# CHAPTER I

# INTRODUCTION

Bezafibrate is one of the prominent lipid-lowering drugs in a fibric acid derivatives group. It has been used predominantly to treat lipid abnomalities in patients with hyperlipoproteinemia types IIa, IIb or IV[1]. In such patients bezafibrate significantly reduces total plasma cholesterol and triglyceride concentrations with the associated increases in high-density lipoprotein (HDL) cholesterol [2,3]. In this chapter the general pharmacological and toxicological informations of bezafibrate as well as a brief account on the mitochondrial respiratory chain and oxidative phosphorylation are presented.

## Pharmacological and Toxicological Actions of Bezafibrate

Hyperlipoproteinemia is a major risk factor for coronary heart disease (CHD)[4]. A raised level of low density lipoprotein (LDL) cholesterol indicates a particular risk; conversely, the level of high density lipoprotein (HDL) cholesterol represents a protective factor [5]. Treatment with hypolipidemic drugs is frequently initiated in hyperlipoproteinemic patients who do not respond adequately to dietary recommendations[6].

Among such drugs, bezafibrate, one of the fibric acid derivatives, has been widely used for many years. It is recommended as treatment in patients with primary hyperlipoproteinemia mainly types II a (increased levels of LDL), IIb(increased levels of both very low density lipoprotein- VLDL - and LDL), and IV (increased levels of VLDL), as well as in hyperlipoproteinemic patients with diabetes [7,8]. The Fredrickson classification of hyperlipoproteinemia is given in table 1.

## 1. Chemical structure and properties

Bezafibrate, 2{4-[2-(4-chlorobenzamido)ethyl] phenoxy}-2-methyl-propionic acid (figure 1), is structurally related to clofibrate and other fibric acid derivatives.

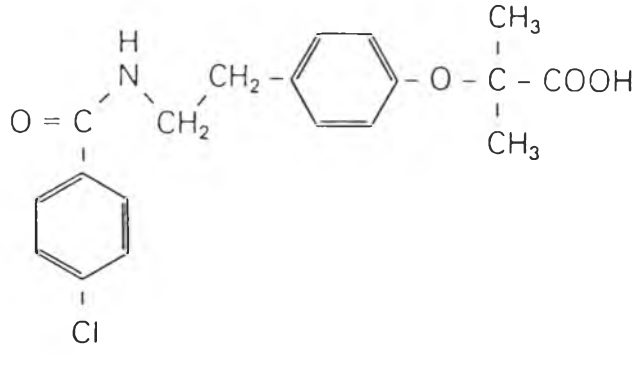


Figure 1. Chemical structure of bezafibrate

## Classification of primary hyperlipidaemias

| Type | Lipoprotein changes | Causes |
|---|---|---|
| I | CM increased<br>HDL, LDL low | Lipoprotein lipase deficiency<br>Apo C-II deficiency |
| IIa | LDL increased | Familial hypercholesterolaemia<br>Common 'polygenic' hypercholesterolaemia<br>Multiple type hyperlipidaemia |
| IIb | LDL, VLDL, increased<br>HDL may be low | Multiple type hyperlipidaemia<br>Familial hypercholesterolaemia |
| III | IDL, CM remnants increased<br>LDL, HDL low | Genetic abnormality of apo E |
| IV | VLDL increased<br>HDL low | Multiple type hyperlipidaemia<br>Familial hypertriglyceridaemia<br>Common 'polygenic' hypertriglyceridaemia |
| V | VLDL, CM increased<br>HDL, LDL low | Multiple type hyperlipidaemia<br>Familial hypertriglyceridaemia<br>Lipoprotein lipase deficiency |

Table 1. Classification of primary hyperlipidaemias.

The compound has a mild acidic properties and a molecular weight of 361.5. Bezafibrate is a white odorless crystalline powder, moderately soluble in water and easily soluble in organic solvents(e.g.,ethanol and chloroform)[7].

## 2. Effects on serum lipids.

Bezafibrate considerably reduces serum concentrations of VLDL(precursors of LDL which transport endogenous triglycerides), LDL(the principle transport particle for cholesterol) and of total cholesterol and triglycerides in patients with hyperlipoproteinemias [9,10]. The concentration of the protective HDL (transport cholesterol back to the liver for excretion) are also increased by bezafibrate[10,11].

## 3. Mechanism of action.

Figure 2 shows the pathway of lipid metabolism. At present, the mechanism of hypolipidemic action of bezafibrate remains controversial. It is generally accepted that bezafibrate reduces the level of triglyceride by stimulation of lipoprotein lipase and to some extent of hepatic lipase leading to an accelerated degradation of plasma triglyceride-rich lipoproteins (VLDL) [12]. In addition, bezafibrate causes an increased concentration of apolipoprotein (apo) CII, a known

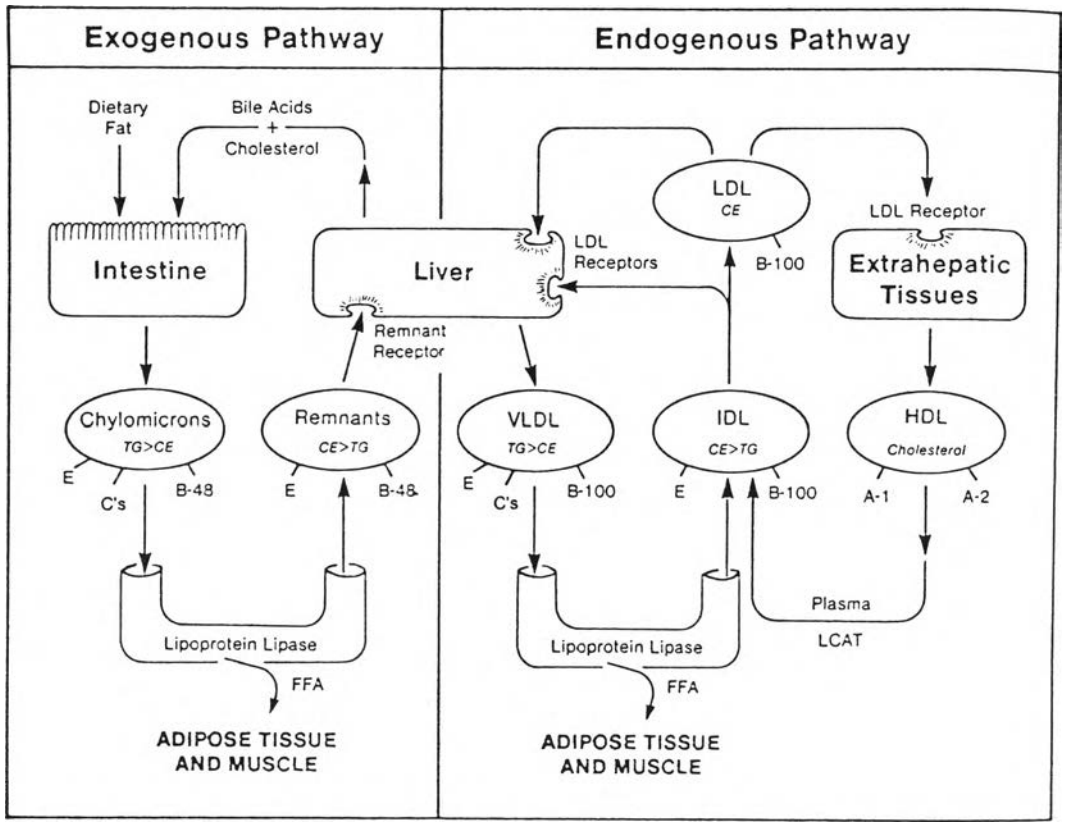


Figure 2. Lipid metabolism

CE denotes cholesteryl esters; FFA, free fatty acids; TG, triglycerides; HDL, high-density lipoprotein; IDL, intermediate-density lipoprotein; LCAT, lecithin: cholesterol acyltransferase; LDL, low-density lipoprotein; and VLDL, very-low-density lipoprotein. A-1, A-2, B-48, B-100, C's, and E represent the apoproteins associated with the indicated lipoprotein particle.

stimulator of the lipoprotein lipase, while the inhibitor apo CIII remain unchanged [7]. The drug may also decrease the hepatic synthesis and secretion of VLDL.

Bezafibrate is believed to raised HDL-cholesterol indirectly as a result of the decrease in the concentration of VLDL which normally exchange lipids with HDL, the triglycerides of VLDL moving to HDL and the cholesteryl esters of HDL moving to VLDL. When VLDL concentrations are reduced, this exchange is slowed. Cholesteryl esters remain in HDL and thus the concentration of HDL-cholesterol increases [13].

The hypocholesterolemic effect of bezafibrate is not fully understood. It is considered to be mediated by an enhanced hepatic clearance of VLDL and IDL (intermediate density lipoproteins) that results in the reduction of LDL synthesis [1], and the increased expression of LDL receptors on the hepatocyte resulting in increased clearance of LDL-cholesterol from the circulation[1,13].

The promoted LDL-receptors are probably a response to a fall in the intracelluar cholesterol pool that follows from inhibition of the endogenous cholesterol synthesis[14]. In rats, bezafibrate seems to inhibit the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG Co A)

reductase, the rate-limiting enzyme in cholesterol synthesis[1,7], but this mechanism in humans is not yet well documented.

## 4. Pharmacokinetics.

Bezafibrate is almost completely absorbed from the gastrointestinal tract, with a peak plasma concentration about 10 mg/L attained 2 hours after administration of 300 mg tablets to healthy volunteers, and 200 mg to hyperlipoproteinemic patients [15]. The drug is 95% protein bound and has the volume of distribution about 17 L[16]. Elimination is rapid and almost exclusively by renal excretion with approximately 50% as unchanged drug. The elimination half life is about 2 hours. The rate of renal clearance is about 4 L/hour.

Bezafibrate is metabolized via hydroxylation and glucuronidation; hydrolytic cleavage by microsomal mixed-function oxidases may also be involved. The main metabolite is glucuronide conjugate (more than 20%) with the remainder consisting of several more polar compound [1,15].

The elimination of bezafibrate is reduced in patients with renal insufficiency and dosage adjustments are necessary to prevent drug accumulation and toxic effects[7].

## 5. Dosage and administration.

Bezafibrate is indicated in hyperlipoproteinemic patients where response to diet and other nonpharmacological methods has proved inadequate. The recommended dose in patients with normal renal function is 200 mg of the standard tablet 3 times daily or 400 mg of the sustained-release formulation once daily. Dosage adjustments are necessary in patients with renal disorders and in geriatric patients. Efficacy and tolerability have not been established in children. When bezafibrate is administered to patients receiving anticoagulation with coumarins, the dose of anticoagulant should be reduced by about 30%[1,7].

## 6. Side effects and toxicity.

During treatment with bezafibrate, there may be gastro-intestinal symptoms, such as transient loss of appetite, nausea, vomiting, and diarrhea or constipation. Other less common side effects are cutaneous reactions (pruritus and urticaria), dizziness, headache, insomnia, and loss of libido. These adverse effects tend to be mild, occur within the first few months of therapy and normally do not require withdrawal of medication [1,7].

Rhabdomyolysis, a muscular syndrome characterized by variable degrees of muscular cramps and weakness accompanied by marked elevation of creatine phosphokinase, has been reported in a few patients with reduced renal function treated with bezafibrate [1,7].

Small reductions in haemoglobin, white blood cell count and plasma viscosity have been reported. Bezafibrate also has a potential to increase biliary lithogenicity with a forecast long-term increase in the incidence of gallstones. However, a real increase has not been shown.

## 7. Literature reviews of the bezafibrate effects on liver functions and hepatotoxicity.

### 7.1 Animal studies

The hepatotoxicity of bezafibrate has been studied in rats, dogs and monkeys, and in each of these species dose-related clinical and chemical signs of liver abnormality were noted. In rats and dogs histological changes of the liver included increased liver cell size, pigment inclusion, and necrosis of the liver parenchyma[7,17]. These findings have not been observed in humans [7]. Hepatomegaly, associated with peroxisomal proliferation and enzyme induction, was seen following

administration of bezafibrate to rats but was not observed in non-human primates [17,18].

The carcinogenicity of bezafibrate has been studied in rats and mice. A dose-related increase in frequency of testicular tumors in rats, and a dose-independent increase in the occurence of liver tumors in male mice, were noted. Liver tumors were found in rats at very high dosage levels. These changes are, however, specific for rodents and were not observed with other animal species [7,17].

It is generally known that several hypolipidemic agents including bezafibrate cause peroxisome proliferation in the rodent liver [19]. Eacho and Foxworthy [20] have found that bezafibrate inhibited mitochondrial fatty acid oxidation and mitochondrial carnitine palmitoyltransferase (I)-the enzyme involving the transport of long chain fatty acid into mitochondria [21]-in rat liver, therefore suggesting that peroxisome proliferation may be an adaptive response to the inhibition in mitochondria. A recent in vitro study with isolated rat liver mitochondria has also shown that gemfibrozil, another fibric acid hypolipidemic agent , has uncoupling action and inhibits complex I of the respiratory chain[22]. Whether these interferences of mitochondrial energy metabolism are involved in the lipid-lowering effect

is presently a matter of conjecture.

### 7.2 Human Studies.

Hepatotoxicity found in rats such as hepatomegaly does not appear to develop following bezafibrate administration in man. However, there are reports of bezafibrate effect on liver enzymes which include a reduction of serum alkaline phosphatase, ɣ-glutamyl transferase and, in long-term studies ($\geq$ 24 months), alanine aminotransferase, but the significance and role of which have not been completely clarified [1,11]. Increases in transaminases have been observed occasionally in bezafibrate-treated patients [23,24]. Cholestasis -stoppage or suppression of bile flow, having intrahepatic or extrahepatic causes-has been described in 1 patient [1], although a cause-effect relationship was not established.

In the present time, bezafibrate is one of the conspicuous hypolipidemic drugs widely used in many hyperlipoproteinemic patients. Such patients have to use bezafibrate for long periods ; accordingly complete blood counts and renal/hepatic function tests should be performed. In patients with renal insufficiency, close monitoring of renal/hepatic functions and creatine phosphokinase levels are required ; clinical signs of muscle damage which potentially indicate rhabdomyolysis

are muscle cramps, pain or weakness. Bezafibrate is contraindicated in patients with liver disease (except fatty liver), gallbladder disease, severe disorders of renal function, pregnancy, and lactation.

The Mitochondrial Respiratory Chain and Oxidative Phosphorylation System.

All the processes involved 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[25,26].

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 3). The energy liberated as these reducing

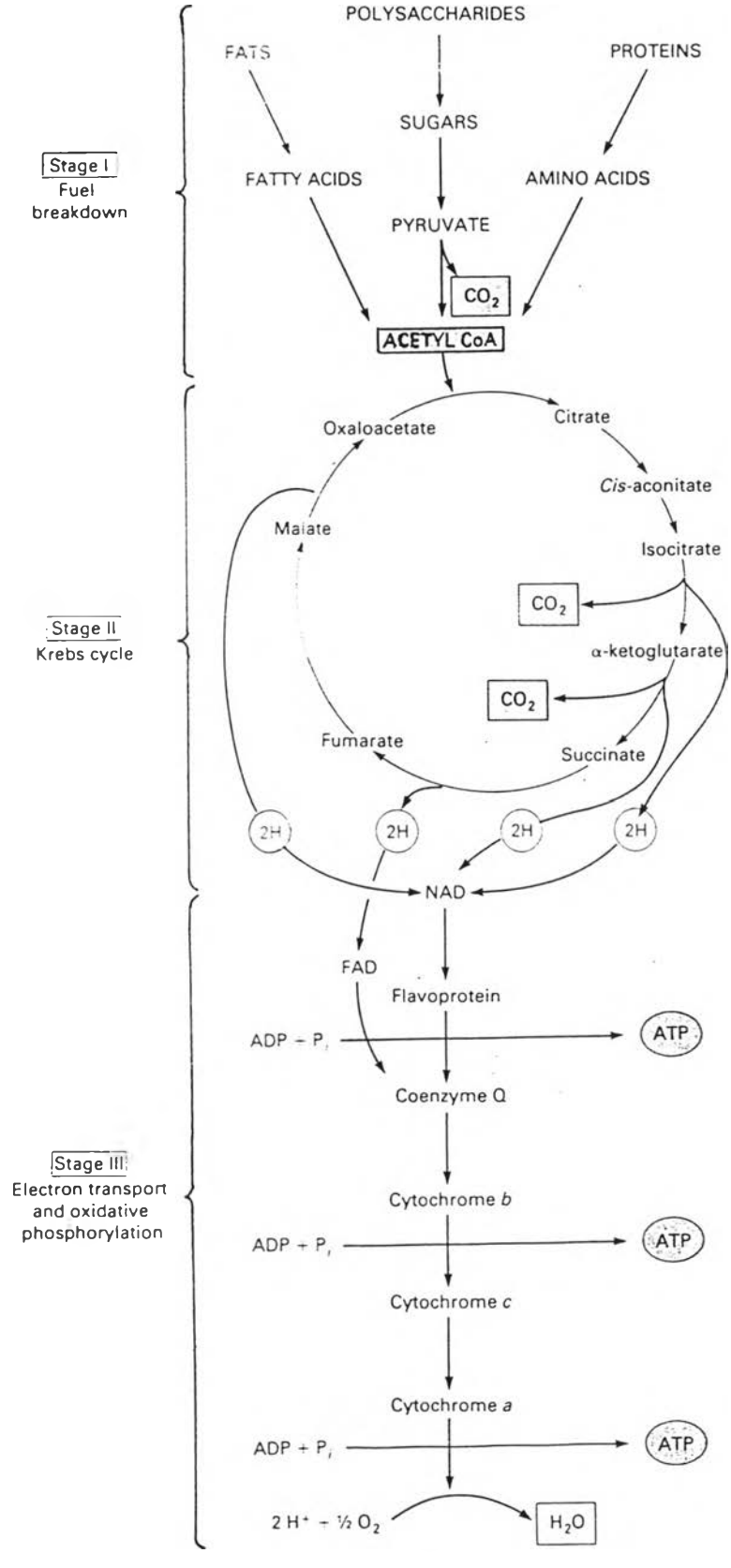


Figure 3. Correlation of fuel breakdown, Krebs cycle, electron transport chain and oxidative phosphorylation

equivalents are oxidized by the mitochondrial respiratory chain is used to drive ATP synthesis from ADP+Pi by the mitochondrial ATP synthase complex [27]. A concise 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 4. 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 numerous electron carriers 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, and $\alpha$-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 [28,29,30].

Experimentally the components of the mitochondrial respiratory chain can be separated into four complexes by using mild detergents. Such complexes have a

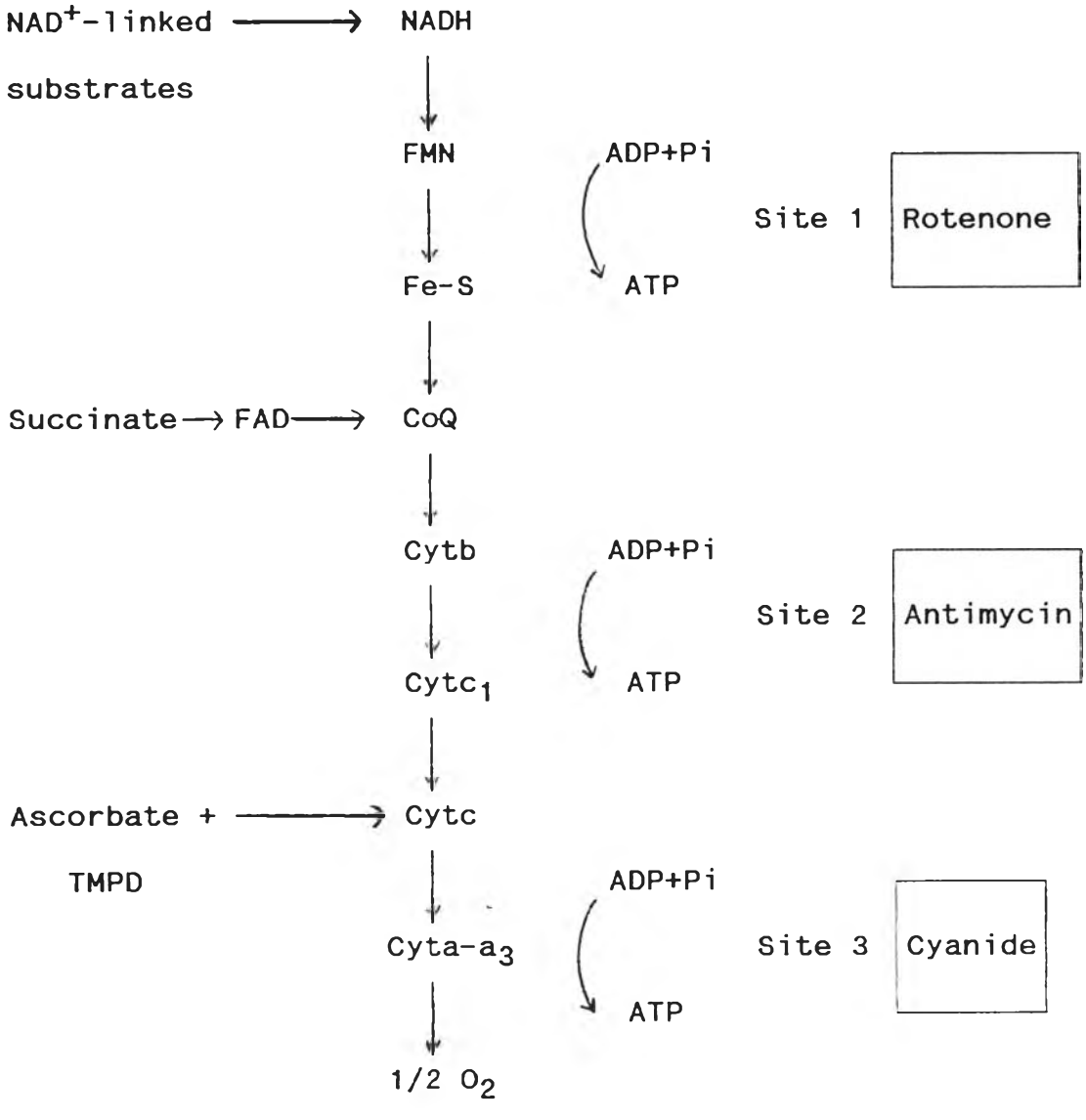


Figure 4. Electron transport chain, coupling sites, and inhibitors.

considerable amount of lipid, which is essential for their activity [25,30].

**1. Complex I or NADH-ubiquinone oxidoreductase.**

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

**2. Complex II or succinate-ubiquinone oxidoreductase.**

This complex, catalyzing the transfer of eletrons from succinate to Co Q, contains two largest polypeptides 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

centers.

**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 eletrons 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 [30].

## 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 [28]. The measured redox potentials and their corresponding free energy changes greatly drop as electrons are transfered through each three sites (figure 5). These sites of ATP synthesis also called coupling sites are regions whereby energy from oxidation or electron transport is coupled to the phosphorylation of ADP. The overall process is called oxidative phosphorylations. Site I, Site II, and Site III in electron transport chain are designated in figure 4.

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 [29].

The electron transport and phosphorylation are

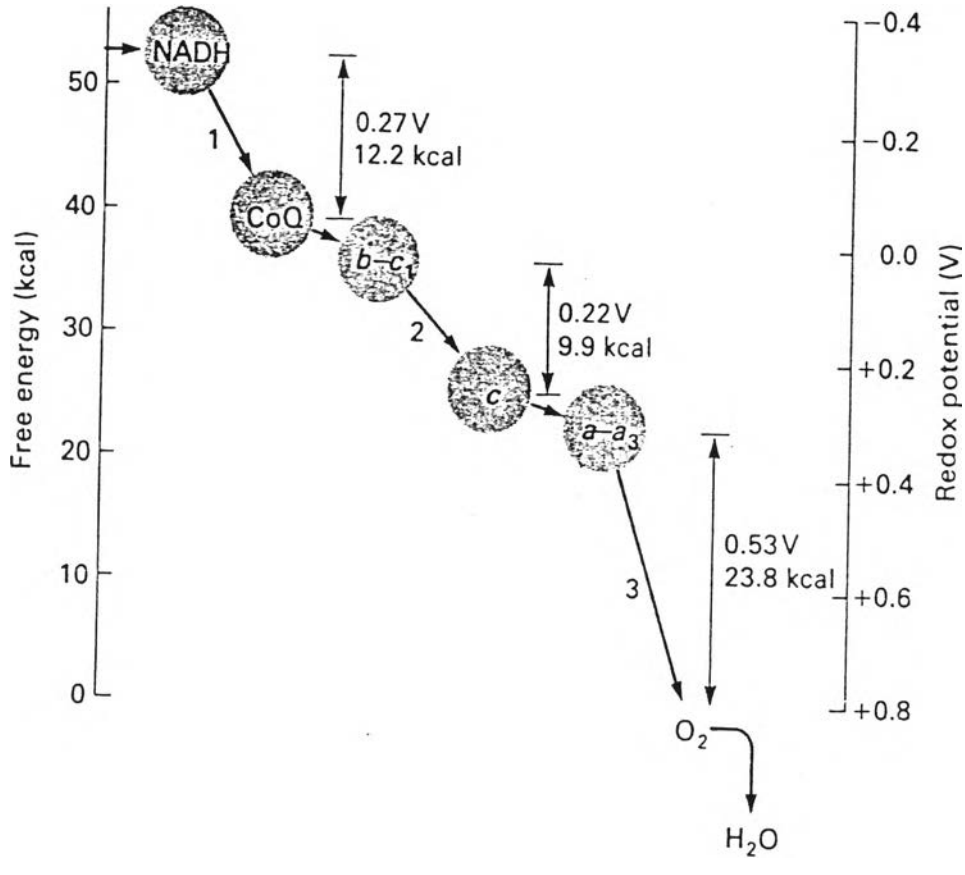


Figure 5. The measured redox potentials and their corresponding free-energy changes in each steps of electron transport from NADH to oxygen.

uncoupled in aged mitochondria or by the uncouplers such as 2,4-dinitrophenol (DNP). In the pressence of DNP, ATP synthesis stops but electron transport continues at a higher rate. All the energy is released as heat under this condition.

### 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 [27,28,30]. The main points of the chemiosmotic hypothesis are as follows:

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

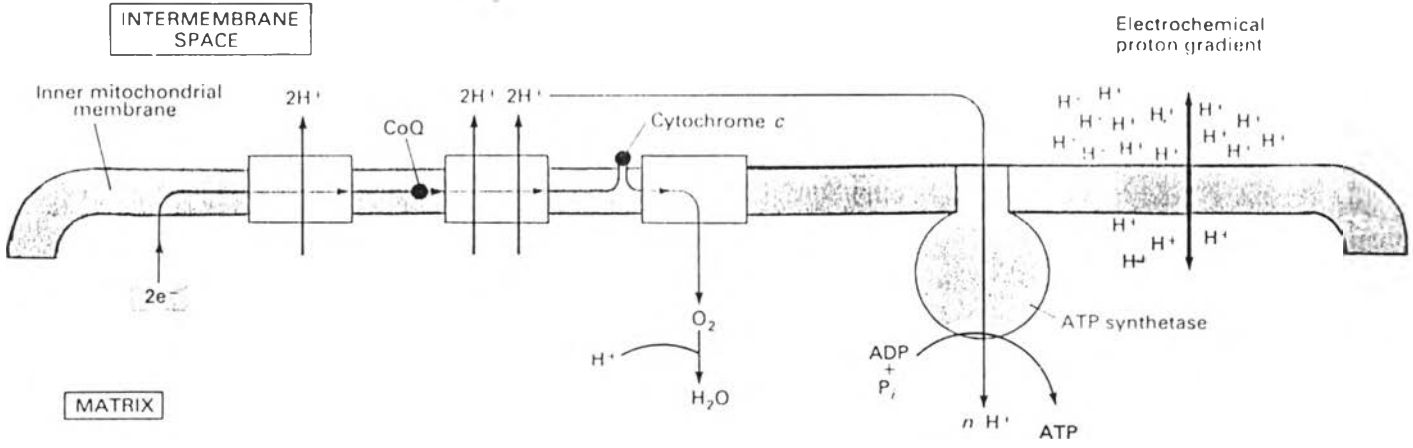


Figure 6. Schematic illustration of the coupled processes of electron transport and oxidative phosphorylation.

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 (figure 6)

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 [26,28,30].

## 4. The mitochondrial ATP synthase.

The enzyme responsible for ATP synthesis in mitochondria, ATP synthase or $F_1F_0$-ATPase, is a component of the mitochondrial inner membrane. It consists of two principal portions, $F_1$ and $F_0$ (figure 7)

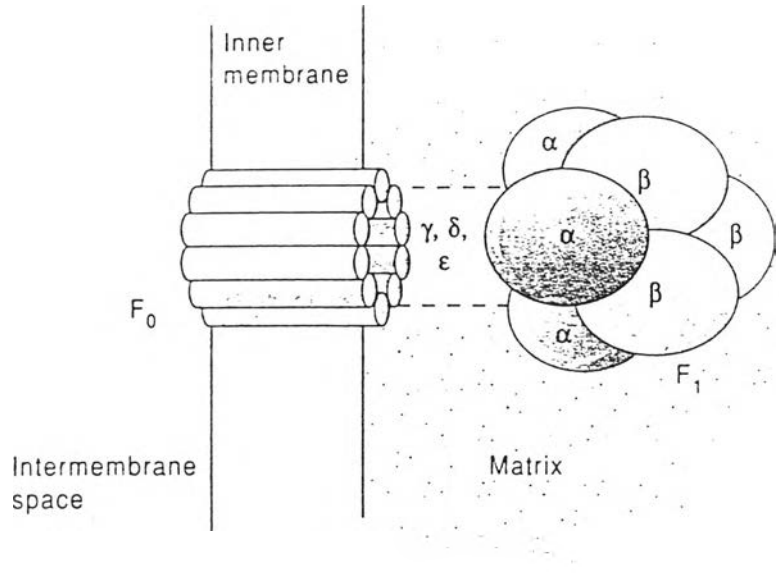


Figure 7. Components of the $F_1F_0$-ATPase complex [30].

$F_1$ 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. $F_0$, the membrane sector, is a 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 confering 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 protrons back into the mitochondrial matrix through the $F_0$ portion; the $F_1$ portion catalyzes ATP synthesis from ADP and Pi. Conversely, under conditions 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 $F_1F_0$-ATPase is a reversible system that can catalyze both ATP synthesis and ATP hydrolysis depending on metabolic state of the mitochondria (figure 8)[28].

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

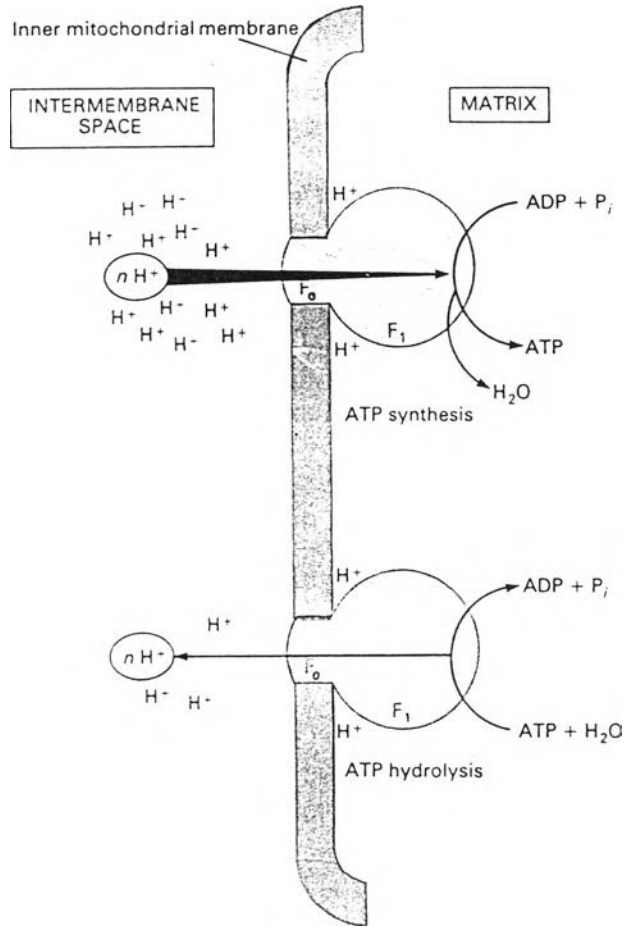


Figure 8. The mitochondrial $F_1$-$F_0$ ATPase may act reversibly as a synthetase or as a hydrolase.

- Oligomycin and dicyclohexylcarbodiimide (DCCD) inhibit ATP synthesis by binding with a subunit of $F_0$ portion and consequently block proton conduction through $F_0$. Oligomycin shows inhibitory action only in the presence of oligomycin sensitivity confering protein (OSCP) which contains solely in mitochondrial $F_1$ $F_0$-ATPase. Bacteria and chloroplast $F_1F_0$-ATPase do not contain OSCP, therefore they are not inhibited by oligomycin[31].

- 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 $P_i$ into mitochondria, and consequently deplete $P_i$ 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 uncouplers (figure 9)[29].

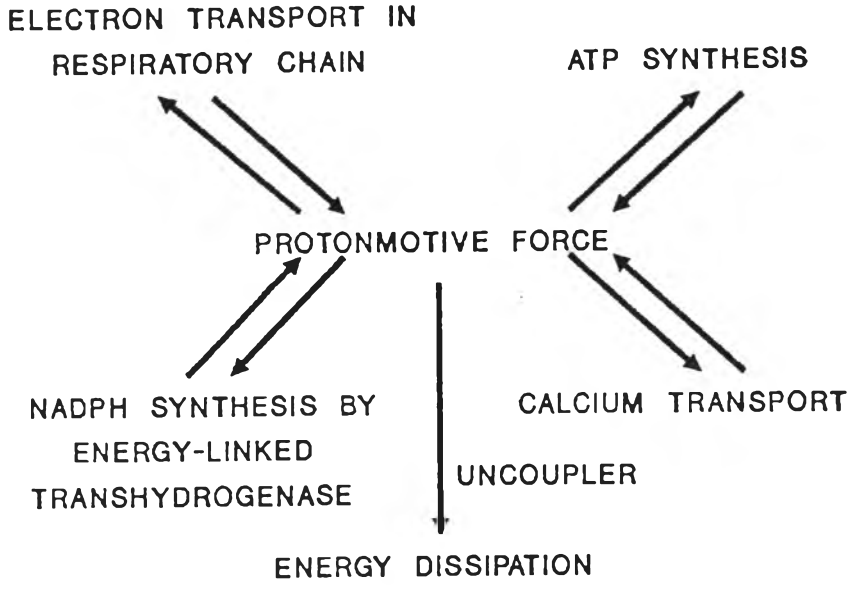


Figure 9 Utilization of mitochondrial protonmotive force in various processes.

In view of the importance of the mitochondria in cell metabolism and functions, the purpose of the present study is to investigate the effect of bezafibrate on mitochondrial bioenergetics. The study has been performed on isolated mitochondria from the liver because hepatic tissue plays an important role in lipid metabolism. Three mitochondrial bioenergetic functions namely, oxidative phosphorylation, ATPase activity, and calcium trasport were studied. In as much as the mechanism of the lipid-lowering effect of bezafibrate is not well understood, the study of bezafibrate effect on mitochondrial functions may shed some lights on the mechanism of the pharmacological and/or toxicological action of this drug.