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# EFFECTS OF PYRIDINE ON RABBIT LIVER, KIDNEY AND LUNG MICROSOMAL CYTOCHROME P450 DEPENDENT DRUG METABOLIZING ENZYMES

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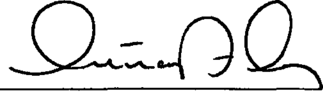

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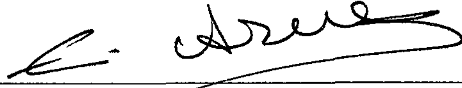

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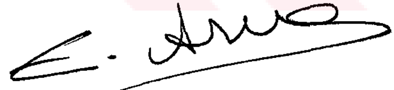

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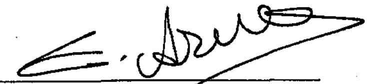
Prof. Dr. Emel ARINÇ 

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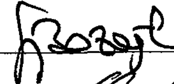
Prof. Dr. Faruk BOZOĞLU 

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ABSTRACT

EFFECTS OF PYRIDINE ON RABBIT LIVER, KIDNEY AND LUNG MICROSOMAL CYTOCHROME P450 DEPENDENT DRUG METABOLIZING ENZYMES

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Pyridine, a natural constituent of coal tar occurs in several naturally occuring products and in combustion-by products. It is used widely as a solvent in pharmaceutical and chemical industry and it undergoes cytochrome P450 mediated oxidation to yield N-oxides. In this study, *in vivo* effects of pyridine treatment (i.p. at a dose of 250 mg/kg body weight on day 1, 5 and 8) on cytochrome P450 levels, microsomal cytochrome P450 dependent drug metabolizing enzymes and on the biomarkers used to measure chemical-induced toxicity including LDH and GOT in rabbit liver, kidney and lung were examined.

Results obtained in this study showed that pyridine treatment elevated microsomal cytochrome P450 contents of liver, kidney and lung by 2.04 -, 1.6 - and 1.4- fold, respectively. Induction of the cytochrome P450 isozymes was observed with the high intensity bands corresponding to approximate Mr of 51 000 and 53 000 in the SDS-PAGE profiles of both liver and kidney microsomes obtained

from the pyridine treated rabbits. Pyridine treatment of rabbits caused 5.8- and 4.5-fold increases in aniline 4-hydroxylation rates of liver and kidney microsomes, respectively, while no change was observed in the activity of enzyme of lung microsomes. Administration of pyridine markedly enhanced hydroxylation rates of *p*-nitrophenol by liver and kidney microsomes about 4.4- and 4.9- fold, respectively. Liver microsomal ethylmorphine N-demethylase activity was not altered upon pretreatment of rabbits with pyridine. On the other hand, pyridine injections caused 3.2- and 1.8-fold increases in rabbit kidney and lung microsomal ethylmorphine N-demethylase activities. However, these increases were found statistically insignificant. NDMA N- demethylase activity was enhanced by pyridine treatment in all three of these organs. (6.9-fold in liver, 3.4-fold in kidney, 5.15-fold in lung). Pyridine treatment of rabbits caused an increase of the P4502E1 level of microsomes and this was reflected in increased rate of metabolism of aniline, *p*-nitrophenol and NDMA. Pyridine treatment also increased the LDH activities in soluble fraction of liver, kidney and lung and GOT activity only in kidney cytosol (2.87-, 52.53-, 2.5- and 1.58-fold, respectively). On the other hand, neither GOT nor LDH activities increased in serum upon subacute treatment of rabbits with pyridine. This is the first time induction of NDMA metabolism has been reported for kidney and lung microsomes of rabbits pretreated with pyridine.

Key words: Pyridine, drug metabolizing enzymes, cytochrome P450, liver, kidney, lung, microsomes, aniline 4-hydroxylase, ethylmorphine N-demethylase, *p*-nitrophenol hydroxylase, NDMA N-demethylase

## ÖZ

# PİRİDİNİN,TAVŞAN KARACİĞER, BÖBREK VE AKCİĞERİ, SİTOKROM P450'YE BAĞIMLI, İLAÇLARI METABOLİZE EDEN MİKROZOMAL ENZİMLERİ ÜZERİNE ETKİLERİ

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Kömür katranının doğal bir bileşeni olan piridin, çeşitli doğal ürünlerin ve yanma ürünlerinin yapısında bulunur. İlaç ve kimya sanayiinde çözücü olarak yaygın bir şekilde kullanılan piridin sitokrom P450'ye bağımlı oksidasyona girerek, N-oksitlerine dönüşür. Bu çalışmada, piridin uygulanmasının (1., 5. ve 8. günlerde 250 mg/kg vücut ağırlığı dozda, i.p.) tavşan karaciğer, böbrek ve akciğerindeki, sitokrom P450 seviyeleri, mikrozomal P450'ye bağımlı ilaç metabolize eden enzimler ve LDH ve GOT gibi kimyasal toksisite ölçümünde kullanılan biyobelirteçler üzerinde gösterdiği *in vivo* etkileri incelendi.

Bu çalışmada elde edilen sonuçlar, piridin uygulanmasının; karaciğer, böbrek ve akciğer mikrosomal sitokrom P450 miktarlarını sırasıyla 2.04-, 1.6- ve 1.4- kat arttırdığını gösterdi. Sitokrom P450 izozimlerinin indüksiyonu, piridin uygulanmış tavşanlardan elde edilen karaciğer ve böbrek mikrozomlarının

SDS-PAGE profillerinde yaklaşık olarak 51 000 ve 53 000 molekül ağırlıklarına denk gelen bölgelerdeki koyu bandlar ile gözlendi. Tavşanlara piridin uygulanması akciğer mikrozomal anilin 4-hidroksilaz aktivitesinde hiçbir değişiklik yapmazken, karaciğer ve böbrek mikrozomlarındaki enzim aktivitesinin sırasıyla 5.8- ve 4.5- kat artmasına neden oldu. Piridin enjeksiyonu *p*-nitrofenolün karaciğer ve böbrek mikrozomları tarafından hidroksilasyon hızını, farkedilir bir şekilde; sırasıyla 4.4- ve 4.9-kat arttırdı. Karaciğer mikrozomal etilmorfin N-demetilaz aktivitesi, tavşanların piridinle ön-muamelesi üzerine değişmedi. Diğer taraftan, piridin enjeksiyonu, istatistiksel olarak önemsiz olmakla beraber, tavşan böbrek ve akciğer mikrozomal etilmorfin N-demetilaz aktivitelerinde 3.2- ve 1.8- kat artışa neden oldu. Piridin uygulanması bu üç organda da NDMA N-demetilaz aktivitesini arttırdı. (Karaciğerde 6.9-kat, böbrekte 3.4-kat, akciğerde 5.15-kat). Tavşanlara piridin uygulanması, mikrozomların P4502E1 seviyesinde bir artışa neden oldu ve bu artış anilin, *p*-nitrofenol ve NDMA metabolizmasındaki artış şeklinde de gözlendi. Karaciğer, böbrek ve akciğerin çözünür fraksiyonlarının (sitosol) LDH ve sadece böbrek sitosolü GOT aktivitelerini arttırdı. (sırasıyla; 2.87-, 52.53-, 2.5- ve 1.58-kat). Diğer yandan, tavşanlara subakut piridin uygulanması kan serum GOT ve LDH aktivitelerinde herhangi bir değişikliğe sebep olmadı. Piridin verilmiş tavşanların böbrek ve akciğer mikrozomları için NDMA metabolizmasının indüksiyonu ilk kez bu çalışmada gösterildi.

Anahtar Kelimeler: Piridin, ilaçları metabolize eden enzimler, sitokrom P450, karaciğer, böbrek, akciğer, mikrozomlar, anilin 4-hidroksilaz, etilmorfin N-demetilaz, *p*-nitrofenol hidroksilaz, NDMA N-demetilaz

To My Parents

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## LIST OF SYMBOLS AND ABBREVIATIONS

| | |
|---|---|
| APS | Ammonium per sulphate |
| BSA | Bovine serum albumine |
| CO | Carbon monoxide |
| DNPH | 2,4-Dinitrophenyl hydrazine |
| ε-ACA | ε-Amino caproic acid |
| EDTA | Ethylenediamine tetra acetic acid |
| EROD | Ethoxyresorufin-*o* -deethylase |
| FAD | Flavine adenine dinucleotide |
| $FADH_2$ | Flavine adenine dinucleotide, reduced form |
| GOT | Glutamate oxaloacetate transaminase |
| HEPES | N-2-hydroxyethyl piperazine-N'-2 ethane sulfonic acid |
| LDH | Lactate dehydrogenase |
| MC | 3-Methylcholantrene |
| MFO | Mixed function oxidases |
| Mr | Molecular weight |
| NADH | Nicotineamideadenine dinucleotide, reduced form |
| NADP+ | Nicotineamideadenine dinucleotide phosphate |
| NADPH | Nicotineamideadenine dinucleotide phosphate, reduced form |
| NDMA | N-nitrosodimethylamine |
| pAp | *p*-Aminophenol |
| PAGE | Polyacrylamide gel electrophoresis |
| PB | Phenobarbital |
| PMSF | Phenylmethylsulfonyl floride |
| PY | Pyridine |
| SDS | Sodium dodecyl sulfate |
| TCA | Trichloro acetic acid |
| TCDD | 2,3,7,8-tetrachloro dibenzo-*p* -dioxin |
| TEMED | N-N-N'-N'-tetramethylenediamine |
| TRIS | Tris (hydroxymethyl ) aminomethane |

# CHAPTER I

# INTRODUCTION

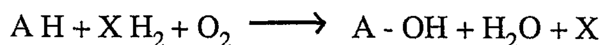
Pyridine and other N-heterocyclic compounds are generated during the processing of fossil fuels. These processes include the conversion of coal to liquid and gas, and the production of fuel from oil shale. In addition, these compounds that constitute an important class of xenobiotics are used as solvents, intermediates in the production of agricultural chemicals (insecticides, herbicides), pharmaceuticals, rubber dyes, explosives etc...

The N-heterocyclic compound moiety also occurs very close to the daily life with the existence of 379 compounds of this kind in cigarette/cigar smoke. So, with regard to their toxicity and abundance, these xenobiotics are of considerable interest. They are usually transformed *in vivo* by mixed function oxidases (MFO) into metabolites which may be less or more toxic than the parent compounds.

## 1.1. Mixed Function Oxidases (MFO)

MFO are among the most ubiquitous enzymes in living organisms. They are present in just every phyla; in procaryotes, unicellular eucaryotes, vertebrates and even in higher plants. In mammalian system MFO are found in several tissues and are complex in their action.

They catalyze the insertation of one oxygen atom of molecular oxygen into the organic substrate; the other oxygen atom is reduced to water. The general equation of such reaction is given below;



$$AH + XH_2 + O_2 \longrightarrow A\text{-}OH + H_2O + X$$

Where, AH is the substrate which accepts oxygen atom; $XH_2$ is the second substrate (cofactor) that furnishes electrons to reduce one atom of oxygen molecule to water. The enzymes catalyzing these reactions are also called as "Monooxygenases " and " Hydroxylases".

1.1.1. Components of Mixed Function Oxidases

It has been established that MFO system functions as a multicomponent electron transport system. Lu and Coon (1968), for the first time, demonstrated that liver microsomal cytochrome P450 dependent MFO system has three components; cytochrome P450 (EC 1.14.14.1), NADPH, cytochrome P450 reductase (EC 1.6.2.4.), and a heat stable factor subsequently shown to be phosphatidylcholine (Strobel *et al.,* 1970).

Cytochrome P450, terminal oxidase of NADPH dependent MFO system, contains $Fe^{3+}$ and protoporphyrin IX and is a b type cytochrome. Recently, it has been shown that it exists as a family of isozymes. Up to now, at least 220 genes for cytochrome P450 have been identified in different species (Schenkman, 1995). Their monomer molecular weights range from 48 000 to 60 000 (Philpot and Arınç, 1976; Lu and West, 1980; Arınç and Adalı, 1983; Black and Coon, 1986).

Some of these forms are inducible, i.e., the levels are elevated by challenge of the animals with xenobiotics. Other forms have not been inducible yet. However, such forms have been shown to be responsive to developmental changes *in vivo*, sexual development, and pathophysiological conditions (Schenkman, 1991).

Some of the P450 isozymes are fairly specific in their choice of substrates, but many, and particularly those in the hepatic endoplasmic reticulum, catalyze a surprisingly large number of chemical reactions with an almost unlimited number of biologically occuring and xenobiotic compounds. Alterations in cytochrome P450 activities toward some of the physiologically important substrates including steroids, prostanoids and other eicosanoids, fat-soluble vitamins, fatty acids and the mammalian alkaloids have already been implicated in diseases that affect human health. Examples of xenobiotics that serve as P450 substrates are drugs (including antibiotics), procarcinogens, antioxidants, organic solvents, anesthetics, dyes, pesticides, alcohols, odorants and a variety of unusual substances in plants and microorganisms. From biochemical and toxicological viewpoint, in vivo studies carried out with chemical carcinogens have demonstrated that different isozymes of P450 are responsible for the activation and toxicity of the most of the chemical agents ( Nebert and Felton, 1976; Wolf *et al.*, 1979; Nebert and Gonzales, 1987; Arınç *et al.*, 1991 ).

NADPH dependent cytochrome P450 reductase enzyme functions in the transfer of electrons from NADPH to cytochrome P450. It is a membrane-bound amphipathic protein containing both hydrophobic peptide and hydrophilic peptide. Monomer molecular weight of P450 reductase was determined to be 78 000 (Gum and Strobel, 1981; Black and Coon, 1982; İşcan and Arınç, 1986 and 1988). Hydrophilic peptide having Mr of 71 000 contains 1 mol each of FAD and FMN. Hydrophobic peptide is responsible for proper interaction of reductase with cytochrome P450 and anchoring the reductase to endoplasmic reticulum.

### 1.1.2. Mechanisms of Cytochrome P450 Dependent Mixed Function Oxidases

The mechanisms postulated for the hydroxylation of organic substances by microsomal cytochrome P450 dependent MFO is illustrated in Figure 1.1. (taken from Lehninger, 1975). The reducing equivalents from NADPH are transferred through NADPH-cytochrome P450 reductase to cytochrome P450 during

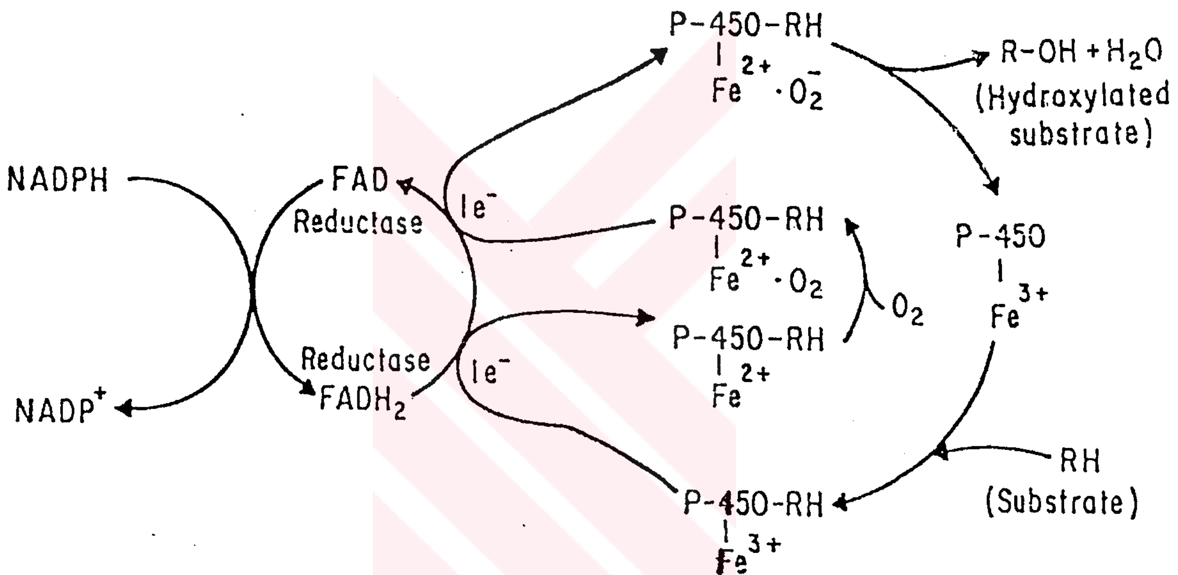



Figure 1.1. Mechanism postulated for the hydroxylation of organic substrates by microsomal cytochrome P450 dependent MFO system

hydroxylation of various compounds. The substrate RH first combines with $Fe^{3+}$ form. The latter is then oxygenated and a second electron from NADPH converts bound oxygen to $O_2$ radical. An internal oxidoreduction takes place by the formation of the hydroxylated substrate and $H_2O$, which contained the oxygen atoms introduced as $O_2$. Free cytochrome P450 is regenerated in its $Fe^{3+}$ form (Figure 1.1.). It has been proposed that, lipid facilitates transfer of electrons from NADPH-cytochrome P450 reductase to cytochrome P450 (Lu and Levin, 1974). All of the three components, cytochrome P450, NADPH-cytochrome P450 reductase, and lipid (phosphatidylcholine) are required to reconstitude the full hydroxylation activity. (Lu and Coon, 1968; Lu and Levin, 1974; Arınç and Philpot, 1976; Black and Coon, 1986; Adalı and Arınç, 1990 ).

### 1.1.3. Classification and Nomenclature of Cytochromes P450

It has been shown that versatility of MFO system is associated with cytochrome P450 (Lu and Levin, 1974). Multiple cytochrome P450 isozymes have been purified and characterized in different laboratories by various purification methods and have been named at these laboratories. Spectral and catalytic properties and molecular weights are not enough to distinguish between cytochrome P450 isozymes.

Because of the limitations in biochemical methodology only a limited number of P450 enzymes was characterized 10-20 years ago. But, with the development of recombinant DNA and some special techniques during 1980s, we come to know the magnitude and complexity of the P450 gene superfamily. In 1985, it became apparent that the amino acid sequence could be aligned and classified on the basis of proposed divergent evolutionary relationships of corresponding genes. A committee was formed to carry out this task (Nelson *et al.*, 1993).

Nebert and Gonzales (1987) made a classification of liver P450 isozymes from various species according to the sequence homology of P450 genes. They divided the P450 genes into 7 main family called P450I, P450II, P450III etc. The P450I gene family in rat, rabbit, mouse and human having two genes that were inducible by polycyclic hydrocarbons and TCDD, contained two groups of P450 isozymes; one contained rabbit LM6, rat c, mouse Pi and human Pi, and another group contained the isozymes rabbit LM4, rat d, mouse P3 and human P3. The gene family of P450II also divided into five subfamily. Subfamily II inducible by phenobarbital contained rabbit LM2 and rat b and rat e. Another subfamily of P450II family which was inducible by ethanol contained the rabbit LM3a and rat j.

The revision that was conducted by Nelson *et al.*, in 1993, canceled the previous works (Nebert *et al.*, 1987, 1989 and 1991) in which a nomenclature system based on divergent evolution of the superfamily, has been described. For naming a P450 gene or cDNA, they recommended to use the italicized root symbol *"CYP"* (*"Cyp"* for the mouse) denoting cytochrome P450, an Arabic number designating the P450 family, a letter indicating the subfamily, when two or more subfamilies are known to exist within that family, an Arabic numeral representing the individual gene. Thus, if rabbit liver isozyme 2 (other common names include, LM2, and PB-1) was being designated, it would be 2B4, with the prefix CYP it would changed to the form CYP2B4.

Naming a P450 gene can be quite simple. The protein sequence is aligned with a representative sequence from each family and subfamily, and the percent identity is determined. This percentage only reflects comparisons of overlapping portions of the sequences; gaps and unmatched ends are not counted in the overall length. If the sequence is less than 40% identical to all other sequences, the new sequence constitutes the first member of a new family. If the new sequence is at least 40% identical to any other sequence, then the new sequence belongs in that family.

Once the family or subfamily is identified, the new sequence is compared with that of all other members in the group. If the new sequence is only a few (≤3%)

amino acids different from a known sequence, it is given the same name-unless it can be shown to be a distinct gene. If the sequence is a new member of the subfamily or family, it is given the next available number in the group. One expection to this rule is the CYP2D subfamily which is the most distant subfamily within the CYP2 family, having some cytochromes P450 that are slightly less than 40% similar to other CYP2 genes.

Multiple forms of cytochrome P450 have been purified from hepatic microsomes of the rabbit and extensively characterized. Forms of cytochrome P450 purified from rabbits seem to be fewer than those from rats. Some of the cytochromes P450 from rabbits rats and human are given Table 1.1.

Table 1.1. Nomenclature and properties of some rabbit, rat and human cytochromes P450

| Gene | Rabbit P450s Protein | Trivial Names | | | | | Rabbit P450s Mr | Induction | Substrate |
|---|---|---|---|---|---|---|---|---|---|
| | | Rabbit | | | Rat | Human | | | |
| | | Isozyme | Liver | Lung | Liver | Liver | | | |
| 1 A | 1A1 | Isozyme 6 | LM6 | LgM6 | P450C | P450HLc | 57.500 | TCDD, MC Benzoflavone Isosafrole | Benzol(a)-pyrene Ethoxyresorufin |
| 2B | 2B4 | Isozyme 2 | LM2 | LgM2 | P450b P450e | - | 49.500 | PB | Benzphetamine 7. ethoxycoumarin |
| 2E | 2E1 | Isozyme 3a | LM3a | – | P450j RML6 | P450HLj | 51.000 | Alcohol, Acetone | Acetone, Benzene Nitrosamines NDMA |
| 4B | 4B1 | Isozyme 5 | LM5 | LgM5 | - | - | 57.000 | PB | Aromatic amines |

CYP 1A1(form 6 or LM6) was induced in rabbit liver by TCDD, benzoflavone, isosafrole and MC. It had a subunit molecular weight of 57 500 and it was very active in metabolism of benzo-(a)-pyrene and ethoxyresorufin (Norman *et al.*, 1978; Koop and Coon, 1984).

CYP 2B4 (form 2, LM2) is a major phenobarbital-inducible form. It was the first cytochrome P450 to be purified (Imai and Sato 1974; Van Der Hoeven *et al.,* 1974). Its monomer molecular weight was reported as 49 500 on SDS-PAGE (Black and Coon 1986). CYP 2B4 was the most active isozyme in catalyzing the metabolism of substrates such as benzphetamine and 7-ethoxycoumarin (Haugen and Coon. 1976; Coon *et al.*, 1978).

CYP2E1(P4502E1), the alcohol-inducible cytochrome P450, previously known as P450 LM3a, P450j, P450ac or P450alc, has been identified in rat, rabbit and man (Ryan *et al.*, 1986; Lasker *et al.*, 1987; Palakodaty *et al.*, 1989). It is also induced by the treatment with acetone, isoniazid, isopropanol, pyridine, dimethyl sulfoxide and by starvation, high-fat feeding and diabetes (Koop and Casazza, 1985; Hong *et al.*, 1987; Song *et al.*, 1987; Kim *et al.*, 1990).

CYP2E1 has received a great deal of attention in recent years because of its vital role in the activation of many toxic chemicals. Its possible role in the activation of xenobiotics to electrophilic, potentially mutagenic metabolities and in tumour development has been demonstrated in studies with benzene (Mehlman, 1991; Nakajima *et al..*, 1992), low molecular weight halogenated hydrocarbon species, like $CCl_4$ (Guengerich *et al..*, 1991) and nitrosamines (Yoo *et al.*, 1988; Yang *et al..*, 1990; Yamazaki *et al..*, 1992).

P4502E1 is among the most conserved forms in the CYP2 family and the catalytic activites of P4502E1 across species are quite similar, suggesting its possible physiological importance. Acetone, a ketone body, is metabolized by P4502E1 to acetal and then to methylglyoxal (Koop and Casazza, 1985) which can be used for the synthesis of glucose. It has been estimated that under physiological conditions, most of the acetone is metabolized via this oxidative pathway (Landau and Brunengraber, 1987). However, NADPH and ATP molecules are consumed to drive this pathway. One may hypothesize that, during fasting, P4502E1 is induced for the acceleration of this pathway for making glucose from acetone, for critical physiological functions.

Among all the P450s studied, P4502E1 is most active in catalyzing the NADPH-dependent formation of $H_2O_2$ and $O_2$ in vitro (Ekstrom and Ingelman-Sundberg, 1989; Gorsky *et al.*, 1984; Persson *et al.*, 1990). Antibodies to P4502E1 almost completely inhibited the NADPH-dependent lipid peroxidation in microsomes (Ekstrom and Ingelman-Sundberg, 1989). It was postulated that induction of P4502E1 in the liver results in increased oxygen stress by generating higher levels of $H_2O_2$ and $O_2$. So, it has also been postulated that CYP2E1 is very important factor in the development of alcohol-induced liver disease, possibly through this increasing lipid peroxidation(Ingelman-Sundberg *et al.*, 1993).

With respect to its physiological and catalytic functions, CYP2E1 is an interesting and challenging topic to investigate. Also, the high similarity of rodent P4502E1 to human P4502E1 gives the chance to extrapolating many of the observations in animals to humans.

CYP4B1(isozyme 5 or LM5) was purified from liver microsomes of rabbits by Slaughter *et al.* , (1981) and Robertson *et al.* , (1983). It is also present in microsomes of rabbit lung (Wolf *et al.*, 1979). It was found that phenobarbital treatment of rabbits resulted in increased content of CYP4B1 and also increased in the metabolism of aromatic amines such as 2-aminoanthracene and 2-amino fluorene to mutagenic products. Its molecular weight has been reported as 57 000 on SDS-PAGE.

## 1.2. Pyridine

Among xenobiotics, N-heterocylic compounds, especially pyridine and several pyridine derivatives are of considerable interest. Because pyridine is found in a variety of industrial and laboratory settings. It is also a natural constituent of coal tar and occurs in several naturally occuring products and in combustion-by products. Pyridine is a heterocyclic compound containing a triunsaturated, six-membered ring of five carbon atoms and one nitrogen atom (Figure 1.2.). It is a colorless, hygroscopic liquid with a pungent, unpleasant odor. When

anhydrous, it boils at 115.2 - 115.3 °C, its density (20/4) is 0.98272 and $n^{20}_{4}$ is 1.50920. Pyridine is miscible with organic solvents as well as with water. It is a good solvent for many organic and inorganic compounds (Merck Index 1989).

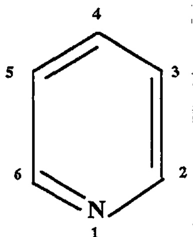



Figure 1.2. Molecular formula of pyridine

Pyridine is irritant to skin (eczema) and other tissues (conjunctivities) and chronic exposure has been known to cause liver and kidney damage. $LD_{50}$ orally in rats is 1.58 g/kg. Repeated exposure to atmospheric levels greater than 5 parts per million is considered hazardous. It may cause Central Nerveous System (CNS) depression (Gensler, 1983).

Pyridine and several pyridine derivatives have been reported to undergo cytochrome P450-mediated oxidation to yield N-oxides (Damani and Crooks, 1982). The P450 iron oxene is a strong oxidant and may be capable of abstracting an electron from the pyridine nitrogen generating an intermediate amine radical cation will then yield pyridine N-oxide. Whereas, N-oxidation serves generally as a mechanism for detoxification, some N-oxides such as 4-nitroquinoline N-oxide and various purine N-oxides have been reported to be carcinogenic (Endo *et al.*, 1971).

The pyridine moiety also occurs in several naturally occuring products such as nicotinamide, nicotine and in combustion-byproducts such as cigarette smoking. Indeed 79 of the 379 nitrogen heterocycles identified in cigarette/cigar smoke are imidazol and pyridine derivatives (Schumacher *et al.*, 1977; Moree-Testa *et al.*, 1984) and several have been shown to possess either carcinogenic, tumor-promoting or teratogenic activities (Landaver and Salem, 1973).

Pyridine and its derivatives are widely used as industrial solvents and as constituents of a number of herbicides (e.g. paraquat, diquat, picloram) (Goe, 1978). Contamination of ground water and soil by industrial agricultural waste creates severe health hazards as a result of acute toxicity and teratogenic effects of pyridine compounds (Jori *et al.*, 1983 ).

In 1988, Kim *et al.*, demonsrated that pyridine administration (100 mg/kg, i.p., 4 days) to rats elevated hepatic microsomal cytochrome P450 content approximately 2.5 - fold as compared to controls. SDS-polyacrylamide gel electrophoresis revealed a protein band of enhanced intensity migrating in the region of P4502E1, the major ethanol-inducible form of rat liver cytochrome P450. *p*-Nitrophenol hydroxylase activity which is associated with primarily by the ethanol-inducible form of P450, P4502E1, was elevated approximately 4-fold in pyridine-induced rat liver microsomes relative to control rat liver microsomes. These conclusions were supported by the substantial increase in NDMA N-demethylase and aniline hydroxylase activities, approximately 5- and 8-fold, respectively. In the same laboratory, it was also shown that the rate of pyridine N-oxide production was enhanced 4-fold in pyridine-induced microsomes relative to untreated controls. Morever, the effect of *p*-nitrophenol on the rate of pyridine N-oxide production was characterized. In the *in vitro* studies, when hepatic microsomes obtained from pyridine treated rats were used, *p*-nitrophenol (400 μM) competitively inhibited the production of pyridine N-oxide by 70% at 0.5 mM pyridine concentration. This result suggested that P4502E1 (P450j) isozyme of P450 was induced in rats by pyridine treatment and that this form is a principal catalyst of pyridine N-oxide production.

The effects of acute and chronic administration of pyridine on rabbit hepatic microsomal cytochrome P450 catalyzed drug metabolism have been examined by Kaul and Novak in 1987. They found that pyridine (1.0 mM) addition to the incubation mixture decreased N-demethylation of NDMA and *O*-demethylation of *p*-nitroanisole approximately 50% for both. They also reported that pyridine administration (100 mg/kg i.p. for 5 days) to rabbits increased hepatic microsomal cytochrome P450 content over 2-fold relative to uninduced animals. SDS gel

electrophoresis of pyridine-induced microsomes revealed protein bands of enhanced intensity occuring in the regions of P450LM3(2E1) and P450LM4(1A2). Pyridine induced microsomes were effective in the production of pyridine N-oxide. The metabolism of NDMA, ethanol, butanol and aniline was also examined in pyridine-induced microsomal suspensions. Markedly enhanced catalytic activities were observed toward each of these substrates (Kaul and Novak, 1987).

In 1990, Kim and Novak also characterized the dose- and time-dependent induction of P4502E1 in rat liver by pyridine. They found that, a single injection of pyridine (100 mg/kg, i.p.) increased P4502E1 levels 2-, 3- and 4-fold at 6, 10 and 24 hours respectively, relative to controls as evidenced by *p*-nitrophenol hydroxylase activity which has been shown to be specific for P4502E1 and by Western blot analysis. Induction of P4502E1 was dose-dependent over the range 10 to 200 mg/kg. The results they obtained suggest that induction of P4502E1 at early times following acute pyridine exposure involves protein synthesis possibly through increased translational efficiency.

In a more recent study in 1991, Kim and his coworkers carried out some laboratory investigations to show the expression and molecular regulation of the P4501A gene subfamily in rat hepatic tissue after treatment with pyridine. Their investigation demonstrated that the EROD activity, which has been shown to be specific for the P4501A subfamily, was increased approximately 2- and 3.5-fold over control values, after a single dose of pyridine (100 mg/kg,i.p.) at 10 and 16 hours, respectively. Thus, they suggested that pyridine is capable of elevating 1A1 and 1A2 forms of P450 simultaneously (Kim *et al.*, 1991b).

The effects of pyridine exposure on expression of cytochromes P4502E1, 2B and 4B in rabbit hepatic microsomes and their respective role in pyridine N-oxide production has been examined by Kim, Philpot and Novak in 1991. Immunoblot analysis revealed that pyridine administration caused a substantial increase in P4502E1 levels, failed to affect P4502B content and marginally increased the expression of P4504B. *p*-Nitrophenol hydroxylase activity elevated about 6-fold in

pyridine-induced microsomes consistent with increased levels of P4502E1. Immunochemical titration experiments revealed only about 15% inhibition of pyridine N-oxide production by goat antirabbit P4502B IgG in PB induced rabbit liver microsomes. In contrast goat antirabbit P4504B IgG decreased pyridine N-oxide production by 80% in PB-induced rabbit liver microsomes suggesting that P4504B is also highly active in the catalysis of pyridine N-oxide production.

In a more recent study, Day, Carlson and De Nicola (1993) examined the induction of P4502E1 by pyridine and compared with that by ethanol. They also investigated the resulting potentiation of pneumotoxicity and hepatotoxicity following $CCl_4$ inhalation by pyridine. Rats were treated with ethanol as either a 10% solution in the drinking water or as a daily bolus (3 ml/kg, i.p.) dose for 7 days or one bolus dose of pyridine (200 mg/kg, i.p.) and compared for P4502E1 apoprotein content by immunoblot analysis. The results showed that ethanol in the drinking water and pyridine elevated both hepatic and pulmonary P4502E1 apoprotein content, but bolus dose ethanol did not. The induction was greatest in the pyridine group. Next, the interaction study was conducted and rats were treated with pyridine (200 mg/kg, i.p.) and 12 hours later were exposed to $CCl_4$ (8000 ppm for 3 hours). Pulmonary injury and hepatic damage were observed 24 hours later by bronchoalveolar lavage fluid (BALF) analysis, which is proven useful in detecting chemically induced pulmonary damage and by light microscopic evaluation. Pyridine induced pulmonary and hepatic microsomal apoprotein levels of cytochrome P4502E1 2- and 2- to 6-fold respectively. Exposure to $CCl_4$ decreased hepatic, but not pulmonary P4502E1 levels. The results showed that induction of cytochrome P4502E1 by pyridine increases the bioactivation of $CCl_4$ in both liver and lung, leading to enhanced toxicity.

In 1992, Carlson and Day conducted another research to show the induction of pyridine on rat hepatic microsomal cytochrome P4502E1 and P4502E1 dependent activities. In this study, a single dose of pyridine (200 mg/kg body weight, i.p.) induced cytochrome P4502E1 in both liver and lung. The pyridine administration resulted in increase of hepatic NDMA metabolism and *p*-nitrophenol hydroxylase

activities 2- to 3-fold those of control and 3- to 4-fold incerase in ethoxyresorufin deethylation. In the lung, there was an approximate doubling each of these activities. Their investigation demonstrated that pyridine appears to be an excellent inducer of P4502E1 isozyme also in the lung.

### 1.3. Aim of This Work

The purpose of our research was to determine the influence of pyridine on liver, kidney and lung microsomal system and associated enzyme activities and to indicate the existence of tissue specificity in the induction of rabbit P450 isozyme by pyridine. In this study, this possibility was evaluated for the first time , by analyzing effects of pyridine especially on a carcinogenic compound N-nitrosodimethylamine (NDMA) metabolism in rabbit kidney and lung. Since, its hazardous effects were increased with its high solubility in soil solutions; and its significant vapor pressure facilitates its absorption through the lung when inhaled, pyridine was choosen for this research. Also pyridine, which occurs very close to the daily life, can serve as a representative of N-heterocyclic compounds to investigate.

# CHAPTER II

# MATERIALS AND METHODS

## 2.1.Materials

Formaldehyde, sodium dodecyl sulphate (SDS), *p*-aminophenol were obtained from Fluka A.G., Switzerland. Carbon monoxide (CO), ammonium per sulphate (APS), N, N, N', N'-tetramethylenediamine (TEMED), aniline were obtained from Fisher Scientific Company, Chemical Manufacturing Division, Fair Lown, New Jersey, USA. Glycerol, ethylene diamine tetraacetic acid (EDTA), phenyl methyl sulfonyl floride (PMSF), methanol, potassium chloride, trichloro acetic acid (TCA), sodium carbonate, sodium hydroxide, phenol, dithionate, magnesium chloride, *p*-nitrophenol and glacial acetic acid were the products of E.Merck, Darmstadt, Germany. Acrylamide, bovine serum albumine (BSA), nicotineamideadenine dinucleotide, reduced form (NADH), egg albumine, bovine liver glutamate dehydrogenase, ε-amino caproic acid (ε-ACA), coommassie brilliant blue, N-nitrosodimethylamine (NDMA), pyridine (PY), glucose 6-phosphate, glucose 6-phosphate dehydrogenase (Type XI), ammonium acetate, nicotineamide adenine dinucleotide phosphate ($NADP^+$), perchloric acid, α-keto glutarate, aspartate, sodium pyruvate, dithiothreithol (DTT), 2-mercaptoethanol were purchased from Sigma Chemical Company, Saint Louis Missouri, USA. Acetyl acetone and bromophenol blue were the products of Riedel-de Haen Ag Seelze, Hannover. Ethylmorphine HCl was obtained from Toprak Mahsulleri Ofisi, İstanbul, Turkey.

All the other chemicals used in this study were of analytical grade and were from commercial sources at the highest grade of purity.

### 2.2. Animals and Treatments

Adult male New Zealand white rabbits (1.7-2.35 kg) purchased from Institute of Poultry Investigations, Ankara were used for pyridine treatment. The rabbits were housed for 7 days prior to the treatment in light and temperature controlled rooms with free access to food. Rabbits were injected 3 times intraperitoneally with 20% pyridine in saline solution at a dose of 250 mg/kg body weight on day 1, day 5 and day 8. Control rabbits were administered with saline. The animals were killed by decapitation 24 hour after the last treatment.

### 2.3. Methods

#### 2.3.1. Preparation of Rabbit Liver Microsomes

Adult male New Zealand rabbits purchased from Institute of Poultry Investigations, Ankara, were killed by decapitation. The livers each weighing about 40 to 60 g were removed immediately after killing. Gall bladders were excised from the livers and livers were placed on crushed-ice. After the removal of connective and fatty tissues, the organs were washed with cold distilled water and 1.15% KCl containing 2 mM EDTA several times to remove the excess blood. All subsequent steps were carried out at $0^0$C to 4 $^0$C. The tissues were blotted by the help of a filter paper. Then livers were weighed, chopped with scissors and homogenized in 1.15% KCl solution contaning 2 mM EDTA, 0.25 mM $\varepsilon$ -ACA and 0.25 mM PMSF at a volume equal to 3 times the weight of liver by using a blender. Five times blending for a period of 20 seconds, each with intervals of 40 seconds, were used for homogenization. The homogenate was centrifuged at 9 500 rpm (10 800 g) (Sorvall RC-2B Automatic Refrigerated Centrifuge lvan Sorvall Inc. , Newton, Connecticut, 06740, USA) with SS-34 rotor for 25 minutes to remove cell debris, nuclei and mitochondria. The supernatant fraction containing endoplasmic reticulum and soluble fraction of the cell

was filtered through double layers of cheese-cloth. The microsomes were sedimented from 10 800 g supernatant by centrifugation at 30 000 rpm (70 000 g) for 110 minutes using a Beckman Type 35 rotor in Beckman L-2 65 B ultracentrifuge (Spinco Division of Beckman Instruments, Palo Alto, California, 94304, USA ). The supernatant fraction was discarded and the firmly packed microsomal pellet was suspended in 1.15% KCl solution containing 2 mM EDTA and centrifuged again as above using Beckman L-2 65 B ultracentrifuge. The supernatant fraction was discarded again. The washed microsomes were finally resuspended in 25% glycerol containing 1mM EDTA at a volume of 0.5 ml for each gram of liver tissue. In order to get a homogenous microsomal suspension, resuspended microsomes were homogenized manually using the teflon-glass homogenizer. The homogenized microsomal suspensions, usually at a concentration of 20-40 mg of protein per ml were gassed with nitrogen in eppendorf tubes and stored in liquid nitrogen for enzymatic assays.

### 2.3.2. Preparation of Rabbit Kidney and Lung Microsomes

Rabbit kidney and lung microsomes were prepared essentially with the same procedure used for the preparation of rabbit liver microsomes with some modifications.

The kidney and lungs were removed immediately after killing and placed on crushed ice. All the following steps were carried out at 0-4 °C. After removing fatty and connective tissues, organs were washed several times by using cold distilled water. Then, the tissues were drained by a filter paper, minced with scissors to get a homogenous kidney and lung samples and homogenized in 1.15% KCl solution containing 2 mM EDTA, 0.25 mM ε-ACA and 0.25 mM PMSF by a Potter-Elvehjem homogenizer coupled with a motor (Black and Decker, V850, multispeed drill) -driven teflon pestle at 2400 rpm. Fifteen passes were used for homogenization of the minced kidney and lung. The volume of 1.15% KCl used was equal to 2.2 times the weight of kidney and lungs.

The homogenate was centrifuged at 9500 rpm (10 800 g) (Sorvall RC-2B Automatic Refrigerated Centrifuge) by using SS-34 rotor for 25 minutes to remove cell debris, nuclei and mitochondria. The supernatant containing endoplasmic reticulum and other soluble fraction of the cell was filtered through double layers of cheese-cloth. The microsomes were sedimented from this supernatant by centrifugation at 30 000 rpm for 110 minutes by using a Beckman Type 35 rotor in Beckman L-2 65B ultracentrifuge.

The supernatant fraction was discarded and the firmly packed microsomal pellet was suspended in 1.15% KCl solution and again centrifuged as above using Beckman L-2 65B ultracentrifuge. The supernatant fraction was discarded again. The washed microsomes were finally resuspended in 25% glycerol containing 1 mM EDTA at a volume of 0.3 ml for each gram of kidney and lung tissue. Resuspended microsomes were homogenized manually using the teflon-glass homogenizer to get a homogenous suspension. The homogenized microsomal suspensions having approximately 20 mg/ml protein concentration was gassed with nitrogen in eppendorf tubes and stored in liquid nitrogen.

### 2.3.3. Protein Determination

The protein concentrations of microsomes were determined by the method of Lowry *et al.* ( 1951 ). Crystalline bovine serum albumin was used as a standard.

### 2.3.4. Determination of Mixed Function Oxidase (MFO) Enzyme Activities

#### 2.3.4.1. Determination of Aniline 4 -Hydroxylase Activity

Aniline 4-hydroxylase activity of rabbit liver and kidney microsomes was determined by measuring the quantity of *p*-aminophenol (pAp) formed as described by Imai *et al.,* (1966). Aniline 4-hydroxylation reaction is given in Figure 2.1.

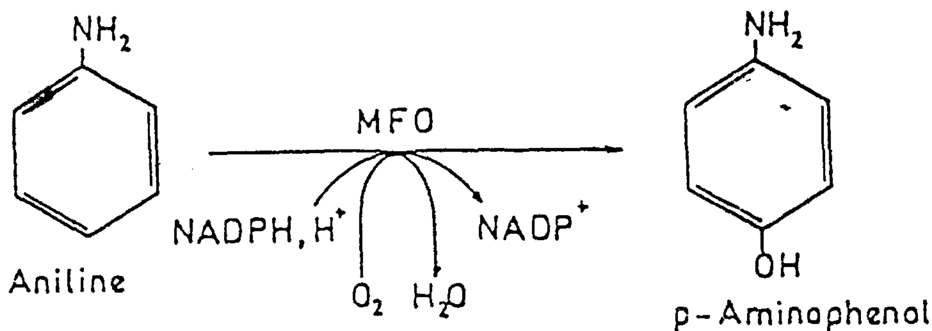



Figure 2.1. Aniline 4-Hydroxylation Reaction

The assay conditions optimized for microsomal aniline 4-hydroxylase by Arınç and İşcan (1983) were also used for determination of rabbit liver and kidney microsomal aniline 4-hydroxylase with some modifications.

A typical assay mixture given in Table 2.1. contained 100 mM HEPES buffer, pH 7.6, 10 mM aniline, 1 mg microsomal protein for liver , 4 mg microsomal protein for kidney and 4 mg microsomal protein for lung and 0.5 mM NADPH-generating system in a final volume of 1.0 ml.

NADPH generating system was prepared by adding 0.5 units of glucose 6-phosphate dehydrogenase into a test tube containing 2.5 mM glucose 6-phosphate, 2.5 mM $MgCl_2$, 14.6 mM HEPES buffer, PH 7.8, and 0.5 mM $NADP^+$. Then the test tube containing generating system was incubated at 37 °C for 5 minutes and finally kept in crushed ice. 1 unit of glucose 6-phosphate dehydrogenase is equal to the amount of enzyme reducing 1 µmole of $NADP^+$ in one minute at 25 °C.

*p*-Aminophenol solution was used as standard. Since it is light-sensitive, 0.5 mM *p*-aminophenol solution was prepared freshly and kept in the dark. Standards at four different *p*-aminophenol concentrations (2.5, 5.0, 12.5 and 25.0 nmoles ) containing aniline and other incubation constituents were run under the same conditions as for reaction mixture.

The reaction was started by the addition of 0.15 ml of NADPH generating system to microsomal incubation mixtures and to zero time blanks to which 0.5 ml of 20% TCA was added before addition of cofactor. Reaction was carried out at 37 °C for 25 minutes aerobically with moderate shaking in a water bath (Nüve Instruments Ltd., 06640 Ankara, Türkiye ). At the end of the incubation time, 0.5 ml of 20% TCA was added to stop the reaction and incubation mixtures were transferred to eppendorf tubes. Denatured proteins were removed by centrifugation at 13 500 rpm for 20 minutes using Heraeus Sepatech Centrifuge 17RS automatically refrigerated centrifuge.

Finally, 1 ml of supernatant was mixed with 0.5 ml 20% sodium carbonate, and with 0.5 ml of 0.4 N NaOH containing 4% phenol. The mixture was incubated at 37 °C for 30 minutes. The intensity of blue color developed was measured at 630 nm using Schimadzu UV-1201 spectrophotometer (Schimadzu Corporation, Analytical Instruments Division, Kyoto, Japan). A standard curve of *p*-aminophenol was constructed and amount of *p*-aminophenol formed was calculated from standard graph.

Table 2.1. The constituents of the incubation mixture for determination of aniline 4-hydroxylase activity in rabbit liver , kidney and lung microsomes

| Constituents | Stock solution | Volume to be added(ml) | Final concentration in 1.0 ml. incubation mixture |
|---|---|---|---|
| HEPES buffer, pH 7.6 | 400 mM | 0.25 | 100 mM |
| Aniline | 100 mM | 0.10 | 10 mM |
| Microsomal protein | | | 1 mg for liver<br>4 mg for kidney<br>4 mg for lung |
| NADPH-Generating System | | | |
| • Glucose-6-phosphate | 100 mM | 0.025 | 2.5 mM |
| • $MgCl_2$ | 100 mM | 0.025 | 2.5 mM |
| • HEPES Buffer, pH 7.8 | 200 mM | 0.073 | 14.6 mM |
| • $NADP^+$ | 20 mM | 0.025 | 0.5 mM |
| • Glucose-6-phosphate dehydrogenase | 350 U | 0.0014 | 0.5 U |
| •Distilled water | | to 1ml | |

2.3.4.2. Determination of Ethylmorphine N-Demethylase Activity

Ethylmorphine N-demethylase activity was determined by measuring the quantity of formaldehyde formed as described by Nash (1953), as modified by Cochin and Axelrod (1959). The assay conditions optimized for liver microsomal ethylmorphine N-demethylase by Arınç (1985) were also used for determination of ethylmorphine N-demethylase activity of rabbit liver , kidney and lung microsomes with slight modifications. Ethylmorphine N-demethylation reaction is shown in Figure 2.2.

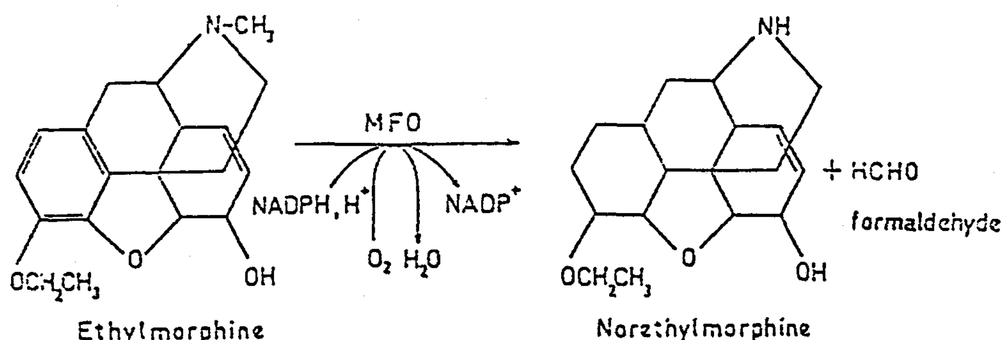



Figure 2.2. Ethylmorphine N-Demethylation Reaction

As it is shown in Table 2.2., a typical assay mixture contained 100 mM HEPES buffer pH 7.7, 100 mM ethylmorphine-HCl, 1.5 mg, 1.5 mg and 3 mg microsomal protein for liver, lung and kidney, respectively, and 0.5 mM NADPH generating system in a final volume of 1ml. NADPH generating system was composed of 2.5 mM $MgCl_2$, 14.6 mM HEPES buffer, pH 7.8, 0.5 mM $NADP^+$ and 0.5 units of glucose 6-phosphate dehydrogenase.

A 0.5 mM freshly prepared formaldehyde solution was used as standard. The tubes containing standards at four concentrations (12.5, 25.0, 50.0, and 100.0 nmoles) , zero time blanks to which 1ml of 0.75 N perchloric acid was added before the cofactor and the microsomal incubation mixtures were placed in a moderate shaking water bath at 37 °C.

The reaction was initiated by the addition of 0.15 ml of NADPH generating system and carried out for 15 minutes aerobically with moderate shaking. At the end of this time, the enzymatic reaction was stopped by addition of 1ml of 0.75 N perchloric acid. The contents of the tubes were transferred into eppendorf tubes and were centrifuged at 13 500 rpm for 20 minutes for removal of denatured microsomal proteins.

Finally, 1 ml aliquots of supernatant solution was transferred to the test tubes and was mixed with freshly prepared 0.75 ml Nash reagent (prepared by the addition of 0.4 ml of acetylacetone, just before use, to 100 ml solution containing 30.8 g ammonium acetate and 0.6 ml of glacial acetic acid ). The mixture was incubated at 50 °C for 10 minutes in a water bath and the intensity of yellow color developed was measured at 412 nm using Schimadzu UV-1201 spectrophtometer. A standard formaldehyde calibration curve was constructed and used for calculation of enzyme activities.

Table 2.2. The constituents of the incubation mixture for the determination of ethylmorphine N-demethylase activity of rabbit liver, kidney and lung microsomes

| **Constituents** | **Stock solution** | **Volume to be added(ml)** | **Final concentration in 1.0 ml. incubation mixture** |
|---|---|---|---|
| HEPES Buffer, pH 7.7 | 400 mM | 0.25 | 100 mM |
| Ethylmorphine HCl | 100 mM | 0.20 | 20 mM |
| Microsomal protein | | | 1.5 mg for liver<br>3 mg for kidney<br>1.5 mg for lung |
| NADPH-Generating System | | | |
| • Glucose-6-phosphate | 100 mM | 0.025 | 2.5 mM |
| • $MgCl_2$ | 100 mM | 0.025 | 2.5 mM |
| . HEPES buffer, pH 7.8 | 200 mM | 0.073 | 14.6 mM |
| • $NADP^+$ | 20 mM | 0.025 | 0.5 mM |
| • Glucose-6-phosphate dehydrogenase | 350 U | 0.0014 | 0.5 U |
| Distilled Water | | to 1 ml | |

2.3.4.3. Determination of *p*-Nitrophenol Hydroxylase Activity

The hydroxylation of *p*-nitrophenol to 4-nitrocatechol (1,2-dihydroxy-4 -nitrobenzene) was determined essentially using the method of Reinke and Moyer (1985). The assay conditions that were optimized for lung microsomal *p*-nitrophenol hydroxylase by Arınç and Aydoğmuş (1990) were also used for the determination of *p*-nitrophenol hydroxylase activity of rabbit liver, kidney and lung microsomes. *p*-Nitrophenol hydroxylation reaction is given in Figure 2.3.

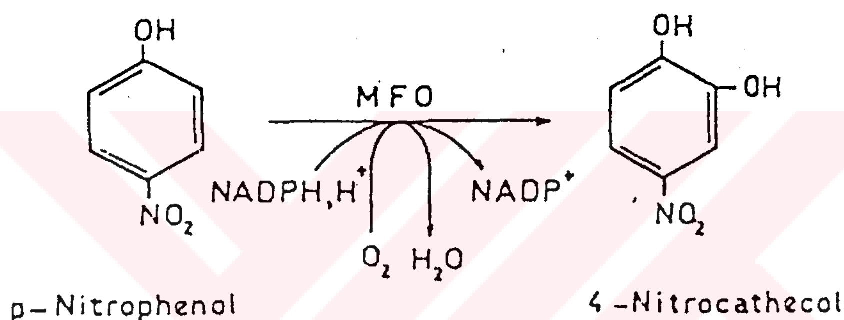



Figure 2.3. *p*-Nitrophenol Hydroxylation Reaction

As given in Table 2.3., the typical optimized reaction mixture contained 0.25 mM *p*-nitrophenol, 100 mM Tris-HCl buffer, pH 6.8, 2 mg microsomal protein for liver, 4 mg microsomal protein for kidney, 2 mg microsomal protein for lung and 0.5 mM NADPH generating system in a final volume of 1.0 ml.

The reaction was started by the addition of 0.15 ml NADPH generating system and carried out for 10 minutes at 37 °C with moderate shaking in a shaking water bath. The reaction was terminated by the addition of 0.5 ml of 0.6 N perchloric acid. Denatured proteins were removed by centrifugation at 13 500 rpm for 20 minutes. Then 1.0 ml aliquot of the supernatant was mixed with 0.1 ml of 10 N NaOH for complete ionization of 4-nitrocatechol.

Then 4-nitrocatechol formed was determined spectrally at 546 nm by using an extinction coefficient of 9.53 $mM^{-1}$ $cm^{-1}$ (Koop *et al.,* 1986 ).

Table 2.3. The constituents of incubation mixture for the determination of *p*-nitrophenol hydroxylase activity of rabbit liver, kidney and lung microsomes.

| **Constituents** | **Stock solution** | **Volume to be added(ml)** | **Final concentration in 1.0 ml. incubation mixture** |
|---|---|---|---|
| Tris Buffer , pH 6.8 | 400 mM | 0.25 | 100 mM |
| *p* -Nitrophenol | 2.5 mM | 0.1 | 0.25 mM |
| Microsomes | | | 2 mg for liver<br>4 mg for kidney<br>2 mg for lung |
| NADPH Generating System | | | |
| • Glucose-6-phosphate | 100 mM | 0.025 | 2.5 mM |
| • $MgCl_2$ | 100 mM | 0.025 | 2.5 mM |
| • HEPES Buffer, pH 7.8 | 200 mM | 0.073 | 14.6 mM |
| • $NADP^+$ | 20 mM | 0.025 | 0.5 mM |
| • Glucose-6-phosphate dehydrogenase | 350 U | 0.0014 | 0.5 U |
| Distilled water | | to 1 ml | |

2.3.4.4. Determination of N-Nitrosodimethylamine (NDMA) N-Demethylase Activity

NDMA N-demethylase activity of rabbit liver, kidney and lung microsomes was determined according to the method of Gorsky and Hollenberg (1989) and formaldehyde formed was measured by the method of Nash (1953) as modified by Cochin and Axelrod (1959). The incubation mixture given in Table 2.4. contained 100 mM potassium phosphate buffer, pH 7.7, 2.5 mM NDMA, 1.5 mg microsomal protein for liver and 2 mg microsomal protein for kidney and lung and 0.5 mM NADPH generating system in a final volume of 1 ml. NDMA N-demethylase reaction is shown in Figure 2.4.

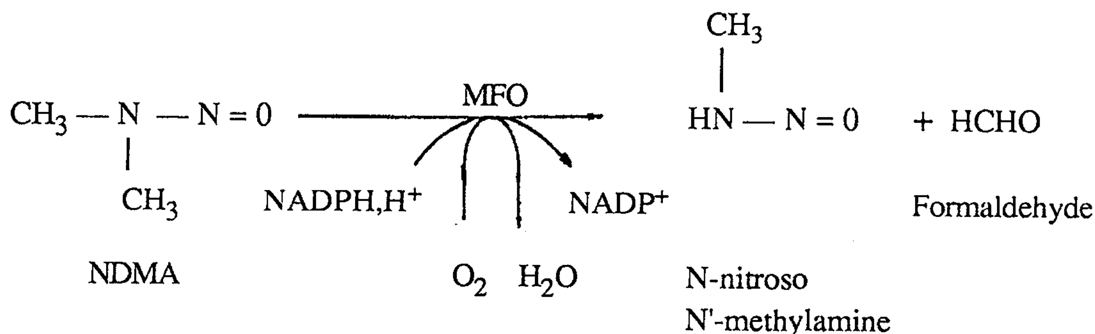



Figure 2.4. NDMA N- Demethylation Reaction

The reaction was initiated by addition of 0.15 ml NADPH generating system to incubation mixtures and to zero time blanks to which 1ml of 0.75 N perchloric acid was added before the cofactor and was carried out at 37 °C for 20 minutes under the air with constant moderate shaking in a water-bath.

After exact period of 20 minutes enzymatic reaction was stopped by the addition of 1 ml of 0.75 N perchloric acid solution. Then, the contents of the tubes were transferred to the eppendorf centrifuge tubes. The precipitated proteins were spin down by the centrifugation at 13 500 rpm for 20 minutes.

Finally, 1 ml aliquots were mixed with 0.75 ml Nash reagent and the mixture was incubated for 10 minutes at 50 °C in a water bath. Formaldehyde amount was determined by measuring the absorbance at 412 nm using Schimadzu UV-1201 spectrophotometer. A standard formaldehyde calibration curve was constructed and used for calculation of enzyme activity.

Table 2.4. The constituents of the incubation mixture for the determination of - NDMA N-demethylase activity of rabbit liver, kidney and lung microsomes

| Constituents | Stock solution | Volume to be added(ml) | Final concentration in 1.0 ml. incubation mixture |
|---|---|---|---|
| HEPES Buffer, pH 7.7 | 400 mM | 0.25 | 100 mM |
| NDMA | 12.5 mM | 0.2 | 2.5 mM |
| Microsomal protein | | | 1.5 mg for liver<br>2 mg for kidney<br>2 mg for lung |
| NADPH-Generating System | | | |
| • Glucose-6- phosphate | 100 mM | 0.025 | 2.5 mM |
| • $MgCl_2$ | 100 mM | 0.025 | 2.5 mM |
| .HEPES Buffer, pH 7.8 | 200 mM | 0.073 | 14.6 mM |
| • $NADP^+$ | 20 mM | 0.025 | 0.5 mM |
| • Glucose-6-phosphate dehydrogenase | 350 U | 0.0014 | 0.5 U |
| Distilled Water | | to 1 ml | |

### 2.3.5. Determination of Glutamate Oxaloacetate Transaminase (GOT) and Lactate Dehydrogenase (LDH) Activities

#### 2.3.5.1.Determination of Glutamate Oxaloacetate Transaminase (GOT) Activity

The transaminases constitute a group of enzymes which catalyze the interconversion of amino acids and α-ketoacids by transfer of amino groups. The α-ketoglutarate/L-glutamate couple serves as the amino group acceptor and donor in pair in all amino transfer reactions. Animal cells contain a variety of amino transferases. GOT is found in practically every tissue of the body, including red blood cells. It is particularly high concentration in cardiac muscle and liver, intermediate in skeletal muscle and in much lower concentrations in others.

In this work, the GOT enzyme activity of blood serum and soluble fraction of liver, kidney and lung was determined according to the method of Reitman and Frankel (1957). The method involves the direct combination of oxalate with DNPH and the measurement of the color in alkaline solution. The GOT catalyzed reaction and measurement of GOT activity are given in Figure 2.5. and 2.6.

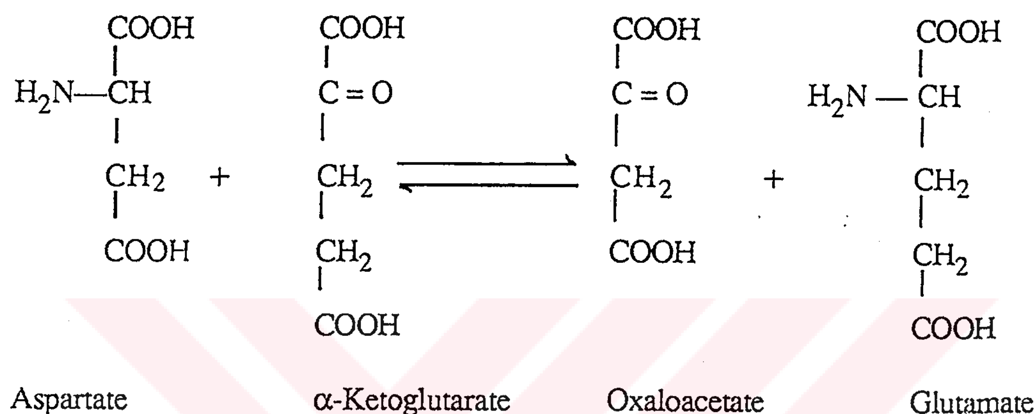



Figure 2.5. The reaction catalysed by GOT

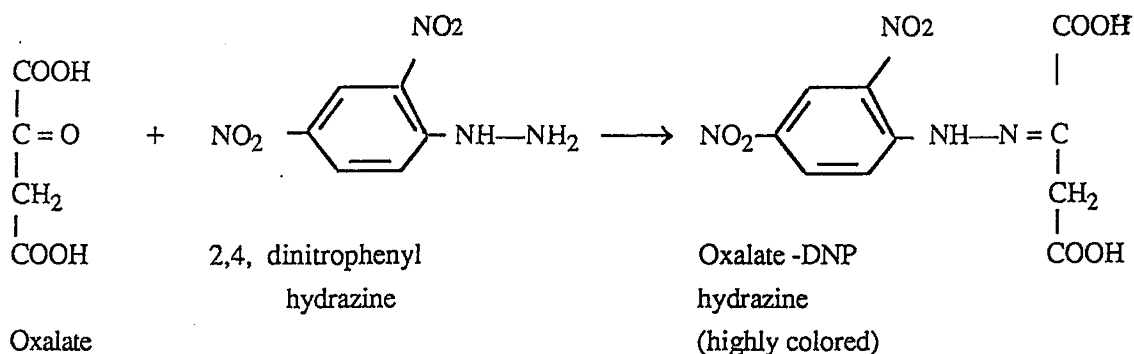



Figure 2.6. The reaction used for the determination of GOT activity

The incubation mixture contained GOT substrate (prepared by the addition of 40 ml of 1 N NaOH to the mixture of 0.0584 g of α-ketoglutarate and 5.32 g D-L-aspartate. The pH of the solution was adjusted to 7.4±0.1 by adding 1N NaOH dropwise, with stirring. Then the solution was diluted to 200 ml with phosphate buffer, pH 7.4) and serum or soluble fractions of liver, kidney and lung. The contents of the tubes are shown in Table 2.5.

Pyruvate standard was prepared freshly by dissolving 20 mg pure Na-pyruvate in 100 ml phosphate buffer, pH 7.4. Five different concentrations of pyruvate which refer to 24, 61, 114, 190 GOT units were used for the construction of standard curve. The enzyme activities of samples were calculated using this linear standard curve.

All the tubes were mixed and only the test tubes were incubated in a water bath at 37 °C for 1 hour. After the incubation, the test tubes were removed from the water bath and 1ml of color reagent which was prepared by dissolving 0.039 g DNPH in 200 ml of 1N HCl was added to all tubes including blanks and standards. Then, all tubes were incubated at room temperature for 20 minutes.

Table 2.5. The constituents of the incubation mixtures for the determination of GOT activity in blood serum and in soluble fraction of liver, kidney and lung

| Tubes | | GOT substrate | | Distilled water (ml) | Standard (ml) | Serum and soluble fractions (ml) |
|---|---|---|---|---|---|---|
| | | ml | units | | | |
| Blank | 1 | 1.0 | - | 0.2 | - | - |
| Standards | 2 | 0.9 | 24 | 0.2 | 0.1 | - |
| | 3 | 0.8 | 61 | 0.2 | 0.2 | - |
| | 4 | 0.7 | 114 | 0.2 | 0.3 | - |
| | 5 | 0.6 | 190 | 0.2 | 0.4 | - |
| Tests | 6> | 1.0 | - | - | - | 0.2 |

Finally, 10 ml of 0.4 N NaOH was added to all tubes and they were allowed to stand for at least 5 minutes. Then, the blank was set to 0.25 absorbance in spectrophotometer at 505 nm. The absorbances of the tubes developing brown color were read. A standard curve was constructed by using the absorbances and GOT units of the standards. Blank was plotted at zero concentration and 0.25 absorbance directly. The GOT units of the samples were calculated by using this calibration curve. One transaminase unit is defined, in terms of the UV procedure, as the amount of enzyme activity in 1 ml of serum that will lower the absorbance by 0.001 in 1 minute under the described conditions of that method (Reitman and Frankel, 1957).

2.3.5.2. Determination of Lactate Dehydrogenase (LDH) Activity

Oxidation-reduction reactions are among the most fundamental in the living organisms. LDH represents an enzyme that catalyses such a reaction shown in Figure 2.7.

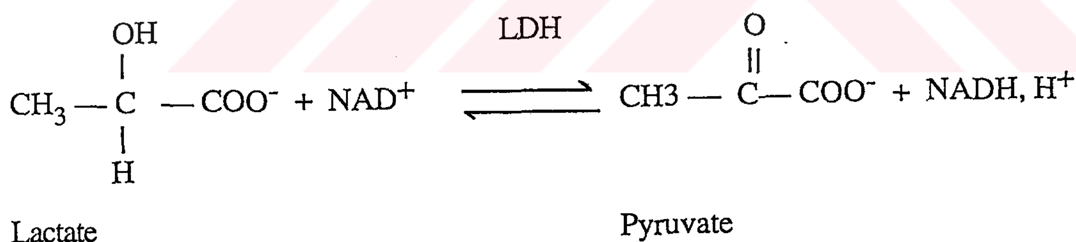



Figure 2.7. LDH reaction

The enzyme is distributed in all human tissues, but is present in high concentrations in liver, cardiac and skeletal muscles, red blood cells and other tissues. In this work, LDH activity determination of blood serum samples and soluble fractions was carried out using the spectrophotometric method of Wroblewski and La Due (1955) who adapted the classical assay of Kubowitz and Ott (1943) to

serum. The activity of LDH was measured by monitoring the rate at which the substrate, pyruvate is reduced to lactate. The reduction is coupled with the oxidation of NADH , which is followed spectrophotometrically in terms of reduced absorbance at 340 nm.

The reaction mixture was contained 2.85 ml of 0.1M phosphate buffer, pH 7.5, 0.05 ml serum or soluble fractions of liver, kidney and lung and 0.2 mg NADH. All the tubes were mixed and left in 25 °C for 20 minutes. After the exact period of 20 minutes, 0.1 ml of 22.7 µM Na-pyruvate in 0.1 M phosphate buffer, pH 7.5 was added to the tubes. They were mixed well and the content of tubes was transferred to a cuvette of 1cm lightpath. Then the absorbance was read at 340 nm in each 30-second intervals for 3 minutes against water as reference.

Calculation;

The LDH activity of samples were determined by selecting a period where the decrease in absorbance is linear with time and $\Delta\ OD_{340}$ /min was calculated for this period. Then the following formula was used for the calculation of enzyme activity in terms of units/ml.

Serum LDH activity (units/ml) =[$OD_{340}$ / min x TCF**]/[0.001x0.05x1(cm)]

where:

0.001=$OD_{340}$ equivalent to 1 unit of LDH activity in a 3 ml volume with 1 cm light path at 25 °C

0.05 =serum volume in cuvette

TCF =Temperature correction factor (1 at 25 °C)

Therefore, if a 1 cm lightpath cuvette is used, the above equation reduces to ;

Serum LDH activity (units/ml) =$OD_{340}$x 20 000 xTCF

### 2.3.6. Determination of Cytochrome P450 Content of Rabbit Liver, Kidney and Lung Microsomes

Cytochrome P450 concentrations were determined by the method of Johannasen and De Pierre (1978), using a Hitachi 220A double beam spectrophotometer with cuvettes of 1 cm optical path length. This method is based on the difference spectrum of dithionite - reduced microsomes minus non-reduced microsomes when both sample and reference have been bubbled with carbonmonoxide.

Cytochrome P450 samples were diluted with 0.1M potassium phosphate buffer, pH 7.7, containing 30% glycerol and 0.1 mM EDTA and placed both in the sample and the reference cuvettes. Carbon monoxide was bubbled through the sample cuvette for 1 minute and during that period the sample was reduced by the addition of solid dithionite. The sample in the reference cuvette was also bubbled with carbon monoxide. The dithionite difference spectrum was recorded after 5-10 minutes. The cytochrome P450 concentration was calculated using an extinction coefficient of 0.105 $mM^{-1}$ $cm^{-1}$ for the difference in absorption between 450 - 475 nm.

### 2.3.7. Sodium dodecylsulphate (SDS) - Polyacrylamide Gel Electrophoresis

Polyacrylamide slab gel electrophoresis in the presence of detergent, SDS, was performed on 4% stacking gel and 7.5% separating gel in a discontinuous buffer system as described by Laemmli (1970). BSA (Mr.68 000), L-glutamate dehydrogenase (Mr.53 000) and egg albumine (Mr.45 000), cytochrome c (Mr.11 700) were used as molecular weight standards. The molecular weight of the polypeptide chains were taken from Weber and Osborn (1969).

Vertical slab gel electrophoresis was carried out using Bio-Rad Protean II Slab Electrophoresis cell. Polyacrylamide slab gels were prepared between two glass plates (long plate 18.3 x 20 cm ; short plate 16 x 20 cm ; spacer 1mm) The

separating gel solution containing 7.5 % acrylamide, 0.375 M Tris -HCl, pH 8.8 and 0.1% SDS was prepared and chemical polymerization was achieved by the addition of ammonium per sulfate (APS) and TEMED. The solution was poured into the glass plates from one edge of the spacers using a 10 ml pipette until the desired height of the solution (12-13 cm) is achieved. Using a syringe, the top of the gel polymerizing solution was covered with a layer of distilled water, approximately 0.5 cm thick, by gentle squirting against the edge of one of the spacers to ensure the formation of a smooth gel surface. The gel, then was allowed for polymerization at room temperature for about 30 minutes. After polymerization, the layer of water was poured off completely using filter paper.

Meanwhile the stacking gel solution containing 4% acrylamide, 0.125 M Tris-HCl, pH 6.8 , 0.1 % SDS, APS and TEMED was prepared and poured on top of the seperating gel along an edge of one of the spacers until the sandwich was filled completely. The 1 mm teflon comb with 15 wells was inserted into the stacking gel polymerization solution without trapping air bubbles in the tooth edges of comb and left to polymerization at room temperature. After the teflon comb was removed and the gel sandwich was placed on the cooling core, together they were placed in to the lower buffer chamber filled with 2 liters of running gel buffer, pH 8.3 which contained 0.025 M Tris, 0.192 M glycine and 0.1% SDS. Upper buffer chamber was also filled with the same buffer freshly prepared. Protein samples and standards were prepared in the same dilution buffer consisting of 0.0625 M Tris - HCl buffer, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.05% bromophenol blue and were immersed in a boiling water bath for 2 minutes then they were applied to SDS-polyacrylamide slab gel in a volume of 5-30 μl containing approximately 50 μg protein.

Electrophoresis was carried out first at 10 mA current until the tracking dye entered into the separating gel. Then the current was increased up to the 20 mA. Electrophoresis was terminated when the dye had traveled a distance of about 8 cm. The total running time was 4 hours.

The slab gel was removed from the glass plates after electrophoresis and stained in a solution containing 0.25% Coomassie Brilliant Blue, 10% acetic acid and 45% methanol for one hour by gentle shaking at room temperature. The destaining of the gel was then achieved with a solution containing 30% methanol for approximately 48 hours. The destained gel was stored in 7% acetic acid solution.

# CHAPTER III

# RESULTS

## 3.1. Effects of Pyridine on Enzyme Activities

The effect of pyridine on hepatic, renal and pulmonary microsomal MFO activities of rabbit was studied by measuring the aniline 4-hydroxylase, ethylmorphine N-demethylase, NDMA N-demethylase, and *p*-nitrophenol hydroxylase activities. In addition to MFO enzyme activities, LDH and GOT activities were also determined in blood serum and soluble fraction of liver, kidney and lung. The MFO enzyme activities have been shown to be valuable criteria in characterizing the specific cytochrome P450 isozymes in mammals.

### 3.1.1. Effects of Pyridine on Mixed Function Oxidase Enzyme Activities

#### 3.1.1.1. Preliminary Studies

Preliminary studies were carried out to establish a dose-effect relationship as well as the time course of pyridine treatment. These studies were conducted in two different experimental sets. In the first set of experiments, pyridine was administered i.p. to rabbits in doses of 250 mg/kg and 500 mg/kg. Following these single dose regimens, the animals were sacrificed at two different times, at 15 hours and 24 hours, after treatment.

The results are shown in Table 3.1. As seen in Table 3.1., these pretreatments (i.p. 250 mg/kg for 15h or 24h or i.p. 500 mg/kg for 15h or 24h) resulted in similar effects in activities of hepatic microsomal enzymes. Hepatic microsomal ethylmorphine N-demethylase activities were not affected by *in vivo* pyridine treatment. On the other hand, pyridine treatment resulted about 1.54- to 2.80-fold and 2.27- to 2.90-fold increases in hepatic aniline 4-hydroxylase and p-nitrophenol hydroxylase activities, respectively. In addition, hepatic microsomal NDMA activity enhanced 2.69- to 4.96-fold by pyridine treatment.

Since i.p. administration of pyridine either in 250 mg/kg or 500 mg/kg doses, resulted in similar effects in hepatic microsomal enzyme activities, for the second set of experiments pyridine in 250 mg/kg body weight dose was choosen. In this second set of experiments, route of administration (i.p. or s.c. or both ), number of doses (2 or 3) and the duration of time between the doses were varied. All the rabbits were killed 24h after the last treatment. The results are tabulated in Table 3.2.. These pretreatments resulted about 2.48- to 7.82- fold and 2.74- to 5.33-fold increases in hepatic aniline 4-hydroxylase and *p*-nitrophenol hydroxylase activities, respectively. Hepatic microsomal cytochrome P450 content and ethylmorphine N-demethylase activities were not altered significantly whereas hepatic NDMA activity elevated 2.00- to 10.83-fold by pyridine treatment. Since pyridine in dose of 250 mg/kg, i.p. for 1, 5 and 8 days resulted in highest fold increase in microsomal enzyme activities, this protocol was used for the further studies.

In order to determine the effects of pyridine on rabbit hepatic, pulmonary and renal MFO system, in detail, the rabbits were treated i.p. with 20% pyridine in saline solution at dose of 250 mg/kg on day 1, 5 and 8.

Table 3.1. Preliminary experiment I. Effects of pyridine on hepatic microsomal aniline 4-hydroxylase, ethylmorphine N-demethylase, NDMA N-demethylase and *p*-nitrophenol hydroxylase activities of rabbits

| Rabbits | $T_1$ | $T_2$ | $T_3$ | $T_4$ | $C_1$ | $C_2$ | Control average |
|---|---|---|---|---|---|---|---|
| Treatment<br>Dose<br>time interval | i.p.<br>250 mg/kg<br>24h | i.p.<br>500 mg/kg<br>24h | i.p.<br>250 mg/kg<br>15h | i.p.<br>500 mg/kg<br>15h | i.p.<br>saline<br>24h | - | - |
| Aniline 4-hydroxylase nmol pAP/min/mg | 0.70 | 0.73 | 0.40 | 0.65 | 0.26 | 0.26 | 0.26 |
| Increase (fold) | 2.69 | 2.80 | 1.54 | 2.50 | - | - | - |
| Ethylmorphine N-demethylase nmol HCHO/min/mg | 1.60 | 1.20 | 0.94 | 1.30 | 1.39 | 1.53 | 1.46 |
| NDMA N-demethylase nmol HCHO/min/mg | 1.29 | 0.94 | 0.70 | 1.03 | 0.21 | 0.31 | 0.26 |
| Increase (fold) | 4.96 | 3.61 | 2.69 | 3.96 | - | - | - |
| *p*-Nitrophenol hydroxylase nmol product/min/mg | 0.59 | 0.56 | 0.50 | 0.64 | 0.19 | 0.25 | 0.22 |
| Increase (fold) | 2.68 | 2.54 | 2.27 | 2.90 | - | - | - |

T : Treated, C : Control

Table 3.2. Preliminary experiment II. Effects of pyridine on hepatic aniline 4-hydroxylase, ethylmorphine N-demethylase, NDMA N- demethylase and *p*-nitrophenol hydroxylase activities and cytochrome P450 levels of rabbits

| Rabbits | $T_1$ | $T_2$ | $T_3$ | $T_4$ | $C_1$ | $C_2$ | $C_3$ | Control average |
|---|---|---|---|---|---|---|---|---|
| Treatments<br>Dose<br>Injections | 3xi.p.<br>250 mg/kg<br>Days 1,5, 8 | 2xi.p.<br>250 mg/kg<br>Days 1, 8 | 3xs.c.<br>250 mg/kg<br>Days 1,5, 8 | 1xs.c. and 1xi.p.<br>250 mg/kg<br>Days 1, 8 | 3xi.p.<br>saline<br>Days 1,5, 8 | - | - | - |
| Aniline 4-hyroxylase<br>nmol pAP/min/mg | 3.60 | 1.60 | 1.90 | 1.14 | 0.44 | - | 0.49 | 0.46 |
| Increase (fold) | 7.82 | 3.47 | 4.13 | 2.48 | - | - | - | - |
| Ethylmorphine<br>N-demethylase<br>nmol HCHO/min/mg | 1.78 | 1.03 | 1.30 | 0.86 | 0.82 | 1.04 | 1.50 | 1.12 |
| NDMA<br>nmol HCHO/min/mg | 2.6 | 0.77 | 0.93 | 0.48 | 0.21 | 0.28 | - | 0.24 |
| Increase (fold) | 10.83 | 3.21 | 3.80 | 2.00 | - | - | - | |
| p-Nitrophenol hydroxylase<br>nmol product/min/mg | 1.44 | 0.87 | 0.94 | 0.74 | 0.32 | 0.22 | - | 0.27 |
| Increase (fold) | 5.33 | 3.22 | 3.48 | 2.74 | - | - | - | - |
| Cytochrome P450 | 1.19 | 0.89 | 1.26 | 0.70 | 0.50 | 0.90 | 0.85 | 0.75 |

T : Treated, C : Control

### 3.1.1.2. Effects of *in vivo* Pyridine Treatment on Mixed Function Oxidase Enzyme Activities of Rabbit Liver Microsomes

On Aniline 4-Hydroxylase Activity;

Aniline 4-hydroxylase activity was determined using 1mg hepatic microsomal protein from both pyridine treated and control rabbits. As shown in Table 3.3., the specific enzyme activities of hepatic microsomes obtained from the treated animals were found to be 2.71, 1.40, 1.30, 1.63, 2.20 nmol pAp/min/mg prot. which gave an average value of 1.85±0.60 nmol pAp/min/mg prot. (mean±SD, N=5). The specific activities of the enzyme from the control rabbits were found as; 0.37, 0.29, 0.29 nmol pAp/min/mg prot. which gave an average value of 0.32±0.05 nmol pAp/min/mg prot. (mean±SD, N=3). Thus, in vivo treatment of rabbits with pyridine i.p., at dose of 250 mg/kg body weight on day 1, 5 and 8 caused 5.8-fold increase in aniline 4-hydroxylation rates of liver microsomes (Table 3.3.).

Table 3.3. Effects of pyridine on hepatic microsomal aniline 4-hydroxylase activity

| Specific Activity (nmol pAp/min/mg protein) | | | | | | |
|---|---|---|---|---|---|---|
| Rabbits | 1 | 2 | 3 | 4 | 5 | Average |
| Treated | 2.71 | 1.40 | 1.30 | 1.63 | 2.20 | 1.85±0.60* |
| Control | 0.37 | 0.29 | 0.29 | - | - | 0.32±0.05 |

*$p < 0.005$

On Ethylmorphine N-Demethylase Activity;

Ethylmorphine N-demethylase activity was determined using 1.5 mg hepatic microsomal protein from both pyridine treated and control rabbits. As shown in Table 3.4., the specific enzyme activities of hepatic microsomes obtained from the treated animals were found to be 1.15, 1.12, 0.82, 1.14, 0.70 nmol HCHO/min/mg prot. which gave an average value of 0.99±0.21 nmol HCHO/min/mg prot. (N=5). The specific activities of the enzyme from the control rabbits were found as; 0.64, 0.98, 1.10 nmol HCHO/min/mg prot. which resulted in an average value of 0.91±0.24 nmol HCHO/min/mg prot. (N=3). These results indicate that pyridine treatment of rabbits caused no significant changes in N-demethylation rates of ethylmorphine of liver microsomes.

Table 3.4. Effects of pyridine on hepatic microsomal ethylmorphine N-demethylase activity

| Specific Activity (nmol HCHO/min/mg protein) | | | | | | |
|---|---|---|---|---|---|---|
| Rabbits | 1 | 2 | 3 | 4 | 5 | Average |
| Treated | 1.15 | 1.12 | 0.82 | 1.14 | 0.70 | 0.99±0.21 |
| Control | 0.64 | 0.98 | 1.10 | - | - | 0.91±0.24 |

On NDMA N-Demethylase Activity;

NDMA N-demethylase activity was determined using 1.5 mg hepatic microsomal protein from both pyridine treated and control rabbits. As shown in Table 3.5., the specific enzyme activities of hepatic microsomes obtained from the treated animals were found to be 1.90, 1.09, 1.13, 1.23, 1.55 nmol

HCHO/min/mg prot. which gave an average value of 1.38±0.34 nmol HCHO/min/mg prot. (N=5). The specific activities of the enzyme from the control rabbits were found as; 0.35, 0.12, 0.13 nmol HCHO/min/mg prot. which resulted in an average value of 0.20±0.13 nmol HCHO/min/mg prot. (N=3). Thus, in vivo treatment of rabbits with pyridine, i.p., at dose of 250 mg/kg body weight on day 1, 5 and 8 caused 6.9-fold increase in NDMA N-demethylase activites of liver microsomes.

Table 3.5. Effects of pyridine on hepatic microsomal NDMA N-demethylase activity

| Specific Activity (nmol HCHO/min /mg protein ) | | | | | | |
|---|---|---|---|---|---|---|
| Rabbits | 1 | 2 | 3 | 4 | 5 | Average |
| Treated | 1.90 | 1.09 | 1.13 | 1.23 | 1.55 | 1.38±0.34* |
| Control | 0.35 | 0.12 | 0.13 | - | - | 0.20±0.13 |

*$p < 0.005$

On *p* -Nitrophenol Hydroxylase Activity;

The hydroxylation of *p*-nitrophenol to 4-nitrocatechol was determined in a reaction mixture containing 2 mg microsomal protein. As observed in the Table 3.6., the specific *p*-nitrophenol hydroxylase activities were found as 1.06, 0.72, 0.65, 0.75, 0.77 nmol product/min/mg prot. which resulted in an average value of 0.79±0.16 nmol product/min/mg prot. (N=5) for liver microsomes obtained from the pyridine treated animals and 0.24, 0.18, 0.12 nmol product/min/mg prot. for the controls which gave average value of 0.18±0.06 nmol product/min/mg prot. (N=3). These results showed that; pyridine treatment (250 mg/kg body weight for 3 days) caused 4.4-fold increase in *p*-nitrophenol hydroxylation rates of rabbit liver microsomes.

Table 3.6. Effects of pyridine on hepatic microsomal *p*-nitrophenol hydroxylase activity

| Specific Activity (nmol product /min /mg protein ) | | | | | | |
|---|---|---|---|---|---|---|
| Rabbits | 1 | 2 | 3 | 4 | 5 | Average |
| Treated | 1.06 | 0.72 | 0.65 | 0.75 | 0.77 | 0.79±0.16* |
| Control | 0.24 | 0.18 | 0.12 | - | - | 0.18±0.06 |

*$p < 0.001$

### 3.1.1.3. Effects of *in vivo* Pyridine Treatment on Mixed Function Oxidase Enzyme Activities of Rabbit Kidney Microsomes

On Aniline 4-Hydroxylase Activity;

Aniline 4-hydroxylase activity of rabbit kidney microsomes was determined by measuring the amount of *p*-nitrophenol formed as described by Imai *et. al.* (1966). 4 mg microsomal protein from both treated and control animals was used for aniline 4- hydroxylation reaction. The specific activities of renal aniline 4-hydroxylase were found; 0.033, 0.036, 0.030, 0.097, 0.076 nmol pAp/min /mg protein which gave an average of 0.054±0.030 nmol pAp/min /mg protein (N=5) for pyridine treated rabbits and 0.013, 0.015, 0.009 nmol pAp/min /mg protein which resulted in an average of 0.012±0.003 nmol pAp/min /mg protein (N=3) for control rabbits as given in Table 3.7. Pyridine treatment of rabbits at a dose of 250 mg/kg body weight on day 1, 5 and 8 caused 4.5-fold increase in aniline 4-hydroxylase activity in rabbit kidney microsomes.

Table 3.7. Effects of pyridine renal microsomal aniline 4-hydroxylase activity

| Specific Activity (nmol pAp/min/mg protein) | | | | | | |
|---|---|---|---|---|---|---|
| Rabbits | 1 | 2 | 3 | 4 | 5 | Average |
| Treated | 0.033 | 0.036 | 0.030 | 0.097 | 0.076 | 0.054±0.030* |
| Control | 0.013 | 0.015 | 0.009 | - | - | 0.012±0.003 |

*$p < 0.05$

On Ethylmorphine N-Demethylase Activity;

The N-demethylation of ethylmorphine in rabbit kidney was determined in a reaction mixture containing 3 mg microsomal protein per ml. As shown in Table 3.8., the specific ethylmorphine N-demethylase activities were found as; 0.94, 0.31, 0.50 nmol HCHO/min/mg prot. which resulted in an average value of 0.58±0.32 nmol HCHO/min/mg prot. (N=3), for the kidney microsomes obtained from the pyridine treated rabbits and 0.14, 0.25, 0.15 nmol HCHO/min/mg prot. (average was 0.18±0.06 nmol HCHO/min/mg prot., N=3) for the microsomes from the control animals.Thus, pyridine treatment of rabbits at a dose of 250 mg/kg body weight on day 1, 5 and 8 caused 3.2-fold increase in ethylmorphine N-demethylase activity in rabbit kidney microsomes.

Table 3.8. Effects of pyridine on renal microsomal ethylmorphine N-demethylase activity

| Specific Activity (nmol HCHO/min/mg protein) | | | | |
|---|---|---|---|---|
| Rabbits | 1 | 2 | 3 | Average |
| Treated | 0.94 | 0.31 | 0.50 | 0.58±0.32 |
| Control | 0.14 | 0.25 | 0.15 | 0.18±0.06 |

On NDMA N-Demethylase Activity;

The N-demethylation of NDMA was determined by using 2 mg/ml microsomal protein for treated and 3mg/ml microsomal protein for control animals. As shown in Table 3.9., the specific enzyme activities of renal microsomes obtained from the pyridine treated rabbits were found to be; 0.40, 0.29, 0.30 nmole HCHO/min/mg prot. which gave an average value of 0.33±0.06 nmole HCHO/min/mg prot. (N=3). The specific activities of the enzyme from the control animals were found as; 0.10, 0.09, 0.10 nmole HCHO/min/mg prot. which resulted in an average value of 0.097±0.006 nmole HCHO/min/mg prot. (N=3). Thus, pyridine treatment of rabbits enhanced NDMA N-demethylase activity of kidney microsomes by 3.44-fold.

Table 3.9. Effects of pyridine on renal microsomal NDMA N-demethylase activity

| Specific Activity (nmol HCHO/min/mg protein) | | | | |
|---|---|---|---|---|
| Rabbits | 1 | 2 | 3 | Average |
| Treated | 0.40 | 0.29 | 0.30 | 0.33±0.06* |
| Control | 0.10 | 0.09 | 0.10 | 0.097±0.006 |

*$p < 0.05$

On *p*-Nitrophenol Hydroxylase Activity;

The hydroxylation of *p*-nitrophenol was determined by using 4 mg/ml microsomal protein concentration. The specific activities of pyridine treated rabbits were found as; 0.078, 0.036, 0.031, 0.086, 0.097 nmol product/min/mg prot. (average was 0.064±0.032, N=5). The specific activities of control rabbits were 0.010, 0.018, 0.013 nmol product/min/mg prot. (average was 0.013±0.006 nmol

product/min/mg prot., N=3). Thus, 250 mg pyridine/kg body weight treatment in the days 1, 5 and 8 caused 4.9-fold increase in *p*-nitrophenol hydroxylase activity of kidney microsomes (Table 3.10.).

Table 3.10. Effect of pyridine on renal microsomal *p*-nitrophenol hydroxylase activity

| Specific Activity (nmol product/min/mg protein) | | | | | | |
|---|---|---|---|---|---|---|
| Rabbits | 1 | 2 | 3 | 4 | 5 | Average |
| Treated | 0.078 | 0.036 | 0.031 | 0.086 | 0.097 | 0.064±0.032* |
| Control | 0.010 | 0.018 | 0.013 | - | - | 0.013±0.006 |

*$p < 0.05$

### 3.1.1.4. Effects of *in vivo* Pyridine Treatment on Mixed Function Oxidase Enzyme Activities of Rabbit Lung Microsomes

On Aniline 4-Hydroxylase Activity;

Microsomal aniline 4-hydroxylase activity in rabbit lung was determined using 4 mg/ml microsomal protein concentration. The specific enzyme activities of pulmonary microsomes obtained from the pyridine treated animals were found as; 0.22, 0.20, 0.18, 0.18 nmol pAp/min/mg prot. (the average was 0.20±0.02 nmol pAp/min/mg prot, N=4). The specific activities of this enzyme from the control rabbits were found to be 0.23, 0.19, 0.17 nmol pAp/min/mg prot. (the average was 0.20±0.03 nmol pAp/min/mg prot., N=3). These results indicate that, *in vivo* pyridine treatment of rabbits at a dose of 250 mg/kg body weight on day 1, 5 and 8 caused no significant changes in microsomal aniline 4-hydroxylase activity in rabbit lung (Table 3.11.).

Table 3.11. Effects of pyridine treatment on pulmonary aniline 4-hydroxylase activity

| Specific Activity (nmol pAp/min/mg protein) | | | | | |
|---|---|---|---|---|---|
| Rabbits | 1 | 2 | 3 | 4 | Average |
| Treated | 0.22 | 0.20 | 0.18 | 0.18 | 0.20±0.02 |
| Control | 0.23 | 0.19 | 0.17 | - | 0.20±0.03 |

On Ethylmorphine N-Demethylase Activity;

Ethylmorphine N-demethylase activity of rabbit lung microsomes was determined in a reaction mixture containing 1.5 mg/ml microsomal protein. As shown in Table 3.12., the specific enzyme activities of pulmonary microsomes of the treated rabbits were found to be 1.70, 3.39, 2.90 nmol HCHO/min/mg prot. which gave an average value of 2.67±0.87 nmol HCHO/min/mg prot. (N=3). The specific ethylmorphine N-demethylase activities of control rabbit microsomes were calculated to be 0.31, 2.40, 1.63 nmol HCHO/min/mg prot. which resulted in an average value of 1.45±1.06 nmol HCHO/min/mg prot. (N=3). Thus, it can be postulated that, pyridine treatment of rabbits resulted in 1.85-fold increase in ethylmorphine N-demethylase activity of lung microsomes.

Table 3.12. Effects of pyridine on pulmonary microsomal ethylmorphine N-demethylase activity

| Specific Activity (nmol HCHO/min/mg protein) | | | | |
|---|---|---|---|---|
| Rabbits | 1 | 2 | 3 | Average |
| Treated | 1.70 | 3.39 | 2.90 | 2.67±0.87 |
| Control | 0.31 | 2.40 | 1.63 | 1.45±1.06 |

On NDMA N-Demethylase Activity;

NDMA N-demethylase activity of rabbit lung microsomes was determined using 2 mg microsomal protein from both pyridine treated and control rabbits. As can be seen in Table 3.13., specific enzyme activities of pulmonary microsomes of the treated rabbits were found to be 0.21, 0.25, 0.12, 0.06, 0.19 nmol HCHO/ min/mg prot. which gave an average value of 0.17±0.08 nmol HCHO/min/mg prot.(N=5). The specific activities of the enzyme from the control rabbits were calculated to be 0.02, 0.05, 0.03 nmol HCHO/min/mg prot. which resulted in an average value of 0.033±0.015 nmol HCHO/min/mg prot. (N=3). Thus, *in vivo* treatment of rabbits with pyridine (i.p.) at a dose of 250 mg/kg body weight on day 1, 5 and 8 resulted in 5.15-fold increase in NDMA N-demethylation rates of lung microsomes.

Table 3.13. Effect of pyridine on pulmonary microsomal NDMA N-demethylase activity

| Specific Activity (nmol HCHO/min/mg protein) | | | | | | |
|---|---|---|---|---|---|---|
| Rabbits | 1 | 2 | 3 | 4 | 5 | Average |
| Treated | 0.21 | 0.25 | 0.12 | 0.06 | 0.19 | 0.17±0.08* |
| Control | 0.020 | 0.050 | 0.030 | - | - | 0.033±0.015 |

*$p < 0.05$

On *p*-Nitrophenol Hydroxylase Activity;

Activity of microsomal *p*-nitrophenol hydroxylase in rabbit lung was determined with an incubation mixture containing 2 mg/ml microsomal protein . As seen in Table 3.14., the specific enzyme activities of pulmonary microsomes

obtained from the treated animals were found to be 0.24, 0.14, 0.19, 0.10, 0.14 nmol product/min/mg prot. (the average was 0.16±0.05 nmol product/min/mg prot., N=5). The specific activities of the enzyme from the control rabbits were found as; 0.12, 0.13, 0.12 (the average was 0.12±0.006 nmol product/min/mg prot., N=3). Pyridine treatment of rabbits at a dose of 250 mg/kg body weight on day 1, 5 and 8 did not alter the activity of lung microsomal *p*-nitrophenol hydroxylase activity with respect to the microsomal enzymes obtained from the control animals.

Table 3.14. Effect of pyridine on pulmonary microsomal *p*-nitrophenol hydroxylase activity

| Specific Activity (nmol product/min/mg protein) | | | | | | |
|---|---|---|---|---|---|---|
| Rabbits | 1 | 2 | 3 | 4 | 5 | Average |
| Treated | 0.24 | 0.14 | 0.19 | 0.10 | 0.14 | 0.16±0.05 |
| Control | 0.12 | 0.13 | 0.12 | - | - | 0.12±0.006 |

### 3.1.2. Effects of Pyridine on Glutamate Oxaloacetate Transaminase and Lactate Dehydrogenase Enzyme Activites of Rabbit Blood Serum and Soluble Fractions of Rabbit Liver, Kidney and Lung

Table 3.15. tabulates the effect of pyridine on GOT and LDH enzyme activities of rabbit blood serum and of soluble fractions of rabbit liver, kidney and lung. The GOT enzyme activities of blood serum obtained from the treated rabbits were noted as; 0.28, 0.45, 0.28, 0.43, 0.57 unit/min/mg prot. which gave an average value of 0.40±0.12 unit/min/mg prot. (mean ±SD, N=5). The activities of the enzyme from the control rabbits were found as; 0.67, 0.56, 0.61 unit/min/mg

prot. which resulted in an average value of 0.61±0.05 unit/min/mg prot. (mean ± SD, N=3). LDH enzyme activities were found to be 5.10, 7.79, 6.19, 9.03, 8.87 unit/min/mg prot. which resulted in an average value of 7.40±1.71 unit/min/mg prot. (N=5) for the blood serum of treated rabbits and 5.91, 6.65, 7.80 unit/min/mg prot. which gave an average value of 6.79±0.95 unit/min/mg prot. (N=3) of the control rabbits. Thus, pyridine treatment of the animals, i.p. at a dose of 250 mg/kg body weight did not alter the activities of GOT and LDH enzymes of blood serum.

After the administration of pyridine to the rabbits in dose of 250 mg/kg body weight, i.p. on the first, fifth and eighth day; the GOT enzyme activities of soluble fraction of rabbit liver were found to be 85.44, 83.63, 97.55, 91.45, 105.69 unit/min/mg prot. which resulted in an average value of 92.75±9.07 unit/min/mg prot. (N=5). The GOT enzyme activities of the control rabbits were found as; 82.70, 77.38 and 73.36 unit/min/mg prot. which resulted in an average specific activity of 77.81±4.70 unit/min/mg prot. (N=3). Thus, pyridine treatment caused not much alteration (1.19 fold) in GOT activity of soluble fraction of rabbit liver. On the other hand, in pyridine treated rabbits the LDH enzme activities of soluble fraction of liver were found as 77.17, 83.19, 108.63, 69.46, 186.52 unit/min/mg prot. The average specific activity was calculated to be 105±48 unit/min/mg prot. The enzyme activities recorded for the control rabbits were 52.93, 18.62 and 37.92 unit/min/mg prot. which gave an average of 36.50±17.20 unit/min/mg prot. Therefore, with pyridine treatment, the LDH enzyme activity of the soluble fraction of liver was increased by 2.87-fold with respect to the control rabbits.

The effect of pyridine treatment on GOT and LDH enzyme activities of soluble fractions of kidney was also studied. The GOT activities obtained from the soluble fraction of kidney of treated rabbits were found to be, 161.45, 226.15, 121.39, 121.50, 185.71 unit/min/mg prot. which resulted in an average value of 163.20±44.60 unit/min/mg prot. and 85.36, 118.72, 105.81 unit/min/mg prot. which gave an average value of 103.30±16.80 unit/min/mg prot. of the control rabbits. As can be observed, pyridine injection i.p. at a dose of 250 mg/kg body

weight on day 1, 5 and 8 caused 1.58-fold increase in GOT activities of the soluble fraction of kidney, with respect to the control animals. LDH enzyme activities of the soluble fraction of kidney in response to pyridine treatment were 1250, 1230.76, 1462, 1192.30, 2024.48 unit/min/mg prot. which resulted in an average value of 1432±347 unit/min/mg prot. The results obtained from the control rabbits were 10.26, 65.01 and 6.53 unit/min/mg prot. which gave an average value of 27.26±32.73 unit/min/mg prot. Thus; upon pyridine treatment of rabbits, LDH enzyme activity of soluble fraction of kidney increased significantly (about 52.53-fold).

There were no significant change in the GOT activity of the soluble fraction of lung by the pyridine administration. The enzyme activities of the soluble fraction of lung were found to be 11.81, 6.35, 12.40, 8.57, 11.99 unit/min/mg prot., the average specific activity was calculated to be 10.22±2.65 unit/min/mg prot. for the treated animals and 10.12, 7.94, 9.56 unit/min/mg prot. which gave an average value of 9.20±1.13 unit/min/mg prot. for the control animals. On the other hand, with pyridine treatment, the LDH enzyme activities of the soluble fraction of lung was increased by 2.5-fold as compared to the values obtained from control animals. The enzyme activities were found to be 15.58, 18.76, 105.34, 85.46, 66.54 unit/min/mg prot. which gave an average of 58.30±40 unit/min/mg prot. for the treated animals and 25.13, 14.69, 30.03 unit/min/mg prot. which resulted in an average value of 23.30±7.83 unit/min/mg prot. for the control animals.

Table 3.15. Effects of pyridine on GOT and LDH enzyme activities of rabbit blood serum and of soluble fractions of rabbit liver, kidney and lung.

| | | GOT (unit/min/mg protein) | | | | | | LDH (unit/min/mg protein) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Tissue | Rabbits | 1 | 2 | 3 | 4 | 5 | Average | 1 | 2 | 3 | 4 | 5 | Average |
| Blood Serum | Trtd. | 0.28 | 0.45 | 0.28 | 0.43 | 0.57 | 0.40±0.12[a] | 5.10 | 7.79 | 6.19 | 9.03 | 8.87 | 7.40±1.71 |
| | Cont. | 0.67 | 0.56 | 0.61 | – | – | 0.61±0.05 | 5.91 | 6.65 | 7.80 | – | – | 6.79±0.95 |
| Liver | Trtd. | 85.44 | 83.63 | 97.55 | 91.45 | 105.69 | 92.75±9.07[a] | 77.17 | 83.19 | 108.63 | 69.46 | 186.52 | 105±48[a] |
| | Cont. | 82.70 | 77.38 | 73.36 | – | – | 77.81±4.70 | 52.93 | 18.62 | 37.92 | – | – | 36.50±17.20 |
| Kidney | Trtd. | 161.45 | 226.15 | 121.39 | 121.50 | 185.71 | 163.20±44.60[a] | 1250 | 1230.76 | 1462 | 1192.30 | 2024.48 | 1432±347[b] |
| | Cont. | 85.36 | 118.72 | 105.81 | – | – | 103.30±16.80 | 10.26 | 65.01 | 6.53 | – | – | 27.26±32.73 |
| Lung | Trtd. | 11.81 | 6.35 | 12.40 | 8.57 | 11.99 | 10.22±2.65 | 15.58 | 18.76 | 105.34 | 85.46 | 66.54 | 58.30±40 |
| | Cont. | 10.12 | 7.94 | 9.56 | – | – | 9.20±1.13 | 25.13 | 14.69 | 30.03 | – | – | 23.30±7.83 |

[a] $p < 0.05$

[b] $p < 0.001$

## 3.2. Effects of Pyridine on Cytochrome P450 Contents of Rabbit Liver, Kidney and Lung

Determination of cytochrome P450 contents of rabbit liver, kidney and lung microsomal preparations was carried out as described under "Materials and Methods". As shown in Table 3.16., cytochrome P450 contents of liver microsomal preparations of pyridine treated rabbits were found as; 2.05, 1.28, 1.16, 1.35 and 1.79 nmoles per mg of microsomal protein. The average specific content was calculated to be 1.53±0.38 nmole/mg prot. (N=5). The P450 contents of microsomal preparations of control rabbits were found to be 0.64, 0.80, 0.80 nmol/mg prot. which gave an average value of 0.75±0.09 nmole/mg prot. (N=3).

The specific contents of the pyridine treated rabbit kidney microsomes were found as; 0.29, 0.18, 0.12, 0.21, 0.25 nmole/mg prot. (the average value was 0.21±0.07 nmole/mg/prot N=5) and of the control microsomal preparations were calculated to be; 0.12, 0.15, 0.12 nmole/mg prot. (the average value was 0.13±0.01 nmole/mg prot., N=3).

The results obtained from the microsomal preparations of treated rabbit lungs were found as 0.53, 0.27, 0.53, 0.33, 0.42 nmole/mg prot.which resulted in an average value of 0.42±0.12 nmole/ mg/ prot (N=5). Finally 0.31, 0.35, 0.24 nmole/mg prot. were the cytochrome P450 content values observed in the microsomal preparations of control rabbit lungs which gave an average of 0.30±0.06 nmole/mg prot. (N=3). As a result, it can be postulated that, the cytochrome P450 contents of liver, kidney and lung microsomal preparations increased 2.04-, 1.6- and 1.4 - fold, respectively, after the pyridine treatment of rabbits at a dose of 250 mg/kg body weight on day 1,5 and 8.

Table 3.16. Effects of pyridine on hepatic, renal and pulmonary cytochrome P450 levels

| Tissue | | Cytochrome P450 (nmol / mg protein) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | Average | Fold |
| LIVER | Trtd. | 2.05 | 1.28 | 1.16 | 1.35 | 1.79 | 1.53±0.38* | 2.04 |
| | Cont. | 0.64 | 0.80 | 0.80 | - | - | 0.75±0.09 | |
| KIDNY | Trtd. | 0.29 | 0.18 | 0.12 | 0.21 | 0.25 | 0.21±0.07* | 1.60 |
| | Cont. | 0.12 | 0.15 | 0.12 | - | - | 0.13±0.01 | |
| LUNG | Trtd. | 0.53 | 0.27 | 0.53 | 0.33 | 0.42 | 0.42±0.12 | 1.40 |
| | Cont. | 0.31 | 0.35 | 0.24 | - | - | 0.30±0.06 | |

* $p < 0.05$

### 3.3. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

Figure 3.1. shows the SDS-PAGE patterns of lung, kidney and liver microsomes prepared from control and pyridine treated rabbits.Lanes 2; 5, 7; 9 show the typical SDS-PAGE patterns for control rabbit lung, kidney and liver microsomal proteins, whereas lanes 3, 4; 8 and 10, 11 show SDS-PAGE patterns of microsomal proteins obtained from pyridine treated lung, kidney and liver, respectively. As seen in the figure, pyridine treatment changed the SDS-PAGE profiles of liver and kidney microsomal proteins, while those of lung microsomes were not effected by pyridine. In the profile of liver microsomes, the intensity of the band corresponding to approximately Mr of 51 000 was markedly increased after pyridine treatment (Compare lane 9 with lanes 10 and 11). In the same profile, the intensity of the band corresponding to approximately Mr of 53 000 was also seemed to be increased. Similar to liver, pyridine treatment also caused an increase in intensity of protein bands corresponding to Mr of 51 000 and 53 000 in kidney microsomes. However, the magnitude of increase in kidney microsomal proteins (Mr of 51 000 and 53 000) by pyridine treatment was found to be lower compared to liver.

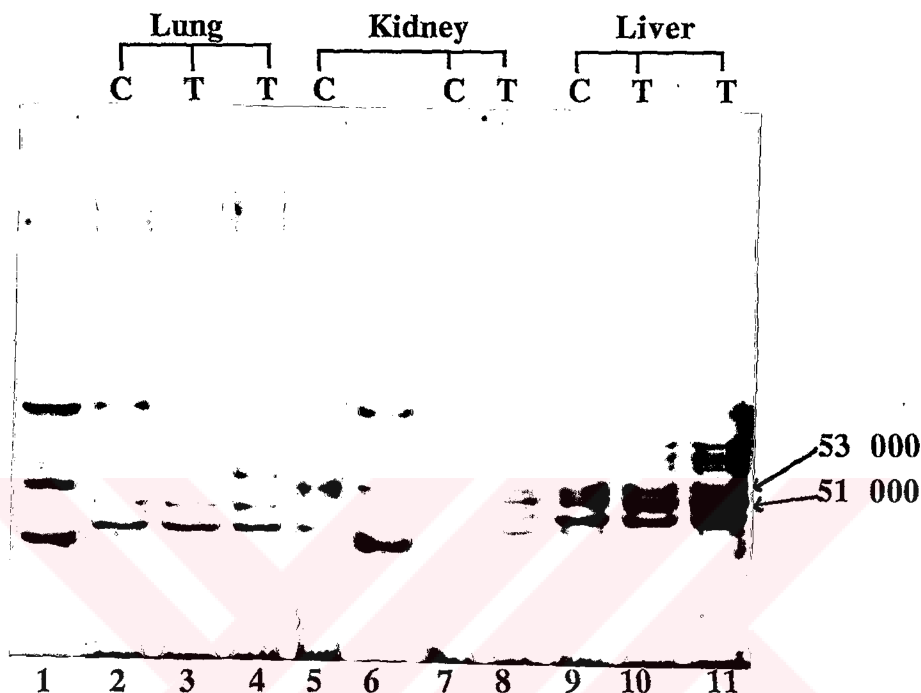



Figure 3.1. SDS-PAGE of lung, kidney and liver microsomes from control animals (C) or rabbits treated with pyridine (T). Lanes 1 and 6 contain three standard proteins. (each 3.75 μg) : BSA (Mr 68 000), glutamate dehydrogenase (Mr 53 000), egg albumin (Mr 45 000). Lanes 2 ; 5, 7; 9 show the typical SDS-PAGE patterns for control rabbit lung, kidney and liver microsomal proteins, lanes 3, 4; 8 and 10, 11 show SDS-PAGE patterns of microsomal proteins obtained from pyridine treated lung, kidney and liver, respectively (50 μg each). Arrows show the 51 000 and 53 000 Mr protein bands.

# CHAPTER IV

# DISCUSSION

Over the past four decades, induction of MFO enzymes has been shown to significantly alter pharmacologic or toxicologic responses *in vivo* in laboratory animals and humans (Conney, 1967, 1982; Goldberg, 1980; Park and Breckenridge, 1981; Okey *et al.* 1986). There are two primary practical concerns related to cytochrome P450 dependent MFO induction: (1) induction may alter the efficacy of therapeutic agents (2) induction may cause an undesirable imbalance between rates of "toxification" versus "detoxification" in organisms exposed to drugs or environmental chemicals (Okey *et al.* 1986; Okey, 1990). Altered pharmacological responses *in vivo* generally are in proportion to the magnitude of increase in P450 dependent catalytic activities that can be measured *in vitro* on microsomes from the "induced" animals.

In pharmacological and toxicological studies, the liver has received a great attention because it is one of the major target organs for many foreign compounds. Although the ability of the lung to metabolize xenobiotics is less than that of liver, the lung may still be important because of its role as a portal for inhaled compounds and high blood flow in relation to other organs. There have been fewer reports on studies of kidney MFO enzymes in species.

Isolation and characterization of the microsomal cytochrome P450 isozymes of rabbit lung have not resulted in the identification of any isozymes that are distinct from forms in liver (Arınç and Philpot, 1976; Serabjit-Singh *et al.*, 1983; Philpot *et al.*, 1991). Despite this similarity, the cytochrome P450 monooxygenase systems of lung and liver differ in two significant respects; the proportion of the total cytochrome P450 that each isozyme comprises and their apparent response to inducers. Both of these factors may reflect marked differences in the mechanisms by which the synthesis of an individual isozyme of cytochrome P450 is controlled (Serabjit-Singh *et al.*, 1983). In untreated rabbits, pulmonary cytochrome P450 is comprised of primarily two isozymes of approximately equal proportion (each 40-45 % of total) P4502B4 (LgM2) and P4504B1 (LgM5) (Philpot *et al.*, 1991). Both cytochromes P4502B4 and P4504B1 are major constitutive components of rabbit lung microsomes -that is phenobarbital or any other chemical treatment of rabbits do not induce these cytochrome P450 isozymes and their respective enzyme activities. In the untreated rabbit liver these forms make up a small fraction (P4502B4 in ~10 %, P4504B1 in ~1 %) of total cytochrome P450 (Philpot *et al.*, 1985), which is comprised of at least 9 forms (Lu and West, 1980; Funae and Imaoka, 1993). Pretreatment of rabbits with PB induces P4502B4 and P4504B1 6-12 and 4-6 fold, respectively (Philpot *et al.*, 1985.; Parandoosh, *et al.*, 1987).

A third form of lung cytochrome P450 (P4501A1) was identified in TCDD- (Liem *et al.*, 1980; Philpot *et al.*, 1985) and a forth form (4A4) was identified in progesteron-treated rabbits (Powell, 1978; Yamamato *et al.*, 1984) and in pregnant rabbits (Powell, 1978; Williams *et al.*, 1984). On the other hand, up to now, neither the presence nor the induction of cytochrome P4502E1 has been shown in rabbit lung.

Cytochrome P4502E1 has received a great deal of attention in recent years because of its vital role in the activation of many toxic chemicals. Its possible role in the activation of xenobiotics to electrophilic, potentially mutagenic metabolites and in tumour development has been demonstrated in studies with benzene (Mehlman,

1991; Nakajima *et al.*, 1992), low molecular weight halogenated hydrocarbon species, like $CCl_4$ (Guengerich *et al.*, 1991) and nitrosamines (Yoo *et al.*, 1988; Yang *et al.*, 1990; Yamazaki *et al.*, 1992)

Cytochrome P4502E1, the alcohol-inducible cytochrome P450, previously known as P450LM3a, P450j, P450ac or P450alc, has been identified in the livers of rat, rabbit and man by immunoblotting (Ryan *et al.*, 1986; Lasker *et al.*, 1987; Palakodaty *et al.*, 1989). It is also induced by the treatment with alcohol, acetone benzene, isoniazid, isopropanol, dimethylsulfoxide and by starvation, high-fat feeding and diabetes (Koop and Casazza, 1985; Hong *et al.*, 1987; Song *et al.*, 1987; Kim *et al.*, 1990). Induction of hepatic cytochrome P4502E1 was also observed after *in vivo* treatment of rats (Kim *et al.*, 1988, Kim and Novak, 1990) and rabbits (Carlson and Day, 1992; Kim *et al.*, 1991a) with pyridine.

Heterocyclic aromatic amines such as pyridine, are used as solvents, intermediates in the production of herbicides, insecticides, and in the manufacture of pharmaceuticals. The pyridine ring is present in the molecular structures of numerous drugs including, isoniazid, amrinone and milrinone (Kim *et al.*, 1991a). The pyridine moiety also occurs in naturally occuring products (e.g. nicotinamide, nicotine) and also in cigarette smoke. Interestingly, 79 of the 379 nitrogen heterocycles identified in cigarette/cigar smoke are imidazole or pyridine derivatives (Schumacher *et al.*, 1977, Moree-Testa *et al.*, 1984).

This study was undertaken to investigate *in vivo* effects of pyridine treatment on drug metabolizing enzymes that is, aniline 4-hydroxylase, *p*-nitrophenol hydroxylase, NDMA N-demethylase, ethylmorphine N-demethylase and on the cytochrome P450 levels and on the biomarkers used to measure chemical-induced toxicity including LDH and GOT in rabbit liver, kidney and lung which have not been adressed in this context before. The results of this study are given in Tables 3.1. to 3.16 and summarized in Table 4.1.

Table 4.1.Effects of pyridine on rabbit liver, kidney and lung cytochrome P450 levels, microsomal drug metabolizing enzyme activities and on GOT and LDH enzyme activities of rabbit blood serum and of soluble fractions of rabbit liver kidney and lung.

| | Tissue or serum | Cytochrome P 450 (nmol/mg prot) | Aniline 4-hydroxylase (nmol pAp/ min/mg prot.) | p-Nitrophenol hydroxylase (nmole product/ min/mg prot.) | NDMA N-demethylase (nmol HCHO min/mg prot.) | Ethylmorphine N-Demethylase (nmol HCHO/min/ mg prot.) | GOT (unit /mg prot.) | LDH (unit /min/ mg prot.) |
|---|---|---|---|---|---|---|---|---|
| LIVER | Control | 0.75±0.09 (3) | 0.32±0.05 (3) | 0.18±0.06 (3) | 0.20±0.13 (3) | 0.91±0.24 (3) | 77.81±4.70 (3) | 36.50±17.20 (3) |
| | Pyridine | 1.53±0.38[a] (5) | 1.85±0.60[b] (5) | 0.79±0.16[c] (5) | 1.38±0.34[b] (5) | 0.99±0.21 (5) | 92.75±9.07[a] (5) | 105±48[a] (5) |
| KIDNEY | Control | 0.13±0.01 (3) | 0.012±0.003 (5) | 0.013±0.006 (5) | 0.097±0.006(3) | 0.18±0.06 (3) | 103.30±16.80(3) | 27.26±32.73 (3) |
| | Pyridine | 0.21±0.07[a] (5) | 0.054±0.030[a] (5) | 0.064±0.032[a] (5) | 0.33±0.06[a] (3) | 0.58±0.32 (3) | 163.20±44.60[a] (5) | 1432±347[c] (5) |
| LUNG | Control | 0.30±0.06 (3) | 0.20±0.03 (3) | 0.12±0.006(3) | 0.033±0.015 (3) | 1.45±1.06 (3) | 9.20±1.13 (3) | 23.30±7.83 (3) |
| | Pyridine | 0.42±0.12 (5) | 0.20±0.02 (4) | 0.16±0.05 (5) | 0.17±0.08[a] (5) | 2.67±0.87 (3) | 10.22±2.65 (5) | 58.30 ±40 (5) |
| SERUM | Control | – | – | – | – | – | 0.61±0.05 (3) | 6.79±0.95 (3) |
| | Pyridine | – | – | – | – | – | 0.40±0.12[a] (5) | 7.40± 1.71 (5) |

Male New Zealand rabbits weighing 1.7-2.35 kg were administered with pyridine (250 mg/kg body weight) i.p., on day 1,5 and 8. Each value represents the mean±SD (number of animals)

[a]Significantly different from control, $p<0.05$

[b]Significantly different from control, $P<0.005$

[c]Significantly different from control, $P<0.001$

Results obtained in this study indicated that microsomal cytochrome P450 contents of liver, kidney and lung were elevated 2.04- , 1.6- and 1.4-fold, respectively by pyridine treatment. In accordance with the results obtained with the effect of pyridine on liver cytochrome P450 content in this study it was demonstrated that pyridine administration to rabbits (Kaul and Novak, 1987) and rats (Kim *et al.,* 1988) elevated hepatic microsomal cytochrome P450 content approximately 2-fold and 2.5-fold as compared to that of controls.

Figure 3.1. shows the SDS-PAGE protein patterns of liver, kidney and lung microsomes prepared from control and pyridine treated rabbits. As seen in the figure, pyridine pretreatment changed the SDS-PAGE profiles of both liver and kidney microsomal proteins, while those of lung microsomes were not affected by pyridine. The intensity of protein band corresponding to approximately Mr of 51 000 was increased both in liver and kidney microsomes markedly (Figure 3.1.). In addition, the protein staining of the bands corresponding to approximately Mr of 53 000 was also increased both in liver and kidney microsomes after pyridine treatment although the observed intensity of the bands was not as high as those observed in the region of 51 000 Mr proteins.

It was shown by immunoblot analysis that administration of pyridine to rabbits (Kim *et al.*, 1991a) and to rats (Kim *et al.*, 1988; Kim and Novak, 1990; Carlson and Day, 1992) apparently induced liver microsomal cytochrome P4502E1 which had Mr of 51 000 on SDS-PAGE. Although, in this study immunoreactivity studies were not carried out, SDS-PAGE studies, in parallel with MFO activity studies demonstrated that cytochrome P4502E1 was induced in both liver and kidney microsomes in rabbits.

Induction of a protein having Mr of 51 400, associated with 5.7-fold and 4.2 -fold increases in aniline 4-hydroxylase and *p*-nitrophenol hydoxylase activities over the uninduced rabbit kidney microsomes was observed from the kidney microsomes of rabbits treated with benzene (Arınç *et al.*, 1991). These results implicated that benzene induced P4502E1 in rabbit kidney (Arınç *et al.*, 1991).

Very recently Roberts *et al.* (1994) demonstrated by Western-Blot analysis that chronic ethanol administration resulted in 4-5-fold intense staining of cytochrome P4502E1 (Mr 51 000) in kidney microsomes.

It has been shown that aniline 4-hydroxylase, *p*-nitrophenol hydroxylase and NDMA N-demethylase activities of the MFOs are associated with cytochrome P4502E1 (Koop, 1986; Tu and Yang, 1985; Kim *et al.*, 1991a) The MFO enzyme activity results shown in Table 4.1. and Figure 4.1., indicate that aniline 4-hydroxylase of liver and kidney microsomes are enhanced by 5.8- and 4.5-fold, respectively. Pyridine treatment increased hydroxylation rates of *p*-nitrophenol in liver and kidney by 4.4- and 4.9- fold, respectively. As seen in Table 4.1. and Figure 4.1. treatment of rabbits with pyridine also enhanced NDMA N-demethylase enzyme activities of liver and kidney by 6.9- and 3.4- fold, respectively. Except the studies carried out with benzene (Arınç *et al.*, 1991), induction of cytochrome P4502E1 associated MFO enzyme activities in kidney has not been investigated in animals. Thus, comparison of the results obtained in this study with those of others could not be made.

The results obtained in this study with P4502E1 associated MFO enzyme activities in liver are in accordance with those of Kim *et al.* (1988) who observed approximately 8-, 4- and 5-fold increase in microsomal aniline 4-hydroxylase, *p*-nitrophenol hydroxylase and NDMA N-demethylase activities of liver obtained from pyridine treated rats. Kim, Philpot and Novak (1991a) also found that pyridine administration to rabbits caused 6-fold increase in microsomal *p*-nitrophenol hydroxylase activitiy of liver. As noted before, pyridine treatment of rabbits increased the total cytochrome P450 content of lung by 1.4-fold (Table 3.16 and Figure 4.2) whereas SDS-PAGE protein profiles of lung microsomes were not affected by pyridine treatment as shown in Figure 3.1. Of the three MFO enzymes namely aniline 4-hydroxylase, *p*-nitrophenol hydroxylase and NDMA N-demethylase, associated with cytochrome P4502E1, a marked 5.15-fold increase was observed only in microsomal NDMA N-demethylase activity of lung upon pretreatment of rabbits with pyridine which was significantly different from control,

$p<0.05$, (Table 4.1., Figure 4.2.) Up to now, neither the presence nor the induction of cytochrome P4502E1 has been detected in rabbit lung.

Carlson and Day (1992) demonstrated by immunoblotting that pyridine induced cytochrome P4502E1 in both rat liver and lung in parallel with induction of NDMA N-demethylase (2.4-fold in liver, 1.7-fold in lung) and *p*-nitrophenol hydroxylase (2.3-fold in liver, 1.7 fold in lung) activities in both tissues. Ethylmorphine is not a specific substrate for cytochrome P4502E1 thus, as expected liver microsomal ethylmorphine N-demethylase activity was not altered upon pretreatment of rabbits with pyridine. On the other hand, pyridine treatments caused 3.2- and 1.8-fold increases in rabbit kidney and lung microsomes (Figure 4.1 and 4.2), although these increases were found statistically insignificant (Table 4.1).

As noted before, among all the P450s studied, P4502E1 is most active in catalyzing the NADPH dependent formation of $H_2O_2$ and $O_2$ *in vitro* (Ekstrom and Ingelman-Sundberg, 1989; Gorsky *et al.* 1984; Persson *et al.*, 1990). Antibodies to P4502E1 almost completely inhibited the NADPH dependent lipid peroxidation in microsomes (Ekstrom and Ingelman-Sundberg, 1989). It was postulated that induction of P4502E1 in the liver results in increased oxygen stress by generating higher levels of $H_2O_2$ and $O_2$. Thus, at the beginning of this study it has been speculated that some degree of membrane damage may occur as a result of lipid peroxidation which could result in enhancement of transaminase and LDH activities in serum. However, as shown in Table 3.15. in "Results" neither transaminase nor the LDH activities have increased in serum upon subacute treatment of rabbits with pyridine. On the other hand, pyridine treatment increased the LDH activities in soluble fraction of liver, kidney and lung and GOT activity only in kidney cytosol. LDH activities of cytosol of liver and lung of pyridine treated rabbits were found to be increased 2.9 - and 2.5 - fold, respectively, compared to control animals. This degree of increase in LDH activity of liver and lung can be attributed to the starvation of animals due to pyridine injection stress. However, LDH activity of cytosol of kidney was found to be 52.53-fold higher in pyridine treated rabbits compared to control animals.

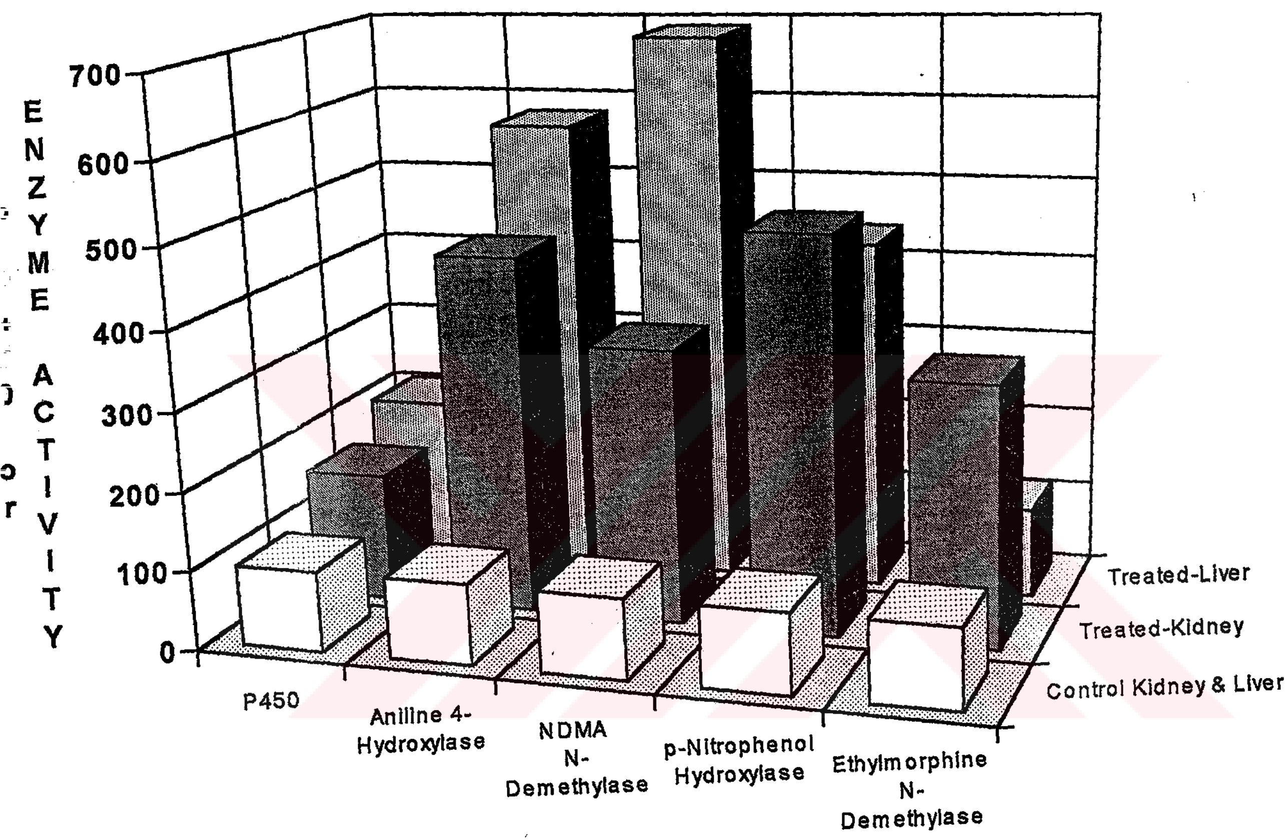



Figure 4.1. The effects of pyridine treatment on rabbit liver and kidney cytochrome P450 levels and on microsomal drug metabolizing enzyme activities. Control values are given as 100%.

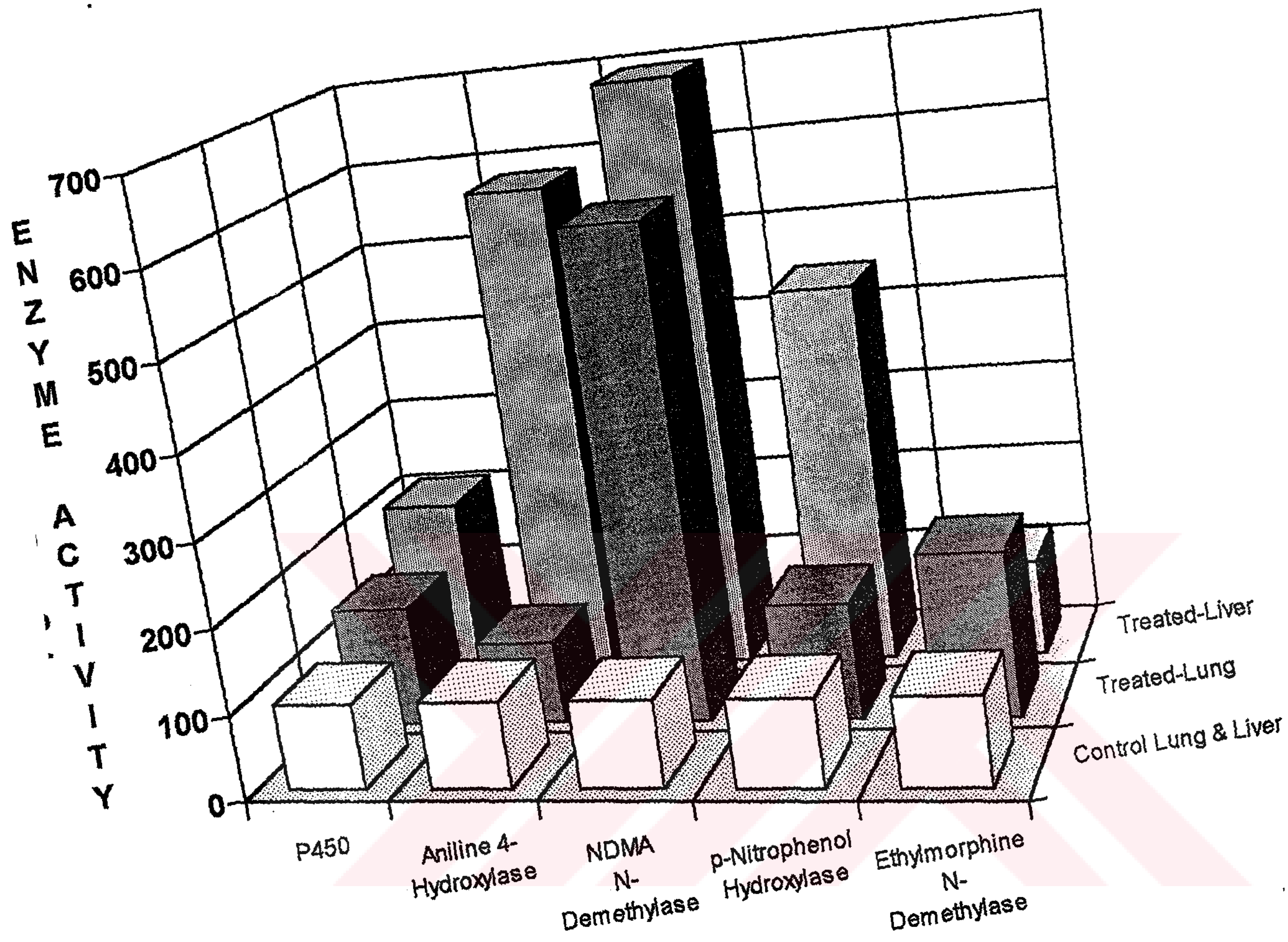



Figure 4.2. The effects of pyridine treatment on rabbit liver and lung cytochrome P450 levels and on microsomal drug metabolizing enzyme activities. Control values are given as 100%.

LDH found in the cytoplasmic portion of cells is distributed in all tissues, but it is present in high concentration in liver, cardiac and skeletal muscles, red blood cells and other tissues. In pathological states involving tissue necrosis and neoplasia, leakage of enzyme from even a small amount of damaged tissue can significantly raise levels in various body fluids including blood serum. GOT is found practically every tissue of the body, including red blood cells. It is particularly high concentration in cardiac muscle and liver, intermediate in skeletal muscle and in much lower concentrations in others. GOT is present in both the cytoplasm and the mitochondria of the cells. The predominant form in serum, in conditions associated with a mild degree of tissue injury, is that from the cytoplasm, although some mitochondrial enzyme is present in severe tissue damage. In this study we did not observe such an increase in LDH and GOT activities of blood serum of pyridine treated rabbits.

LDH occurs as five different isozymes in the tissues. All contain four polypeptide chains, each of consist of five different combinations of two different kinds of polypeptide chains, designated A and B. The isozyme predominating in muscle and liver has four identical A chains (M4 or LD5) ; another which predominates in heart and kidney has four identical B chains (H4 or LD1). All LDH isozymes catalyze the same reaction, but they differ in their dependence on substrate concentration, particularly pyruvate, as well as their $V_{max}$ values when pyruvate is the substrate.

The kidneys have an extremely high rate of respiratory metabolism and also show considerable metabolic flexibility. From two-thirds to four-fifths of the total energy generated by the kidneys is utilized in the formation of urine. The LDH isozyme, characteristic of the kidney reduces pyruvate at a relatively low rate. Moreover, the dehydrogenation of lactate catalyzed by the kidney isozyme is strongly inhibited by pyruvate. We can postulate that pyridine treatment of rabbits caused an increase of LDH activity of kidneys by abolishing the pyruvate inhibition of enzyme and by increasing gluconeogenetic pathway. It can be suggested that, this is the condition, observed in LDH activity of kidney cytosol in this work.

It has been thought that the most important finding of this study is related to the induction of NDMA N- demethylase activity in the rabbit kidney and lung. The results obtained in this study demonstrated for the first time, a significant 5.1-fold induction of NDMA N-demethylase activity in the rabbit lung over the controls. Pyridine is readily absorbed by inhalation and is a constituent of tobacco and tobacco smoke. Thus, induction of NDMA N-demethylase suggests that in the lung as in the liver, pyridine may influence the metabolic activation of this nitrosamine and in turn formation of lung cancer.

# CHAPTER V

# CONCLUSION

The *in vivo* effects of pyridine treatment (i.p., at a dose of 250 mg/kg body weight on day 1, 5 and 8) on cytochrome P450 levels, microsomal cytochrome P450 dependent drug metabolizing enzymes and on the biomarkers used to measure chemical-induced toxicity including LDH and GOT in rabbit liver, kidney and lung were examined.

Results obtained in this study showed that pyridine treatment elevated microsomal cytochrome P450 contents of liver, kidney and lung by 2.04- 1.6- and 1.4-fold, respectively. Induction of the two different cytochrome P450 isozymes was observed with the high intensity of the bands corresponding to approximate Mr of 51 000 and 53 000 in the SDS-PAGE profiles of both liver and kidney microsomes obtained from the pyridine treated rabbits. The profiles of lung microsomes were not affected by pyridine administration. Pyridine treatment of rabbits caused 5.8- and 4.5- fold increases in aniline 4-hydroxylation rates of liver and kidney microsomes, respectively, while no change was observed in the activity of enzyme of lung microsomes. Administration of pyridine markedly enhanced hyroxylation rates of *p*-nitrophenol only by liver and kidney about 4.4- and 4.9-fold, respectively. Both of these enzymes are associated with cytochrome P4502E1. Ethylmorphine is not a specific substrate for cytochrome P4502E1 thus, as expected liver microsomal ethylmorphine N-demethylase activity was not altered upon pretreatment of rabbits with pyridine. On the other hand, pyridine treatments

caused 3.2- and 1.8- fold increases in rabbit kidney and lung microsomal ethylmorphine N-demethylase activities. However, although these increases were found statistically insignificant. NDMA N-demethylase activitiy was enhanced by pyridine treatment in all three of these organs and this increase is the most likely associated with cytochrome P4502E1 induction. (6.9-fold in liver, 3.4-fold in kidney. 5.15-fold in lung). In the case of lung this 5.15-fold increase in NDMA N-demethylase activity is remarkable, because up to now neither the presence nor the induction of cytochrome P4502E1 has been detected in rabbit lung.

Pyridine treatment also increased the LDH activities in soluble fraction of liver, kidney and lung and GOT activity only in kidney cytosol (2.87-, 52.45-, 2.5- and 1.58-fold, respectively). On the other hand, neither GOT nor LDH activities have increased in serum upon subacute treatment of rabbits with pyridine.

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