ENZYME-CATALYZED REDUCTIVE ACTIVATION OF ANTICANCER DRUGS IDARUBICIN AND MITOMYCIN C

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ABSTRACT

ENZYME-CATALYZED REDUCTIVE ACTIVATION OF ANTICANCER DRUGS IDARUBICIN AND MITOMYCIN C

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Idarubicin (IDA) and mitomycin C (MC) are clinically effective quinone-containing anticancer agents used in the treatment of several human cancers. Quinone-containing anticancer drugs have the potential to undergo bioreduction by oxidoreductases to reactive species, and thereby exert their cytotoxic effects. In the present study, we investigated, for the first time, the potential of IDA, in comparison to MC, to undergo reductive activation by NADPH-cytochrome P450 reductase (P450R), NADH-cytochrome b5 reductase (b5R) and P450R-cytochrome P4502B4 (CYP2B4) system by performing both in vitro plasmid DNA damage experiments and enzyme assays. In addition, we examined the potential protective effects of some antioxidants against DNA-damaging effects of IDA and MC resulting from their reductive activation. To achieve these goals, we obtained P450R from sheep lung, beef liver and PB-treated rabbit liver microsomes, b5R from beef liver microsomes and CYP2B4 from PB-treated rabbit liver microsomes in highly purified forms.

The plasmid DNA damage experiments demonstrated that P450R is capable of effectively reducing IDA to DNA-damaging species. The effective protections

provided by antioxidant enzymes, SOD and catalase, as well as scavengers of hydroxyl radical**,** DMSO and thiourea, revealed that the mechanism of DNA damage by IDA involves the generation of ROS by redox cycling of IDA with P450R under aerobic conditions. The extent of DNA damages by both IDA and MC were found to increase with increasing concentrations of the drug or the enzyme as well as with increasing incubation time. IDA was found to have a greater ability to induce DNA damage at high drug concentrations than MC. The plasmid DNA experiments using b5R, on the other hand, showed that, unlike P450R, b5R was not able to reduce IDA to DNA-damaging reactive species. It was also found that in the presence of b5R and cofactor NADH, MC barely induced DNA strand breaks. All the purified P450Rs reduced IDA at about two-fold higher rate than that of MC as shown by the measurement of drug-induced cofactor consumption. This indicates that IDA may be a more potent cytotoxic drug than MC in terms of the generation of reactive metabolites. The results obtained from enzyme assays confirmed the finding obtained from plasmid DNA experiments that while MC is a very poor substrate for b5R, IDA is not a suitable substrate for this enzyme unlike P450R. The reconstitution experiments carried out under both aerobic and anaerobic conditions using various amounts of CYP2B4, P450R and lipid DLPC revealed that reconstituted CYP2B4 produced about 1.5-fold and 1.4-fold rate enhancements in IDA and MC reduction catalyzed by P450R alone, respectively. The present results also showed that among the tested dietary antioxidants, quercetin, rutin, naringenin, resveratrol and trolox, only quercetin was found to be highly potent in preventing DNA damage by IDA.

These results may have some practical implications concerning the potential use of P450R as therapeutic agent on their own in cancer treatment strategies. Selective targeting of tumor cells with purified P450R by newly developed delivery systems such as using polymers, liposomes or antibodies may produce greater reductive activation of bioreductive drugs in tumor cells. Consequently, this strategy has a high potential to increase the efficacy and selectivity of cancer chemotherapy.

Keywords: Idarubicin, mitomycin C, bioreductive activation, P450 reductase, b5 reductase, CYP2B4, DNA strand breaks, NAD(P)H oxidation

ÖZ

ANTİKANSER İLAÇLAR İDARUBİSİN VE MİTOMİSİN C'NİN ENZİM-KATALİZLENMİŞ REDÜKTİF AKTİVASYONU

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İdarubisin (IDA) ve mitomisin C (MC) çeşitli insan kanserlerinin tedavisinde kullanılan klinik olarak etkili kinon-içeren antikanser ajanlardır. Kinon-içeren antikanser ilaçlar, oksidoredüktazlar yoluyla reaktif türlere biyoredüktif aktivasyona uğrama potansiyeli taşırlar ve böylece sitotoksik etkilerini gösterirler. Bu çalışmada, ilk defa, hem in vitro plazmid DNA hasar deneyleri hem de enzim assayleri yaparak, IDA'nin MC'ye kıyasla, NADPH-sitokrom P450 redüktaz (P450R), NADH-sitokrom b5 redüktaz (b5R) ve sitokrom P450 redüktaz-sitokrom P4502B4 (CYP2B4) sistemi tarafından redüktif aktivasyona uğrama potansiyelini araştırdık. Ayrıca, bazı antioksidanların, IDA ve MC'nin redüktif aktivasyonlarından kaynaklanan DNA'ya hasar verici etkilerine karşı olası koruyucu etkilerini inceledik. Bu amaçlar için, P450R'ı koyun akciğer, sığır karaciğer ve FB-verilmiş tavşan karaciğer mikrozomlarından, b5R'ı sığır karaciğer mikrozomlarından ve CYP2B4'ü FB-verilmiş tavşan karaciğer mikrozomlarından oldukça saf bir halde elde ettik.

Plazmid DNA hasar deneyleri, P450R'ın, IDA'ni etkin olarak DNA'ya hasar veren türlere indirgeyebildiğini gösterdi. Antioksidan enzimler, SOD ve katalaz ve hidroksil radikal süpürücüleri, DMSO ve tiyoüre ile sağlanan etkin korumalar,

IDA'le olan DNA hasar mekanizmasının, IDA'nin aerobik şartlar altında P450R ile redoks döngüsüne girmesi yoluyla ROT'nin oluşumunu gerektirdiğini gösterdi. IDA ve MC'le olan DNA hasarlarının büyüklüğünün artan ilaç veya enzim konsantrasyonlarının yanısıra artan inkübasyon zamanı ile de arttığı bulundu. IDA'nin, yüksek ilaç konsantrasyonlarında, DNA zararını indüklemede MC'den daha etkin olduğu bulundu. Diğer taraftan, b5R kullanılarak yürütülen plasmid DNA deneyleri, b5R'ın, P450R'ın aksine, IDA'ni DNA'ya hasar veren reaktif türlere indirgeyemediğini gösterdi. Aynı zamanda, b5R ve kofaktör NADH varlığında, MC plazmid DNA iplik kırılmalarını güçlükle indükledi. Saflaştırılmış tüm P450R'lar, ilaç-indüklenmiş kofaktör tüketiminin ölçülmesi ile gösterildiği üzere, IDA'ni MC'ninkinden yaklaşık iki kat daha yüksek hızda indirgedi. Bu, IDA'nin MC'den, reaktif metabolitlerin oluşumuna dayanarak, daha potent bir sitotoksik ilaç olabileceğini gösterir. Enzim assaylerinden elde edilen sonuçlar, plazmid DNA deneylerinden elde edilen, MC'nin, P450R'ın aksine, b5R için çok zayıf bir sübstrat olduğu, IDA'nin ise bu enzim için uygun bir sübstrat olmadığı bulgusunu doğruladı. Değişen miktarlarda CYP2B4, P450R ve lipit DLPC ile aerobik ve anaerobik şartlar altında yürütülen rekonstitüsyon deneyleri, rekonstitüe olmuş CYP2B4'ün, yalnız P450R ile katalizlenen IDA ve MC indirgenmelerinde sırasıyla yaklaşık 1.5-kat ve 1.4-katlık bir artış doğurdunu gösterdi. Mevcut sonuçlar, test edilen diyetle alınan antioksidanlar, kuersetin, rutin, naringenin, resveratrol ve troloks içinde, yalnız kuersetinin IDA'le olan DNA hasarını engellemede çok güçlü olduğunu da gösterdi.

Bu sonuçların, P450R'ın kendi başına, terapatik ajan olarak, kanser tedavi stratejilerinde potansiyel kullanımına dair birtakım pratik uygulamaları olabilir. Tümör hücrelerinin saflaştırılmış P450R ile, yeni geliştirilmiş iletim sistemleri yoluyla örneğin lipozomlar, polimerler veya antikorlar kullanılarak selektif olarak hedeflenmesi, biyoredüktif ilaçların tümör hücrelerinde daha fazla redüktif aktivasyonuna neden olur. Dolayısıyla, bu strateji kanser kemoterapisinin etkinliğinin ve spesifisitesinin arttırılmasında yüksek bir potansiyel taşır.

Anahtar kelimeler: İdarubisin, mitomisin C, biyoredüktif aktivasyon, P450 redüktaz, b5 redüktaz, CYP2B4, DNA iplik kırılmaları, NAD(P)H oksidasyonu

Dedicated to my father

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TABLE OF CONTENTS

LIST OF TABLES

TABLE

LIST OF FIGURES

FIGURE

LIST OF ABBREVIATIONS

ε-ACA	ε-Amino caproic acid
ADEPT	Antibody-directed enzyme prodrug therapy
APS	Ammonium persulfate
b5R	NADH-cytochrome b5 reductase
BIS	N,N'-methylene bisacrylamide
BSA	Bovine serum albumin
CO	Carbon monoxide
CYP	Cytochrome P450
DEAE-cellulose	Diethylaminoethyl-cellulose
DLPC	Dilauroyl phosphatidylcholine
DMSO	Dimethylsulfoxide
DTT	DL-dithiothreitol
EDTA	Ethylenediaminetetraacetic acid disodium salt
FAD	Flavin adenine dinucleotide
FB	Fenobarbital
FMN	Flavin adenine mononucleotide
GDEPT	Gene-directed enzyme prodrug therapy
GPAT	Genetic prodrug activation therapy
HEPES	N-2-hydroxyethylpiperazine-N'-2, ethane sulfonic acid
HTP	Hydroxylapatite
IDA	Idarubicin
KPi	Potassium phosphate
MC	Mitomycin C
MFO	Mixed function oxidase
NADH	β-Nicotinamide adenine dinucleotide reduced form
$NADP^+$	β-Nicotinamide adenine dinucleotide phosphate
NADPH	β-Nicotinamide adenine dinucleotide phosphate reduced form
PAGE	Polyacrylamide gel electrophoresis
PB	Phenobarbital
PDEPT	Polymer-directed enzyme prodrug therapy
PELT	Polymer enzyme liposome therapy
PMSF	Phenylmethane sulfonyl fluoride
OC	Open circular
P450R	NADPH-cytochrome P450 reductase
ROS	Reactive oxygen species
ROT	Reaktif oksijen türleri
SC	Supercoiled
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
TEMED	N, N, N', N'-Tetramethylethylene diamine
VDEPT	Virus-directed enzyme prodrug therapy

CHAPTER I

INTRODUCTION

Idarubicin and mitomycin C are clinically effective quinone-containing antineoplastic agents currently used alone or in combination chemotherapy regimens to treat several types of human cancers. Idarubicin is effective against breast cancer and some haematological malignancies including acute myelogenous leukemia, multiple myeloma and non-Hodgkin's lymphoma, while mitomycin C is commonly used in the treatment of cancers of the bladder, breast, cervix, stomach, head and neck, lung and pancreas. The quinone-containing anticancer drugs have the potential to undergo bioreductive activation by a number of oxidoreductases to free radicals and thus exert their cytotoxic actions. The role of bioreductive activation in the cytotoxic action mechanisms of quinone-containing anticancer drugs as well as their other features will be discussed in detail in the following sections.

1.1 Drugs Used in Cancer Chemotherapy

Cancer is a disease of worldwide importance. Its incidence rate is rising and it is the second leading cause of death in the industrialized countries. In developing countries, a similar tendency can also be observed (Eckhardt, 2002). Various factors like the gradual improvement in the life expectancy, environmental changes and also the socio-economic situation around the world affect this tendency. For example, it is now well recognized that 1 in 8 women in the industrialized world will develop breast cancer in their lifetime and it is commonly accepted that a strong correlation exists between age and diet and the incidence of colon cancer. Among the three

established strategies currently used for the treatment of cancer, surgery, irradiation and chemotherapy, the last one remains an important therapeutic option in the clinic (Novotny and Szekeres, 2003). However, the major problem that the patients encounter during the cancer chemotherapy is the unwanted severe toxic side effects of anticancer drugs towards the normal body cells. These side effects of chemotherapy might be so severe that the patient may suffer from even life threatening situations (i.e. infections) which can lead to the limitation of the quality of his/her life. Thus, a major problem of the anticancer chemotherapy today is the selective killing of the tumor cells without causing any or with only minimal toxicity to normal healthy organs and cells.

Since the well-known side effects of cytotoxic agents on normal cells limit their application in chemotherapy, much interest has been focused on identification of the specific drug targets in tumor cells, the function of which is essential to tumor but not to normal organs and tissues. Therefore, intensive studies are carried out to find new and better mechanism-based drugs that would specifically kill malignant tumor cells. This strategy theoretically gives a higher selectivity to tumor cells than seen for classical cytotoxic drugs and results in elimination or reducing of the damage to normal healthy organs and cells thereby increases the overall success of the cancer chemotherapy (Novotny and Szekeres, 2003; Bradbury, 2007). Consequently, in order to increase the efficacy of cancer chemotherapy, there is an urgent need for finding less toxic and more specific compounds possessing broader spectrum of antitumor activity as well as finding new targets for cancer chemotherapeutic agents with novel mechanisms of action (Garrett and Workman, 1999; Verweij and de Jonge, 2000; Eckhardt, 2002; Novotny and Szekeres, 2003; Colevas *et al.*, 2006). Elucidating the still unknown cytotoxic action mechanisms of the conventionally used anticancer agents together with a better understanding of the molecular events accompanying malignant transformation of a normal cell is, therefore, of crucial importance in the design and development of new anticancer drugs.

Traditionally, anticancer drugs were classified as chemotherapy, hormonal therapy and immunotherapy. Among these, chemotherapy contained a number of different groups of drugs defined by both their chemical structure and mechanisms of action: alkylating agents, antibiotics, antimetabolites, topoisomerase I and II inhibitors, mitosis inhibitors, platinum compounds and others. However, a large number of anticancer drugs have appeared in clinic in the last years, many of which cannot be placed in any of the existing classes in this classification (Wu, 2006). Therefore, in order to meet this requirement, new classification systems based on either therapeutic targets or cell biological mechanisms were proposed in recent years by Espinosa (2003) and Wu (2006), respectively. In the classification system proposed by Wu (2006), chemotherapeutic agents were divided into cytotoxic drugs and modifiers, which could regulate the interaction of tumor, patient and drug (Table 1.1). In his classification system, the modifiers were further subdivided into three groups: cell biological modifier, biological response modifier and biochemical modulator. Cell biological modifier can reverse the abnormal biological behavior of tumor cells, biological response modifier can regulate the host response of carcinogenesis, and biochemical modulator enhances the chemosensitivity of cytotoxic drug via affecting the metabolic pathway of it or reduces host cell cytotoxicity caused by these agents (Table 1.1). In the clinic use, cytotoxic drugs and cell biological modifiers are usually used as primary drugs, whereas biological response modifiers and biochemical modulators have found utility mainly as adjuvant drugs in combination cancer chemotherapy (Wu, 2006).

1.2 Quinone-Containing Bioreductive Anticancer Drugs

Quinone-containing antitumor agents represent one of the largest classes of cytotoxic drugs which are referred to as bioreductive anticancer agents. These drugs have attracted significant scientific interest as anticancer drugs since late 1950s. They are among the most widely used drugs to treat human cancer. For example, the anthracycline quinone antibiotics are among the most frequently used anticancer drugs ever developed. Quinone-based anticancer drugs in clinical practice are

Table 1.1 Classification of anticancer drugs based on cell biological mechanisms (taken from Wu, 2006)

Cytotoxic Drug	
Non-specific cytotoxic drug	Alkylating Agents
	Antibiotics
	Topoisomerase inhibitors
	Mitosis inhibitors
	Platinum Compounds
	Others
Tumor tissue specific cytotoxic drug	
Molecular targeted cytotoxic drug	Cytotoxic monoclonal antibody
	Antibody guided therapy
	Hormone guided chemotherapy
Cell Biological Modifier	
Cytostatic agent	Antagonist of growth factor
	Growth signal transduction inhibitor
	Cell cycle active drugs
Differentiating agent	
Apoptosis inducing agent	
Biological Response Modifier	
Immune modifier	Cytokines
	Active specific immunotherapy
	Adoptive immunotherapy
Incretion modifier	Hormonal supplementary therapy
	Hormonal antagonist
Microenvironment modifier	
Biochemical Modulator	
Chemosensitizer	
Chemoprotectant	

commonly used in chemotherapy regimens along with combination with other groups of drugs generally having a different biochemical mechanism of action to effectively treat several types of malignancies. Besides anthracycline antibiotics such quinone-containing anticancer agents include mitomycin C, aziridinylbenzoquinones, benzoquinone mustard and many others that are used in clinic (Begleiter, 2000; Gutierrez, 2000; Beall and Winski, 2000; Hargreaves *et al.*, 2000; Asche, 2005).

Quinone-containing anticancer drugs have the potential to undergo bioreductive activation by one or two-electron reductases to reactive species, and thus exert their toxic effects. This bioreduction results in the formation of either the semiquinone form or the hydroquinone form of the anticancer drug. In 1963, it was shown by Nishibayashi *et al*. that a microsomal flavoprotein enzyme, NADPH-cytochrome P450 reductase, is capable of catalyzing the one-electron reduction of quinones and later on it was demonstrated by Iyanagi and Yamazaki (1969) that this reduction occurs via a single-electron transfer through the flavoprotein producing a semiquinone product (Bachur *et al.*, 1979). Studies in 1960's also demonstrated that cellular or chemical reduction of quinone-containing anticancer agents mitomycin C and streptonigrin was essential for the activation of these compounds to become cytotoxic and bind DNA (Schwartz *et al*., 1963; Iyer and Szybalski, 1964; White and White, 1966; White and White, 1968; referred by Bachur *et al*., 1979). During this period and the next decade, the studies carried out with some quinone-containing anticancer drugs have suggested that superoxide radical, peroxide and hydroxyl radical formed via redox cycling in the aerobic environment during one-electron reduction of these agents may be responsible for their toxic actions (White and White, 1968; Gregory and Fridovitch, 1973; Handa and Sato, 1975; Cone *et al*., 1976; Tomasz, 1976; Goodman and Hochstein, 1977; referred by Bachur *et al*., 1979). On the other hand, Bachur *et al.* in 1979 proposed that it was the free radical forms of the anticancer drugs that bind to DNA and thereby exerting their cytotoxic effects upon reductive activation by NADPH-cytochrome P450 reductase and other quinone reductases in the cell. The cell damage and death caused by these agents were attributed to the production of site-specific free radicals which provides the mechanism for selective damage of essential cellular macromolecules. It was

proposed that the semiquinone forms of the quinone anticancer agents, when brought into close proximity of DNA, may either directly bind to it or generate reactive oxygen species within the DNA helix which could damage the DNA. It was also suggested that these free radicals may bind to other critical macromolecules in the cell such as RNA and protein (Figure 1.1) (Bachur *et al.*, 1979).

During the following years, a considerable amount of studies have been carried out on quinone-containing anticancer agents in order to elucidate the underlying molecular mechanisms for the cytotoxic actions of these drugs. However, although there are general and well-established mechanisms for quinone toxicity, the exact contribution of the quinone moiety to the cytotoxic effect of these drugs remains often questionable (Asche, 2005). It is apparent that no single mechanism is able to fully explain all of the cytotoxic effects and several mechanisms may exist (Butler and Hoey, 1987).

1.2.1 One- and Two-Electron Reduction of Quinone-Containing Anticancer Drugs; Redox Cycling and Oxidative Stress

In the process of redox cycling of quinone anticancer drugs, the first step is the one-electron reduction catalyzed by a number of flavoenzymes such as NADPH-cytochrome P450 reductase, NADH-cytochrome b5 reductase, xanthine oxidase and endothelial nitric oxide synthase. Bioreduction by these enzymes leads to the formation of semiquinone free radical which is a highly reactive intermediate form under aerobic conditions (Figure 1.1). It is rapidly back oxidized by molecular oxygen to the parent compound, a process that results in the concomitant production of superoxide radical anion ($O_2^{\cdot-}$) (Gutierrez, 2000). The $O_2^{\cdot-}$ formed in the cell can undergo a variety of reactions. It dismutates to hydrogen peroxide (H_2O_2) either spontaneously or by the action of superoxide dismutase (SOD); $2O_2^{\cdot-} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$. Thus, the formation of $O_2^{\cdot-}$ is accompanied by the formation of significant amounts of H_2O_2. H_2O_2 thus formed can be removed by antioxidant defense systems of the cell, catalase or glutathione peroxidase (Kappus, 1986).

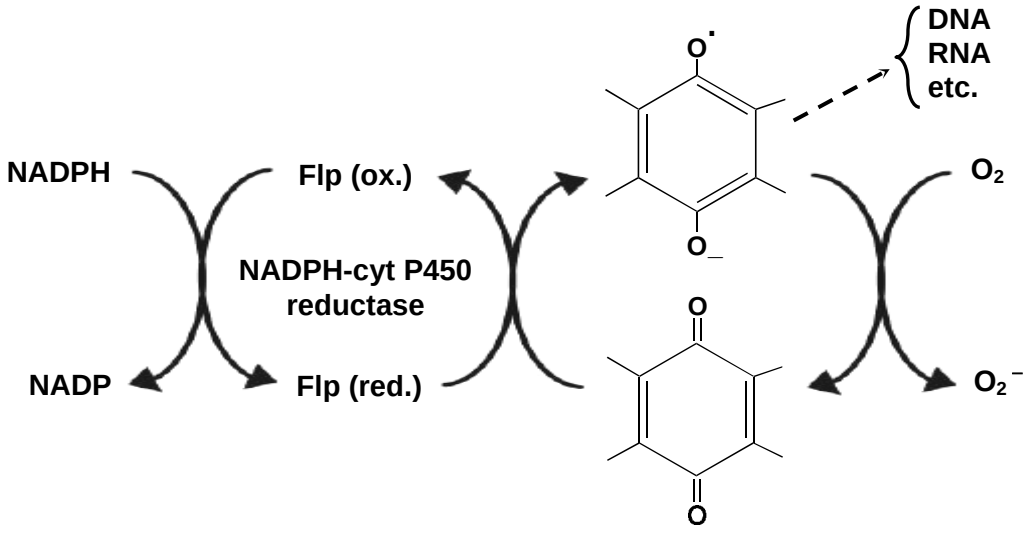


Figure 1.1 Proposed scheme for the electron transfer sequence and production of semiquinone intermediates. Flp, flavoprotein (taken from Bachur *et al.*, 1979).

Hydrogen peroxide and superoxide anion radical are relatively unreactive and react with only very few molecules in the cell. However, in the presence of transition metal ions, hydrogen peroxide rapidly decomposes to a highly reactive hydroxyl radical and hydroxide anion, a process known as the Haber-Weiss or Fenton reaction. The Fenton reaction (Figure 1.2) is discovered almost 120 years ago, but the precise mechanism of it is still unknown and the intermediates formed during the reaction are still unidentified. Although other transition metal ions can also participate in hydroxyl radical formation *in vitro*, studies *in vivo* have largely concentrated on ferrous and cuprous ions in which superoxide catalyzes the reduction of oxidized metal ion thusly perpetuating the catalytic cycle (Figure 1.3) (Kovacic and Osuna, 2000).

$$Fe(II) + H_2O_2 \longrightarrow Fe(III) + OH^{\bullet} + HO^{-}$$

Figure 1.2 The Fenton reaction

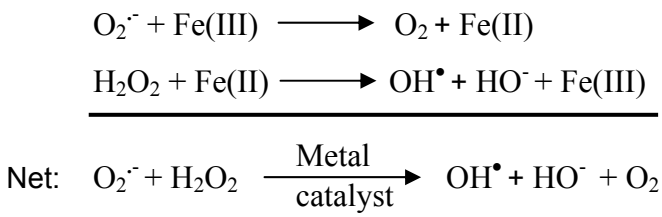


Figure 1.3 Superoxide assisted Fenton reaction

Thus, the futile redox cycling of quinone-containing anticancer drugs and activation of molecular oxygen result in the formation of reactive oxygen species (ROS) which are commonly involved in the formation of "oxidative stress" in the cell. The term "oxidative stress" is defined as a disruption of the prooxidant-antioxidant balance of the cell in favor of the former, leading to potential damage (Sies, 1991). The hydroxyl radical is the most reactive, unstable and powerful oxygen metabolite among other reactive oxygen species and may contribute to the formation of serious molecular damage in living systems such as oxidative DNA and protein damage and membrane lipid peroxidation. Lipid peroxidation causes structural membrane damage which results in the disruption of cellular integrity and the release of cell components. It can also release toxic reaction products which can cause DNA damage. The oxidation of proteins by oxygen radicals can generate a range of stable as well as reactive products and may result in conformational changes and enzyme inactivation. Hydroxyl radicals can also cause strand breaks, base modification and deoxyribose fragmentation in DNA which results in cytotoxicity, carcinogenicity and mutagenicity (Kappus, 1986; Halliwell and Aruoma, 1991; Wiseman and Halliwell, 1996; Kehrer, 2000).

Unlike one-electron reduction, two-electron reduction of a quinone anticancer agent to its corresponding hydroquinone form results in a more complicated cycle for reduction and oxidation of that drug (Gutierrez, 2000). The two-electron reduction of quinone anticancer drugs is catalyzed by flavoprotein DT-diaphorase or other two-electron reductases which reduce quinones to their corresponding hydroquinones

(diols) (Figure 1.4). This reductive pathway is generally regarded as a detoxification pathway as it is generally not associated with oxidative stress unlike one-electron reduction reactions of quinones. However, there are exceptions and some hydroquinones, such as that of diaziquone, can lead to the induction of oxidative stress in the cell. Diaziquone is a diaziridinylbenzoquinone antitumor drug that can be bioactivated by DT-diaphorase. The two-electron reduction of diaziquone results in the formation of the corresponding hydroquinone which can then be auto-oxidized one electron at a time in the presence of molecular oxygen. This process leads to the formation of semiquinone radical and toxic oxygen radicals, thereby inducing oxidative stress (Fisher and Gutierrez, 1991 referred by Rooseboom *et al.*, 2004). The detoxifying enzyme DT-diaphorase, paradoxically, also catalyzes the bioactivation of chemotherapeutic quinones such as mitomycin C, orthonaphtoquinones and aziridinylbenzoquinones via two-electron reduction to cytotoxic species which can bind DNA (Danson *et al.*, 2004).

All quinone-containing antitumor agents undergo redox cycling at different rates (Gutierrez, 2000). Reactive oxygen species formed via redox cycling are generally thought to be involved in undesired cytotoxic properties of quinone-containing anticancer drugs. For example, it is generally accepted that induction of oxidative stress is the main causative factor for the cardiotoxicity of doxorubicin, an anthracycline quinone-containing anticancer drug. Unfortunately, the undesired adverse side effects caused by this class of drugs in particular cardiotoxicity limit their clinical usefulness in many cases (Kovacic and Osuna, 2000). Pulmonary toxicity associated with mitomycin C is also believed to be mediated by induction of oxidative stress and lipid peroxidation (Doll *et al*., 1985).

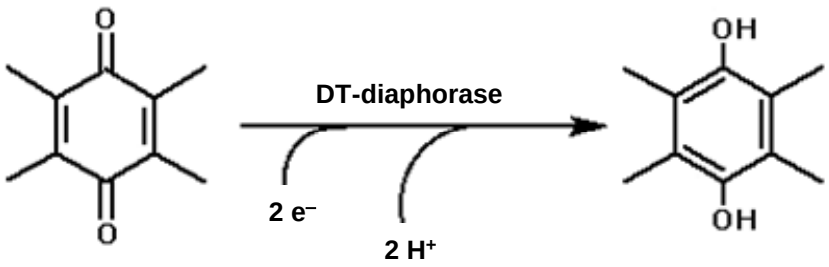


Figure 1.4 The two-electron reduction of quinones by DT-diaphorase

1.2.2 Bioreductive Alkylation and the Problem of Tumor Hypoxia

Tumor hypoxia is a very important aspect of cancer chemotherapy due to its prognostic importance. Tumor hypoxia is the situation where tumor cells have been deprived of oxygen and it is a therapeutic problem as it makes solid tumors resistant to ionizing radiation and some types of chemotherapy. The failure of radiotherapy in killing the hypoxic tumor cells is largely because of DNA damage produced by ionizing radiation can be repaired by hydrogen donation from cellular nonprotein sulfhydryls. Whereas, in the well-oxygenated aerobic cells, oxygen reacts extremely rapidly with the single electron of the free radical, formed on DNA by ionizing radiation, and converts it into a permanent damage which is lethal to the cell (see the reviews by Brown, 1999; Brown and Wilson, 2004; Ahn and Brown, 2007). Tumor hypoxia is also a problem for chemotherapy. Because the hypoxic tumor cells which are usually far away from nutritive blood vessels do not receive adequate anticancer drug concentrations. In addition, because hypoxic tumor cells are nonproliferating cells, the chemotherapeutic agents that target rapidly dividing cells may be less effective on this population of cells (Ahn and Brown, 2007). Furthermore, hypoxia has been shown to promote tumor progression through inducing gene amplification that results in resistance to common antineoplastic agents (Sartorelli, 1988). This is mainly the result of overexpression of hypoxia-inducible factor 1 (HIF-1) transcription factor which stimulates the increased expression of a large number of

genes involved in cellular metabolism and survival under hypoxic conditions (Ahn and Brown, 2007).

One of the strategies to overcome the problem of hypoxic tumor cells in cancer therapy is selective targeting of them by using bioreductive anticancer prodrugs. Hypoxic cells, therefore, have been a major target by many, but not all bioreductive antitumor drugs. Among the different classes of bioreductive anticancer prodrugs that can act as hypoxic cytotoxins, quinone-containing ones such as mitomycin C, porfiromycin and EO9 (a synthetic analogue of mitomycin C) occupy an important place (Rauth *et al.*, 1998; Ahn and Brown, 2007; McKeown *et al*., 2007). Numerous attempts have been made to exploit the enzymatic reduction of quinones in the design and synthesis of more effective bioreductive antitumor agents showing greater degree of preferential toxicity towards hypoxic cells.

The concept of bioreductive activation was first developed by Sartorelli and his coworkers (Lin *et al*., 1972). Bioreductive alkylating agents refer to the drugs which generate electrophilic species upon reductive activation which then bind covalently to cellular macromolecules. This concept was based on the expectation that the oxygen deficiency of hypoxic cells leads to an environment which is conducive to reductive reactions (Sartorelli, 1988 referred by Beall and Winski, 2000). Under hypoxic conditions, one-electron reduction of bioreductive anticancer prodrugs results in the formation of one-electron reduced intermediates (in the case of quinones, it is semiquinone intermediate), which may themselves be cytotoxic, or they are further reduced to other cytotoxic species that kill the cell. The selective cytotoxicity of these drugs to hypoxic tumor cells is usually a result of futile redox cycling under aerobic conditions in which molecular oxygen reacts rapidly with the one-electron reduced intermediate, thereby regenerating the non-toxic or less toxic parent drug and producing superoxide anion radical as byproduct (Figure 1.5) (Ahn and Brown, 2007). Reactive oxygen species generated by redox cycling leads to DNA strand breaks, lipid peroxidation and other oxidative damage, but cellular defense mechanisms like superoxide dismutase, glutathione peroxidase and catalase may limit their toxicity. Therefore, differential cytotoxicity of these agents to

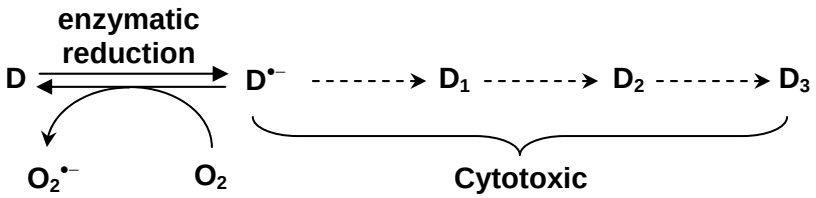


Figure 1.5 A scheme of activation for bioreductive prodrug (D) by enzymatic reduction. The reduced intermediate of the bioreductive prodrug ($D^{\bullet-}$) can be back-oxidized by the presence of oxygen (O_2) with the formation of superoxide anion ($O_2^{\bullet-}$). $D^{\bullet-}$ may itself be cytotoxic or be further reduced (D_1, D_2, or D_3) to produce cytotoxins killing hypoxic cells (taken from Ahn and Brown, 2007).

hypoxic cells results from generation of more toxic intermediates than the oxygen radicals under hypoxic conditions upon bioreductive activation (Beall and Winski, 2000; Ahn and Brown, 2007).

One-electron reduction is not the only route for hypoxia-selective quinone-containing bioreductive anticancer drugs. Mitomycin C and other agents within this class such as RH1 (a novel diaziridinylbenzoquinone) and apaziquone (EO9; a synthetic analogue of mitomycin C) can also be reduced to their corresponding hydroquinone forms by two-electron reducing enzyme DT-diaphorase. However, since two-electron reduction by DT-diaphorase is an oxygen-independent pathway, bioactivation can also lead to formation of toxic drug species under aerobic conditions that can severely compromise hypoxic selectivity of such drugs and lead to some unwanted host tissue toxicity (Mckeown *et al.,* 2007). DT-diaphorase is overexpressed in a range of tumors, and its level in these tumors is markedly elevated. This property of the enzyme makes it an attractive target for enzyme-directed bioreductive drug design. This enzyme-directed approach constitutes another strategy different from hypoxia-based approach for achieving selective toxicity through bioreductive activation. By this way, bioreductive drug activation becomes independent of tumor oxygenation status, and tumors rich in this enzyme

are targeted by bioreductive prodrugs which are good substrates for DT-diaphorase enzyme (see the reviews by Workman, 1994; Beall and Winski, 2000; Seddon *et al*., 2004; McKeown *et al.,* 2007). Therefore, knowing the oxygenation status together with type and levels of the enzymes necessary for reduction in individual tumors are very important in bioreductive anticancer prodrug therapy.

1.3 Mitomycin C

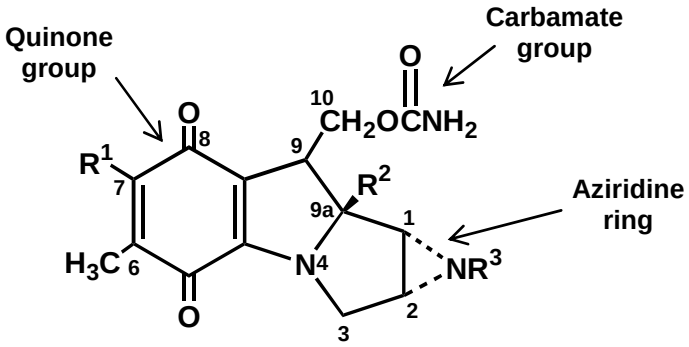
Mitomycins are a group of closely related antibiotics that have potent antitumor activity against a variety of human tumors. The mitomycins have attracted significant scientific interest because of their complex chemical structure and proven clinical utility. The extensive study of molecular pharmacology and chemistry of mitomycins has provided a basis for the design and synthesis of highly specific and effective bioreductive anticancer drugs (Lown, 1983; Powis, 1987; Spanswick *et al.*, 1998; Begleiter, 2000; Beall and Winski, 2000; Bradner, 2001).

1.3.1 Origin and Chemical Structure of Mitomycin C

The mitomycins are a group of potent antibiotics that were first isolated in 1956 from the fermentation cultures of the microorganisms *Streptomyces caespitosis* by Hata and his coworkers (Hata *et al.*, 1956). Two compounds were isolated from this strain and they were designated as mitomycins A and B. It was shown that they were potent antibacterial and antitumor agents but, were also very toxic in mice. In 1958, Wakaki and his collaborators isolated a third mitomycin designated as mitomycin C from *S. caespitosis.* This mitomycin was found to have superior antitumor activity and less toxicity as compared to other mitomycins (Wakaki *et al*., 1958). Two years later, an N-methyl derivative of mitomycin C, called as porfiromycin, was isolated from a different strain, *S.ardus* (DeBoer *et al*., 1960; see the review by Powis, 1987). All these mitomycins were found to be effective against both gram positive and gram negative bacteria (Szybalski and Iyer, 1967). However,

it was the antitumor properties of these compounds that aroused significant scientific interest (see the review by Lown, 1983). Among these mitomycins, it was found that only mitomycin C and porfiromycin have appreciable antitumor activity (see the review by Begleiter, 2000). Clinical trials showed that mitomycin C was an effective antitumor agent and it was started to be used clinically in Japan in the early 1960s. However, this agent was not approved for standard clinical use in North America until 1974. Clinical trials of porfiromycin carried out in 1970s, on the other hand, showed that it was less active than mitomycin C and it was not marketed commercially (Lown, 1983; Doll *et al*., 1985; Powis, 1987; Begleiter, 2000). Furthermore, in a recently completed Phase III study, porfiromycin has been found to be inferior to mitomycin C as an adjunct to radiation therapy in the management of squamous cell cancer of the head and neck despite its promising preclinical data and an acceptable toxicity profile observed in a previous Phase I study (Haffty *et al.*, 2005). Although mitomycin C is an active anticancer drug, its use in the clinic has been severely restricted by its toxicity and resistance (Begleiter, 2000). Therefore, a large number of synthetic derivatives were prepared in order to find better drugs with improved pharmacological profiles. However, several of them (such as KW-2149, BMS-181174, KW-2083 and EO9) achieved to enter early clinical trials, but none have been approved for use in clinic (Begleiter, 2000; Beall and Winski, 2000).

The chemical structure of all the mitomycins was revealed in 1962 by Webb *et al.* (1962) (referred by Powis, 1987). This study showed that mitomycins have a unique chemical structure containing three recognized parts necessary for antitumor activity; aziridine, quinone and carbamate groups (Figure 1.6) (see the review by Lown, 1983). These groups are arranged around a pyrrolo [1,2-a]indole nucleus. Mitomycins were the first known naturally occurring compounds containing an aziridine ring. The absolute configuration of mitomycins was also studied in detail by x-ray crystallographic studies (Tulinsky, 1962) and later on by an independent analysis (Stevens *et al*., 1965). Structure-activity studies have shown the intact mitosane structure is essential for the activity of mitomycins. The analogues with good water solubility, low lipophilicity and low quinone reduction potential, which favor enzymatic reduction, showed enhanced antitumor activities (Powis, 1987).



Antibiotic	R^1	R^2	R^3
Mitomycin A	CH_3O	OCH_3	H
Mitomycin B	CH_3O	OH	CH_3
Mitomycin C	H_2N	OCH_3	H
Porfiromycin	H_2N	OCH_3	CH_3

Figure 1.6 Structures of the principal members of the mitosane group of antitumor antibiotics (taken from Lown, 1983)

1.3.2 Clinical Activity of Mitomycin C

It has been well demonstrated that mitomycin C as a single agent has shown activity against a number of neoplastic diseases including bladder, breast, cervix, gastric, head and neck, lung, colon and pancreatic cancers (Powis, 1987; Bradner, 2001). Response rates to mitomycin C are usually low and of short duration. However, the activity of mitomycin C can range from marginal to substantial. While mitomycin C is possibly the most active cytotoxic drug available for local intravesical treatment of early stage disease of bladder cancer, it has minimal activity in colon cancer and probably has no longer therapeutic use in the treatment of this disease. Although mitomycin C has been used for a long time in the treatment of cervical, gastric and pancreatic cancer and has been quite active in these cancers, it is being displaced by other drugs. Mitomycin C is active in breast, head and neck and non-small cell lung cancer, but as the new chemotherapy regimens are developed, its

role in the treatment of these cancers needs to be redefined. This is critical, because the development of new therapies and chemotherapy regimens makes it necessary to redefine the role and use of older drugs like mitomycin C in the treatment of a particular cancer (Bradner, 2001).

The common toxicities of mitomycin C include anorexia, vomiting, nausea, diarrhea and myelosupression (Doll, 1985). Hematological toxicity of mitomycin C is usually dose related and cumulative, therefore, great caution must be given if it will be used with other myelotoxic drugs (Bradner, 2001). Anemia is a common complication of mitomycin C administration, but it is less severe than thrombocytopenia or leukopenia. The usual dose-limiting effect of mitomycin therapy is the myelosupression. During mitomycin C administration if drug extravasation occurs, local tissue necrosis and ulceration, which sometimes requires skin grafting, may occur. Another side-effect of mitomycin C therapy is the pulmonary toxicity which does not appear to be dose related and sometimes followed by a lethal renal failure syndrome (Doll, 1985).

1.3.3 Mechanism of Bioreductive Activation of Mitomycin C

The molecular mechanisms underlying the cytotoxic properties of mitomycin C is unprecedented. Studies carried out in 1960s about the molecular pharmacology of this drug have shown that mitomycin C and other members of this class were able to cross-link the complementary strands of DNA (Iyer and Szybalski, 1963). Mitomycins are the only antibiotics that work in this way except carzinophillin, the structure of which has not been fully characterized (Armstrong *et al.,* 1992). It was found that mitomycins can also alkylate the DNA monofunctionally through attachment of the drug molecule to only one strand of DNA which accompanies the cross-linking (Szybalski and Iyer, 1964; Weissbach and Lisio, 1965; Tomasz *et al*., 1974; Lown *et al*., 1976). These studies have demonstrated that the primary reason for the cytotoxic effects of mitomycins is the inhibition of DNA replication, and mitomycin C-induced cross-links are fundamentally responsible for this inhibition. It

was shown that a single cross-link per genome was sufficient to cause death of a bacterial cell (Szybalski and Iyer, 1964). There are several hypotheses for the high cytotoxicity of cross-link type DNA damage. It has been proposed that during replication of DNA, cross-links present at the replication fork generate more severe impediments to replication when compared with a comparable monofunctional drug-DNA adduct. Alternatively, the reason may be that the cellular repair mechanisms for the cross-linked DNA adduct is less efficient as compared with that for monofunctional adducts (see the reviews by Tomasz, 1995; Tomasz and Palom, 1997).

The most important feature of the molecular mechanism action of mitomycin C is that this drug exits as a prodrug, and it requires reduction of the quinone moiety in order to exert its DNA cross-linking and alkylating activities (Iyer and Szybalski, 1964). This property of the drug gives it the potential for the preferential killing of hypoxic tumor cells (see the review by Seow *et al.*, 2004).

The mechanism of bioreductive activation of mitomycin C is shown in Figure 1.7. Mitomycin C can be reduced by cellular reductases through either one- or two-electron reduction to produce mitomycin C semiquinone anion radical or mitomycin C hydroquinone, respectively. Both pathways have been proposed in the literature for the reductive bioactivation of mitomycin C and alkylation of DNA (see the reviews by Powis, 1987 and 1989; Seow *et al.*, 2004). Mitomycin C semiquinone anion radical, under aerobic conditions, is a very short-lived species having a calculated $T_{1/2}$ of less than 0.1 ms at 37 °C (Penketh *et al.*, 2001 referred by Seow *et al.*, 2004). Thus, highly reactive semiquinone radical under aerobic conditions enters into a redox cycle with molecular oxygen at near diffusion controlled rates ($k = 10^9\ M^{-1}\ s^{-1}$) which results in the regeneration of the non-toxic parent drug and concomitant production of superoxide radicals. Therefore, due to rapid redox cycling of mitomycin C semiquinone radical under aerobic conditions, no significant alkylation of DNA by semiquinone radical is expected to occur. However, under hypoxic conditions, the mitomycin C semiquinone radical becomes much more stable and can participate in several nucleophilic reactions with first-order kinetics, with an

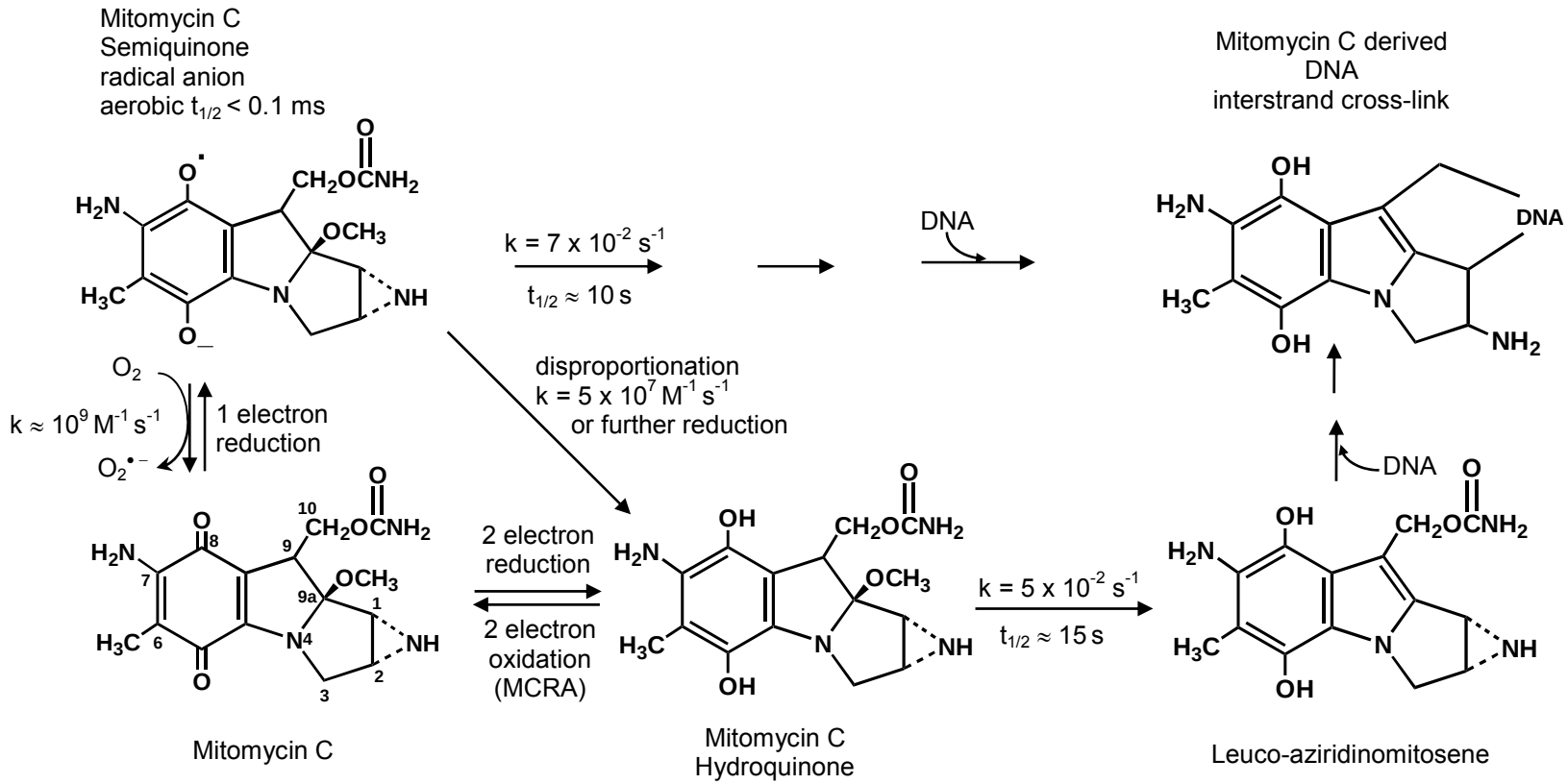


Figure 1.7 Reduction/oxidation pathways of mitomycin C (taken from Seow *et al*., 2004)

estimated rate constant of k = 7 x 10^{-2} s^{-1} (Nagata and Matsuyama, 1970 referred by Seow *et al.*, 2004). Mitomycin C semiquinone radical can alkylate DNA through a proposed mechanism in which one-electron reduction of mitomycin to mitomycin C semiquinone radical is followed by the loss of the C-9a methoxy group to give an indole semiquinone radical which in turn can undergo a nucleophilic attack at the C-1 position to form a monofunctional mitomycin C adduct (Pan *et al*., 1984; Sartorelli, 1986). Monofunctional *cis* or *trans* mitomycin C adducts of DNA can also undergo secondary enzymatic reduction to give a semiquinone radical followed by a second nucleophilic attack by the DNA, presumably at the C-10 position with loss of carbamate side chain which results in the formation of cross-linked DNA adducts (Figure 1.7) (Pan *et al*., 1984; for details see the reviews by Powis, 1987 and 1989).

In addition, mitomycin semiquinone radical could also undergo disproportionation to the hydroquinone and this pathway is more likely to occur based on the calculated kinetic parameters (k = 5 x 10^{7} M^{-1} s^{-1}) (Hoey *et al*., 1988 referred by Seow *et al.*, 2004). Alternatively, the semiquinone can be further reduced by other biomolecules to produce mitomycin C hydroquinone. Under aerobic conditions, mitomycin C hydroquinone is a relatively stable species with respect to semiquinone radical and its $T_{1/2}$ was estimated as 15 s at 37 °C and pH 7.4 *in vitro* (Penketh *et al.,* 2001 referred by Seow *et al.*, 2004). Like mitomycin C semiquinone, the hydroquinone can also back oxidized to non-toxic parent drug, mitomycin C. In *Streptomyces lavandulae* which produces mitomycin C and is resistant to this prodrug, an oxygen-requiring enzyme known as mitomycin C resistance protein A (MCRA) is responsible for this back oxidation (Figure 1.7) (Seow *et al.*, 2004).

Mitomycin C can also be activated by two-electron reducing enzymes through direct production of hydroquinone form (Figure 1.7). The two-electron addition to mitomycin C results in resonance in the quinone ring structure which favors the loss of methoxy group to produce the leuco-aziridinomitosene followed by the opening of the aziridine ring structure and the leaving of carbamyl group resulting in the formation of DNA-reactive sites at the C-1 and C-10 positions of mitomycin C. Thus, mitomycin C can act as a bifunctional cross-linking agent

through the formation of C-1 and C-10 reactive sites (Seow *et al.*, 2004). This activation pathway is observed at more acidic pHs, however, at neutral pHs an alternative pathway is activated from leuco-aziridinomitosene which results in the formation of mostly monofunctional minor groove DNA adducts (Tomasz and Palom, 1997; Cummings *et al.*, 1998). In the formation of mitomycin C cross-linked DNA, the first, monoalkylation step (between C-1 position of mitomycin C and 2-NH_2 group of guanine) is selective for guanine nucleosides in the sequence 5'-C_pG-3', while the second step (between C-10 position of mitomycin C and 2-NH_2 group of guanine) is absolutely specific for the same sequence in the opposite strand (Tomasz, 1995; Tomasz and Palom, 1997).

The hypoxic tumor selectivity of mitomycin C comes from the fact that under aerobic conditions, one-electron reducing enzymes participate in a futile redox cycle in which mitomycin C semiquinone radical formed upon one-electron reduction of mitomycin C is rapidly back-oxidized to its parent compound, mitomycin C, at near diffusion-limited rates. Therefore, although ROS are formed under aerobic conditions, the two-electron reducing pathway is expected primarily to be involved in the generation of the toxic mitomycin C hydroquinone species. On the contrary, both mitomycin C hydroquinone and mitomycin C semiquinone species by undergoing a series of spontaneous rearrangements are responsible for the observed cell kill under hypoxic conditions through alkylation of DNA. Thus, under hypoxic conditions, one- and two-electron reducing pathways combine to enhance the ability of mitomycin C to alkylate DNA by producing an increase in the levels of toxic mitomycin C species. It is this property that allows the mitomycin C to become differentially cytotoxic to hypoxic tumor cells (Seow *et al.*, 2004).

1.3.4 Enzymes Involved in Bioreductive Activation of Mitomycin C

A growing list of enzymes has been shown to catalyze the reduction of mitomycin C *in vitro*. These reductive enzymes that require a reduced pyridine nucleotide cofactor include NADPH-cytochrome P450 oxidoreductase (EC 1.6.2.4)

(Pan *et al*., 1984; Tomasz *et al*., 1986), NADH-cytochrome b5 oxidoreductase (EC 1.6.2.2) (Hodnick and Sartorelli, 1993), DT-diaphorase (EC 1.6.99.2) (Siegel *et al*., 1990), xanthine:oxygen oxidoreductase (EC 1.17.3.2) (Pan *et al*., 1984; Tomasz *et al.,* 1986), xanthine:NAD^+ oxidoreductase (EC 1.17.1.4) (Gustafson and Pritsos, 1992), nitric oxide synthase (EC 1.14.13.39) (Jiang *et al.*, 2000) and NADPH-ferrodoxin reductase (EC 1.18.1.2) (Jiang *et al*., 2001). Several studies have shown that cytochrome P450 (EC 1.14.14.1) is also involved in one-electron reduction of mitomycin C (Kennedy *et al.*, 1982; Vromans *et al*., 1990; Goeptar *et al.,* 1994). However, among these enzymes only NADPH:cytochrome P450 oxidoreductase, NADH-cytochrome b5 oxidoreductase, DT-diaphorase, xanthine:oxygen oxidoreductase, xanthine:NAD^+ oxidoreductase have been studied extensively *in vivo*. Only two of these enzymes, DT-diaphorase and xanthine dehydrogenase (xanthine:NAD^+ oxidoreductase) are capable of catalyzing two-electron reduction of mitomycin C, while the others catalyze the one-electron reduction. It appears that NADPH-cytochrome P450 reductase, NADH-cytochrome b5 reductase and DT-diaphorase are the major enzymes responsible for reductive activation of mitomycin C in tumor cells (Cummings *et al*., 1998; Seow *et al.*, 2004).

1.3.4.1 The Role of DT-Diaphorase in the Bioreductive Activation of Mitomycin C

The enzyme DT-diaphorase (DTD, NAD(P)H:quinone oxidoreductase, EC 1.6.99.2) is a homodimeric flavoprotein with each unit having a molecular weight of 32,000 kD and contains a flavin adenine dinucleotide (FAD) as prosthetic group. It catalyzes the two-electron reduction of several substrates including quinones and aromatic nitro compounds. It is a predominantly cytosolic enzyme and more than 90% of it is found in the cytoplasm. However, some studies have shown that DT-diaphorase is present in the endoplasmic reticulum, mitochondria, Golgi body and nucleus (see the review by Danson *et al*., 2004). DT-diaphorase is characterized by its capacity to use either NADH or NADPH as reducing cofactors and its inhibition by dicumarol (Beall and Winski, 2000). In addition to its role in two-electron

reduction of chemotherapeutic quinones, DT-diaphorase is also involved in the reduction of dietary and environmental quinones. It has multiple physiological roles in the cell, but many of which are understood poorly. It can recycle the membrane antioxidants ubiquinone and vitamin E, and plays important roles in vitamin K, glucose and fatty acid metabolism (Danson *et al.*, 2004). DT-diaphorase is generally categorized as a Phase II detoxification enzyme since it can protect the cell from the cytotoxic effects of a broad range of chemically reactive metabolites. It bypasses the formation of potentially toxic semiquinone radical intermediates through two-electron reduction of quinones to their corresponding stable hydroquinones. However, as mentioned before not all hydroquinones are redox-stable. Some redox-labile hydroquinones like diaziquone can react with molecular oxygen to form semiquinones and generate reactive oxygen species. Alternatively, in the presence of quinone and its hydroquinone form, semiquinones can be generated via comproportionation reactions (Beall and Winski, 2000). DT-diaphorase levels are strongly elevated in many tumor tissues compared with normal tissues, especially in non-small cell lung carcinoma. This provides the opportunity for designing enzyme-directed bioreductive drugs that will target DT-diaphorase (Danson *et al*., 2004; Rooseboom *et al*., 2004).

Mouse, rat and human DT-diaphorases appear to exist as two, three and four different forms, respectively. The enzyme NADPH-quinone oxidoreductase-1 (NQO1) comprises the majority of human DT-diaphorase activity present in tissues but the roles of the other three forms are poorly understood. Another DT-diaphorase enzyme in humans called as NQO2 (NRH:quinone oxidoreductase) is coded by a different genetic loci. Although the gene product has been described, the role of NQO2 in the detoxification of quinones is unknown (Rooseboom *et al.,* 2004; Danson *et al*., 2004). However, recently it was found that NQO2 is capable of catalyzing the reduction of mitomycin C with NADH as a cofactor (Jamieson *et al*., 2006). Unlike NQO1, NQO2 requires dihydronicotinamide riboside (NRH) rather than NADH or NADPH as the electron donor (Danson *et al*., 2004).

The enzyme DT-diaphorase is responsible for the reductive activation of some chemotherapeutic quinones such as mitomycin C, orthonaphtoquinones and aziridinylbenzoquinones (Danson *et al.,* 2004). It is usually regarded as the major enzyme responsible for reductive activation of mitomycin C in aerobic cancer cells (Cummings *et al.,* 1998). However, the role of DT-diaphorase in the reductive activation of mitomycin C has remained controversial and unclarified for several years (Spanswick *et al.*, 1998). Some workers have shown that mitomycin C sensitivity is associated with high DT-diaphorase levels, but others have demonstrated contradictions between *in vitro* and *in vivo* cytotoxicity (Danson *et al*., 2004). Studies carried out with purified DT-diaphorases showed minimal or no reductive activation and DNA cross-linking at physiological pH (Cummings *et al.,* 1998), but the use of cell lines with contrasting high and low DT-diaphorase levels demonstrated that this enzyme may have a role for aerobic bioactivation of mitomycin C (Spanswick *et al.*, 1998). In addition, pH studies have shown that DT-diaphorase catalyzed the reduction of mitomycin C in a pH dependent manner, with the activity increasing as the pH of the reaction buffer decreased from 7.8 to 5.8. DNA-cross linking events also followed the same pH pattern (Seow *et al*., 2004). Thus, these discrepancies observed between *in vitro* and *in vivo* conditions may be caused by the lack of physiological conditions present in the cells (low drug concentrations/high reductase levels/more acidic pH) in cell-free systems. Although mitomycin C is accepted as a poor substrate for DT-diaphorase with no doubt, the physiological conditions present in the cell may create a suitable environment to enhance the ability of DT-diaphorase to activate mitomycin C. It is now obvious that the enzyme DT-diaphorase either as the pure protein or in cells with high enzyme content, or in CHO transfectants expressing a high level of the enzyme is capable of reductively activating mitomycin C to cytotoxic species under both aerobic and hypoxic conditions with equal facility. It was observed that cell lines with high DT-diaphorase levels showed similar sensitivity to mitomycin C under both aerobic and anaerobic conditions indicating a predominant role for this enzyme under such conditions. Whereas cell lines with low in DT-diaphorase content displayed a greater difference in mitomycin C cytotoxicity under aerobic and hypoxic conditions because under these conditions one-electron reductases such as cytochrome P450

reductase becomes the major enzyme responsible for mitomycin C bioactivation (Cummings *et al.*, 1998).

1.3.4.2 The Role of NADH-Cytochrome b5 Reductase in the Bioreductive Activation of Mitomycin C

NADH-cytochrome b5 reductase is a FAD containing flavoprotein and catalyzes the reduction of cytochrome b5 through the transfer of electrons from NADH cofactor. Besides its physiologic partner, cytochrome b5, NADH-cytochrome b5 reductase can also reduce a number of nonphysiologic electron acceptors such as 2,6-dichlorophenolindophenol, ferricyanide, indigo tri and tetrasulfanotes. NADH-cytochrome b5 reductase exists as two isoforms. These include the membrane-associated form and soluble form. The membrane-bound form of NADH-cytochrome b5 reductase is present in both endoplasmic reticulum and outer membrane of the mitochondria. In endoplasmic reticulum, membrane-bound NADH-cytochrome b5 reductase transfers electrons from NADH cofactor to membrane-bound cytochrome b5 which in turn catalyzes several reactions of lipid metabolism including desaturation and elongation of fatty acids, plasmologen biosynthesis, cholesterol biosynthesis and drug metabolism. The membrane-bound forms of NADH-cytochrome b5 reductase and cytochrome b5 possess an amphipathic structure having a cytosolic domain and additional hydrophobic membrane segment. The hydrophobic tails of the enzymes are essential for their proper interaction, and anchoring to the biological membranes. Cytochrome b5 belongs to a family of hemoproteins (Arinç *et al.*, 1987). This hemoprotein can also accept electrons from an alternative microsomal reductase, NADPH-cytochrome P450 reductase (Enoch and Strittmatter, 1979). In addition, cytochrome b5 can participate in drug metabolism through interacting with some (but not all) forms of cytochrome P450 (Arinç *et al.*, 1994 and 1995). Thus, cytochrome b5 can act as a link between these two microsomal NADH- and NADPH-dependent electron transport systems. The soluble form of cytochrome b5 reductase, on the other hand, is localized in a soluble fraction of circulating erythrocytes where it plays a crucial role for the maintenance

of hemoglobin in the reduced state (Arinç *et al.*, 1992; for details see the review by Arinç, 1991).

NADH-cytochrome b5 reductase alone also has an important role in the bioreductive activation of therapeutically important anticancer drugs in addition to its electron carrier role in cytochrome b5 mediated reactions. NADH-cytochrome b5 reductase together with cytochrome b5 has been shown to be involved in the reductive activation of heterocyclic mono-N-oxide bioreductive drug, RB90740, (Barham and Stratford, 1996) and bleomycin (Mahmutoglu and Kappus, 1988, Kappus *et al*., 1990) which is a glycopeptide antibiotic used in chemotherapy. It was also shown that soluble NADH-cytochrome b5 reductase purified from rabbit erythrocytes can metabolically activate mitomycin C and adriamycin (doxorubicin) in a pH-dependent manner (Hodnick and Sartorelli, 1993 and 1994). The role of NADH-cytochrome b5 reductase in the reductive bioactivation of mitomycin C has been studied in detail by Sartorelli and coworkers. In contradictory to above *in vitro* data obtained with purified enzyme, it was found by the same research group that overexpression of NADH-cytochrome b5 reductase in CHO cells results in a decrease in sensitivity to mitomycin C under aerobic conditions and similar sensitivity under hypoxia with respect to parental cell lines (Belcourt *et al*., 1998a). Similarly, in other studies it was observed that overexpression of NADH-cytochrome b5 reductase in CHO cells caused no increase in sensitivity to mitomycin C over that seen with the parental cell line under either aerobic or hypoxic conditions (Holtz *et al*., 2003). However, CHO cells transfectants overexpressing the soluble form of the NADH-cytochrome b5 reductase (generated through removal of the membrane anchor) were restored to parental line sensitivity to mitomycin C under aerobic conditions with marked increases (25-fold) in drug sensitivity under hypoxic conditions (Belcourt *et al*., 1998a). Similarly, when the NADH-cytochrome b5 reductase is directed to the nuclear region of CHO cells through the incorporation of a nuclear localization signal to cytochrome b5 reductase gene, sensitivity to mitomycin C is more pronounced under both aerobic and hypoxic conditions with respect to CHO cells transfectants overexpressing cytochrome b5 reductase in the mitochondrial/endoplasmic reticulum compartments (Holtz *et al*., 2003). All these

studies show that subcellular localization of NADH-cytochrome b5 reductase affects mitomycin C cytotoxicity and bioactivation of it in close proximity to the DNA target results in greater cytotoxicity (see the review by Seow *et al*., 2004).

1.3.4.3 The Role of NADPH-Cytochrome P450 Reductase in the Bioreductive Activation of Mitomycin C

The second electron transport chain of endoplasmic reticulum is NADPH-dependent where the reducing equivalents are transferred from NADPH to cytochrome P450 through a flavoprotein, NADPH-cytochrome P450 reductase (Lu and Coon, 1968; Schenkman and Voznesensky, 1995). NADPH-cytochrome P450 reductase is an integral membrane flavoprotein containing two flavin molecules, FAD and FMN, per molecule of enzyme (Iyanagi and Mason, 1973) and localized in the endoplasmic reticulum. Besides transferring electrons to cytochrome P450, it can also catalyze the reduction of nonphysiologic electron acceptors such as cytochrome *c*, ferricyanide, menadione and 2,6-dichlorophenolindophenol (Williams and Kamin, 1962). It is an essential component of the microsomal cytochrome P450 monooxygenase system. NADPH-cytochrome P450 reductase is an amphipathic protein having a large cytosolic domain and additional hydrophobic segment (Figure 1.8). The hydrophilic peptide containing both FAD and FMN retains the spectral characteristic of the native enzyme, and can catalyze the reduction of cytochrome *c*. The hydrophobic peptide, however, is essential for the proper interaction of reductase with cytochrome P450 and, thus, for its participation in cytochrome P450-mediated monooxygenation reactions. The cytochrome P450-dependent monooxygenase system in the endoplasmic reticulum is a coupled electron transport system and consists of NADPH-cytochrome P450 reductase, lipid and a family of heme proteins called cytochrome P450 (Lu and Levin, 1974). This system is involved in oxidative metabolism of both endogenous and exogenous compounds. In these monooxygenase reactions, in order to function, cytochrome P450 must be bound to oxygen. However, the oxidized form of cytochrome P450 (ferric form, Fe^{+3}) can not bind to oxygen but the ferrous form (Fe^{+2}) does. Cytochrome P450 alone is unable to

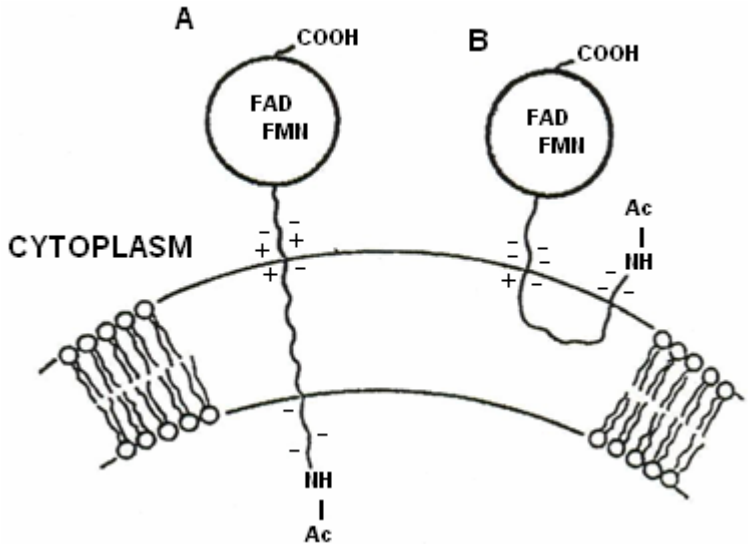


Figure 1.8 Possible orientations of NADPH-cytochrome P450 reductase, where the NH_2 – and COOH- terminal regions are either (A) on opposite sides or (B) on the same side of the microsomal membrane (taken from Black and Coon, 1982).

take these electrons directly from NADPH but does so indirectly via NADPH-cytochrome P450 reductase, which is facilitated by lipids. Cytochrome P450 monooxygenases can catalyze the metabolism of a wide range of different types of exogenous and endogenous compounds. Exogenous substrates include many drugs, chemotherapeutic agents, industrial solvents, chemical carcinogens, polycyclic aromatic and halogenated hydrocarbons, insecticides, herbicides, food additives, mutagens, antioxidants etc. Cytochrome P450s catalyze the key steps in steroid biosynthesis in animals (e.g. glucocorticoids, cortisol, estrogens and androgens). Besides cytochrome P450 isozymes, NADPH-cytochrome P450 reductase can also transfer electrons in endoplasmic reticulum to another hemoprotein called cytochrome b5 (Enoch and Strittmatter, 1979) as mentioned in previous section (for details see the review by Arinç, 1991 and 1995).

In addition to its electron carrier role in cytochrome P450 and cytochrome b5 mediated reactions, cytochrome P450 reductase alone can also catalyze the one-

electron reduction of foreign chemicals including therapeutically important quinone-containing anticancer drugs such as adriamycin (doxorubicin), mitomycin C, daunorubicin, bleomycin, porfiromycin, other environmental quinones, nitroimidazoles, aromatic nitro compounds, herbicide paraquat and certain azo dyes (Lu, 1991). Cytochrome P450 reductase is generally accepted as the enzyme responsible for reductive bioactivation of mitomycin C in hypoxic cancer cells (Cummings *et al.*, 1998). Utilizing CHO cells transfected with cDNAs of cytochrome P450 reductase and DT-diaphorase, it was shown that in comparison with the parental cell line, the transfected cells were more sensitive toward mitomycin C. However, CHO cells transfected with DT-diaphorase cDNA showed no difference in cytotoxicity of mitomycin C under both hypoxic and aerobic conditions, whereas cytochrome P450 reductase expressing cells showed greater sensitivity to mitomycin C under hypoxia than under in air (Belcourt *et al*., 1998b referred by Rooseboom *et al.,* 2004). Similarly, while only a modest increase (2.1 to 3.0-fold) in cytotoxicity of mitomycin C was observed for cytochrome P450 reductase overexpressing CHO cells under aerobic conditions (Sawamura *et al.*, 1996), a much larger increase occurred under hypoxic conditions (Belcourt *et al*., 1996). On the contrary, Hoban *et al.* (1990) claimed that cytochrome P450 reductase contributes to the cytotoxicity of mitomycin C in CHO cells only under aerobic conditions, in which cytotoxicity of mitomycin C is mainly attributed to the formation of toxic oxygen radicals rather than alkylating species (see the review by Cummings *et al*., 1998).

The role of oxygen radicals in tumor cell killing activity of mitomycin C remains to be clarified. Although some authors proposed that oxygen radicals contribute only in a minor way to the antitumor activity of quinone anticancer drugs (Powis, 1987, Butler and Hoey, 1987; referred by Cummings *et al*., 1998), others showed that there was an excellent correlation between tumor cell kill and formation of reactive oxygen species (Sinha, 1989 referred by Kovacic and Osuna, 2000). It appears that the formation of toxic oxygen radicals contribute to the cytotoxicity of mitomycin C in some systems (but not all) under aerobic conditions (Hoban *et al*., 1990).

1.4 Anthracyclines

Anthracyclines represent a large group of antibiotics which are among the most effective anticancer drugs ever developed to treat human cancer (see the reviews by Powis, 1987; Minotti *et al*., 2004; Asche, 2005; Doroshow, 2006). There is much interest on these agents primarily owing to the still uncertain molecular mechanisms of their action and identification and development of their better analogues (see the reviews by Gewirtz, 1999; Minotti, 2004).

1.4.1 Origin and Chemical Structure of Anthracyclines

The first anthracycline antibiotic designated later as daunorubicin was simultaneously isolated in 1963 from *S.peucetius* and *S.coerolerubidus* by Di Marco *et al*. and DuBost *et al*., respectively. The finding that this compound had antitumor activity against leukemia stimulated the interest to isolate other anthracyclines with better anticancer activity. In 1969, a structurally related anthracycline called as doxorubicin (adriamycin) was isolated from a chemically mutated strain of S.*peucetius* var. *caesisus* (Arcamone *et al.,* 1969). This compound was found to show a broader spectrum of activity and increased efficacy than seen for daunorubicin against solid tumors and started to be used in the clinic in the early 1970s. A great number of synthetic and semisynthetic analogues chemically related to the parent anthracycline compounds have been obtained and tested in experimental tumor models in order to find compounds with improved pharmacological and biological properties (see Powis, 1987). However, only a few of them such as epirubicin and idarubicin have reached clinical trials and approved for clinical use (see Minotti *et al*., 2004).

The chemical structures of some anthracycline antibiotics are shown in Figure 1.9. As shown in the figure, all these anthracyclines composed of an aglyconic part linked to a sugar moiety. The aglycone consists of a tetracycline ring structure which is usually red, orange or yellow in color with quinone and hydroquinone moieties on

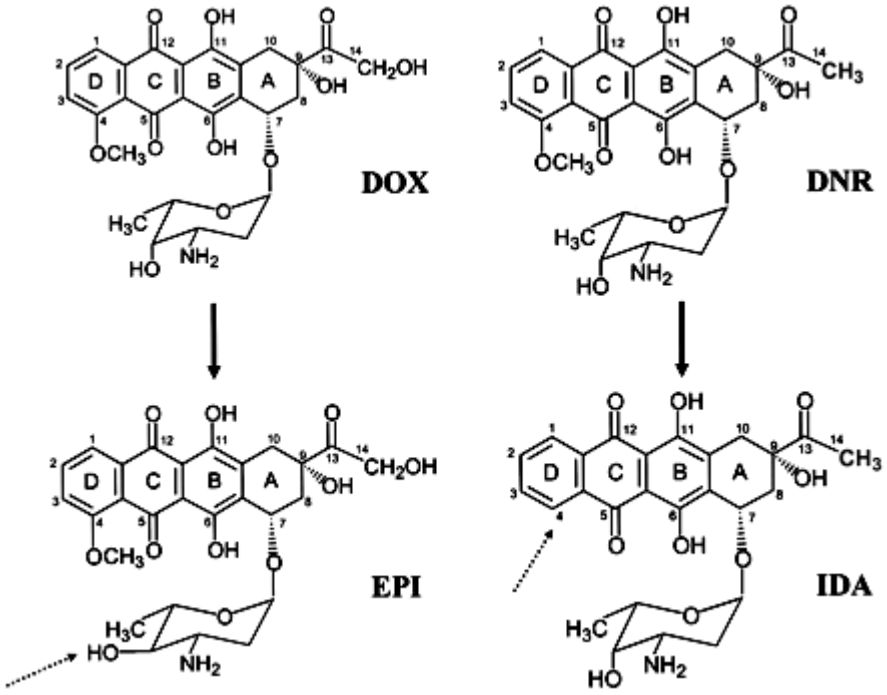


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Figure 1.9 Structures of doxorubicin (adriamycin), daunorubicin, epirubicin and idarubicin. Dotted arrows indicate structural modifications in epirubicin compared with doxorubicin or in idarubicin compared with daunorubicin (taken from Minotti *et al*., 2004).

adjacent rings C-B. An unusual amino sugar called daunosamine is attached to this aglycone part by a glycosidic linkage to the C-7 atom of the ring A. The natural anthracyclines doxorubicin and daunorubicin possess a methoxy group at C-4 and a side chain at C-9 with a carbonyl group at C-13. Doxorubicin differs from daunorubicin only by the presence of hydroxymethyl group at C-14 of side chain which is occupied by a methyl group in daunorubicin. Epirubicin is the epimer of doxorubicin and obtained by changing the axial position of the C4' hydroxyl group of daunosamine sugar of doxorubicin to the equatorial position. Idarubicin, on the other hand, is a synthetic analogue of daunorubicin and obtained by the removal of methoxy group at C-4 atom in ring D (see Powis, 1987; Minotti *et al*., 2004).

1.4.2 Clinical Activity of Anthracyclines

The anthracycline antibiotics are among the most widely used antineoplastic agents currently used in clinic. Doxorubicin and daunorubicin show activity against a variety of human tumors including hematological malignancies such as acute lymphoblastic and acute myeloblastic leukemias, multiple myeloma, Hodgkin's and non-Hodgkin's lymphoma and childhood solid tumors as well as breast, lung, ovary, stomach and thyroid cancers, bone and soft tissue sarcomas. On the other hand, the minor difference in the structure of doxorubicin and daunorubicin affects their spectrum of activity. While doxorubicin is widely used in current clinical practice for the treatment of solid tumors, especially breast cancer and lymphoma as well as childhood solid tumors and soft tissue sarcomas, daunorubicin is routinely used in the treatment of acute lymphoblastic (ALL) and acute myeloblastic leukemias (AML). The semisynthetic derivative of doxorubicin, epirubicin, shows similar spectrum of activity with the parent compound but displays less cardiotoxicity. Idarubicin has broader spectrum of activity than its parent drug daunorubicin and is an essential component of treatment of acute myelogenous leukemia, multiple myeloma, non-Hodgkin's lymphoma and breast cancer. Apart from these agents, only a few more anthracyclines including pirarubicin, aclacinomycin A and mitoxanthrone (a substituted aglyconic anthraquinone) have been approved for clinical use (see Minotti *et al*., 2004; Doroshow, 2006).

The common toxicities of anthracyclines include myelosupression, mucositis, alopecia, cardiac toxicity, nausea, vomiting and severe local tissue damage if the drug extravasates accidentally during administration. Myelosuppression, mostly in the form of neutropenia, constitutes the acute dose-limiting toxicity of doxorubicin and daunorubicin while cardiac toxicity constitutes the cumulative dose-limiting toxicity with anthracyclines. Cardiac toxicity produced by anthracycline drugs has a unique pathology and mechanism (see Powis, 1987; Doroshow, 2006).

1.4.3 General Mechanism of Action of Anthracyclines

Although anthracycline anticancer drugs have been used extensively and for a long time in the clinic, their mechanisms of action remain a subject of considerable controversy. Several mechanisms have been proposed for their cytotoxic and cytostatic properties. These include intercalation into DNA resulting in inhibition of macromolecular biosynthesis, free radical formation resulting in induction of DNA damage or lipid peroxidation, DNA binding and alkylation, DNA cross-linking, interference with DNA unwinding or DNA strand separation and helicase activity, direct membrane effects, initiation of DNA damage via inhibition of topoisomerase II and induction of apoptosis in consequence of upstream events such as inhibition of topoisomerase II (for a review see Gewirtz, 1999).

The ability of anthracyclines to intercalate into DNA is the well-known properties of these agents. It was shown that daunorubicin has a preferential affinity for dGdC-rich regions in DNA flanked by A:T base pairs, whereas, doxorubicin showed highest affinity for the 5'-TCA consensus sequence. The functional groups of the anthracycline molecule responsible for the DNA interaction are; the planar ring structure, which actually intercalates into DNA; the side chain (and its associated cyclohexane ring A), which forms hydrogen bond with DNA bases; and the daunosamine sugar, which interacts with minor groove of DNA and plays a critical role in base recognition and sequence selectivity. The capability of anthracyclines to intercalate in DNA was assumed initially to have a major role in the mechanism of action of these agents. A number of proposals including inhibition of DNA and RNA polymerase have been suggested in order to explain how DNA intercalation might cause tumor cell killing. However, no correlation was observed between inhibition of RNA and DNA synthesis and the cytotoxicity of doxorubicin which might be caused by the requirement of higher concentrations of the drug for the inhibition of these enzymes than can be achieved in *vivo* conditions (see Doroshow, 2006).

Another mechanism suggested to be involved in the cytotoxic activity of anthracyclines is the inhibition of topoisomerase II enzyme by these agents. Topoisomerases are the enzymes that modify the topology of the DNA during its synthesis or transcription. They can modulate the degree of DNA supercoiling by inducing either transient single strand breaks (topoisomerase I) or transient double strand breaks (topoisomerase II) without altering DNA structure and sequence. Anthracyclines inhibit topoisomerase II by stabilizing a ternary complex formed by anthracycline, topoisomerase II and DNA in which the enzyme is covalently linked to the broken DNA strands via tyrosine residues, eventually leading to blocking of DNA resealing (see Minotti *et al*., 2004). Some workers claimed that anthracyclines inhibit topoisomerase II through intercalation into DNA and structural determinants of the anthracyclines especially amino sugar part seem to have important roles in the formation and stabilization of this ternary complex (see Minotti *et al*., 2004). In contrast, studies revealed that anthracyclines may stimulate topoisomerase II-mediated DNA cleavage by a nonintercalative mechanism. Anthracycline analogues that do not intercalate into DNA as well as doxorubicin at concentrations well below the dissociation constant for DNA intercalation effectively stimulated the formation of topoisomerase II-mediated DNA cleavage. In addition, the demonstration of inhibition of purified topoisomerase II by doxorubicinone, an aglyconic form of doxorubicin suggested that sugar moiety of anthracyclines is not required for enzyme inhibition (see Droshow, 2006). DNA topoisomerase II inhibition by anthracyclines clearly occurs in many mammalian cells, however the role of topoisomerase-mediated DNA damage in antitumor activity of these agents remains uncertain. It appears that the formation of protein associated DNA breaks is only potentially lethal but not sufficient for tumor cell killing (see Doroshow, 2006). The reasons for this uncertainty include the lack of any correlation between topoisomerase-mediated DNA damage and cytotoxicity of the drug in some cells; failure to detect significant DNA damage at drug concentrations equal to that of the IC_{50} value; rapid repair of topoisomerase-mediated DNA damage after removal of the cytotoxic agent; not understanding of the process of how reversible damage leads to cell death; the absence of relationship between the level of topoisomerase II and the sensitivity of human cell lines to doxorubicin *in vitro*; lack of correlation between topoisomerase

IIα content or activity in primary tumors and clinical response of breast cancer patients to anthracyclines (see Cullinane *et al*., 1994; Doroshow, 2006).

In addition to intercalation into DNA and inhibiting topoisomerase II activity, anthracyclines form complexes with DNA that interfere with the activity of a specific class of nuclear enzymes, the helicases, by increasing the duplex DNA stability thereby preventing the dissociation of duplex DNA into DNA single strands and limiting replication. The studies carried out with purified enzymes showed that this process occurs at clinically relevant drug concentrations and correlates, at least in part, with the cytotoxic spectrum of a number of anthracycline analogues (see Gewirtz, 1999; Doroshow, 2006).

It has also been demonstrated that doxorubicin redox cycling with molecular oxygen leads to the formation of detectable levels of potentially cytotoxic, oxidized DNA bases which can occur at clinically relevant drug concentrations. This observation provides an alternative mechanism unrelated to strand cleavage to explain the action of doxorubicin under pharmacokinetic conditions as well as indicate a potential mechanism for anthracycline-related mutagenicity and carcinogenicity because of the inhibitory effects of oxidized bases on the action of DNA polymerases and other DNA repair mechanisms (see Minotti *et al*., 2004; Doroshow, 2006). In addition to their effects on DNA, anthracyclines can also interact with cell membranes and alter a variety of membrane functions which could be involved in their cytotoxic mechanism of action. The membrane interaction of anthracyclines indicates that these agents potentially could also act through interacting with signal transduction pathways of the cell. Doxorubicin and daunorubicin exposure has been shown also to induce morphological changes associated with apoptosis in a number of cell lines. Furthermore, it has been demonstrated recently that exposure of mammalian cells to anthracycline antibiotics may result in cellular senescence in addition to necrotic and apoptotic death phenotypes (see Doroshow, 2006).

1.4.3.1 Bioreductive Activation of Anthracyclines

Anthracyclines act as chemically inert compounds during intercalation into DNA and binding to topoisomerase II enzyme. However, these agents are also chemically reactive compounds and can be reduced by one- and two-electron reductases present in the cell which may lead to the formation of reactive oxygen species and their reactive intermediate forms capable of making covalent bonds with cellular macromolecules like DNA. The bioreductive activation of anthracyclines, thus, constitutes an important part of the mode of action of these agents (see Doroshow, 2006).

A large number of enzymes are involved in the one-electron reductive activation of doxorubicin. These include NADPH-cytochrome P450 reductase (Pan *et al*., 1981; Komiyama *et al*., 1982; Kappus, 1986; Powis, 1987), NADH-cytochrome b5 reductase (Hodnick and Sartorelli, 1994), xanthine:oxygen oxidoreductase (xanthine oxidase) (Pan *et al*., 1981; Kappus, 1986; Powis, 1987), all three isoforms of nitric oxide synthase (Vasquez-Vivar *et al*., 1997; Garner *et al*., 1999), NADH dehydrogenase (EC 1.6.5.3, complex I of the mitochondrial electron transport chain) (Doroshow and Davies, 1986; Kappus, 1986; Powis, 1987), NADH:lipoamide oxidoreductase (EC 1.8.1.4) (Pan *et al*., 1981), NADPH:nitrate oxidoreductase (EC 1.7.1.3) (Pan *et al*., 1981; Powis, 1987), ferrodoxin:$NADP^+$ oxidoreductase (Kappus, 1986; Powis, 1987), NADH-dependent cytochrome *c* and flavin oxidoreductase (EC 1.6.99.3) (Pan *et al*., 1981) and a cardiac specific exogenous NADH:oxidoreductase (Nohl, 1988). Furthermore, rat liver cytochrome P4502B1 has been shown to be involved in one-electron reduction of adriamycin (doxorubicin) by a proposed mechanism in which cytochrome P450 takes the electrons from NADPH cofactor indirectly via NADPH-cytochrome P450 reductase, and thus catalyzes the one-electron reduction of adriamycin (Goeptar *et al.,* 1993).

One-electron reduction of anthracyclines leads to the formation of the corresponding unstable and highly reactive semiquinone radical. Under aerobic conditions, the semiquinone radical rapidly undergoes redox cycle with molecular

oxygen to generate superoxide anion radical, which in turn leads to the formation of reactive oxygen species such as hydrogen peroxide and a highly reactive hydroxyl radical (Sinha *et al*., 1987). Here, it needs to mention that the chelation of metal ions particularly iron by anthracyclines provides also another mechanism for the generation of reactive oxygen species which is independent from the reductive activation and formation of semiquinone radical (Sinha, 1989). There have been many reports that one electron reduction of anthracyclines to the semiquinone radical may contribute to the cytotoxic mechanism of anthracyclines via covalent binding of anthracycline residues to cellular macromolecules (Sinha, 1980; Ghezzi *et al*., 1981; Scheulen *et al*., 1982; Cummings *et al*., 1991; Zeman *et al*., 1998; for details see Powis, 1989). It has been shown by Scheulen *et al.* (1982) that aerobic reduction of doxorubicin resulted in the covalent binding of drug metabolites to bovine serum albumin and microsomal proteins which suggested that direct covalent binding of cellular macromolecules by semiquinone radical could compete effectively with redox cycling under aerobic conditions (see Powis, 1989). Interestingly, it was demonstrated that under hypoxic conditions one-electron reduction may also lead to C-7 reductive cleavage of anthracyclines to generate 7-deoxyaglycone through the reduction and cleavage of glycosidic bond (see Powis, 1989). This reductive deglycosidation pathway has been generally accepted as a detoxification pathway (Cummings *et al*., 1992). However, a C-7 carbon centered anthracycline radical has been proposed to be responsible for both covalent binding to cellular macromolecules and formation of the biologically inactive 7-deoxyaglycone under anaerobic conditions (Mason, 1979; Sinha, 1980; referred by Powis 1989).

As mentioned in Section 1.2.1, the formation of antracycline-induced oxidative stress and reactive oxygen species formation is generally accepted as the main causative factor for the cardiac toxicity of anthracyclines (see Kovacic and Osuna, 2000; Minotti *et al*., 2004). However, the role of reactive oxygen species formation on the antitumor activity of these agents remains a matter of speculation and debate (for details see Doroshow, 2006). For example, Gewirtz (1999) raised the question that "whether free radicals are generated at clinically relevant concentrations of anthracyclines and at normal (i.e hypoxic) oxygen tension in the

tumor cell and whether such free radicals could be responsible for anthracycline toxicity to the tumor". In contradictory to above opinion, anthracycline-induced oxidative stress has been proposed by Koch and coworkers to be responsible for most but not all of the biological activity of anthracyclines (Taatjes *et al.*, 1998). These authors demonstrated that anthracycline-induced oxidative stress formed via redox cycling leads to the release of formaldehyde from cellular carbon sources, which in turn is utilized by the drug to produce a drug-formaldehyde conjugate. Such conjugates have been shown *in vitro* experiments to have a unique ability to form novel DNA crosslinks called as "virtual cross-link" which contribute significantly to the antitumor activity of anthracyclines (Taatjes *et al*., 1998; reviewed in Kovacic and Osuna, 2000; Minotti *et al*., 2004; Doroshow, 2006).

Doxorubicin can also be reduced to its corresponding hydroquinone directly via two-electron reduction. It has been shown that DT-diaphorase (Cummings *et al*., 1992) and xanthine dehydrogenase (Yee and Pritsos, 1997) can catalyze the two-electron reduction of doxorubicin. Alternatively, the hydroquinone form of the doxorubicin may be formed by sequential one-electron transfers under hypoxic conditions. It is also likely that under hypoxic conditions, doxorubicin semiquinone radical could undergo disproportionation to the hydroquinone. Two-electron reduction of doxorubicin leads to glycosidic cleavage with the production of unstable transient quinone methide. The quinone methide intermediate has been proposed to be a potential monofunctional alkylating agent. However, DNA adducts formed by this transient form has not been yet verified structurally (Taatjes *et al.*, 1997). In addition, there exists little evidence that quinone methide intermediate plays an important cytotoxic role in tumor cells (see Doroshow, 2006). The major fate of this intermediate in tumor cells is progression via a second arrangement to produce corresponding 7-deoxyaglycone. 7-deoxyaglycones are biologically less active compounds than the parent drug, therefore, this pathway is generally regarded as a detoxification pathway (Powis, 1989; Cummings *et al*., 1992; Yee and Pritsos, 1997; Doroshow, 2006).

1.4.4 Idarubicin

Idarubicin (4-demethoxydaunorubicin) is a semisynthetic drug that was first developed in 1976 by Arcamone *et al.* in an approach to obtain anthracycline analogues with better antitumor activities and less cardiotoxic properties. It is a relatively new drug and approved by the US FDA in 1990 for the treatment of acute myelogenous leukemia. Idarubicin is a daunorubicin derivative which differs from the parental drug only in that C-4 methoxy group in the D ring of the aglycone moiety is replaced with a hydrogen atom. However, this minor modification of the molecule creates significant differences in the oral bioavailability, pharmacokinetics, toxicity and antitumor activity of the two drugs which makes the idarubicin unique among the anthracyclines (see the reviews by Fields and Koeller, 1991; Hollingshead and Faulds, 1991; Robert, 1993; Goebel, 1993; Borchmann *et al*., 1997, Crivellari *et al*., 2004).

Idarubicin after its development drew much attention from the scientists because of its high potency against several experimental tumors in mice (Arcomone *et al.,* 1976; Formelli *et al*., 1979; Broggini *et al*., 1984) and antitumor activity of its orally administered form (Di Marco *et al*., 1977) (referred by Robert, 1993). Idarubicin is more lipophilic than its parent drug, daunorubicin which leads to improved absorption of the drug across the gastrointestinal mucosa (a better oral bioavailability) and significantly enhanced cellular uptake (see Hollingshead and Faulds, 1991; Fields and Koeller, 1991; Goebel, 1993; Crivellari *et al*., 2004). The higher lipophilicity of the drug, thus, allows it to accumulate faster in the nuclei and to bind DNA with higher affinity which consequently results in a greater cytotoxicity when compared with daunorubicin (see Borchmann *et al*., 1997). The greater cytotoxicity of idarubicin compared to daunorubicin and doxorubicin shown in *in vitro systems* (Salmon *et al*., 1981; Dodion *et al*., 1987; Schott and Robert, 1989) has been attributed to the superior DNA binding capacity and consequently to the much higher activity of idarubicin in inducing topoisomerase II-mediated DNA breaks (Capranico *et al*., 1990; Gieseler *et al*., 1994; Robert, 1995; referred by Robert, 1993 and Borchmann *et al.,* 1997).

Anthracyclines in the body are rapidly and extensively metabolized by NADPH-dependent cytosolic carbonyl reductases or aldo-keto reductases which catalyze the two-electron reduction of the ketone at C-13 to the corresponding alcohol (see Figure 1.9) (see Minotti *et al.,* 2004). Anthracycline secondary alcohol metabolites constitute the primary metabolites of these drugs in the body. This pathway is generally viewed as a detoxification pathway, since C-13 metabolites generally display much less antitumor activity (Kuffel *et a*l., 1992). However, it has been found by several workers that the secondary alcohol metabolite of idarubicin, idarubicinol, demonstrated equipotent cytotoxic activity with idarubicin and a much greater activity than the other anthracycline alcohol metabolites. The significant cytotoxic activity shown by idarubicinol *in vitro* suggests that it may play important role in the antitumor activity of idarubicin *in vivo* (Ganzina *et al*., 1986; Kuffel *et al*., 1992; reviewed in Fields and Koeller, 1991; Robert, 1993; Goebel, 1993; Borchmann *et al*., 1997). Idarubicinol is the only metabolite of all anthracyclines showing this kind of effect (Borchmann *et al*., 1997). Furthermore, it has been demonstrated that idarubicin, and mainly idarubicinol can cross the blood-brain barrier and can be detected in cerebrospinal fluid. This property of the drug makes it particularly important for clinical use (Goebel, 1993; Borchmann *et al*., 1997). Idarubicin has been also shown to be less cardiotoxic than other anthracyclines (Hollingshead and Faulds, 1991; Goebel, 1993; Borchmann *et al.,* 1997; Crivellari *et al*., 2004). In addition, idarubicin appears to be less susceptible than other anthracyclines to P-glycoprotein (P-gp) and the so-called MRP (multidrug resistance-associated proteins) which act as a pump for xenobiotics and involved in the cellular resistance to several unrelated drugs including anthracyclines (Robert, 1993; Borchmann *et al.,* 1997; Crivellari *et al*., 2004). The presence of its oral form and being less cardiotoxic than other anthracyclines makes the idarubicin an attractive drug for clinical use in elderly patients (Leone *et al*., 1999; Crivellari *et al*., 2004). Idarubicin is the only anthracycline that is bioavailable both in the injectable and oral forms (Crivellari *et al*., 2004). Oral idarubicin has been mainly used in patients affected by metastatic breast cancer and these studies showed that idarubicin is an active cytotoxic drug against advanced breast cancer (Crivellari *et al*., 2004). The use of oral idarubicin in breast cancer, non lymphoid leukemia, non-Hodgkin's lymphoma, multiple myeloma

and AIDS-associated Kaposi's sarcoma is currently under investigation (Hollingshead and Faulds, 1991; Goebel, 1993; Crivellari *et al*., 2004). Intravenous idarubicin has shown high activity in acute myelogenous leukemia (AML) and moderate to good activity in acute lymphoblastic leukemia (ALL). It may also have potential in the treatment of other leukemias or breast cancer (Hollingshead and Faulds, 1991).

1.5 Alternative Strategies for the Treatment of Cancer

The major drawbacks of the conventional cancer chemotherapy in the treatment of cancer patients includes dose-limiting toxicity of chemotherapeutic agents to host tissues and cells of the body, development of resistance to multiple anticancer drugs in the tumors and limited accessibility of the tumors to the therapeutic regimen. Therefore, one of the major goals of cancer therapy has been selective and specific targeting of tumor cells using cytotoxic drugs while preventing or limiting damage to normal tissue. For this purpose, several approaches have been developed including gene-directed enzyme prodrug therapy (GDEPT), virus-directed enzyme prodrug therapy (VDEPT), antibody-directed enzyme prodrug therapy (ADEPT), polymer-directed enzyme prodrug therapy (PDEPT), genetic prodrug activation therapy (GPAT) and polymer enzyme liposome therapy (PELT), which are currently under investigation and still at the early stages of development. In general, strategies that promote selective activation of prodrugs by enzymes in tumor cells can be classified into two major classess; selective delivery of a gene encoding an enzyme and selective delivery of an antibody-enzyme immunoconjugate to tumor cells as discussed below in detail (Blau *et al.*, 2006).

1.5.1 Gene-Directed Enzyme Prodrug Therapy

Gene-directed enzyme prodrug therapy (GDEPT) consists of three components. These include the prodrug to be activated, the enzyme required for

activation of the prodrug and the delivery system for the corresponding gene of the enzyme (Denny, 2003). In this approach, the gene directed to the target tumors encode an enzyme which is not toxic on its own but activates an injected prodrug into a highly toxic product which leads to selective killing of tumor cells (Greco and Dachs, 2001). A number of vector systems including RNA and DNA viruses, peptides, cationic lipids and naked DNA together with antibody-directed and ligand-directed delivery of DNA and use of an anaerobic bacterium, *Clostridium,* for targeting hypoxic tumors have been suggested as delivery systems for gene therapy (Niculescu-Duvaz *et al.,* 1998). This approach requires a number of criteria to be met to achieve therapeutic benefit. These requirements include; the gene should be expressed exclusively in tumor cells, the expressed enzyme should have high enough catalytic activity to activate the prodrug at physiological conditions of the tumor cells, expression of the enzyme itself should not lead to cytotoxic effects, the prodrug activation pathway of the enzyme should be different from any endogenous enzyme in order to avoid cytotoxic activation of the prodrug in normal cells, the prodrug should be a systemic agent, metabolically stable and readily diffusible throughout the tumor as well as an effective and selective substrate for the enzyme, the toxic agent released from prodrug should also have ability to diffuse and kill neighboring tumor cells (bystander effect) because transfection of all the cells in a tumor population with the prodrug activating enzyme gene is not possible (Niculescu-Duvaz *et al*., 1998; Greco and Dachs, 2001; Denny, 2003). It is important that, after gene transfection, the prodrug must be administered after a while in order to allow the expression and accumulation of the enzyme in tumor cells (Greco and Dachs, 2001). Prodrugs for this approach can be designed in such a way that the actual toxic or potentially toxic compound is joined, sometimes via a linker, to a molecule that is the substrate for the activating enzyme and is subject to selective metabolism (Denny, 2003). In general, enzymes which are not of human origin are preferred, otherwise, normal healthy tissues can be damaged due to the potential endogenous activation of the prodrug. However, the products of the foreign genes may create complications with the immune system of the body. Alternatively, an endogenous enzyme that is expressed at low levels in tumors and is able to activate the prodrug can also be used in this approach (Niculescu-Duvaz *et al*., 1998; Greco and Dachs, 2001).

A number of enzyme-prodrug combinations have been suggested for GDEPT including the prodrugs of anthracyclines such as doxorubicin and daunorubicin (Deny, 2003; Rooseboom *et al.,* 2004) in which the release of actual drug from the prodrug may require further cellular metabolism by endogenous enzymes in order to show its toxic effects. For example, activation of prodrugs of anthracyclines such as doxorubicin and daunorubicin glutamates by bacterial enzyme carboxypeptidase G2 to release doxorubicin and daunorubicin (Niculescu-Duvaz *et al*., 1999), activation of anthracycline glucuronides by *E. coli* beta-glucuronidase (Bakina *et al*., 1997) and anthracycline beta-galactosides by *E. coli* beta-galactosidase (Bakina and Farquhar, 1999) to release corresponding anthracycline carbinolamines have been suggested as prodrug/enzyme combinations for GDEPT (see Deny, 2003 for details). Furthermore, activation of quinones such as EO9 (a synthetic analogue of mitomycin C) and diaziquone by *E.coli* nitroreductase has been suggested; but this combination was found to be less effective as compared to *E.coli* nitroreductase combinations with other prodrugs such as CB1954 (an aromatic nitro compound) and nitrofurazone (Bailey *et al.,* 1996). Apart from the use of exogenous enzymes in GDEPT, the endogenous mammalian enzymes such as cytochrome P450 and NADPH-cytochrome P450 reductase have also been suggested for their use with appropriate chemotherapeutic prodrugs such as the oxazaphosphorines, cyclophosphamide and ifosfamide, and bioreductive prodrugs tirapazamine and mitomycin C as enzyme/prodrug or enzyme/bioreductive prodrug combinations in GDEPT (Waxman *et al*., 1999; Jounaidi and Waxman, 2000; Cowen *et al.*, 2003; Roy and Waxman, 2006).

1.5.2 Antibody-Directed Enzyme Prodrug Therapy

Antibody-directed enzyme prodrug therapy (ADEPT) is based on selective targeting of tumor cells with a specific antibody conjugated to a prodrug activating enzyme, thereby providing the activation of non toxic prodrugs exclusively at tumor sites. A number of tumor specific antigens have been exploited for their potential use in ADEPT. With the use of chimerized or humanized antibodies, it is possible to

target tumor-specific antigens without the risk of a human immune response to the antibodies themselves. In this approach, the antibody can be linked to an enzyme by chemical and other means. It is important that the prodrug can be administered to the body after ensuring that the circulating antibody-enzyme conjugates that were not bound to the target tissue were eliminated from the bloodstream. Otherwise undesired systemic effects may occur (for details see Jung, 2001).

Several prodrug/enzyme combinations including the prodrugs of quinone anticancer agents such as mitomycin C and anthracyclines have been suggested for ADEPT (Rooseboom *et al.,* 2004). These include the activation of mitomycin C phosphate and N-(4-phosphonooxy)phenylacetyl)-doxorubicin by alkaline phosphatase to release mitomycin C and doxorubicin, respectively (Senter *et al*., 1989; Vrudhula *et al*., 1993), activation of DPO (a doxorubicin prodrug) and N-(phenylacetyl)doxorubicin by bacterial penicillin G amidase to release doxorubicin (Kerr *et al*., 1990; Vrudhula *et al.,* 1993), activation of C-DOX, PRODOX (doxorubicin analogues) and cephalosporin mitomycin C by bacterial β-lactamase to release doxorubicin and mitomycin C, respectively (Hudyma *et al.*, 1993; Jungheim *et al.*, 1993; Vrudhula *et al.,* 1997; referred by Rooseboom *et al.,* 2004).

1.6 The Aim of This Study

Despite the extensive use of quinone-containing anticancer agents in cancer chemotherapy, the cytotoxic action mechanisms of these drugs are still not well understood and need to be further clarified. For this purpose, intensive studies are being carried out in different laboratories all over the world to try to understand the molecular mechanisms underlying the cytotoxic actions of these agents. Therefore, a detailed elucidation of these mechanisms together with a better understanding of the molecular events leading to the malignant transformation of a normal healthy cell will aid in the design of more effective and highly selective novel chemotherapeutic drugs.

Anthracyclines represent a large group of quinone-containing antibiotics which are among the most potent and widely used classes of anticancer agents. There is much interest on these agents primarily owing to the still uncertain molecular mechanisms of their action and identification and development of their better analogues with improved antineoplastic activities and less cardiotoxic properties. Idarubicin is one of such analogues, used in the treatment of certain types of human cancers. Several mechanisms have been proposed for the antitumor effects of anthracycline agents as mentioned before in detail. Among the suggested mechanisms, bioreductive activation by cellular oxidoreductases, resulting in the formation of free radicals, is considered as an important mechanism contributing to the therapeutic effectiveness of these agents. However, the studies on the bioreductive activation of anthracyclines have been generally conducted with natural anthracycline doxorubicin as mentioned in the previous sections. A large number of articles, for example, have been published considering the reduction of doxorubicin by various purified enzymes. Although the semisynthetic drug idarubicin has unique features with respect to its oral bioavailability, pharmacokinetics, toxicity and antitumor activity that distinguish it from other natural anthracyclines, to our knowledge, no reports on the bioreductive activation of idarubicin by purified NADPH-cytochrome P450 reductase, NADH-cytochrome b5 reductase and cytochrome P450 isozymes have appeared. The bioactivation studies on anthracycline analogues are particularly important, given the fact that modification or removal of functional groups of a drug molecule may affect its physicochemical and biological properties including its binding affinity to cellular enzymes. In addition, identification of the cellular enzymes involved in the reductive bioactivation of currently used as well as newly developed anthracycline drugs and elucidation of their antitumor action mechanisms are especially important for the chemotherapeutic use of these drugs. Therefore, the aims of this study are the following:

1. to examine the ability of idarubicin to undergo bioreduction by NADPH-cytochrome P450 reductase to DNA-damaging species
2. to investigate the mechanism of DNA damage induced by idarubicin in the presence of NADPH-cytochrome P450 reductase

3. to examine the possible participation of purified NADH-cytochrome b5 reductase in the bioreductive activation of idarubicin to DNA-damaging species, and to compare its ability with that of NADPH-cytochrome P450 reductase in promoting the DNA strand cleavage in the presence of idarubicin
4. to evaluate the protective potential of some dietary antioxidants against DNA damage induced as a consequence of P450 reductase-catalyzed reductive bioactivation of idarubicin
5. to further examine and compare the abilities of purified NADPH-cytochrome P450 reductase and NADH-cytochrome b5 reductase to catalyze the reduction of idarubicin by measuring drug-induced NAD(P)H oxidation using either microsomes or purified enzymes
6. to assess the involvement of cytochrome P4502B4 in the bioreductive activation of idarubicin to induce DNA strand scission, and to determine the role of cytochrome P4502B4 in the reduction of idarubicin, relative to NADPH-cytochrome P450 reductase, using reconstituted systems of purified cytochrome P4502B4 and cytochrome P450 reductase

In order to achieve these goals, the enzymes used in the present study were highly purified from different species and tissues. NADPH-cytochrome P450 reductase was purified from phenobarbital-treated rabbit liver, beef liver and sheep lung microsomes. The sheep lung and sheep liver P450 reductases were purified previously in our laboratory and their some kinetic, catalytic and structural properties were compared (İşcan and Arinç, 1986 and 1988). It has been found that sheep lung P450 reductase is more stable than liver P450 reductase against proteolytic cleavage. That is why we purified P450 reductase from sheep lung microsomes and used in this study. Since we purified P450 reductase from beef liver microsomes before in our laboratory and described its some biochemical characteristics (Arinç and Çelik, 2002), we also obtained P450 reductase from beef liver microsomes in highly purified form and used in bioreduction studies of idarubicin and mitomycin C. In addition, although beef liver microsomes have relatively less NADPH-cytochrome P450 reductase activity per mg of microsomal protein compared to rabbit liver and sheep lung microsomes, the relatively larger liver tissue of beef allows to purify a

high amount of P450 reductase. NADPH-cytochrome P450 reductase from phenobarbital-treated rabbit liver microsomes, on the other hand, is the enzyme which has been extensively used in bioactivation studies of a large number of drugs and chemicals in the literature. Therefore, in addition to sheep lung and beef liver P450 reductase, we purified P450 reductase from phenobarbital-treated rabbit liver microsomes and used in our studies. Besides cytochrome P450 reductases, we purified microsomal NADH-cytochrome b5 reductase and cytochrome P4502B4 from beef liver tissue and phenobarbital-treated rabbit liver microsomes, respectively. In this study, in order to determine whether redox cycling of idarubicin by P450 reductase is involved in the induction of DNA damage under aerobic conditions, the antioxidant enzymes superoxide dismutase (SOD) and catalase as well as scavengers of hydroxyl radicals ($OH^{\cdot}$), dimethyl sulfoxide (DMSO) and thiourea were employed. Their protective effects against strand breaks may indicate whether reactive oxygen species produced during redox cycling of idarubicin with molecular oxygen catalyzed by P450 reductase can play a role in DNA-damaging activity of idarubicin. In addition, the DNA-damaging capacity of idarubicin was characterized with respect to increasing concentrations of P450 reductase or drug as well as increasing incubation time using the *in vitro* plasmid DNA damage assay.

In this study, all of the above experiments were also repeated under the same reaction conditions using mitomycin C, and the results were compared. We have chosen mitomycin C as a model compound, since it has been extensively studied with respect to its molecular pharmacology and chemistry. Mitomycin C has been shown previously by others to undergo bioreductive activation by P450 reductase and b5 reductase as well as by cytochrome P450 as mentioned before (see Section 1.3.4). The list of the enzymes involved in the reductive activation of mitomycin C has also been growing steadily with the appearance of "new" enzymes in the literature (Barak *et al.*, 2006). However, there is still inadequate information concerning the exact roles and involvements of cytochrome P450 isozymes on the bioreduction and cytotoxicity of mitomycin C. Besides, to our knowledge, there appears to be only one published detailed study about *in vitro* reductive activation of mitomycin C by the purified NADH-cytochrome b5 reductase, which, in this study,

was the soluble form of the enzyme purified from rabbit erythrocytes (Hodnick and Sartorelli, 1993). Therefore, the results obtained from this study will also demonstrate whether there exists a kinetic difference between microsomal and soluble forms of NADH-cytochrome b5 reductase in catalyzing reductive activation of mitomycin C.

The results obtained from this study will also provide:

- ✓ a crucial comparative data on the bioreductive activation of idarubicin and mitomycin C catalyzed by NADPH-cytochrome P450 reductase, NADH-cytochrome b5 reductase and cytochrome P4502B4
- ✓ an important contribution to a better understanding of cytotoxic efficacy of idarubicin and mitomycin C in various tumor and normal cells containing different reductive enzyme profiles and levels
- ✓ some insights into the novel anticancer drug development studies
- ✓ practical implications concerning the potential use of these purified reductive enzymes as therapeutic agents on their own in cancer treatment strategies in combination with bioreductive anticancer drugs like idarubicin, mitomycin C or some other potential quinone anticancer drugs
- ✓ insights concerning the possible use of these purified reductive enzymes in gene directed enzyme prodrug therapy (GDEPT) strategy.

CHAPTER II

MATERIALS AND METHODS

2.1 Materials

Hydroxylapatite (Bio-Gel HTP; 130-0420) and N, N, N', N'-tetramethylethylene diamine (TEMED; 161-0801) were purchased from Bio-Rad Laboratories, Richmond, California, USA. Emulgen 913 (polyoxyethylene nonyl phenyl ether) was a gift from Kao-Atlas Co., Tokyo, Japan. Carbon monoxide (CO; 010-0264) was purchased from Matheson Gas Products, Divisie van Will Ross, Oevel, Belgium. Adenosine 2', 5'-diphosphate-Sepharose 4B (170081-001), octyl-Sepharose CL-4B and Sephadex G-100 were obtained from Pharmacia Fine Chemicals, Inc., Uppsala, Sweden.

Cupper sulfate ($CuSO_4$; 102787), potassium chloride (KCl; 104935), sodium chloride (NaCl; 106400), sodium dithionite ($Na_2S_2O_4$; 106507), sodium hydroxide (NaOH; 106462), magnesium chloride ($MgCl_2$; 105833), potassium dihydrogen phosphate (KH_2PO_4; 104873), dipotassium hydrogen phosphate (K_2HPO_4; 105101), sodium dodecyl sulfate (SDS; 113760), polyethylene glycol 6000 (PEG 6000; 807491), glycerol (104093), dimethylsulfoxide (DMSO; 2951), ethylenediaminetetraacetic acid disodium salt (EDTA; 108418), sodium carbonate (Na_2CO_3; 106392), sodium dihydrogen phosphate (NaH_2PO_4; 106349), disodium hydrogen phosphate (Na_2HPO4; 106576) and hydrochloric acid (HCl; 100314) were purchased from E.Merck, Darmstadt, Germany.

Acrylamide (A8887), adenosine 5'-diphosphate-agarose (A4398), idarubicin hydrochloride (I1656), adenosine 2'-monophosphate (A9396), cytochrome *c* (C7752), phenylmethane sulfonyl fluoride (PMSF; P7626), ε-amino caproic acid (ε-ACA; A2504), bovine serum albumin (BSA; A7511 or A7906), ammonium persulfate (APS; A3678), ammonium acetate (A7262), egg albumin (A7642), bovine liver catalase (C100), 3α, 7α, 12α-trihydroxy-5β-cholon-24-oic acid, sodium salt (sodium cholate; C1254), coomassie brilliant blue R (B0149), D-glucose-6-phosphate dehydrogenase (G8289), bovine liver L-glutamate dehydrogenase type IV (G4008), N-2-hydroxyethylpiperazine-N'-2, ethane sulfonic acid (HEPES; H3375), β-mercapto-ethanol (M7154), β-nicotinamide adenine dinucleotide reduced form (NADH; N8129), dilauroyl phosphatidylcholine (C12:0) (DLPC; P1263), 2-amino-2-(hydroxymethyl)-1, 3-propanediol (Trizma Base; T1378), N,N'-methylene bisacrylamide (BIS; M7256), sodium potassium tartrate (Rochelle salt; S2377), phenobarbital (P5178), superoxide dismutase (SOD) from bovine erythrocytes (S2515), bromophenol blue (B5525), thiourea (T7875), methanol (24229), acetic acid (27225), potassium ferricyanide ($K_3Fe(CN)_6$; 244023), boric acid (11607), quercetin (Q0125), rutin (R5143), naringenin (N5893) and trolox (56510) were purchased from Sigma-Aldrich Chemical Company, Saint Louis, Missouri, USA.

Agarose (A2114), β-nicotinamide adenine dinucleotide phosphate ($NADP^+$; A1394), β-nicotinamide adenine dinucleotide phosphate reduced form (NADPH; A1395), DL-dithiothreitol (DTT; A2948) and D-glucose-6-phosphate monosodium salt (A3789) were purchased from Applichem, Darmstadt, Germany. Diethylaminoethyl (DEAE)-cellulose (DE52 microgranular preswollen; 4057050) was purchased from Whatman Biochemicals Ltd., Kent, England. Benzphetamine-HCL was kindly provided by Dr. J. F. Stiver of UpJohn Co., USA. Mitomycin C was obtained from Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan. pBR322 plasmid DNA (SD0041) was purchased from Fermentas International Inc., Ontario, Canada.

All the other chemicals were of analytical grade and were obtained from commercial sources at the highest grade of purity available.

2.2 Methods

2.2.1 Preparation of Beef Liver Microsomes

The livers from well-bled healthy bovine (about 1-2 years old) were obtained from a local slaughterhouse immediately after killing. The gall bladders were removed in the slaughterhouse carefully in order to avoid the spoilage of its contents which are known to be inhibitory to monooxygenase activities. The livers were placed in plastic bags packed in crushed ice. All subsequent steps were carried out at 0-4°C or cold room. Beef liver microsomes were prepared as described by Arinç and Çelik (2002). Upon reaching the laboratory, the fatty and connective tissues were removed from livers, respectively. They were washed first with distilled water and then with cold 1.15% KCl solution to remove as much as blood as possible. After draining and blotting on a filter paper, tissues were weighed to the nearest 0.1 g and were cut into small pieces with scissors. The resulting minced tissues were homogenized in 1.15% KCl solution containing 2 mM EDTA, 0.25 mM ε-ACA and 0.1 mM PMSF using a Commercial Waring blender at high speed by blending four or five times for a period of 15 seconds each with intervals of 60-120 seconds. The volume of homogenization solution used was equal to 3 times of the weight of beef liver tissue.

Then the liver homogenates were centrifuged at 9928 rpm (10800xg) at Sigma 3K30 refrigerated centrifuge, Sigma Laborzentrifugen, Osterode am Harz, Germany by using 12159 rotor for 25 minutes to remove cell debris, nuclei and mitochondria. The supernatant fractions containing endoplasmic reticulum and other soluble fraction of the cells were filtered through two layers of cheese cloth in a Buchner funnel while avoiding the loose pellet.

The microsomes were sedimented from the supernatant solution by centrifugation at 45000 rpm (145215xg) for 50 minutes using T-880 rotor or at

40000 rpm (115632xg) for 60 minutes using A-841 rotor in Sorvall-Combi ultracentrifuge (Du Pont Company, Newton, Connecticut, USA). The supernatant fractions were discarded and the microsomal pellets were suspended in 1.15% KCl solution containing 2 mM EDTA and re-sedimented by ultracentrifugation at 145215xg for 50 minutes or at 115632xg for 60 minutes. The supernatant fractions were discarded again.

The washed microsomal pellets were resuspended in 25% glycerol containing 1 mM EDTA. For each gram of liver, 0.5 ml of suspensions were used. In order to obtain a homogenous microsomal suspension, resuspended microsomes were homogenized manually using the Teflon-glass homogenizer.

Microsomal suspensions containing approximately 35 to 40 mg protein per milliliter were gassed with nitrogen in small plastic bottles and stored at -70°C in a deep freezer for the purification of NADPH-cytochrome P450 reductase and NADH-cytochrome b5 reductase.

2.2.2 Preparation of Sheep Lung Microsomes

Sheep lung microsomes were prepared as described by Adalı and Arinç (1990). The procedure used for the preparation of sheep lung microsomes was essentially the same as the one described above for the preparation of beef liver microsomes, except that the volume of 1.15% KCl solution containing 2 mM EDTA, 0.25 mM ε-ACA and 0.1 mM PMSF used for homogenization was equal to 2.5 times of the weight of lung tissue and the volume of 25% glycerol containing 1 mM EDTA used for suspension of sedimented microsomes was 0.3 ml for each gram of lung tissue.

2.2.3 Phenobarbital Treatment of Rabbits and Preparation of Rabbit Liver Microsomes

A total of 6 male New Zealand rabbits, 3 months old and each weighing 2.5-3.5 kg were housed for 5 days under a 12 h/12 h light/dark cycle in a temperature and humidity controlled room and fed with a standard laboratory pellet chow and tap water. After adaptation to the lighting conditions for 5 days, they were divided into two groups. While the rabbits in the experimental group were treated with 0.1% (1 mg/ml) sodium phenobarbital in drinking water for seven days, the rabbits in the control group were given tap water. Approximately a total of 1.3-1.4 g sodium phenobarbital was administered orally in drinking water to each rabbit during this period (about 60-70 mg/kg body weight/day). Animals were fasted for 24 hours after last day of treatment and they were killed by decapitation. The procedures involving animals and their care were carried out in accordance with the Declaration of Helsinki.

Liver microsomes from phenobarbital-treated rabbits were prepared by differential centrifugation as described by Adalı and Arinç (1990). The livers, each weighing 70-100 g, were removed immediately after killing and gall bladders were removed carefully. They were washed first with distilled water and then with cold 1.15% KCl solution to remove excess blood. The connective and fatty tissues were removed. The procedure used for the preparation of phenobarbital-treated rabbit liver microsomes was essentially the same as the one described above for the preparation of beef liver microsomes.

2.2.4 Purification of Beef Liver NADPH-Cytochrome P450 Reductase

NADPH-cytochrome P450 reductase of beef liver microsomes was purified to apparent homogeneity from the detergent solubilized microsomes according to the method described by Arinç and Çelik (2002) with slight modifications. The purification procedure involved anion exchange chromatography of the detergent

solubilized microsomes on two successive DEAE-cellulose columns, affinity chromatography of the partially purified reductase on adenosine 2', 5'-diphosphate-Sepharose 4B column and further concentration and purification of the reductase on a final hydroxylapatite column. The key step in purification of beef liver microsomal cytochrome P450 reductase was the affinity chromatography on adenosine 2', 5'-diphosphate-Sepharose 4B as first introduced by Yasukochi and Masters (1976). The overall procedure used to purify NADPH-cytochrome P450 reductase from beef liver microsomes is given in Figure 2.1. During washing step in first DEAE-cellulose column chromatography, the eluted fractions containing high quantities of NADH-cytochrome b5 reductase activity and low cytochrome P450 content were pooled and used for the subsequent purification of NADH-cytochrome b5 reductase.

2.2.5 Purification of Beef Liver Microsomal NADH-Cytochrome b5 Reductase

Since some difficulties were encountered during the purification of NADH-cytochrome b5 reductase from phenobarbital-treated rabbit liver microsomes, NADH-cytochrome b5 reductase was purified from detergent solubilized beef liver microsomes by slight modifications of the methods described by Güray and Arinç (1990) and Arinç and Çakir (1999) for the purification of sheep lung and sheep liver NADH-cytochrome b5 reductases, respectively. The overall procedure used to purify NADH-cytochrome b5 reductase from beef liver microsomes is given in Figure 2.2. NADH-cytochrome b5 reductase was purified from the same crude microsomal sample that was used for the purification of NADPH-cytochrome P450 reductase from beef liver as described before. Thus the first two steps – solubilization and first DEAE-cellulose column chromatography – in the purification of both NADPH-cytochrome P450 reductase and NADH-cytochrome b5 reductase were identical. The overall procedure for the purification of NADH-cytochrome b5 reductase from detergent solubilized beef liver microsomes involved two successive anion-exchange chromatography using DEAE-cellulose and affinity chromatography on adenosine 5'-diphosphate-agarose. The key step in the purification procedure was the use of adenosine 5'-diphosphate-agarose affinity column chromatography.

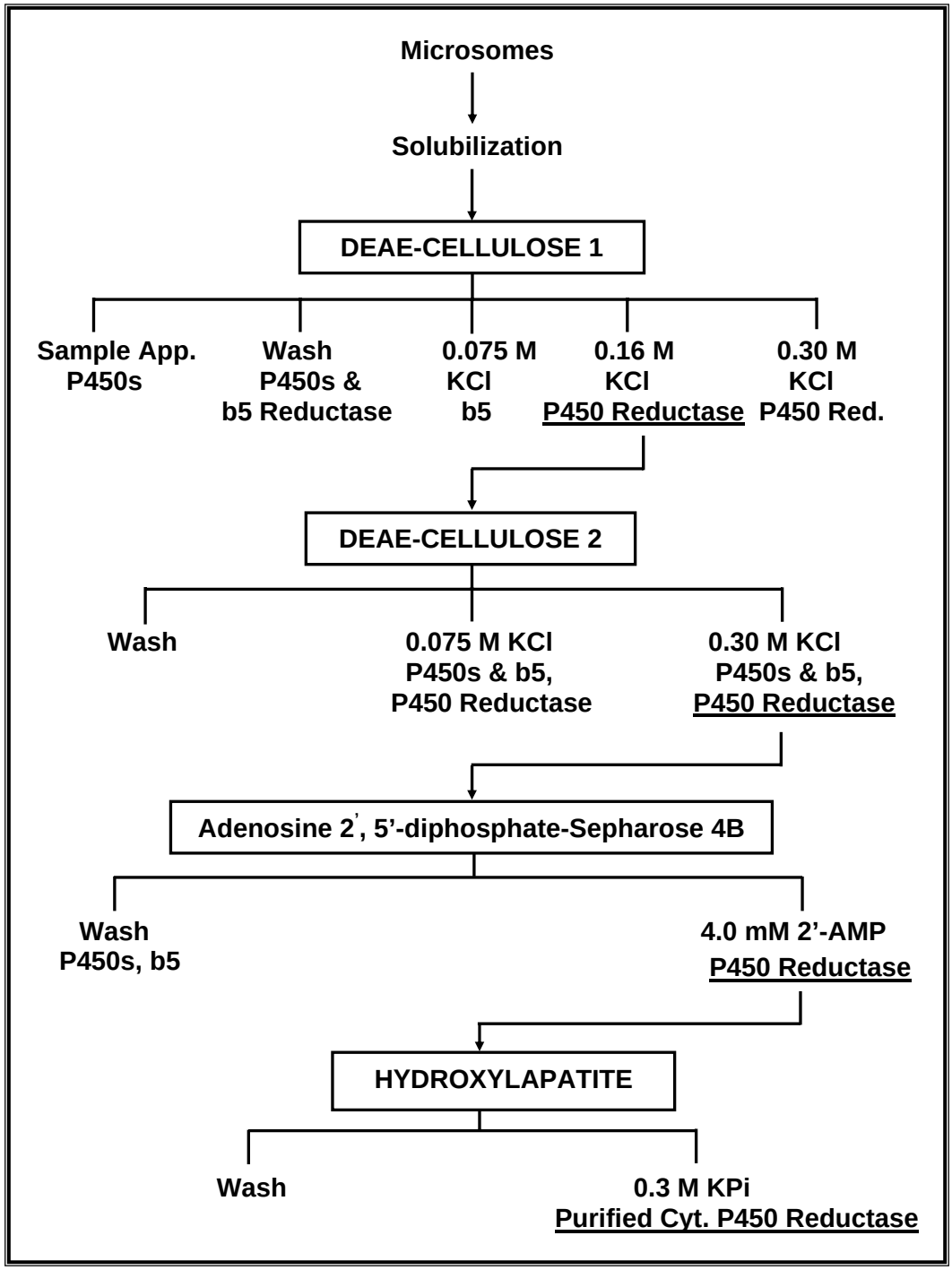


Figure 2.1 Flow chart for the purification of NADPH-cytochrome P450 reductase from beef liver microsomes

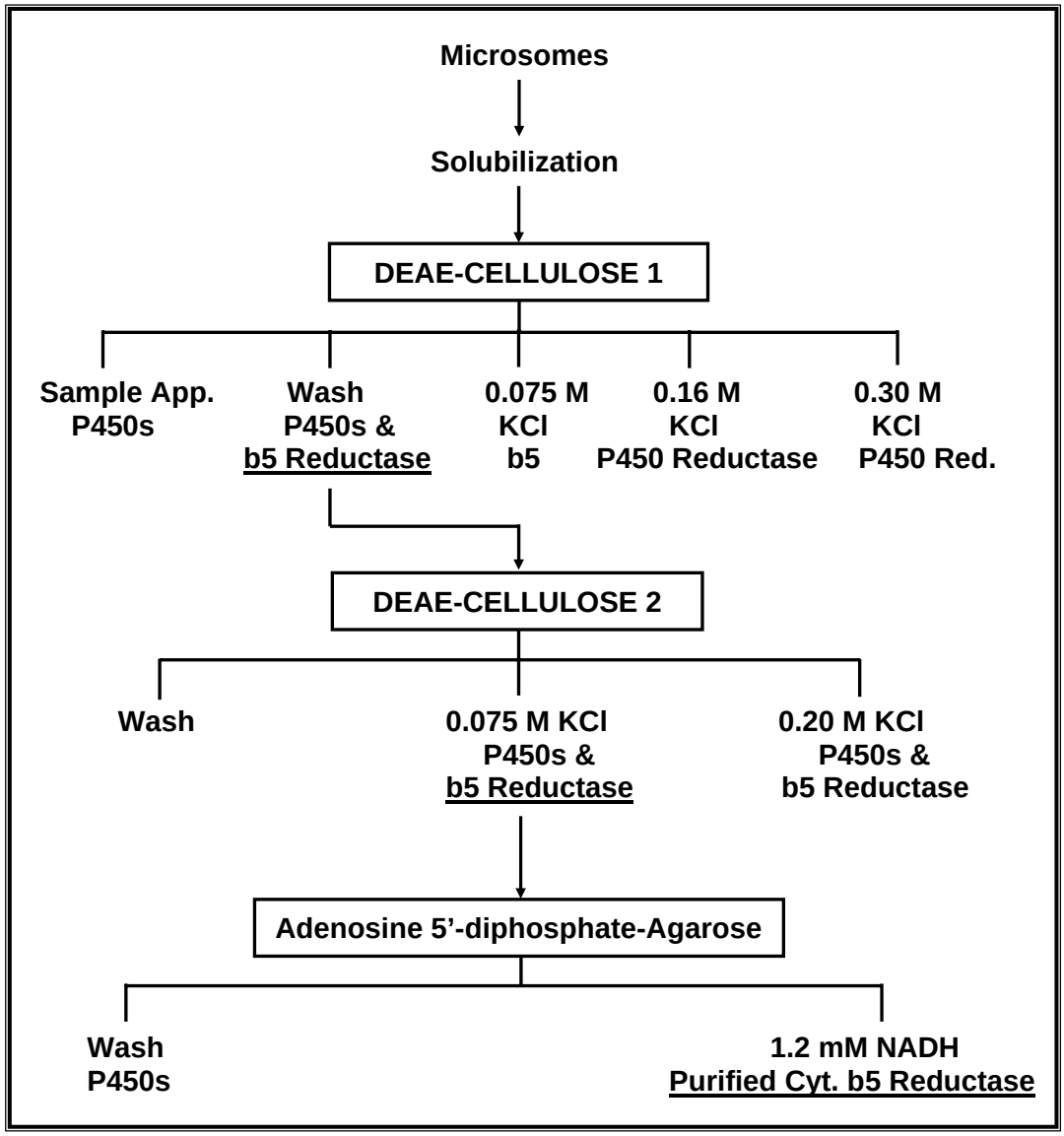


Figure 2.2 Flow chart for the purification of NADH-cytochrome b5 reductase from beef liver microsomes

2.2.6 Purification of Cytochrome P4502B4 from Phenobarbital-Treated Rabbit Liver Microsomes

Cytochrome P4502B4 (LM2) was purified from phenobarbital-treated rabbit liver microsomes by slight modifications of the method used before in our laboratory as described by Adalı and Arinç (1990). The method described by Adalı and Arinç (1990) was developed from the combination of the procedures of Arinç and Philpot (1976), Haugen and Coon (1976) and Wolf *et al.* (1980) with some modifications. The purification procedure involved anion exchange chromatography of the detergent solubilized and polyethylene glycol fractionated rabbit liver microsomes on an initial DEAE-cellulose column, adsorption chromatography on first hydroxylapatite column and hydrophobic interaction chromatography on octyl-Sepharose CL-4B column. Partially purified cytochrome P4502B4 obtained from octyl-Sepharose CL-4B column was then subjected to a second hydroxylapatite column which was followed by anion exchange chromatography on a second DEAE-cellulose column. Finally, cytochrome P4502B4 was further purified and concentrated on a third hydroxylapatite column. The overall procedure used to purify cytochrome P4502B4 from phenobarbital-treated rabbit liver microsomes is outlined in Figure 2.3. It has been reported that rabbit liver and lung microsomal CYP2B4 have similar structural, biocatalytical and immunological properties (for a review see Arinç, 1993).

2.2.7 Purification of NADPH-Cytochrome P450 Reductase from Phenobarbital-Treated Rabbit Liver Microsomes

NADPH-cytochrome P450 reductase from phenobarbital-treated rabbit liver microsomes was purified from the detergent solubilized microsomes by slight modifications of already existing methods developed in our laboratory for the purification of NADPH-cytochrome P450 reductases from a variety of tissues and species (İşcan and Arinç, 1986; İşcan and Arinç, 1988; Arinç and Aydoğmuş, 1990; Şen and Arinç, 1998; Arinç and Çelik, 2002). The overall procedure used to purify

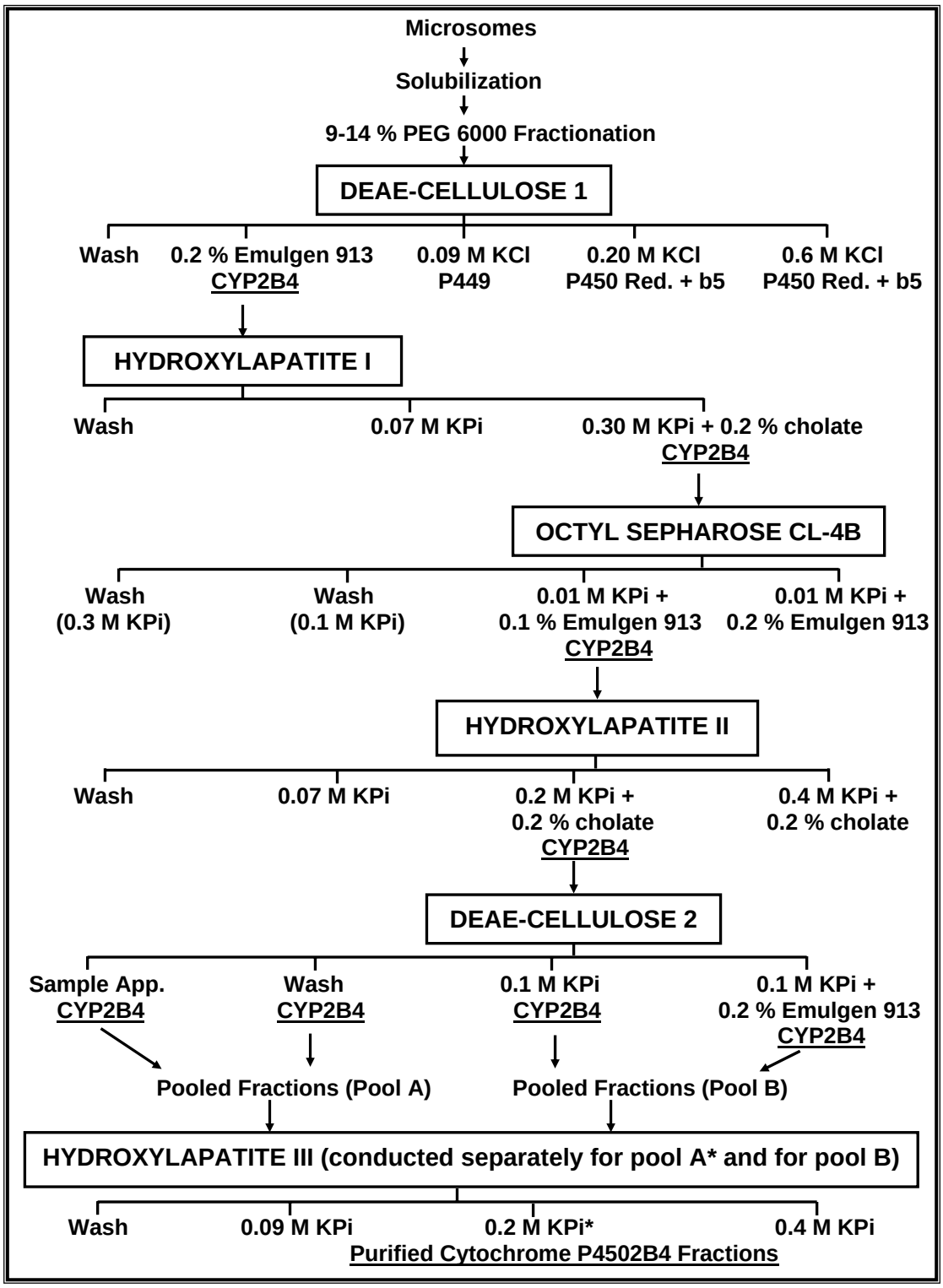


Figure 2.3 Flow chart for the purification of cytochrome P4502B4 from phenobarbital-treated rabbit liver microsomes. * The highly purified CYP2B4 fraction eluted from the third HTP column (loaded with pool A) with 0.2 M KPi was used in all the experiments.

NADPH-cytochrome P450 reductase from phenobarbital-treated rabbit liver microsomes is outlined in Figure 2.4. During the elution step in first DEAE-cellulose column chromatography, fractions containing high cytochrome b5 content and low quantities of NADPH-cytochrome P450 reductase activity were pooled and used for the subsequent purification of cytochrome b5.

2.2.8 Purification of Microsomal Cytochrome b5 from Phenobarbital-Treated Rabbit Liver

Purification of cytochrome b5 from phenobarbital-treated rabbit liver microsomes was accomplished according to the combination of the methods described by Başaran and Arinç (1998) and Arinç and Çakir (1999) for the purification of cytochrome b5 from sheep lung and liver microsomes, respectively, with slight modifications. The method involved the use of three successive DEAE-cellulose column and Sephadex G-100 column chromatographies in the presence of detergents Emulgen 913 and cholate. Cytochrome b5 was purified from the same crude microsomal sample that was used for the purification of NADPH-cytochrome P450 reductase from phenobarbital-treated rabbit liver microsomes as described before. Thus, the first two steps – solubilization and first DEAE-cellulose column chromatography – in the purification of both cytochrome b5 and NADPH-cytochrome P450 reductase were identical. The overall procedure used to purify cytochrome b5 from phenobarbital-treated rabbit liver microsomes is outlined in Figure 2.5.

2.2.9 Purification of Sheep Lung NADPH-Cytochrome P450 Reductase

The purification of NADPH-cytochrome P450 reductase from detergent solubilized sheep lung microsomes was achieved by slight modifications of the already existing methods developed in our laboratory for the purification of sheep lung NADPH-cytochrome P450 reductase (İşcan and Arinç; 1986 and 1988; Arinç

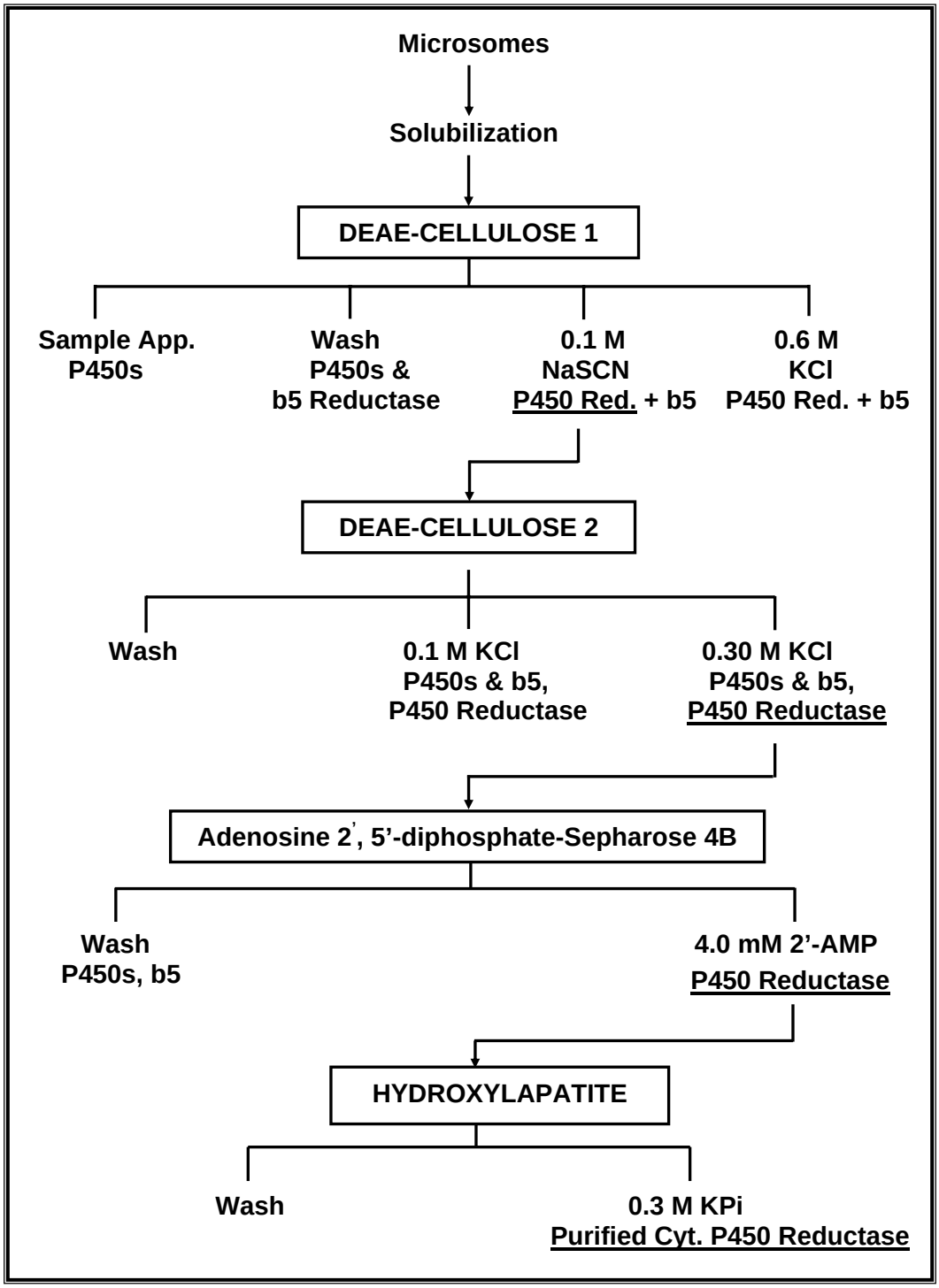


Figure 2.4 Flow chart for the purification of NADPH-cytochrome P450 reductase from phenobarbital-treated rabbit liver microsomes

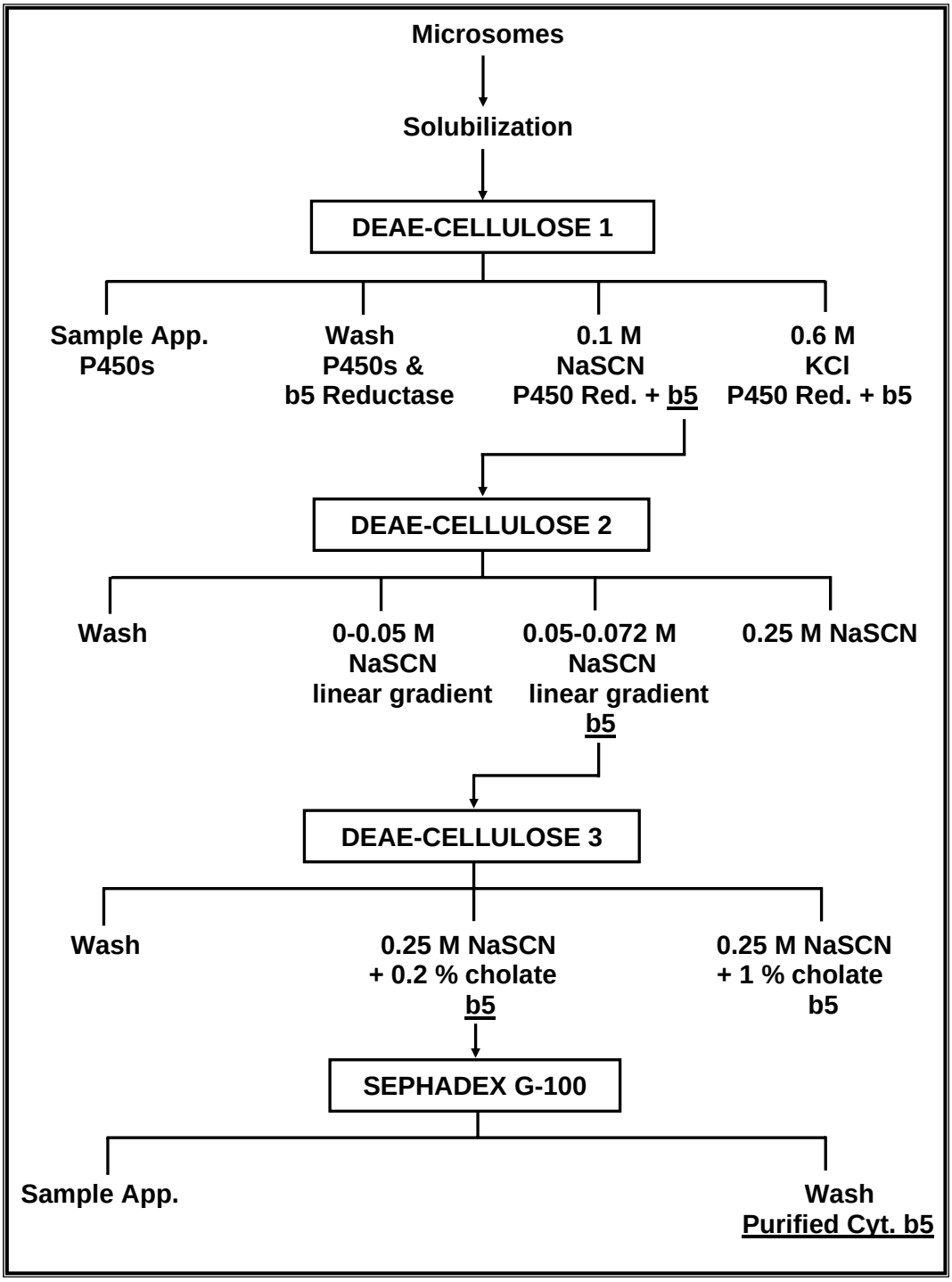


Figure 2.5 Flow chart for the purification of cytochrome b5 from phenobarbital-treated rabbit liver microsomes

and Aydoğmuş; 1990). Except some modifications, the key steps in the purification of sheep lung NADPH-cytochrome P450 reductase were essentially the same as those described above for the purification of beef liver and rabbit liver P450 reductases as shown in Figure 2.1 and Figure 2.4, respectively.

2.2.10 Analytical Procedures

2.2.10.1 Protein Determinations

The protein concentrations of the microsomes and of the purified or partially purified fractions obtained at various stages of purification studies were determined using the method described by Lowry *et al*. (1951). The samples were initially diluted and aliquots of 0.1 ml, 0.25 ml and 0.5 ml taken from the appropriately diluted samples were poured into test tubes. Their volumes were then completed to a final volume of 0.5 ml with distilled water. After that, they were mixed with 2.5 ml of alkaline copper reagent which was prepared by mixing 2% copper sulfate, 2% sodium potassium tartrate and 0.1 N NaOH containing 2% sodium carbonate in a ratio of 1:1:100 in their written order. All the tubes were let stand 10 minutes at room temperature (24-25°C) for copper reaction to take place in alkaline medium. Then, 2 N Folin-Phenol reagent was diluted with distilled water with a ratio of 1:1 and 0.25 ml of this reagent was added to each tube and mixed immediately within 8 seconds by vortex. The tubes were incubated for 30 minutes at room temperature for color development. The intensity of color developed was measured at 660 nm. Finally, a standard curve was constructed using crystalline bovine serum albumin at five different concentrations (20, 50, 100, 150 and 200 μg) in order to calculate the unknown protein concentrations of the samples.

2.2.10.2 Determination of Cytochrome P450 Content

The concentrations of cytochrome P450 either in microsomes or in purified fractions were determined according to the method of Omura and Sato (1964). Cytochrome P450 content of the samples was measured by the detection of a peak around 447-452 nm when samples were reduced and gassed with CO using Hitachi U-2800 UV/Vis double beam recording spectrophotometer (Hitachi Ltd., Tokyo, Japan) with cuvettes of 1.0 cm light path length.

Aliquots of individual fractions obtained at various stages of purification studies and of purified samples (0.1 – 2.0 ml) were poured into test tubes and diluted to 6.0 ml with cytochrome P450 dilution buffer (0.1 M potassium phosphate buffer, pH 7.5 containing 30% glycerol and 1 mM EDTA). If the sample was microsomes, 0.2-0.5 ml aliquots were diluted to 5.4 ml with cytochrome P450 dilution buffer and then 0.6 ml cholate from 10% stock solution was added to get 1% final concentration. The tube content was then mixed well by vortex. The diluted sample was divided equally into two cuvettes and then placed both in sample and reference chambers of spectrophotometer and baseline was recorded. Carbon monoxide was then bubbled through the sample in sample cuvette for about 20 seconds and the samples in both cuvettes were reduced by the addition of a pinch of sodium dithionite, $Na_2S_2O_4$. Finally, CO was bubbled again through the reduced sample (Fe^{+2}) in sample cuvette for about additional 40 seconds and the CO-difference spectrum [(Fe^{+2} –CO)– (Fe^{+2})] was recorded at 7^{th} or 8^{th} minutes after bubbling.

The cytochrome P450 amounts were calculated by measuring the absorbance difference between 450 nm and 490 nm in the CO-induced difference spectrum samples using an extinction coefficient of 91 $mM^{-1}.cm^{-1}$ (Omura and Sato, 1964).

2.2.10.3 Determination of NADPH-Cytochrome P450 Reductase Activity

NADPH dependent cytochrome P450 reductase activity was measured spectrophotometrically by the method of Masters *et al.* (1967) except that the reaction was carried out in 0.3 M potassium phosphate buffer, pH 7.7 at 25°C.

The assay depends on the measurement of the rate of reduction of artificial substrate, cytochrome *c*, at 550 nm. Reaction mixture contained 0.7 ml of cytochrome *c* (final concentration: 80.2 nmol per 0.775 ml reaction mixture; stock: 1.1 mg cytochrome *c* per ml in 0.3 M potassium phosphate buffer, pH 7.7), 0.025 ml NADPH (final concentration: 127.7 nmol per 0.775 ml of reaction mixture; stock: freshly prepared 3.3 mg NADPH per ml in cold distilled water) and appropriate amounts of cytochrome P450 reductase sample (0.01-0.05 ml). Before the addition of NADPH, baseline was recorded. The reaction was then initiated by the addition of NADPH and followed for 60-120 seconds at 550 nm at 25°C using Hitachi U-2800 double beam spectrophotometer with cuvettes of 1.0 cm light path length. The enzyme activities were calculated using the extinction coefficient of 19.6 $mM^{-1}.cm^{-1}$ for the difference in absorbance between the reduced minus the oxidized form of cytochrome *c* at 550 nm as described by Yonetani (1965). One unit of reductase is defined as the amount of enzyme catalyzing the reduction of 1.0 μmole of cytochrome *c* per minute under the above conditions.

2.2.10.4 Determination of Cytochrome b5 Content

The method described by Nishibayashi and Sato (1968) was used for the determination of cytochrome b5 concentrations of individual fractions and of purified samples. Aliquots of samples were diluted to 6.0 ml with 0.1 M potassium phosphate buffer, pH 7.5 containing 1 mM EDTA and divided equally into two cuvettes placed into sample and reference chambers of the spectrophotometer. After recording the baseline, the sample in sample cuvette was reduced by the addition of a

pinch of solid sodium dithionite and dithionite-reduced minus oxidized difference spectrum of cytochrome b5 was recorded immediately using Hitachi U-2800 double beam spectrophotometer with cuvettes of 1.0 cm light path length.

The concentration of cytochrome b5 was estimated from the initial dithionite-reduced minus oxidized difference spectrum using an extinction coefficient of 185 $mM^{-1}.cm^{-1}$ for the difference in absorption between 424 nm and 410 nm.

2.2.10.5 Determination of NADH-Ferricyanide Reductase Activity

The NADH-ferricyanide reductase activity was measured spectrophotometrically according to the procedure reported by Strittmatter and Velick (1957) by measuring the rate of potassium ferricyanide reduction at 420 nm at 25°C.

The reaction mixture contained 0.9 ml of 0.1 M potassium phosphate buffer, pH 7.5, 0.04 ml of 3 mM NADH (final concentration: 0.12 mM), 0.04 ml of 5 mM potassium ferricyanide (final concentration: 0.2 mM) and appropriate amounts of microsomal enzyme in a final volume of 1.0 ml. The reaction was started by the addition of NADH and the reduction of ferricyanide was followed by recording the absorbance decrease at 420 nm using Hitachi U-2800 double beam spectrophotometer with cuvettes of 1.0 cm light path length. Since potassium ferricyanide was also reduced with NADH chemically without the enzyme, controls were carried out under similar conditions and the reaction rate was corrected by subtracting the background value from the rate of enzymatic reaction. The enzyme activity was calculated using the extinction coefficient of 1.02 $mM^{-1}.cm^{-1}$ for the difference in absorbance between the reduced minus the oxidized form of ferricyanide at 420 nm. One unit of enzyme activity is defined as the amount of enzyme causing the reduction of 1.0 μmole of potassium ferricyanide per minute under above conditions.

2.2.10.6 Determination of NADH-Cytochrome b5 Reductase Activity

The biocatalytic activity of the purified beef liver NADH-cytochrome b5 reductase was determined according to its ability to catalyze the reduction of purified rabbit liver cytochrome b5 in the presence of NADH cofactor. The assay was based upon the measurement of reduction of cytochrome b5 at 423 nm (Strittmatter and Velick, 1956). The reaction medium contained 0.1 M potassium phosphate buffer, pH 6.8 or pH 7.5, 0.111 mM NADH, 0.01% Emulgen 913, 2.0 nmol of purified rabbit liver cytochrome b5 and 0.036 units (based on ferricyanide reduction) of purified beef liver cytochrome b5 reductase in a final volume of 1.0 ml at 25°C. The enzyme activity was calculated by taking the molar extinction coefficient increment between the reduced and the oxidized form of cytochrome b5 as 100 $mM^{-1}.cm^{-1}$ (Strittmatter and Velick, 1956). One unit of reductase is defined as the amount of enzyme catalyzing the reduction of 1.0 nmol of cytochrome b5 per minute under the described conditions, unless otherwise indicated.

2.2.10.7 Determination of NADH-Cytochrome *c* Reductase Activity

The NADH-cytochrome *c* reductase activity of purified beef liver NADH-cytochrome b5 reductase was measured spectrophotometrically by following the cytochrome b5 coupled reduction of cytochrome *c* at 550 nm at 25°C. The reaction mixture contained 0.3 M potassium phosphate buffer, pH 7.5, 0.111 mM NADH, 89 nmol of cytochrome *c*, 0.35 nmol of purified rabbit liver cytochrome b5 and appropriate amounts of purified beef liver cytochrome b5 reductase (0.05 or 0.1 units based on ferricyanide reduction) in a final volume of 1.0 ml. The molar extinction coefficient for cytochrome *c* was taken as 19.6 $mM^{-1}.cm^{-1}$ (Yonetani, 1965). One unit of reductase is defined as the amount of enzyme catalyzing the reduction of 1.0 μmole of cytochrome *c* per minute under the described conditions, unless otherwise indicated.

2.2.10.8 Determination of the Total Flavin Content of the Purified NADPH-Cytochrome P450 Reductases

The concentrations of the highly purified NADPH-cytochrome P450 reductases were determined by measuring the total flavin content of the enzymes spectrophotometrically according to the method of Cerletti and Siliprandi (1958) assuming that the purified enzyme contained equal amounts of FMN and FAD. The total flavin content was calculated using an extinction coefficient of 11.3 $mM^{-1}.cm^{-1}$. The absorbance values used in calculation were the difference between the absorbance at 450 nm and 600 nm.

2.2.10.9 Preparation of Dilauroyl Phosphatidylcholine Vesicles for Reconstitution Studies

Dilauroyl phosphatidylcholine (DLPC) micelles were prepared in order to create a lipid membrane environment for reconstitution studies as described below:

In a typical preparation, the lipid vesicles were prepared by suspending 2.0 mg DLPC in 1.0 ml of 0.05% cholate. In order to obtain lipid micelles, the suspension was subjected to sonication five times for a period of 20 seconds each, with 15 seconds intervals between sonications, at 50 watt output in an ice bath using a sonic dismembrator (Fisher Scientific Artec Systems Corporation, Farmingdale, New York, USA) equipped with a suitable microtip.

2.2.10.10 Determination of Benzphetamine N-Demethylase Activity in Reconstituted Systems Containing Purified Beef Liver Cytochrome P450 Reductase and Rabbit Liver CYP2B4

The biocatalytic activities of highly purified beef liver NADPH-cytochrome P450 reductase and rabbit liver cytochrome P4502B4 were determined in

reconstituted systems in the presence of dilauroyl phosphatidylcholine as a synthetic lipid according to the ability of beef liver cytochrome P450 reductase to transfer electrons from NADPH to rabbit liver cytochrome P4502B4 which in turn catalyze the N-demethylation reaction of benzphetamine (Figure 2.6). The method described by Nash (1953) and modified by Cochin and Axelrod (1959) was used for the determination of benzphetamine N-demethylase activity in reconstituted systems by measuring the quantity of formaldehyde formed.

Cytochrome P4502B4 and cytochrome P450 reductase were reconstituted in the presence of 0.08 mg/ml DLPC and preincubated at room temperature (24-25°C) for 20 minutes with occasional shaking.

A typical reaction mixture contained 100 mM HEPES buffer, pH 7.7, 1.5 mM benzphetamine-HCL, reconstituted system and 0.5 mM NADPH generating system in a final volume of 0.5 ml as shown in Table 2.1. NADPH generating system was prepared by adding 0.25 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 $NADP^+$. The test tube containing the generating system then was incubated at 37ºC for 5 minutes. One unit of glucose-6-phosphate dehydrogenase is defined as the amount of enzyme reducing 1 μmole of $NADP^+$ in one minute at 25ºC. A 0.5 mM freshly prepared formaldehyde solution was used as standard. Standards at four concentrations (6.25, 12.5, 25.0 and 50.0 nmol) were prepared and were made up to 0.5 ml with distilled water and were run under the same conditions.

The reaction was initiated by the addition of 0.075 ml NADPH generating system to incubation mixtures. 0.5 ml of 0.75 N perchloric acid was added to zero time blank tubes before the addition of cofactor. The reaction was carried out at 37°C under the air with constant and moderate shaking in a shaking water bath. After exact period of 10 minutes, enzymatic reaction was stopped by the addition of 0.5 ml of 0.75 N perchloric acid solution. The contents of the tubes were transferred into eppendorf tubes which were then spin down by centrifugation at 14000xg for 20

Table 2.1 A typical constituents of the reaction mixture for reconstitution of benzphetamine N-demethylase activity

Constituents	Stock Solutions	Volume to be taken (µl)	Final Concentration in 0.5 ml Reaction Mixture
Reconstitution System:			
Cytochrome P450	Rabbit Liver P4502B4 (19.1 nmol/ml)	5 or 10 µl	0.1 or 0.19 nmol
Reductase	Beef Liver Reductase (5.56 units/ml)	18 or 36 µl	0.1 or 0.2 units
Lipid	2 mg/ml	20	0.04 mg
HEPES Buffer, pH 7.7	400 mM	125	100 mM
Benzphetamine-HCl	7.5 mM	100	1.5 mM
NADPH Generating System:			
Glucose-6-phosphate	100 mM	12.5	2.5 mM
$MgCl_2$	100 mM	12.5	2.5 mM
HEPES Buffer, pH 7.8	200 mM	36.5	14.6 mM
NADP+	20 mM	12.5	0.5 mM
Glucose-6-phosphate dehydrogenase	365 units/mg protein 4.8 mg protein/ml	0.14	0.25 units
Distilled water		to 0.5 ml	

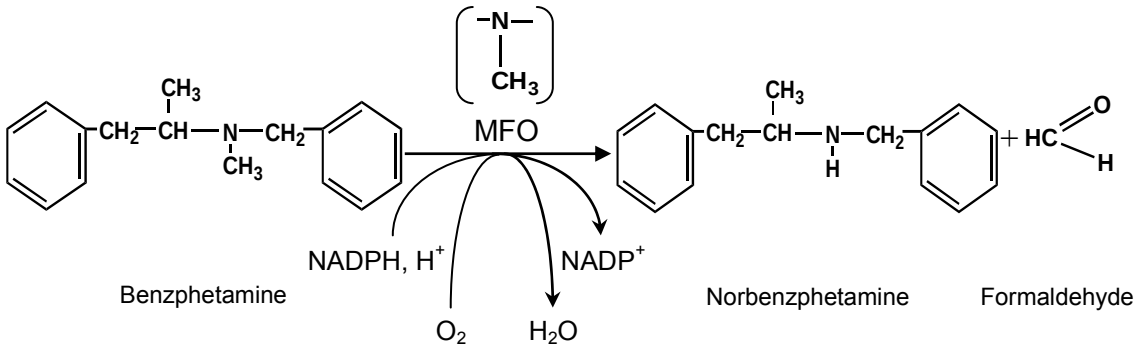


Figure 2.6 Benzphetamine N-demethylation reaction catalyzed by cytochrome P450 dependent monooxygenases

minutes, using Thermo Scientific Microlite RF refrigerated microcentrifuge (Thermo Scientific, Milford, Massachusetts, USA) in order to remove denatured proteins.

Finally, 0.5 ml aliquots of supernatant solution were transferred to test tubes and were mixed with freshly prepared 0.375 ml Nash reagent (prepared by the addition of 0.1 ml of acetylacetone just before use to 25 ml solution containing 7.7 g ammonium acetate and 0.15 ml of glacial acetic acid). The mixture was incubated 10 minutes at 50°C in a water bath and the intensity of yellow color developed was measured at 412 nm using Schimadzu UV-1201 spectrophotometer (Schimadzu Co., Analytical Instruments Division, Kyoto, Japan). A standard calibration curve was constructed and used for calibration of enzyme activities.

Figure 2.6 shows the benzphetamine N-demethylase reaction catalyzed by cytochrome P450 dependent monooxygenases.

2.2.10.11 Determination of Idarubicin and Mitomycin C Reduction Rates by Phenobarbital-Treated and Untreated Rabbit Liver Microsomes in the Presence of Cofactor NADPH and by Purified NADPH-Cytochrome P450 Reductases under Aerobic Conditions

The initial rates of idarubicin and mitomycin C reduction in phenobarbital-treated and untreated rabbit liver microsomes and of highly purified NADPH-cytochrome P450 reductases were determined by measuring NADPH oxidation at 340 nm based on the methods of Kumagai *et al.* (1997), Goeptar *et al.* (1993) and Ramji *et al.* (2003). The reactions catalyzed by P450 reductase in the presence of either drug and cofactor NADPH are shown in Figure 2.7. All reactions were performed at 25°C under aerobic conditions. The reaction mixture contained 0.3 M potassium phosphate buffer, pH 7.7, 0.1 mM EDTA, pH 7.7, idarubicin (25 μM, dissolved in distilled water) or mitomycin C (25 μM, dissolved in distilled water), 0.2 mg microsomal protein or appropriate amounts of purified cytochrome P450 reductases and 0.1 mM NADPH in a final volume of 1.0 ml. The reactions were initiated by the addition of NADPH cofactor. Reduction of drugs was measured by following NADPH consumption at 340 nm in a Hitachi U-2800 double beam spectrophotometer using an extinction coefficient of 6.22 $mM^{-1}.cm^{-1}$. Control incubations were carried out by performing identical incubations without enzyme or either drug. Reaction rates were corrected by subtracting the very low rates of NADPH consumption catalyzed by the purified P450 reductases measured in the absence of drugs.

If microsomes were used as enzyme source, the non-ionic detergent Triton X-100 was added to incubation mixtures at a final concentration of 0.5% in order to eliminate the contribution of cytochrome P450 isozymes to NADPH consumption and thus to determine the microsomal cytochrome P450 reductase-dependent cofactor oxidation (Kumagai *et al.*, 1997). Moreover, mitomycin C or idarubicin reduction rates in phenobarbital-treated or untreated rabbit liver microsomes were corrected further by subtracting microsomal NADPH consumptions measured in the absence of drugs (if any) from microsomal NADPH consumptions measured in the

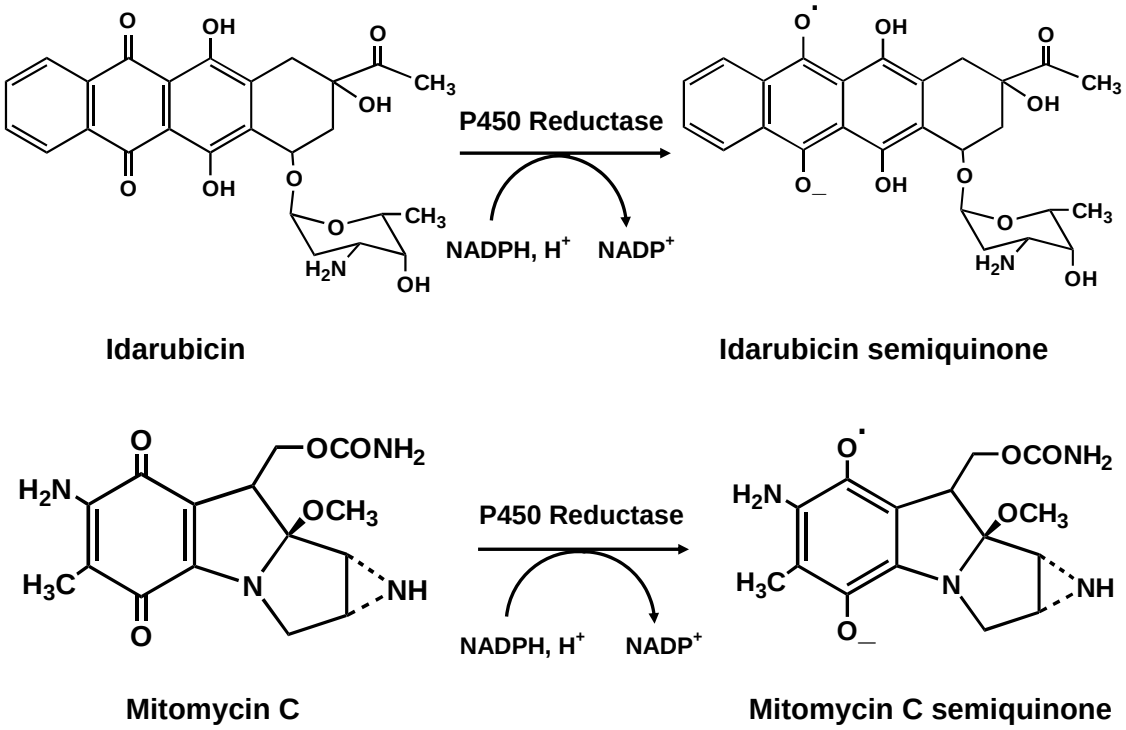


Figure 2.7 NADPH-cytochrome P450 reductase-catalyzed reduction of idarubicin and mitomycin C in the presence of cofactor NADPH

presence of drugs (Ramji *et al.,* 2003). The calculations of microsomal NADPH consumptions in the presence or absence of anticancer drugs are explained below;

Microsomal NADPH oxidations in the absence of mitomycin C or idarubicin were calculated by subtracting the reaction rates measured in samples lacking both microsomes and anticancer drugs from the reaction rates measured in samples lacking only anticancer drugs. Microsomal NADPH consumptions in the presence of mitomycin C or idarubicin were calculated by subtracting the reaction rates measured in samples lacking microsomes from the reaction rates measured in the complete incubation mixtures (Ramji *et al*., 2003).

2.2.10.12 Determination of Idarubicin and Mitomycin C Reduction Rates by Phenobarbital-Treated and Untreated Rabbit Liver Microsomes in the Presence of Cofactor NADH and by Purified Beef Liver NADH-Cytochrome b5 Reductase under Aerobic Conditions

The same method described above was used for the determination of initial rates of idarubicin and mitomycin C reduction in phenobarbital-treated and untreated microsomes under aerobic conditions in the presence of NADH cofactor except that, 0.1 M potassium phosphate buffer, pH 7.5 was used in all incubation mixtures and 0.1 mM EDTA was excluded from the reaction mixture. For determination of the microsomal cytochrome b5 reductase-dependent cofactor oxidation, the non-ionic detergent Triton X-100 was also added to the incubation mixtures at a final concentration of 0.5% in order to block the contribution of cytochrome b5 to NADH consumption (Kumagai *et al*., 1997). While different concentrations of microsomal protein (0.02-0.8 mg) and mitomycin C (12.5 to 200 μM) were tested in order to be able to measure the reduction of mitomycin C in phenobarbital-treated and untreated microsomes, only microsomal protein at 0.4 mg amount and idarubicin at 25.0 μM concentration were tested in reaction mixtures to measure the reduction of idarubicin.

When highly purified beef liver NADH-cytochrome b5 reductase was used as enzyme source, the reaction mixture contained 10 mM potassium phosphate buffer, pH 6.6 or pH 7.5, idarubicin (12 μM) or mitomycin C (25 μM), appropriate amounts of purified beef liver NADH-cytochrome b5 reductase and 0.1 mM NADH cofactor in a final volume of 1.0 ml. The idarubicin or mitomycin C reduction rate of the purified beef liver NADH-cytochrome b5 reductase was expressed as the disappearance of NADH cofactor measured at 340 nm based on oxidation of NADH to NAD^+, in a Hitachi U-2800 double beam spectrophotometer using an extinction coefficient of 6.22 $mM^{-1}.cm^{-1}$ (Hodnick and Sartorelli, 1993 and 1994). All reactions were performed at 25°C under aerobic conditions. Control incubations in which enzyme or either drug omitted were also carried out under identical conditions.

Reaction rates were corrected by subtracting the very low rates of NADH oxidation catalyzed by the purified b5 reductase measured in the absence of anticancer drugs.

2.2.10.13 Determination of Idarubicin and Mitomycin C Reduction Rates in Reconstituted Systems Containing Purified Rabbit Liver Cytochrome P450 Reductase and Rabbit Liver CYP2B4 under Aerobic Conditions

The initial rates of idarubicin and mitomycin C reduction were determined in reconstituted systems containing highly purified rabbit liver cytochrome P450 reductase, cytochrome P4502B4 and artificial lipid dilauroyl phosphatidylcholine based on the method of Goeptar *et al.* (1993) with some modifications. The reaction velocities were expressed as the disappearance of NADPH cofactor measured at 340 nm spectrophotometrically as described above. All reactions were performed at 25°C under aerobic conditions. The reaction mixture contained 0.3 M potassium phosphate buffer, pH 7.5, 0.5 mM EDTA pH 7.5, 5.0 mM $MgCl_2$, 0.2 mM NADPH and appropriate amounts of reconstituted enzymes in 2.0 ml. Reconstituted systems were prepared by mixing the appropriate amounts of purified rabbit liver cytochrome P4502B4 and cytochrome P450 reductase with dilauroyl phosphatidylcholine (50 μg/ml) in a test tube. The test tube was incubated at room temperature (24-25°C) for 6 minutes with thorough mixing. Then, all reaction constituents except substrate were added to it. After mixing the content of the tube, it was divided equally into two cuvettes which were then placed in sample and reference chambers of spectrophotometer and baseline was recorded. The reaction was then started by the addition of either drug (40 μM) to sample cuvette and the same volume of water was added to reference cuvette to complete the cuvette content to 1.0 ml. The rate of NADPH oxidation at 340 nm was recorded for several minutes and the activities were calculated using an extinction coefficient of 6.22 $mM^{-1}.cm^{-1}$.

2.2.10.14 Determination of Mitomycin C Reduction Rates in Reconstituted Systems Containing Purified Beef Liver Cytochrome P450 Reductase and Rabbit Liver CYP2B4 under Anaerobic Conditions

The reduction of mitomycin C in reconstituted systems containing highly purified beef liver cytochrome P450 reductase, rabbit liver cytochrome P4502B4 and artificial lipid dilauroyl phosphatidylcholine was assayed spectrophotometrically under anaerobic conditions at 25°C by measuring the decrease in absorbance at 375 nm based on disappearance of the quinone moiety of the drug in a Hitachi U-2800 double beam spectrophotometer using an extinction coefficient of 13.2 $mM^{-1}.cm^{-1}$ (Belcourt *et al.,* 1998a; Jiang *et al.,* 2000). The constituent of the reaction mixture was the same as written in Section 2.2.10.13 except that reconstituted system contained beef liver cytochrome P450 reductase instead of the rabbit liver cytochrome P450 reductase. The enzymes were incubated in a test tube for 6 minutes at room temperature (24-25°C) for reconstitution with thorough mixing as mentioned in the previous section. Then, all reaction constituents except substrate were added to the test tube. After mixing the content of the tube, it was divided equally into two cuvettes. The content of the sample cuvette was then made anaerobic. Anaerobic conditions were achieved by flushing the reaction mixture in anaerobic sealable cuvettes (Hellma GmbH & Co., Müllheim, Germany) with pure nitrogen gas containing less than 5 ppm of oxygen for 10 minutes at 25°C before the initiation of the reaction. The reaction mixture was flushed with N_2 gas by puncturing the silicone septum of the cuvette with 18-gauge (inflow) and 26-gauge (outflow) needles. Both cuvettes were then placed in sample and reference chambers of spectrophotometer and baseline was recorded. Mitomycin C solution was also made anaerobic in the same way in a separate anerobic cuvette. The reaction was then started by the addition of mitomycin C to the sample cuvette (40 μM) without interrupting hypoxia by using a Hamilton syringe previously flushed with anaerobic water. The same volume of water was added to reference cuvette to complete the cuvette content to 1.0 ml. The rate of mitomycin C reduction at 375 nm was recorded for several

minutes and the activities were calculated using an extinction coefficient of 13.2 $mM^{-1}.cm^{-1}$.

2.2.10.15 Determination of Idarubicin Reduction Rates in Reconstituted Systems Containing Purified Beef Liver Cytochrome P450 Reductase and Rabbit Liver CYP2B4 under Anaerobic Conditions

Idarubicin reduction rates were also measured in reconstituted systems containing highly purified beef liver cytochrome P450 reductase, rabbit liver cytochrome P4502B4 and artificial lipid dilauroyl phosphatidylcholine based on the method of Goeptar *et al.* (1993) with some modifications under similar conditions as written in Section 2.2.10.13 except that the reactions were carried out under anaerobic conditions. The achievement of anaerobic conditions is described in Section 2.2.10.14. The reactions were started by the addition of idarubicin, which was made anaerobic in a separate vessel, to the sample cuvette without interrupting hypoxia as described in detail in the previous section.

2.2.11 DNA Strand Cleavage Assay

DNA strand breakage was detected by the method based on the conversion of supercoiled form of plasmid DNA (SC, form I) to the nicked (open) circular (OC, form II) and linear forms (form III) and their differential mobility on agarose gel as reported previously by others (Fisher and Gutierrez, 1991; Walton *et al.*, 1991; Shen and Hollenberg, 1994; Kukielka and Cederbaum, 1994, Garner *et al.,* 1999).

2.2.11.1 Induction of DNA Strand Breaks by Purified Sheep Lung NADPH-Cytochrome P450 Reductase-Catalyzed Bioactivation of Idarubicin and Mitomycin C

The experiments were performed in a volume of 60 µl reaction mixture containing supercoiled pBR322 (1.0 µg), idarubicin (100 µM) or mitomycin C (100 µM), highly purified sheep lung NADPH-cytochrome P450 reductase (0.1 µg), NADPH (2 mM) and 100 mM sodium phosphate buffer, pH 7.4 under aerobic conditions. The samples were incubated at 37°C for 30 minutes. Untreated pBR322 plasmid DNA alone and reaction mixtures in which one of the reaction components omitted were included as controls in each run of gel electrophoresis. For hydroxyl radical-induced DNA damage, a typical $OH^{\cdot}$ generating system consisting of 10 µM ferric chloride, 20 µM EDTA and 1 mM ascorbate was employed (Kumagai *et al.*, 1997). After incubating the samples at 37°C for 30 minutes under dimmed light, 5 µl of loading buffer (0.25% bromophenol blue, 0.5% SDS, 60% glycerol and 5 mM EDTA) was mixed with 20 µl aliquots of reaction mixtures. The samples were then analyzed by electrophoresis on a 1% agarose horizontal slab gel containing final concentration of 0.5 µg/ml of ethidium bromide in Tris-borate-EDTA buffer (45 mM Tris-borate, 1 mM EDTA). Gels were photographed using a computer-based gel imaging instrument system (Infinity 3000-CN-3000 darkroom) (Vilber Lourmat, Marne-la-Vallee Cedex 1, France) with Infinity-Capt Version 12.9 software, and DNA damage was quantified using Scion Image Version Beta 4.0.2 software. A correction factor of 1.22 was applied to values obtained from densitometric analysis of the bands corresponding to the supercoiled form of plasmid DNA to account for decreased binding of ethidium bromide into this form as compared to others (Fisher and Gutierrez, 1991).

2.2.11.2 Induction of DNA Strand Breaks by Purified Beef Liver NADH-Cytochrome b5 Reductase-Catalyzed Bioactivation of Idarubicin and Mitomycin C

The same reaction conditions described above for purified sheep lung NADPH-cytochrome P450 reductase were used for detection of DNA strand breaks in the presence of highly purified beef liver NADH-cytochrome b5 reductase and either drug except that reaction mixtures contained various amounts of purified cytochrome b5 reductase up to 1.09 units (based on ferricyanide reduction) and cofactor NADH (2 mM) in a volume of 60 µl reaction mixture. In addition, experiments were repeated under above conditions using two different concentrations of mitomycin C (100 µM and 25 µM) and 25 µM concentration of idarubicin in reaction mixtures containing 10 mM sodium phosphate buffer, pH 6.6 instead of 100 mM sodium phosphate buffer, pH 7.4. The samples were analyzed by electrophoresis and DNA damage was quantified densitometrically as described in detail in the previous section.

2.2.11.3 Induction of DNA Strand Breaks by Purified Rabbit Liver Cytochrome P4502B4-Catalyzed Bioactivation of Idarubicin

The DNA strand breakage was detected under aerobic conditions using reconstituted systems containing highly purified rabbit liver cytochrome P4502B4 and rabbit liver NADPH-cytochrome P450 reductase and artificial lipid dilauroyl phosphatidylcholine. Appropriate amounts of cytochrome P4502B4 and cytochrome P450 reductase were reconstituted in an eppendorf tube at room temperature (24-25°C) for 20 minutes in the presence of synthetic lipid dilauroyl phosphatidylcholine (50 µg/ml) with mixing thoroughly. Then, all reaction constituents except cofactor NADPH were added to it. The reaction constituents and their concentrations were the same as written in Section 2.2.11.1. The reaction was then started by the addition of cofactor NADPH to incubation mixtures and the samples were incubated at 37°C for

30 minutes under dimmed light. DNA breakage was detected by the method described in Section 2.2.11.1.

2.2.11.4 Effects of Dietary Antioxidants against DNA Strand Breaks Induced by Purified Rabbit Liver NADPH-Cytochrome P450 Reductase-Catalyzed Bioactivation of Idarubicin and Mitomycin C

The effects of antioxidants quercetin, naringenin, rutin, resveratrol and trolox (a water-soluble derivative of vitamin E) on the protection of supercoiled pBR322 plasmid DNA against strand breaks induced by highly purified rabbit liver NADPH-cytochrome P450 reductase-catalyzed bioactivation of idarubicin and mitomycin C were studied in reaction mixtures containing supercoiled pBR322 plasmid DNA (1.0 μg), idarubicin (100 μM) or mitomycin C (100 μM), appropriate amounts of rabbit liver NADPH-cytochrome P450 reductase, NADPH (2 mM), 100 mM sodium phosphate buffer, pH 7.4 and appropriate concentrations of antioxidants in a final volume of 60 μl under aerobic conditions. All the stock solutions of antioxidants were prepared in methanol in eppendorf tubes wrapped by aluminum foil in order to protect the chemicals from light. The volume of methanol in incubation mixtures was 2% of the reaction volume. pBR322 plasmid DNA-alone control and solvent control incubations were also carried out in each run of gel electrophoresis. The reaction mixtures were incubated at 37°C for 30 minutes in eppendorf tubes wrapped by aluminum foil and under dimmed light in order to protect the samples from light. The samples were then analyzed by electrophoresis and DNA damage was quantified densitometrically as described in detail in Section 2.2.11.1.

2.2.11.5 Quantification of DNA Damage

DNA damage produced by enzyme-catalyzed bioactivation of idarubicin and mitomycin C was quantified by densitometric analysis (see Section 2.2.11.1) and expressed as SBI (DNA strand breaking index, OC%) which was calculated by using the below formula (Ashikaga *et al.,* 2000; Rajagopalan *et al.,* 2002):

SBI = (open circular (OC) DNA / total DNA) x 100

An important point in the calculation of DNA damage was the application of a correction factor of 1.22 to the values obtained from densitometric analysis of the bands corresponding to the supercoiled form of plasmid DNA as mentioned in Section 2.2.11.1.

The protective effects of antioxidants against DNA strand breaks induced by purified NADPH-cytochrome P450 reductase-catalyzed bioactivation of idarubicin and mitomycin C were expressed as protection% and calculated as follows (Ashikaga *et al.,* 2000; Rajagopalan *et al.,* 2002):

$$\text{Protection\%} = \left(1 - \frac{\text{SBI in the presence of antioxidant} - \text{control SBI}^{\,a}}{\text{SBI in the absence of antioxidant}^{\,b} - \text{control SBI}} \right) \times 100$$

[a] SBI for pBR322 plasmid DNA alone

[b] For the antioxidants prepared in methanol, SBI for solvent control was used to eliminate any effect coming from the solvent itself.

2.2.12 SDS-Polyacrylamide Gel Electrophoresis

Polyacrylamide slab gel electrophoresis in the presence of detergent, SDS, was performed in a discontinuous buffer system as described by Laemmli (1970) on 4% stacking gel and 8.5% separating gel for NADPH-cytochrome P450 reductase and cytochrome P4502B4 and on 4% stacking gel and 12.5% separating gel for NADH-cytochrome b5 reductase and cytochrome b5.

Vertical slab gel electrophoresis was carried out using Bio-Rad Protean II Slab and Bio-Rad Protean II Electrophoresis Cell. Polyacrylamide slab gels were prepared using the gel sandwich. The gel sandwich was assembled between two glass plates (long plate 18.3 x 20 cm; short plate 16 x 20 cm; spacer 1 mm). The glass plates were then screwed with the clamps from both sides. In order to prevent the leakage of separating gel from the bottom of the glass plates, the gel sandwich was placed in melted agar and both sides were sealed with melted agar.

The separating gel solution (30 ml) containing 8.5 ml (8.5%) or 12.5 ml (12.5%) gel solution, 0.375 M Tris-HCl, pH 8.8 and 0.1% SDS was prepared and chemical polymerization was achieved by the addition of 0.15 ml of 10% ammonium persulfate (APS) and 0.015 ml TEMED in their written order. The solution was poured into the glass plates from one edge of the spacers using 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 thin layer of 2-methyl propan-1-ol, approximately 0.1 cm thick, to ensure the formation of a smooth gel surface. The gel was then allowed for polymerization at room temperature (24-25°C) for about 30 minutes. After polymerization, the layer of alcohol was poured off completely. Meanwhile the stacking gel solution (10 ml) containing 4 ml (4%) gel solution, 0.125 M Tris-HCl, pH 6.8, 0.1% SDS, 0.05 ml of 10% APS and 0.01 ml TEMED was prepared and poured on top of the separating 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. The gel was allowed for polymerization at room temperature for about 30 minutes. After the teflon comb was removed carefully without tearing the wells, wells were filled with freshly prepared electrode running buffer which contained 25 mM Tris, 192 mM glycine and 0.1% SDS using a syringe with a fine needle to remove any air bubbles in the wells if present. The gel sandwich was then placed on the cooling core and upper chamber of the cooling core was filled with freshly prepared electrode running buffer. Then, protein samples about 15-75 µl and molecular weight standards about 15 µl were loaded into the wells carefully by a

Hamilton syringe as a thin layer at the bottom of the wells. If necessary, the protein samples were initially diluted. Aliquots of appropriately diluted protein samples and standards were diluted 1:3 (3 part sample and 1 part sample dilution buffer) with 4x sample dilution buffer consisting of 0.25 M Tris-HCl buffer, pH 6.8, 8% SDS, 40% glycerol, 20% β-mercaptoethanol and 0.01% bromophenol blue and were immersed in a boiling water bath for 90 seconds. The stock solutions of molecular weight standards were prepared at a concentration of 2 mg per ml. BSA (M_r 68000), catalase (M_r 60000), L- glutamate dehydrogenase (M_r 53000), egg albumin (M_r 45000) and cytochrome *c* (11700) were used as molecular weight standards. The molecular weight of the polypeptide chains were taken from Weber and Osborn (1969).

After the wells were loaded with protein samples and standards, gel sandwich together with cooling core were placed into the lower buffer chamber of Bio-Rad Protean II Cell filled with 2 liters of electrode running buffer. The cell was then connected to the power supply Bio-Rad model 2 (Bio-Rad Laboratories, Richmond, California, USA) and electrophoresis was carried out at 7 mA constant current overnight. The power supply was turned off when the dye reached to the bottom (approximately 8-9 cm from the beginning of the separating gel). The total run time was about 13-14 hours.

After electrophoresis was completed, the slab gel was removed from the glass plates and stained and fixed in a solution containing 0.1% Coomassie Brilliant blue R, 50% methanol and 12% glacial acetic acid for one hour by moderate shaking at room temperature. The gel was then destained with a solution of 30% methanol containing 7% acetic acid glacial to remove the unbound dye for at least 1.5 hours by moderate shaking. The destaining solution was refreshed at the end of each 30 minutes. Finally, the destained gels were stored in destaining solution and gels were photographed by using computer based gel imaging instrument (Infinity 3000-CN-3000 darkroom) (Vilber Lourmat, Marne-la-Vallee Cedex 1, France). Gels were analyzed and photographed by using Infinity-Capt Version 12.9 software.

CHAPTER III

RESULTS

NADPH-cytochrome P450 reductase, NADH-cytochrome b5 reductase and cytochrome P4502B4 were highly purified from lung and liver tissues of sheep, beef and phenobarbital-treated rabbit and their roles and involvements in the reductive bioactivation of idarubicin were assessed. Because mitomycin C was used as a model compound in this study, all the experiments were also repeated using it under same reaction conditions and the results were compared. The abilities of the purified oxidoreductases to catalyze the bioreductive activation of idarubicin and mitomycin C to DNA-damaging species were examined and compared. The mechanism of DNA damage induced by idarubicin in the presence of NADPH-cytochrome P450 reductase was investigated. The *in vitro* DNA-damaging capacity of idarubicin was characterized with respect to increasing concentrations of enzyme or drug as well as increasing incubation time. In addition, it was assessed whether differences exist in the reductive bioactivation of idarubicin and mitomycin C to generate strand breaks in DNA under the above incubation conditions. Furthermore, the potential protective effects of some dietary antioxidants against idarubicin- and mitomycin C-induced DNA strand breaks were studied. The involvement of these enzymes in the bioreduction of idarubicin, in comparison to mitomycin C, was further investigated by measuring drug-induced NAD(P)H oxidation using microsomes or purified enzymes. Finally, the role of CYP2B4 in the reduction of idarubicin, relative to NADPH-cytochrome P450 reductase, was determined using reconstituted systems of purified CYP2B4 and cytochrome P450 reductase. The results for the purification of each enzyme are given briefly in the following sections.

3.1 Purification of Beef Liver NADPH-Cytochrome P450 Reductase

The purification of NADPH-cytochrome P450 reductase from beef liver microsomes was achieved through anion exchange chromatography of the detergent solubilized microsomes on two successive DEAE-cellulose columns followed by affinity chromatography of the partially purified reductase on adenosine 2', 5'-diphosphate-Sepharose 4B column. Further purification and concentration of the reductase was achieved on a final hydroxylapatite column. The elution profile of first DEAE-cellulose column chromatography for the purification of NADPH-cytochrome P450 reductase from beef liver microsomes is given in Figure 3.1. During washing step in first DEAE-cellulose column chromatography, the eluted fractions containing high quantities of NADH-cytochrome b5 reductase activity and low cytochrome P450 content were pooled and used for the subsequent purification of NADH-cytochrome b5 reductase.

The results for the purification of NADPH-cytochrome P450 reductase from beef liver microsomes are given in Table 3.1. It was found that NADPH-cytochrome P450 reductase from beef liver microsomes was purified about 262-fold with an overall yield of 10.9% with respect to microsomes. The specific activity of purified cytochrome P450 reductase was 30.9 units/mg of protein when cytochrome *c* reduction was assayed spectrophotometrically at 550 nm as described in "Methods".

The purity of beef liver NADPH-cytochrome P450 reductase was evaluated by polyacrylamide gel electrophoresis under denaturing conditions. Figure 3.2 shows the SDS-PAGE patterns of the purified beef liver NADPH-cytochrome P450 reductase and the fractions obtained at different stages of the purification study. As seen in Figure 3.2, purified beef liver cytochrome P450 reductase was highly pure with respect to microsomes. The purity of beef liver NADPH-cytochrome P450 reductase was further confirmed by its absolute absorption spectrum. As seen in Figure 3.3, the absorption spectrum of the purified beef liver P450 reductase gave two peaks at 455 nm and 378 nm and a shoulder around 478 nm, which are characteristics for flavoproteins. There was no shoulder around 420 nm region

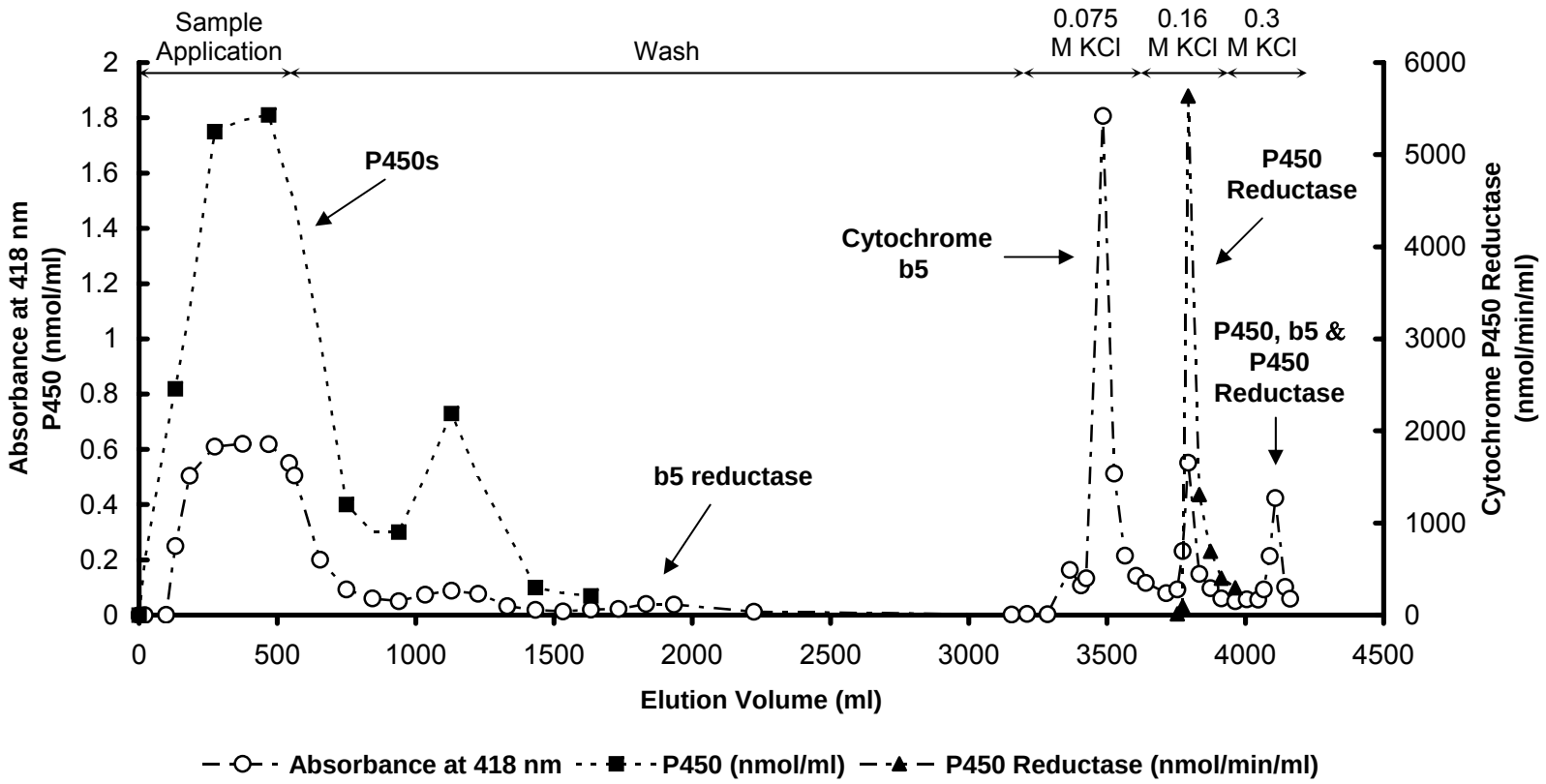


Figure 3.1 Elution profile of first DEAE-cellulose column chromatography for beef liver NADPH-cytochrome P450 reductase and NADH-cytochrome b5 reductase. About 3016.0 mg detergent solubilized beef liver microsomes were chromatographed on a 3.0 x 40 cm DEAE-cellulose column. Absorbances at 418 nm, cytochrome P450, cytochrome b5 amounts, cytochrome P450 reductase and cytochrome b5 reductase activities of fractions were measured. After sample application, column was extensively washed with equilibration buffer and cytochrome b5 reductase was eluted during this step. Cytochrome P450 reductase was eluted with 0.16 M KCl in equilibration buffer.

Table 3.1 Purification of NADPH-cytochrome P450 reductase from beef liver microsomes

Fractions	Volume (ml)	Protein Amount (mg/ml)	Cytochrome P450 Reductase			Recovery %	Purification Fold
			Activity (nmol/min/ml)	Specific Activity (nmol/min/mg)	Total Activity (nmol/min)		
Microsomes	80.0	37.7	4450.1	118.0	356008.0	100.0	1.0
Solubilized Microsomes	488.0	5.90	920.7	156.1	449301.6	126.2	1.32
DEAE-Cellulose-1	186.0	1.12	1350.5	1205.8	251193.0	70.6	10.2
DEAE-Cellulose-2	33.0	2.87	5885.9	2050.8	194234.7	54.6	17.4
Adenosine 2', 5'-diphosphate-Sepharose 4B	36.0	0.08	2334.0	29175.0	84024.0	23.6	247.2
Hydroxylapatite	7.0	0.18	5560.5	30891.7	38923.5	10.9	261.8

NADPH- dependent cytochrome *c* reductase activities were assayed at 25 °C, in 0.3 M potassium phosphate buffer, pH 7.7.

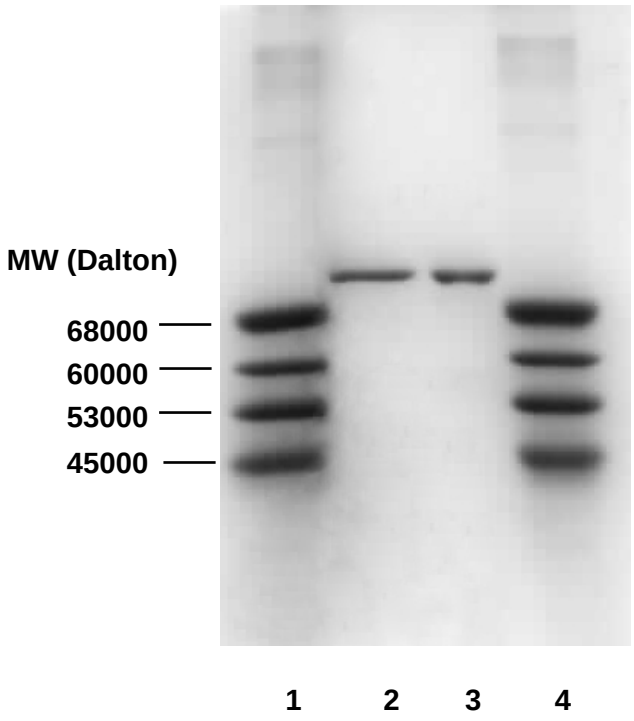


Figure 3.2 SDS-PAGE showing the different stages for the purification of beef liver NADPH-cytochrome P450 reductase. **Lanes 1 and 4**, four reference proteins (BSA, catalase, glutamate dehydrogenase and egg albumin, 3.8 µg each); **lane 2,** cytochrome P450 reductase fraction obtained from affinity column (1.2 µg); **lane 3,** cytochrome P450 reductase fraction obtained from hydroxylapatite column (0.68 µg).

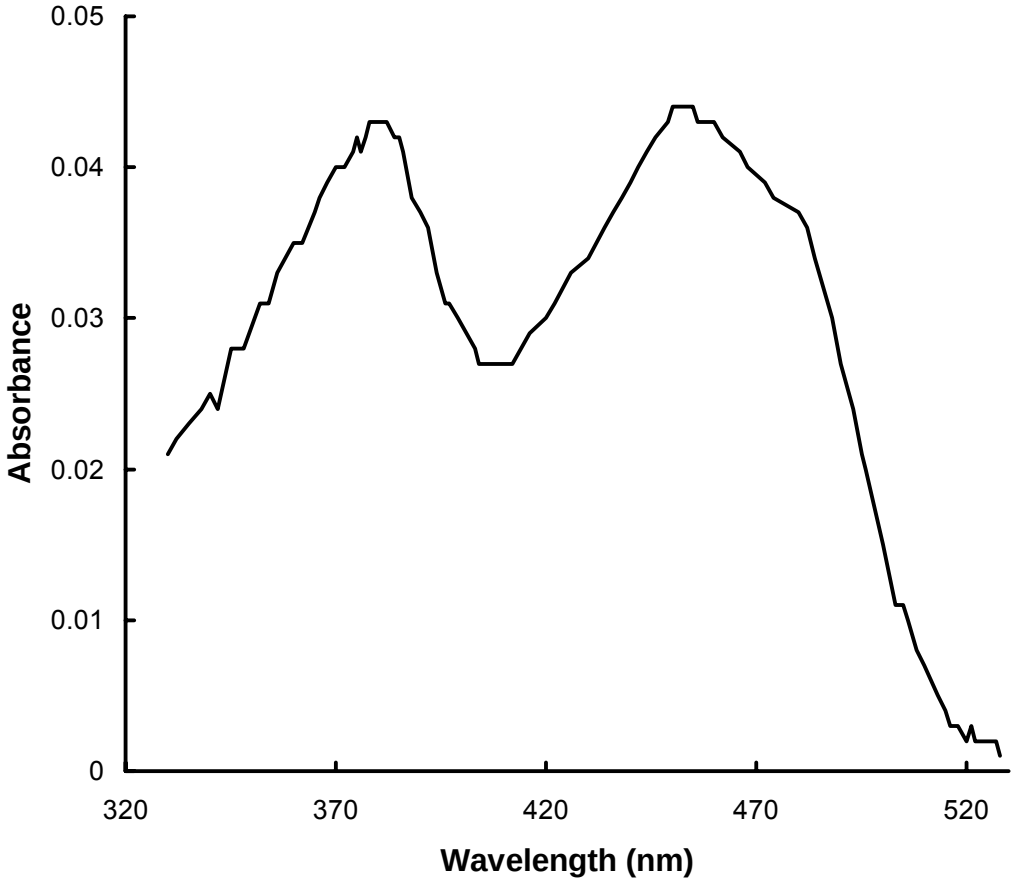


Figure 3.3 Visible absolute absorption spectrum of the final highly purified preparation of beef liver NADPH-cytochrome P450 reductase eluted from hydroxylapatite column. Absolute absorption spectrum of the enzyme was recorded without diluting the final purified preparation against elution buffer in a double beam spectrophotometer using 1.0 cm pathlength cuvettes.

suggesting that the purified enzyme preparation was not contaminated with hemoproteins like cytochrome b5 and cytochrome P450.

3.2 Purification of Beef Liver Microsomal NADH-Cytochrome b5 Reductase

In order to obtain a purified NADH-cytochrome b5 reductase preparation, firstly, the enzyme was tried to be purified from phenobarbital-treated rabbit liver microsomes. However, some difficulties were encountered during the purification of NADH-cytochrome b5 reductase from phenobarbital-treated rabbit liver microsomes. The fractions showing the highest NADH-cytochrome b5 reductase activities eluted from first DEAE-cellulose column during the sample application and washing steps also contained high amounts of cytochrome P450s. In addition, cytochrome b5 reductase could not be bound to the second DEAE-cellulose column and almost all of the cytochrome b5 reductase and cytochrome P450s eluted from the column together during the sample application and washing steps. Therefore, NADH-cytochrome b5 reductase could not be isolated from cytochrome P450 isozymes and could not be concentrated at this step which was necessary before the affinity chromatography on adenosine 5'-diphosphate-agarose.

For this reason, NADH-cytochrome b5 reductase fractions eluted from the first DEAE-cellulose column during the purification of NADPH-cytochrome P450 reductase from beef liver were pooled and used for the subsequent purification of NADH-cytochrome b5 reductase as described in "Methods". Thus, the first two steps – solubilization and first DEAE-cellulose column chromatography – in the purification of both NADPH-cytochrome P450 reductase and NADH-cytochrome b5 reductase were identical. The overall procedure for the purification of NADH-cytochrome b5 reductase from detergent solubilized beef liver microsomes involved two successive anion-exchange chromatography using DEAE-cellulose and affinity chromatography on adenosine 5'-diphosphate-agarose as outlined in Figure 2.2. The elution profiles of first DEAE-cellulose and adenosine 5'-diphosphate agarose column chromatographies for the purification of beef liver microsomal NADH-

cytochrome b5 reductase are shown in Figure 3.1 and Figure 3.4, respectively. The activity of final purified cytochrome b5 reductase preparation was 93.5 units/ml when potassium ferricyanide reduction was assayed spectrophotometrically at 420 nm as described in "Methods". The purified cytochrome b5 reductase preparation was homogenous and gave a single protein band on 12.5% slab gel (Figure 3.5).

3.3 Purification of Cytochrome P4502B4 from Phenobarbital-Treated Rabbit Liver Microsomes

The purification of cytochrome P4502B4 (LM2) from phenobarbital-treated rabbit liver microsomes involved anion exchange chromatography of detergent solubilized and polyethylene glycol fractionated microsomes on an initial DEAE-cellulose column, adsorption chromatography on first hydroxylapatite column and hydrophobic interaction chromatography on octyl-Sepharose CL-4B column. The partially purified cytochrome P4502B4 was then applied onto a second hydroxylapatite column which was followed by anion exchange chromatography on second DEAE-cellulose column. Finally, cytochrome P4502B4 was further purified and concentrated on a third hydroxylapatite column. The elution profiles of first DEAE-cellulose and first hydroxylapatite column chromatographies for the purification of cytochrome P4502B4 from phenobarbital-treated rabbit liver microsomes are shown in Figure 3.6 and Figure 3.7, respectively. A summary of the results for cytochrome P4502B4 purification from phenobarbital-treated rabbit liver microsomes is presented in Table 3.2. It was found that cytochrome P4502B4 was purified 8.5-fold with an overall yield of 6% with respect to phenobarbital-treated rabbit liver microsomes. The specific content of highly purified cytochrome P4502B4 was 14.0 nmol of P450 per mg of protein. The final enzyme preparation by this procedure was highly pure with respect to microsomes as judged by SDS-PAGE (Figure 3.8) and essentially free of cytochrome b5, NADPH-cytochrome P450 reductase and NADH-cytochrome b5 reductase.

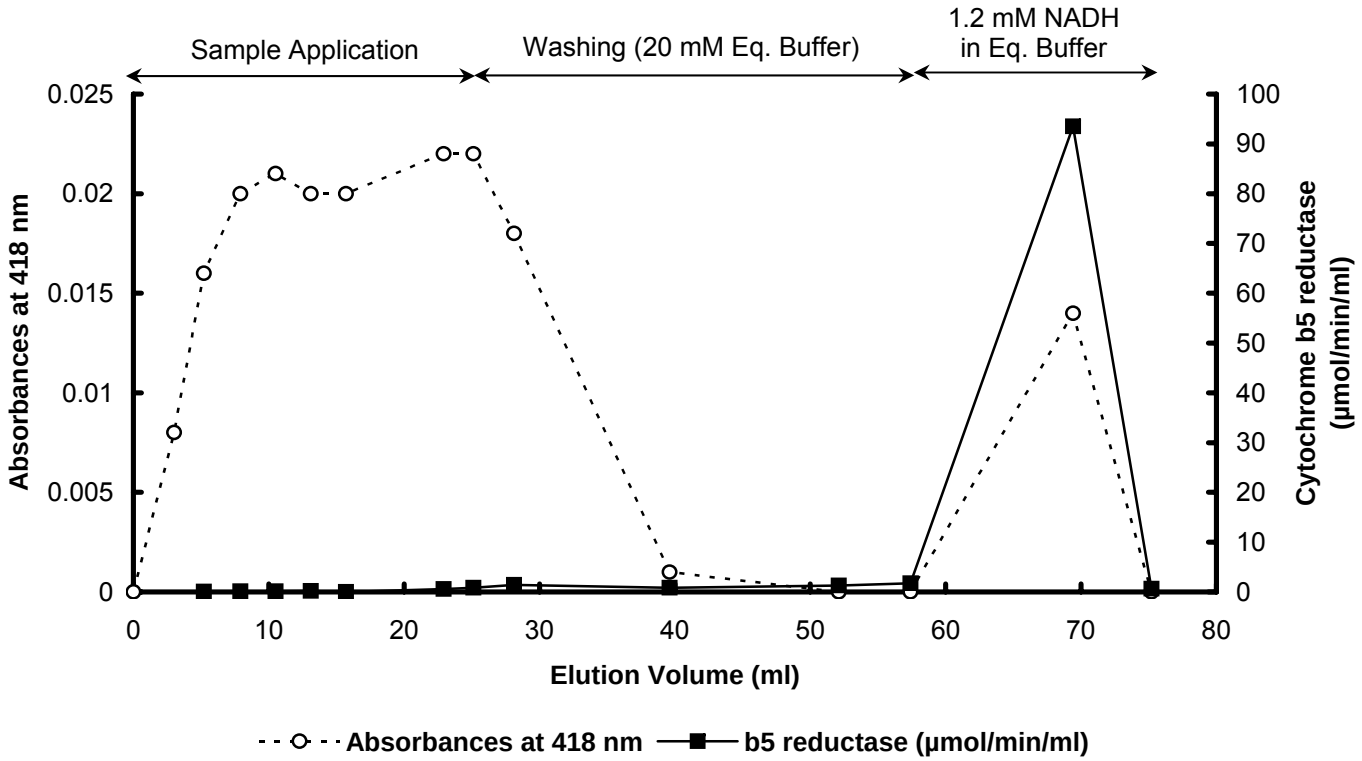


Figure 3.4 Adenosine 5'-diphosphate-agarose column (0.7 x 6 cm) chromatography of the partially purified beef liver cytochrome b5 reductase obtained from second DEAE-cellulose column. Absorbances at 418 and b5 reductase activities of fractions were measured. Cytochrome b5 reductase was eluted with 1.2 mM NADH in equilibration buffer.

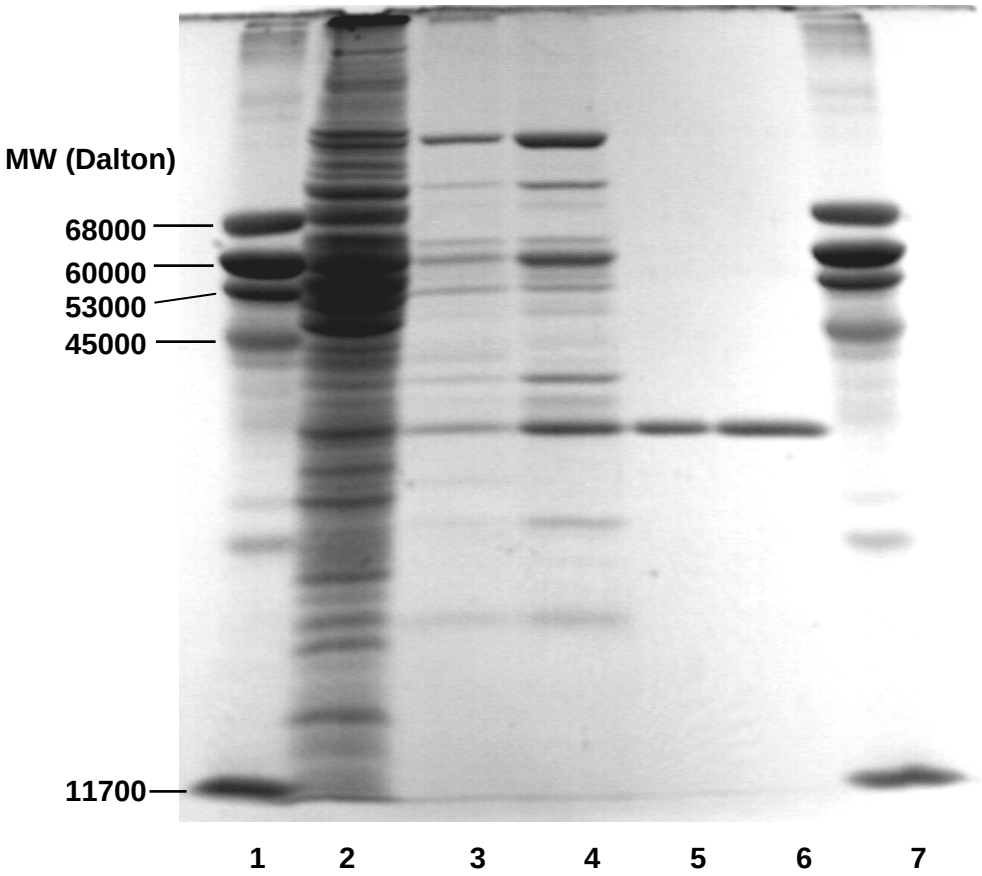


Figure 3.5 A 12.5% SDS-polyacrylamide gel showing the different steps for the purification of the beef liver NADH-cytochrome b5 reductase along with five reference proteins. **Lanes 1 and 7**, five reference proteins (BSA, catalase, glutamate dehydrogenase, egg albumin, and cytochrome *c,* 3.3 μg each); **lane 2,** microsomes (90.9 μg); **lane 3,** cytochrome b5 reductase fraction obtained from first DEAE-cellulose column; **lane 4,** cytochrome b5 reductase fraction obtained from second DEAE-cellulose column; **lanes 5 and 6**, cytochrome b5 reductase fraction obtained from adenosine 5'-diphosphate-agarose column (enzyme was diluted with sample dilution buffer, 3 part sample : 1 part sample dilution buffer and 30 μl and 50 μl were applied to the wells 5 and 6, respectively).

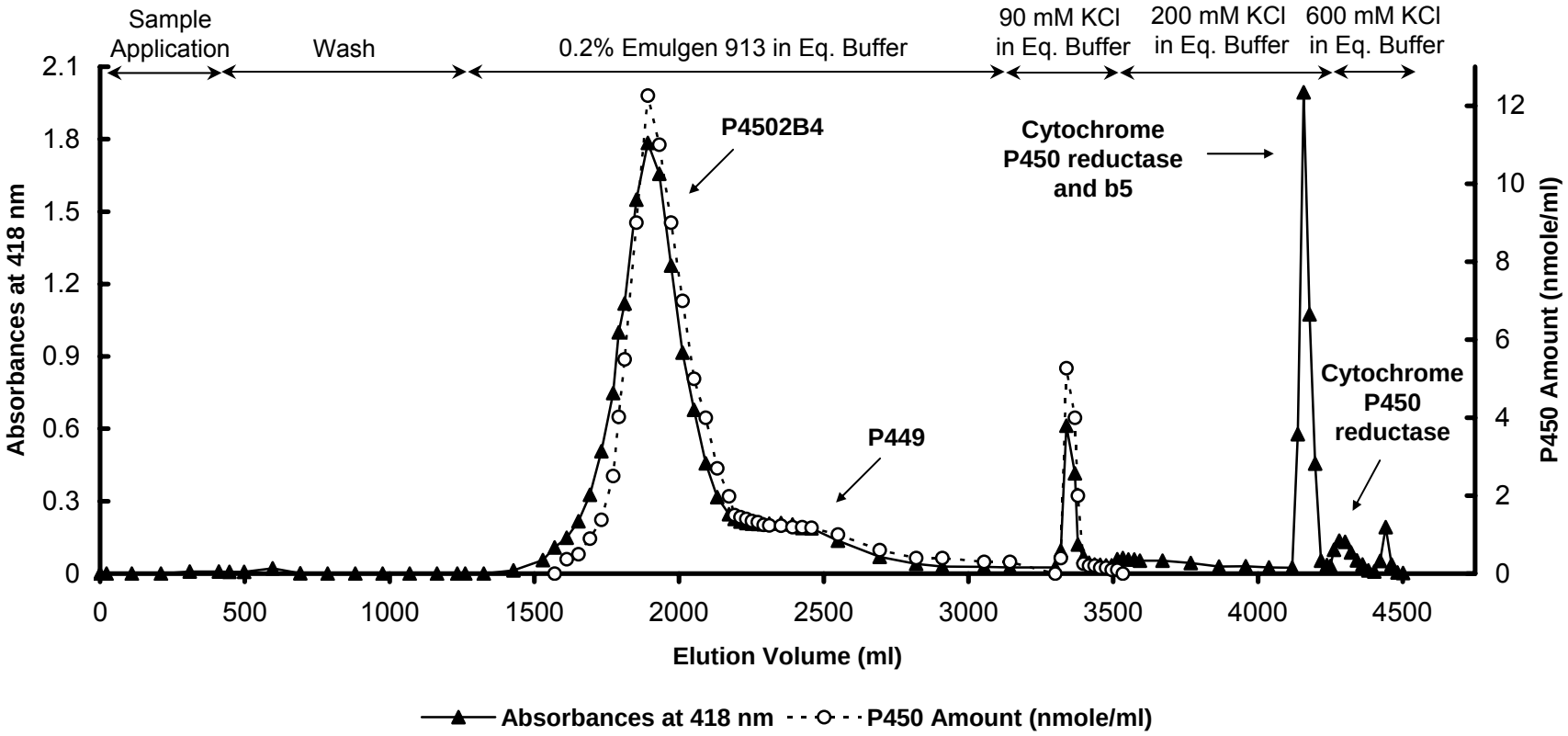


Figure 3.6 Elution profile of first DEAE-cellulose column chromatography for phenobarbital-treated rabbit liver cytochrome P4502B4. About 2880.0 mg cholate solubilized and PEG 6000 fractionated rabbit liver microsomes containing 3950.2 nmoles of P450 were chromatographed on a 2.5 x 45 cm DEAE-cellulose column. Absorbances at 418 nm, cytochrome P450, cytochrome b5 amounts and cytochrome P450 reductase activities were measured. After sample application, column was washed with equilibration buffer and then CYP2B4 was eluted with 0.2% Emulgen 913 in equilibration buffer.

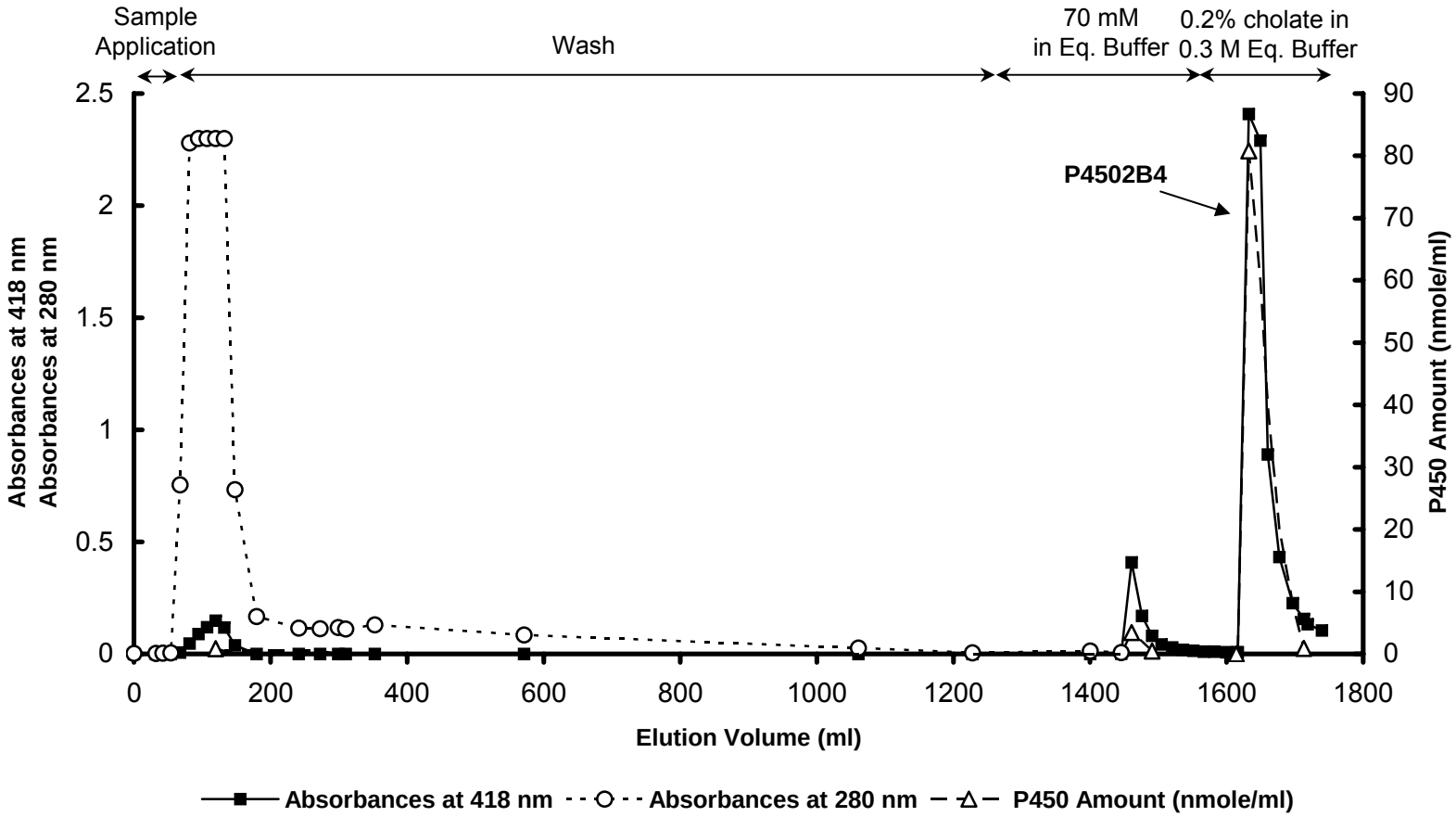


Figure 3.7 First hydroxylapatite column (3.2 x 7.0 cm) chromatography of the partially purified cytochrome P4502B4 obtained from first DEAE-cellulose column. Absorbances of fractions were measured at 280 nm and 418 nm. Cytochrome P4502B4 was eluted with equilibration buffer containing 0.2% cholate and 300 mM KPi, pH 7.7.

Table 3.2 Purification of cytochrome P4502B4 from phenobarbital-treated rabbit liver microsomes

Fractions	Volume (ml)	Protein Amount (mg/ml)	Cytochrome P450			Recovery %	Purification Fold
			P450 Amount (nmol/ml)	Specific P450 Content (nmol/mg prt.)	Total P450 Content (nmol)		
Microsomes	50.0	57.6	94.8	1.65	4740.0	100.0	1.0
9-14% PEG 6000 fraction	99.5	8.48	39.7	4.68	3950.2	83.3	2.84
DEAE-Cellulose-1	427.5	0.95	646.0	6.80	2761.7	58.3	4.12
Hydroxylapatite-1	77.5	3.45	26.6	7.72	2064.6	43.6	4.68
Octyl-Sepharose CL-4B	422.0	0.27	2.14	7.93	903.1	19.1	4.81
Hydroxylapatite-2	51.8	2.04	16.5	8.08	852.8	18.0	4.90
DEAE-Cellulose-2	840.0	0.05	0.66	13.2	554.4	11.7	8.0
Hydroxylapatite-3	15.0	1.36	19.1	14.0	286.5	6.0	8.5

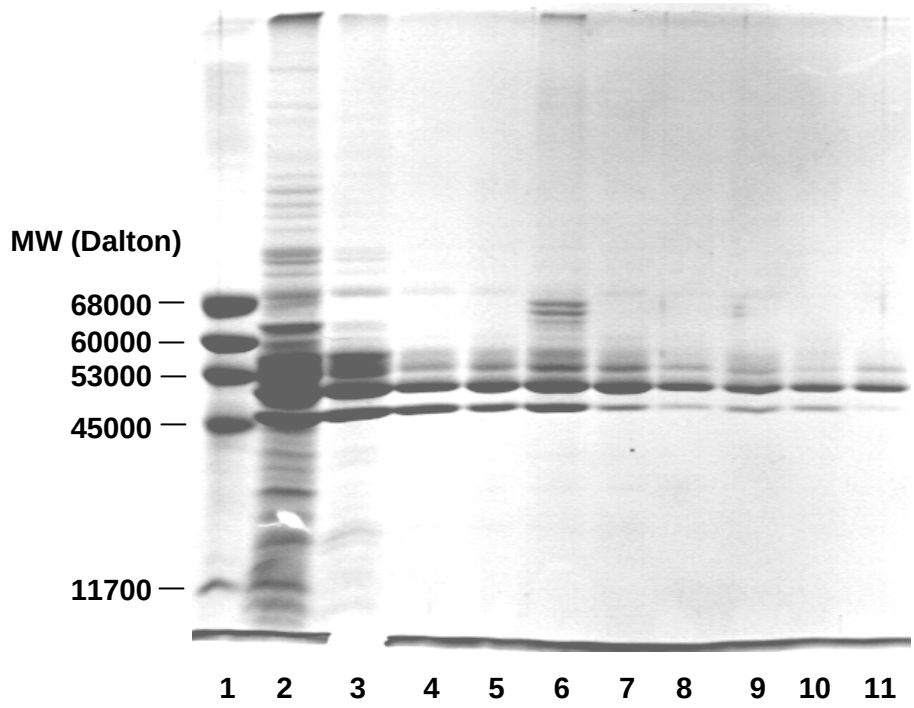


Figure 3.8 A 8.5% SDS-polyacrylamide gel patterns of the highly purified cytochrome P4502B4 and the fractions obtained at various stages of the purification procedure. **Lanes 1 and 15,** five reference proteins (BSA, catalase, glutamate dehydrogenase, egg albumin, and cytochrome *c*, 3.3 µg each); **lane 2,** microsomes (36.5 µg); **lane 3,** 9-14% PEG 6000 fraction (12.9 µg); **lane 4,** cytochrome P450 pool obtained from first DEAE-cellulose column (4.4 µg); **lane 5,** cytochrome P450 pool obtained from first hydroxylapatite column (3.9 µg); **lane 6,** cytochrome P450 pool obtained from octyl-Sepharose CL-4B column (3.7 µg); **lane 7,** cytochrome P450 pool obtained from second hydroxylapatite column (3.7 µg); **lane 8,** pooled cytochrome P4502B4 fractions (pool A) eluted from the second DEAE-cellulose column during the sample application and washing steps (1.1 µg); **lane 9,** pooled cytochrome P4502B4 fractions (pool B) eluted from the second DEAE-cellulose column with 0.1 M KPi and 0.1 M KPi + 0.2% Emulgen 913; **lanes 10 and 11,** highly purified cytochrome P4502B4 fractions eluted from the third HTP column (loaded with pool A obtained from second DEAE-cellulose column) with 0.09 M KPi and 0.2 M KPi (1.1 µg) (see Figure 2.3). The highly purified CYP2B4 fraction (P450 Amount: 19.1 nmol/ml) eluted from the third HTP column with 0.2 M KPi (lane 11) was used in all the experiments.

3.4 Purification of NADPH-Cytochrome P450 Reductase from Phenobarbital-Treated Rabbit Liver Microsomes

The purification of NADPH-cytochrome P450 reductase from phenobarbital-treated rabbit liver microsomes involved anion exchange chromatography of the detergent solubilized microsomes on two successive DEAE-cellulose columns followed by affinity chromatography of the partially purified enzyme on adenosine 2', 5'-diphosphate-Sepharose 4B column. Further purification and removal of Emulgen 913 and 2'-AMP from final enzyme preparation were carried out on a final hydroxylapatite column and the enzyme was concentrated at this step. The elution profile of first DEAE-cellulose column chromatography for the purification of NADPH-cytochrome P450 reductase from phenobarbital-tretaed rabbit liver microsomes is given in Figure 3.9. During the elution step in first DEAE-cellulose column chromatography, fractions containing high cytochrome b5 content and low quantities of NADPH-cytochrome P450 reductase activity were pooled and used for the subsequent purification of cytochrome b5.

The results for the purification of NADPH-cytochrome P450 reductase from phenobarbital-treated rabbit liver microsomes are shown in Table 3.3. It was found that cytochrome P450 reductase was purified 115.9-fold with a final yield of 16.6% with respect to phenobarbital-treated rabbit liver microsomes. The specific activity of purified P450 reductase was 31.7 units/mg of protein when cytochrome *c* reduction was assayed spectrophotometrically at 550 as described in "Methods". The absolute absorption spectrum of the highly purified rabbit liver P450 reductase gave the characteristic peaks of flavoproteins and further confirmed the purity of the enzyme (data not shown).

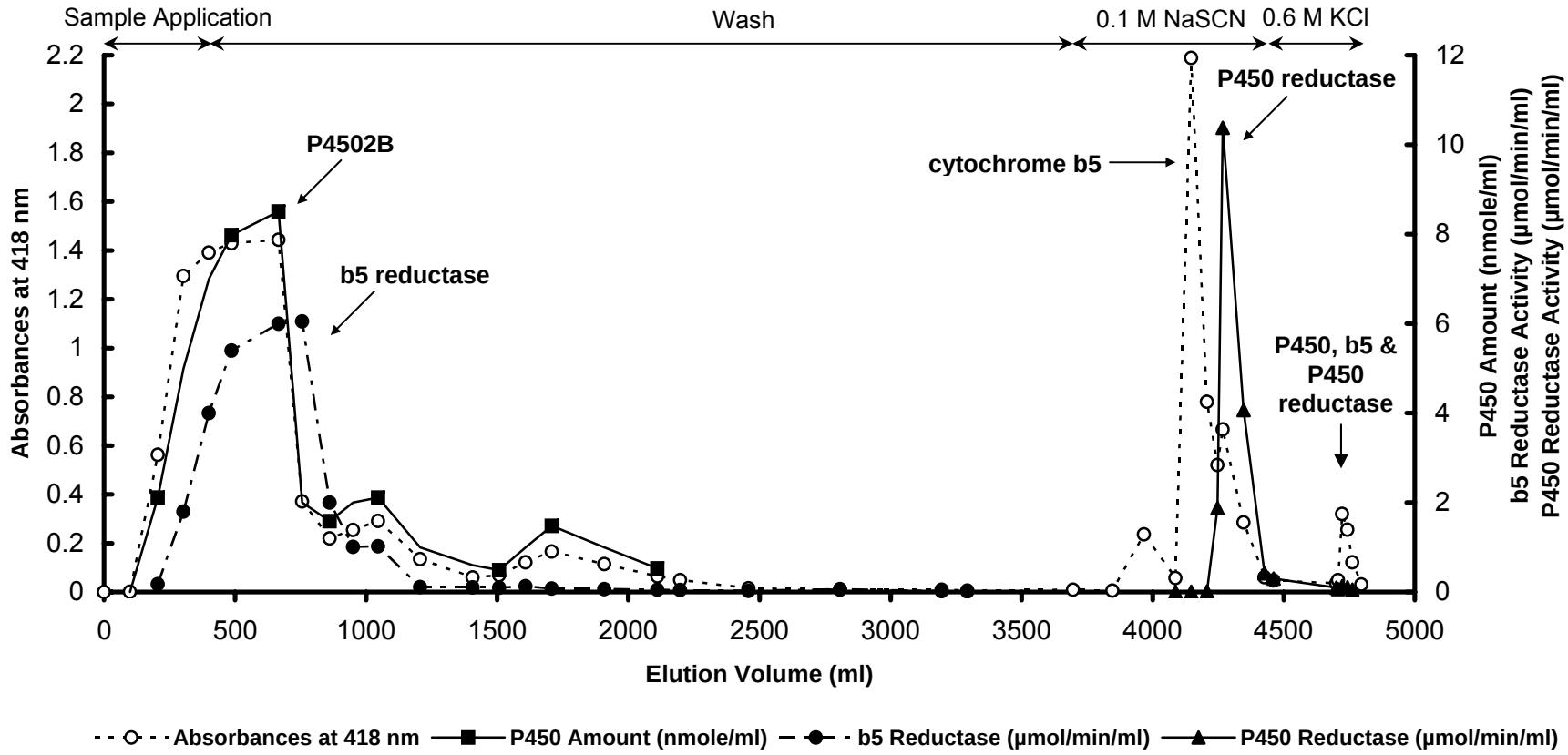


Figure 3.9 Elution profile of first DEAE-cellulose column chromatography for phenobarbital-treated rabbit liver NADPH-cytochrome P450 reductase and cytochrome b5. About 2992.0 mg detergent solubilized rabbit liver microsomes were chromatographed on a 3.2 x 48.5 cm DEAE-cellulose column. Absorbances at 418 nm, cytochrome P450, cytochrome b5 amounts, cytochrome P450 reductase and cytochrome b5 reductase activities of fractions were measured. Cytochrome P450 reductase and cytochrome b5 were eluted with 0.1 M NaSCN in equilibration buffer.

Table 3.3 Purification of NADPH-cytochrome P450 reductase from phenobarbital-treated rabbit liver microsomes

Fractions	Volume (ml)	Protein Amount (mg/ml)	Cytochrome P450 Reductase			Recovery %	Purification Fold
			Activity (nmol/min/ml)	Specific Activity (nmol/min/mg)	Total Activity (nmol/min)		
Microsomes	49.7	60.2	16485.1	273.8	819309.5	100.0	1.0
Solubilized Microsomes	458.0	5.26	1941.8	369.2	889344.4	108.5	1.35
DEAE-Cellulose-1	206.5	2.86	3948.4	1380.6	815344.6	99.5	5.04
DEAE-Cellulose-2	62.5	1.48	6908.5	4667.9	431781.3	52.7	17.05
Adenosine 2', 5' diphosphate-Sepharose 4B	85.0	0.09	2178.9	24210.0	185206.5	22.6	88.4
Hydroxylapatite	6.8	0.63	19993.4	31735.6	135955.1	16.6	115.9

NADPH- dependent cytochrome *c* reductase activities were assayed at 25 °C, in 0.3 M potassium phosphate buffer, pH 7.7.

3.5 Purification of Microsomal Cytochrome b5 from Phenobarbital-Treated Rabbit Liver

Cytochrome b5 was purified from phenobarbital-treated rabbit liver microsomes in order to be used in reconstituted systems for determination of the biocatalytic activity of the purified beef liver NADH-cytochrome b5 reductase according to its ability to catalyze the reduction of cytochrome b5. The purification of cytochrome b5 from phenobarbital-treated rabbit liver microsomes was achieved by using three successive DEAE-cellulose and Sephadex G-100 column chromatographies as shown in Figure 2.5. Cytochrome b5 was purified from the same crude microsomal sample that was used for the purification of NADPH-cytochrome P450 from rabbit liver tissue as mentioned in the previous section. Thus, the first two steps – solubilization and first DEAE-cellulose column chromatography - in the purification of both cytochrome b5 and cytochrome P450 reductase from phenobarbital-treated rabbit liver microsomes were identical. The elution profiles of first and second DEAE-cellulose and Sephadex G-100 column chromatographies for the purification of microsomal cytochrome b5 from phenobarbital-treated rabbit liver are given in Figures 3.9, 3.10 and 3.11, respectively.

The results for the purification of microsomal cytochrome b5 from phenobarbital-treated rabbit liver microsomes are given in Table 3.4. It was found that cytochrome b5 from rabbit liver microsomes was purified 25.3-fold and the recovery in the final purified fraction was 22.2% with respect to microsomes. The specific activity of purified cytochrome b5 was 36.5 nmol per mg protein. SDS-PAGE carried out at the end of the Sephadex G-100 column chromatography to evaluate the purity of the enzyme. It was found that the final purified cytochrome b5 preparation was homogenous and gave a single protein band on 12.5% slab gel (Figure 3.12).

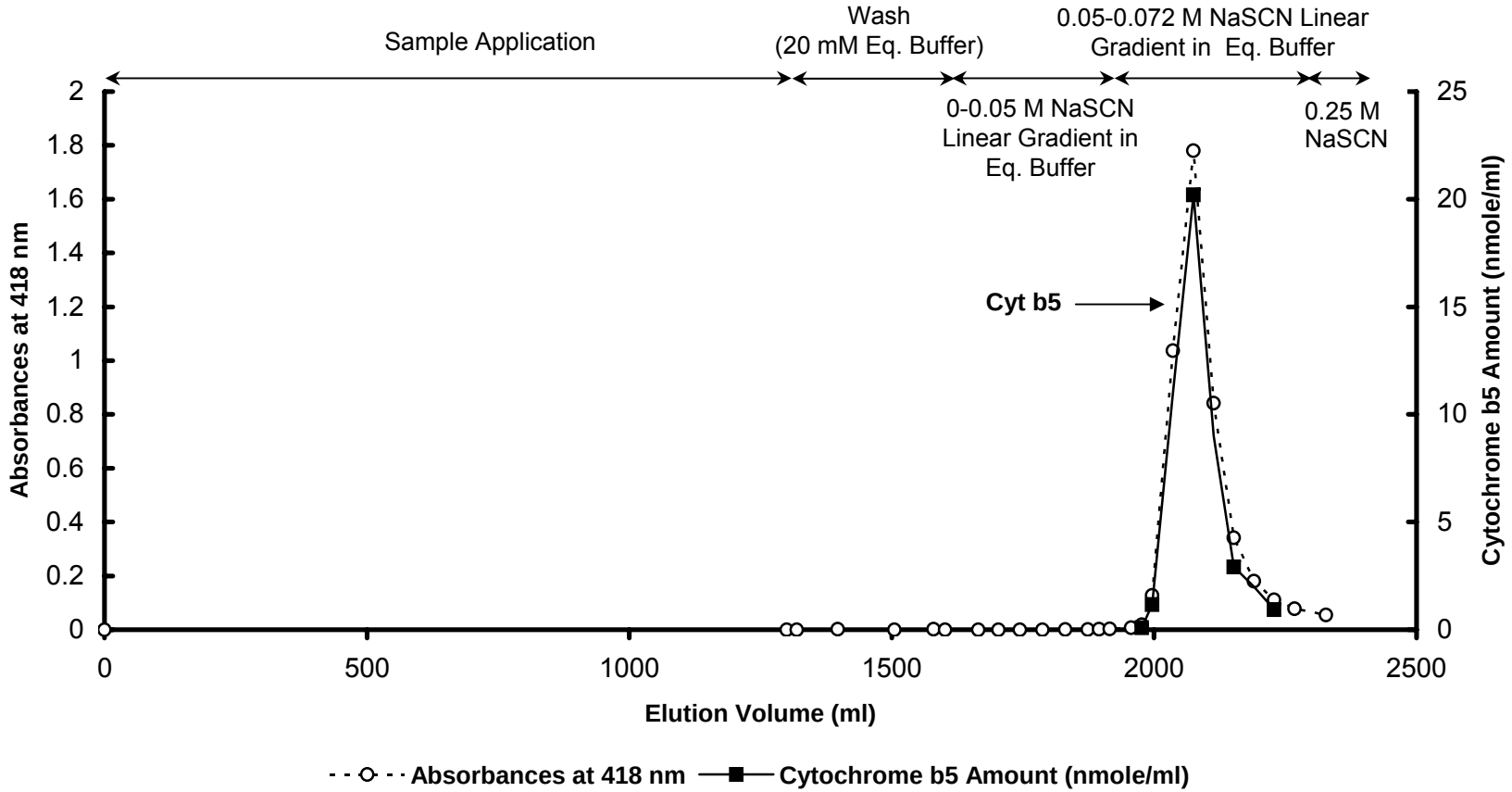


Figure 3.10 Second DEAE-cellulose column (2.8 x 12.5 cm) chromatography of the partially purified phenobarbital-treated rabbit liver cytochrome b5 obtained from first DEAE-cellulose column. Absorbances at 418 nm and cytochrome b5 amounts of fractions were measured. Cytochrome b5 was eluted with 0.05-0.072 M NaSCN linear gradient in equilibration buffer.

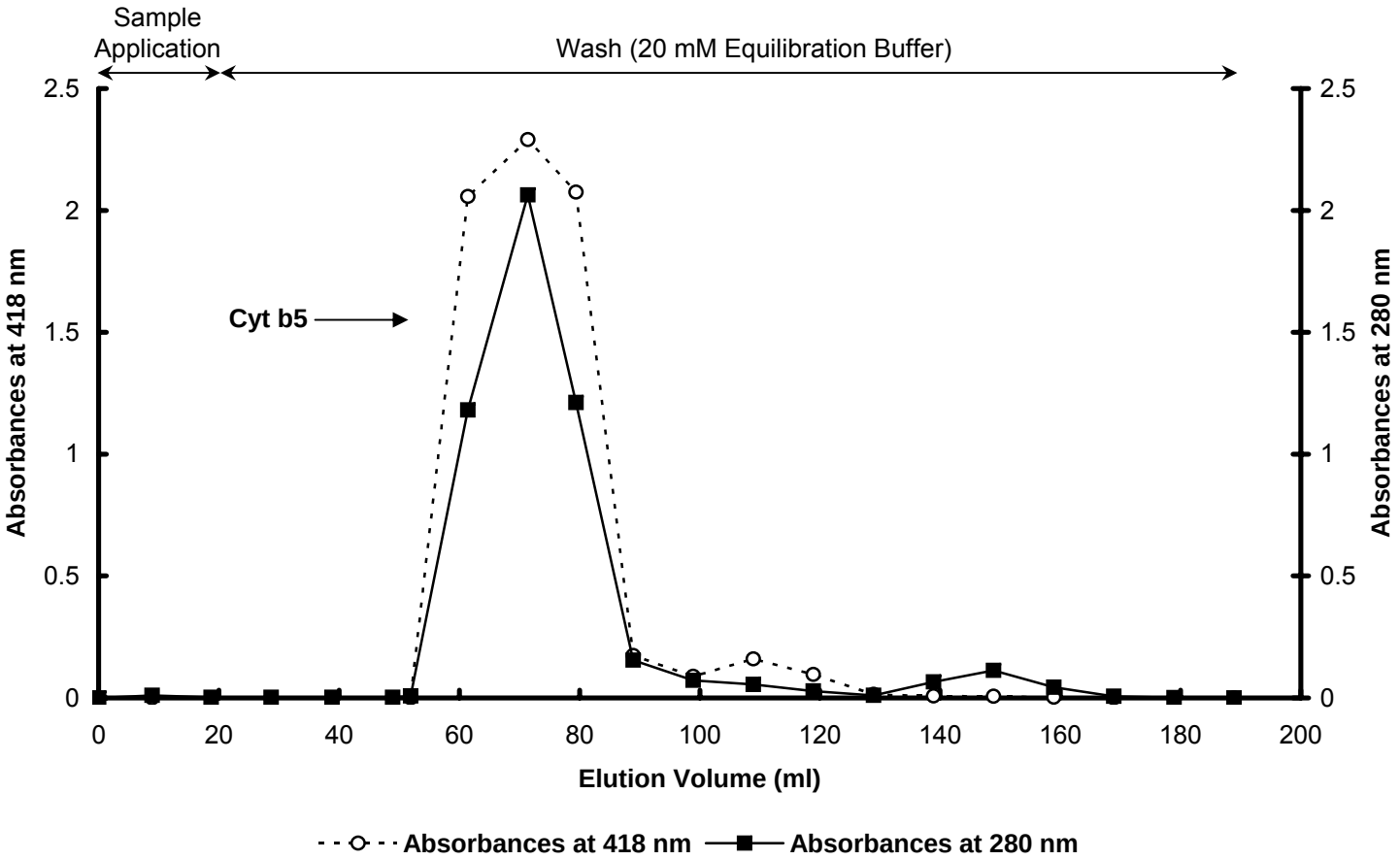


Figure 3.11 Sephadex G-100 column (1.5 x 84 cm) chromatography of the partially purified phenobarbital-treated rabbit liver cytochrome b5 obtained from third DEAE-cellulose column. Absorbances of fractions at 418 nm and 280 nm were measured.

Table 3.4 Purification of cytochrome b5 from phenobarbital-treated rabbit liver microsomes

Fractions	Volume (ml)	Protein Amount (mg/ml)	Cytochrome b5			Recovery %	Purification Fold
			b5 Amount (nmol/ml)	Specific b5 Content (nmol/mg prt.)	Total b5 Content (nmol)		
Microsomes	49.7	60.2	86.4	1.44	4294.1	100.0	1.0
Solubilized Microsomes	458.0	5.26	8.10	1.54	3032.0	70.6	1.07
DEAE-Cellulose-1	129.5	0.90	16.5	18.3	2134.2	49.7	12.7
DEAE-Cellulose-2	230.5	0.20	4.80	24.0	1106.4	25.8	16.7
DEAE-Cellulose-3	19.5	1.52	54.5	35.9	1062.8	24.8	24.9
Sephadex G-100	27.5	0.95	34.7	36.5	954.3	22.2	25.3

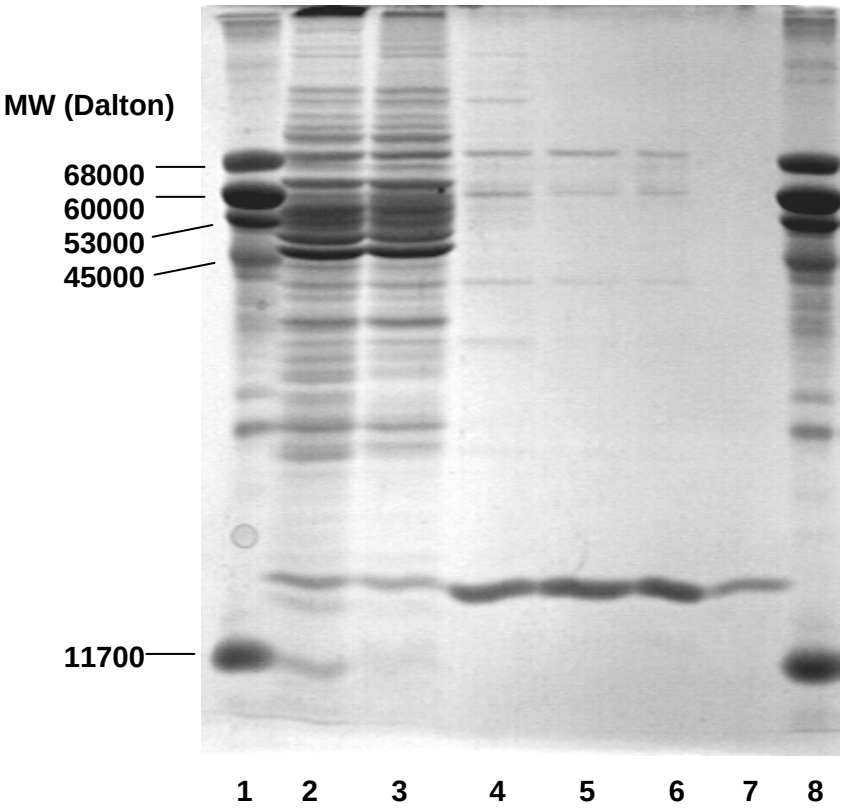


Figure 3.12 A 12.5% SDS-polyacrylamide gel showing the different steps for the purification of cytochrome b5 from phenobarbital-treated rabbit liver microsomes. **Lanes 1 and 8,** five reference proteins (BSA, catalase, glutamate dehydrogenase, egg albumin, and cytochrome *c,* 3.3 μg each); **lane 2,** microsomes (36.1 μg); **lane 3,** solubilized microsomes (32.9 μg); **lane 4,** cytochrome b5 fraction obtained from first DEAE-cellulose column (6.8 μg); **lane 5,** cytochrome b5 fraction obtained from second DEAE-cellulose column (3.6 μg); **lane 6,** cytochrome b5 fraction obtained from third DEAE-cellulose column (3.9 μg); **lane 7,** cytochrome b5 fraction obtained from Sephadex G-100 column (1.1 μg).

Figure 3.13 shows the absolute spectra of the purified rabbit liver cytochrome b5 in its oxidized state (Fe^{+3}). The absorption spectrum of cytochrome b5 (Fe^{+3}) showed a maximum absorbance at 413 nm. Cytochrome b5 in its oxidized state gave a peak at 358 nm which is characteristic for hemoproteins and another peak at 280 nm due to aromatic amino acid residues in the protein.

3.6 Purification of Sheep Lung Microsomal NADPH-Cytochrome P450 Reductase

The procedure for the purification of sheep lung NADPH-cytochrome P450 reductase involved anion-exchange chromatography of the detergent solubilized microsomes on first DEAE-cellulose column. Further purification and concentration of sheep lung P450 reductase was carried out with a second DEAE-cellulose column followed by affinity chromatography of the partially purified reductase on adenosine 2', 5'-diphosphate-Sepharose 4B column. Finally, hydroxylapatite column chromatography was carried out to remove the non-ionic detergent Emulgen 913 and to further purify and concentrate the enzyme. A summary of the results for the purification NADPH-cytochrome P450 reductase from sheep lung microsomes is shown in Table 3.5. NADPH-cytochrome P450 reductase was purified 198-fold with a final yield of 16.5% with respect to sheep lung microsomes. The specific activity of purified P450 reductase was 31.1 units/mg of protein when cytochrome *c* reduction was assayed spectrophotometrically at 550 nm as described in "Methods". The final preparation of enzyme produced a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Figure 3.14). The gel photograph shows that the lanes 6, 7 and 8 were overloaded with the purified enzyme. The minor protein band observed just beneath the major band represents the small amounts of proteolytically cleaved biocatalytically inactive P450 reductase present in the final purified preparation. The visible absolute absorption spectrum of the purified sheep lung NADPH-cytochrome P450 reductase also further confirmed the purity of the enzyme (data not shown).

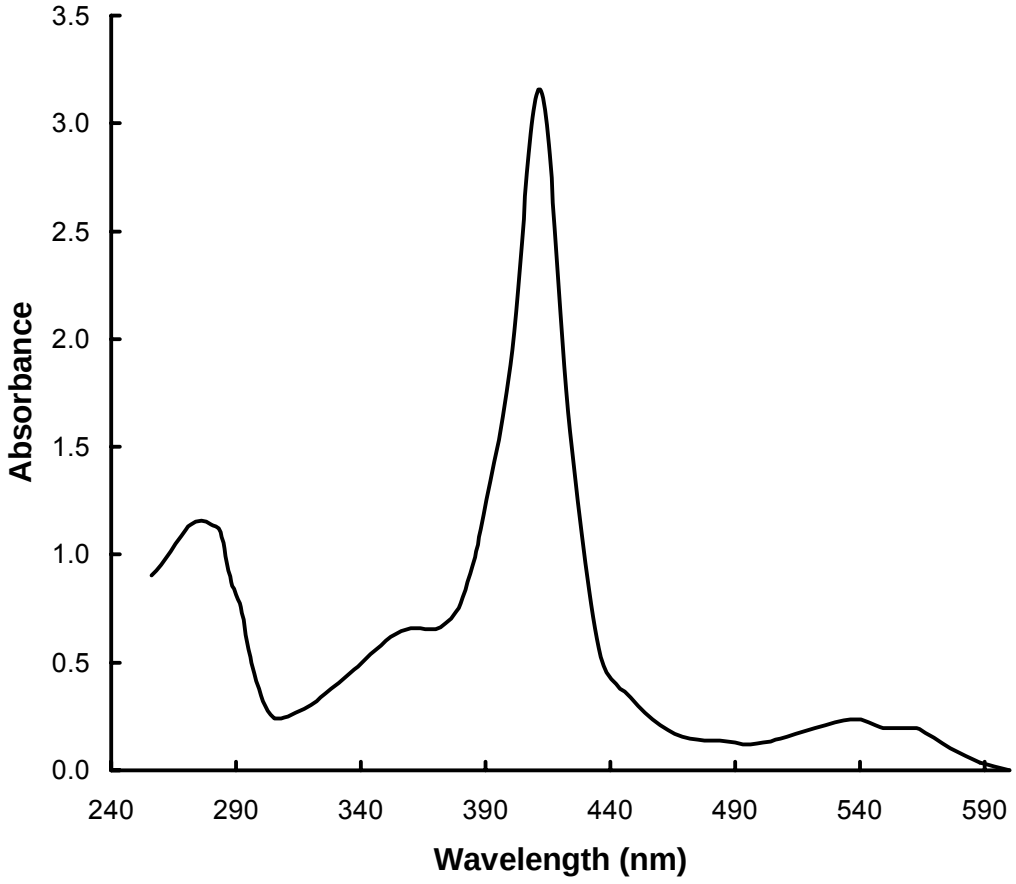


Figure 3.13 The absolute absorption spectrum of the highly purified rabbit liver microsomal cytochrome b5 in its oxidized state (Fe^{+3}). Absolute absorption spectrum of the cytochrome b5 was recorded without diluting the final preparation of the enzyme eluted from the Sephadex G-100 column against equilibration buffer in a double beam spectrophotometer using 1.0 cm pathlength cuvettes.

Table 3.5 Purification of NADPH-cytochrome P450 reductase from sheep lung microsomes

Fractions	Volume (ml)	Protein Amount (mg/ml)	Cytochrome P450 Reductase			Recovery %	Purification Fold
			Activity (nmol/min/ml)	Specific Activity (nmol/min/mg)	Total Activity (nmol/min)		
Microsomes	195.0	26.9	4232.4	157.3	825318.0	100.0	1.0
Solubilized Microsomes	835.0	4.24	1280.7	302.1	1069384.5	129.6	1.92
DEAE-Cellulose-1	173.0	5.13	4545.4	886.0	786354.2	95.3	5.63
DEAE-Cellulose-2	80.0	3.44	4352.7	1265.3	348216.0	42.2	8.04
Adenosine 2', 5'-diphosphate-Sepharose 4B	45.0	0.13	3903.1	30023.8	175639.5	21.3	190.9
Hydroxylapatite	4.70	0.93	28911.2	31087.3	135882.6	16.5	197.6

NADPH- dependent cytochrome *c* reductase activities were assayed at 25 °C, in 0.3 M potassium phosphate buffer, pH 7.7.

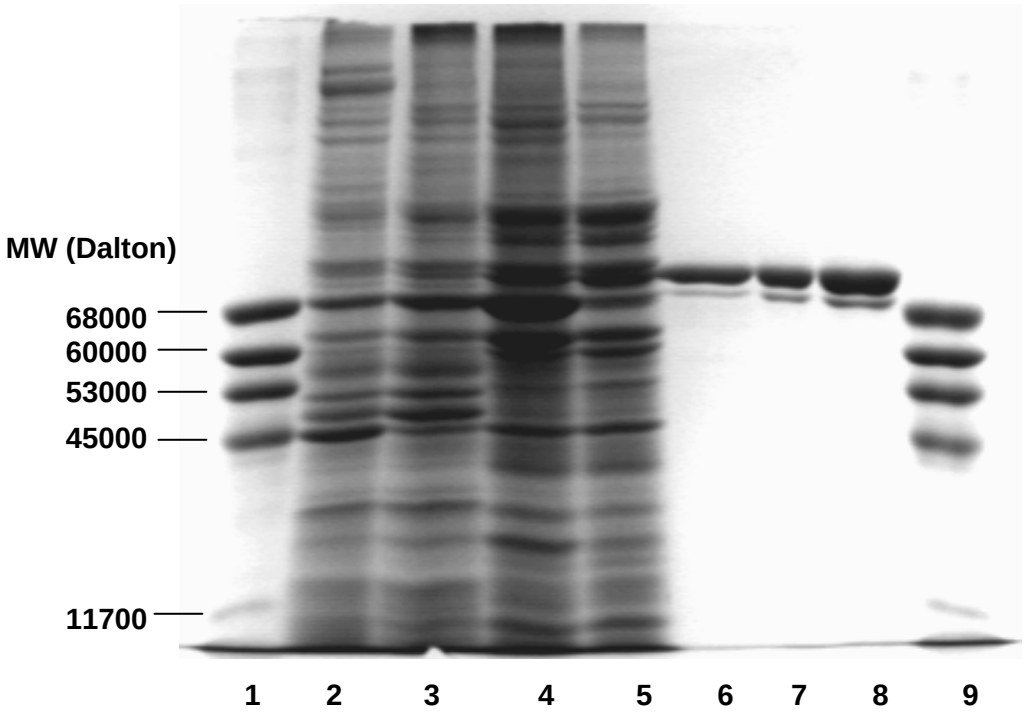


Figure 3.14 SDS-PAGE showing the different stages for the purification of sheep lung NADPH-cytochrome P450 reductase. **Lanes 1 and 9,** five reference proteins (BSA, catalase, glutamate dehydrogenase, egg albumin and cytochrome *c*, 3.3 µg each); **lane 2,** microsomes (60.5 µg); **lane 3,** solubilized microsomes (95.4 µg); **lane 4,** cytochrome P450 reductase fraction obtained from first DEAE-cellulose column (115.1 µg); **lane 5,** cytochrome P450 reductase fraction obtained from second DEAE-cellulose column (64.5 µg); **lane 6,** cytochrome P450 reductase fraction obtained from affinity column (4.9 µg); **lanes 7 and 8,** cytochrome P450 reductase fraction obtained from hydroxylapatite column 3.5 µg and 7.0 µg, respectively.

3.7 Biocatalytic Activities of Purified Beef Liver NADPH-Cytochrome P450 Reductase and Rabbit Liver Cytochrome P4502B4 in Reconstituted Systems

In order to be able to determine the relative contributions of higly purified beef liver P450 reductase and rabbit liver CYP2B4 to the reduction of idarubicin and mitomycin C under anaerobic conditions in reconstituted systems (Sections 3.13.2 and 3.13.3), first of all it was necessary to show that beef liver P450 reductase and rabbit liver CYP2B4 were biocatalytically active and could couple in the presence of a synthetic lipid in catalyzing a monooxygenation reaction in reconstituted systems. For this reason, benzphetamine N-demethylation reaction, a monooxygenation reaction catalyzed primarily by CYP2B isozyme, was chosen to assess the biocatalytic activities of purified enzymes. The relative contributions of rabbit liver P450 reductase and rabbit liver CYP2B4 to the reduction of idarubicin and mitomycin C under aerobic conditions were also investigated in detail in reconstituted systems (Section 3.13.1) and it was assumed that rabbit liver P450 reductase was biocatalytically active and could couple with the rabbit liver CYP2B4 in the presence of dilauroyl phosphatidylcholine as a synthetic lipid in reconstituted systems.

Benzphetamine N-demethylase activities of reconstituted systems containing various amounts of rabbit liver CYP2B4 and beef liver P450 reductase are shown in Table 3.6. As seen in Table 3.6, neither CYP2B4 nor P450 reductase alone were effective in catalyzing the N-demethylation of benzphetamine and lipid was necessary for reconstitution of the purified enzymes to catalyze the benzphetamine N-demethylation reaction. It was shown that beef liver P450 reductase and rabbit liver CYP2B4 were biocatalytically active and coupled in in the presence of synthetic lipid in reconstituting the benzphetamine N-demethylation reaction. Table 3.6 shows that the rate of benzphetamine N-demethylase activity increased with increasing amounts of both CYP2B4 and P450 reductase. However, this increase in the rate of benzphetamine N-demethylase activity was not proportional to increases in the amounts of added purified enzymes at the chosen concentrations (Table 3.6).

Table 3.6 Benzphetamine N-demethylase activities in reconstituted systems containing purified beef liver NADPH-cytochrome P450 reductase and rabbit liver cytochrome P4502B4 in the presence of dilauoryl phosphatidylcholine as a synthetic lipid

Components	BND Activity[a] (nmol $min^{-1}ml^{-1}$)
Rabbit CYP2B4 (0.1 nmol) + Lipid	0
Beef Reductase (0.1 units) + Lipid	0.52
Rabbit CYP2B4 (0.1 nmol) + Beef Reductase (0.1 units)	0.90
Rabbit CYP2B4 (0.1 nmol) + Beef Reductase (0.1 units) + Lipid	4.14
Rabbit CYP2B4 (0.1 nmol) + Beef Reductase (0.2 units) + Lipid	6.27
Rabbit CYP2B4 (0.19 nmol) + Beef Reductase (0.1 units) + Lipid	5.93
Rabbit CYP2B4 (0.19 nmol) + Beef Reductase (0.2 units) + Lipid	9.79

BND-Benzphetamine N-demethylase activities were determined as described in "Methods". Data represent the average of duplicate determinations.

[a] Incubation mixtures contained 100 mM HEPES buffer, pH 7.7, 1.5 mM benzphetamine-HCL, appropriate amounts of reconstituted enzymes and 0.5 mM NADPH generating system in a final volume of 0.5 ml.

3.8 Biocatalytic Activities of Purified Beef Liver Microsomal NADH-Cytochrome b5 Reductase

In order to determine the ability of idarubicin, in comparison to mitomycin C, to undergo bioreductive activation by highly purified beef liver microsomal NADH-cytochrome b5 reductase (Sections 3.9.3 and 3.12), it was crucial to show that the enzyme was biocatalytically active and purified from beef liver microsomes in its native amphipathic form. Biocatalytic properties of the purified beef liver cytochrome b5 reductase were determined according to its ability to reduce

cytochrome b5 and cytochrome *c* (through cytochrome b5) in reconstituted systems as described in "Methods".

Table 3.7 shows the ability of purified beef liver cytochrome b5 reductase to catalyze the transfer electrons from NADH to its endogenous substrate cytochrome b5 at two different pHs. As shown in Table 3.7, the purified beef liver cytochrome b5 reductase was biocatalytically active and effectively reduced the purified rabbit liver cytochrome b5. The rates of cytochrome b5 reductions by purified beef liver cytochrome b5 reductase were found to be almost similar at both pHs.

The purified enzyme was also found to be effective in catalyzing the cytochrome b5 coupled reduction of cytochrome *c* in reconstituted systems through the transfer of electrons from NADH to cytochrome b5 which in turn reduce cytochrome *c* (Table 3.8). The data in Table 3.8 demonstrate that individual components of the reconstituted system did not possess any cytochrome *c* reductase activity unless they came together in a reaction mixture. When 0.05 units of cytochrome b5 reductase were reconstituted with cytochrome b5 at an amount of 0.35 nmol per assay, the rate of cytochrome *c* reduction was found as 19.9 nmol per min. A two fold increase in the amount of cytochrome b5 reductase resulted in a proportional increase in cytochrome *c* reduction rate (Table 3.8). All these data indicated that NADH-cytochrome b5 reductase was biocatalytically active and obtained from beef liver microsomes in its native amphipathic form.

Table 3.7 NADH-cytochrome b5 reductase activities of purified beef liver NADH-cytochrome b5 reductase

Components	NADH-Cytochrome b5 Reductase Activity[a] (nmol min^{-1} ml^{-1})	
	pH 6.8	pH 7.5
Beef b5 Reductase (0.036 units) + Rabbit b5 (2.0 nmol)	526.5	567.0

The activities were calculated as described in "Methods". Data represent the average of duplicate determinations.

[a] The reaction medium contained 0.1 M potassium phosphate buffer pH 6.8 or pH 7.5, 0.111 mM NADH, 0.01% Emulgen 913, 2.0 nmol of purified rabbit liver cytochrome b5 and 0.036 units (based on ferricyanide reduction) of purified beef liver cytochrome b5 reductase in a final volume of 1.0 ml at 25°C. The enzyme activity is expressed as nmol of cytochrome b5 reduced per minute per ml of purified NADH-cytochrome b5 reductase sample.

Table 3.8 NADH-cytochrome *c* reductase activities of purified beef liver NADH-cytochrome b5 reductase

Components of Reconstituted System	NADH-Cytochrome *c* Reductase Activity[a] (nmol min^{-1})
Rabbit Cyt. b5 (0.35 nmol)	0
Beef b5 Reductase (0.05 units)	0.13
Beef b5 Reductase (0.05 units) + Rabbit Cyt. b5 (0.35 nmol)	19.9
Beef b5 Reductase (0.1 units) + Rabbit Cyt. b5 (0.35 nmol)	37.0

The activities were determined as described in "Methods". Data represent the average of duplicate determinations.

[a] The reaction mixture contained 0.3 M potassium phosphate buffer pH 7.5, 0.111 mM NADH, 89 nmol of cytochrome *c*, 0.35 nmol of purified rabbit liver cytochrome b5 and appropriate amounts of purified beef liver cytochrome b5 reductase (0.05 or 0.1 units based on ferricyanide reduction) in a final volume of 1.0 ml 25°C. The enzyme activity is expressed as nmol of cytochrome *c* reduced per minute per ml of reaction mixture.

3.9 DNA Strand Break Induction

The double stranded pBR322 plasmid DNA exists in a compact supercoiled form (SC, form I) in its native state which is converted to nicked circular or open circular DNA (OC, form II) upon single-strand cleavage. When double-strand breaks or two opposing single-strand breaks in close proximity are formed, the supercoiled circular DNA molecule is converted into linear form (form III). As the intensity of damage to closed circular DNA molecule increases, the DNA molecule is broken down ultimately into small DNA fragments which results in the complete degradation of pBR322 DNA. These three forms of plasmid DNA have different electrophoretic mobilities on the agarose gel due to their different tertiary structures. Supercoiled form of plasmid DNA moves faster in the gel compared to the open-circular form which has a reduced electrophoretic mobility, whereas, linear form of plasmid DNA migrates as a single band between the bands corresponding to supercoiled and open circular forms of plasmid DNA. The conversion of supercoiled form of plasmid DNA to the open circular form and their subsequent separation by agarose gel electrophoresis was used, therefore, as a sensitive assay in this study to examine whether and to what degree the purified oxidoreductases are involved in the bioactivation of idarubicin, to examine the possible involvement of redox cycling in the generation of DNA strand breaks as a consequence of enzyme-catalyzed bioactivation of idarubicin, and to investigate whether differences exist in the reductive activation of idarubicin and mitomycin C to generate strand breaks in DNA.

3.9.1 Redox-Cycling and Induction of DNA Damage during Bioreductive Activation of Idarubicin by Purified Sheep Lung P450 Reductase

As shown in Figure 3.15, incubation of plasmid DNA with idarubicin in the presence of highly purified sheep lung NADPH-cytochrome P450 reductase and NADPH cofactor under aerobic conditions as described in Section 2.2.11.1 resulted in loss in the intensity of bands corresponding to the supercoiled form with

concomitant increase in those associated with the open circular form but not linear form (lanes 6 and 10). The linear form of pBR322 plasmid DNA was obtained by digestion with PstI (lane 16). The plasmid-alone control incubation showed that approximately 10% of pBR322 plasmid DNA was already in the open circular form as seen in Figure 3.15 (lane 1). Control incubations in which either enzyme, NADPH cofactor or drug were omitted produced no DNA strand breaks over plasmid-alone control (lanes 7, 8 and 9), which indicates that the purified sheep lung P450 reductase catalyzes the bioreductive activation of idarubicin to DNA-damaging species.

In order to investigate the mechanism of DNA damage by idarubicin and the identity of radical species involved in this process, antioxidant enzymes, SOD and catalase, and scavengers of hydroxyl radicals, DMSO and thiourea, were employed. For this purpose, first of all, the effects of hydroxyl radicals generated via a typical $OH^{\cdot}$ generating system (ferric chloride-EDTA-ascorbate) on the induction of DNA damage were demonstrated. As shown in Figure 3.15, exposure of the pBR322 plasmid DNA to $OH^{\cdot}$ generating system lead to a complete conversion of supercoiled form into open circular and linear forms (lane 2). The yield of $OH^{\cdot}$-induced DNA strand breaks was found to be reduced by the addition of DMSO and thiourea (lanes 3 and 4). It was found that thiourea at a concentration of 10 mM was less protective than DMSO at 50 mM concentration in preventing $OH^{\cdot}$-induced DNA damage. Thiourea provided a 65% protection against $OH^{\cdot}$-induced DNA strand breaks, whereas, treatment of plasmid DNA with DMSO resulted in a 86% reduction in strand scission (Table 3.9).

Figure 3.15 also demonstrates that DNA strand breaks produced as a consequence of P450 reductase-catalyzed bioreductive activation of idarubicin were significantly inhibited by the treatment of pBR322 plasmid DNA with DMSO and thiourea (lanes 13 and 14). While 50 mM DMSO produced a 71% reduction in DNA strand breaks, treatment of plasmid DNA with 10 mM thiourea lead to a 58% protection (Table 3.9). Similarly, both SOD and catalase were found to be very effective in protecting DNA against strand scission induced by idarubicin (Figure

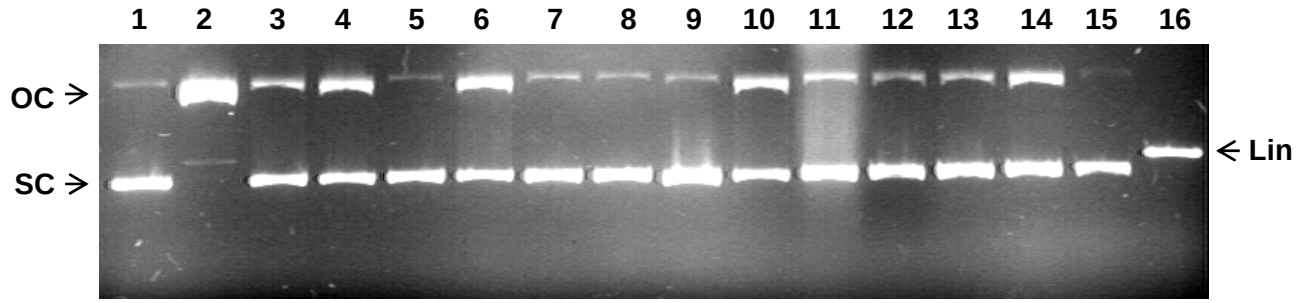


Figure 3.15 Agarose gel electrophoresis showing the protective effects of radical scavengers against plasmid DNA strand breaks induced by purified sheep lung NADPH-cytochrome P450 reductase (P450R)-catalyzed reductive activation of idarubicin in the presence of cofactor NADPH. Supercoiled pBR322 DNA (1.0 µg) was incubated for 30 minutes at 37°C in the presence of P450R (0.1 µg), NADPH (2 mM) and idarubicin (100 µM) with various radical scavengers at indicated concentrations in a final volume of 60 µl reaction mixture as described in "Materials and Methods". Agarose gel electrophoresis, **lane 1,** plasmid DNA control; **lane 2,** plasmid DNA + hydroxyl radical generating system (10 µM ferric chloride-20 µM EDTA-1 mM ascorbate); **lane 3,** plasmid DNA + hydroxyl radical generating system + 50 mM DMSO; **lane 4,** plasmid DNA + hydroxyl radical generating system + 10 mM thiourea; **lane 5,** plasmid DNA + idarubicin only; **lane 6,** complete system (plasmid DNA + idarubicin + P450R + NADPH); **lane 7,** no P450R control (plasmid DNA + idarubicin + NADPH); **lane 8,** no NADPH control (plasmid DNA + idarubicin + P450R); **lane 9,** no idarubicin control (plasmid DNA + P450R + NADPH); **lane 10,** complete system (plasmid DNA + idarubicin + P450R + NADPH); **lane 11,** complete system + SOD (42 units); **lane 12,** complete system + catalase (42 units); **lane 13,** complete system + 50 mM DMSO; **lane 14,** complete system plus + 10 mM thiourea; **lane 15**, plasmid DNA control (0.25 µg); **lane 16**, plasmid DNA (0.25 µg) + PstI (20 units). SC, supercoiled (form I); OC, open circular (form II).

Table 3.9 Protective effects of radical scavengers against hydroxyl radical ($OH^{\cdot}$)- and idarubicin-induced DNA strand breaks

Treatment	SBI% (OC%)[a]	Protection%[a]
pBR322 plasmid DNA-alone control	5.3	—
pBR322 + $OH^{\cdot}$ radical generating system[b]	100	—
pBR322 + $OH^{\cdot}$ radical gen. sys. + DMSO (50 mM)	18.7	85.9
pBR322 + $OH^{\cdot}$ radical gen. sys. + thiourea (10 mM)	38.7	64.7
No idarubicin control	6.9	—
Complete system[c] including idarubicin	38.4	—
Complete system + SOD (42 units per assay)	11.4	85.7
Complete system + catalase (42 units per assay)	14.6	75.6
Complete system + DMSO (50 mM)	15.9	71.4
Complete system + thiourea (10 mM)	20.3	57.5

Experimental conditions are described in detail under “Materials and Methods”.

[a] Calculations for SBI (DNA strand breaking index, OC%) and protection% values are described in Section 2.2.11.5.

[b] Hydroxyl radical ($OH^{\cdot}$) generating system consists of 10 μM ferric chloride-20 μM EDTA-1 mM ascorbate.

[c] Complete system consists of pBR322 plasmid DNA (1.0 μg) + idarubicin (100 μM) + purified sheep lung NADPH-cytochrome P450 reductase (0.1 μg) + NADPH (2 mM) in a final volume of 60 μl reaction mixture.

3.15, lanes 11 and 12). Treatment of pBR322 plasmid DNA with SOD and catalase at 42.0 units per assay concentrations provided 86 and 76% protections against idarubicin-induced single-strand breaks, respectively (Table 3.9).

The above plasmid DNA experiments were also repeated under same reaction conditions using mitomycin C and the results were compared. We have chosen mitomycin C as a model compound in this study, since it is an effective redox cycling quinone-containing anticancer drug that produces oxygen radicals in the presence of cytochrome P450 reductase (Seow *et al.*, 2004). Figure 3.16 and Table 3.10 demonstrate that essentially similar results were obtained when mitomycin C was used in place of idarubicin.

As a result, all the presented data above strongly suggested that reactive oxygen species produced during redox cycling of idarubicin by purified sheep lung cytochrome P450 reductase appear to promote DNA damage. The production of superoxide anion, hydrogen peroxide and hydroxyl radicals during this process was confirmed by the treatment of plasmid DNA with superoxide dismutase, catalase and other radical scavengers, DMSO and thiourea which effectively protected pBR322 plasmid DNA against idarubicin-induced strand breaks.

3.9.2 Comparison of DNA-Damaging Potentials of Idarubicin and Mitomycin C in the Presence of Purified Sheep Lung P450 Reductase

In order to evaluate the *in vitro* capacity of idarubicin to redox cycle with cytochrome P450 reductase, and thus to induce DNA damage, the effects of increasing incubation time, drug concentration and enzyme amount on the generation of single-strand DNA breaks were examined. Typical results demonstrating the effects of increasing incubation time, enzyme amount and drug concentration on the generation of idarubicin-induced DNA strand breaks in the presence of purified sheep lung P450 reductase are presented in Figures 3.17-3.19, respectively. It was shown that the degree of DNA damage increased as a function of increasing

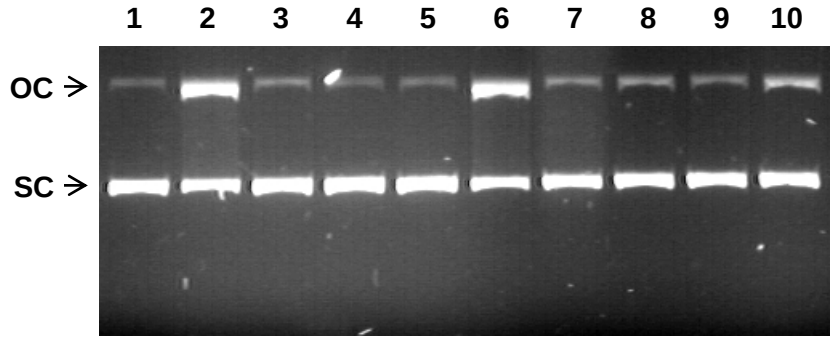


Figure 3.16 Agarose gel electrophoresis showing the protective effects of radical scavengers against plasmid DNA strand breaks induced by purified sheep lung NADPH-cytochrome P450 reductase (P450R)-catalyzed reductive activation of mitomycin C in the presence of cofactor NADPH. Supercoiled pBR322 DNA (1.0 μg) was incubated for 30 minutes at 37°C in the presence of P450R (0.1 μg), NADPH (2 mM) and mitomycin C (100 μM) with various radical scavengers at indicated concentrations in a final volume of 60 μl reaction mixture as described in "Materials and Methods". Agarose gel electrophoresis, **lane 1,** plasmid DNA + mitomycin C only; **lane 2,** complete system (plasmid DNA + mitomycin C + P450R + NADPH); **lane 3,** no P450R control (plasmid DNA + mitomycin C + NADPH); **lane 4,** no NADPH control (plasmid DNA + mitomycin C + P450R); **lane 5,** no mitomycin C control (plasmid DNA + P450R + NADPH); **lane 6,** complete system (plasmid DNA + mitomycin C + P450R + NADPH); **lane 7,** complete system + SOD (42 units); **lane 8,** complete system + catalase (42 units); **lane 9,** complete system + 50 mM DMSO; **lane 10,** complete system + 10 mM thiourea. SC, supercoiled (form I); OC, open circular (form II).

Table 3.10 Protective effects of radical scavengers against mitomycin C-induced DNA strand breaks

Treatment	SBI% (OC%)[a]	Protection%[a]
No mitomycin C control	6.4	—
Complete system[b]	38.3	—
Complete system + SOD (42 units)	10.6	86.8
Complete system + catalase (42 units)	12.7	80.3
Complete system + DMSO (50 mM)	10.9	85.9
Complete system + thiourea (10 mM)	18.0	63.6

Experimental conditions are described in detail under "Materials and Methods".

[a] Calculations for SBI (DNA strand breaking index, OC%) and protection% values are described in Section 2.2.11.5.

[b] Complete system consists of pBR322 plasmid DNA (1.0 µg) + mitomycin C (100 µM) + purified sheep lung NADPH-cytochrome P450 reductase (0.1 µg) + NADPH (2 mM) in a final volume of 60 µl reaction mixture.

incubation time (5-90 minutes) (Figure 3.17) or enzyme amount (0.025-1.25 µg) (Figure 3.18) as well as with increasing concentrations of drug (1-400 µM) (Figure 3.19). There was no DNA damage when NADPH, enzyme or drug were omitted from incubation mixtures (Figures 3.17-3.19).

Idarubicin's ability to generate reactive oxygen species during its redox cycling by cytochrome P450 reductase was then compared with mitomycin C as determined by the assessment of DNA damage under above conditions (for mitomycin C, the results are shown in Figures 3.20-3.22). As shown in Figure 3.17, aerobic incubation of plasmid DNA with idarubicin for 30 minutes in the presence of purified sheep lung P450 reductase and cofactor NADPH resulted in about 40% increase in OC form over control, whereas incubation for 90 minutes produced about 75% increase over control. The time-course experiment for the generation of DNA strand breaks induced by mitomycin C produced essentially similar results with

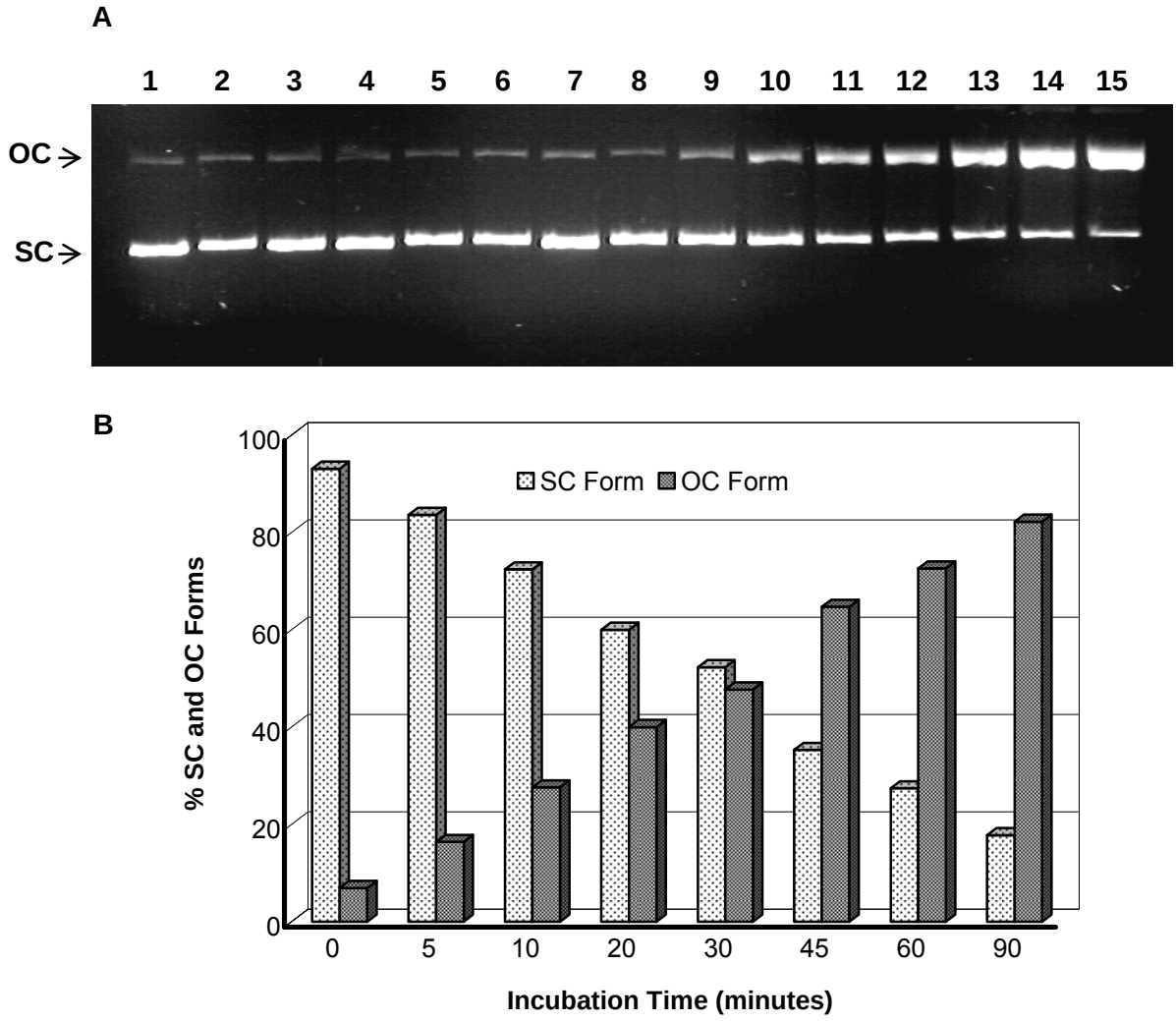


Figure 3.17 Effect of increasing incubation time on the formation of plasmid DNA strand breaks induced by purified sheep lung NADPH-cytochrome P450 reductase (P450R)-catalyzed reductive activation of idarubicin in the presence of cofactor NADPH. Supercoiled pBR322 DNA (1.0 µg) was incubated for various incubation times (0-90 minutes) at 37°C in the presence of idarubicin (100 µM), NADPH (2 mM) and P450R (0.1 µg) in a final volume of 60 µl reaction mixture as described in "Methods". **(A) Agarose gel electrophoresis, lane 1**, plasmid DNA control; **lane 2**, plasmid DNA + idarubicin; **lane 3**, plasmid DNA + NADPH; **lane 4**, plasmid DNA + P450R; **lane 5**, no NADPH control (plasmid DNA + P450R + idarubicin); **lane 6**, no P450R control (plasmid DNA + idarubicin + NADPH); **lane 7**, no idarubicin control (plasmid DNA + P450R + NADPH); **lanes 8 to 15**, complete system incubations for increasing incubation time (0, 5, 10, 20, 30, 45, 60, 90 minutes, respectively). **(B)** Percentage of detected SC (form I) and OC (form II) forms of pBR322 plasmid DNA represented as column chart. Light colored columns represent % SC form of DNA and dark colored columns represent % OC form of DNA. Data correspond to lanes 8 to15 (complete system incubations for increasing incubation time, 0, 5, 10, 20, 30, 45, 60, 90 minutes, respectively). OC, open circular; SC, supercoiled.

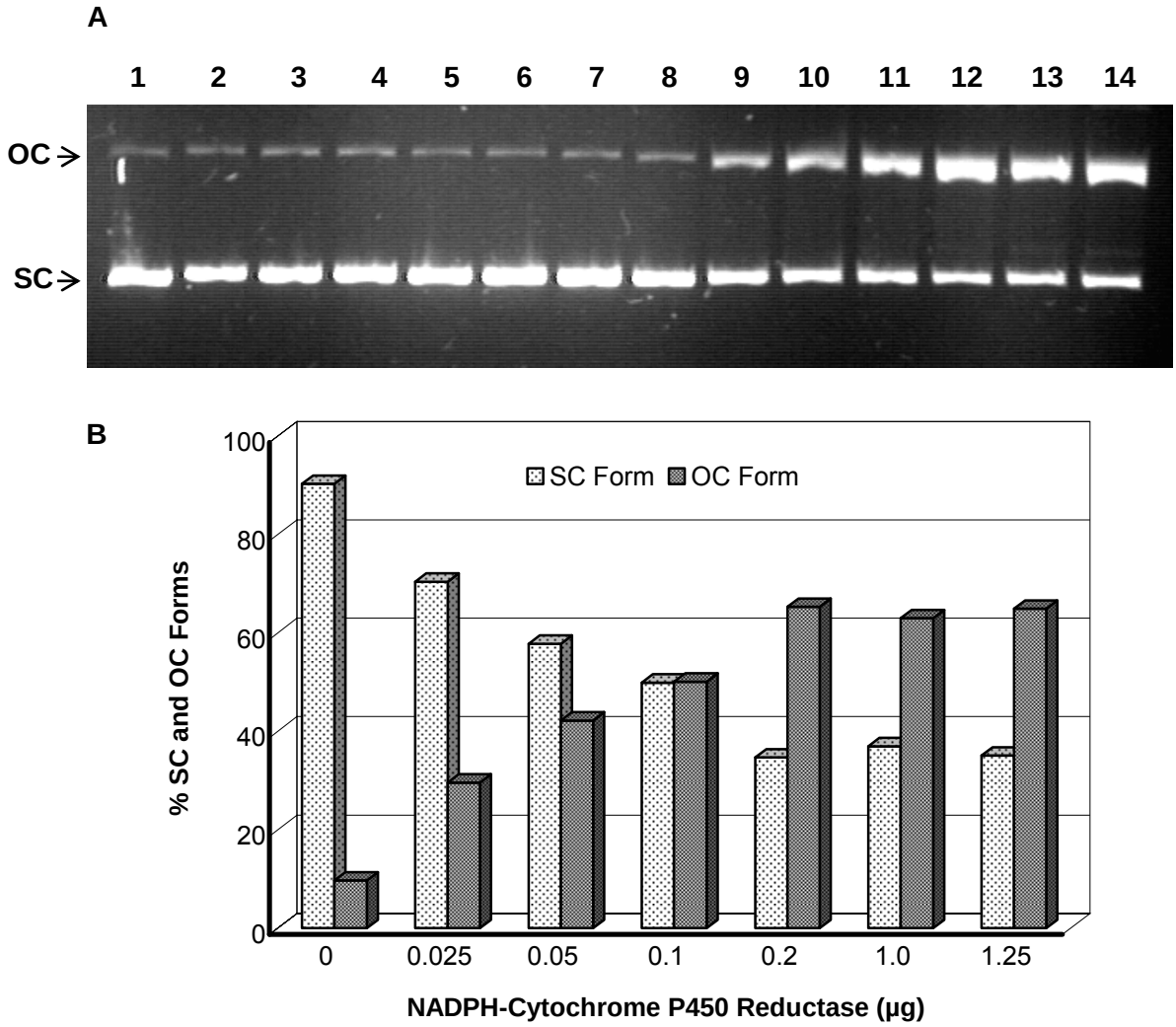


Figure 3.18 Effect of increasing enzyme concentration on the formation of plasmid DNA strand breaks induced by purified sheep lung NADPH-cytochrome P450 reductase (P450R)-catalyzed reductive activation of idarubicin in the presence of cofactor NADPH. Supercoiled pBR322 DNA (1.0 µg) was incubated for 30 minutes at 37°C in the presence of idarubicin (100 µM) and NADPH (2 mM) with various concentrations of P450R (0-1.25 µg) in a final volume of 60 µl reaction mixture as described in "Methods". **(A) Agarose gel electrophoresis, lane 1,** plasmid DNA control; **lanes 2 to 7,** no NADPH controls for increasing P450R concentrations (0.025, 0.050, 0.1, 0.2, 1.0, 1.25 µg, respectively); **lanes 8 to 14,** complete system incubations for increasing P450R concentrations (0, 0.025, 0.050, 0.1, 0.2, 1.0, 1.25 µg, respectively) including NADPH. **(B)** Percentage of detected SC (form I) and OC (form II) forms of pBR322 plasmid DNA represented as column chart. Light colored columns represent % SC form of DNA and dark colored columns represent % OC form of DNA. Data correspond to lanes 8 to14 (complete system incubations for increasing P450R concentrations, 0, 0.025, 0.050, 0.1, 0.2, 1.0, 1.25 µg, respectively). OC, open circular; SC, supercoiled.

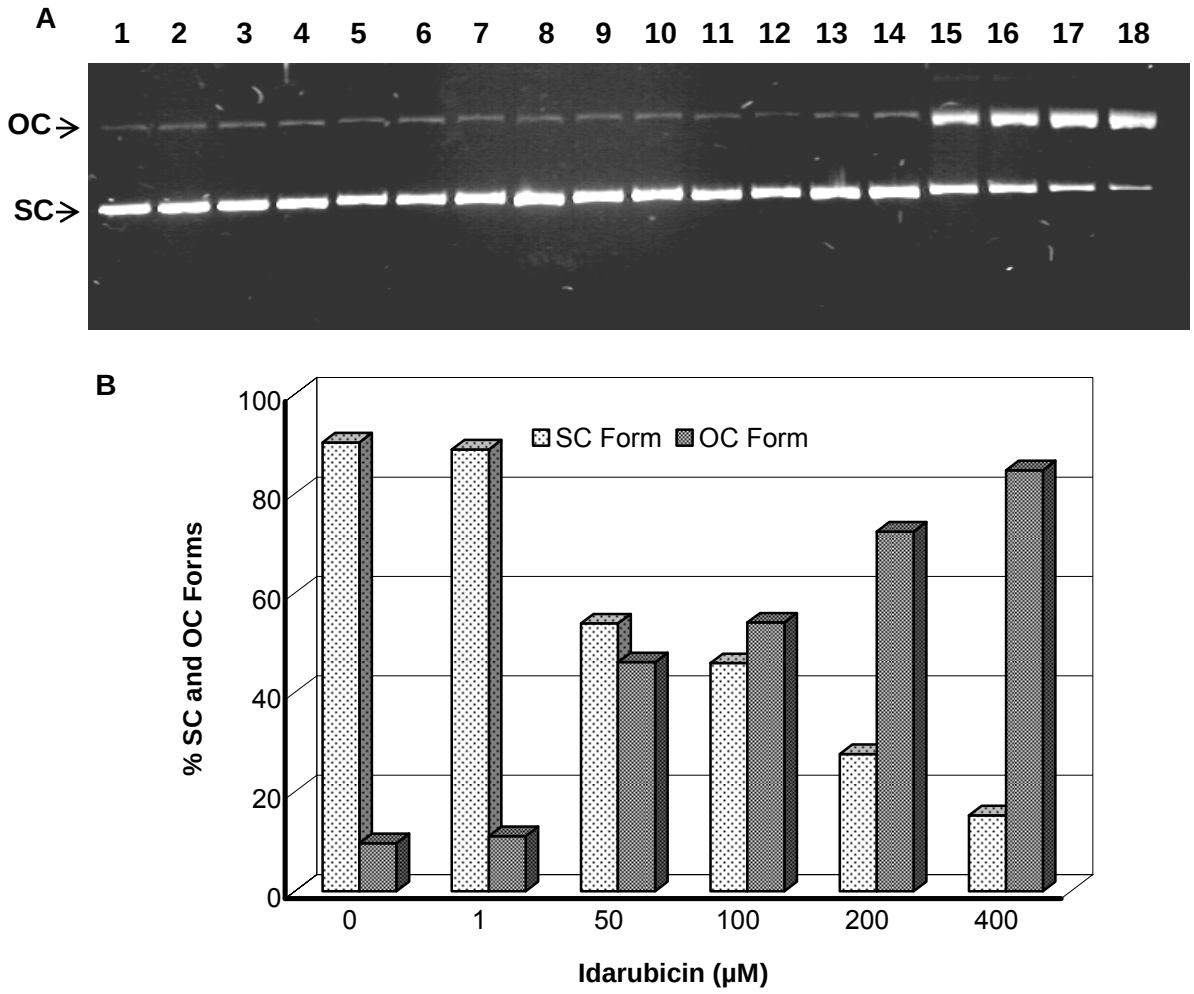


Figure 3.19 Effect of increasing drug concentration on the formation of plasmid DNA strand breaks induced by purified sheep lung NADPH-cytochrome P450 reductase (P450R)-catalyzed reductive activation of idarubicin in the presence of cofactor NADPH. Supercoiled pBR322 DNA (1.0 μg) was incubated for 30 minutes at 37°C in the presence of P450R (0.1 μg) and NADPH (2 mM) with various concentrations of idarubicin (0-400 μM) in a final volume of 60 μl reaction mixture as described in "Methods". **(A) Agarose gel electrophoresis, lane 1,** plasmid DNA control; **lane 2,** plasmid DNA + P450R; **lane 3,** plasmid DNA + NADPH; **lane 4,** no idarubicin control (plasmid DNA + P450R + NADPH); **lane 5,** plasmid DNA + idarubicin; **lane 6,** no P450R control (plasmid DNA + idarubicin + NADPH); **lane 7,** no NADPH control (plasmid DNA + P450R + idarubicin); **lanes 8 to 12,** no NADPH controls for increasing idarubicin concentrations (1, 50, 100, 200 and 400 μM, respectively); **lanes 13 to 18**, complete system incubations for increasing idarubicin concentrations (0, 1, 50, 100, 200 and 400 μM, respectively) including NADPH. **(B)** Percentage of detected SC (form I) and OC (form II) forms of pBR322 plasmid DNA represented as column chart. Light colored columns represent % SC form of DNA and dark colored columns represent % OC form of DNA. Data correspond to lanes 13 to 18 (complete system incubations for increasing idarubicin concentrations, 0, 1, 50, 100, 200 and 400 μM, respectively). OC, open circular; SC, supercoiled.

idarubicin (Figure 3.20). Aerobic incubation of plasmid DNA with mitomycin C for 30 minutes in the presence of purified lung P450 reductase and cofactor NADPH resulted in a 43.6% increase in % OC form over control, while incubation for 90 min lead to a 74.3% increase in % OC form over control.

The results for the characterization of idarubicin- and mitomycin C-induced DNA strand cleavage with respect to increasing sheep lung P450 reductase concentration are depicted in Figure 3.18 and Figure 3.21, respectively. The extent of idarubicin-induced DNA damage was found to increase with increasing P450 reductase amount up to 0.2 μg beyond which saturation occurred whereas this saturation was reached at a higher amount of P450 reductase (1.0 μg) in the presence of mitomycin C.

The effects of increasing drug concentration on the generation of idarubicin- and mitomycin C-induced plasmid DNA strand breaks during their reductive activation by P450 reductase in the presence of NADPH are shown in Figure 3.19 and Figure 3.22, respectively. It was found that at 100 μM concentration, both drugs in the presence of sheep lung P450 reductase induced about 40% increase in DNA scissions over control while at 200 and 400 μM concentrations idarubicin was 20 and 24% more effective in promoting DNA damage (Table 3.11).

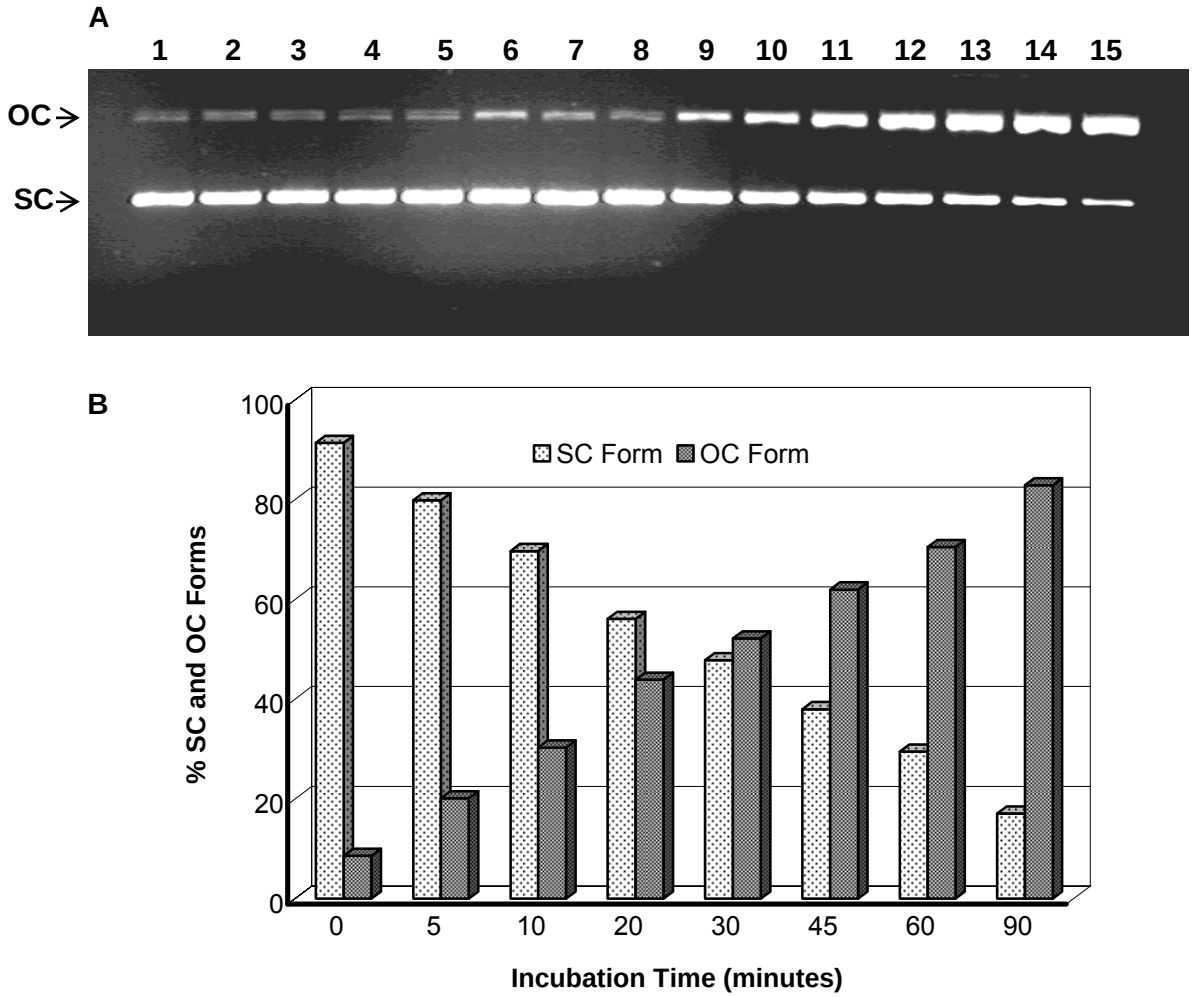


Figure 3.20 Effect of increasing incubation time on the formation of plasmid DNA strand breaks induced by purified sheep lung NADPH-cytochrome P450 reductase (P450R)-catalyzed reductive activation of mitomycin C in the presence of cofactor NADPH. Supercoiled pBR322 DNA (1.0 µg) was incubated for various incubation times (0-90 minutes) at 37°C in the presence of mitomycin C (100 µM), NADPH (2 mM) and P450R (0.1 µg) in a final volume of 60 µl reaction mixture as described in "Methods". **(A) Agarose gel electrophoresis, lane 1**, plasmid DNA control; **lane 2**, plasmid DNA + mitomycin C; **lane 3**, plasmid DNA + NADPH; **lane 4**, plasmid DNA + P450R; **lane 5**, no NADPH control (plasmid DNA + P450R + mitomycin C); **lane 6**, no P450R control (plasmid DNA + mitomycin C + NADPH); **lane 7**, no mitomycin C control (plasmid DNA + P450R + NADPH); **lanes 8 to 15**, complete system incubations for increasing incubation time (0, 5, 10, 20, 30, 45, 60, 90 minutes, respectively). **(B)** Percentage of detected SC (form I) and OC (form II) forms of pBR322 plasmid DNA represented as column chart. Light colored columns represent % SC form of DNA and dark colored columns represent % OC form of DNA. Data correspond to lanes 8 to15 (complete system incubations for increasing incubation time, 0, 5, 10, 20, 30, 45, 60, 90 minutes, respectively). OC, open circular; SC, supercoiled.

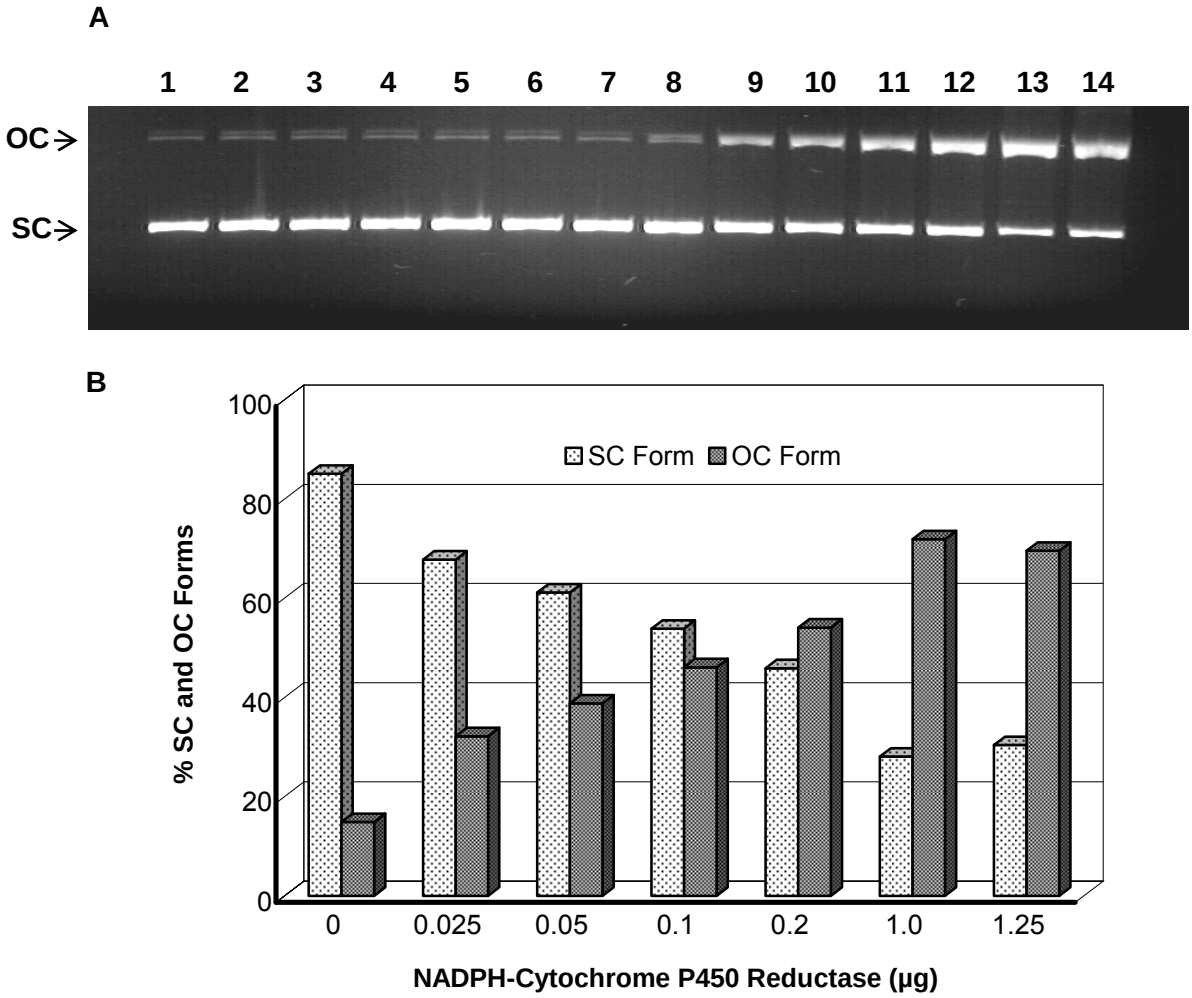


Figure 3.21 Effect of increasing enzyme concentration on the formation of plasmid DNA strand breaks induced by purified sheep lung NADPH-cytochrome P450 reductase (P450R)-catalyzed reductive activation of mitomycin C in the presence of cofactor NADPH. Supercoiled pBR322 DNA (1.0 µg) was incubated for 30 minutes at 37°C in the presence of mitomycin C (100 µM) and NADPH (2 mM) with various concentrations of P450R (0-1.25 µg) in a final volume of 60 µl reaction mixture as described in "Methods". **(A) Agarose gel electrophoresis, lane 1,** plasmid DNA control; **lanes 2 to 7,** no NADPH controls for increasing P450R concentrations (0.025, 0.050, 0.1, 0.2, 1.0, 1.25 µg, respectively); **lanes 8 to 14,** complete system incubations for increasing P450R concentrations (0, 0.025, 0.050, 0.1, 0.2, 1.0, 1.25 µg, respectively) including NADPH. **(B)** Percentage of detected SC (form I) and OC (form II) forms of pBR322 plasmid DNA represented as column chart. Light colored columns represent % SC form of DNA and dark colored columns represent % OC form of DNA. Data correspond to lanes 8 to14 (complete system incubations for increasing P450R concentrations, 0, 0.025, 0.050, 0.1, 0.2, 1.0, 1.25 µg, respectively). OC, open circular; SC, supercoiled.

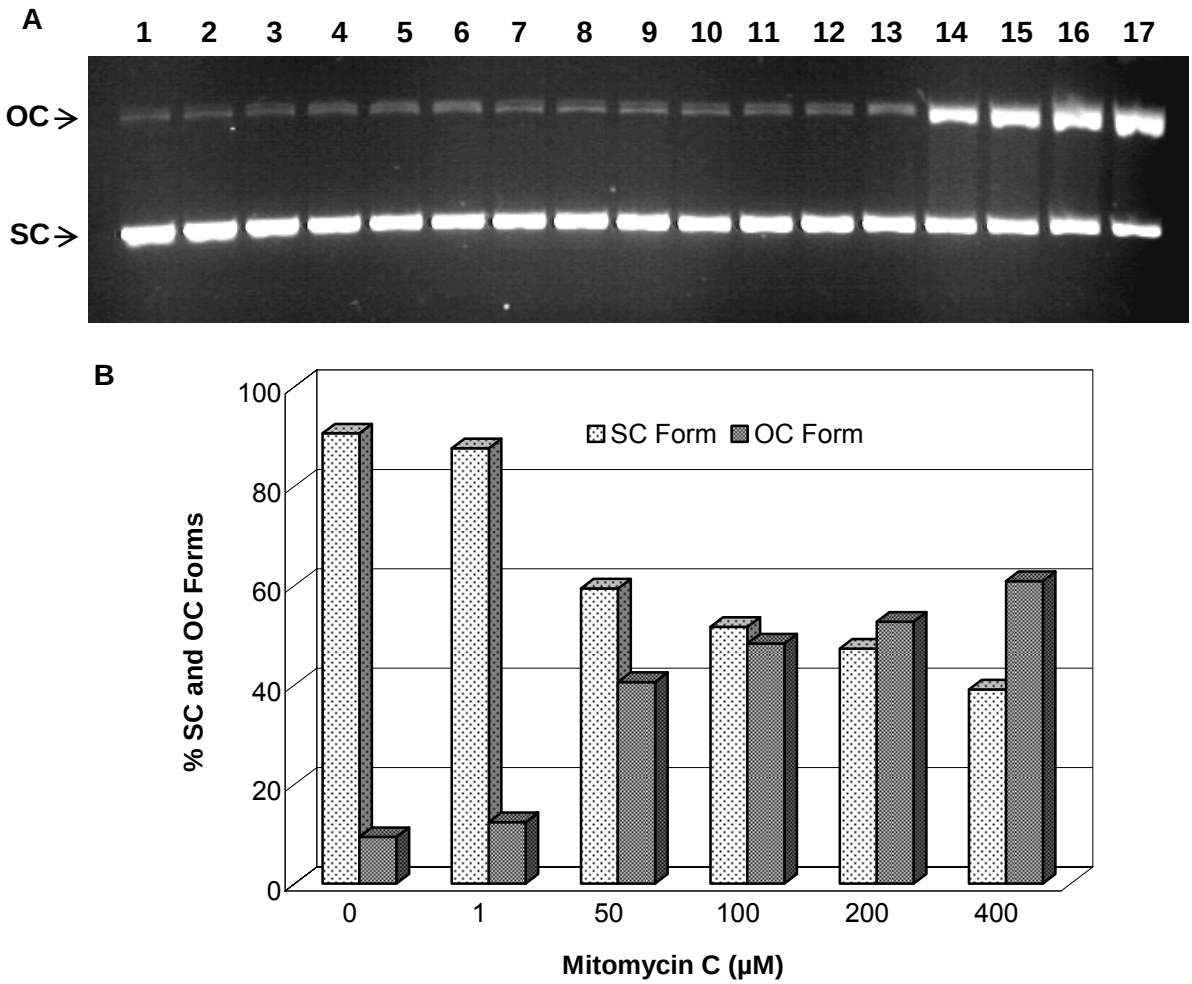


Figure 3.22 Effect of increasing drug concentration on the formation of plasmid DNA strand breaks induced by purified sheep lung NADPH-cytochrome P450 reductase (P450R)-catalyzed reductive activation of mitomycin C in the presence of cofactor NADPH. Supercoiled pBR322 DNA (1.0 µg) was incubated for 30 minutes at 37°C in the presence of P450R (0.1 µg) and NADPH (2 mM) with various concentrations of mitomycin C (0-400 µM) in a final volume of 60 µl reaction mixture as described in "Methods". **(A) Agarose gel electrophoresis, lane 1,** plasmid DNA control; **lane 2,** plasmid DNA + P450R; **lane 3,** plasmid DNA + NADPH; **lane 4,** no mitomycin C control (plasmid DNA + P450R + NADPH); **lane 5,** plasmid DNA + mitomycin C; **lane 6,** no P450R control (plasmid DNA + mitomycin C + NADPH); **lane 7,** no NADPH control (plasmid DNA + P450R + mitomycin C); **lanes 8 to 12,** no NADPH controls for increasing mitomycin C concentrations (1, 50, 100, 200 and 400 µM, respectively); **lanes 13 to 17**, complete system incubations for increasing mitomycin C concentrations (1, 50, 100, 200 and 400 µM, respectively) including NADPH. **(B)** Percentage of detected SC (form I) and OC (form II) forms of pBR322 plasmid DNA represented as column chart. Light colored columns represent % SC form of DNA and dark colored columns represent % OC form of DNA. Data correspond to lanes 4 and 13 to 17 (complete system incubations for increasing mitomycin C concentrations, 0, 1, 50, 100, 200 and 400 µM, respectively). OC, open circular; SC, supercoiled.

Table 3.11 Effect of increasing drug concentration on the generation of idarubicin- and mitomycin C-induced plasmid DNA strand breaks in the presence of purified sheep lung NADPH-cytochrome P450 reductase and cofactor NADPH

Drug Concentration (μM)	SBI% (OC%)[a]	
	Idarubicin-Induced	Mitomycin C-Induced
0	9.7	9.4
1	11.1	12.4
50	46.1	40.6
100	54.1	48.3
200	72.4	52.7
400	84.7	60.9

[a] Supercoiled pBR322 DNA (1.0 μg) in 100 mM sodium phosphate buffer, pH 7.4, was incubated for 30 minutes at 37°C in the presence of purified sheep lung cytochrome P450 reductase (0.1 μg) and NADPH (2 mM) with various concentrations of either drug (0-400 μM) in a final volume of 60 μl reaction mixture and subjected to agarose gel electrophoresis. Gels were photographed and the amount of DNA damage was quantified. SBI (DNA strand breaking index, OC%) was calculated as described in Section 2.2.11.5.

3.9.3 Involvement of Purified Beef Liver Microsomal NADH-Cytochrome b5 Reductase in Idarubicin- and Mitomycin C-Induced Plasmid DNA Breakage

In order to determine the ability of idarubicin to undergo bioreductive activation by highly purified beef liver microsomal NADH-cytochrome b5 reductase to generate strand breaks in DNA, various reaction conditions were tested as described in detail under "Methods". Figure 3.23 shows the typical results of plasmid DNA assay in which pBR322 plasmid DNA was incubated with idarubicin at 25 μM concentration, various amounts of either purified sheep lung NADPH-cytochrome P450 reductase or purified beef liver microsomal NADH-cytochrome b5 reductase

and 10 mM sodium phosphate buffer, pH 6.6 in the presence of cofactors NADPH or NADH. Interestingly, as shown in Figure 3.23, the purified beef liver microsomal cytochrome b5 reductase was found to be not effective in promoting DNA strand breaks in the presence of idarubicin and cofactor NADH. Plasmid DNA incubations with purified beef liver b5 reductase even at high concentrations (0.14-1.1 units, based on ferricyanide reduction) in the presence of idarubicin and cofactor NADH produced no DNA strand breaks (lanes 5-9), whereas, addition of purified sheep lung P450 reductase to incubation mixture in the presence of cofactor NADPH effectively generated single-strand breaks under the same conditions described above (lanes 10 and 11) (Figure 3.23). The aerobic incubation of plasmid DNA with purified sheep lung P450 reductase at an amount of 1.0 and 1.25 μg under these conditions in the presence of cofactor NADPH resulted in about 59% and 50% increase in % OC form over control, respectively (Figure 3.23, lanes 10 and 11). The previous results have also shown that sheep lung P450 reductase was effective even at lower concentrations as low as 0.025 μg in promoting plasmid DNA strand breaks albeit at a lower efficiency (Figure 3.18). Plasmid DNA incubations were performed also with various amounts of beef liver b5 reductase (0.14-1.1 units) and idarubicin at 100 μM concentration in 100 mM sodium phosphate buffer, pH 7.4 (not at pH 6.6). It was found that under these conditions purified beef liver b5 reductase did not promote plasmid DNA strand breaks unlike purified sheep lung P450 reductase also.

Figure 3.24 shows the typical results of plasmid DNA assay for mitomycin C in which pBR322 plasmid DNA was incubated with various amounts of purified beef liver b5 reductase (0.08-0.38 units, based on ferricyanide reduction), mitomycin C at 100 μM concentration and 10 mM sodium phosphate buffer pH 6.6 in the presence of cofactor NADH. Similar with idarubicin, incubations of plasmid DNA with mitomycin C in the presence of various amounts of purified beef liver b5 reductase and cofactor NADH barely produced DNA single-strand breaks over control incubation without enzyme (Figure 3.24, lanes 7-12). Plasmid DNA incubations with various amounts of purified b5 reductase (0.78×10^{-3}-1.1 units) were also carried out in 100 mM sodium phosphate buffer, pH 7.4 (not at pH 6.6) in the presence of 100 μM mitomycin C and cofactor NADH. However, no DNA strand breaks over control

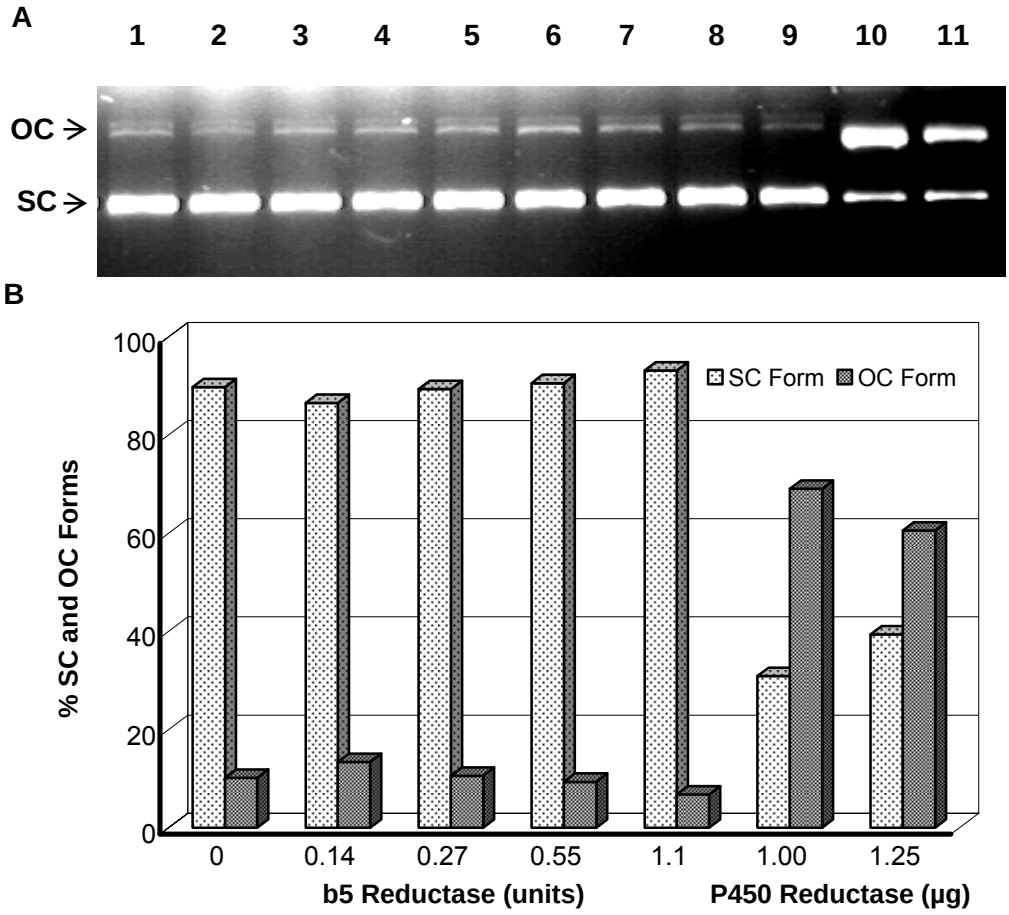


Figure 3.23 Involvement of purified beef liver NADH-cytochrome b5 reductase (b5R) in idarubicin-mediated generation of plasmid DNA strand breaks in comparison to purified sheep lung NADPH-cytochrome P450 reductase (P450R). Supercoiled pBR322 DNA (1.0 µg) was incubated for 30 minutes at 37°C in the presence of idarubicin (25 µM), either cofactor (2 mM) and 10 mM sodium phosphate buffer (pH 6.6) with various concentrations of b5R or P450R in a final volume of 60 µl reaction mixture as described in detail under "Methods". **(A) Agarose gel electrophoresis, lanes 1 and 2**, no NADH controls for increasing b5R concentrations (0.55, 1.1 units, based on ferricyanide reduction, respectively); **lanes 3 and 4**, no NADPH controls for increasing P450R concentrations (1.0 and 1.25 µg, respectively); **lanes 5 to 9**, complete system incubations for increasing b5R concentrations (0, 0.14, 0.27, 0.55, 1.1 units, respectively) including NADH; **lanes 10 and 11**, complete system incubations for increasing P450R concentrations (1.0 and 1.25 µg, respectively) including NADPH. **(B)** Percentage of detected SC (form I) and OC (form II) forms of pBR322 plasmid DNA represented as column chart. Light colored columns represent % SC form of DNA and dark colored columns represent % OC form of DNA. Data correspond to lanes 5 to 9 (complete system incubations for increasing b5R concentrations; 0, 0.14, 0.27, 0.55, 1.1 units, respectively) and lanes 10 and 11 (complete system incubations for increasing P450R concentrations; 1.0 and 1.25 µg, respectively). OC, open circular; SC, supercoiled.

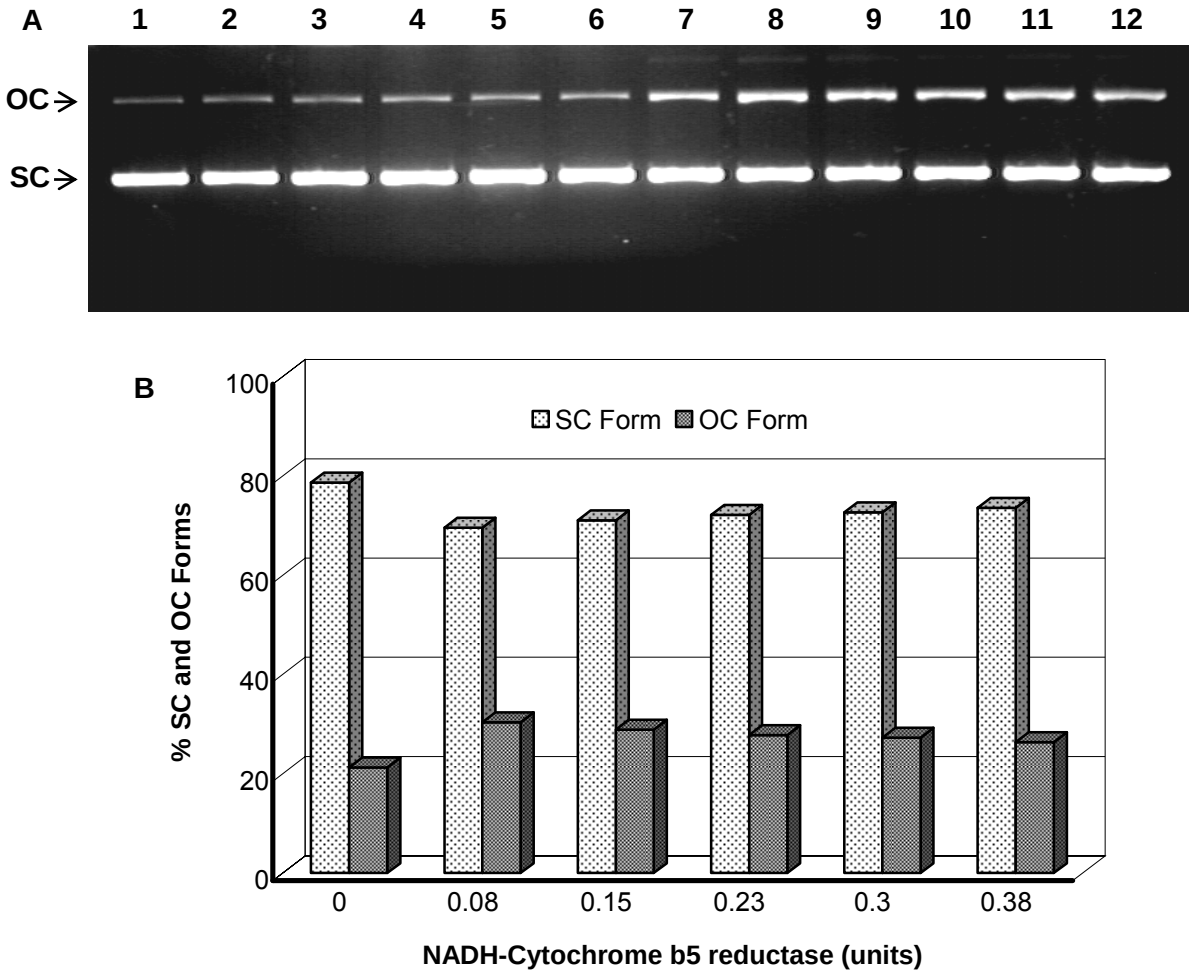


Figure 3.24 Involvement of purified beef liver NADH-cytochrome b5 reductase (b5R) in mitomycin C-mediated generation of plasmid DNA strand breaks in the presence of cofactor NADH. Supercoiled pBR322 DNA (1.0 µg) was incubated for 30 minutes at 37°C in the presence of mitomycin C (100 µM), NADH (2 mM) and 10 mM sodium phosphate buffer (pH 6.6) with various concentrations of b5R (0.08-0.38 units, based on ferricyanide reduction) in a final volume of 60 µl reaction mixture as described in detail under "Methods". **(A) Agarose gel electrophoresis, lane 1**, plasmid DNA control; **lanes 2 to 6**, no NADH controls for increasing b5R concentrations (0.08, 0.15, 0.23, 0.30, 0.38 units, respectively); **lanes 7 to 12**, complete system incubations for increasing b5R concentrations (0, 0.08, 0.15, 0.23, 0.30, 0.38 units, respectively) including NADH. **(B)** Percentage of detected SC (form I) and OC (form II) forms of pBR322 plasmid DNA represented as column chart. Light colored columns represent % SC form of DNA and dark colored columns represent % OC form of DNA. Data correspond to lanes 7 to12 (complete system incubations for increasing b5R concentrations; 0, 0.08, 0.15, 0.23, 0.30, 0.38 units, respectively). OC, open circular; SC, supercoiled.

were observed at any concentrations of beef liver b5 reductase tested unlike sheep lung P450 reductase under these conditions (data not shown).

3.9.4 Involvement of Purified Rabbit Liver Microsomal Cytochrome P4502B4 in Idarubicin-Induced Plasmid DNA Breakage

In order to examine whether rabbit liver CYP2B4 is involved in the bioreductive activation of idarubicin to DNA-damaging species, DNA strand breakage was detected under aerobic conditions in reconstituted systems containing highly purified rabbit liver cytochrome P4502B4 and rabbit liver NADPH-cytochrome P450 reductase in the presence of dilauroyl phosphatidylcholine as a synthetic lipid as described in detail under "Methods". For this purpose, both the effect of increasing amounts of P450 reductase in the presence of a fixed amount of CYP2B4 (50.0 nM) and the effect of increasing amounts of CYP2B4 in the presence of a fixed amount of P450 reductase (4.5 nM) were studied.

The effect of increasing amounts of P450 reductase on idarubicin-induced generation of plasmid DNA strand breaks in the presence of a fixed amount of CYP2B4 (50.0 nM) is shown in Figure 3.25 and Table 3.12. As seen in Figure 3.25, rabbit liver CYP2B4 alone was not effective in promoting idarubicin-induced DNA strand breaks in the presence of cofactor NADPH (lane 2). It was found that rabbit liver P450 reductase effectively promoted idarubicin-induced plasmid DNA strand breaks in the presence of cofactor NADPH (lanes 3-12). Incubation of plasmid DNA with rabbit liver cytochrome P450 reductase at a concentration of 1.5 nM (0.016 μg) in the presence of idarubicin and cofactor NADPH resulted in about 30% increase in % OC (open circular) form over plasmid-alone control (lane 3). This increase remained nearly constant with additional increase in P450 reductase amount up to 5.0 nM (0.052 μg). Addition of rabbit liver CYP2B4 at 50.0 nM concentration (0.22 μg) to a reconstituted system containing rabbit liver P450 reductase alone at varying amounts produced just between 1.11-1.24-fold increases in idarubicin-induced formation of DNA strand breaks (Table 3.12). However, if the agarose gel shown in

Figure 3.25 was carefully examined, it was seen that incubations of plasmid DNA with varying amounts of P450 reductase in the presence of a fixed amount of CYP2B4 produced detectable increases in the relative densities of the bands corresponding to the linear form of the plasmid DNA compared to that produced by P450 reductase alone. In addition, as shown in Figure 3.25 incubation of plasmid DNA with reconstituted system consisting of P450 reductase and CYP2B4 in the presence of idarubicin and cofactor NADPH resulted in a formation of a different band with a slightly slower mobility and a lower density as compared to linear form. The relative densities of these bands corresponding to the linear form and the other were not included in the calculations which caused some underestimation of the damage produced in the presence of CYP2B4 (Table 3.12).

The effect of increasing amounts of rabbit liver CYP2B4 on the generation of idarubicin-induced plasmid DNA strand breaks in the presence of a fixed amount of rabbit liver P450 reductase (4.5 nM) is also shown in Figure 3.26 and Table 3.13. Addition of increasing amounts of rabbit liver CYP2B4 to a reconstituted system consisting of P450 reductase alone at 4.5 nM concentration resulted in just between 1.15-1.23-fold increases in the generation of single-strand breaks on DNA (Table 3.13). Similarly, there were detectable increases in the relative densities of the bands corresponding to the linear form of plasmid DNA when CYP2B4 was added to reconstituted system consisting of cytochrome P450 reductase alone (Figure 3.26). It was also shown that further increasing the CYP2B4 amount to 200 nM in reconstituted systems lead to a decrease in the amount of single-strand breaks as shown by a decrease in the relative density of the band corresponding to the open circular form (lane 12).

All the presented data above indicate that the contribution of rabbit liver CYP2B4 relative to P450 reductase to idarubicin-induced generation of plasmid DNA strand breaks might be considered as negligible or the *in vitro* plasmid DNA damage assay employed under the conditions described above might not be sensitive enough to determine exactly whether and to what degree rabbit liver CYP2B4 is involved in the bioactivation of idarubicin to DNA-damaging species. Therefore, the

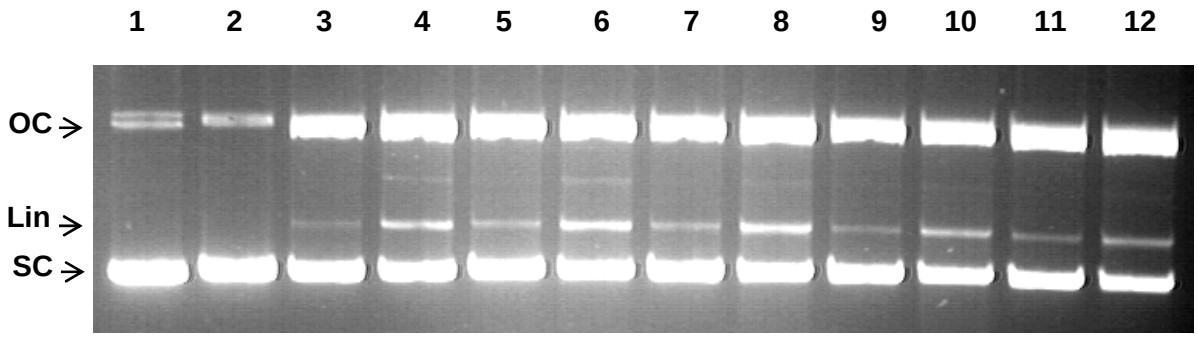


Figure 3.25 Agarose gel electrophoresis showing the effect of increasing amounts of purified rabbit liver NADPH-cytochrome P450 reductase (P450R) on idarubicin-mediated formation of plasmid DNA strand breaks in the presence of a fixed amount of highly purified rabbit liver CYP2B4 (50.0 nM). Supercoiled pBR322 DNA (1.0 μg) was incubated for 30 minutes at 37°C in the presence of idarubicin (100 μM) and NADPH (2 mM) with appropriate amounts of reconstituted enzymes in a final volume of 60 μl reaction mixture as described in detail under "Methods". **Agarose gel electrophoresis, lane 1**, plasmid DNA control; **lane 2**, reconstituted system omitting P450R (50.0 nM CYP2B4 + lipid); **lane 3**, reconstituted system omitting CYP2B4 (1.5 nM P450R + lipid); **lane 4**, complete reconstituted system containing 1.5 nM P450R + 50.0 nM CYP2B4 + lipid; **lane 5**, reconstituted system omitting CYP2B4 (3.0 nM P450R + lipid); **lane 6**, complete reconstituted system containing 3.0 nM P450R + 50.0 nM CYP2B4 + lipid; **lane 7**, reconstituted system omitting CYP2B4 (4.5 nM P450R + lipid); **lane 8**, complete reconstituted system containing 4.5 nM P450R + 50.0 nM CYP2B4 + lipid; **lane 9,** reconstituted system omitting CYP2B4 (5.0 nM P450R + lipid); **lane 10**, complete reconstituted system containing 5.0 nM P450R + 50.0 nM CYP2B4 + lipid; **lanes 11 and 12,** same with the lanes 9 and 10, respectively. OC, open circular; SC, supercoiled; Lin, linear.

Table 3.12 Effect of increasing amounts of purified rabbit liver cytochrome P450 reductase on idarubicin-mediated generation of plasmid DNA strand breaks in the presence of a fixed amount of purified rabbit liver cytochrome P4502B4

Components of Reconstituted System	**SBI% (OC%)[a]**
Plasmid-alone Control **(Lane 1)**	10.4
Rabbit CYP2B4 (50.0 nM) + Lipid **(Lane 2)**	11.0
Rabbit P450 Reductase (1.5 nM) + Lipid **(Lane 3)**	41.5
Rabbit P450 Reductase (1.5 nM) + Rabbit CYP2B4 (50.0 nM) + Lipid **(Lane 4)**	48.6
Rabbit P450 Reductase (3.0 nM) + Lipid **(Lane 5)**	45.6
Rabbit P450 Reductase (3.0 nM) + Rabbit CYP2B4 (50.0 nM) + Lipid **(Lane 6)**	49.5
Rabbit P450 Reductase (4.5 nM) + Lipid **(Lane 7)**	45.4
Rabbit P450 Reductase (4.5 nM) + Rabbit CYP2B4 (50.0 nM) + Lipid **(Lane 8)**	52.8
Rabbit P450 Reductase (5.0 nM) + Lipid **(Lane 9)**	45.6
Rabbit P450 Reductase (5.0 nM) + Rabbit CYP2B4 (50.0 nM) + Lipid **(Lane 10)**	50.8
Rabbit P450 Reductase (5.0 nM) + Lipid **(Lane 11)**	51.7
Rabbit P450 Reductase (5.0 nM) + Rabbit CYP2B4 (50.0 nM) + Lipid **(Lane 12)**	58.2

[a] SBI (DNA strand breaking index, OC%) was calculated as described in Section 2.2.11.5. Experimental conditions are described in detail under “Methods”.

role and involvement of rabbit liver CYP2B4 in the bioactivation of idarubicin in comparison to mitomycin C were further investigated in detail in reconstituted systems by enzymatic assays under both aerobic and anaerobic conditions (see Sections 3.13.1 to 3.13.3)

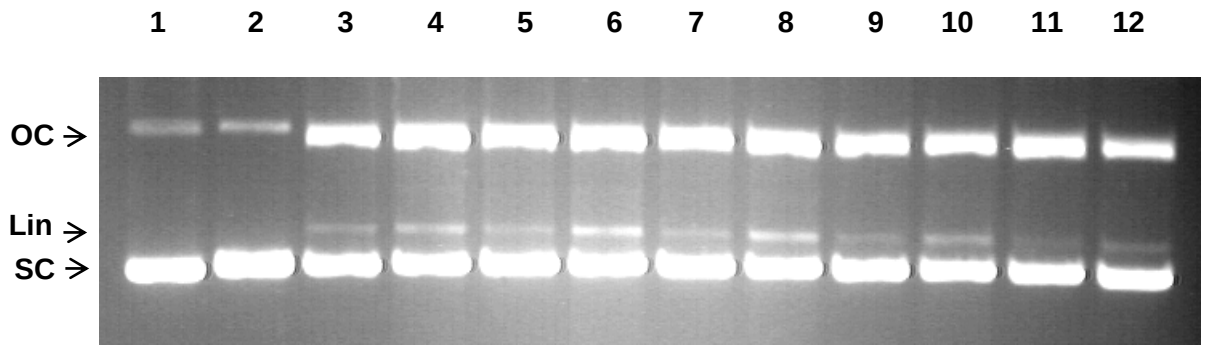


Figure 3.26 Agarose gel electrophoresis showing the effect of increasing amounts of purified rabbit liver cytochrome P4502B4 on idarubicin-mediated formation of plasmid DNA strand breaks in the presence of a fixed amount (4.5 nM) of highly purified rabbit liver NADPH-cytochrome P450 reductase (P450R). Supercoiled pBR322 DNA (1.0 µg) was incubated for 30 minutes at 37°C in the presence of idarubicin (100 µM) and NADPH (2 mM) with appropriate amounts of reconstituted enzymes in a final volume of 60 µl reaction mixture as described in detail under "Methods". **Agarose gel electrophoresis, lane 1**, plasmid DNA control; **lane 2**, reconstituted system omitting P450R; **lanes 3**, **5, 7 and 9**, reconstituted system omitting CYP2B4 (4.5 nM P450R + lipid); **lane 4**, complete reconstituted system containing 4.5 nM P450R + 12.5 nM CYP2B4 + lipid; **lane 6**, complete reconstituted system containing 4.5 nM P450R + 25.0 nM CYP2B4 + lipid; **lanes 8 and 10**, complete reconstituted system containing 4.5 nM P450R + 50.0 nM CYP2B4 + lipid; **lane 11,** complete reconstituted system containing 4.5 nM P450R + 100 nM CYP2B4 + lipid; **lane 12,** complete reconstituted system containing 4.5 nM P450R + 200.0 nM CYP2B4 + lipid. OC, open circular; SC, supercoiled; Lin, linear.

Table 3.13 Effect of increasing amounts of purified rabbit liver cytochrome P4502B4 on idarubicin-mediated generation of plasmid DNA strand breaks in the presence of a fixed amount of purified rabbit liver cytochrome P450 reductase

Components of Reconstituted System	SBI% (OC%)[a]
Plasmid–alone Control **(Lane 1)**	9,0
Rabbit CYP2B4 (50.0 nM) + Lipid **(Lane 2)**	10,9
Rabbit P450 Reductase (4.5 nM) + Lipid **(Lane 3)**	40.8
Rabbit P450 Reductase (4.5 nM) + Rabbit CYP2B4 (12.5 nM) + Lipid **(Lane 4)**	48.3
Rabbit P450 Reductase (4.5 nM) + Lipid **(Lane 5)**	44.2
Rabbit P450 Reductase (4.5 nM) + Rabbit CYP2B4 (25.0 nM) + Lipid **(Lane 6)**	47.3
Rabbit P450 Reductase (4.5 nM) + Lipid **(Lane 7)**	44.5
Rabbit P450 Reductase (4.5 nM) + Rabbit CYP2B4 (50.0 nM) + Lipid **(Lane 8)**	48.2
Rabbit P450 Reductase (4.5 nM) + Lipid **(Lane 9)**	43.0
Rabbit P450 Reductase (4.5 nM) + Rabbit CYP2B4 (50.0 nM) + Lipid **(Lane 10)**	45.5
Rabbit P450 Reductase (4.5 nM) + Rabbit CYP2B4 (100.0 nM) + Lipid **(Lane11)**	44.3
Rabbit P450 Reductase (4.5 nM) + Rabbit CYP2B4 (200.0 nM) + Lipid **(Lane 12)**	37.4

[a] SBI (DNA strand breaking index, OC%) was calculated as described in Section 2.2.11.5. Experimental conditions are described in detail under “Methods”.

3.9.5 Protective Potentials of Dietary Antioxidants against DNA Strand Breaks Induced by Purified Rabbit Liver NADPH-Cytochrome P450 Reductase-Catalyzed Bioactivation of Idarubicin and Mitomycin C

The protective potentials of phenolic phytochemicals quercetin, naringenin, rutin, resveratrol and trolox (a water-soluble derivative of vitamin E) were evaluated against DNA single-strand breaks induced by highly purified rabbit liver cytochrome P450 reductase-catalyzed reductive activation of idarubicin. For mitomycin C, the protective effect of only quercetin was assessed as explained below. The results of these experiments are shown in Figures 3.27 to 3.31. It was found that incubation of plasmid DNA with rabbit liver P450 reductase at a concentration of 0.2 μg in the

presence of idarubicin and cofactor NADPH produced between 61-69% increases in % OC form over plasmid-alone control (Figures 3.27 and 3.28). Since all the stock solutions of antioxidants were prepared in methanol, solvent control incubations were also carried out in each run of gel electrophoresis. It was found that methanol itself, at a 2% final concentration in incubation mixtures, produced between 34-45% protections against idarubicin- and mitomycin C-induced generation of single-strand breaks (Figures 3.27, 3.28 and 3.31). In the presence of quercetin the extent of idarubicin-induced DNA damage was found to decrease significantly in a concentration dependent manner. As shown in Figure 3.27 and Figure 3.29, at a concentration of 50 μM, quercetin produced about a 58% protection against idarubicin-induced plasmid DNA damage and 100 μM of quercetin was enough for almost complete inhibition of single-strand breaks. As mentioned in Section 2.2.11.5, since all the antioxidants were prepared in methanol, solvent control incubations were used as reference for the calculation of protection (%) values in order to eliminate the protective effect coming from the solvent itself. Unlike quercetin, Figures 3.27 to 3.30 show that the protective effects of other tested compounds were less pronounced even at high concentrations. Both resveratrol and naringenin, at a concentration of 2 mM, protected DNA against idarubicin-induced formation of single-strand breaks only to the same extent of about 30% (Figures 3.27 and 3.29). While 5 mM concentration of resveratrol provided about a 62% protection against DNA damage, naringenin at the same concentration showed only a 41% reduction in idarubicin-induced formation of single-strand breaks. Trolox was almost ineffective in protecting DNA against idarubicin-induced generation of single-strand breaks even at high concentrations. A 5 mM concentration of trolox reduced idarubicin-induced plasmid DNA damage only by 13.2% (Figures 3.28 and 3.30). Similarly, for rutin, the used range of 50-750 μM did not show any protection against idarubicin-induced single-strand breaks. Surprisingly, 2 mM concentration of rutin was found to provide almost complete protection against idarubicin-induced single-strand breaks.

Since the antioxidant capacities of resveratrol, naringenin, trolox and rutin against idarubicin-induced generation of single-strand breaks were significantly low as compared with quercetin, it was decided to test the protective effect of only

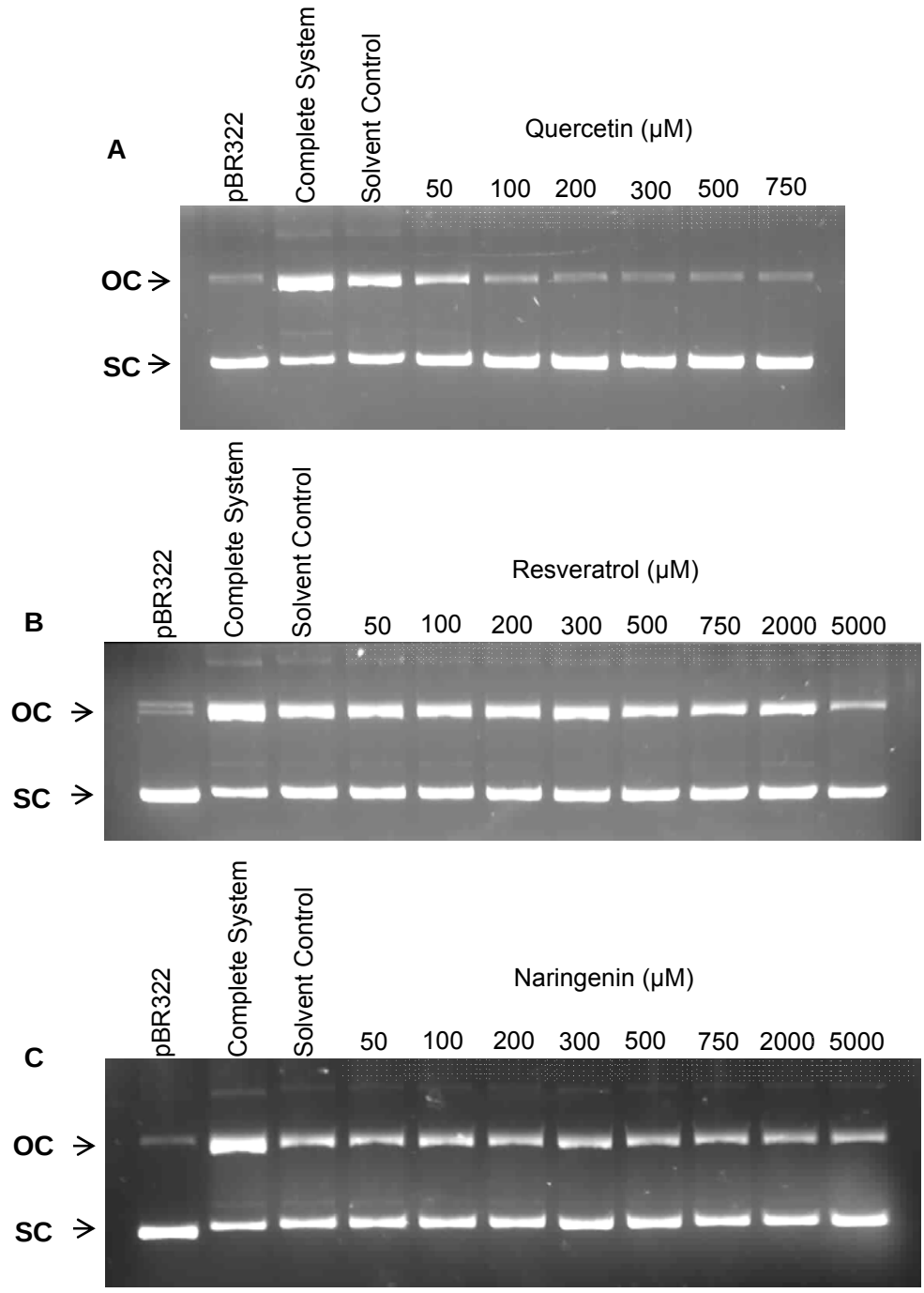


Figure 3.27 Agarose gels showing the protective effects of quercetin (A), resveratrol (B) and naringenin (C) against DNA single-strand breaks induced by purified rabbit liver NADPH-cytochrome P450 reductase (P450R)-catalyzed reductive activation of idarubicin in the presence of cofactor NADPH. Supercoiled pBR322 DNA (1.0 μg) was incubated for 30 minutes at 37°C in the presence of P450R (0.2 μg), NADPH (2 mM) and idarubicin (100 μM) with various concentrations of antioxidants in a final volume of 60 μl reaction mixture as described in "Methods". (Complete system: pBR322 plasmid DNA + P450R + idarubicin + NADPH; Solvent control: pBR322 plasmid DNA + P450R + idarubicin + NADPH + 2% Methanol). OC, open circular; SC, supercoiled.

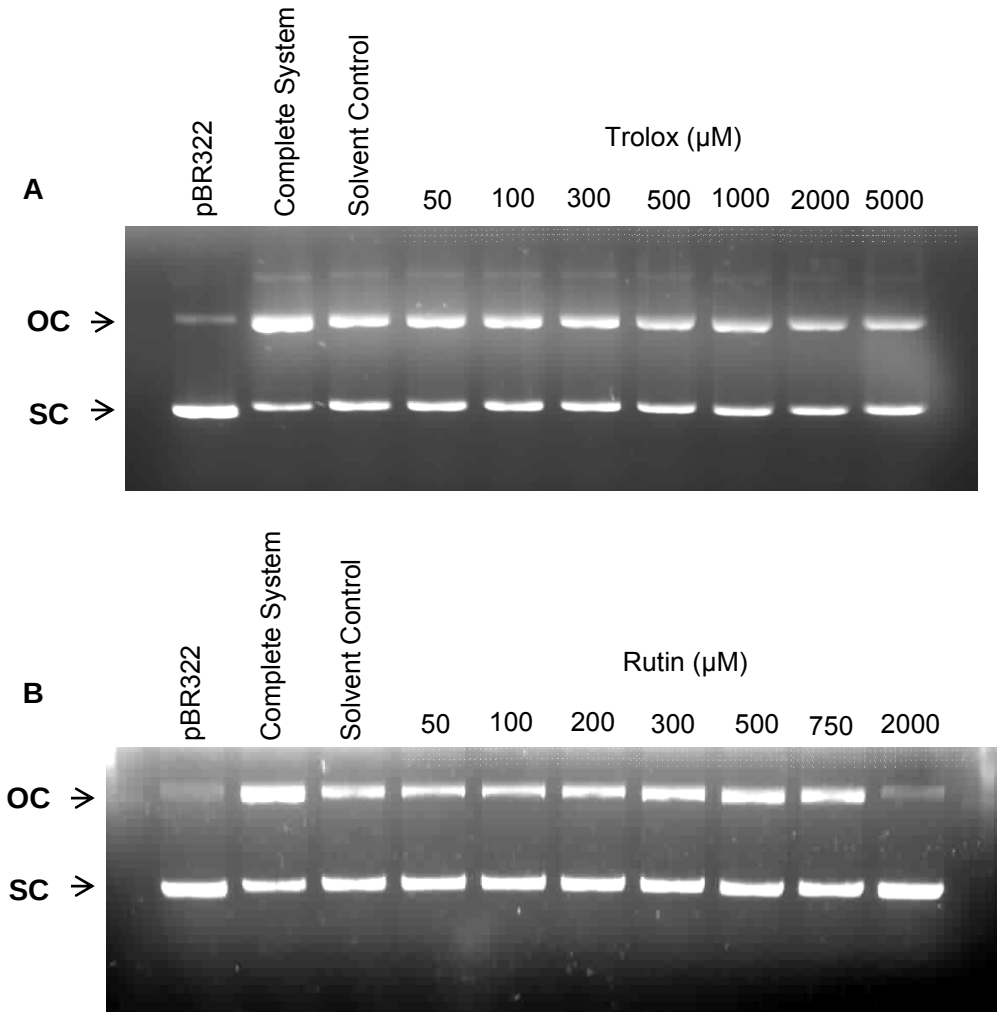


Figure 3.28 Agarose gels showing the protective effects of trolox (A) and rutin (B) against DNA single-strand breaks induced by purified rabbit liver NADPH-cytochrome P450 reductase (P450R)-catalyzed reductive activation of idarubicin in the presence of cofactor NADPH. Supercoiled pBR322 DNA (1.0 μg) was incubated for 30 minutes at 37°C in the presence of P450R (0.2 μg), NADPH (2 mM) and idarubicin (100 μM) with various concentrations of antioxidants in a final volume of 60 μl reaction mixture as described in "Methods". (Complete system: pBR322 plasmid DNA + P450R + idarubicin + NADPH; Solvent control: pBR322 plasmid DNA + P450R + idarubicin + NADPH + 2% Methanol). OC, open circular; SC, supercoiled.

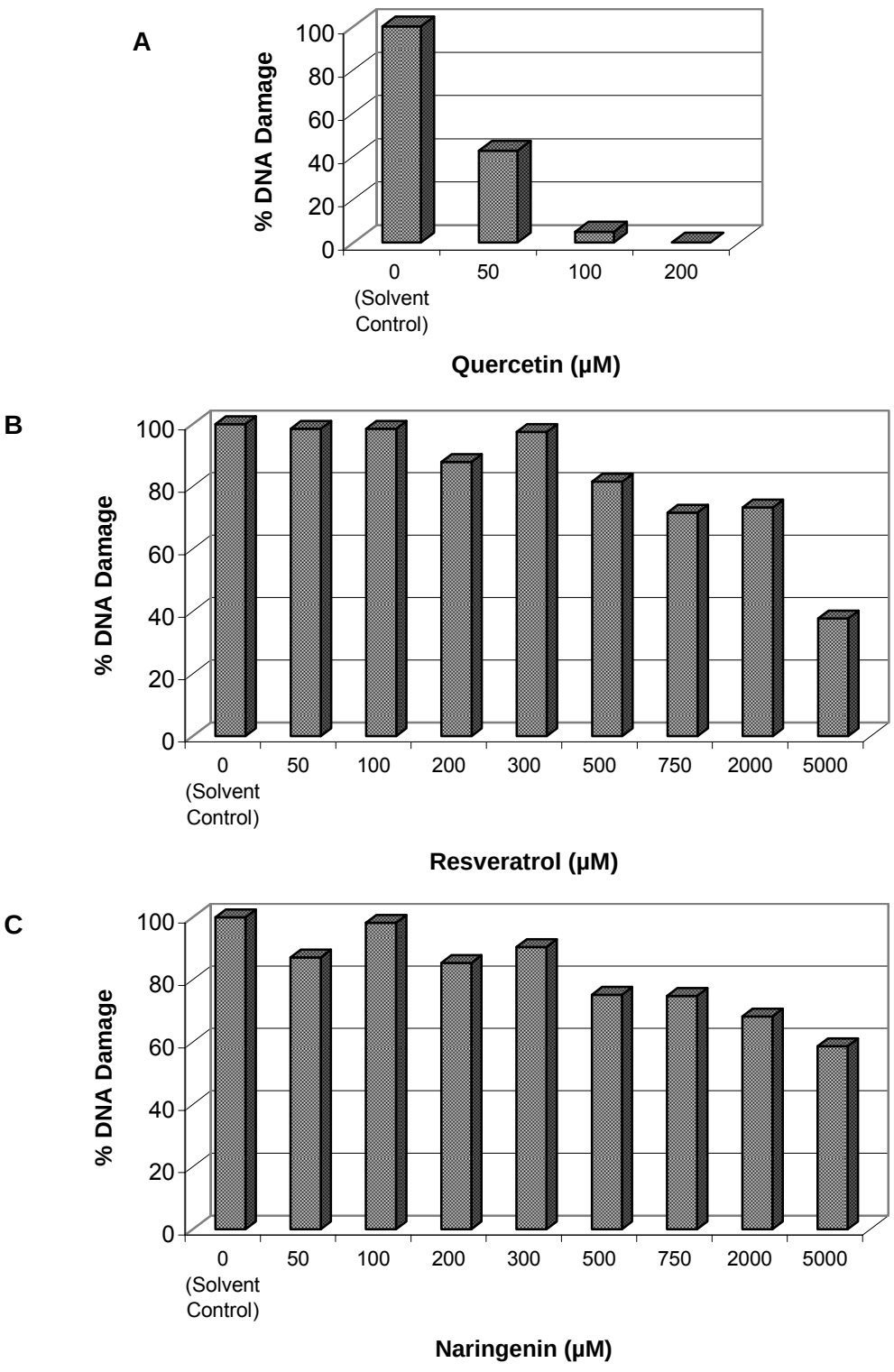


Figure 3.29 The protective effects of quercetin (A), resveratrol (B) and naringenin (C) against DNA single-strand breaks induced by purified rabbit liver NADPH-cytochrome P450 reductase-catalyzed reductive activation of idarubicin in the presence of cofactor NADPH. Calculations for protection (%) values are described in Section 2.2.11.5. Experimental conditions are described in detail under “Methods”.

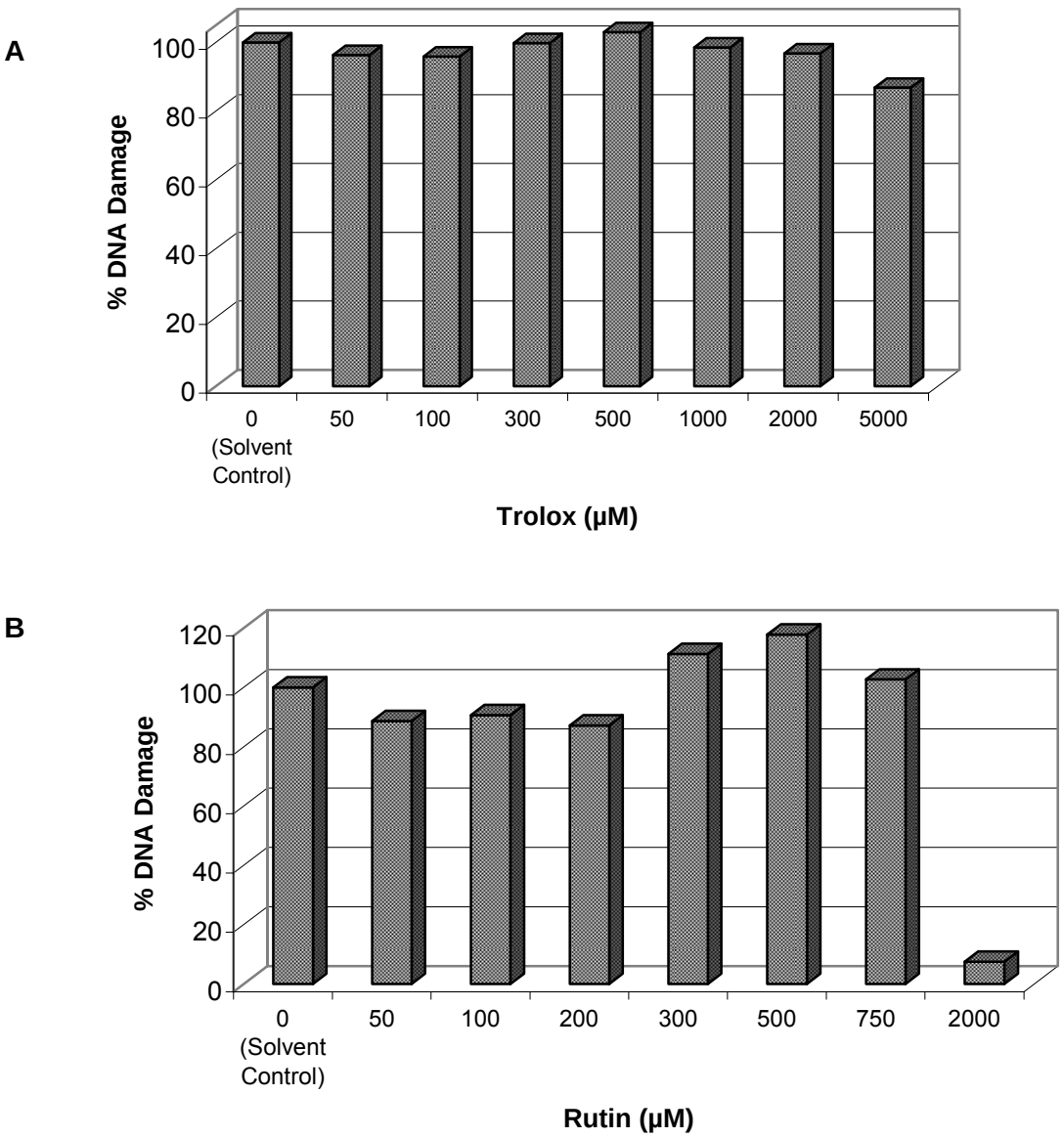


Figure 3.30 The protective effects of trolox (A) and rutin (B) against DNA single-strand breaks induced by purified rabbit liver NADPH-cytochrome P450 reductase-catalyzed reductive activation of idarubicin in the presence of cofactor NADPH. Calculations for protection (%) values are described in Section 2.2.11.5. Experimental conditions are described in detail under “Methods”.

quercetin against plasmid DNA damage induced as a consequence of rabbit liver P450 reductase-catalyzed reductive activation of mitomycin C. While the purified enzyme at an amount of 0.2 μg per volume of reaction mixture caused significant DNA damage in the presence of idarubicin as mentioned before, incubation of plasmid DNA with the same amount of enzyme in the presence of mitomycin C produced lower amounts of single-strand breaks. Therefore, a higher concentration of rabbit liver P450 reductase was used in incubation mixtures to produce more mitomycin C-induced DNA single-strand breaks which made it much easier to clearly observe the protective effect of quercetin against mitomycin C-induced plasmid DNA damage. It was found that incubation of plasmid DNA with rabbit liver P450 reductase at a concentration of 2.0 μg per volume of reaction mixture in the presence of mitomycin C and cofactor NADPH produced about a 55.0% increase in % OC form over control (Figure 3.31). The flavonoid quercetin protected pBR322 plasmid DNA against mitomycin C-induced single-strand breaks in a concentration dependent manner similarly as observed in idarubicin-induced plasmid DNA damage (Figure 3.31). At 50 μM concentration quercetin produced about a 45% reduction in mitomycin C-induced formation of single-strand breaks on plasmid DNA. However, a higher concentration of quercetin was required for almost complete inhibition of mitomycin C-induced DNA damage compared to idarubicin-induced DNA damage. While quercetin at 200 μM concentration provided about a 77% protection, 750 μM concentration was required to significantly reduce the formation of mitomycin C-induced generation of strand breaks by about 94% (Figure 3.31).

The IC_{50} values of quercetin against idarubicin- and mitomycin C-induced DNA damage were almost the same and calculated as 43.5μM and 49.8 μM, respectively. The IC_{50} is the concentration that inhibits the formation of DNA damage by 50%. IC_{50} values of the other tested antioxidants were in the mM range except trolox since even at very high concentration it was not effective (5.0 mM) in protecting DNA against idarubicin-induced strand breaks. These results show that quercetin was a more potent antioxidant with respect to resveratrol, naringenin, rutin and trolox in protecting DNA against the strand breakage induced as a consequence of P450 reductase-catalyzed reductive activation of idarubicin and mitomycin C.

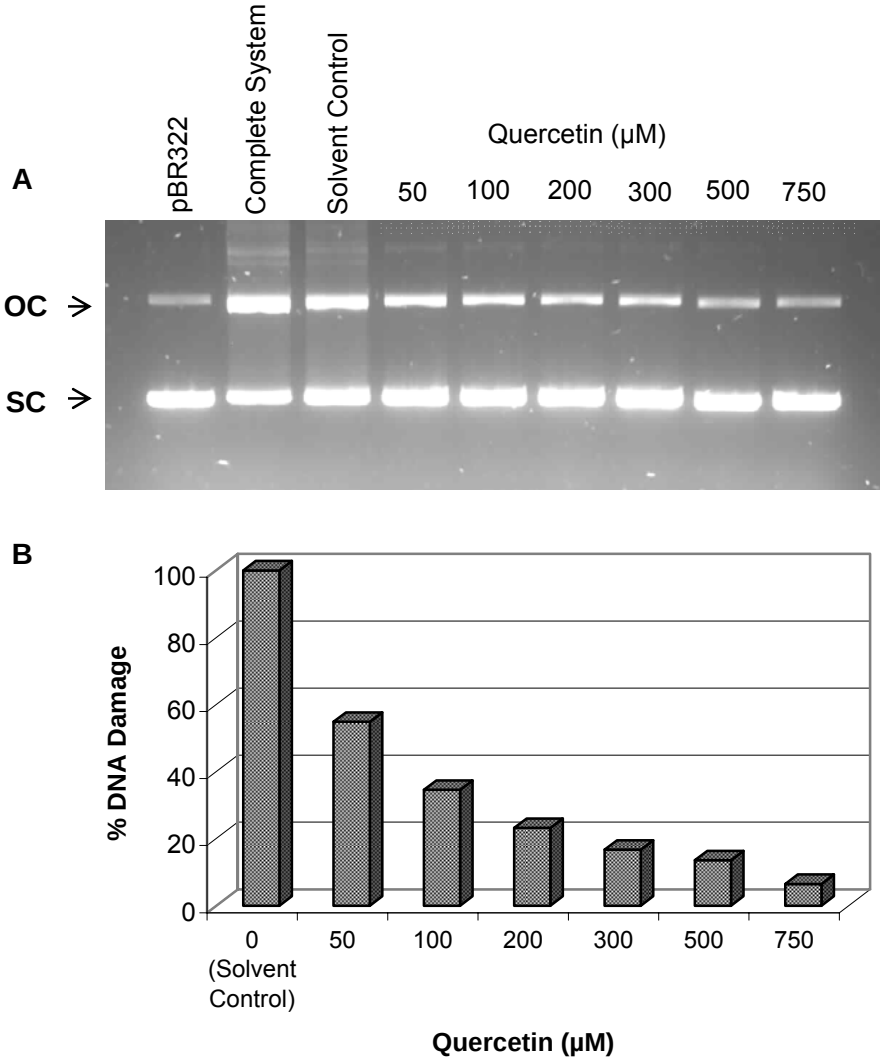


Figure 3.31 The protective effect of quercetin against DNA single-strand breaks induced by purified rabbit liver NADPH-cytochrome P450 reductase-catalyzed reductive activation of mitomycin C in the presence of cofactor NADPH. **(A) Agarose gel;** Supercoiled pBR322 DNA (1.0 μg) was incubated for 30 minutes at 37°C in the presence of P450R (2.0 μg), NADPH (2 mM) and mitomycin C (100 μM) with various concentrations of quercetin in a final volume of 60 μl reaction mixture as described in "Methods". (Complete system: pBR322 plasmid DNA + P450R + mitomycin C + NADPH; Solvent control: pBR322 plasmid DNA + P450R + mitomycin C + NADPH + 2% Methanol) **(B)** Protection of pBR322 plasmid DNA by quercetin against single-strand breaks induced by rabbit liver P450 reductase-catalyzed reductive activation of mitomycin C represented as column chart. Calculations for protection (%) values are described in Section 2.2.11.5. OC, open circular; SC, supercoiled.

3.10 Reduction of Idarubicin and Mitomycin C by Phenobarbital-Treated and Untreated Rabbit Liver Microsomes under Aerobic Conditions

The initial rates of idarubicin and mitomycin C reductions were determined in phenobarbital-treated and untreated rabbit liver microsomes by measuring NAD(P)H consumption under aerobic conditions as described in detail under "Methods" and the results are shown in Table 3.14. Cytochrome b5 and cytochrome P450 amounts, NADPH-dependent cytochrome *c* reductions and NADH-dependent ferricyanide reductions by phenobarbital-treated and untreated rabbit liver microsomes were also determined (Table 3.14). It was found that there were statistically significant increases in cytochrome b5 and cytochrome P450 amounts and NADPH-dependent cytochrome *c* reductase activities in phenobarbital-treated microsomes as compared to untreated control microsomes which indicates that phenobarbital treatment of the rabbits resulted in the induction of cytochrome b5, cytochrome P450 and NADPH-cytochrome P450 reductase levels in the liver tissues (Table 3.14). However, NADH-dependent ferricyanide reduction activities were not affected by phenobarbital treatment and showed no significant difference between two groups.

Table 3.14 shows that incubations of phenobarbital-treated or untreated control microsomes with idarubicin or mitomycin C in the presence of NADPH cofactor under aerobic conditions resulted in a measurable rate of NADPH consumption as a function of time. As shown in Table 3.14, phenobarbital treatment of rabbits produced a low increase in idarubicin and mitomycin C reduction rates with NADPH cofactor compared to untreated group, however, this difference was not statistically significant. The reason may be the low induction of cytochrome P450 reductase enzyme by phenobarbital treatment which might have resulted in the observable but not statistically significant increase in idarubicin and mitomycin C reduction rates. The relative contributions of cytochrome P450 isozymes to idarubicin or mitomycin C reduction, if any, by a mechanism involving transfer of electrons from NADPH to cytochrome P450 via cytochrome P450 reductase was almost markedly eliminated with the addition of non ionic detergent Triton X-100 to incubation mixtures. The relative contribution of cytochrome P4502B4, the major

phenobarbital inducible form of rabbit liver microsomal cytochrome P450, to the reduction of idarubicin, in comparison to mitomycin C, was also studied in detail in reconstituted systems of highly purified enzymes as will be mentioned later.

Although various microsomal proteins and drug concentrations were tested in incubation mixtures as mentioned in “Methods”, neither idarubicin nor mitomycin C reduction was detected in phenobarbital-treated or untreated control rabbit liver microsomes in the presence of NADH cofactor on the contrary to what was observed in the presence of NADPH cofactor under reaction conditions described in “Methods” (Table 3.14). These results indicate that microsomal rabbit liver NADH-cytochrome b5 reductase might be either a very weak or ineffective drug activator for idarubicin and mitomycin C as compared to P450 reductase under these conditions. The involvement of microsomal cytochrome b5 (if any) in idarubicin and mitomycin C reduction under these conditions was almost markedly suppressed due to the addition of non-ionic detergent Triton X-100 to incubation mixtures.

Table 3.14 Cytochromes b5 and P450 amounts and NAD(P)H-dependent enzyme activities of phenobarbital-treated and untreated rabbit liver microsomes

Enzyme Source	Cytochrome b5 Amount[a] (nmol mg^{-1})	Cytochrome P450 Amount[a] (nmol mg^{-1})	NADH-$K_3Fe(CN)_6$ Reduction (nmol min^{-1} mg^{-1})	NADPH-Cytochrome *c* Reduction[a] (nmol min^{-1} mg^{-1})
PB-Treated Mic.				
Rabbit 1	1.36	1.37	1830.0	295.0
Rabbit 2	1.44	1.57	2250.0	308.9
Rabbit 3	1.30	1.66	2810.0	320.9
Untreated Mic.				
Rabbit 4	0.93	0.40	2700.0	238.7
Rabbit 5	0.97	0.73	3730.0	230.6
Rabbit 6	0.97	0.76	2850.0	221.7

Enzyme Source	NADH Oxidation (nmol min^{-1} mg^{-1})		NADPH Oxidation (nmol min^{-1} mg^{-1})	
	Idarubicin-Induced	Mitomycin C-Induced	Idarubicin-Induced	Mitomycin C-Induced
PB-Treated Mic.				
Rabbit 1	ND[b]	ND	14.9	13.9
Rabbit 2	ND	ND	19.7	16.0
Rabbit 3	ND	ND	18.0	15.5
Untreated Mic.				
Rabbit 4	ND	ND	10.8	9.7
Rabbit 5	ND	ND	12.5	13.7
Rabbit 6	ND	ND	13.0	14.0

[a] $p < 0.05$. [b] ND, not detectable. Experimental conditions for each enzyme activity determination are described in detail under “Methods”. All the data in the table represent the average of duplicate determinations.

3.11 Reduction of Idarubicin and Mitomycin C by Purified NADPH-Cytochrome P450 Reductases

Since the extent of reactive oxygen species production by idarubicin and mitomycin C depends on their reduction rates by NADPH-cytochrome P450 reductase, the initial relative rates of idarubicin and mitomycin C reductions by P450 reductases highly purified from phenobarbital-treated rabbit liver, beef liver and sheep lung microsomes were determined by measuring the disappearance of NADPH at 340 nm under aerobic conditions as described in detail under "Methods". The results are shown in Table 3.15. The NADPH-dependent cytochrome *c* reduction rates of purified enzymes were also shown in Table 3.15. The specific activities of NADPH-dependent cytochrome *c* reductions by purified P450 reductases were found to be similar around 31000 nmol/min/mg of purified protein (Table 3.15). As shown in Table 3.15, it was found that all the purified P450 reductases catalyzed the reduction of idarubicin at between 2.1 and 2.3-fold greater rates compared to mitomycin C reduction. When rabbit liver P450 reductase was used as enzyme source, the specific activities for idarubicin- and mitomycin C-induced NADPH oxidations were found to be 5112 and 2426 nmol/min/mg of protein, respectively, which were almost similar to those catalyzed by beef liver P450 reductase. However sheep lung P40 reductase was found to be somewhat less effective than other P450 reductases in catalyzing the reduction of both idarubicin and mitomycin C (Table 3.15). This might be caused by the differences in three-dimensional structures of P450 reductases purified from different species which occur as a result of differences in amino acid sequences.

Control incubations were carried out also by performing identical incubations without enzyme or either drug. In the absence of enzyme, idarubicin or mitomycin C was not reduced by NADPH. A very small endogenous rate was observed in the absence of either drug. This small background rate was subtracted from the rate of enzymatic reactions observed in complete incubation mixtures, hence reaction rates were corrected.

Table 3.15 Idarubicin and mitomycin C reduction by NADPH-cytochrome P450 reductases purified from phenobarbital-treated rabbit liver, beef liver and sheep lung microsomes as determined by NADPH oxidation

Purified Enzyme Source	Cytochrome *c* Reduction[a] (nmol min^{-1} mg^{-1})	NADPH Oxidation[b] (nmol min^{-1} mg^{-1})	
		Idarubicin-Induced	Mitomycin C-Induced
Rabbit Liver P450 Reductase	31735.6	5112.2	2425.7
Beef Liver P450 Reductase	30891.7	5673.9	2452.8
Sheep Lung P450 Reductase	31087.3	3111.7	1461.6

Experimental conditions for each enzyme activity determination are described in detail under "Methods". Data represent the averages of duplicate determinations.

[a] NADPH- dependent cytochrome *c* reductase activities were assayed at 25 °C, in 0.3 M potassium phosphate buffer, pH 7.7.

[b] The reaction mixture contained 0.3 M potassium phosphate buffer pH 7.7, 0.1 mM EDTA, pH 7.7, idarubicin (25 μM) or mitomycin C (25 μM), appropriate amounts of purified cytochrome P450 reductases and 0.1 mM NADPH in a final volume of 1.0 ml at 25 °C.

3.12 Reduction of Idarubicin and Mitomycin C by Purified Beef Liver Microsomal NADH-Cytochrome b5 Reductase

Since the ability of idarubicin to induce DNA damage in the presence of highly purified beef liver microsomal NADH-cytochrome b5 reductase is expected to depend on its reduction by this flavoenzyme, the initial rate of idarubicin reduction was determined by measuring the disappearance of NADH at 340 nm under aerobic conditions as described in detail under "Methods". The results were compared with those obtained using mitomycin C. The purified beef liver microsomal b5 reductase was not effective in catalyzing the reduction of idarubicin. Idarubicin reduction by beef liver b5 reductase was measured at two different pHs (pH 7.5 and 6.6), however, no reduction was detected even at either pH (Table 3.16). Similar with

idarubicin, beef liver b5 reductase exhibited hardly measurable mitomycin C reduction activities in the presence of cofactor NADH. Table 3.16 shows that the rate of mitomycin C reduction at pH 6.6 was found to be about 1.5-fold higher than the rate at pH 7.5. The results of these enzyme assays were found to be consistent with those obtained from plasmid DNA breakage assays described in Section 3.9.3. All these results suggested that while mitomycin C is a very poor substrate, idarubicin is not a substrate for purified beef liver microsomal cytochrome b5 reductase unlike cytochrome P450 reductase.

Control incubations in which enzyme or either drug omitted were also carried out under same conditions. No reduction of idarubicin or mitomycin C by NADH was observed in the absence of enzyme. A very low endogenous rate seen in the absence of anticancer drugs was subtracted from the rate of enzymatic reactions observed in complete incubation mixtures to correct the reaction rate.

The activity of NADH-dependent ferricyanide reduction by purified microsomal beef liver b5 reductase was also determined and found as 93.5 μmoles of ferricyanide reduced per min per ml of purified sample under conditions described in Section 2.2.10.5 (Table 3.16). This value was found to be much higher than that reported for the purified sheep lung b5 reductase under same conditions by Güray and Arinç (1990) which indicates that b5 reductase was obtained from beef liver microsomes in a very concentrated form. In addition, it was previously demonstrated that the purified beef liver b5 reductase was biocatalytically active and obtained from beef liver microsomes in its native amphipathic form since it effectively catalyzed the reduction of cytochrome b5 and cytochrome *c* (through cytochrome b5) in reconstituted systems (Table 3.7 and Table 3.8).

Table 3.16 Idarubicin and mitomycin C reduction by purified beef liver microsomal NADH-cytochrome b5 reductase as determined by NADH oxidation

Purified Enzyme Source	$K_3Fe(CN)_6$ Reduction[a] ($\mu mol\ min^{-1}\ ml^{-1}$)	NADH Oxidation[b] ($nmol\ min^{-1}\ ml^{-1}$)			
		Idarubicin-Induced		Mitomycin C-Induced	
		pH 6.6	pH 7.5	pH 6.6	pH 7.5
Beef Liver b5 Reductase	93.5	ND[C]	ND	6.10 ± 2.48	3.95 ± 0.13

Experimental conditions for each enzyme activity determination are described in detail under "Methods".

[a] NADH- dependent ferricyanide reductase activity was assayed at 25 °C. The reaction mixture contained 0.1 M potassium phosphate buffer, pH 7.5, 0.12 mM NADH, 0.2 mM potassium ferricyanide and appropriate amounts of microsomal enzyme in a final volume of 1.0 ml. Data represents the average of duplicate determinations. The enzyme activity is expressed as µmol of $K_3Fe(CN)_6$ reduced per minute per ml of purified sample.

[b] The reaction mixture contained 10 mM potassium phosphate buffer pH 6.6 or pH 7.5, idarubicin (12 µM) or mitomycin C (25 µM), purified beef liver NADH-cytochrome b5 reductase (2.81 units) and 0.1 mM NADH cofactor in a final volume of 1.0 ml at 25 °C. Data were presented as the mean ± standard error of mean (SEM) of four separate determinations. The enzyme activity is expressed as nmol of NADH oxidized per minute per ml of purified sample.

[c] ND, not detectable.

3.13 Involvement of Rabbit Liver Cytochrome P4502B4 in Idarubicin and Mitomycin C Reduction

In order to examine whether rabbit liver CYP2B4 is involved in the bioreductive activation of idarubicin, the ability of idarubicin to undergo bioreduction was examined under both aerobic and anaerobic conditions in reconstituted systems containing highly purified rabbit liver CYP2B4 and either highly purified rabbit liver P450 reductase or beef liver P450 reductase in the presence of dilauroyl phosphatidylcholine as a synthetic lipid as described in detail under "Methods". The results were then compared with those obtained using mitomycin C, because the role of cytochrome P450 in the reductive bioactivation of

this quinone drug has been shown previously by others in rat liver microsomes or rat hepatocytes as well as in reconstituted systems containing purified rat liver P450s (Kennedy *et al.*, 1982; Vromans *et al.*, 1990; Goeptar *et al.*, 1994).

3.13.1 Reduction of Idarubicin and Mitomycin C in Reconstituted Systems Containing Purified Rabbit Liver Cytochrome P450 Reductase and CYP2B4 under Aerobic Conditions

In order to determine the involvement of rabbit liver CYP2B4 in the reductive bioactivation of idarubicin, the initial rates of idarubicin reduction, under aerobic conditions, were determined by measuring NADPH oxidation at 340 nm in reconstituted systems containing highly purified rabbit liver CYP2B4 and rabbit liver P450 reductase as described in detail under "Methods". The experiments were also repeated using mitomycin C under same reaction conditions and the results were compared (Table 3.17). The rabbit liver CYP2B4 alone was not capable of catalyzing idarubicin or mitomycin C reduction. The initial rate of idarubicin reduction by P450 reductase (18.2 nM) alone was 11.9 nmol/min. It was found that idarubicin reduction increased about 1.5-fold in a fully reconstituted system containing rabbit liver CYP2B4 (20.0 nM) and P450 reductase (18.2 nM) as compared with that in a system containing P450 reductase only (Table 3.17). The rate of mitomycin C reduction by rabbit liver P450 reductase (20.0 nM) alone, on the other hand, was found to be 6.4 nmol/min which was about 2-fold lower than idarubicin reduction rate. The rate of mitomycin C reduction in a fully reconstituted system containing rabbit liver CYP2B4 and P450 reductase was found to be 8.2 nmol/min indicating just a 1.3-fold stimulation of mitomycin C reduction by the purified rabbit liver CYP2B4 (Table 3.17).

Figure 3.32 (A) shows the idarubicin reduction rates of the reconstituted systems containing varying amounts of rabbit liver CYP2B4 in the presence of a fixed amount of rabbit liver P450 reductase (18.2 nM). As shown in Figure 3.32 (A), the rate of idarubicin reduction by P450 reductase increased by the addition of

Table 3.17 Idarubicin- and mitomycin C-induced NADPH oxidation rates in reconstituted systems containing purified rabbit liver cytochrome P4502B4 and NADPH-cytochrome P450 reductase under aerobic conditions

Components of Reconstituted System	NADPH Oxidation (nmol min^{-1})	
	Idarubicin-Induced	**Mitomycin C-Induced**
Rabbit Liver CYP2B4 + Lipid	0.15	0.14
Rabbit Liver P450 Reductase + Lipid	11.9	6.4
Rabbit Liver CYP2B4 + Rabbit Liver P450 Reductase + Lipid	17.6[a]	8.2[b]

The activities were determined as described in "Methods". Data represent the average of duplicate determinations.

[a] Complete mixture contained purified rabbit liver CYP2B4 (20.0 nM), rabbit liver cytochrome P450 reductase (18.2 nM), idarubicin (40 μM) and NADPH (0.2 mM).

[b] Complete mixture contained purified rabbit liver CYP2B4 (20.0 nM), rabbit liver cytochrome P450 reductase (20.0 nM), mitomycin C (40 μM) and NADPH (0.2 mM).

increasing amounts of CYP2B4 to the reconstituted systems. The reaction rate increased from 11.9 to 17.6 nmol/min with increasing amounts of added CYP2B4 up to 20.0 nM. With further increase in CYP2B4 concentration, reaction rate became constant. Thus, the maximum activity was observed at nearly equimolar concentrations of CYP2B4 and P450 reductase. The initial rates of idarubicin reduction in reconstituted systems containing varying concentrations of P450 reductase and a fixed amount of CYP2B4 (20.0 nM) were also compared with those in reconstituted systems without CYP2B4 (Figure 3.32 B). As shown in Figure 3.32 (B) the rate of idarubicin reduction in reconstituted systems without CYP2B4 increased proportionally as a function of P450 reductase concentration up to 40.0 nM. Whereas a steeper curve of idarubicin reduction rate was obtained in the presence of a fixed amount of CYP2B4 with increasing amounts of P450 reductase. While the idarubicin reduction rate in reconstituted system containing only P450

reductase at 40.0 nM concentration was 25.0 nmol/min, addition of 20.0 nM CYP2B4 to the reconstituted system resulted in the stimulation of drug reduction to a rate of 36.5 nmol/min (Figure 3.32 B). From the calculations of the slopes of these two curves, the rates of idarubicin reduction by P450 reductase alone and by P450 reductase in the presence of 20.0 nM CYP2B4 were found to be 626.5 and 920.2 nmol of NADPH/nmol of P450 reductase/min, respectively. Thus, the rate of idarubicin reduction by P450 reductase increased 1.47-fold in the presence of 20.0 nM rabbit liver CYP2B4. Since P450 reductase alone was able to catalyze idarubicin reduction, a maximal activity was not observed at nearly equimolar concentrations (20.0 nM) of P450 reductase and CYP2B4 in Figure 3.32 B on the contrary to what was observed in Figure 3.32 A. As shown in Figure 3.32 B, idarubicin reduction rate still increased with increasing amounts of P450 reductase up to 40.0 nM in the presence of 20.0 nM CYP2B4.

The same kinds of experiments were carried out with mitomycin C in order to determine the relative contributions of rabbit liver P450 reductase and CYP2B4 in the reduction of this drug as compared to that of idarubicin under described conditions. The results are shown in Figure 3.33. Figure 3.33 (A) shows that the initial rate of mitomycin C reduction in the presence of a fixed amount of P450 reductase (20.0 nM) increased slowly as a function of added rabbit liver CYP2B4 amount up to 20.0 nM beyond which saturation occurred. Similarly, the maximum rate of mitomycin C reduction was obtained at equimolar concentrations of CYP2B4 and P450 reductase. In addition, the initial rate of mitomycin C reduction by purified P450 reductase alone was found to be 318.8 nmol of NADPH/nmol of P450 reductase/min. This rate was obtained from the calculation of the slope of the curve corresponding to mitomycin C reduction rate vs. P450 reductase amount in the absence of CYP2B4 shown in Figure 3.33 (B). In the presence of 20.0 nM purified rabbit liver CYP2B4, whereas, the rate of mitomycin C reduction by P450 reductase increased to 440.3 nmol of NADPH/nmol of P450 reductase/min (Figure 3.33 B). Thus, in the presence of rabbit liver CYP2B4, the rate of mitomycin C reduction by P450 reductase increased by 1.38-fold.

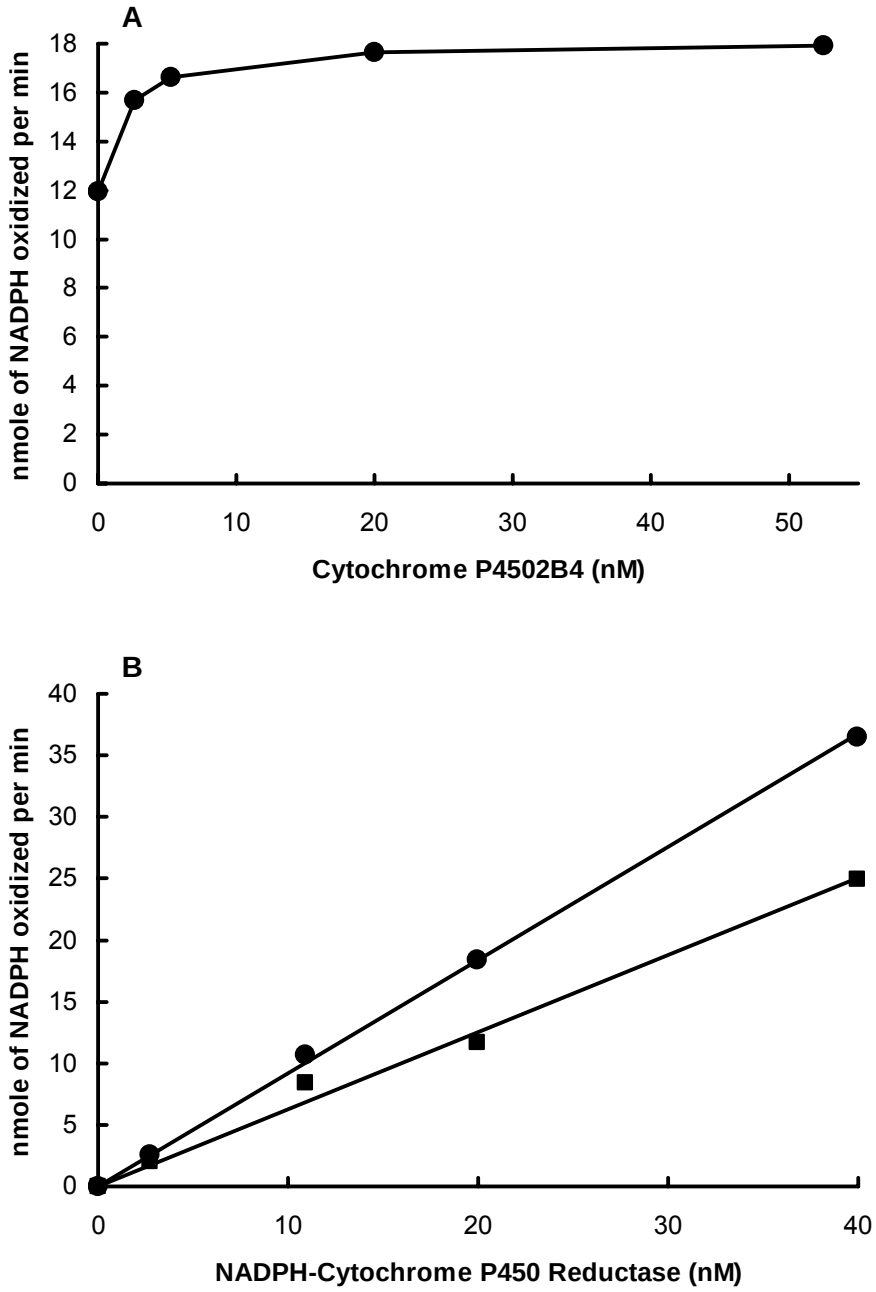


Figure 3.32 Idarubicin-induced NADPH oxidation in reconstituted systems containing purified rabbit liver CYP2B4 and cytochrome P450 reductase under aerobic conditions. The activities were calculated as described in "Methods". **A,** Idarubicin-induced NADPH oxidation vs. increasing amounts of CYP2B4 in the presence of 18.2 nM cytochrome P450 reductase. **B,** Idarubicin-induced NADPH oxidation vs. increasing amounts of cytochrome P450 reductase in the absence (—■—) or presence (—●—) of 20.0 nM CYP2B4. Each point represents the average of duplicate determinations.

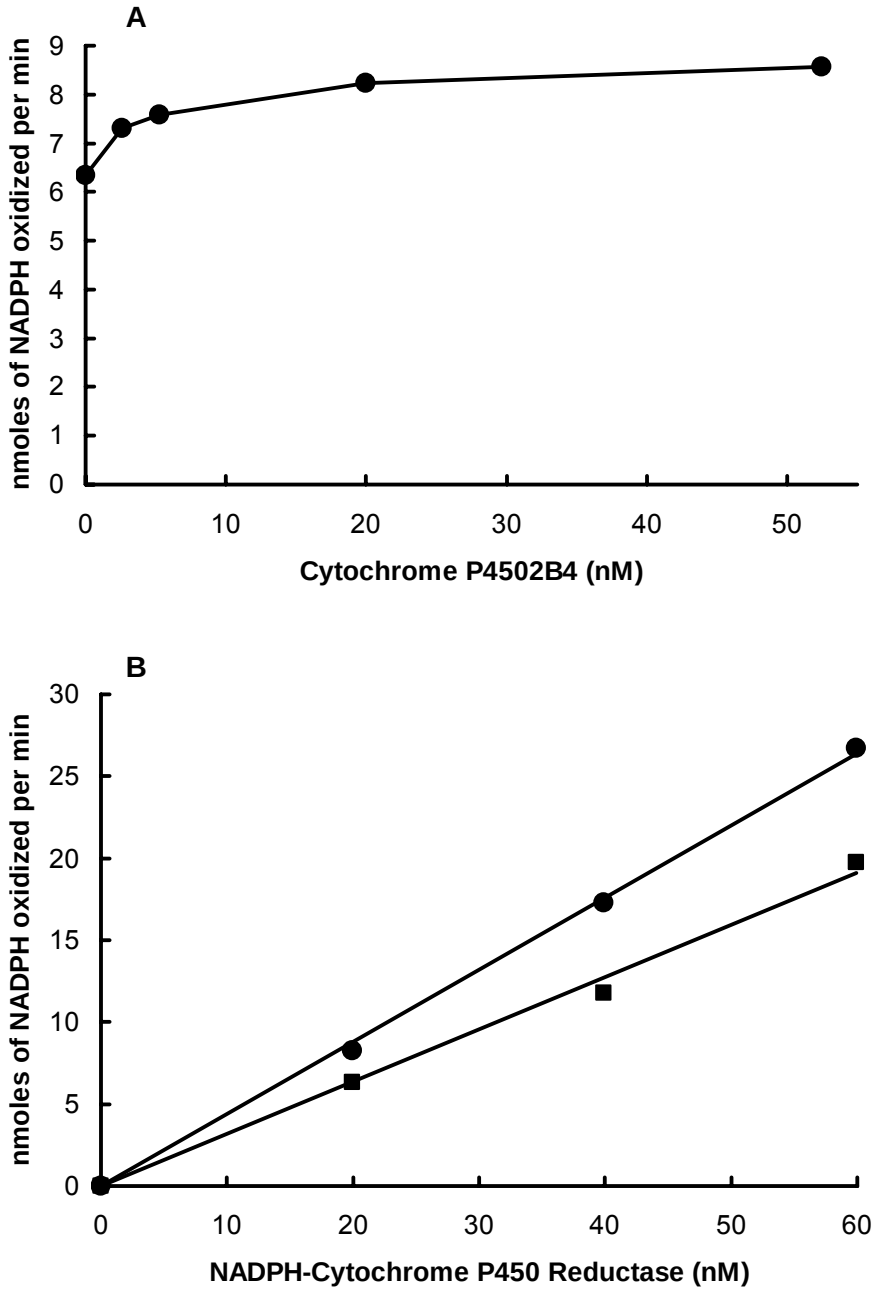


Figure 3.33 Mitomycin C-induced NADPH oxidation in reconstituted systems containing purified rabbit liver CYP2B4 and cytochrome P450 reductase under aerobic conditions. The activities were calculated as described in "Methods". **A,** Mitomycin C-induced NADPH oxidation vs. increasing amounts of CYP2B4 in the presence of 20.0 nM cytochrome P450 reductase. **B,** Mitomycin C-induced NADPH oxidation vs. increasing amounts of cytochrome P450 reductase in the absence (—■—) or presence (—●—) of 20.0 nM CYP2B4. Each point represents the average of duplicate determinations.

3.13.2 Reduction of Idarubicin in Reconstituted Systems Containing Purified Beef Liver Cytochrome P450 Reductase and Rabbit Liver CYP2B4 under Anaerobic Conditions

To further clarify the involvement of rabbit liver cytochrome P4502B4 in idarubicin reduction, the initial rate of idarubicin-induced NADPH oxidation in fully reconstituted system containing highly purified beef liver P450 reductase and rabbit liver CYP2B4, under anaerobic conditions, was compared with that in a reconstituted system of P450 reductase alone as described in detail under "Methods". It was previously shown that the purified beef liver P450 reductase was biocatalytically active and coupled with purified rabbit liver CYP2B4 in reconstituting the benzphetamine N-demethylation reaction (Table 3.6).

Table 3.18 shows that while beef liver P450 reductase (40.0 nM) alone catalyzed the idarubicin reduction at a rate of 144.2 nmol of NADPH/nmol of P450 reductase/min, in the presence of rabbit liver CYP2B4 (20.0 nM), this rate increased to 204.0 nmol of NADPH/nmol of P450 reductase/min. Thus, under anaerobic conditions, idarubicin reduction in a fully reconstituted system containing rabbit liver CYP2B4 (20.0 nM) and beef liver P450 reductase (40.0 nM) was found to be 1.41-fold higher than that in a system containing P450 reductase alone. All these results show that the rate of idarubicin reduction by beef liver P450 reductase (40.0 nM) under anaerobic conditions and by rabbit liver P450 reductase (40.0 nM) under aerobic conditions increased almost same fold (1.41-fold and 1.47-fold, respectively) in the presence of 20.0 nM purified rabbit liver CYP2B4 (Table 3.18). Although it was previously shown that both beef liver P450 reductase and rabbit liver P450 reductase were equally effective in catalyzing the idarubicin reduction (Table 3.15), the data in Table 3.18 show that the rate of idarubicin reduction by beef liver P450 reductase under anaerobic conditions was much more lower as compared to that by rabbit liver reductase under aerobic conditions. This discrepancy was obviously due to the differences in assay conditions as written in detail under "Methods".

Table 3.18 Idarubicin-induced NADPH oxidation rates in reconstituted systems under aerobic and anaerobic conditions

	Components of Reconstituted System	Idarubicin-Induced NADPH Oxidation (nmol/nmol of P450 reductase/min)
Aerobic (+ O_2)	Rabbit Liver P450 Reductase + Lipid	624.5
	Rabbit Liver P450 Reductase + Rabbit Liver CYP2B4 + Lipid	914.9[a]
Anaerobic (- O_2)	Beef Liver P450 Reductase + Lipid	144.2
	Beef Liver P450 Reductase + Rabbit Liver CYP2B4 + Lipid	204.0[b]

The activities were determined as described in "Methods". Data represent the average of duplicate determinations.

[a] Complete mixture contained purified rabbit liver CYP2B4 (20.0 nM), rabbit liver cytochrome P450 reductase (40.0 nM), idarubicin (40 μM) and NADPH (0.2 mM). The data for idarubicin-induced NADPH oxidation under aerobic conditions were obtained from the graph given in Figure 3.26 B.

[b] Complete mixture contained purified rabbit liver CYP2B4 (20.0 nM), beef liver cytochrome P450 reductase (40.0 nM), idarubicin (40 μM) and NADPH (0.2 mM).

3.13.3 Reduction of Mitomycin C in Reconstituted Systems Containing Purified Beef Liver Cytochrome P450 Reductase and Rabbit Liver CYP2B4 under Anaerobic Conditions

In order to make an accurate comparison with idarubicin and to obtain a reliable comparative data**,** the role of rabbit liver cytochrome P4502B4 in the reduction of mitomycin C was further determined, under anaerobic conditions, in reconstituted systems containing highly purified rabbit liver CYP2B4 and beef liver P450 reductase as described in detail under "Methods". Table 3.19 shows that the

initial rate of mitomycin C reduction by beef liver P450 reductase (40.0 nM), as measured directly by the decrease in absorbance at 375 nm based on the disappearance of quinone moiety of the drug, increased from 177.1 to 237.7 nmol of mitomycin C/nmol of P450 reductase/min in the presence of rabbit liver CYP2B4 (20.0 nM). Thus, mitomycin C reduction by P450 reductase (40.0 nM) occurred at a 1.34-fold greater rate in the presence of CYP2B4 (20.0 nM) as compared with that by P450 reductase alone under anaerobic conditions. Likewise, Table 3.19 shows that, in the presence of rabbit liver CYP2B4 (20.0 nM), the enhancements (fold increases) in the rates of mitomycin C reduction by beef liver P450 reductase (40.0 nm) under anaerobic conditions and of mitomycin C-induced NADPH oxidation by rabbit liver P450 reductase (40.0 nM) under aerobic conditions were almost the same (1.34-fold and 1.47-fold, respectively) (Table 3.19). Also, from the calculations of the slope of the curves in Figure 3.33 B, it was shown that in the presence of 20.0 nM rabbit liver CYP2B4, the rate of mitomycin C-induced NADPH oxidation by rabbit liver P450 reductase under aerobic conditions increased by 1.38-fold. This was a more close value to that obtained under anaerobic conditions.

As shown in Table 3.19, the initial rate of mitomycin C reduction by beef liver P450 reductase alone was also determined under aerobic environment by measuring decrease in absorbance at 375 nm based on the disappearance of quinone moiety under the same reaction conditions applied for the rate measurements under anaerobic environment. It was found that the rate of mitomycin C reduction by P450 reductase alone measured under aerobic conditions was 15.0% of that measured under anaerobic conditions which indicates that redox cycling of mitomycin C semiquinone with oxygen results in the regeneration of the parent quinone under aerobic conditions (see Discussion in Chapter IV).

Table 3.19 Mitomycin C-induced NADPH oxidation and mitomycin C reduction (quinone reduction) rates in reconstituted systems under aerobic and anaerobic conditions

Components of Reconstituted System	Mitomycin C-Induced NADPH Oxidation (nmol of NADPH oxidized/nmol of P450 reductase/min)
	Aerobic (+ O2)
Rabbit Liver P450 Reductase + Lipid	294.8
Rabbit Liver P450 Reductase + Rabbit Liver CYP2B4 + Lipid	433.5[a]

Components of Reconstituted System	Mitomycin C Reduction (nmol of mitomycin C reduced/nmol of P450 reductase/min)	
	Anaerobic (- O_2)	Aerobic (+ O_2)
Beef Liver P450 Reductase + Lipid	177.1	26.1
Beef Liver P450 Reductase + Rabbit Liver CYP2B4 + Lipid	237.7[b]	ND[c]

The activities were determined as described in "Methods". Data represent the average of duplicate determinations.

[a] Complete mixture contained purified rabbit liver CYP2B4 (20.0 nM), rabbit liver cytochrome P450 reductase (40.0 nM), mitomycin C (40 μM) and NADPH (0.2 mM). The data for mitomycin C-induced NADPH oxidation under aerobic conditions were obtained from the graph given in Figure 3.27 B.

[b] Complete mixture contained purified rabbit liver CYP2B4 (20.0 nM), beef liver cytochrome P450 reductase (40.0 nM), mitomycin C (40 μM) and NADPH (0.2 mM).

[c] ND, not determined

CHAPTER IV

DISCUSSION

Idarubicin and mitomycin C are clinically important quinone-containing anticancer agents used in the treatment of several human neoplasms. Idarubicin is a second-generation anthracycline drug which is clinically effective against breast cancer and some haematological malignancies including acute myelogenous leukemia, multiple myeloma and non-Hodgkin's lymphoma (Goebel, 1993; Borchman *et al.*, 1997; Crivellari *et al.*, 2004). Mitomycin C, on the other hand, shows antitumor activity as a single agent against a number of neoplastic diseases including bladder, breast, cervix, gastric, head and neck, lung, colon and pancreatic cancers (Powis, 1987; Bradner, 2001). Bioreductive activation of mitomycin C by oxidoreductases is a prerequisite for its DNA cross-linking and alkylating activities and thereby for exerting its antitumor effects (Ross *et al.*, 1993; Seow *et al.*, 2004). Among the mechanisms proposed for the antitumor effects of anthracyclines, free radical generation via bioreductive activation and subsequent redox cycling under aerobic conditions is considered as having an important contributing role on the effectiveness of these chemotherapy agents (Sinha, 1989; Powis, 1989; Cullinane *et al.*, 1994; Taatjes *et al.*, 1997; Kostrzewa-Nowak *et al.*, 2005; Doroshow, 2006). Free radical generation by anthracyclines involves their bioreductive activation by cellular oxidoreductases (Kappus, 1986). To the best of our knowledge, there have been no previous reports demonstrating the bioreductive activation of idarubicin by purified NADPH-cytochrome P450 reductase, NADH-cytochrome b5 reductase and cytochrome P4502B4 isozyme. Thus, in the present study, for the first time, we showed that purified cytochrome P450 reductase is capable of effectively catalyzing the bioreductive activation of idarubicin to DNA-damaging species, whereas purified

microsomal b5 reductase is not involved in this process using *in vitro* plasmid DNA damage assay. Our results demonstrated that bioreductive activation of idarubicin by highly purified cytochrome P450 reductase results in the formation of redox active metabolites which causes DNA strand breaks under aerobic conditions through generating reactive oxygen species. In addition, we characterized the DNA-damaging capacity of idarubicin with respect to increasing enzyme or drug concentration as well as increasing incubation time using the above method. The results obtained from spectrophotometric enzyme assays with highly purified enzymes and microsomes were also found to be consistent with those obtained from *in vitro* plasmid DNA damage experiments. Besides, the contribution of rabbit liver CYP2B4 to idarubicin reduction, relative to cytochrome P450 reductase, was determined in reconstituted systems. The studies in highly pure reconstituted sytems showed that the reconstituted rabbit liver CYP2B4 isozyme produced a higher idarubicin reduction rate as compared to that catalyzed by P450 reductase alone. Finally, among the tested antioxidants, only quercetin was found to be highly potent in protecting DNA against strand breaks induced by P450 reductase-catalyzed bioreductive activation of idarubicin. All the above experiments in this study were also repeated under the same reaction conditions using mitomycin C and the results were compared. Thus, our results provided crucial comparative data on the bioreductive activation of idarubicin and mitomycin C by NADPH-cytochrome P450 reductase, NADH-cytochrome b5 reductase and cytochrome P4502B4. Interestingly, our findings showed that mitomycin C seems to be a very poor substrate of microsomal cytochrome b5 reductase while it is effectively reduced by cytochrome P450 reductase. The details of our results are discussed below.

In this study, it was demonstrated that purified cytochrome P450 reductase is capable of effectively catalyzing the reductive activation of idarubicin like its parent drug daunorubicin. Daunorubicin has been shown previously by others to undergo one-electron reduction by cytochrome P450 reductase (Pawlowska, 2003). The plasmid DNA experiments and spectrophotometric enzyme assays carried out with highly purified enzymes showed that the deletion of methoxy group at C-4 position of the D ring in the aglycone moiety of daunorubicin does not impede the ability of

cytochrome P450 reductase to catalyze the reduction of this synthetic derivative. This is especially important since it has been shown that the structural differences of anthraquinones may influence their ability to undergo reduction by flavoenzymes including cytochrome P450 reductase to generate reactive oxygen species (Tarasiuk, 1992; Pawlowska, 2003).

The plasmid DNA assays have demonstrated that the purified sheep lung cytochrome P450 reductase can catalyze the formation of DNA-damaging products in the presence of idarubicin as shown by the conversion of supercoiled form of pBR322 into open circular conformation which resulted from the induction of single-strand breaks in DNA. The *in vitro* plasmid DNA damage assay used in this study is a very useful and sensitive method for detecting strand breaks in DNA exposed to damaging agents. It has been used effectively by researchers as a method for evaluating the role of different reductive enzymes on the bioactivation of various anticancer drugs and compounds (Fisher and Gutierrez, 1991; Shen and Hollenberg, 1994; Kumagai *et al.*, 1997; Garner *et al.*, 1999; Çelik and Arinç, 2006a, b and c).

We used cytochrome P450 reductase purified from sheep lung tissue in plasmid DNA experiments, since previous studies carried out in our laboratory have shown that sheep lung P450 reductase was more resistant to proteolytic cleavage compared to the P450 reductase purified from liver tissue (İşcan and Arinç, 1988). It was demonstrated that antioxidant enzymes, SOD and catalase, as well as scavengers of hydroxyl radicals, DMSO and thiourea, provided effective protections against DNA strand scission induced by idarubicin (Figure 3.15 and Table 3.9). Based on these results, we proposed that the mechanism of DNA damage induced by idarubicin appears to involve redox cycling of idarubicin with P450 reductase under aerobic conditions to generate reactive oxygen species as shown in Figure 4.1. This figure indicates that one-electron reductive activation of idarubicin by purified P450 reductase results in the formation of corresponding semiquinone radical which undergoes redox cycling with molecular oxygen under aerobic conditions to generate superoxide. The $O_2^{\cdot -}$ formed by this process could then undergo spontaneous or enzymatic dismutation to produce hydrogen peroxide (H_2O_2) which in the presence

of trace amounts of ferric ions rapidly decomposes to very reactive hydroxyl radical via Fenton reaction. The possible presence of trace amounts of Fe^{+3} ions as a contaminant in one of the reaction components such as sodium potassium buffer in plasmid DNA assays may have been responsible for the production of these highly reactive hydroxyl radicals (Shen and Hollenberg, 1994). The highly potent $OH^{\cdot}$ then causes the formation of DNA strand breaks (Kappus, 1986; Brawn and Fridovich, 1981; Kovacic and Osuna, 2000). The plasmid DNA experiments using mitomycin C (Figure 3.16 and Table 3.10), a model redox cycling quinone with P450 reductase, also confirmed that the proposed mechanism shown in Figure 4.1 appears to be responsible for the generation of DNA strand breaks by idarubicin in the presence of cytochrome P450 reductase and cofactor NADPH. This mechanism is considered to be one of the most important pathways contributing to the antitumor effect of idarubicin.

The idarubicin concentrations used in plasmid DNA experiments were higher than its clinically achievable concentrations. However, for example in the case of doxorubicin, several studies have shown that reactive oxygen species could be detected also at very low concentrations of this drug in cancer cells (Ubezio and Civoli, 1994; Bustamante *et al.*, 1990; referred by Doroshow, 2006). Thus, it may be suggested based on these results that reactive oxygen species generated by cytochrome P450 reductase-catalyzed reductive activation of idarubicin may contribute both to the chemotherapeutic effects of idarubicin in the treatment of tumor cells as well as its toxic side effects in normal healthy cells. The reactive oxygen species formed in the presence of cytochrome P450 reductase may also be responsible for the genotoxic effects of this antineoplastic drug to induce secondary malignancies.

When the ability of idarubicin to induce DNA damage was compared with that of mitomycin C at varying incubation times and drug concentrations as well as at different enzyme amounts, it was shown that both drugs exhibited almost similar DNA-damaging potentials under aerobic conditions (Figures 3.17-3.22). The only marked difference observed was the greater ability of idarubicin versus mitomycin C

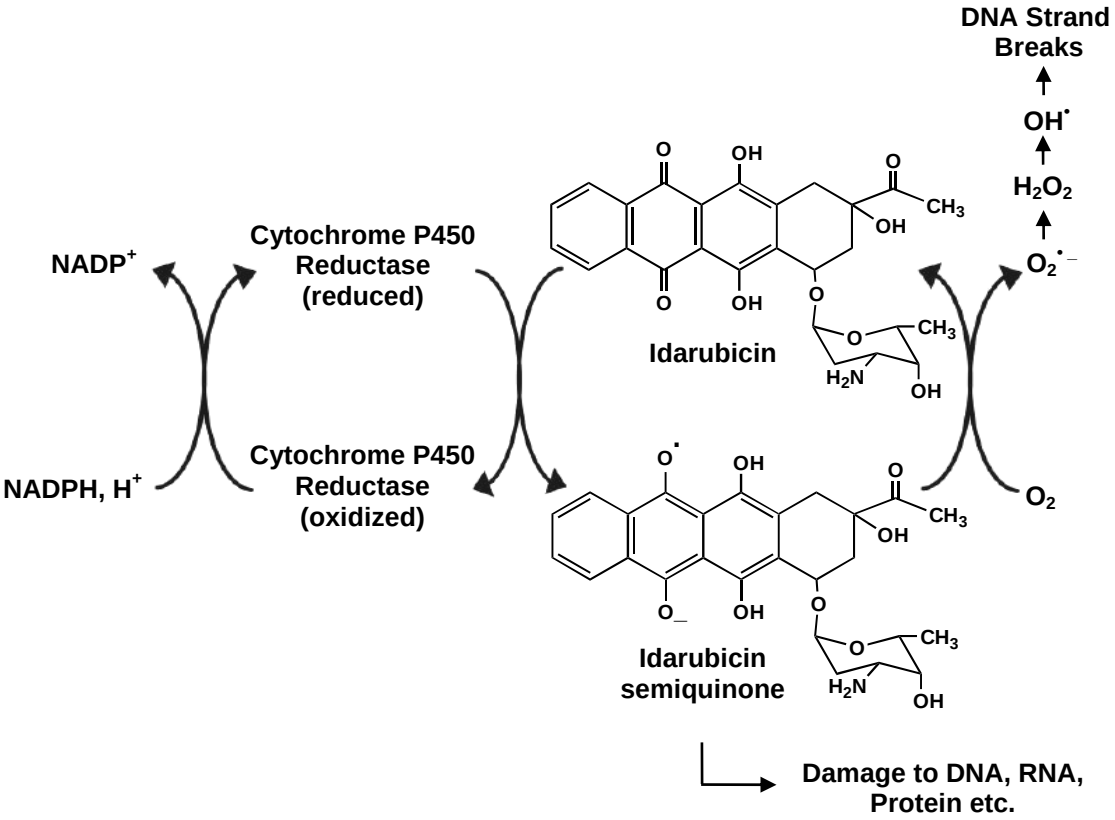


Figure 4.1 Reductive activation of idarubicin by NADPH-cytochrome P450 reductase and the mechanism of DNA damage

to induce DNA strand breaks at higher drug concentrations (200 and 400 μM) (Table 3.11). This finding may suggest that these structurally related compounds might have similar abilities to redox cycle with cytochrome P450 reductase, and thus to induce single-strand breaks in DNA.

The enzyme assay experiments with P450 reductases purified from phenobarbital-treated rabbit liver, beef liver and sheep lung microsomes revealed that idarubicin exhibited about two-fold higher rate of reduction, as measured by NADPH consumption at 340 nm, than mitomycin C by all the purified P450 reductases under aerobic conditions (Table 3.15). Mitomycin C shows an absorption peak at 363 nm,

and the absorption at this wavelength declines upon reduction of the quinone moiety of the drug. This property has been used by others to measure the metabolism of mitomycin C under anaerobic conditions (Kennedy *et al.*, 1982). A question might be raised as to whether such property of the drug had an effect on the absorbance measurements at 340 nm due to overlap of peaks corresponding to NADPH and mitomycin C, which might cause an overestimation of the rates of mitomycin C-induced NADPH oxidation by purified P450 reductases. However, since 340 nm is close to the isobestic point (the wavelength at which two or more substance absorb the light to the same extent) for mitomycin C at 331 nm, the loss of the absorbance at 363 nm due to the reduction of quinone should interfere very little with the absorbance at 340 nm (Hodnick and Sartorelli, 1993). In addition, under aerobic conditions, mitomycin C semiquinone undergoes redox cycle with molecular oxygen and thereby regenerates the parent quinone. Therefore, no significant loss of absorbance at 363 nm is expected under aerobic conditions. The rate of toxic oxygen radical production by these drugs is expected to be proportional to their one-electron reduction rates by cytochrome P450 reductase. Thus, based on these results, idarubicin appears to be a more potent cytotoxic drug than mitomycin C in terms of the generation of reactive and/or redox active metabolites by P450 reductase. However, despite the two-fold difference in their reduction rates, the reason for observing no major difference in the DNA-damaging potentials of idarubicin and mitomycin C at various incubation conditions remains unclear and needs further detailed investigation. This may be related to the sensitivity of the plasmid DNA assay or to the differences in the assay conditions. Nevertheless, the difference in the reduction rates of idarubicin and mitomycin C by purified sheep lung P450 reductase may account for the increased ability of idarubicin to induce DNA strand breaks at higher drug concentrations (200 and 400 μM) compared to mitomycin C. Another point is that the purified sheep lung P40 reductase, when compared to P450 reductases purified from beef liver and phenobarbital-treated rabbit liver microsomes, was found to be somewhat less effective in catalyzing the reduction of both idarubicin and mitomycin C (Table 3.15). This functional difference may be caused by the differences in three-dimensional structures of P450 reductases purified from different species that occur as a result of differences in amino acid sequences.

The absolute absorption spectra and SDS-polyacrylamide gel electrophoresis analysis have shown that our purified cytochrome P450 reductase enzyme preparations were highly pure and not contaminated with hemoproteins like cytochrome b5 and cytochrome P450.

It has been previously shown that soluble form of NADH-cytochrome b5 reductase purified from rabbit erythrocytes catalyzes the reduction of mitomycin C and adriamycin with the production of reactive and/or redox active metabolites (Hodnick and Sartorelli, 1993 and 1994). Based on these observations, it was expected that NADH-cytochrome b5 reductase purified from beef liver microsomes in this study would catalyze the reduction of idarubicin as well. Therefore, the ability of idarubicin to undergo reductive activation by the purified NADH-cytochrome b5 reductase was also examined. NADH-cytochrome b5 reductase used in these experiments was purified from beef liver microsomes. Indeed, initially, the enzyme was tried to be purified from phenobarbital-treated rabbit liver microsomes. However, due to some difficulties encountered during the purification of NADH-cytochrome b5 reductase from phenobarbital-treated rabbit liver microsomes (see Section 3.2), it was decided to purify the enzyme from beef liver microsomes in the hope of obtaining a homogenous preparation.

The *in vitro* plasmid DNA damage assays, interestingly, revealed that the purified beef liver microsomal b5 reductase is not capable of catalyzing the reduction of idarubicin to DNA-damaging species with the resulting formation of strand breaks in DNA (Figure 3.23). The previous studies have shown that the soluble rabbit erythrocytic b5 reductase reduces mitomycin C and adriamycin in a pH dependent manner, with reduction occurring at a greater rate at pH 6.6 than at pH 7.6 for mitomycin C and with reduction occurring at pH 6.6, but not at pH 7.6 for adriamycin (Hodnick and Sartorelli, 1993 and 1994). For this reason, in our experiments, the pBR322 plasmid DNA was incubated with the purified cytochrome b5 reductase and idarubicin at two different pHs, pH 6.6 and pH 7.4. However, no DNA strand breaks were observed over plasmid-alone control at either pH, even if different reaction conditions and various amounts of drug or enzyme, as described in

Section 2.2.11.2 and Section 3.9.3, were employed. The finding that the purified beef liver microsomal b5 reductase did not reduce idarubicin as well, as measured by NADH oxidation at 340 nm at either pH (pH 6.6 or pH 7.5) (Table 3.16), indicated that NADH-cytochrome b5 reductase purified from beef liver microsomes is not a catalyst for the reduction of idarubicin. In our studies, we employed similar reaction conditions for the reduction of idarubicin by microsomal beef liver b5 reductase as those used for the reduction of adriamycin by soluble rabbit erythrocytic b5 reductase (Hodnick and Sartorelli, 1994). The specific activity of adriamycin reduction by soluble rabbit cytochrome b5 reductase at pH 6.6 has been found as 33.4 ± 9.2 nmol of NADH oxidized/min/mg (Hodnick and Sartorelli, 1994). Actually, this value was very low as compared with those of idarubicin reductions catalyzed by our purified cytochrome P450 reductases (5112.2 nmol of NADPH oxidized/min/mg for rabbit liver P450 reductase), which indicates that rabbit soluble erythrocytic b5 reductase is also not an efficient drug activator for adriamycin unlike P450 reductase.

In our *in vitro* plasmid DNA damage experiments and enzyme assays, the microsomal form of the NADH-cytochrome b5 reductase purified from beef liver tissue was used. In the studies by Hodnick and Sartorelli (1993 and 1994), soluble NADH-cytochrome b5 reductase purified from rabbit erythrocytes was used to characterize the reduction of mitomycin C and adriamycin. Microsomal NADH-cytochrome b5 reductase present in a variety of tissues is a membrane-bound flavoprotein which has an amphipathic structure having a large cytosolic domain and additional hydrophobic membrane segment. While the hydrophilic peptide contains FAD and retains the spectral characteristics of the native enzyme, the hydrophobic peptide is essential for the proper interaction of the reductase with cytochrome b5, and anchoring of the enzyme to the biological membranes. The enzyme in erythrocytes, on the other hand, exists as a soluble protein and catalyzes the reduction of methemoglobin via transferring electrons to cytochrome b5 (Arinç, 1991). NADH-cytochrome b5 reductase in this system appears to be very similar to the enzyme in endoplasmic reticulum (microsomal form). The comparison of the amino acid sequences of the soluble and microsomal forms of the cytochrome b5

reductase revealed that the soluble reductase lacks a hydrophobic segment at the NH_2 terminus, which is present in the membrane-bound reductase. It has been proposed that soluble b5 reductase is generated through the posttranslational proteolytic cleavage of membrane-bound protein during erythrocyte maturation. In the rat, however, it was shown that two different mRNAs generated from the same reductase gene by an alternative promoter mechanism encode the soluble and membrane-bound forms of the cytochrome b5 reductase (Borgese *et al.*, 1993). In addition, the complete amino acid sequence analysis of steer liver b5 reductase demonstrated that the limited tryptic cleavage of the membrane-bound protein produces a soluble peptide lacking the fist 28 amino acid residues of the N-terminal hydrophobic segment (Ozols *et al.*, 1985). This peptide was found to retain its structural features necessary for enzymatic activity. When the amin oacid sequences of human erythrocyte cytosolic b5 reductase and steer liver microsomal b5 reductase were compared, it was found that a high degree of homology greater than 90% exists between them (Yubisui *et al*., 1986).

The finding that purified beef liver microsomal b5 reductase did not reduce idarubicin contrary to what was observed in the studies by Hodnick and Sartorelli (1993 and 1994) with soluble b5 reductase raised some concern that there might be some differences between the soluble and membrane-bound forms of b5 reductase in catalyzing the quinone-containing anticancer drug substrates. The existence of some kinetic differences between the soluble and membrane-bound forms of b5 reductase supports this hypothesis (Williams, 1976 referred by Hodnick and Sartorelli, 1993). In addition, species-specific differences (Yubisui and Takeshita, 1982 referred by Hodnick and Sartorelli, 1993) may also be responsible for this observed discrepancy.

NADH-cytochrome b5 reductase used in our study was obtained from beef liver microsomes in homogenous form as judged by SDS-polyacrylamide gel electrophoresis (Figure 3.5). Besides, the purified NADH-cytochrome b5 reductase was found to be biocatalytically active as determined by its ability to reduce cytochrome b5 and cytochrome *c* (through cytochrome b5) in reconstituted systems, indicating that the enzyme was purified from beef liver microsomes in its native

amphipathic form. Our purified enzyme was much more pure than the soluble rabbit erythrocytic b5 reductase used in the study by Hodnick and Sartorelli (1993), since it contained hemoglobin as a contaminant in some enzyme preparations. However, the authors reported that purified hemoglobin did not catalyze the reduction of mitomycin C under identical conditions with the purified b5 reductase, therefore was not responsible for the reductive activation of mitomycin C (Hodnick and Sartorelli, 1993).

Our results have also shown that, while plasmid DNA incubations with purified beef liver b5 reductase even at high concentrations in the presence of idarubicin and cofactor NADH produced no DNA strand breaks, the addition of purified sheep lung P450 reductase to incubation mixtures in the presence of cofactor NADPH effectively generated single-strand breaks under the same conditions (Figure 3.23). This result clearly demonstrated that unlike b5 reductase, cytochrome P450 reductase is an efficient enzyme in catalyzing the reductive bioactivation of idarubicin to redox active metabolites which induce strand breaks in DNA.

Another interesting point was that our purified beef liver NADH-cytochrome b5 reductase enzyme was also found to be a very weak catalyst for the reduction of mitomycin C as shown by both *in vitro* plasmid DNA damage experiments and enzyme assays. Although different incubation conditions and various amounts of enzyme were tested (see Sections 2.2.11.2 and 3.9.3), DNA strand breaks were detected only at very low amounts over plasmid-alone control in the presence of cytochrome b5 reductase and cofactor NADH (Figure 3.24). Our findings obtained from plasmid DNA experiments were also verified by enzyme assays, as measured by the consumption of NADH cofactor at 340 nm (Table 3.16). The purified beef liver microsomal b5 reductase was found to barely catalyze the reduction of mitomycin C and this rate was assumed negligible compared to those catalyzed by purified NADPH-cytochrome P450 reductases (Table 3.15 and Table 3.16).

In our experiments, similar reaction conditions to those used in the study by Hodnick and Sartorelli (1993) were employed for mitomycin C reduction by purified

beef liver b5 reductase. The finding that our purified microsomal beef liver b5 reductase did not effectively catalyze the reductive activation of mitomycin C under these conditions in contrast to rabbit erythrocytic b5 reductase may be explained by the reasons discussed above. However, if the specific activities of mitomycin C reductions obtained using soluble rabbit cytochrome b5 reductase (75.3 ± 2.8 and 48.2 ± 8.1 nmol of NADH oxidized/min/mg at pH 6.6 and at pH 7.6, respectively) (Hodnick and Sartorelli, 1993) were compared with those catalyzed by our purified cytochrome P450 reductases (2425.7 nmol of NADPH oxidized/min/mg, for rabbit liver P450 reductase), it seems that soluble rabbit b5 reductase is also not an effective drug activator as P450 reductase for mitomycin C.

The rate measurements of idarubicin and mitomycin C reduction in the presence of NAD(P)H cofactor in phenobarbital-treated and untreated rabbit liver microsomes also suggested that microsomal NADH-cytochrome b5 reductase is either a very weak catalyst or ineffective in catalyzing the reduction of mitomycin C and idarubicin as compared to P450 reductase (Table 3.14). These possibilities were tested using purified enzymes as mentioned above and the results obtained raised the conclusion that mitomycin C is a very poor substrate for purified microsomal cytochrome b5 reductase, whereas idarubicin is not a suitable substrate. NADPH-cytochrome P450 reductase, on the other hand, effectively catalyzes the reduction of both anticancer drugs.

In addition to the lack of any information regarding the roles of NAPDH-cytochrome P450 reductase and NADH-cytochrome b5 reductase in the reductive bioactivation of idarubicin, to our knowledge, there also exists no report on the possible role of cytochrome P450 in this reduction reaction. Therefore, in this study, we investigated the role of cytochrome P450 in the reductive bioactivation of idarubicin, relative to the role of P450 reductase in highly pure reconstituted systems. The results were then compared with those obtained using mitomycin C, since several studies have demonstrated its reductive bioactivation in the presence of cytochrome P450 (Kennedy *et al.*, 1982; Vromans *et al.*, 1990; Goeptar *et al.*, 1994). In a study conducted by Vromans and co-workers (1990) using rat liver microsomes

and reconstituted systems of purified rat liver cytochrome P450 and cytochrome P450 reductase, it has been shown that cytochrome P450 is involved in the one-electron reduction of mitomycin C under anaerobic conditions and in the reduction of molecular oxygen under aerobic conditions. However, the mechanism underlying CYP-mediated reductive bioactivation of mitomycin C is subject to discussion (Keyes *et al.,* 1985 and 1984).

The plasmid DNA experiments demonstrated that rabbit liver CYP2B4 contributes very little to the idarubicin-mediated generation of DNA strand breaks as compared to P450 reductase when DNA damage was assessed using highly pure reconstituted systems containing either increasing amounts of rabbit liver P450 reductase and a fixed amount of rabbit CYP2B4 or increasing amounts of CYP2B4 and a fixed amount of P450 reductase (Figures 3.25 and 3.26; Tables 3.12 and 3.13). This finding may also suggest that the *in vitro* plasmid DNA damage assay employed under these conditions might not be sensitive enough to determine exactly whether and to what degree rabbit liver CYP2B4 is involved in the bioactivation of idarubicin to DNA-damaging species. In order to test this possibility, the relative rates of idarubicin reduction were measured in reconstituted systems containing highly purified enzymes under both aerobic and anaerobic conditions and the results were compared with those obtained using mitomycin C.

The enzymatic assays carried out using highly pure reconstituted systems clearly demonstrated that rabbit liver CYP2B4 is a potential candidate enzyme for the reduction of idarubicin. The experiments performed with increasing amounts of P450 reductase in the presence or absence of a fixed amount of CYP2B4 under aerobic conditions revealed that, while rabbit liver P450 reductase alone catalyzed the reduction of idarubicin at a rate of 626.5 nmol of NADPH/nmol of P450 reductase/min, this rate increased to 920.2 nmol of NADPH/nmol of P450 reductase/min (calculated from the slope of curves in Figure 3.32 B) in the presence of CYP2B4. These results indicated that reconstituted rabbit liver CYP2B4 produced about 1.5-fold rate enhancement in idarubicin reduction catalyzed by P450 reductase alone.

The experiments with increasing amounts of rabbit liver CYP2B4 in the presence of a fixed amount of rabbit liver P450 reductase, on the other hand, showed that the maximum activity, expressed as nmol of NADPH oxidized per minute, was observed at 1:1 stoichiometry of P450 reductase and CYP2B4 (Figure 3.32 A). The reason why this saturation was not observed in Figure 3.32 B with further addition of P450 reductase beyond equimolar concentrations of P450 reductase and CYP2B4 is simply because P450 reductase alone is able to catalyze idarubicin reduction. Since CYP2B4 is not capable of catalyzing the reduction of idarubicin alone, a further increase in CYP2B4 amount did not increase the reaction rate further beyond a point as seen in Figure 3.32 A.

The experiments carried out using mitomycin C under the same above conditions revealed that the initial rate of mitomycin C reduction by rabbit P450 reductase alone was 318.8 nmol of NADPH/nmol of P450 reductase/min, whereas in the presence CYP2B4 this rate increased to 440.3 nmol of NADPH/nmol of P450 reductase/min (Figure 3.33 B). Consistent with the previous findings (Table 3.15), the rate of mitomycin C reduction by rabbit P450 reductase alone (318.8 nmol of NADPH/nmol of P450 reductase/min) was found to be almost half that of idarubicin reduction (626.5 nmol of NADPH/nmol of P450 reductase/min). These results also indicated that reconstituted rabbit liver CYP2B4 produced about 1.4-fold rate enhancement in mitomycin C reduction catalyzed by P450 reductase alone. Thus, the relative contributions of rabbit liver P450 reductase and CYP2B4 to idarubicin and mitomycin C reductions were found to be almost the same.

The studies by Goeptar and co-workers (Goeptar *et al.,* 1993) using the same above experimental approach revealed that the contribution of CYP2B1 purified from phenobarbital-treated rat liver microsomes to the one-electron reduction of adriamycin was similar to that of P450 reductase. The same research group also showed that reconstituted CYP2B1 produced a 4-5-fold higher rate of one-electron reduction of 2,3,5,6-tetramethylbenzoquinone at equimolar concentrations of CYP2B1 and P450 reductase compared to P450 reductase alone (Goeptar *et al.*, 1992). The differences between these results and our results suggest that structural

differences of quinone compounds and/or differences in the properties between different mammalian cytochrome P450 isozymes and NADPH-cytochrome P450 reductases may affect CYP-mediated bioactivation of quinone-containing compounds.

The rate enhancement of idarubicin reduction by cytochrome P450 may be explained by a mechanism in which cytochrome P450 reductase, besides its direct role in catalyzing the reduction of idarubicin, can also transfer electrons from NADPH cofactor to cytochrome P450 which in turn may catalyze the reduction of idarubicin as well. The cytochrome P450 alone is unable to take these electrons directly from NADPH but does so indirectly via NADPH-cytochrome P450 reductase, which is facilitated by lipids. Thus, in reconstituted systems, cytochrome P450 reductase can transfer electrons from NADPH cofactor to idarubicin either directly or indirectly via cytochrome P450. The maximal activity observed at 1:1 stoichiometry of P450 reductase and CYP2B4 suggests that both enzymes act together in the reduction of idarubicin. However, in order to understand the exact mechanism behind the rate enhancement of idarubicin reduction by cytochrome P450 further studies are required. Such a mechanism for idarubicin has also been proposed for CYP-mediated one-electron reduction of 2,3,5,6-tetramethylbenzoquinone and adriamycin (Goeptar *et al.*, 1992 and 1993).

It has been reported that cytochrome P450 catalyze the reduction of a variety of organic compounds such as carbon tetrachloride, halothane, gentian violet, and benznidazole to free radicals under anaerobic conditions (De Groot and Sies, 1989). This bioactivation occurs at a site of P450 where oxygen usually binds and becomes activated during the monooxygenase cycle. Thus, there occurs a competition between these xenobiotics and oxygen for the electrons and the reduction of xenobiotics to free radicals occurs at a maximal rate under anaerobic conditions (De Groot and Sies, 1989). For mitomycin C and adriamycin, the difference spectra revealed that these drugs interact with cytochrome P450 at a binding site on the protein moiety of cytochrome P450 and an interaction with heme iron is unlikely as in the case of halogenated alkanes (Vromans *et al.*, 1990; Goeptar *et al.*, 1993). Therefore,

molecular oxygen is not expected to inhibit the reduction of these drugs by CYP under aerobic conditions. That's why the researchers studied the role of cytochrome P450 in the reductive biotransformation of adriamycin and mitomycin C under aerobic conditions by measuring the production of H_2O_2 as well, which may be generated through redox cycling of one-electron reduced semiquinone forms of these drugs with molecular oxygen (Vromans *et al.*, 1990; Goeptar *et al.*, 1993). A number of quinone compounds including quinoneimines such as N-acetyl-para-benzoquinoneimine (NAPQI) and 3,5-dimethyl-NAPQI have been shown to stimulate the oxidase or oxygen reductase activity of cytochrome P450 via inducing uncoupling of the cytochrome P450 reaction cycle, which also results in the production of H_2O_2 under aerobic conditions. The molecular mechanisms underlying this oxygen reductase activity as well as xenobiotic reductase activity of cytochrome P450 are not well-understood (Goeptar *et al.*, 1995). For this reason, in the studies by Vromans *et al.* (1990) and Goeptar *et al.* (1993), the authors speculated that either the formation of H_2O_2 under aerobic conditions is caused by redox cycling of adriamycin and mitomycin C semiquinones formed by CYP through one-electron reduction, or it is due to the adriamycin or mitomycin C-induced uncoupling of the P450 monooxygenase cycle. Alternatively, both pathways may have combined effect for the production of H_2O_2 under aerobic conditions.

In our study, we determined the relative contributions of rabbit liver CYP2B4 and rabbit liver P450 reductase to idarubicin and mitomycin C reductions under aerobic conditions as measured by drug-induced NADPH oxidation. Therefore, to what extent the electrons from NADPH are utilized for the reduction of idarubicin and mitomycin C or for the reduction of oxygen through uncoupling of the monooxygenase cycle is also a matter of speculation. In order to clear these issues and to further clarify the involvement of rabbit liver CYP2B4 in the reduction of idarubicin, the initial rates of idarubicin-induced NADPH oxidation were measured under anaerobic conditions in highly pure reconstituted systems containing beef liver P450 reductase and rabbit liver CYP2B4. In order to make an accurate comparison with idarubicin**,** the involvement of rabbit liver CYP2B4 in the reduction of mitomycin C was also determined under the same conditions, as measured by the

decrease in absorbance at 375 nm based on the disappearance of quinone moiety of the drug. It has been reported that, under anaerobic conditions, P450 may be involved in the reduction of xenobiotics through forming ferrous xenobiotic complex (Goeptar *et al.*, 1995).

Before these reconstitution experiments, it was shown that cytochrome P450 reductase was purified from beef liver microsomes in its biocatalytically active form and could couple effectively with purified rabbit liver CYP2B4 in reconstituting the benzphetamine N-demethylation reaction (Table 3.6). The results of the reconstitution experiments performed under anaerobic conditions using beef liver P450 reductase and rabbit liver CYP2B4 revealed that reconstituted CYP2B4 produced about 1.4-fold rate enhancement in idarubicin reduction catalyzed P450 reductase alone (Table 3.18). This finding indicated that the rate enhancement (fold increase) of idarubicin reduction by rabbit liver CYP2B4 under anaerobic conditions in reconstituted systems containing beef liver P450 reductase or under aerobic conditions in reconstituted systems containing rabbit liver P450 reductase were almost the same (Table 3.18). The equal effectiveness of both beef liver P450 reductase and rabbit liver P450 reductase in catalyzing the reduction of idarubicin, as shown previously in Table 3.15, and their probable similar efficiencies in transferring the electrons from NADPH to rabbit liver CYP2B4 may be the reasons for this finding. In addition, the data obtained from experiments under anaerobic conditions strongly suggested that, under aerobic conditions, the electrons from NADPH are most probably utilized for the reduction of idarubicin but not for the reduction of molecular oxygen through idarubicin-stimulated uncoupling of the monooxygenase cycle.

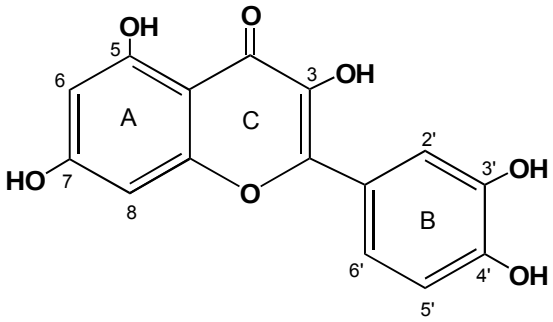
The above experiments under anaerobic environment were also repeated using mitomycin C under the same reaction conditions applied for idarubicin in the reconstituted systems. The reconstitution experiments performed using rabbit liver CYP2B4 and beef liver P450R under anaerobic conditions demonstrated that the relative contribution of reconstituted rabbit liver CYP2B4 to the reduction of mitomycin C was almost the same with that observed in reconstituted systems under

aerobic conditions using rabbit liver P450 reductase as in the case of idarubicin (see Table 3.19). Since the reduction of mitomycin C under anaerobic conditions was determined directly by measuring the decrease in absorbance at 375 nm due to the disappearance of quinone moiety, the data obtained under anaerobic conditions suggest that the rate enhancement in mitomycin C-induced NADPH consumption by CYP2B4 under aerobic conditions is probably due to the combined action of P450 reductase and CYP2B4 to catalyze the reduction of mitomycin C as explained by the above mechanism. However, further studies are required in order to understand the molecular events underlying the rate enhancement of mitomycin C or idarubicin reduction by CYP under both anaerobic and aerobic conditions.

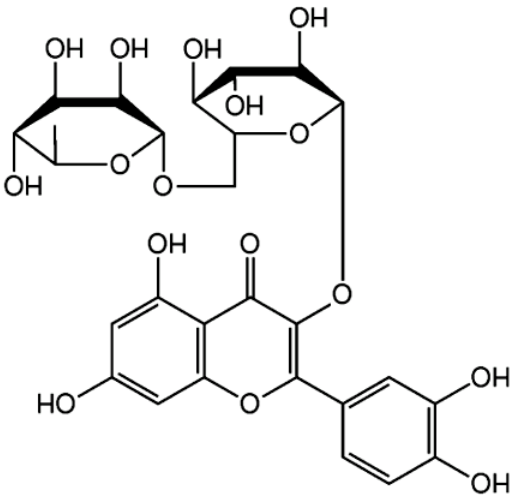
The initial rate of mitomycin C reduction by beef liver P450 reductase alone was also determined under aerobic environment by measuring the decrease in absorbance at 375 nm under the same reaction conditions applied for the rate measurements under anaerobic environment. Actually, this method for the measurement of the rate of mitomycin C reduction based on the disappearance of quinone moiety is carried out only under anaerobic conditions. It was found that the rate of mitomycin C reduction by P450 reductase alone measured under aerobic conditions was 15.0% of that measured under anaerobic conditions (Table 3.19). This data indicated that, under aerobic conditions, mitomycin C semiquinone undergoes redox cycle with molecular oxygen and regenerates the parent quinone. However, why a 15% of the rate measured under anaerobic conditions could be still observed under aerobic conditions may be explained in a way that the formation of mitomycin C semiquinone via one-electron reduction by P450 reductase may proceed at a faster rate than the regeneration of the parent quinone via redox cycling of semiquinone with molecular oxygen.

In the present study, the protective potentials of dietary antioxidants, quercetin, naringenin, rutin, resveratrol and trolox (a soluble analogue of vitamin E) (Figure 4.2) against genotoxic effects of idarubicin resulting from its reductive activation by cytochrome P450 reductase were also investigated using *in vitro* plasmid DNA damage assay. Flavonoids are polyphenolic compounds found in

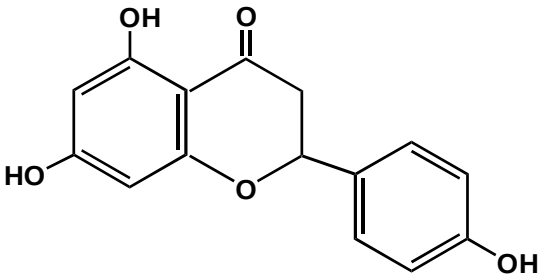
significant quantities in foods of plant origin like vegetables, fruits, seeds, and in plant-derived beverages such as tea and wine (for details see the review by Havsteen, 2002). Several population studies have linked increased consumption of flavonoid-containing foods to protection from certain chronic and degenerative diseases including cancer and cardiovascular diseases. The exact molecular mechanisms underlying the protective mode of action of flavonoids are unknown. Several mechanisms have been proposed for their protective effects. These include the ability of flavonoids to act as antioxidants (reducing agents, hydrogen donors, free radical quenchers or metal ion chelators), carcinogen inactivators, modulators of gene expression and DNA repair, inhibitors of enzyme and inducers of apoptosis (Duthie and Dobson, 1999; Havsteen, 2002). Quercetin (3,3',4',5,7-pentahydroxyflavone) has been reported to be one of the most predominant flavonol. It has been reported that the average intake of all flavonoids combined is 23 mg/day, and quercetin accounts for 70% of total intake in the average diet, with tea, onions and apples being the most important sources (Hertog, 1993). The antioxidant properties and chemopreventive effects of quercetin have been studied in detail (Onuki *et al*., 2005). Paradoxically, quercetin has also been shown to be mutagenic and carcinogenic (see Yamashita *et al.*, 1999). Rutin (quercetin-3-rutinoside) is a glycoside of quercetin and has been shown to be one of the most commonly occurring flavonol glycoside in the human diet. Naringenin (4′,5,7- trihydroxyflavanone) is one of the most abundant citrus bioflavonoids. Naringenin has been shown to have a wide range of pharmacological effects such as inhibition of lipid peroxidation, modulation of cytochrome P450-dependent monooxygenase activities, possessing antioxidant, anticancer, antimutagenic, antiatherogenic, hepatoprotective, antifibrogenic and free radical scavenging activities (Pari and Gnanasoundari, 2006). Resveratrol (3,5,4'-trihydroxy-trans-stilbene), a natural polyphenolic non-flavonoid antioxidant, is a phytoalexin found in the skin of redgrapes, nuts and berries and is a constituent of red wine. Several studies have shown that resveratrol can act as an antioxidant, inhibits platelet aggregation and LDL oxidation and scavenges lipid hydroperoxyl free radicals as well as hydroxyl and superoxide radicals (Sun *et al*., 2002). Trolox is a water soluble analogue of α-tocopherol (vitamin E) and reported to be also a free radical scavenger and protector against oxidative DNA damage (Kumar *et al.*, 1999).



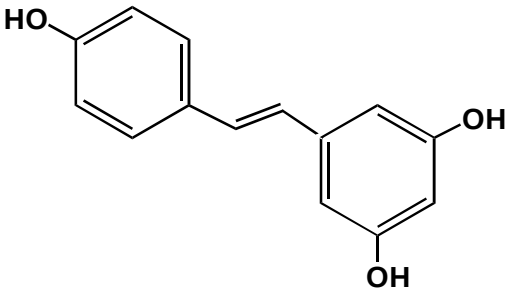
Quercetin



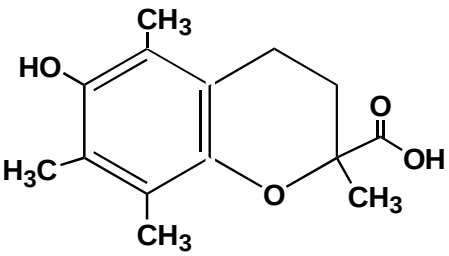
Rutin



Naringenin



Resveratrol



Trolox

Figure 4.2 Structures of antioxidants, quercetin, rutin, naringenin, resveratrol and trolox

Our *in vitro* plasmid DNA damage assays have conclusively demonstrated that among the tested antioxidants only quercetin was highly effective in protecting plasmid DNA against strand breaks induced by purified rabbit liver P450 reductase catalyzed-bioactivation of idarubicin (Figures 3.27-3.30). Quercetin was found to have higher ability to reduce DNA-damaging effect of idarubicin than its conjugate flavonoid, rutin. While, at a concentration of 50 μM, quercetin was found to protect plasmid DNA by 58% against idarubicin-induced generation of strand breaks, rutin up to 750 μM produced no protective effect. Quercetin at 100 μM concentration almost completely inhibited idarubicin-induced single-strand breaks in plasmid DNA. On the other hand, rutin provided almost complete protection at a very high concentration (2 mM) as compared to quercetin. In agreement with our results, Malgorzata *et al.* (2003) have reported that rutin at 100 μM concentration did not show any sign of protection against cumene hydroperoxide-induced oxidative DNA damage in rat C6 glioma cells, whereas quercetin, at a concentration range of 10-100 μM, was effective in protecting DNA. Also, it was found that rutin, at a concentration of 50 μM, was failed to provide any protection against H_2O_2-induced DNA damage in human lymphocytes, whereas quercetin exhibited protection in a concentration range of 3.1-25 μM (Liu and Zheng, 2002). Contrary to our results, Ündeğer *et al.* (2004) reported that, at a concentration range of 80-820 μM, rutin protected lymphocytes from mitomycin C-induced DNA damage, while at the highest concentrations of 1.64 and 3.28 mM and at the lowest concentration of 20 μM, no protective effect was observed. The same authors have also shown that quercetin provided protection against mitomycin C-induced DNA damage in human lymphocytes in a concentration range of 30 μM–3 mM except at the highest concentration (6 mM) at which no protection was observed (Ündeğer *et al.*, 2004). In our study, the observation that quercetin had greater ability to reduce DNA-damaging effect of idarubicin compared to its conjugate flavonoid rutin, is also consistent with the results of several studies obtained using different methods for evaluation of the antioxidant capacities of various flavonoids (see Noroozi *et al.*, 1998).

Our results demonstrated that trolox was unable to protect plasmid DNA against strand breaks under the same experimental conditions. In agreement with this finding, Anderson *et al.* (1994) reported that trolox had no effect on H_2O_2- and bleomycin-induced DNA damage in human lymphocytes. Also, Melidou *et al.* (2005) have shown that trolox did not exhibit any sign of protection against H_2O_2-induced DNA damage in human lymphocytes using the Comet assay.

The IC_{50} value of quercetin against idarubicin-induced plasmid DNA damage in the presence of P450 reductase was calculated as 43.5μM in our study. On the other hand, IC_{50} values for the other tested antioxidants, naringenin, rutin and resveratrol, were estimated to be in the millimolar range. It has been shown that the position and number of the hydroxyl radicals have an important role for the optimal protective effects of individual flavonoids (Noroozi *et al.*, 1998; Melidou *et al.*, 2005). The presence of two hydroxyl groups in the ortho position or a hydroxyl group and an oxo group at proximal carbon positions have been shown as the main structural requirements for the greater capacity of flavonoids to protect DNA against oxidative damage (Figure 4.2) (Melidou *et al.*, 2005). Since quercetin possesses all these properties and contains 5 hydroxyl groups compared to naringenin and resveratrol (contain 3 hydroxyl groups) (Figure 4.2), our results demonstrating the greater protective effect of quercetin with respect to resveratrol and naringenin in pBR322 plasmid DNA system were in agreement with this finding.

The above plasmid DNA experiments were also repeated using mitomycin C under the same incubation conditions. Since antioxidants except quercetin did not provide effective protection even at high concentrations against single-strand breaks in DNA induced as a consequence of reductive activation of idarubicin by P450 reductase, only the antioxidant capacity of quercetin was tested against mitomycin C-induced DNA damage. Quercetin was found to reduce DNA-damaging effect of mitomycin C in a concentration dependent manner similarly as observed in idarubicin-induced plasmid DNA damage. The IC_{50} value of quercetin against mitomycin C-induced plasmid DNA damage was calculated as 49.8 μM, which was almost same with that observed against idarubicin-induced plasmid DNA damage.

Our finding that quercetin provided effective protection against DNA damage induced by mitomycin C in the presence of P450 reductase is consistent with the results of the studies mentioned above.

The present results emphasized the importance of quercetin, one of the most potent and the most predominant antioxidant present in nature. Although, the exact molecular mechanisms of protection provided by quercetin against idarubicin- and mitomycin C-induced DNA damage is not known with the present results, several mechanisms including chelation of iron (Melidou *et al.*, 2005), direct electron (or hydrogen atom) transfer to ROS-induced radical sites on the DNA (Anderson *et al.*, 2001), and possible involvement of scavenging of toxic oxygen radicals generated in reactions initiated by idarubicin and mitomycin C might have been responsible for the observed effect.

Finally, some practical implications of our findings should be emphasized. The results of the present study suggest that cytochrome P450 reductase may potentially be used as therapeutic agent on their own in cancer treatment strategies. Our results demonstrated the higher ability of NADPH-cytochrome P450 reductase to catalyze the reductive bioactivation of idarubicin and mitomycin C as compared to NADH-cytochrome b5 reductase. Therefore, selective targeting of cancerous cells with purified cytochrome P450 reductase enzyme by some currently used or newly developed delivery methods such as using polymers, liposomes or antibodies (ADEPT, PDEPT, PELT) (Bagshave *et al.*, 1999; Vicent and Duncan, 2006) together with a selective administration of anticancer drugs may thus result in the greater reductive activation of drug molecules in tumour cells. However, our results suggested that selective delivery of NADH-cytochrome b5 reductase enzyme to malignant, transformed cells is likely to be of no therapeutic value in killing these cells due to catalytic inefficiency of this enzyme in the bioactivation process. On the other hand, the present results implicated that the combined administration of cytochrome P450 reductase enzyme together with CYP isozymes selectively to cancerous cells may potentiate the activity of quinone-containing chemotherapy agents including idarubicin and mitomycin C in tumor cells. Further animal and

human studies should be performed in order to clarify these issues. In addition, it needs to be mentioned here that determination of endogenous reductive enzyme levels and profiles in cancerous cells will be of crucial importance for such enzyme-based therapy of cancer. It has been reported that cytochrome P450 reductase activity is generally lower in tumor tissue than the corresponding normal tissue, and correlates with P450 activity (see Rooseboom *et al.*, 2004).

Cytochrome P450 reductase may possibly be used also in other cancer treatment strategies like gene-directed enzyme prodrug therapy (GDEPT) in combination with bioreductive anticancer drugs such as idarubicin, mitomycin C, or some other potential anticancer drugs. For example, in a study by Cowen *et al.* (2003), it was shown that overexpression of cytochrome P450 reductase enzyme in tumor cells by viral delivery of P450 reductase gene led to the sensitization of tumor cells to mitomycin C both *in vitro* and *in vivo*. Also, Jounaidi and Waxman (2000) reported that coexpression of CYP2B6 with cytochrome P450 reductase in tumor cells through transferring P450/P450 reductase genes led to a significant increase in tumor cell cytotoxicity *in vitro* and antitumor activity *in vivo* when P450-activated prodrug cyclophosphamide was administered in combination with the P450 reductase-activated bioreductive prodrug tirapazamine, as compared to the response observed when either drug was administered alone (see also Waxman *et al*., 1999; Roy and Waxman, 2006).

CHAPTER V

CONCLUSION

In summary, in the present study, we demonstrated, for the first time, using *in vitro* plasmid DNA damage assay, that NADPH-cytochrome P450 reductase is capable of effectively reducing idarubicin to DNA-damaging species. The omission of enzyme, NADPH or drug from incubation mixtures did not produce any damage to DNA over plasmid-alone control, which indicates a requirement for an enzymatic process. In order to investigate the mechanism of DNA damage by idarubicin, we employed antioxidant enzymes, SOD and catalase, as well as scavengers of $OH^{\cdot}$ radical**,** DMSO and thiourea. The finding that these antioxidants effectively protected DNA against idarubicin-induced strand breaks strongly suggested that P450 reductase catalyzes the bioreductive activation of idarubicin to redox active metabolites which causes DNA strand breaks under aerobic conditions through generating ROS. The plasmid DNA experiments performed using mitomycin C under the same incubation conditions produced similar results as with idarubicin. Also, in order to characterize and compare the DNA-damaging potentials of idarubicin and mitomycin C, the effects of increasing concentrations of the enzyme or the drug as well as increasing incubation time were studied. The extent of DNA damages by both idarubicin and mitomycin C were found to increase with increasing concentrations of the drug or the enzyme as well as with increasing incubation time. It was shown that both drugs had almost similar DNA-damaging potentials under aerobic conditions. The only marked difference observed was the greater ability of idarubicin versus mitomycin C to induce DNA strand breaks at high drug concentrations. In the present study, we also checked the involvement of microsomal NADH-cytochrome b5 reductase purified from beef liver on the generation of DNA

strand breaks induced by idarubicin and mitomycin C resulting from their reductive activation. Cytochrome b5 reductase was found not to reduce idarubicin to reactive species with the resulting formation of DNA strand breaks in the presence of cofactor NADH, whereas addition of P450 reductase to reaction mixtures in the presence of cofactor NADPH effectively generated strand breaks under the same incubation conditions. It was also found that in the presence of b5 reductase and cofactor NADH, plasmid DNA strand breaks were barely induced by mitomycin C.

In order to assess the roles of these purified enzymes in the reductive activation of idarubicin and mitomycin C exactly, the relative rates of their reduction by the purified P450 reductases and b5 reductase were determined by measuring drug-induced cofactor consumption. It was demonstrated that P450 reductases purified from sheep lung, beef liver and phenobarbital-treated rabbit liver microsomes effectively reduced both idarubicin and mitomycin C. Idarubicin was found to exhibit two-fold higher rate of reduction than mitomycin C by all the P450 reductases, which indicates that idarubicin may be a more potent cytotoxic drug than mitomycin C in terms of the generation of reactive metabolites catalyzed by P450 reductase. On the contrary to P450 reductase, b5 reductase was found not to reduce idarubicin. On the other hand, although b5 reductase was shown to reduce mitomycin C, this activity was hardly measurable and assumed negligible compared to rates of mitomycin C reduction by P450 reductases. Furthermore, in order to determine the contribution of purified rabbit liver CYP2B4, relative to P450 reductase, to the reduction of idarubicin and mitomycin C, the reduction rates of both drugs were measured in reconstituted systems containing P450 reductase and CYP2B4 under both aerobic and anaerobic conditions. The reconstitution experiments with varying amounts of rabbit liver CYP2B4, rabbit liver P450 reductase and lipid DLPC under aerobic conditions revealed that reconstituted CYP2B4 produced about 1.5-fold and 1.4-fold rate enhancement in idarubicin and mitomycin C reduction catalyzed by P450 reductase alone, respectively, as measured by NADPH oxidation at 340 nm. In addition, the reconstitution experiments performed using rabbit liver CYP2B4 and beef liver P450 reductase under anaerobic conditions demonstrated that the relative contribution of reconstituted rabbit liver CYP2B4 to the reduction of both drugs was

almost the same with that observed in reconstituted systems under aerobic conditions using rabbit liver P450 reductase. Under anaerobic conditions, while idarubicin reduction rate was determined by measuring drug-induced NADPH oxidation at 340 nm as in the case of aerobic incubations, mitomycin C reduction rate was determined by measuring the decrease in absorbance at 375 nm based on the disappearance of the quinone moiety of the drug.

In the present study, the potential protective effects of some antioxidants against DNA-damaging effects of idarubicin and mitomycin C resulting from their reductive activation by P450 reductase were also evaluated. The results of the plasmid DNA experiments demonstrated that among the tested dietary antioxidants, quercetin, rutin, naringenin, resveratrol and trolox, only quercetin was found to be highly potent in preventing DNA damage by idarubicin. The ability of quercetin to prevent mitomycin C-induced DNA damage was found to be comparable with that of DNA damage induced by idarubicin.

The results of this study may have some practical implications concerning the potential use of cytochrome P450 reductase as therapeutic agent on their own in cancer treatment strategies (or their genes in GDEPT strategy) in combination with bioreductive anticancer drugs like idarubicin and mitomycin C. Furthermore, the present results led to a conclusion that bioreduction of idarubicin by NADPH-cytochrome P450 reductase resulting in the formation of DNA damage is considered as one of the mechanisms contributing to the antitumor effect of idarubicin. The present results also emphasized the importance of quercetin, one of the most potent and the most predominant antioxidant present in nature.

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PUBLICATIONS

1. MSc Thesis

Çelik, H (2002) Biochemical and Immunological Characterization of Beef Liver NADPH-Cytochrome P450 Reductase.

2. Articles in Science Citation Index

Arinç, E., and Çelik, H. (2002) Biochemical characteristics of purified beef liver NADPH–cytochrome P450 reductase. *J. Biochem. Mol. Toxicol.* 16; 6: 286-297.

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3. Congress and Workshop Presentations and Abstracts

National:

Çelik, H., and Arinç, E. (2002) Multifunctional enzyme cytochrome P450 reductase: its purification, biochemical and immunological characterization. In *Abstracts from the 17th National Biochemistry Congress*, pp. 233-234, June 24-27, Ankara, Turkey.

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