absorption phase, with transient saturation of the metabolising capacity of the liver [5,8].

Tissue Distribution in Man

In man a single oral dose of 200 mg given to healthy subjects produces detectable concentrations of ketoconazole in urine, saliva, sebum and cerumen. In a few patients with various deep mycoses (whether had any fungal meningitis is unclear) peak cerebrospinal fluid concentrations of 2 μg/ml and 7 μg/ml occur 3 to 4 and 1 to

2 hours after doses of 200 and 400 mg, respectively. Concentrations of 1 μg/ml or more persist for 5 to 6 hours [5]. In a patient with candidal meningitis receiving ketoconazole 400 mg twice daily, peak cerebrospinal fluid concentrations of 3.0 and 2.2 μg/ml occur 6 and 8 hours after a 400 mg dose, following 7 days and 30 days of therapy, respectively [9]. These cerebrospinal fluid concentrations correspond with plasma levels of 9.5 and 9.0 μg/ml. Thus, it appears that like other available imidazole antifungal, cerebrospinal fluid penetration of ketoconazole is relatively low, but concentration which might be expected to be effective against many fungal pathogens may be achieved [5,8].

Metabolism and Excretion in Man

Four days after an oral dose of tritiated Ketoconazole administered to 3 healthy males, 70% of the dose had been excreted, about 57% in the faeces and 13% in the urine. Following absorption ketoconazole is extensively metabolized; unchanged drug accounts for 20 to 65% of the faecal radioactivity but for only 2 to 4% of urinary radioactivity. In the 0 to 24 hours urine, 30 to 50% of the metabolites are basic compounds, 5 to 10% are polar acidic compounds and 5 to 10% are conjugates of polar cacidic compounds. About 35% of the urinary metabolites could not be extracted. In faecal methanol extracts, basic

metabolites account for more than 80% of the radioactivity. The various metabolites in the urinary and faecal extracts have been separated and purified by high-performance liquid chromatography and the major metabolites analyzed by mass spectrometry. The main identified metabolic pathways are oxidation of the imidazole ring, degradation of the oxidized imidazole, oxidative O-dealkylation, oxidative degradation of the piperazine ring and aromatic hydroxylation [5,8,9].

In healthy subjects the elimination half-life(t1/2) of ketoconazole are 6.5, 8.1 and 9.6 hours after a single oral dose (given as a tablet) of 100, 200 and 400mg, respectively [5].

Safety Studies In Man

In a number of clinical studies laboratory investigations directed at monitoring biochemical effects of ketoconazole have been performed. No toxicity observed on the hematological system, the eyes, liver or kidney function and electrolyte balance. However, as a result of the tendency toward bone fragility seen in female rats during toxicity studies, a number of parameters related to bone formation have been studied in man. No adverse changes in bone density or formation occur in the small number of patients studied, and there are no abnormal elevations of serum calcium or alkaline phosphatase or decreases in -

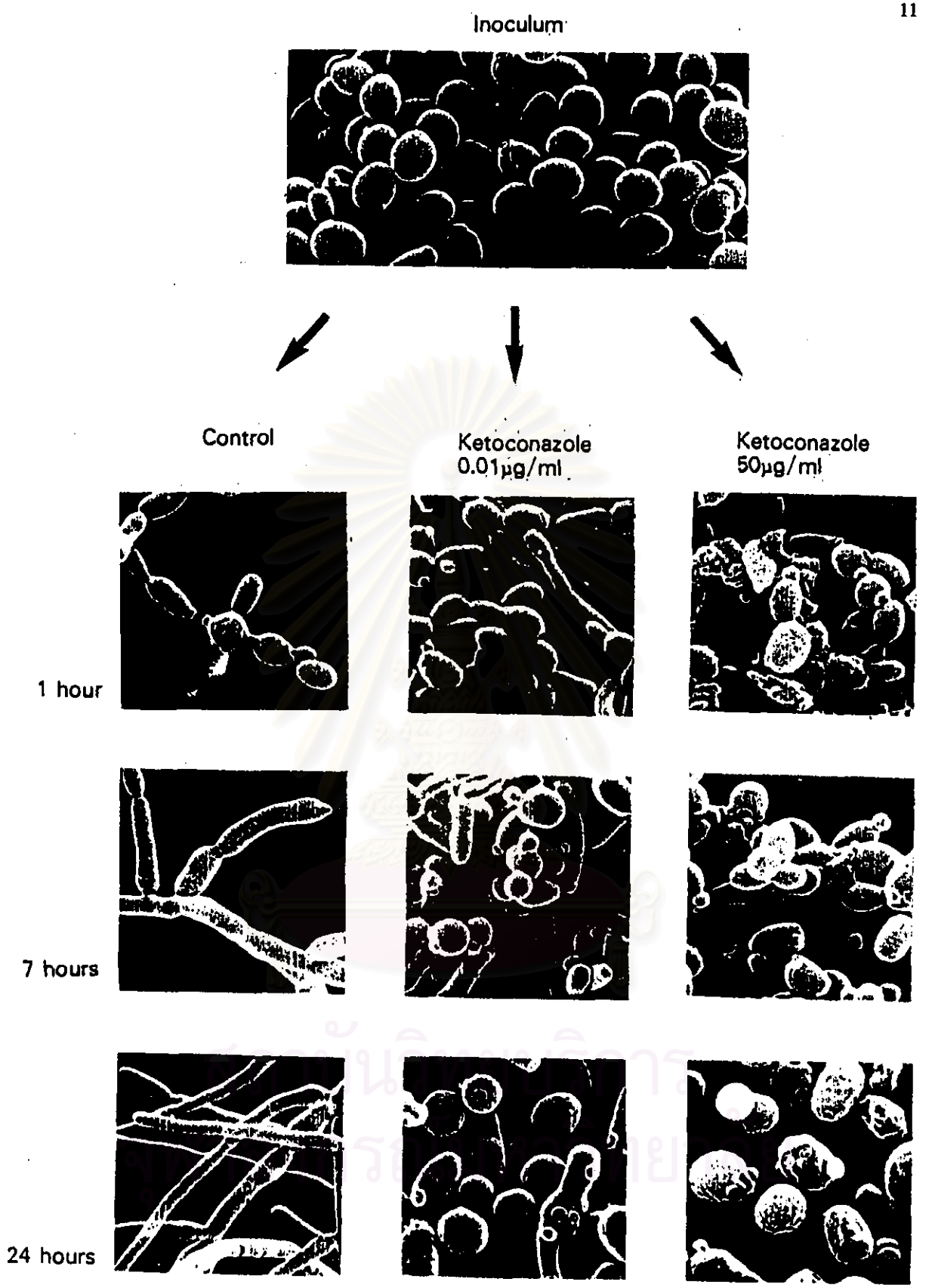

Fig 2. The time-related development of mycelium from the inoculated yeast cells and the influence of low and high dose ketoconazole.

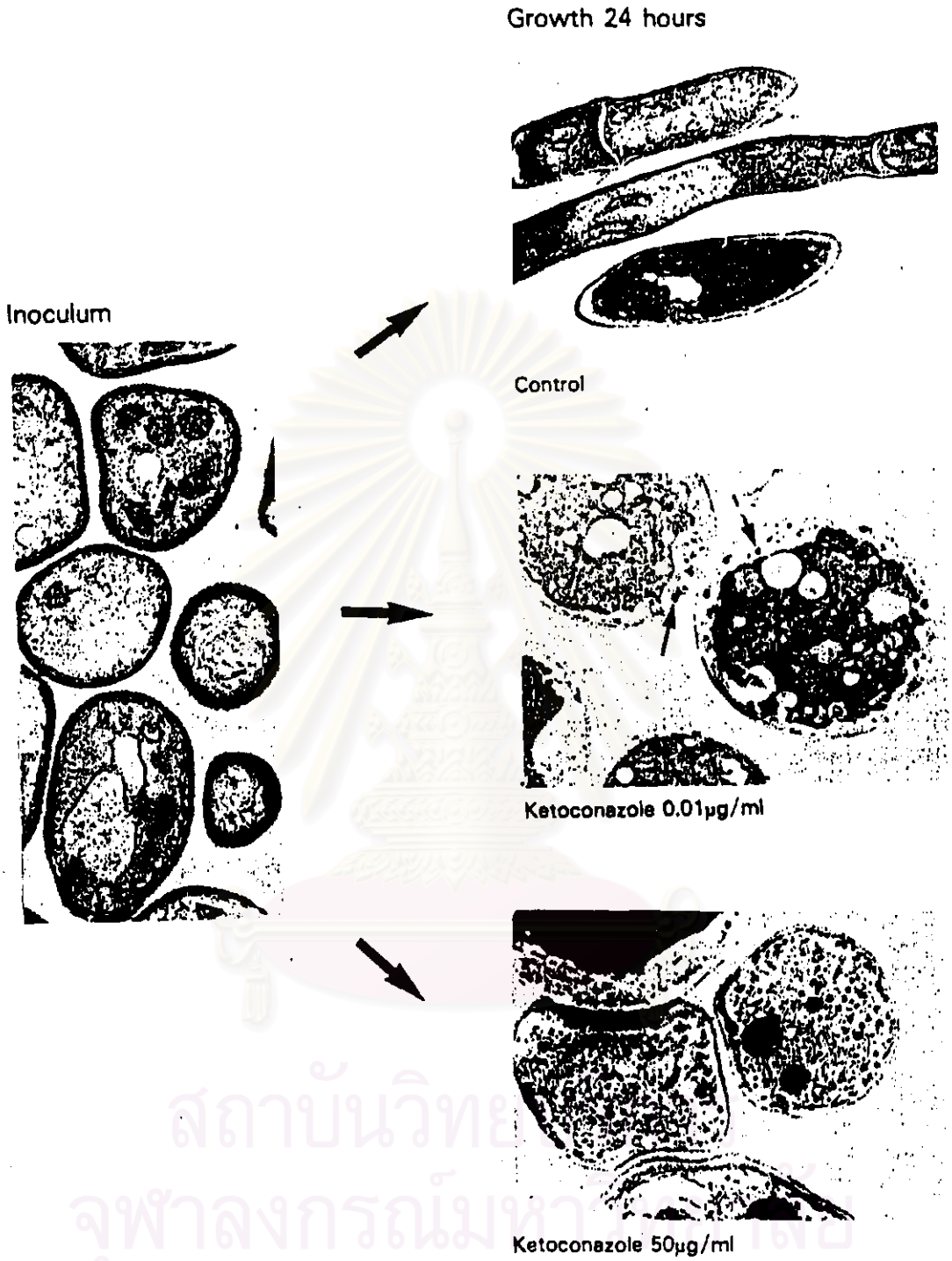

Fig 3. The exposure to low dose of ketoconazole results in the characteristic deposition of osmiophillic vescles(arrow) in the cell wall of growth inhibited yeasts whereas a high dose induces complete necrosis.

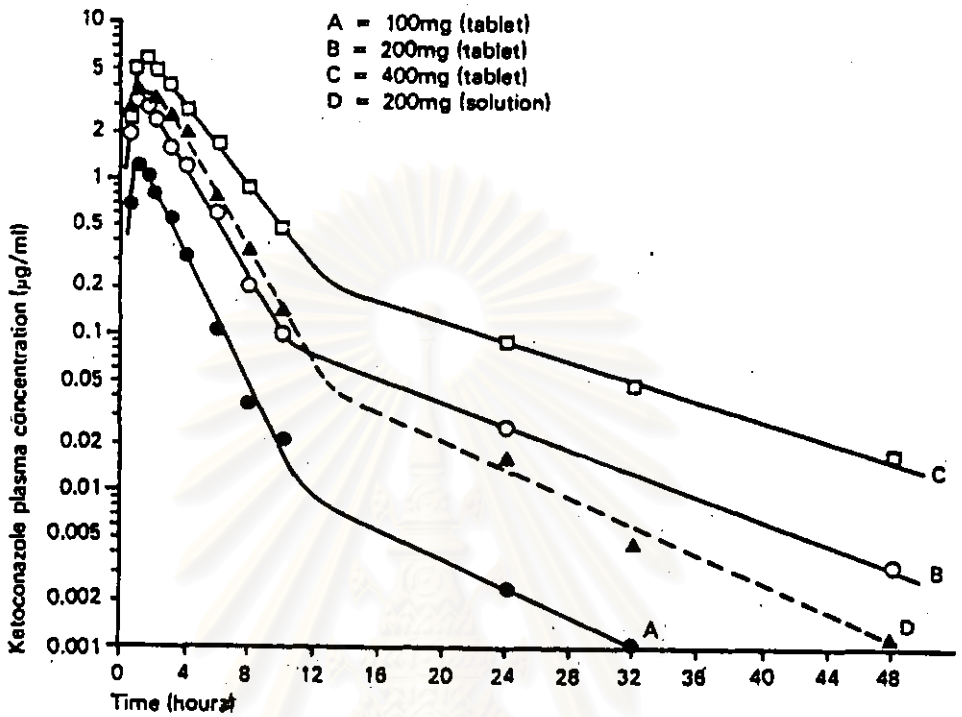

Fig 4. Plasma concentrations of ketoconazole in 12 fasted male volunteers given various doses just before breakfast.

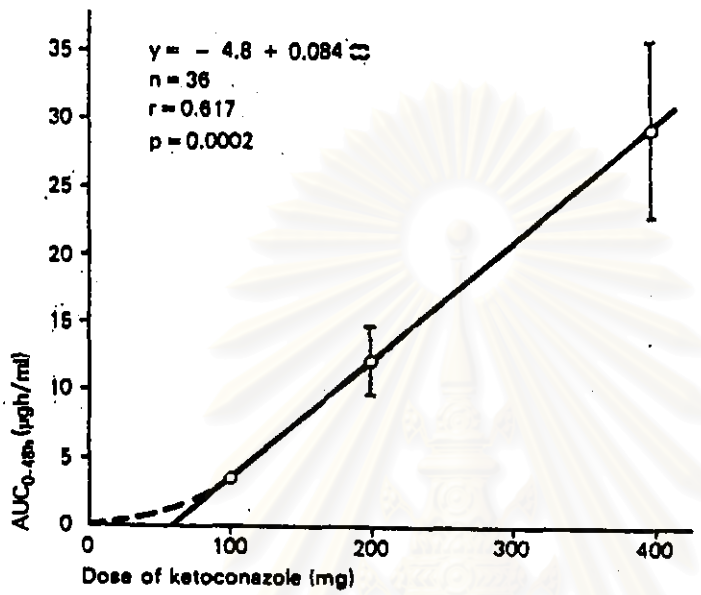

Fig 5. Mean area under the plasma concentration-time curves after ketoconazole administration, as in fig 4. (Unpublished, on file Jansen Research Foundation)

serum phosphorus. Indeed, in a few patients with bone lesions due to fungal infections new bone formation occurred during treatment [10,11].

Gynaecomastia was reported in 3 of 40 men on ketoconazole doses ranging from 200 to 1200 mg daily [12] and 5 of 24 patients on doses of 800 to 1200 mg daily [13]. Gynaecomastia and associated breast pain may remit with continued therapy or persist until the drug is discontinued. Ketoconazole reduces serum testosterone concentration [13,14] and this reduction may play a role in gynaecomastia. Oligospermia and impotence have also occurred [13,14].

A decreased cortisol response has been reported in healthy subjects given ketoconazole 400 mg daily [15] and in patients receiving high doses of ketoconazole (800 to 1200 mg daily) for fungal infections [13] or prostatic carcinoma [16]. In contrast, plasma cortisol concentrations were reported to be above normal in a study of 6 patients with leukemia receiving ketoconazole 400 mg daily, possibly due to disease-associated stress [17,18,19].

Symptomatic adrenal insufficiency shown as hyponatraemia and confusion, was reported by Pillans et al. [20] in a man given ketoconazole 600 mg daily for 2.5 months for the treatment of prostatic carcinoma. McCanee et al.[21], also reported acute hypoadrenalism in woman given ketoconazole 400 mg twice daily for 5 days. Other case reports include persistent adrenal insufficiency in a 61-

years old woman receiving ketoconazole 400 mg daily for blastomycosis [22] and adrenal failure in a 56-year old man with keratitis receiving high dose of ketoconazole [23].

A review of all the cases of silent and symptomatic hepatic reactions occurring world wide during treatment with ketoconazole that had been reported to the manufacturer up to March 1982. Silent reactions (transient elevation of serum transaminases and alkaline phosphatase without symptoms of hepatic disease) have been observed during routine laboratory examination of patients on ketoconazole. These reactions occurred at any time during treatment and usually returned to normal despite continuation of therapy. In 1074 patients receiving ketoconazole in daily doses of 50 mg to 1 g for periods of up to 15 months, 8% had elevated serum liver enzymes before treatment, increasing to 11% during treatment, and decreasing to 6% toward the end of treatment. There were 31 reports of symptomatic hepatic reactions, 25 involving jaundice, in patients being treated with ketoconazole, viral aetiology could not be excluded in all of these. The majority of patients were being treated for onychomycosis and symptoms occurred after a range of 1 to 24 weeks of treatment. Patients were aged between 5 and 90 years, 18 were women and 13 men. At least 9 had a history of idiosyncrasy to other drugs, 8 had a previous history of hepatitis, at least 14 had been treated with griseofulvin,

and at least 19 were receiving concomitant drug therapy. Treatment was discontinued in 30 patients, all of whom recovered except one who died from hepatic necrosis. Two were re-treated and both relapsed, one after approximately 2 weeks but the reaction was less severe than initially . The one patient in whom ketoconazole was not discontinued recovered uneventfully within one month while still taking the drug. By September 1982 a further 46 cases of symptomatic hepatic reactions, one fatal, had been reported, and the incidence of these reactions was estimated to be 1 in 12000 [24,25]

The Mitochondrial Respiratory Chain and Oxidative Phosphorylation System.

All the processes involving in growth and metabolism of aerobic eukaryotic cells require an input of energy. In most cases this energy is supplied by hydrolysis of the high-energy phosphate bonds in adenosine triphosphate (ATP) of which the main source is in the mitochondria [26,27].

ATP synthesis is the most important and fundamental function of the mitochondria. Most of the ATP utilized by cell is synthesized in the mitochondria by the process called oxidative phosphorylation. Energy generating foodstuffs, mainly carbohydrate, lipid, and protein are broken down into glucose, fatty acids, and amino acid respectively and

further metabolized to yield the common end product of fuel breakdown, i.e., acetyl CoA. In the mitochondrial matrix, acetyl CoA enters the Krebs cycle and is fully oxidized to CO_2 with the generation of reducing equivalents, i.e., NADH and $FADH_2$ (figure 6). The energy liberated as these reducing equivalents are oxidized by the mitochondrial respiratory chain, is used to drive ATP synthesis from ADP+Pi by the mitochondrial ATP synthase complex [28]. A brief description of the mitochondrial respiratory chain and oxidative phosphorylation is presented below.

1. Mitochondrial respiratory chain.

Electrons are transported from NADH and $FADH_2$ along a chain of various electron carriers, arranged in a sequential pattern as illustrated in figure 7. Electrons from NADH are transferred sequentially to a flavoprotein with flavin mononucleotide (FMN) as prosthetic group, the iron-sulfur centers and then coenzyme Q (Co Q) which is the most abundant electron carrier in mitochondrial inner membrane and sometimes called ubiquinone. There are several other substrates which donate electrons to Co Q, for instance, succinate, fatty acyl CoA, alpha-glycerol-phosphate. Between Co Q and O_2 is a chain of electron carrying cytochromes, each of which has a heme prosthetic group. At the terminus of the electron transport chain

molecular oxygen accepts electrons from cytochrome a_3 and is thereby reduced to form water [29,30,31].

Experimentally the components of the mitochondrial respiratory chain can be separated into four complexes by using mild detergents. Such complexes have a considerable amount of lipid, which is essential for their activities [26,31].

1. Complex I or NADH-ubiquinone oxidoreductase.

This large complex, catalyzing the transfer of electrons from NADH to Co Q, consists of least 16 polypeptide chains, including a FMN-containing flavoprotein and five to eight iron-sulfur centers. NAD^+-linked substrates such as glutamate, malate, ketoglutarate and pyruvate donate electrons to respiratory chain via complex I.

2. Complex II or succinate-ubiquinone oxidoreductase.

This complex, catalyzing the trans transfer electrons from succinate to Co Q, consists of two largest polypeptide with the catalytically active centers of succinate dehydrogenase, FAD, and iron-sulfur centers.

3. Complex III or ubiquinol-ferri cytochrome c oxidoreductase

This oxidoreductase complex catalyzes the transfer of electrons from reduced Co Q to cytochrome c. It contains cytochromes b, cytochrome c_1, and iron-sulfur center.

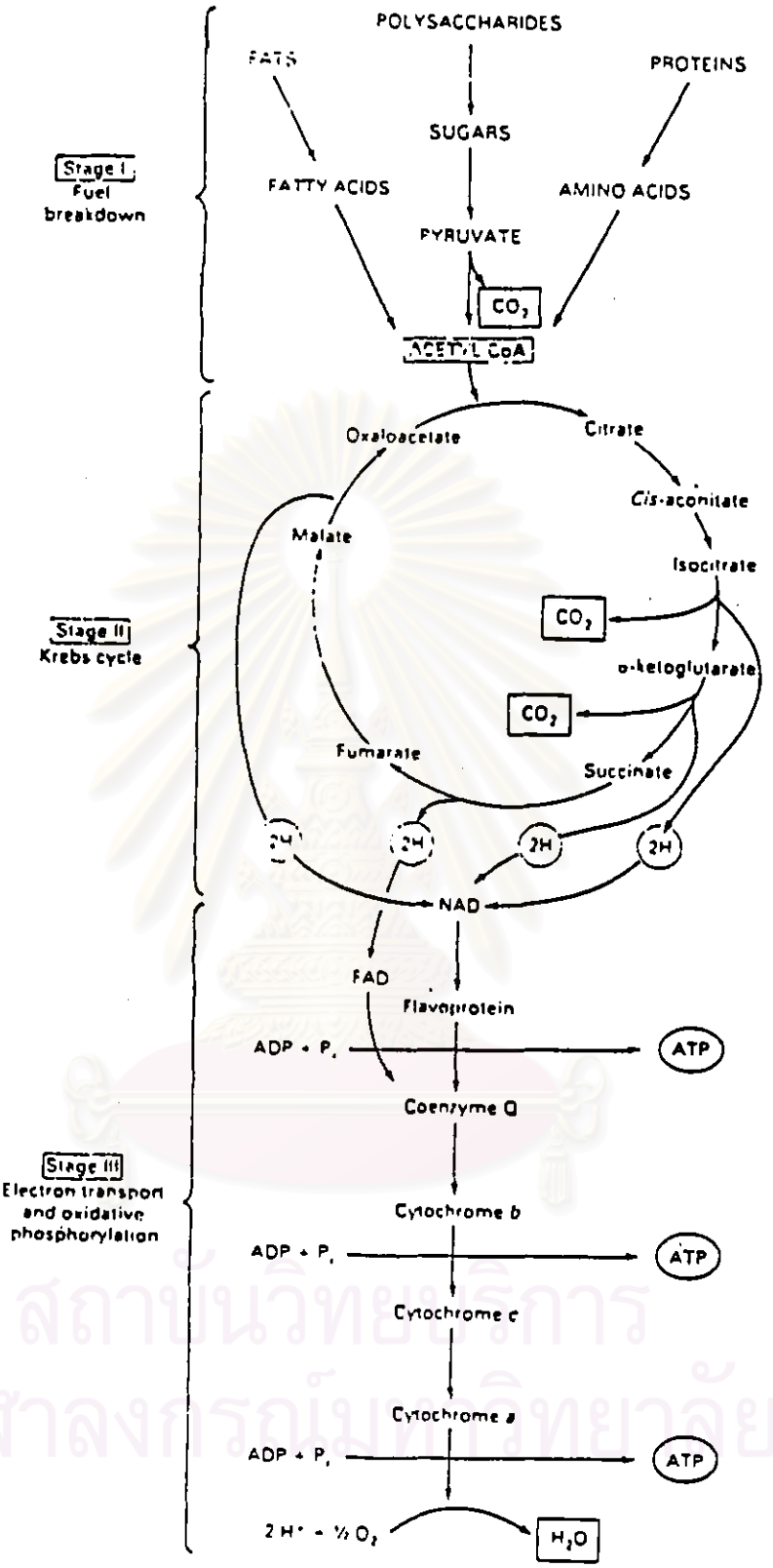

Fig 6. Correlation of fuel breakdown, Krebs cycle, electron transport chain and oxidative phosphorylation.

4. Complex IV or ferrocytochrome c-oxygen oxidoreductase or cytochrome c oxidase complex.

Cytochrome c oxidase, the terminal component of the respiratory chain, transfers electrons from cytochrome c to O_2. It consists of several polypeptides, two cytochromes (a and a_3) and two copper atoms. This complex can also accept electrons from artificial electron donors, ascorbate and N,N,N/,N/-tetramethyl- p- phenylenediamine (TMPD).

Many inhibitors of mitochondrial function are known to block electron transport at specific complexes. Rotenone (a plant-derived toxin that has been used as an insecticide and fish poison), amytal (a barbiturate), and piericidin A specifically block complex I or coupling site I. Malonate blocks complex II. Antimycin, a toxin obtained from Streptomyces griseus, acts on complex III or coupling site II whereas cyanide, azide, and carbon monoxide act on complex IV or coupling site III. These respiratory chain inhibitors have been most useful in confirming the electron carriers sequence from NADH to oxygen [31].

2. Mitochondrial oxidative phosphorylation.

During the passage of a pair of electrons from NADH to oxygen along a gradient of declining free energy level, a large enough change in free energy (at least 9-10 kcal/mole) occurs at three different sites to sponsor ATP synthesis from ADP and Pi [29]. The measured redox potentials and their

corresponding free energy changes greatly drop as electron are transferred though each three sites (fig. 8). These sites of ATP synthesis also called coupling sites, regions whereby energy from oxidation or electron transport is coupled to the phosphorylation of ADP. The overall process is called oxidative phosphorylation. Site I, II and III in electron transport chain are designated in fig. 7.

In isolated intact mitochondria, electron transport is tightly coupled to phosphorylation. The oxidation slightly occurs in the absence of ADP. When ADP is added, the oxidation proceeds rapidly, in other words, the mitochondria can oxidize substrates only in the presence of ADP and Pi to generate ATP. This phenomenon, termed respiratory control, indicates the tightness of couple mechanism and illustrates how one reactant can limit the rate of a complex set of reactions [30].

The electron transport and phosphorylation are uncoupled in aged mitochondria or by the uncoupler such as 2,4-dinitrophenol (DNP). In the presence of DNP, ATP synthesis stops but electron transport continues at a higher rate, all the energy is released as heat under this condition.

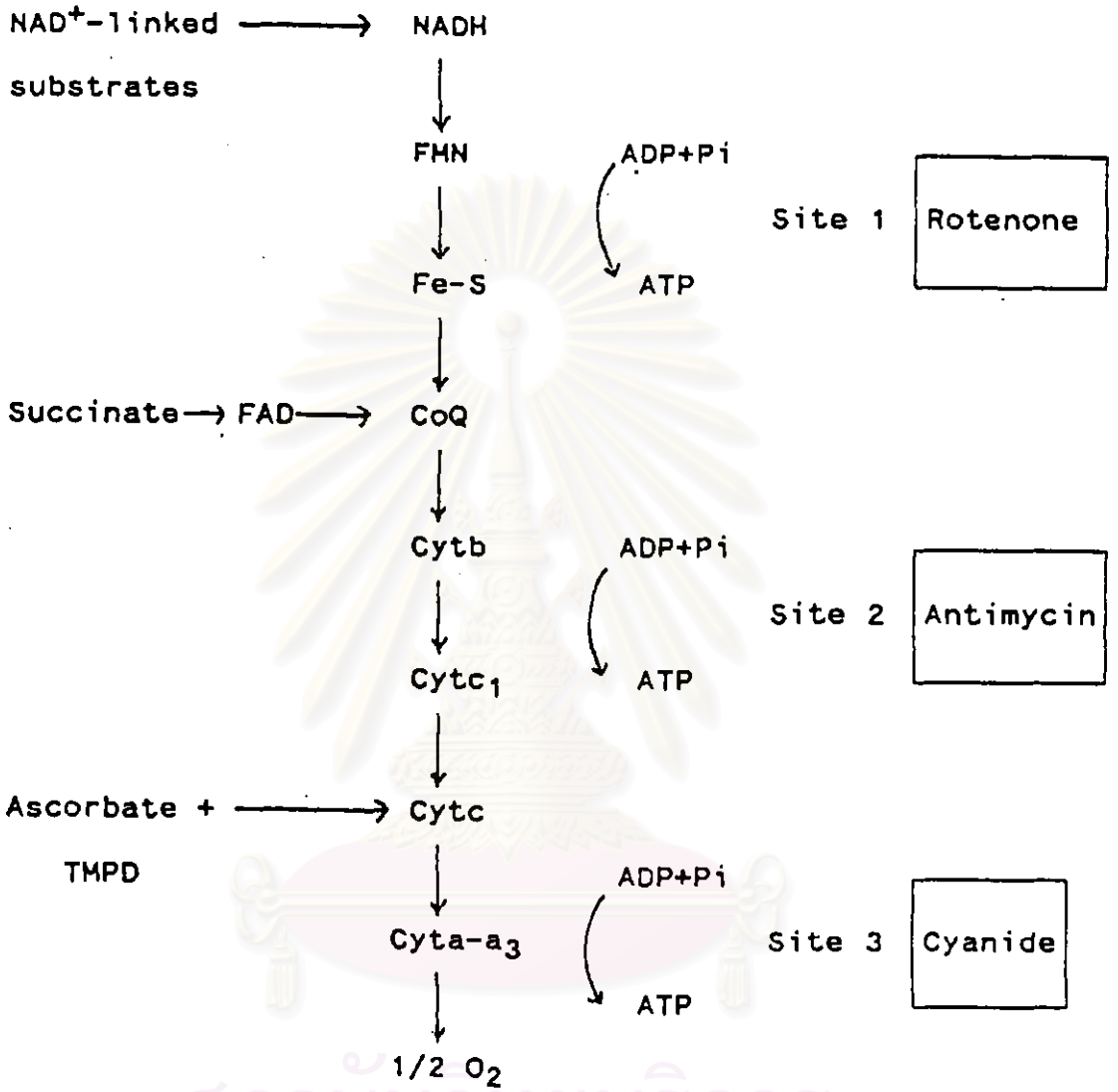

Fig 7. Electron transport chain, coupling sites, and inhibitors.

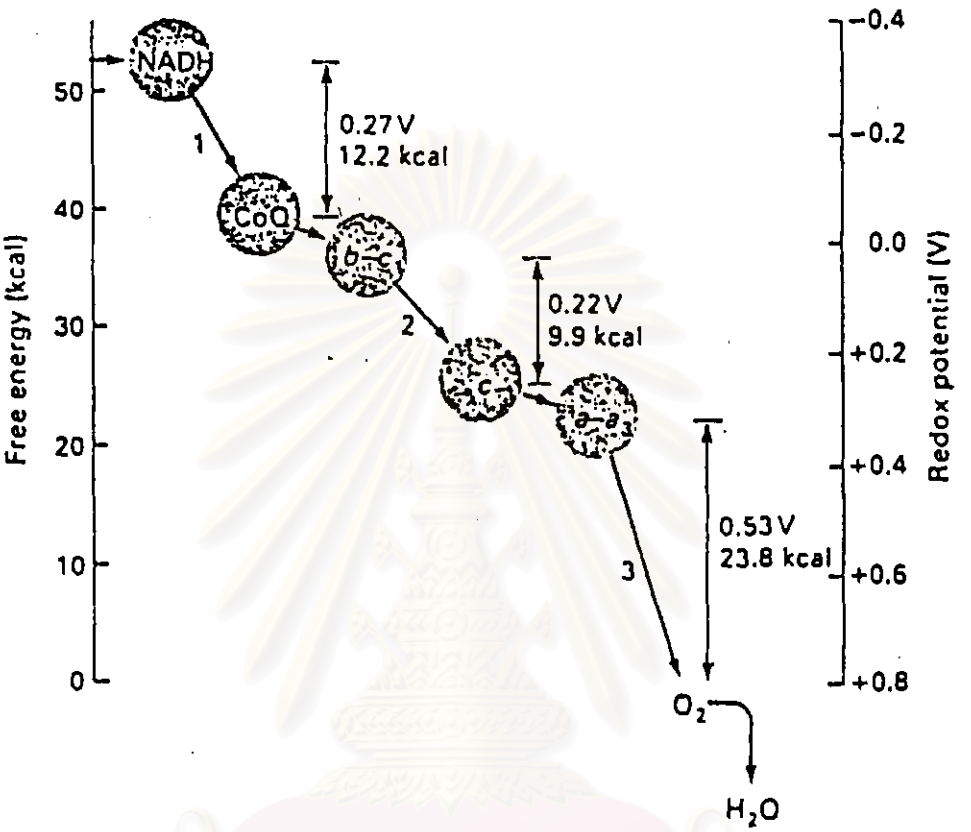

Fig 8. The measured redox potentials and their corresponding free-energy changes in each steps of electron transport from NADH to oxygen.

3. Mechanism of mitochondrial oxidative phosphorylation.

The mechanism by which energy derived from the passage of electrons along the electron transport chain is transduced into the chemical energy as ATP has been extensively studied. Currently, the most widely accepted mechanism is the chemiosmotic hypothesis, first proposed in 1961 by Peter Mitchell [28,29,31]. The main points of the chemiosmotic hypothesis are as follow:

1. The inner mitochondrial membrane is particularly impermeable to H^+ and OH^- and generally to other ionic species.

2. As electrons move down the electron transport chain, the released free energy is used to pump protons out of the matrix into the intermembrane space, across the inner membrane. this proton movement generates proton electrochemical gradient or protonmotive force composed of proton concentration gradient (low proton concentration in matrix side) and membrane potential (matrix side negative) across inner membrane.

3. The free energy released when protons move down electrochemical gradient through ATP synthase complex is used by the enzyme to drive ATP synthesis from ADP and Pi (fig. 9).

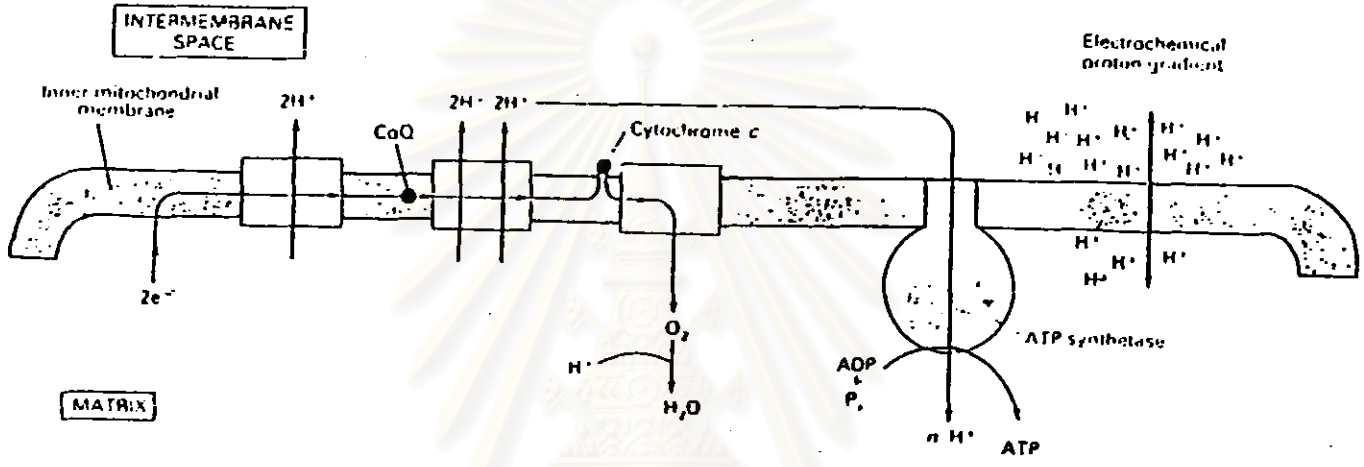

Fig 9. Schematic illustration of the coupled processes of electron transport and oxidative phosphorylation.

4. The membrane contains H^+-linked or OH^--linked carrier systems that transport metabolites across the membrane into or out of the matrix. Thus, in the chemiosmotic theory, energy released from electron transport is first transduced into protonmotive force by enzyme complexes of the respiratory chain, and the protonmotive force is then transduced into chemical energy in ATP by the ATP synthase complex.

As mentioned earlier, DNP and other uncouplers can separate oxidation from phosphorylation which leads to a cessation of ATP synthesis and enhances rate of electron transport. According to the chemiosmotic theory, the DNP-type uncouplers uncouple oxidative phosphorylation by acting as a proton - ionophore transporting protons across inner membrane to the matrix without passing through ATP synthase complex. In other words, the uncouplers make inner membrane freely permeable to protons. The collapsed protonmotive force causes substrate oxidation or, electron flow to proceed rapidly. However, with the uncoupler presents, the energy of the protonmotive force is continuously dissipated as heat and little or no energy is available for ATP formation [27,29,31].

4. The mitochondrial ATP synthase.

The enzyme responsible for ATP synthesis in mitochondria, ATP synthase or F_1 F_0-ATPase, is a

component of the mitochondrial inner membrane. It consists of two principal portions, F_1 and F_0

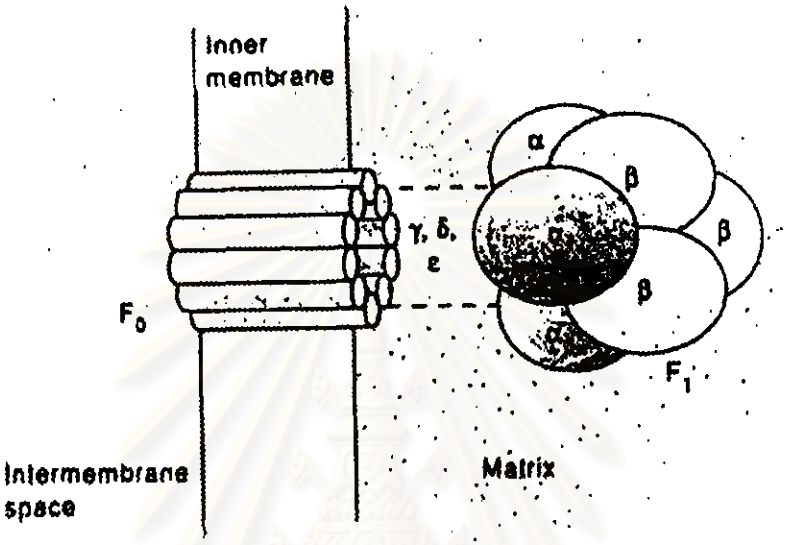

Fig 10. Components of the F_1F_o-ATPase complex [31].

F_1 is composed of five polypeptide subunits bound together in a spherical head portion that protrudes from the matrix-facing surface of the inner membrane. It is a catalytic sector and can be isolated as a soluble highly active ATPase, catalyzing the hydrolysis of ATP. F_o, the membrane sector, is proton channel that renders the inner membrane permeable to protons. It consists of three or four nonidentical subunits, one of which is called the oligomycin sensitivity conferring protein, causing the enzyme to show sensitivity to the inhibitory action of oligomycin.

The enzyme ATP synthase utilizes the energy of the protonmotive force to drive ATP synthesis by harnessing the flow of protons back into the mitochondrial matrix through the F_0 portion; the F_1 portion catalyzes ATP synthesis from ADP and Pi. Conversely, under condition favorable for ATP hydrolysis, for example, when the mitochondria are uncoupled, the enzyme can use the energy of ATP hydrolysis to pump protons across inner membrane in the opposite direction, i.e., from the matrix to outside; Hence the F_1 F_0-ATPase is a reversible system that can catalyze both ATP synthesis and ATP hydrolysis depending on metabolic state of the mitochondria (figure 11) [29].

In addition to the uncoupling agents, several substances can inhibit ATP synthesis. These are :

Oligomycin and dicyclohexylcarbodiimide (DCCD) inhibit ATP synthesis by binding with a subunit of F_0 portion and consequently block proton conduction through F_1 Oligomycin shows inhibitory action only in the presence of oligomycin-sensitivity conferring protein (OSCP) which contains solely in mitochondrial F_1 F_0-ATPase. Bacteria and chloroplast F_1 F_0-ATPase do not contain OSCP, therefore they are not inhibited by oligomycin [32].

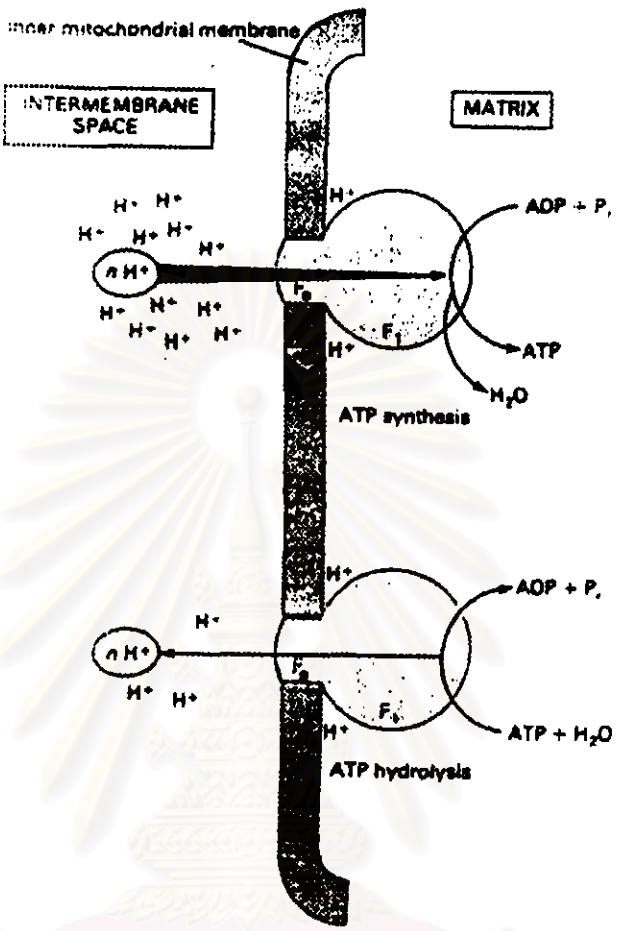

Fig. 11. The mitochondrial F_1F_o-ATPase may act reversibly as a synthetase or as a hydrolase.

Atractyloside inhibits adenine nucleotide translocator in the mitochondrial inner membrane which transports ADP into mitochondria. Thus making ADP unavailable for ATP synthesis.

N-ethylmaleimide (NEM) and mersalyl inhibit phosphate translocator which transports Pi into mitochondria, and consequently deplete Pi for ATP synthesis.

5. Utilization of conserved energy by mitochondria.

It is now generally agreed, that the protonmotive force may be used for other energy-requiring processes in the mitochondria without having first to be converted to ATP, besides ATP synthesis, other processes driven by protonmotive force are cation transport particularly calcium ion and synthesis of NADPH by energy linked transhydrogenase. The protonmotive force can also be dissipated into heat by uncouples (fig. 12) [30].

In view of the importance of the mitochondria in cell metabolism and function, the purpose of the present study is to investigate the effect of ketoconazole on mitochondrial bioenergetics and the possible contribution of these effects that might have on the pharmacological and/or toxicological actions of this antifungal agent.

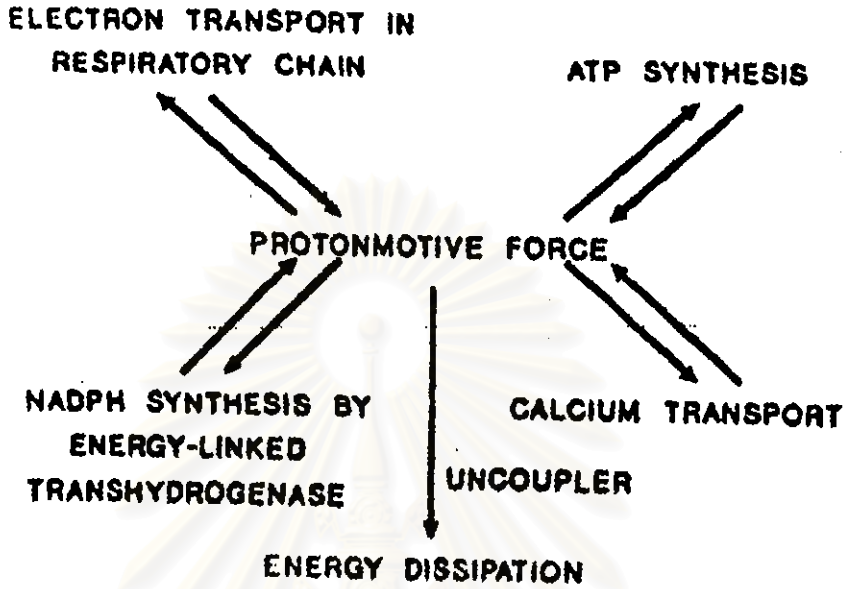

Fig. 12. Utilization of mitochondrial protonmotive force in various processes.