HYDROGEN PEROXIDE/PEROXIDASE-MEDIATED CO-OXIDATION (A POTENTIAL PATHWAY FOR THE ACTIVATION OF CARCINOGENIC AND TOXIC XENOBIOTICS IN THE CELL)

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HYDROGEN PEROXIDE/PEROXIDASE-MEDIATED CO-OXIDATION (A POTENTIAL PATHWAY FOR THE ACTIVATION OF CARCINOGENIC AND TOXIC XENOBIOTICS IN THE CELL)

BY

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ABSTRACT

Products of the metabolism of arylamine carcinogens ^{14}C-benzidine, N-^{14}C-methylaminoazobenzene and ^{14}C-aminofluorene and ^{14}C-phenol bind irreversibly to nuclear DNA in intact polymorphonuclear leukocytes activated by the tumor promoter phorbol myristate acetate. Little binding occurred under anaerobic conditions indicating that the binding was dependent upon oxygen. Sulfhydryl inhibitors p-chloromercuribenzoate and N-ethylmaleimide inhibited oxygen uptake as well as decreased the binding of arylamine carcinogens and phenol to DNA in leukocytes activated by tumor promoter. Phenolic antioxidants butylated hydroxyanisole or nordihydroguaiaretic acid also had a similar inhibitory effect. Azide increased both the oxygen uptake and binding, presumably as a result of intracellular catalase inhibition. However, higher concentrations of azide and cyanide inhibited binding without affecting the oxygen uptake indicating that the binding is catalysed by myeloperoxidase. Granules isolated from the activated leukocytes catalysed a cyanide-sensitive binding of benzidine to calf-thymus DNA in the presence of hydrogen peroxide.

Products of the metabolism of ^{14}C-phenol also bind irreversibly to calf-thymus DNA in the presence of horseradish peroxidase and hydrogen peroxide. Binding also occurred to the homopolyribonucleotides polyriboadenylic acid, polyriboguanylic acid, polyribocytidylic acid and polyribouridylic acid, suggesting that the binding is relatively non-specific with respect to nucleotide bases.

DNA binding of ^{14}C-phenol oxidized with horseradish peroxidase/hydrogen peroxide was prevented by glutathione and N-acetylcysteine and the mechanism was shown to involve reduction of the activated phenol intermediates and the formation of conjugates with glutathione and N-acetylcysteine. Ascorbate prevented binding by reduction of the activated phenol intermediates.

Phenol oxidation by horseradish peroxidase/hydrogen peroxide initially results in p,p′-biphenol and o,o′-biphenol formation and subsequently results in polymer formation. o,o′-Biphenol is the major product formed but is rapidly oxidized to polymer, particularly in the presence of phenol. Phenol oxidation with bone marrow homogenate and hydrogen peroxide also results in o,o′-biphenol as the major product. p,p′-Biphenol is very rapidly oxidized to p,p′-biphenoquinone which can also be involved in polymer formation.

Enzymic or acid-catalyzed hydrolysis of DNA releases the bound products. o,o′-Biphenol, but not p,p′-biphenol binds to DNA following peroxidase-catalysed oxidation. Enzymic hydrolysis of the DNA, to which the products of the oxidation of o,o′-biphenol had bound, resulted in the release of products derived from the biphenol.

Peroxidase also catalysed the formation of active oxygen species in the presence of NADH or GSH and traces of hydrogen peroxide and arylamin

phenolic substrates. Some oxygen activation occurred with some arylamines even in the absence of NADH or GSH. Oxygen consumption was proportional to the NADH oxidized or GSSG formed. Approximately 0.80 and 0.40 moles of oxygen were consumed per mol of NADH or GSH oxidized respectively. The requirement for trace amounts of hydrogen peroxide and arylamine or phenolic substrates suggests that the redox cycling resulted in hydrogen peroxide formation. It is proposed that initially formed phenoxy radicals or arylamine cation radicals oxidize NADH or GSH to radicals which react with oxygen to form superoxide radicals and hydrogen peroxide.

Non-carcinogenic arylamines mesidine, aniline and 1-naphthylamine were poor at initiating redox cycling in the presence of NADH or GSH, whereas carcinogenic 2-naphthylamine, 4-aminobiphenyl, methylaminoazobenzene, N,N'-dimethyl p-toluidine or 2-aminofluorene were highly effective in initiating redox cycling in this system with resultant hydrogen peroxide formation.

Only phenol and o,o'-biphenol were effective among phenolic substrates in redox cycling with resultant hydrogen peroxide formation. p,p'-Biphenol, hydroquinone and catechol were ineffective in activating oxygen. p,p'-Biphenol formed a glutathione conjugate when oxidized with horseradish peroxidase and hydrogen peroxide in the presence of glutathione. The major conjugate formed was identified as 3-S-(glutathion-yl)-p,p'-biphenol using FAB-Mass spectroscopy and NMR spectroscopy.

Acknowledgements

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List of Abbreviations

AB	=	Aminoazobenzene.
4-AB	=	4-Aminobiphenyl.
2-AF	=	2-Aminofluorene.
$ArNH_2$	=	Arylamine substrate.
ATP	=	Adenosine 5'-triphosphate.
BHA	=	Butylated hydroxyanisole.
BSA	=	Bovine serum albumin.
Cetavlon	=	Cetyl trimethylammonium bromide.
CHP	=	Cumene hydroperoxide.
Cpm	=	Counts per minute.
DAB	=	N,N'-Dimethyl-4-aminoazobenzene.
DMPT	=	N,N'-Dimethyl-p-toluidine.
DMSO	=	Dimethyl sulfoxide.
DNA	=	Deoxyribonucleic acid.
DNAase	=	Deoxyribonuclease.
DNFB	=	Dinitrofluorobenzene.
EDTA	=	Ethylenediamine tetra acetate.

FAB	=	Fast atom bombardment.
g	=	Gram.
GSH	=	Glutathione.
GSSG	=	Glutathione disulfide.
HCl	=	Hydrochloric acid.
H.p.l.c.	=	High pressure liquid chromatography.
HRP	=	Horseradish peroxidase.
LP	=	Lactoperoxidase.
MPO	=	Myeloperoxidase.
mRNA	=	Messenger ribonucleic acid.
1-NA	=	1-Naphthylamine.
2-NA	=	2-Naphthylamine.
NADH	=	Reduced nicotinamide adenine dinucleotide.
NADPH	=	Reduced nicotinamide adenine dinucleotide phosphate.
NaCl	=	Sodium chloride.
$NaHCO_3$	=	Sodium bicarbonate.

NaOH	=	Sodium hydroxide.
NDGA	=	Nordihydroguaiaretic acid.
NEM	=	N-Ethylmaleimide.
NMR	=	Nuclear magnetic resonance.
O_2	=	Oxygen.
$\dot{O}_2$	=	Superoxide radical.
OH	=	Hydroxyl radical.
PCMB	=	p-Chloromercuribenzoate.
PMA	=	Phorbol-12-myristate-13-acetate.
PMN's	=	Polymorphonuclear leukocytes.
P_2O_5	=	Phosphorous pentoxide.
PPO	=	2,5 - Diphenyloxazole.
Poly A	=	Polyriboadenylic acid.
Poly C	=	Polyribocytidylic acid.
Poly G	=	Polyriboguanylic acid.
Poly U	=	Polyribouridylic acid.
RNA	=	Ribonucleic acid.
RNAase	=	Ribonuclease.

SDS	=	Sodium dodecyl sulphate.
SOD	=	Superoxide dismutase.
TCA	=	Trichloroacetic acid.
T.l.c.	=	Thin layer chromatography.
TMB	=	3,3',5,5'-Tetramethylbenzidine.
Tris	=	Tris (hydroxymethyl) amiho methane.
t-RNA	=	Transfer ribonucleic acid.
uv	=	Ultraviolet.

OVERVIEW

Much of the emphasis on the biological oxidation mechanisms of chemical carcinogens has been laid on two-electron oxidations of carcinogens to form reactive electrophiles which subsequently interact with cellular nucleophiles. Michaelis (1939) in his theory had generalized that oxidation-reduction in non-biological chemical systems would compulsorily proceed by one-electron transfer mechanisms, which involves the formation of free-radicals. However, as most free-radicals are very unstable, and are often exceedingly reactive, it was proposed that in biological oxidation-reduction, the transfer of reducing equivalents takes place essentially by two-electron transfer so that it controls the potentially destructive effects of free-radicals, and thus conserves the biological function in the respiring cell. Thus, living cells have developed enzymes which are compartmentalized in a manner such that the free-energy of oxidation is efficiently recovered and the intermediates are prevented from reacting randomly with cellular components.

Free-radical formation from chemicals by enzymic action has been explored by many researchers. This mechanism is often taken as a defensive mechanism of the cell to combat against infective bacteria and viruses. Rapid industrialization of modern society has led to the accumulation of newer chemicals in the environment, the reactive metabolic products of which cause extensive damage to cellular macromolecules. Despite vigorous efforts to understand the formation of reactive metabolites (either electro-

philes or free-radicals) by enzymic action, the biological function of such an enzyme system remains elusive. It should be noted that the ubiquitous nature and the persistence during evolution of these enzymes in all living systems suggest that there was enough selective pressure to give obvious advantage to the organism so as to maintain such a metabolic pathway. The formation of reactive metabolites, is therefore a consequence of an unknown biological function of these enzymes, and hence it might be improper to view the action of these enzymes in the current context and extrapolate to its function and evolution. However, the chemical nature of the reactive intermediates which would pose threat to the living organisms and the mechanisms by which the reactive intermediates cause potential damage to the cells are not understood clearly. The majority of the evidence to date suggests that electrophiles (formed by a two-electron oxidation mechanism of a variety of xenobiotics) are the intermediates that cause cellular damage. It is possible that electrophiles formed through two-electron oxidation mechanisms that would normally exist in cells may not be of potential threat to living beings. There is not much literature existing, however, on the formation of the free-radicals which are sometimes more reactive than electrophiles. This thesis is essentially directed towards the biological reactivity of free-radicals and the possible mechanisms by which they can be generated in a cell. Results are presented on the biochemical reactions of free-radicals (generated by a peroxidase/H_2O_2-mediated oxidation of

xenobiotics) with a variety of cellular molecules and the possible role of peroxidase enzyme systems in the initiation of chemical carcinogenesis is discussed.

CHAPTER 1
INTRODUCTION: A REVIEW

(Xenobiotic : denotes all foreign chemicals not found normally in the body)

1.1) INTRODUCTION :

The perception of cancer as a disease primarily related to the environment has been progressively strengthened during the past two decades. Diet, carcinogenic chemicals, radiation and viruses are among the major factors that appear to be involved. Of these, chemicals as carcinogens have been receiving increasing attention as having the greatest role in the genesis of cancer (Doll and Peto, 1981).

The majority of the chemical carcinogens are pre-carcinogens, which must be converted (metabolized), *in vivo*, to ultimate carcinogens or the final reactive forms of these agents (Miller and Miller, 1985). It is believed that covalent reaction of these ultimate carcinogens with specific targets in the cell results in the initiation of carcinogenesis (Miller and Miller, 1985). The nature of these targets still remains unknown but, reactions of chemical carcinogens with DNA continue to receive much attention. Some investigators, suggest that RNA and protein adducts should also be considered (Gronow, 1980). Evidence against genomic DNA as the target in chemical carcinogenesis has been presented (Cairns, 1981). On the other hand, binding of

chemical carcinogens to DNA *in vivo* correlates with carcinogenesis, but not with binding of chemical carcinogens to protein or RNA (Brésnick and Eastman, 1982).

The widely accepted theory for the initiation of carcinogenesis by chemicals, as proposed by the Miller and Miller (1985), is that carcinogens elicit their activity after conversion to electrophilic metabolites which interact with a nucleophilic region of a macromolecule (Figure 1.1). DNA is considered to be the critical nucleophilic target associated with the induction of neoplasia (Miller and Miller, 1985). *An electrophile is a molecule that accepts an electron pair. A nucleophile is a molecule which donates an electron pair to an electrophile.* Electrophiles react nonenzymically to form covalent bonds through the sharing of electron pairs with nucleophilic atoms (Miller and Miller, 1985).

Studies on the metabolic activation of chemical carcinogens have primarily concentrated on two classes of chemicals, a) the polycyclic aromatic hydrocarbons and b) the aromatic amines. Figure 1.2 shows the structures of some representative chemicals of these two classes.

1.2) ENZYMOLOGY FOR THE INDUCTION OF CHEMICAL CARCINOGENESIS

FIGURE 1.1

A CURRENT VIEW OF THE CELLULAR EVENTS IN THE INDUCTION OF CANCER BY A CHEMICAL

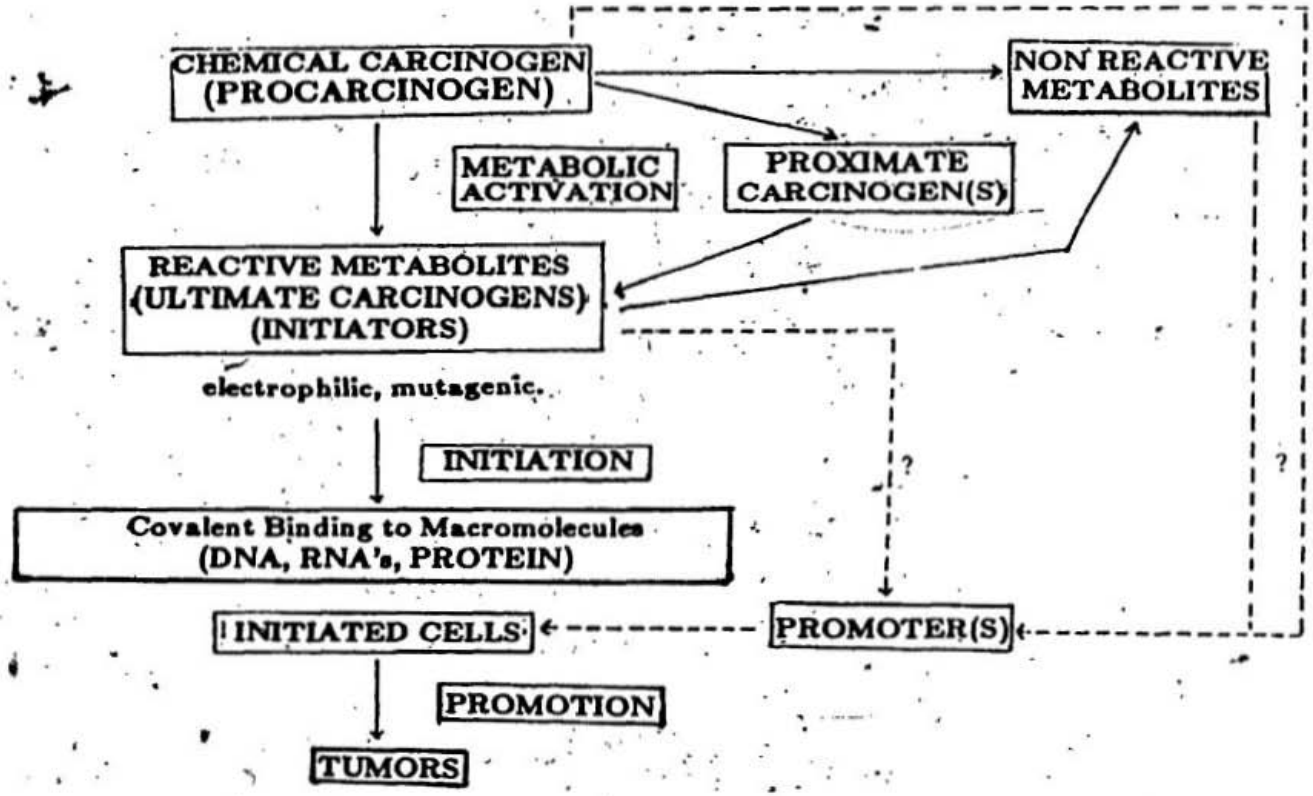
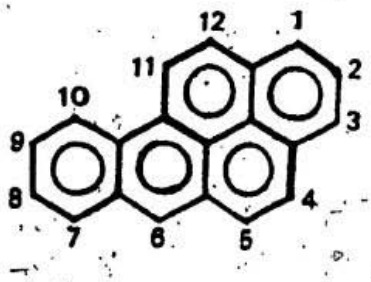


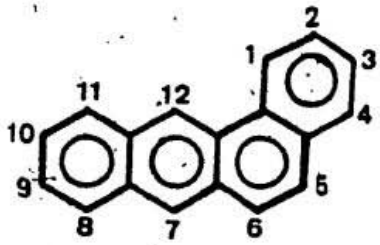
FIGURE 1.2

STRUCTURES OF SOME REPRESENTATIVE CARCINOGENIC CHEMICALS

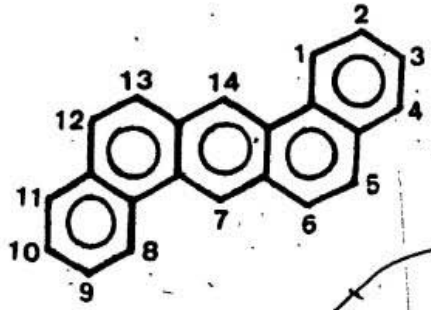
A) POLYCYCLIC AROMATIC HYDROCARBONS:



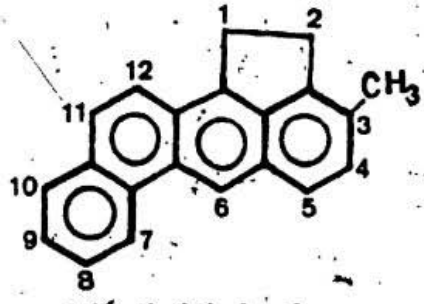
Benzo(a)pyrene



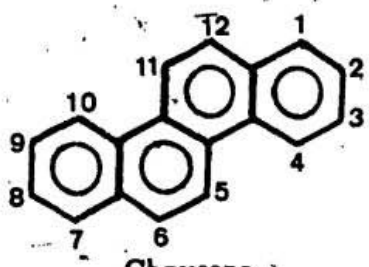
Benzo(a)anthracene



Dibenzo(a,h)anthracene

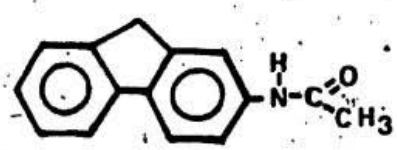


3-Methylcholanthrene

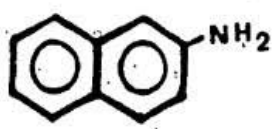


Chrysene

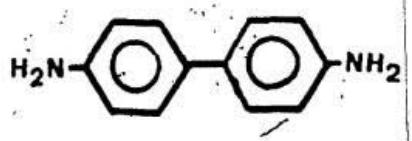
B) AROMATIC AMINES:



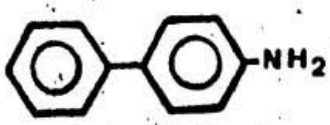
Acetylaminofluorene



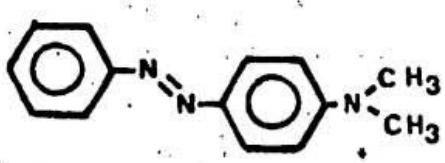
2-Naphthylamine



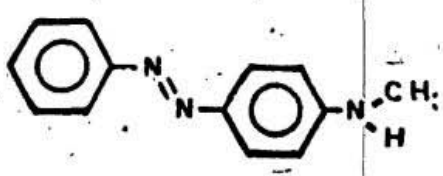
Benzidine



4-Aminobiphenyl



Dimethylaminoazobenzene



Methylaminoazobenzene

The metabolic activation of the carcinogens is believed to occur within the endoplasmic reticulum and the nucleus of mammalian cells. A family of cytochrome P-450 isoenzymes is implicated in the initial oxygenation as part of a mixed-function oxidase activity. In the activation of polycyclic aromatic hydrocarbons, epoxide hydrase is also required whereas, in the activation of arylamine carcinogens various cytosolic or endoplasmic reticular transferases are involved in the formation of the electrophile. The activation by the mixed-function oxidase is considered to be a two-electron oxidation of the polycyclic aromatic hydrocarbon to form an epoxide or of the arylamine to form an N-hydroxy arylamine.

In addition to mixed-function oxidases, prostaglandin H synthase has also been shown to activate a wide variety of xenobiotics during prostaglandin biosynthesis from arachidonic acid (Eling et al., 1983; Krauss and Eling, 1984). This activation system is considered to be an alternative or additional means of reactive metabolite production especially in extrahepatic target tissues, where cytochrome P-450 amounts are very low (Eling et al., 1983; Krauss and Eling, 1984).

1.2.1) CYTOCHROME P-450-CATALYZED ACTIVATION OF CARCINOGENIC CHEMICALS :

Microsomes from various tissues contain an enzyme system capable of carrying out a large number of xenobiotic-metabolizing processes for which the presence of NADPH and molecular oxygen are required (Burke and Orrenius, 1982). This enzyme system contains three components: cytochrome P-450, NADPH-cytochrome P-450 reductase and phosphatidyl choline in which cytochrome P-450 acts as a terminal electron acceptor (Bjorkhem, 1982). This enzyme system can carry out a wide variety of reactions (O'Brien, 1982).

i) Mechanism of substrate hydroxylation by NADPH-dependent cytochrome P-450 : Studies in many laboratories on the mechanistic details of cytochrome P-450-catalyzed reactions, as well as on the behaviour of other hemoproteins and model systems, have led to the scheme proposed as shown in reaction 3 plus reaction 5 of Figure 1.3 (O'Brien, 1982) for substrate hydroxylation by this enzyme system. As indicated in the scheme, initial reduction of ferric P-450 to the ferrous state, allows oxygen binding. A second reduction of the ferrous oxy complex results in splitting of the oxygen-oxygen bond, one atom being lost in water. The other oxygen atom, presumably now an 'activated oxygen' (compound I) is inserted into a carbon-hydrogen bond of the substrate (reaction 5) to produce the corresponding alcohol, which is then released with regeneration of the ferric resting state of the enzyme and completion of the catalytic cycle. During

Figure 1.3

A scheme on the mechanism of reactions carried out by cytochrome P-450

(Adapted from O'Brien, 1982)

Donor can be : (i) reduced cytochrome b_5 or NADPH:cytochrome c reductase; (ii) tetramethylphenylenediamine, dimethylphenylenediamine, diaminobenzidine etc.; (iii) dimethylaniline or aminopyrine etc.; (iv) ROOH (Primary or secondary or tertiary hydroperoxides); (v) unsaturated lipids ; (vi) various antioxidants.

S substrate can be amines, drugs, steroids, carcinogens, antioxidants, alcohols.

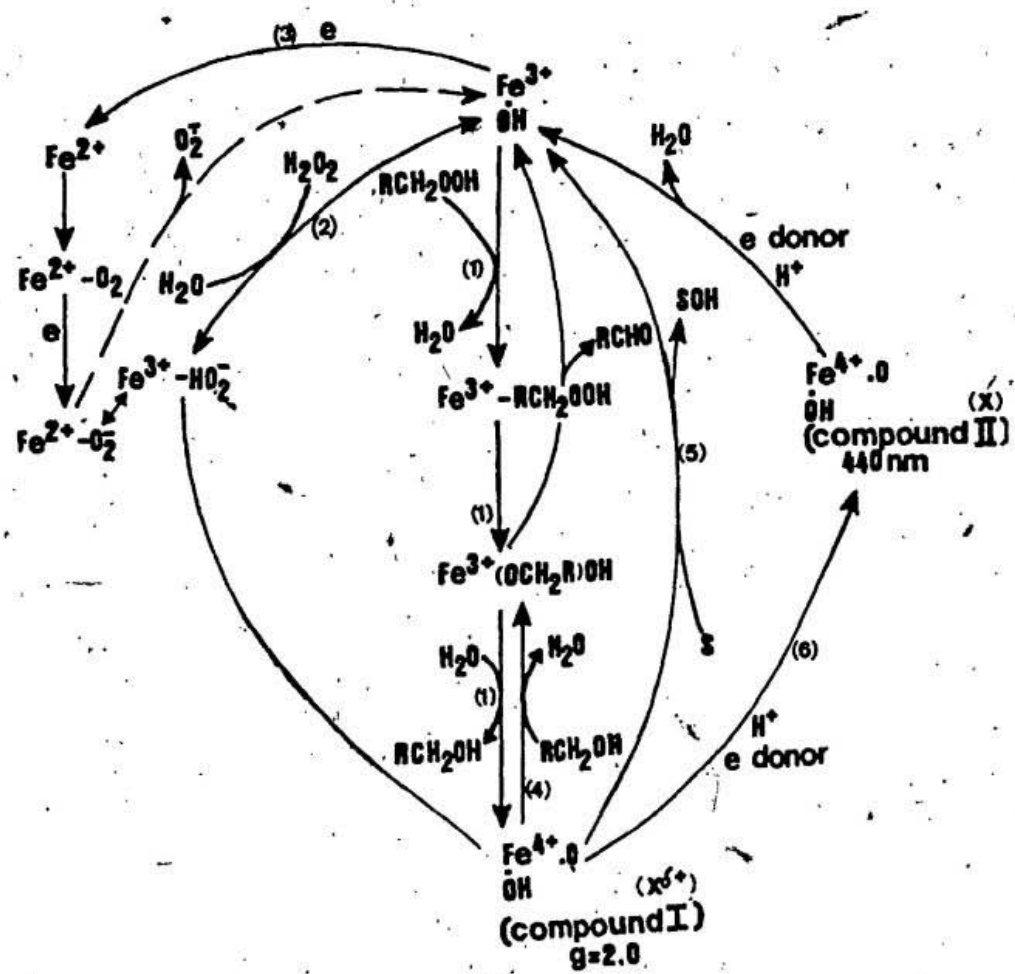
RCHO : Aldehyde product from alcohol oxidation.

FIGURE 1.3

A SCHEME ON THE MECHANISM OF THE REACTIONS

CARRIED OUT BY CYTOCHROME P-450

(taken from O'Brien, 1982)



the second reduction some of the ferrous oxygen complex may dissociate to form superoxy radical (broken line in the Figure 1.3) and native ferric enzyme. The reduction of the ferric enzyme and ferrous oxy complex by NADPH appears to occur *via* NADPH cytochrome P-450 reductase (Bjorkhem, 1982). According to White and Coon (1980), the binding of substrate to the ferric P-450 is the first step in the hydroxylation mechanism, followed by reduction of substrate bound ferric P-450 to ferrous state.

ii) Peroxide-supported hydroxylation reactions : Cytochrome P-450 can also carry out all the reactions mentioned above in the presence of the tertiary hydroperoxides. Cumene hydroperoxide, in particular, is very effective (O'Brien, 1982). These tertiary hydroperoxides substitute for NADPH, NADPH-cytochrome P-450 reductase and molecular oxygen in the mixed-function oxidase reaction for all cytochrome P-450 drug substrates (O'Brien, 1982). The mechanism of peroxide supported hydroxylation is presumed to involve, first, the formation of an enzyme-peroxide complex (reaction 1 in Figure 1.3). This complex may rearrange by an outer sphere electron transfer mechanism. Some of this complex may be dissociated to the corresponding aldehyde and the original enzyme. The complex may also be hydrated to compound I, the 'activated oxygen' species releasing the alcohol. Note that the same 'activated oxygen' species is formed in the NADPH-dependent mixed-function oxidase system. Subsequent steps for

substrate hydroxylation are the same as in NADPH-dependent substrate hydroxylation. In addition, O'Brien (1982) suggested that the hydroperoxide-catalyzed alcohol oxidation to an aldehyde may be explained by a reversal of the reactions starting from compound I (Reaction 4). Alternatively, hydroxylation of the alcohol by the compound I, and rearrangement of the hydroxylated product to an aldehyde may also occur. On the other hand, the hydroperoxide-catalyzed oxidation of hydrogen or electron donors, unsaturated lipids or antioxidants is hypothesized to involve the protein or porphyrin free radical ($X^{\delta}+$) of compound I and the ferryl iron of compound II (Reaction 6). In competition with these donors, hydroperoxide can also convert compound I to compound II and the resulting peroxy radicals can also oxidize the donors. In the absence of these donors, cytochrome P-450 destruction readily occurs as a result of the peroxy or protein or porphyrin radicals formed. Reaction 6 in figure 1.3 is thought to decrease during the NADPH-dependent mixed-function oxidase-catalyzed oxidation, presumably by reduction of the radicals by NADPH.

In addition hydrogen peroxide can also support some substrate hydroxylation reactions (O'Brien, 1982). The proposed reaction mechanism is shown in reaction 2 of Figure 1.3.

1.2.1A) Activation of polycyclic aromatic hydrocarbons : - Several excellent reviews have been published recently on the mechanism of

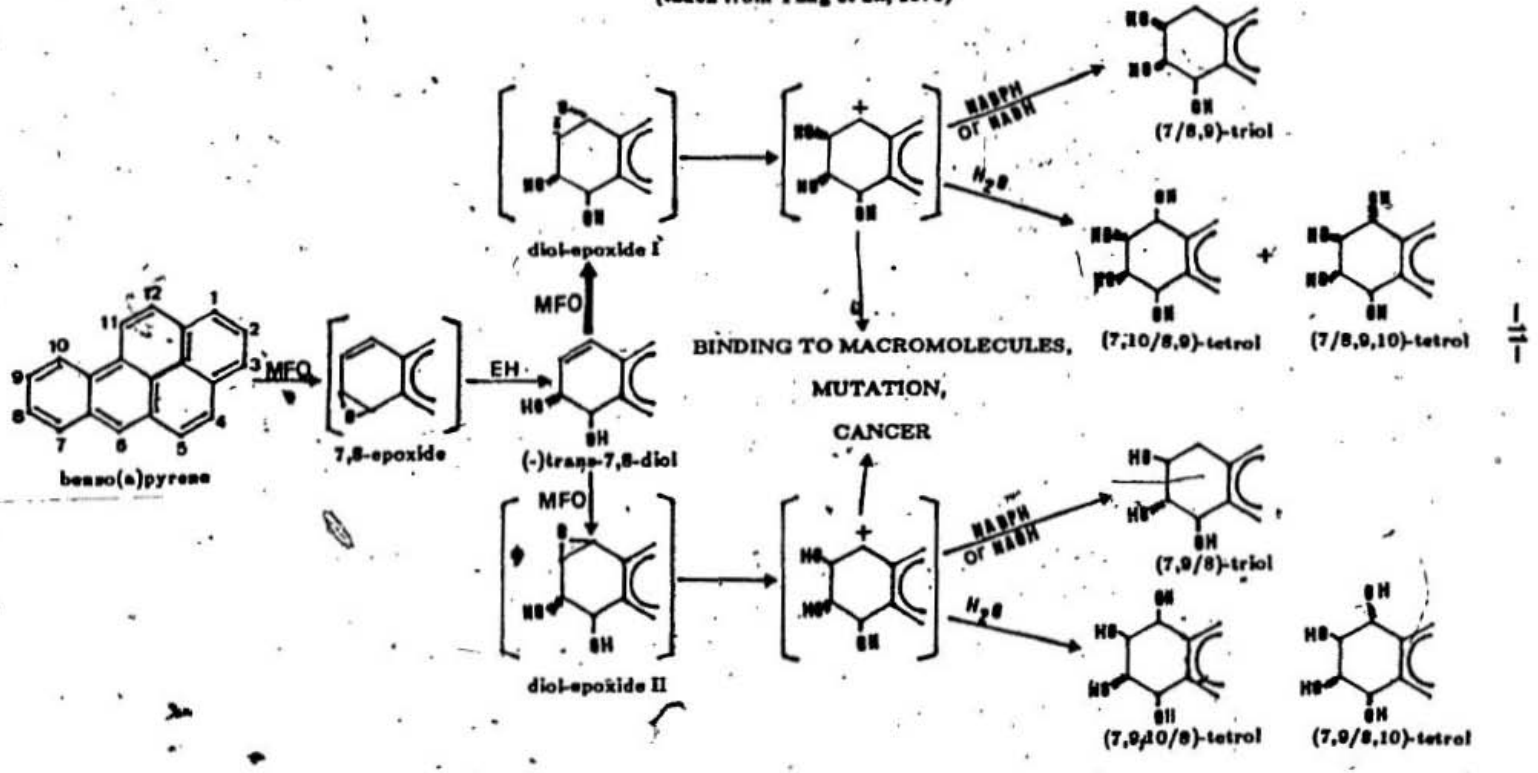
bioactivation of polycyclic aromatic hydrocarbons (Cavalieri and Rogan, 1984; Conney, 1982; Gelboin, 1980; Pelkonen and Nebert, 1982; Phillips, 1983). Benzo(a)pyrene (BP) has been the most well studied representative of polycyclic aromatic hydrocarbons. Activation of BP (Figure 1.4) first requires oxygenation across the 7 and 8 positions of the BP by the mixed-function oxidase to form (+)-BP-7,8-oxide. This epoxide is thought to rearrange non-enzymatically to phenol, and is converted by the epoxide hydrase to an optically pure (-)-*trans*-BP-7,8-dihydrodiol, and the oxygen of the 7-hydroxyl is derived from the molecular oxygen. The (-)-*trans*-BP-7,8-dihydrodiol is further oxygenated by the mixed-function oxidase to form two diol epoxides: the predominant oxygenation is at a 9,10-double bond at a *trans* position to the 7-hydroxyl, forming 7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (*anti* diol epoxide). The (-)-trans-BP-7,8-dihydrodiol, also oxygenated to a smaller extent at the site of 9,10-double bond, which is *cis* to the 7-hydroxyl that forms 7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (*syn* diol epoxide).

Both the diol epoxides form C-10 electrophilic *carbonium ion* intermediates that are responsible for their reactivity toward cellular nucleophiles (Phillips, 1983). The preferred oxygenation site *in vitro* in the formation of *anti* and *syn* diol epoxides appear to depend on the form of mixed-function oxidase catalyzing the reaction (Phillips, 1983); however, the *anti*

FIGURE 1.4

MECHANISM OF ENZYMATIC ACTIVATION OF BP TO BP-7,8-DIOL-9,10-EPOXIDES

(taken from Yang et al., 1976)



diol epoxide is the major isomer formed *in vivo* (Phillips, 1983). The *anti* isomer also appears to be more carcinogenic than the *syn* isomer (Phillips, 1983).

Adducts formed in tissues or cells treated with BP are derived predominantly from the *anti* diol epoxide, with the minor involvement of the *syn* diol epoxide in some cases (Phillips, 1983).

1.2.1B) Activation of aromatic amine carcinogens: -Mechanisms of metabolic activation of aromatic amine carcinogens have also been reviewed (King, 1982; Schut and Costanguay, 1984; O'Brien, 1984). Much evidence suggests that the carcinogenicity of aromatic amines depends on their conversion to N-hydroxy metabolites, as proximate carcinogens, by the liver cytochrome P-450 monooxygenase system (Bresnick and Eastman, 1982; King, 1982). Further metabolism to *activated esters* by sulfotransferases, N,O-acyltransferases or seryltransferases is often required (Bresnick and Eastman, 1982). However, the lack of transferases in non-hepatic target tissues (Irving, Janss and Russell, 1971; Oglesby, Flammang, Tullis and Kadlubar, 1981) has made this pathway less attractive (O'Brien, 1984).

Recent studies suggested that the electrophilic *nitrenium ions* formed on protonation of N-hydroxy arylamines may be the ultimate carcinogens

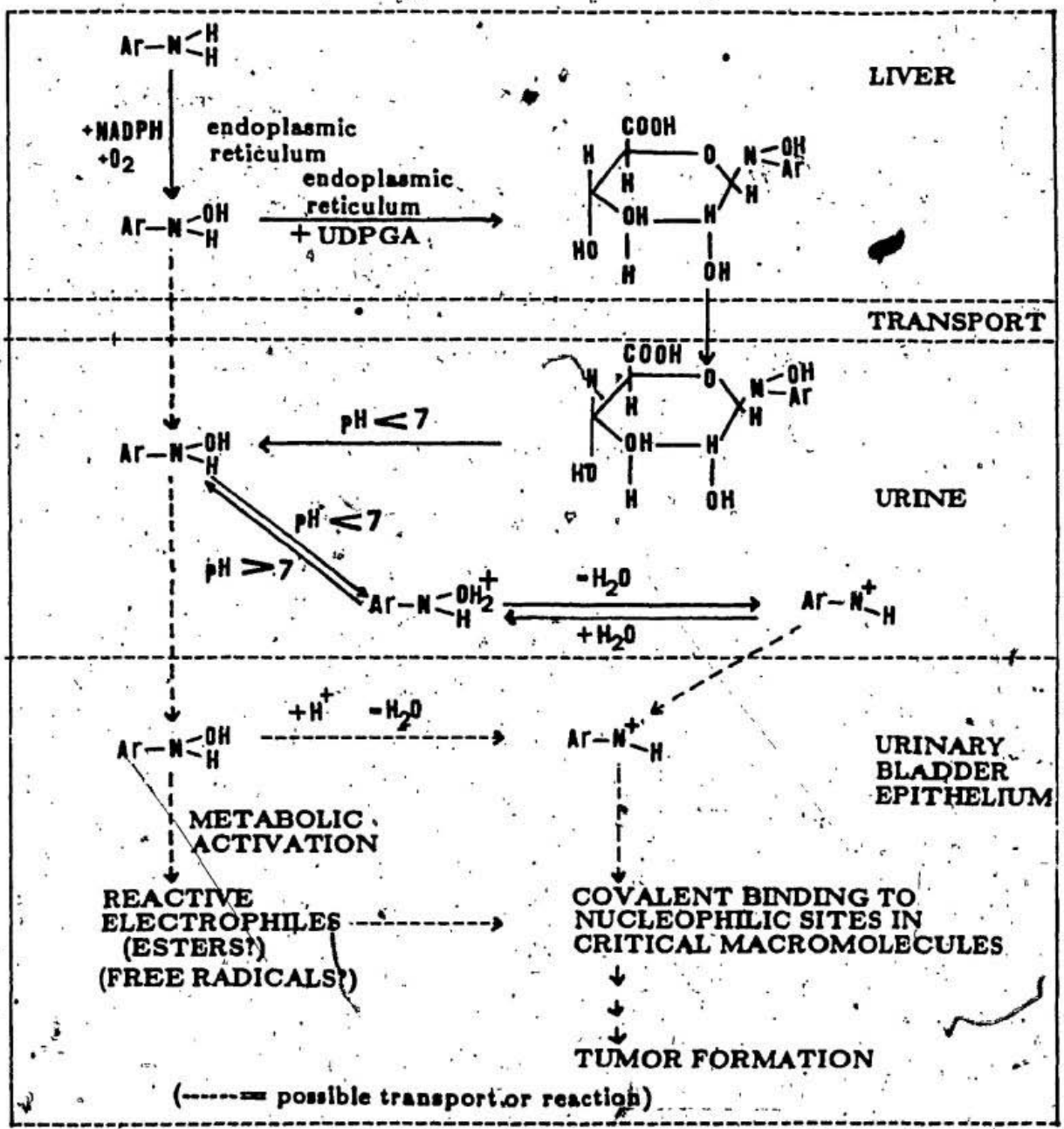
for the induction of urinary bladder tumors in dogs or humans (Bresnick and Eastman, 1982) (Figure 1.5). The *activated esters* can also form *nitrenium ions* (Bresnick and Eastman, 1982). N-hydroxylation is considered to be an obligatory event in this activation scheme. Kadlubar et al. have shown that hydroxylamines are N-glucuronidated by the hepatic endoplasmic reticulum (Kadlubar, Miller and Miller, 1977). They proposed that the further transport of these N-glucuronides to urinary bladder, the target organ, where hydrolysis of N-glucuronides by acidic urine results in the formation of hydroxylamines for the reaction with DNA (Kadlubar, Miller and Miller, 1977). Radomski et al. (1977) characterized the N-glucuronide of N-hydroxy-4-aminobiphenyl as a urinary metabolite in the dog.

The major DNA adducts isolated from the livers of animals treated with the arylamine carcinogens are formed by the linkage of the aromatic amine nitrogen to C-8 of guanine (Bresnick and Eastman, 1982; King 1982; Beland et al., 1983). Minor adducts are formed with other purine bases and in some cases, adducts formed from the N-acetyl derivatives of the amines are also observed (Bresnick and Eastman, 1982; King, 1982; Beland et al., 1983). The DNA adducts formed by aromatic amines in the urinary bladder are not well characterized.

1.2.1C) Cytochrome P-450 in drug and chemical toxicity: - The formation of electrophilic metabolites, by initial activation through

FIGURE 1.5

METABOLIC ACTIVATION OF ARYLAMINE CARCINOGENS



Formation and transport of possible proximate and ultimate carcinogenic metabolites of arylamines for the induction of urinary bladder cancer.

Ar : aryl substituent., UDPGA : uridine diphosphoglucuronic acid

cytochrome P-450 monooxygenases, also appears to be involved in the development of toxicities other than carcinogenesis. For example, the widely used analgesic drug acetaminophen causes hepatic necrosis in high doses (Davidson and Eastham, 1966) and can induce renal damage, even in moderate doses (Mitchell et al., 1977). It has been proposed that the activation of the drug to an electrophilic N-acetyl-p-benzoquinonimine and its binding to protein as the possible pathway for the induction of hepatic necrosis (Mohandas et al., 1981; West et al., 1984). Bromobenzene and 4-ipomeanol also require metabolic activation by cytochrome P-450 to form electrophilic metabolites (Boyd, 1980a and b).

1.2.1D) Cytochrome P-450-catalyzed one-electron oxidation :- An extensive body of literature shows the importance of cytochrome P-450 monooxygenases in the metabolic activation of chemicals to reactive electrophiles (Bresnick and Eastman, 1982; Conney, 1982; Sato and Omura (eds.), 1978). Cytochrome P-450 is also known to catalyze the formation of free-radicals, by a one-electron oxidation, only to a limited extent (reviewed by O'Brien, 1984).

i) Free radical formation during cytochrome P-450 peroxidase function :- Besides functioning as a monooxygenase, cytochrome P-450 also acts as a peroxidase (reviewed by O'Brien, 1982). Tertiary hydroperoxides, particularly cumene hydroperoxide, can substitute for NADPH, NADPH-

cytochrome P-450 reductase and molecular oxygen in the mixed-function oxidase reaction for all cytochrome P-450 drug substrates (reviewed by O'Brien, 1982). This hydroperoxide system was shown to catalyze mainly the normal two-electron oxidations like epoxidation, N-hydroxylation and O-dealkylation (reviewed by O'Brien, 1982). However, a one-electron oxidation to free-radicals occurs with phenylenediamines and aminophenols. Thus, N,N,N',N'-tetramethylphenylenediamine is oxidized to the stable Wurster's blue cation-radical. Most unacetylated benzidines are oxidized by a one-electron oxidation pathway to diimines rather than to the N-hydroxy derivatives with either the mixed-function oxidase activity or peroxidase activity (O'Brien, 1984). N-alkyl compounds readily N-demethylate via a one-electron oxidation (O'Brien, 1984). Some violene cation-radical is formed in the N-demethylation of aminopyrene by cytochrome P-450 and cumene hydroperoxide (Griffin et al., 1980). However, the significance of this reaction has been questioned (O'Brien, 1984).

Benzo(a)pyrene is also oxidized by phenobarbital-induced microsomes/cumene hydroperoxide to a much higher level of quinones than is formed by the mixed-function mixed oxidase (Capdevilla, Estabrook and Prough, 1980) as a result of enhanced one-electron oxidation reaction to the radical cation and further oxidation of the phenols (formed from the epoxides) to quinones. The prostaglandin H synthase/arachidonate system also

appears to form exclusively quinones from BP (Marnett, Reed and Johnson, 1977; Reed and Marnett, 1979). The mechanism of this reaction will be discussed later. Oxidation by microsomes/H_2O_2 forms products similar to those formed with the mixed-function oxidase activity (Renneberg et al., 1981) probably because the phenols are not further oxidized in this system.

ii) Free radical formation by cytochrome P-450 during mixed-function oxidase function :- Cytochrome P-450 as a mixed-function oxidase has also been shown to catalyze the formation of free radicals by one-electron oxidation. Thus, direct N-oxidation of N-hydroxycocaine was shown to be catalyzed by microsomal mixed-function oxidase (Rauckman, Rosen and Cavaguaro, 1982). N-Hydroxy-2-naphthylamine and N-hydroxyacetylaminofluorene are also oxidized to nitroxyl radicals by microsomal mixed-function oxidase (Nagata et al., 1982; Nakayama et al., 1982). Alkane hydroxylation by mixed-function oxidase proceeds via a free-radical oxidation mechanism (Groves, Akinbote and Ovaria, 1980; White and Coon, 1980). The electrophilic N-acetyl-p-benzoquinonimine (ultimate reactive metabolite of acetaminophen) formation was also proposed to proceed via a phenoxy radical (West et al., 1984).

iii) Superoxide-mediated oxidation :- The mixed-function oxidase can also result in superoxide formation, probably as a result of dissociation of the oxy-cytochrome P-450 complex (Kuthan, Ullrich and Estabrook, 1982).

The released superoxide has been shown to oxidize the hydroxylamine TEMPO (2,2,6,6-tetramethyl-4-piperidone-N-oxyl) to a nitroxyl radical. The mixed-function oxidase-catalyzed oxidation of catechol (Dybing et al., 1976), methyl-DOPA (Dybing et al., 1976), 2-hydroxyestradiol (Nelson et al., 1976), or epinephrine (Dybing et al., 1976) is mediated by superoxide and results in protein binding. Binding of 2,2'-dichlorobiphenyl to microsomes also seems to be mediated by superoxide (Hesse, Mezger and Wolff, 1978). Oxidation of alcohol which is catalyzed by microsomal mixed-function oxidase is partly mediated by superoxide (Cederbaum and Cohen, 1980). A hydroxyethyl radical is formed by the oxidation of alcohol (Janzen, 1980).

1.2.1E) Conclusions: In summary, cytochrome P-450 mainly carries out two-electron oxidation of substrate either as a mixed-function oxidase or as a peroxidase. However, in the case of phenylenediamines, aminophenols, unacetylated benzidines, hydroxylamines and alcohols, a one-electron oxidation by a peroxidase type of oxidation appears to be involved. High levels of NADPH in the mixed-function oxidase pathway may prevent one-electron oxidation by reduction of the radicals by NADPH. However, the relevance of these *in vitro* oxidation pathways in terms of cytochrome P-450-mediated oxidations *in vivo* is still an open question.

1.2.2) PROSTAGLANDIN H SYNTHASE-CATALYZED ACTIVATION OF CARCINOGENS:

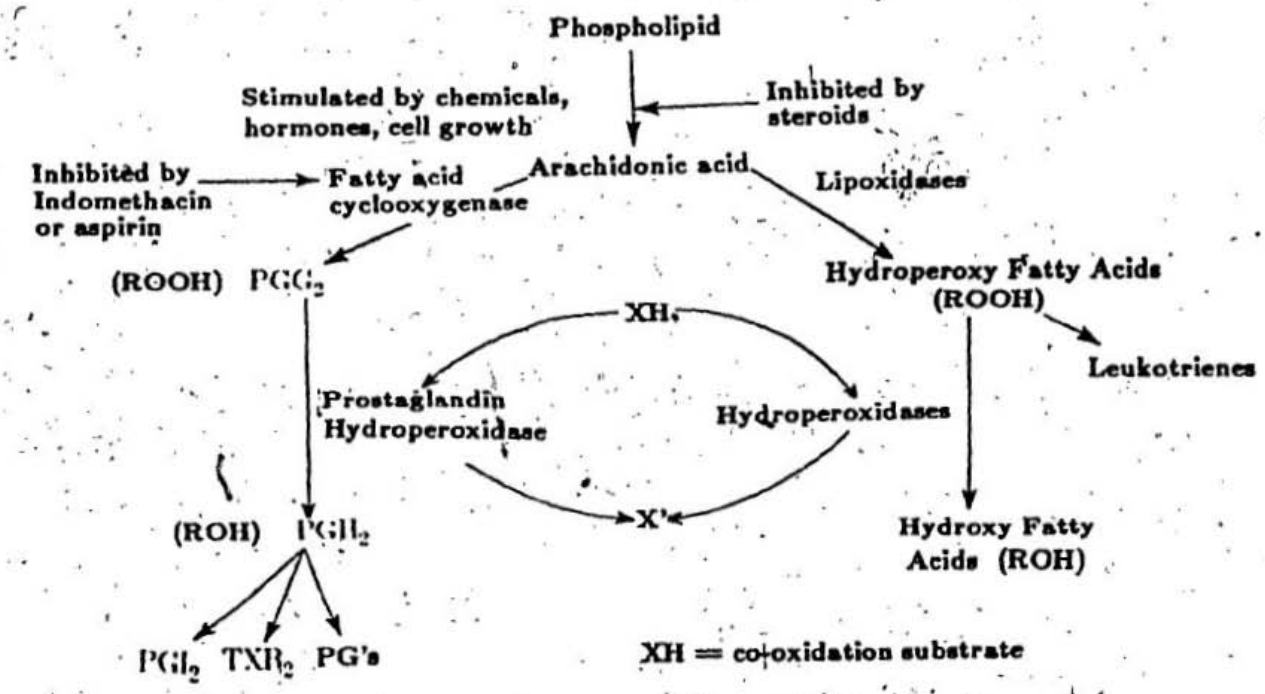
As described above, an extensive body of literature supports the view that cytochrome P-450 monooxygenase-mediated metabolic activation of chemicals by two-electron oxidation to reactive electrophiles could be the important step in the initiation of carcinogenesis. However, Marnett, Wloodawer and Samuelsson (1975) showed that several chemicals, including benzo(a)pyrene, were cooxidized during the oxidative conversion of arachidonic acid to prostaglandins. Prostaglandin H synthase (PHS) activity is high in many extrahepatic tissues, which are low in monooxygenase activity (Eling et al., 1983; Krauss and Eling, 1984). Thus, it has been suggested that PHS could be an alternative or additional enzyme system for the activation of carcinogens in these tissues.

The biosynthesis of prostaglandins and thromboxanes can be divided conceptually into two stages (Figure 1.6). In the first stage, the cyclooxygenase activity of PHS converts arachidonic acid to the hydroperoxy endoperoxide prostaglandin G_2 (PGG_2) which is then reduced by the peroxidase activity of PHS in the presence of a hydrogen donor to the hydroxy-endoperoxide PGH_2. Xenobiotics can act as hydrogen donors and in the process are cooxidized by this reaction. In the second stage, PGH_2 is further metabolized by additional enzymes to the classical prostaglandins (

FIGURE 1.6

METABOLISM OF ARACHIDONIC ACID TO PROSTAGLANDINS AND HYDROXY-FATTY ACIDS

(taken from Eling et al., 1983)



PGD_2, PGE_2, PGF_2), thromboxane A_2 and/or prostacyclin, depending on the tissue in which it is generated. The level of free fatty acids in most cells is quite low, and the release of arachidonic acid from membrane phospholipids is an important controlling event for the regulation of prostaglandin biosynthesis. However, arachidonic acid liberated from phospholipids can be metabolized by lipoxygenases, as well as PHS (Samuelsson, 1983). Lipoxygenase metabolizes arachidonic acid to hydroperoxy fatty acids (HPETEs) that are reduced to hydroxy fatty acids, or further metabolized to leukotrienes (Samuelsson, 1983). The peroxidase responsible for the reduction of HPETE, may be PHS or glutathione peroxidase (Bryant, Simon and Bailey, 1982 and 1983).

The release of arachidonic acid from membrane phospholipids by lipases and subsequent prostaglandin formation occurs in response to a wide variety of chemical, physiological and physical stimuli. For example, stimulation of endothelial cells by bradykinin and angiotensin (Hong, 1980) and stimulation of a variety of cells by the tumor promoter tetradecanoyl phorbol-12-myristate-13-acetate releases arachidonic acid leading to increased prostaglandin formation (Levine, 1981). Mechanical stimulation, including perfusion or inhalation of particulates, or altered respiration rate increases prostaglandin biosynthesis in the isolated perfused lung (Korbut, Boyd and Eling, 1981; Piper and Vane, 1971). Cell division also alters pros-

taglandin production in cell culture. Growing cells produce significant amounts of prostaglandins, while stationary monolayers produce only low amounts of arachidonic acid metabolites (Ali, Barrett and Eling, 1980).

The reduction of PGG_2 (or HPETEs), by PHS peroxidase requires reducing cofactors. These cofactors donate single electrons to the peroxidase and, in turn, are converted to reactive metabolites (Eling et al., 1983, Krauss and Eling, 1984).

The activity of PHS varies dramatically from tissue to tissue. Ram seminal vesicles contain an extremely high level of PHS activity (Christ and Vandorp, 1972). High activity is also found in platelets, kidneys and lungs (Eling et al., 1983). Low levels of activity of PHS are also present in skin, gut and liver (Marcus, 1978; Sivarajah, Lasker and Eling, 1981). The enzyme is membrane bound and appears to be localized in the endoplasmic reticulum and nuclear membranes (Rollins and Smith, 1980).

Many investigations of the cooxidation of chemicals by PHS have focussed on polycyclic aromatic hydrocarbons and aromatic amines. However, a number of other chemicals have been studied but in less detail. For most chemicals, the reaction mechanisms catalyzed by PHS are not known. Benzo(a)pyrene, 7,12-dimethylbenzanthracene, oxophenylbutazone, and phenylbutazone are oxidized by PHS to hydroxylated products, possibly via

corresponding hydroperoxides (Eling et al., 1983). The mechanism for the metabolism of aromatic amines by PHS appears to involve the formation of free radicals (Eling et al., 1983; Krauss and Eling, 1984).

1.2.2A) Cooxidation of polycyclic aromatic hydrocarbons: - BP was metabolized by ram seminal vesicle preparations following the addition of PHS substrate arachidonic acid (Marnett, Wloodawer and Samuelsson, 1975). The three stable products were 1,6-, 3,6-, and 6,12-quinones (Marnett, Reed and Johnson, 1977). On the other hand the proximate carcinogen (±)-BP-7,8-dihydrodiol is epoxidized in the presence of arachidonic acid or hydroperoxy fatty acids to *anti* diol epoxide (Marnett, Johnson and Bienkowski 1979; Sivarajah, Mukhtar and Eling, 1979). Little or no *syn* diol epoxide is formed. This is different from the cytochrome P-450 monooxygenase-catalyzed oxidation where (+)-isomer is converted to the *syn* diol epoxide, and the (-)-isomer to the anti diol epoxide (Conney, 1982; Gelboin, 1980). 7,8-Dihydro-BP is also oxidized by PHS and the stereochemistry of epoxidation is analogous to that of BP-7,8-dihydrodiol (Panthananickal, Weller and Marnett, 1983). However, the mechanism of epoxidation is different from that of the cytochrome P-450 monooxygenase system in that a fatty acid derived peroxy radical is hypothesized to be the epoxidizing agent (Marnett et al., 1983).

1.2.2B) Cooxidation of aromatic amines: - Unlike polycyclic aromatic hydrocarbons, aromatic amines are excellent reducing cofactors for PHS peroxidase. The work in this area has centered around two compounds benzidine and 2-aminofluorene.

Benzidine is metabolized by PHS to a radical cation that is in equilibrium with a charge transfer complex and its two-electron oxidation product, a diimine (Josephy, Eling and Mason, 1983). Azobenzidine is the major organic extractable metabolite. Polymeric material is also formed. When benzidine is metabolized in the presence of DNA or RNA, its activated metabolites bind covalently to the nucleic acids with very high efficiency (Kadlubar et al., 1982). Benzidine is a potent carcinogen in rodents and humans causing primarily liver and mammary tumors in rats and bladder tumors in humans (Clayson and Garner, 1976; Haley, 1975).

PHS and arachidonic acid also oxidize aminofluorene to nitrofluorene and azoaminofluorene (Boyd, Harvan and Eling, 1983) and products that bind to DNA (Kadlubar et al., 1982). A direct one-electron oxidation to cation-radicals appear to be involved (Rogan et al., 1979). Several other carcinogenic aromatic amines, including 2-naphthylamine and 4-aminobiphenyl are metabolized by PHS to reactive or mutagenic metabolites (Kadlubar et al., 1982; Morton et al., 1983; Robertson et al., 1981).

1.2.3) PEROXIDASE-CATALYZED ACTIVATION OF DRUGS AND CARCINOGENS:

Although the peroxidative type of catalytic activation of drugs and carcinogens by cytochrome P-450 and prostaglandin H synthase has been shown, a definite role *in vivo* has not been established. Horseradish peroxidase (HRP), a plant peroxidase has been used as a model for peroxidative type of one-electron oxidation mechanisms of activation of carcinogens (Floyd, Soong and Culver, 1976; Jellinck and Fletcher, 1971; O'Brien, 1984; Norymberski, 1977; Roerig, Reak and Wang, 1976; Rogan et al., 1979). DNA adduct formation mediated by HRP/H_2O_2 occurs with xenobiotics: polynuclear aromatic hydrocarbons (Rogan et al., 1979), diethylstilbestrol (Metzler and Mclahlan, 1978), trans-4-aminostilbene (Osborne, Metzler and Neumann, 1980), benzidine (Tsuruta et al., 1985), N-OH-2-acetylaminofluorene (Floyd, Soong and Culver, 1976), and p-phenetidine (Andersson et al., 1984). Cation radicals formed from polynuclear aromatic hydrocarbons have been implicated as the reactive metabolites binding to DNA (Rogan et al., 1979).

One-electron oxidation of organic substrates (AH_2) catalyzed by HRP is usually depicted by the following well-known mechanism (Saunders, Holmer-Siedle and Stark (eds.), 1964):

$$HRP + H_2O_2 \rightarrow \text{Compound I} \quad (1)$$

$$\text{Compound I} + AH_2 \rightarrow \text{Compound II} + \dot{A}H \quad (2)$$

$$\text{Compound II} + AH_2 \rightarrow HRP + \dot{A}H \quad (3)$$

Peroxidase (or HRP), in the normal ferric state, reacts with hydrogen peroxide to form Compound I (eqn 1). Compound I then undergoes a one-electron reduction by a hydrogen donor, giving Compound II (eqn 2) and finally Compound II undergoes one-electron reduction by a hydrogen donor regenerating ferriperoxidase (eqn. 3). Phenols and aromatic amines are widely known to react *via* such a mechanism (Saunders, Holmer-Siedle and Stark (eds.), 1964). The electronic structures of compound I and compound II have recently been ascertained (Sontum and Case, 1985).

Interestingly, peroxidases also have been implicated as an alternative or additional enzyme systems in carcinogenicity and toxicity of certain chemicals and drugs (O'Brien, 1984). Thus the Zymbal's gland, a sebaceous gland located in the rat external ear duct, contains lactoperoxidase and is the target for trans-4-aminostilbene (Osborne, Metzler and Neumann, 1980), acetylaminofluorene (O'Brien, 1984), monomethylaminoazobenzene (Clayson and Garner, 1976) and benzene (Maltoni et al., 1982). The Harderian gland is a target organ for benzidine in mice (Haley, 1975) and contains high levels of lactoperoxidase (Morrison and Allen, 1967) and low levels of mixed-

function oxidase. The uterus, a target organ for diethylstilbestrol (Metzler and Mclahlan, 1978), contains no mixed-function oxidase activity (Bennett, Marshall and O'Brien, 1982) but contains high levels of peroxidase (Kimura, Elce and Jellinck, 1983) and prostaglandin H synthase (Degen, Eling and Mclahlan, 1982). Liver, skin, colon, rectum, bone marrow, lungs, mammary gland, thyroid gland are also target organs in human cancer and contain peroxidases (Marshall and O'Brien, 1984, O'Brien, 1984). Thus, liver hepatocytes have a very high level of cytochrome P-450 (Hrycay and O'Brien, 1971) but the Kupffer cells of the liver contain a peroxidase (Fahimi, 1970). The skin, colon, rectum and bone marrow also contain cytochrome P-450 and peroxidase (O'Brien, 1984). Target tissues containing all three systems include the lungs, mammary gland, brain and bone marrow (O'Brien, 1984). These data suggest that most of the target organs for carcinogenesis contain a peroxidase. Also, liver which contains high amounts of mixed-function oxidase (Hrycay and O'Brien, 1971) and low amounts of peroxidase (O'Brien, 1984) is an uncommon site for carcinogenesis by chemicals (Craddock, 1976). Interestingly, the mammary gland is readily susceptible for carcinogenesis by polycyclic aromatic hydrocarbons (Dao, 1969) and aromatic amines (Malejka-Giganti, Guttman and Rydell, 1973).

Recently, it has been suggested that particular cell types are often the target organs for carcinogenesis by chemicals (O'Brien, 1984). The cell

types include leukocytes, eosinophils, peritoneal macrophages, bone marrow cells (Bentfeld, Nichols and Bainton, 1977), liver Kupffer cells (Fahimi, 1970), thyroid follicular epithelial cells (Strum and Karnovsky, 1970), salivary gland (Strum and Karnovsky, 1970), uterus endometrium (Brokelman and Fawcett, 1969) colon crypts of Lieberkuhn mucous secretory cells (Venkatachalam, Saltoni and Fahimi, 1970), Zymbal's gland (Osborne, Metzler and Neumann, 1980), submaxillary, sublingual, Harderian (Morrison and Allen, 1967) and mammary gland (Anderson, Trantalis and Kang, 1975). All these cell types are rich in peroxidases. The principal intracellular sites for peroxidases appear to be the cisternae of endoplasmic reticulum and the nuclear envelope in the cells.

1.3) AIMS OF THE THESIS :

Although the peroxidases are widely distributed, as pointed out above, the mechanisms of bioactivation of xenobiotics by peroxidases and the biochemical and carcinogenic effects during the activation of xenobiotics, have been overlooked. It is the aim of this thesis to investigate the reactivity of the oxidation products formed during a peroxidase-mediated oxidation of the xenobiotics, towards various biomolecules, with special emphasis on DNA as it may relate to the potential of DNA damage and induction of carcinogenesis. Several questions may be raised in this regard. For exam-

ple, if the peroxidase oxidation of xenobiotics results in products which can bind to DNA a) what is the nature of the binding? b) can those reactive metabolites be produced in an intact cell? (c) even if the reactive metabolites are formed, can they reach the DNA in the nucleus? Another aspect of the project is to determine the possible and potential source(s) of hydrogen peroxide, that may carry out the peroxidative oxidation of xenobiotics *in vivo* continuously, without any limitations.

CHAPTER 2

MATERIALS AND METHODS

2.1) Materials :

Benzidine dihydrochloride, butylated hydroxyanisole (BHA), N-ethylmaleimide (NEM), nordihydroguaiaretic acid (NDGA), p-chloromercuribenzoate (PCMB), cetyltrimethylammonium bromide (cetavlon), dinitrofluorobenzene (DNFB), 3,3′,5,5′-tetramethyl benzidine (TMB), horseradish peroxidase (HRP) type VI, calf-thymus DNA type I, sodium dodecyl sulfate (SDS), polyriboadenylic acid, polyriboguanylic acid, polyribocytidylic acid, polyribouridylic acid, t-RNA type X, bovine serum albumin (BSA), glutathione (GSH), ascorbic acid, N-acetylcysteine, lysine, proline, lead tetraacetate, o,o′-biphenol, p,p′-biphenol, 2-aminofluorene (2-AF), 1-naphthylamine (1-NA), 2-naphthylamine (2-NA), 4-aminobiphenyl (4-AB), N,N′-dimethyl-p-toluidine (DMPT), N,N′-dimethyl-4-aminoazobenzene (DAB), aminoazobenzene (AB), reduced nicotinamide adenine dinucleotide phosphate (NADPH), reduced nicotinamide adenine dinucleotide (NADH), lactoperoxidase (LP), ribonuclease A type I-A, ribonuclease T_1, protease (type XIV), superoxide dismutase (SOD), tyrosinase (grade III) and ferricytochrome *c* were purchased from Sigma Chemical Company (St. Louis, MO., USA). Phenol (analytical grade) was obtained

from Baker Chemical Company (Phillipsburg, NJ, USA) and was further purified by distillation.

Hydrogen peroxide (H_2O_2; 30%) was obtained from Fisher Chemical Company (Toronto, Ontario, Canada). Sephacryl-300 and Sephadex LH-20 were obtained from Pharmacia Fine Chemicals (Quebec, Canada).

N-(ring- ^{14}C)-Methylaminoazobenzene was synthesized as previously described by Munier and Chauveau (1970) and was purified by thin layer chromatography. The preparation had a specific activity of 5.4 mCi/mmol with a purity of 99%. No [^{14}C]-4-aminoazobenzene was present in the preparation. 2-(9- ^{14}C)-Aminofluorene was prepared from 2-(9- ^{14}C)-acetylaminofluorene (50 mCi/mmol) (New England Nuclear, Boston, Mass., USA) by incubating the latter with carboxyesterase II, and the 2-(9- ^{14}C)-aminofluorene formed was extracted with ethyl acetate and purified by high pressure liquid chromatography (Boyd, Harvan and Eling, 1983). (ring- ^{14}C)-Benzidine (25.7 mCi/mmol) was purchased from New England Nuclear (Boston, Mass., USA). U[^{14}C] Phenol (71.1 mCi/mmol) was obtained from Amersham/Searle (Oakville, Ontario, Canada) with a stated purity of 99%. All the radioactive samples were diluted to the required specific activity with pure non-radioactive samples before use.

2.2) Methods :-

2.2.1) Cell and organelle preparation:-

2.2.1.1) Leukocyte preparation :- Leukocytes containing more than 90% polymorphonuclear leukocytes (PMN's) were obtained from intraperitoneal cavity of guinea pigs as described previously (Takanaka and O'Brien, 1975 a). A guinea pig was injected with 30 ml of 0.9% NaCl solution containing 2% neutralized sodium caseinate solution. After 14 to 16 hours, the intraperitoneal fluid was extracted with 30 ml of 0.9% NaCl solution. The cell suspension was centrifuged at 400 x g for 5 minutes. Supernatant solution was discarded and the cell pellet was resuspended in 3 ml of 100 mM sodium phosphate buffer pH 7.4 containing 50 μM $CaCl_2$ and 0.9% NaCl; 3 ml of ice-cold water was added to remove contaminating erythrocytes. After 30 seconds, 6 ml of 1.8% NaCl solution was added to restore the isotonicity. The suspension was centrifuged again (5 min; 400 x g). The resulting cell pellet was resuspended in 1 ml of the above-mentioned buffer. After the cells were obtained from guinea pigs, all procedures were carried out at 4°C. Microscopic examination after staining (by adding one or two drops of 0.5% bromophenol blue solution to an aliquot of the cell suspension) showed the following average differential count: PMN's 93%, lymphocytes 4%, eosinophils 2% and monocytes 1%. Leukocyte granules were isolated according to the method of

Rossi and Zatti (1968).

2.2.1.2) Preparation of bone marrow homogenate :- Male Sprague-Dawley rats (200-400 gms) were killed by cervical dislocation; both femur and tibia were immediately removed. Adhering tissue was scraped off and bones were opened by cutting both the ends with the scissors and the marrow was flushed out of the cavity with ice-cold 0.9% saline. The resulting bone marrow cells were centrifuged for 5 minutes at 400 x g. The cell pellet was resuspended in 3 ml of 0.1 M Tris-HCl buffer pH 7.4 containing 0.9% NaCl solution. Erythrocyte contamination was removed by adding 3 ml of ice-cold water and centrifugation at 400 x g for 5 minutes. The cell pellet was resuspended in 0.1 M Tris-HCl buffer pH 7.4 and homogenized in glass/Teflon homogenizer and centrifuged for 10 minutes at 4°C and 400 x g. The supernatant was used as bone marrow homogenate. Protein was estimated by the method of Lowry et al. (1951).

2.2.1.3) Isolation of myeloperoxidase :- Myeloperoxidase was isolated from human white blood cells as previously described by Andrews and Krinsky (1981). The enzyme concentration was calculated from an absorbance coefficient of $89nM^{-1}\ cm^{-1}$ at 428 nm. The absorbance ratio (A_{428}/A_{280nm}) of the enzyme was 0.9.

2.2.1.4) Preparation of rat liver microsomes :- Liver microsomes were prepared from livers removed from Male Sprague-Dawley rats (150-250 gms), after perfusion with isotonic saline (0.9% NaCl), as previously described (Hrycay and O'Brien, 1971).

2.2.2) Studies on the binding of arylamine or phenol oxidation products to DNA :-

2.2.2.1) Binding to DNA in leukocytes :- Incubations were performed in 2 ml of 100 mM sodium phosphate buffer pH 7.4 containing 0.9% NaCl and 50μM $CaCl_2$. The following additions were made :- PMN's (5 x 10^7 cells), PMA (1-2 x 10^{-9} M) and either ^{14}C-benzidine (60 μM) or ^{14}C- methylaminoazobenzene (50 μM) or ^{14}C-aminofluorene (5 μM) or ^{14}C-phenol (120 μM). Other chemicals were added where indicated. Anaerobic conditions, as determined with an oxygen electrode, were obtained by using boiled buffers and continuously bubbling argon or oxygen free nitrogen during the incubation period. The mixture was incubated for 20 minutes at 37 °C with shaking and the reaction was terminated by extracting with ethyl acetate (6 x 2 ml). After removal of the excess ethyl acetate from the aqueous phase by bubbling nitrogen, the DNA was subsequently isolated. Binding of arylamine or phenol oxidation products to DNA in leukocytes in the absence of PMA, was also measured.

2.2.2.2) DNA isolation from leukocytes :- DNA from PMN's was isolated essentially by the method of Baird and Brookes (1973). The aqueous phase after ethyl acetate extraction was bubbled with N_2, treated with 400 μl of sodium dodecyl sulfate (final 1%) and incubated with protease (0.5 mg/ml). After 1 hour, an equal volume of water-saturated phenol and water-saturated chloroform were added to each sample and the reaction mixtures were shaken vigorously and centrifuged. The aqueous phase was transferred into another test-tube and the nucleic acids were precipitated by the addition of 2 volumes of ethanol. The nucleic acids were redissolved in 2 ml of 0.1 M Tris-HCl, 1.0 mM EDTA buffer pH 7.0 and incubated with ribonuclease A (10 units) and ribonucleaseT_1 (5 units) for 10 minutes. The DNA was reprecipitated with twice the volume of ethanol, redissolved in 0.1 M Tris-HCl buffer pH 7.4 (1 ml) and aliquots were analyzed for DNA content by measuring absorption at 260 nm and the bound (^{14}C) radioactivity was measured by liquid scintillation counting. Using this technique 300-400 μg DNA was isolated from 5 x 10^7 cells. The 260/280 nm ratio was around 1.7 for both the isolated DNA as well as for pure calf-thymus DNA (used as standard) indicating that protein was completely eliminated from leukocyte DNA during the isolation procedure.

2.2.2.3) In vitro binding to DNA :- 3 ml of 0.1 M Tris-HCl buffer containing 3 mg calf-thymus DNA were incubated with ^{14}C-substrate with H_2O_2 and HRP or with H_2O_2 and MPO or with H_2O_2 and leukocyte granules or with

rat liver microsomes and NADPH or with rat liver microsomes and cumene hydroperoxide (CHP) or tyrosinase (for appropriate conditions see tables 3.3 & 4.1). After incubations under appropriate conditions, the reactions were terminated by extracting with ethyl acetate (3 x 3 ml). The DNA was subsequently isolated from the aqueous phase as described above (Section 2.2.2.2) except that incubation with ribonuclease was omitted. 80-90% of the added DNA was always recovered.

2.2.2.4) DNA and polyribonucleotide hydrolysis, and column chromatography :- DNA adduct analysis was carried out by a modification of the method of Baird and Brookes (1973). DNA after precipitation was redissolved in 3 ml of 5 mM Tris-HCl buffer pH 7.1 containing 0.1 mM EDTA and 10 mM $MgCl_2$ and denatured by heating for 3 minutes in boiling water and rapidly cooled with ice. Deoxyribonuclease I (0.1 mg/mg DNA) was added and the solution was incubated for 12 hours at 37°C. The pH was then adjusted to 8.0 by addition of 1 M tris-base and phosphodiesterase (0.04 units/mg DNA) and alkaline phosphatase (1 unit/mg DNA) were added. The solutions were further incubated at 37°C for another 12 hours after which pH was re-adjusted to 7.0 with 1 M HCl and extracted with butanol. The aqueous layers were then subjected to column chromatography using Sephadex LH-20 on a 2.5 x 40 cm column. Ribonuclease A (10 units) was used for the digestion of polyriboadenylic acid or polyribouridylic acid or polyribocytidylic acid.

Ribonuclease T_1 (5 units) was used for the digestion of polyriboguanylic acid.

2.2.3) Studies on binding of phenol oxidation products to protein :-

2.2.3.1) Incubation procedures:-

2.2.3.1.1) Microsomal incubations :- Incubation mixtures in 3 ml of 0.1 M Tris-HCl buffer pH 7.4 contained the following: Rat liver microsomal protein (1 mg/ml), NADPH (0.5 mM) or CHP (0.5 mM) and ^{14}C-phenol (0.2 mM). Some incubations contained heat-denatured microsomes, or no protein or no co-factor (NADPH or CHP). These samples were used to assess the extent of non-enzymatic conversion and binding of phenol to microsomal protein. Incubations were carried out for 15 minutes at 37 ° C. The reactions were terminated by the addition of ethyl acetate (3 ml) and the organic layers were removed. Repeated extractions were performed (3 times).

2.2.3.1.2) Peroxidase incubations :- ^{14}C-Phenol (0.2 mM) was incubated with HRP (10 μg), H_2O_2 (0.5 mM) and heat denatured microsomes (1 mg/ml) in 3 ml of 0.1 M Tris-HCl buffer pH 7.4 at 37 ° C for 15 minutes.

2.2.3.1.3) Bone marrow incubations :- ^{14}C-Phenol (0.2 mM) was incubated with bone marrow homogenate (25-200 μg protein) and H_2O_2 (10 mM) in 3 ml of 0.1 M Tris-HCl buffer pH 7.4 at 37 ° C.

2.2.3.2) Quantitative determination of irreversible binding to protein :- Quantitative determination of irreversible binding to protein was performed as described by Sivarajah, Anderson and Eling (1978). The protein in the aqueous layer was precipitated with 100% TCA (300 μl), washed with 10% TCA (1 ml) and the protein, after centrifugation, was exhaustively extracted (2 times) with 80% methanol/water and chloroform/methanol (2:1 by vol). The washed protein was solubilized in 1 ml of 1 N sodium hydroxide, radioactivity determined by scintillation counting and protein measured by the method of Lowry et al. (1951). Approximately 75%-80% protein was always recovered.

2.2.3.3) Further examination of nature of binding to protein :- The protein samples obtained after methanol extractions, as described above, were further examined to determine how strongly the products are bound to protein, using the method described by Jollow et al. (1973). The protein samples were suspended in 3 ml of 0.1 M Tris-HCl buffer pH 7.4 and digested with protease for 12 hours at 37 °C. Maximal digestion occurred within 12 hours, after which the further incubation had no effect on amount digested. The digest was extracted with ethyl acetate (3 ml x 2) and the organic layers were removed. The aqueous layers were bubbled with N_2 for a few minutes (to evaporate excess ethyl acetate), and an aliquot was removed for measuring the radioactivity.

Some samples after methanol extractions and digestion with protease, were mixed with dinitrofluorobenzene (DNFB) (5% w/v) in ethanol and the mixtures were kept for four hours at 25°C in the dark, with occasional stirring. The pH was adjusted to 9.0 by the addition of $NaHCO_3$ and the reaction mixtures were extracted with 3 ml ethyl acetate (2 times). An aliquot of the aqueous layer was measured for radioactivity. The remaining aqueous layers were acidified to pH 1.0 with 6.0 M HCl and the reaction mixtures were further extracted with ethyl acetate (3 ml x 2). The radioactivity in the aqueous layers was removed.

2.2.3.4) SDS-Polyacrylamide gel electrophoresis for bone marrow proteins :- SDS-Polyacrylamide gel electrophoresis was performed to analyze which proteins are specifically labelled by reactive phenol metabolites. After incubations (as described above: Section 2.2.3.1.3), the reactions were terminated by adding ice-cold sucrose (0.25 M) and centrifuged at 100,000 g and at 4°C for 1 hour. The supernatant was discarded and the protein pellet was washed with sucrose (0.25 M). More than 90% of the total bound radioactivity was recovered in the protein pellet after centrifugation. The protein was then solubilized in 300 μl of Tris-HCl buffer pH 6.8 containing SDS (1%), glycerol (10%) and 2-mercaptoethanol (10%). 30 μl aliquots were run on 15% polyacrylamide slab gels containing 0.09% bisacrylamide. After the completion of the electrophoresis the gels were stained for 3 hours in 0.25% commas-

sie brilliant blue containing 50% methanol and 10% acetic acid. Destaining was performed in 25% ethanol containing 10% acetic acid for 2 days. The gels were treated with PPO (saturated solution in DMSO) for 2 hours and dried before exposing the gels to Kodak X-Omat films at -70 °C for 4 weeks. Molecular weights of the standards used were 12,300, 30,000, 46,000, 69,000 and 92,000.

2.2.4) Phenol product analysis :-

The ethyl acetate layers (obtained after extraction of the incubation mixtures) were pooled and concentrated to 0.5 ml under N_2 and the products were analyzed by high-pressure liquid chromatography (h.p.l.c.) or thin layer chromatography (t.l.c.) or by mass spectroscopy.

2.2.4.1) H.p.l.c. :- Analysis of 20 μl aliquots was carried out using a Waters Associated system, equipped with a C_{18} μ-Bondapak (0.39 x 30 cm) column. The solvent system consisted of a mixture of 1% acetic acid in water and methanol. A linear gradient of 45-100% methanol with a flow-rate of 1 ml/min was applied during a 15 minute run. The products were detected at 280 nm. Quantitation was achieved by measuring peak heights and plotting a calibration curve for known quantities of phenol, p,p'-biphenol and o,o'-biphenol. Under these conditions phenol recovery was 95-100% and p,p'-

biphenol and o,o'-biphenol was 98-100%. The retention times (minutes) were as follows: phenol (7.0), p,p'-biphenol (9.5) and o,o'-biphenol (11.5). In addition minor unknown peaks at 12.8 minutes and 14.0 minutes were observed.

2.2.4.2) T.l.c. :- All t.l.c. studies were performed using Analtech precoated silica gel HLF (20 x 20 cm., 0.25 mm thick) plates. 200 µl aliquots of the organic layers were spotted on t.l.c. plates and run in a solvent system containing benzene-methanol-acetic acid (70:8:4 by vol.). Quantitation was achieved by measuring the radioactivities of the individual bands. The R_F values were as follows :- p,p'-biphenol (0.42), o,o'-biphenol (0.58), phenol (0.74) and p,p'-biphenoquinone (0.95). In addition some minor bands were also observed with R_F values as follows :- unknown 1 (0.00), unknown 2 (0.50), unknown 3 (0.52) unknown 4 (0.62) (See Results in Chapter 5; Table 5.1).

2.2.4.3) Mass spectral analysis :- The ethyl acetate extracts of the peroxidase oxidized phenol or o,o'-biphenol or p,p'-biphenol reaction mixtures were dried under N_2 and were analyzed directly by a mass spectrometer. The analyses was carried out using a VG Micromass 7070-HS mass spectrometer and PDP8A digital RLO_2 disc data system by direct probe insertion with electron ionization. Scanning was performed at low resolution with m/e in the range of 0-500. Other parameters include 70 eV electron input ionization and source temperature of 350° C.

2.2.4.4) Determination of reduction rate constants for peroxidase compound II by phenolic compounds:- The rate constants for the reduction of peroxidase compound II were determined by the method of Hewson and Dunford (1976) using a Hewlett Packard stopped-flow spectrophotometer model 120 B. Compound II (1 μM) was prepared by mixing HRP (1 μM) and H_2O_2 (2 μM) in 0.1 M Tris-HCl buffer pH 7.4. A 10-fold excess phenolic substrates were used and the rate of the reaction was followed by the decrease in absorbance at 427 nm. All the measurements were performed at 30° C.

2.2.5) Structural analysis p,p'-biphenol-glutathione conjugate :-

2.2.5.1) Incubation conditions :- p,p'-Biphenol (0.05 mM) was incubated with HRP (1 μg), glutathione (0.05 mM) and H_2O_2 (0.06 mM) in 3 ml of 0.1 M Tris-HCl buffer pH 7.4 at room temperature for 5 minutes. Reactions were stopped with the addition of ethyl acetate (3 ml x 3). Large scale incubations for metabolite isolation had a total volume of 500 ml.

2.2.5.2) H.p.l.c.:- H.p.l.c. of the water-soluble metabolites formed from the above incubations was performed using a C_{18} μ-Bondapak column (0.39 x 30 cm) developed with methanol:water:acetic acid (30:7:2 by vol) and with a flow rate of 1 ml/min for 15 minutes.

2.2.5.3) FAB Mass spectrostopy of glutathione conjugate :- A VG Micromass 7702E instrument with Xe atom beam was used at ambient temperature to record spectra of samples prepared in glycerol and deposited on the probe.

2.2.5.4) Nuclear Magnetic Resonance Spectroscopy of glutathione conjugate :- A WH-400 MHz Bruker instrument was used to record spectra of samples.

2.2.5.5) Acid hydrolysis of the isolated glutathione conjugate :- Aliquots of h.p.l.c. purified unknowns were hydrolyzed in 6 N HCl (2 ml) in sealed vials at 100 ° C for 15 hours. After the removal of the acid *in vacuo* the residue was dried overnight over phosphorous pentoxide (P_2O_5) and chromatographed against glutamic acid and glycine standards on silica gel GF plates developed in butanol:acetic acid:water (60:20:20 by vol). After spraying with ninhydrin, the plates were heated at 100 ° C for 5 minutes for colour development.

2.2.5.6) Isolation of p,p'-biphenol-GSH conjugate from bone marrow homogenate :- The GSH conjugate from bone marrow homogenate (200 μg) was isolated as described above following incubation with H_2O_2 (10 mM) and p,p'-biphenol (200 μM), and GSH (400 μM) after precipitation of the protein with an equal volume of TCA (10%). After centrifugation, the

resulting aqueous fraction was neutralized with 1 N NaOH and subjected to h.p.l.c.

2.2.6) Measurement of oxygen consumption :-

2.2.6.1) Oxygen consumption by PMN's :- Oxygen consumption by PMN's was estimated in the reaction medium (2 ml) containing 100 mM phosphate buffer pH 7.4, 50 μM $CaCl_2$, 0.9% NaCl, PMN's (1-2 x 10^7 cells). The electrodes for oxygen (Clarke type) were placed in the reaction mixture which was kept at 37 °C with circulating water. Rapid oxygen uptake occurred following the addition of PMA (1.2 x 10^{-9}M). The oxygen uptake was not affected by the various substrates used.

2.2.6.2) Oxygen consumption by peroxidase :- Oxygen consumption was measured as described above (Section 2.2.6.1) at 20 °C. The standard reaction mixtures contained in 2 ml of 0.1 M Tris-HCl, 1.0 mM EDTA buffer pH 7.4, phenol (100 μM) or arylamine substrate (10 μM), HRP (10 μg), NADH (200 μM) or GSH (400 μM). Reactions were started by the addition of H_2O_2 (10 μM) and followed until complete.

2.2.7) Measurement of NADH oxidation :-

The reaction mixtures, in 2 ml of 0.1 M Tris-HCl, 1.0 mM EDTA buffer pH 7.4, contained phenol (100 μM) or arylamine substrate (10 μM), HRP (10 μg), and NADH (200 μM). Reactions were started by the addition of H_2O_2 (10 μM) and the disappearance of NADH with time was followed at 340 nm using a Shimadzu UV-240 spectrophotometer until the NADH oxidation was complete (usually 1-2 minutes).

2.2.8) Measurement of Rate Constants for NADH oxidation :-

2 ml reaction mixtures of 0.1 M Tris-HCl, 1.0 mM EDTA buffer pH 7.4 contained (unless otherwise stated) the following :- NADH (100 μM), arylamine or phenolic substrate (10 μM), HRP (1 μg) or LP (10 μg). Reactions were started by the addition of H_2O_2 (10 μM) and the rate of NADH oxidation was followed at 340 nm using a Shimadzu UV-240 spectrophotometer.

2.2.9) Measurement of GSSG formation :- The standard 2 ml reaction mixtures in 0.1 M Tris-HCl, 1.0 mM EDTA buffer pH 7.4, contained: phenol (100 μM) or arylamine substrate (10 μM), HRP (10 μg), and GSH (400 μM). The reactions were started by the addition of H_2O_2 (10 μM). After 10 minutes NADPH (200 μM) was added to each reaction mixture and the amount of NADPH oxidized following the addition of glutathione reductase (1 unit) was determined (Srivastava and Beutler, 1967).

2.2.10) Reduction of ferricytochrome *c* :-

The 2 ml reaction mixtures of 0.1 M Tris-HCl buffer pH 7.4 contained NADH (200 μM), HRP (1 μg), ferricytochrome *c* (20 μM) and phenol. Reactions were started by the addition of H_2O_2 (10 μM). Reduction of ferricytochrome *c* was monitored by increase in the absorbance at 550 nm (Takayama and Nakano, 1977).

CHAPTER 3

PEROXIDASE-MEDIATED IRREVERSIBLE BINDING OF ARYLAMINE CARCINOGENS TO DNA IN INTACT POLYMORPHONUCLEAR LEUKOCYTES ACTIVATED BY A TUMOR PROMOTER.

3.1) Introduction :

Whilst much emphasis has been placed in chemical carcinogenesis on the two-electron oxidation catalytic activity of mixed-function oxidases, it is clear that one-electron oxidation pathways mediated by lipoxygenases, prostaglandin synthase and other peroxidases utilizing fatty acid hydroperoxides or H_2O_2 also oxidize carcinogens to metabolites that bind to DNA (Boyd, Harvan and Eling, 1983; Kadlubar et al., 1982; Marshall and O'Brien, 1984; Morton et al., 1983; Zenser et al., 1979). The polymorphonuclear leukocyte has very high levels of myeloperoxidase (Bentfeld, Nichols and Bainton, 1977) as well as prostaglandin synthase (Rossi, Dellabianca and Bellavite, 1981) and lipoxygenase (Rossi, Dellabianca and Bellavite, 1981) and has little or no mixed-function oxidase activity (Busbee, Shaw and Cantrell, 1972). Interaction of the leukocyte plasma membrane with the tumor promoter phorbol-12-myristate-13-acetate (PMA) results in a cyanide-resistant respiratory burst with the formation of

superoxide, hydroxyl radicals and H_2O_2 (Goldstein et al., 1981; Takanaka and O'Brien, 1980) and activation of the arachidonic acid cascade forming prostaglandins, thromboxanes and leukotrienes (Rossi, Dellabianca and Bellavite, 1981). These effects therefore make the leukocyte a useful model for a cellular study of the one-electron oxidation pathway for carcinogen activation and also for the effects of a tumor promoter on chemical carcinogenesis mechanisms.

In this chapter, binding of arylamine carcinogens and phenol to the nuclear DNA of intact PMN's has been demonstrated following treatment with the tumor promoter PMA. Phenol is an excellent substrate for peroxidase (Saunders, Holmer-Siedle and Stark, 1964) and a major *in vivo* metabolite of the leukemogen benzene (Rickert et al., 1979). Evidence will be presented that the mechanism for the activation of arylamines and phenol in PMN's involves H_2O_2 and myeloperoxidase. This study is the first reported example of carcinogen activation by a peroxidase/H_2O_2 system in the intact cell.

3.2) Results :

The respiratory burst initiated in PMN's, on exposure to PMA, is characterized by an increase in cyanide-resistant oxygen consumption leading to superoxide, hydrogen peroxide and hydroxyl radicals and enhanced glucose oxidation through the hexose-monophosphate shunt (Takanaka and O'Brien,

1980). In table 3.1, it can be seen that this can also result in an irreversible binding of radiolabel derived from various arylamine carcinogens and phenol to leukocyte nucleic acids. The binding is proportional to the number of PMN's present (Figure 3.1). Negligible binding occurred in the absence of PMA. Furthermore a drastic inhibition of the binding under anaerobic conditions (Table 3.1) suggests that the binding is coupled to the increased respiration of the PMN's.

The production of the activated oxygen species as a result of the leukocyte stimulation by PMA is believed to be due to the activation of a NAD(P)H oxidase located in the plasma membrane and in the phagosomal membrane formed by invagination of the plasma membrane (Badwey and Karnovsky, 1980; Takanaka and O'Brien, 1975 b; Patriarca et al., 1975). Following a lag of 15-30 seconds, the activation reached a plateau at about 20 minutes and the oxidase activity remained constant for at least 90 minutes (McPhail and Snyderman, 1983). As shown in table 3.1, the respiratory burst is readily inhibited by the thiol inhibitor, p-chloromercuribenzoate (PCMB), even though this inhibitor does not permeate the plasma membrane. This suggests that the oxidase or the activation mechanism has essential thiol groups. The isolated NADPH oxidase is completely inhibited by PCMB (Gabig, Schervish and Santiga, 1982). NEM, another thiol inhibitor, also inhibited the respiratory burst. The inhibition in the binding of products of arylamine carcinogens and phenol

Table 3.1

Effect of various agents on irreversible binding of carcinogenic arylamines and phenol to DNA in polymorphonuclear leukocytes.

Additions [a]	O_2 uptake [b] (nmoles)	^{14}C-bound to DNA (pmol/mg) [c]			
		MAB	Benzidine	Aminofluorene	Phenol
None	74.0±7.1	53.6±6.0	251.3±23.1	90.1±10.6	706.5±50.2
+ p-CMB (0.1 mM)	28.9±4.2	19.3±3.2	63.6±6.7	10.3±3.2	232.4±30.2
+ NEM (1 mM)	26.3±4.9	15.1±4.6	51.2±7.1	30.2±15.1	255.2±27.4
+ Azide (1 mM)	95.1±11.3	70.1±6.9	302.4±31.3	130.1±14.2	1420.6±75.5
+ Azide (10 mM)	93.6±10.9	3.2±2.8	13.1±3.6	1.0±1.1	22.2±2.8
+ Cyanide (1 mM)	78.6±9.3	63.7±7.0	270.2±28.2	104.1±11.8	1210.1±68.4
+ Cyanide (10 mM)	79.1±11.3	7.3±3.6	23.2±2.7	9.1±7.1	423.6±35.1
+ NDGA (0.1 mM)	22.1±4.1	14.3±2.3	63.1±7.2	20.1±2.2	328.6±22.3
+ BHA (0.1 mM)	11.3±9.7	4.6±1.8	33.2±4.1	13.7±1.4	36.1±4.4
PMA absent	1.1±0.6	1.1±1.1	0.7±1.5	0.1±1.6	33.2±3.1
Anaerobiosis		0.5±1.3	21.3±11.3	11.1±9.7	18.7±2.3

a. Chemicals (10-20 µl) were added before the leukocytes were added to the reaction mixture. Reaction conditions are described in Materials and Methods (section 2.2.2.1). The DNA was isolated as described in Materials and Methods (section 2.2.2.2).

b. nmol consumed oxygen/20 min/ 10^7 cells. Means ± Standard deviations of three experiments (using three different leukocyte preparations).

c. pmol (^{14}C)-bound/mg DNA*. Means ± Standard deviations of three experiments (using three different leukocyte preparations).

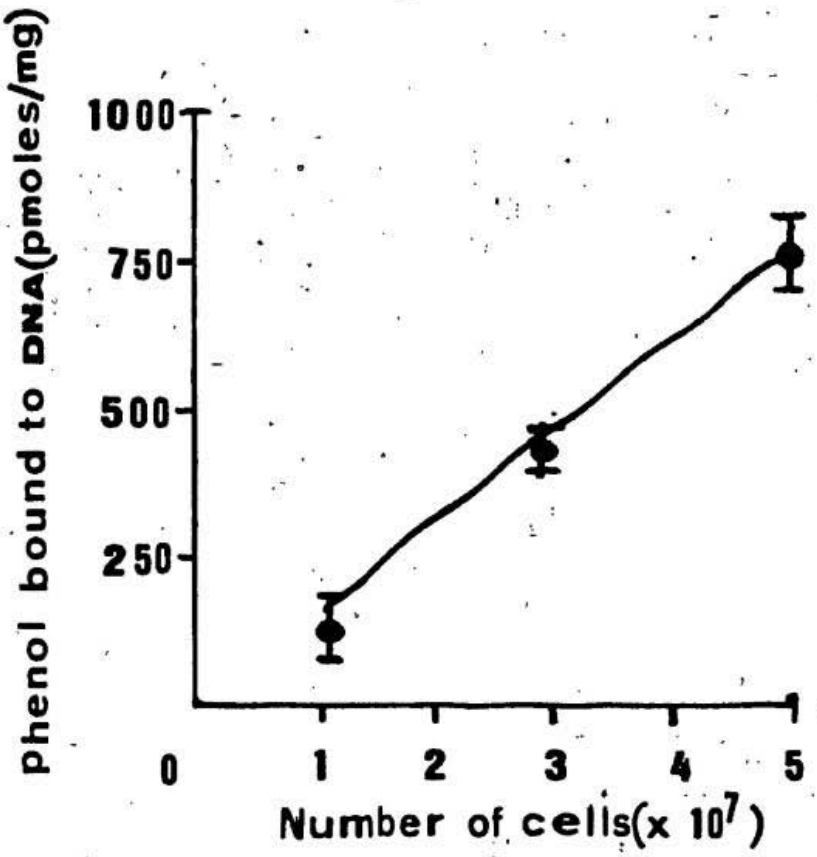
* refers to the amount of radioactive material associated with the DNA expressed in terms of the amount of original labelled compound added to the system.

Figure 3.1

Effect of different concentrations of PMN's on the [U ^{14}C]Phenol binding to DNA :

See Materials and Methods (sections 2.2.2.1 and 2.2.2.2) for experimental procedures. Results are expressed as Means ± S.D., n=3.

FIGURE 3.1


Phenol bound to DNA(pmoles/mg)
1000
750
500
250
0
1
2
3
4
5
Number of cells(x 10^7)

to DNA (table 3.1), in the presence of above-mentioned thiol inhibitors, indicates that the binding of these arylamine carcinogens and phenol to DNA in leukocytes requires the plasma membrane oxygen activating system.

The antioxidants NDGA (Rossi, Dellabianca and Bellavite, 1981), n-propyl gallate (Beswick and Slater, 1978), catechin (Beswick and Slater, 1978) and promethazine (Beswick and Slater, 1978) also inhibit the respiratory burst. The inhibition is reversible and may involve a non-competitive inhibition of the activated NADPH oxidase or membrane activating system (Rossi, Dellabianca and Bellavite, 1981). As shown in table 3.1, the inhibition of the respiratory burst by the antioxidants NDGA and BHA, also resulted in the inhibition of the arylamine and phenol oxidation products binding to leukocyte DNA. This is further evidence that the arylamine and phenol activation involved the cyanide-resistant oxygen activation mechanism.

Low concentrations of azide (1 mM) enhanced binding of arylamine or phenol oxidation products to DNA as well as oxygen uptake. Low concentrations of cyanide (1 mM) also, enhanced binding of phenol oxidation products to DNA. Others have shown that at these concentrations, leukocyte endogenous catalase is inhibited, oxygen uptake is stimulated and the level of intracellular H_2O_2 is raised (Nauseef, Metcalfe and Root, 1983). At higher concentrations of azide and cyanide endogenous myeloperoxidase is also inactivated (Nauseef, Metcalfe and Root, 1983) and binding of arylamine or phenol

oxidation products to DNA was markedly inhibited (Table 3.1), although oxygen uptake was not affected. This implies that endogenous myeloperoxidase is involved in the binding of arylamine or phenol oxidation products to DNA, but not in H_2O_2 formation. Furthermore, the enhanced binding of arylamine or phenol oxidation products to DNA by low concentrations of azide and cyanide, may indicate that H_2O_2 is involved in the activation process of arylamines or phenol to products that bind to DNA. It appears that leukocytes have no mitochondria and they derive their ATP through glycolysis (Murphy, 1976). Furthermore, antimycin A and rotenone, inhibitors of the mitochondrial electron transport chain, had no effect on the oxygen uptake and the binding of arylamine and phenol oxidation products to DNA in leukocytes (results not shown) which suggests that the activation of arylamines and phenol in leukocytes may not involve mitochondria.

Several other lines of evidence also indicated that it is H_2O_2 that is formed in the PMA-stimulated leukocytes, which mediates the oxidation of phenol to products that bind to DNA (Table 3.2). Firstly, addition of catalase to the incubation medium, reduced binding of phenol oxidation products to DNA in leukocytes by 80%. Secondly, addition of SOD which catalyzes the dismutation of O_2^- to produce H_2O_2 (Diguiseppi and Fridovich, 1984) increased the binding nearly two-fold suggesting that the rate of H_2O_2 production was limiting. It is unlikely that catalase and superoxide dismutase per-

Table 3.2
Effect of various agents on ^{14}C-phenol oxidation products binding to DNA in intact polymorphonuclear leukocytes.

Incubation conditions	^{14}C-Phenol bound to DNA (pmol/mg)[O]
Complete system	820.4±32.6
- PMA*	33.2±3.1
+ Catalase (20 μg)	66.6±8.2
+ SOD (20 μg)	1621.9±120.6
+ H_2O_2 * (100 μM)	263.6±22.4
+ Glucose (100 μM)/Glucose oxidase * (20 μg)	952.7±76.2

Each value is a mean of 3 different incubations (different leukocyte preparations) ± standard deviation.

* PMA absent.

[O] refers to the amount of radioactive material associated with the DNA expressed in terms of the amount of original labelled compound added to to the system.

meate the plasma membrane which suggest that PMA causes the release of superoxide and H_2O_2 into the surrounding medium from the plasma membrane. The leukocytes were shown to be intact as they excluded trypan blue and had no latent dehydrogenase activity (measured by the method of Takanaka and O'Brien, 1975 b). In the absence of PMA, addition of H_2O_2 also catalyzed some binding of phenol oxidation products to DNA. Addition of glucose/glucose oxidase, a H_2O_2 generating system (Ohno and Gallin, 1985) also caused significant phenol oxidation products binding to DNA. Similar results were obtained with methylaminoazobenzene as a substrate (Takanaka et al., 1982).

As shown in table 3.3 the isolated leukocyte granules also mediated *in vitro* oxidation of ^{14}C-benzidine to products that bind to calf thymus DNA in the presence of H_2O_2. Binding of ^{14}C-benzidine oxidation products to DNA by a peroxidase system is very rapid (even at very low peroxidase) and reaches a maximum within two minutes (Tsuruta et al., 1985). Addition of myeloperoxidase inhibitors, cyanide and azide, resulted in a drastic inhibition of binding. The detergent cetavlon is known to release myeloperoxidase from the leukocyte granules (Harrison and Shultz, 1976) and this treatment resulted in a 2.5 fold enhanced binding of benzidine oxidation products to DNA. The latter binding amounted to 10% of the benzidine trapped. Other possible pathways of activation were investigated. NADPH did not catalyze binding even though

Table 3.3

Leukocyte granule-catalyzed benzidine oxidation to products that bind calf thymus DNA

Additive	DNAbinding [a] (pmol/mgDNA[Q])
None	<5 [b]
H_2O_2	1442±120
Arachidonate	32±3
NADPH	<5
H_2O_2 + Cetavlon	3861±310
H_2O_2 + KCN	120±21
H_2O_2 +Azide	<5

a : The reaction mixture containing leukocyte granules (0.36 mg protein), calf thymus DNA (2 mg), ^{14}C-benzidine (60 μM) in 2 ml Tris-HCl buffer (0.1 M, pH 7.4) were incubated for 15 minutes at 37° C. The following additives were used as indicated:- H_2O_2 (0.2 mM), arachidonate (0.2 mM), NADPH(0.5 mM), cetavlon (0.025%), cyanide (10 mM) or azide (10 mM). The DNA was isolated as described in Materials and Methods (Section 2.2.2.3). The means of three experiments (leukocyte granules derived from three different leukocyte preparations) are given.

b : Judged to be the limit of detection.

Q : refers to the amount of radioactive material associated with the DNA expressed in terms of the amount of radiolabel material added to the original incubation mixture.

the granules have NADPH oxidase activity (Patriarca et al., 1975). Arachidonate, however, did mediate some binding, presumably as a result of prostaglandin synthase or lipoxygenase activity in the granule fraction (Rossi, Dellabianca and Bellavite, 1981).

Table 3.4 shows that myeloperoxidase/H_2O_2 markedly mediated the *in vitro* oxidation of ^{14}C-arylamines to products that bind to calf-thymus DNA. No binding to DNA, by the arylamine oxidation products, occurred in the absence of peroxidase or H_2O_2. Cyanide or azide also markedly inhibited the peroxidase-mediated binding. From table 3.3 and table 3.4 it can also be seen that the binding of benzidine to DNA catalyzed by peroxidase was ten times higher than that catalyzed by granules presumably because of the competition by granule protein and/or the decomposition of H_2O_2 by the catalase activity associated with the granules.

Since both azide and cyanide are good nucleophiles, the possibility that they competed with DNA, for the intermediates which bound to DNA, was ruled out as the ^{14}C-arylamines or ^{14}C-phenol were recovered from the reaction mixtures after extraction with ethyl acetate. Azide and cyanide, therefore, inhibit binding of arylamine or phenol oxidation products to DNA by inactivating the peroxidase.

Table 3.4
Effect of various agents on irreversible binding of carcinogenic arylamines to calf thymus DNA.

Additions [a]	(^{14}C-Arylamine bound to DNA(pmol/mg) [b]		
	MAB	Benzidine	Aminofluorene
None	<5 [c]	<5	<5
H_2O_2	<5	<5	<5
Myeloperoxidase (MPO)	<5	<5	<5
H_2O_2 + MPO	1,350±110	36,500±2,100	215±19
H_2O_2 + azide	8±1	7±1	<5
H_2O_2 + MPO + cyanide	40±2	33±3	6±1
H_2O_2 + HRP	1,510±110	35,400±2,300	209±20

a : The reaction mixture contained 2 ml Tris-HCl buffer (0.1 M, pH 7.4) calf thymus DNA (2 mg), MPO or HRP (12 nM) and ^{14}C-MAB (25 μM) or ^{14}C-benzidine (25 μM) or ^{14}C-aminofluorene (5 μM). The following additives were used as indicated: - H_2O_2 (0.05 mM), cyanide (10 mM) or azide (10 mM). The mixture was incubated for 15 minutes at 37°C.

b : DNA was isolated as described in Materials and Methods (Section 2.2.2.3). The mean of 3 experiments are given.

c : Judged to be the limit of detection.

3.3) Discussion :

Interaction of the leukocyte plasma membrane with the tumor promoter PMA results in a cyanide-insensitive respiratory burst with the formation of superoxide, hydroxyl radicals and H_2O_2 and the activation of arachidonic acid cascades forming prostaglandins, thromboxanes and leukotrienes. In the present study, evidence has been presented that activated oxygen formation can result in the irreversible binding of oxidation products of carcinogenic arylamines and phenol to leukocyte DNA. The inhibition of binding of arylamine carcinogens and phenol to DNA in leukocytes at higher concentrations of cyanide (10 mM) and azide (10 mM) that result in myeloperoxidase inactivation in leukocytes (Nauseef, Metcalfe and Root, 1983), suggests that the oxidation of carcinogenic arylamines may be catalyzed by myeloperoxidase. The enhancement in binding to DNA, by arylamine or phenol oxidation products in leukocytes, by low concentrations of azide (1 mM) and to some extent by cyanide (1 mM) which inhibit catalase (Nauseef, Metcalfe and Root, 1983) also suggests the involvement of hydrogen peroxide in the activation mechanism.

It has been shown by other investigators that DNA fragmentation occurs in leukocytes following PMA treatment presumably as a result of activated oxygen formation (Rinboim, 1982), although no chromosome aberrations have been found(Kinsella, Gainer and Butler, 1983). The present study suggests

that under certain circumstances the tumor promoter PMA could increase initiation in the carcinogenic process. This has been shown *in vivo* when the carcinogen is administered within 24 hours of PMA administration (Kinsella, Gainer and Butler, 1983; Pound, 1968).

Antioxidants markedly inhibit carcinogenesis *in vivo* (Wattenberg, 1981). Whilst several mechanisms have been suggested with regard to the two-electron oxidation pathway, the above results suggest an additional mechanism, involving inhibition of the oxygen activation mechanism by antioxidants, which prevent activation of carcinogens by a one-electron oxidation.

The intracellular hydrogen donors ascorbate and glutathione prevent the binding of benzidine or phenol oxidation products to DNA following a peroxidase/H_2O_2-catalyzed activation as a result of reduction of the reactive oxidized benzidine (Tsuruta et al., 1985) or phenol species (chapter 4) involved in binding to DNA or conjugate formation with GSH. Presumably the binding of arylamine or phenol oxidation products to DNA in leukocytes, reported in the present study, occurs following the oxidation of these intracellular donors or GSH conjugate formation. The concentration of intracellular GSH and ascorbate has been reported to be 4 mM and 2 mM respectively (Dechatelet et al., 1974; Oliver et al., 1977). However, it is likely that these levels are markedly decreased following leukocyte activation (Mendelson, Metz and Sagone, 1977).

CHAPTER 4

PEROXIDASE-CATALYZED OXIDATION OF PHENOL TO PRODUCTS THAT BIND TO DNA.

4.1) Introduction :

Benzene, a widespread environmental pollutant (Berlin, Cage and Johnson, 1974), induces bone marrow depression and leukemia in human-beings (Laskin and Goldstein, 1977; Snyder and Kocsis, 1975; Snyder et al., 1977) and rodents (Cronkite et al., 1984; Goldstein and Snyder, 1982). Zymbal gland carcinomas have been observed in rats following exposure to benzene by inhalation (Maltoni et al., 1982; 1983). Benzene is a simple aromatic hydrocarbon and, thus, attracted many researchers to investigate the biochemical mechanisms of chemical carcinogenesis (reviewed in Sawahata, Rickert and Greenlee, 1985). Like many other carcinogens, benzene also requires metabolic activation to exert its toxic effects. It is believed that one or more reactive metabolites that interact with the cellular nucleophiles, formed from benzene, could be the likely candidates for the observed toxicity and carcinogenicity. Benzene is metabolized to phenol in liver (Gonasun et al., 1973) and lung (Harper, Drew and Fouts, 1975) by the microsomal cytochrome P-450 monooxygenase system. The metabolism of benzene to phenol has been postulated to proceed through

a reactive epoxide intermediate (Snyder and Kocsis, 1975). However, studies by Tunek et al. (1979) indicated that a metabolite of phenol may be the reactive metabolite which covalently binds to tissue nucleophiles. Phenol was shown to be metabolized to catechol and hydroquinone which were oxidized to reactive benzosemiquinones and quinones which bind to microsomal proteins (Sawahata and Neal, 1983; Tunek et al., 1980). In view of the low amounts of cytochrome P-450 and low metabolism of benzene in bone marrow (Andrews, Sasame and Gillette 1979; Irons et al., 1980), it was proposed that metabolites from liver and lung may be transported to bone marrow. Activation of benzene metabolites in bone marrow could lead to the specific organ toxicity. However, it was recently shown that quantitatively significant amounts of phenol were produced when benzene was incubated with rabbit bone marrow microsomes and that the bone marrow cytochrome P-450 monooxygenase was responsible for the phenol formation (Gollmer, Graf and Ullrich, 1984). Phenol was found to be the major metabolite in bone marrow after inhalation of benzene by rat (Rickert et al., 1979).

Epidemiological studies indicate that benzene causes bone marrow toxicity and leukemia in human-beings. The leukemia most predominantly found is acute myelogenous leukemia (Infante, 1978; Vigliani, 1976). Myeloperoxidase activity has been used as a cytochemical myeloid marker for this type of leukemia (Zittoun et al., 1976). The peroxidase activity of bone marrow is

exclusively associated with leukocytes (Bentfeld, Nichols and Bainton, 1977) and 90% of the leukocytes in the body are present in the bone marrow (Erslev 1974; Sietz, 1969). Benzene also causes Zymbal gland carcinomas in rats (Maltoni et al., 1982). This tissue contains high amounts of lactoperoxidase (Osborne, Metzler and Neumann, 1980) and low mixed-function oxidase activity (Kreig et al., 1978). Sawahata and Neal (1982) reported that the peroxidase of bone marrow can metabolize phenol to biphenols and result in binding of oxidation products to proteins.

Although the relationship between industrial exposure to benzene and leukemia in man is well established (reviewed by Snyder et al., 1977) the understanding of the mechanism of benzene induced carcinogenicity has been hampered, primarily because of lack of a suitable animal model. Earlier studies, on laboratory animals, to induce leukemia with benzene, were consistently negative. However, in recent years, evidence has been presented that benzene can induce leukemia in rodents (Goldstein and Snyder, 1982; Cronkite et al, 1984). Maltoni et al. (1983) have conducted a long-term study in Sprague-Dawley rats after oral dosing with 50 or 250 mg/kg of benzene. From the age of 12-13 weeks, animals were dosed 4-5 days/week for 52 weeks and then observed until death. A dose-related increase in Zymbal gland carcinomas were observed in these rats and small incidences of tumours in mammary glands, liver and oral cavity were also observed. These investigators (Maltoni

et al., 1983) suggested that benzene may be a multi-potential carcinogen. Similar effects were observed in a recently reported bioassay conducted by the U.S. National Technical Programme Report (NTP 289, 1985).

Another problem frequently encountered in understanding benzene induced carcinogenesis is the lack of carcinogenic effects, both in animals and humans, by the metabolites of benzene. As stated above, phenol, hydroquinone and catechol are the metabolites detected *in vivo* of benzene. Phenol was found to be the major metabolite of benzene. Phenol was found to be non-carcinogenic in rats and mice when orally administered in drinking water at 2500 ppm to 5000 ppm for 103 weeks (U.S. National Cancer Institute Bioassay Technical Report, 203, 1980). It should be noted that the majority of the phenol gets conjugated to glucuronides and sulfates and excreted through urine. Hydroquinone and catechol would also be expected to be eliminated this way and thus these hydrophilic metabolites may not reach the bone marrow. An excellent review in this regard has been presented (Snyder et al., 1977). It appears that when benzene was given alone, the concentration of benzene metabolites in bone marrow exceeded that in any other organ including liver, a major site of benzene metabolism. The concentration of benzene metabolites in bone marrow was approximately 10 fold greater than the blood concentration. When metabolites were administered subcutaneously or intraperitoneally, they did not accumulate in bone marrow. Administered phenolic

conjugates also did not accumulate in bone marrow. It was shown in dogs, chronically exposed to benzene, that benzene concentration was approximately 20-fold higher in bone marrow than in blood (Schrenk et al., 1941). These observations, coupled with the reports of Andrews, Sasame and Gillette (1979) and Gollmer, Graf and Ullrich (1984) that benzene hydroxylase (a form of mixed-function oxidase) involved in benzene hydroxylation, was identified in bone marrow, suggest that the bone marrow itself may be the site at which toxic or carcinogenic metabolites of benzene are formed. Assuming the lungs as the primary means of entry of benzene into the body, it was suggested earlier (Cohen, Freedman and Goldstein, 1978), that benzene may pass through the bone marrow before it reaches liver for possible detoxification. Moreover, the increase in benzene concentration found in the marrow may be associated with the high lipid solubility of the agent and the high proportion of fat cells in this location.

Although the evidence points to the production of a specific metabolite(s) that may be responsible for toxicity or carcinogenicity, the exact nature of the metabolite(s) has yet to be determined. Benzene epoxide as a reactive metabolite has been ruled out by Tunek et al. (1979), and they suggested that a further metabolite of hydroquinone may be the reactive metabolite.

Despite some evidence against genomic DNA as the critical target in chemical carcinogenesis (Cairns, 1981), most investigators believe that

alteration of cellular DNA is the critical step in the initiation of carcinogenesis (Bresnick and Eastman, 1982). Metabolites of benzene have been shown to bind to DNA, RNA (Arfellini et al., 1985; Gill and Ahmed, 1981; Lutz and Schlatter, 1977) and proteins (Sawahata and Neal, 1983; Tunek et al., 1980) *in vivo*, both in liver and bone marrow. Relatively higher binding to bone marrow nucleic acids and negligible binding to liver nucleic acids was found when ^{14}C-benzene was administered subcutaneously (Gill and Ahmed, 1981). Binding of benzene oxidation products to protein and glutathione was reported *in vitro* when benzene was activated with liver microsomes (Tunek et al., 1980). Binding to protein was also found in bone marrow homogenate (Sawahata and Neal, 1982), when phenol was activated by bone marrow homogenate and H_2O_2. Binding to nucleic acids *in vitro* by benzene metabolites has been reported to occur by Arfellini et al. (1985), but this report is contradictory to an earlier report by Tunek et al. (1979).

In vitro mutagenic studies using Salmonella/S-9 or microsomal systems, have resulted in conflicting results with benzene as well as benzene metabolites, phenol, hydroquinone and catechol (reviewed by Dean, 1985). Thus, the understanding of benzene metabolism in relation to its toxicity or carcinogenicity has been hampered by the lack of a suitable *in vitro* model system.

Horseradish peroxidase has been used as a model peroxidase for peroxidase-catalyzed drug and carcinogen oxidations (Floyd, Soong and Culver,

1976; Jellinck and Fletcher, 1971; O'Brien, 1984; Norymberski, 1977; Roerig, Reak and Wang, 1976; Rogan et al., 1979). In this chapter it is shown that extensive binding of ^{14}C-phenol metabolites to DNA occurs following activation with horseradish peroxidase and H_2O_2. However, no binding of ^{14}C-phenol metabolites to DNA occurred following metabolism of phenol with liver microsomes and NADPH or cumene hydroperoxide. The reactivity of peroxidase/H_2O_2-catalyzed oxidation of phenol to products that bind to various biomolecules are also compared.

4.2) Results:

Table 4.1 shows a comparison of mixed-function oxidase and peroxidase-catalyzed ^{14}C-phenol binding to DNA and protein. Rat liver microsomes in the presence of NADPH catalyzed the binding of some radioactivity to protein but not to DNA. In the absence of NADPH or with heat denatured microsomes, there was little radioactivity associated with protein indicating very little non-enzymic oxidation of phenol. These results indicated that rat liver microsomes in the presence of NADPH activate ^{14}C-phenol to reactive metabolites which bind to protein. However, the reactive products did not bind to DNA under the conditions studied.

Organic hydroperoxides in the presence of cytochrome P-450 can also oxi-

Table 4.1

A comparison of mixed-function oxidase and peroxidase-catalyzed binding of ^{14}C-phenol to DNA and protein.

Incubation system	Binding to DNA (nmol/mg)	Binding to Protein (nmol/mg)
RLM + ----	0.00	0.03±0.01
BRLM + NADPH	0.00	0.02±0.01
RLM + NADPH	0.00	5.80±0.40
BRLM + CHP	0.00	0.05±0.02
RLM + CHP	0.00	20.5±3.54
TYR	0.00	42.4±5.6
HRP + ----	0.00	*0.03±0.01
---- + H_2O_2	0.00	*0.03±0.01
HRP + H_2O_2	135.5±12.5	*160.6±15.4

RLM: Rat Liver Microsomes; BRLM: Boiled Rat Liver Microsomes; NADPH: Reduced Nicotinamide Adenine Dinucleotide; CHP: Cumene Hydroperoxide; TYR: Tyrosinase.

^{14}C-Phenol (0.2 mM) was incubated with rat liver microsomes (1 mg/ml.) and NADPH (0.5 mM) or with rat liver microsomes (1 mg/ml.) and cumene hydroperoxide or with tyrosinase (50 μg) or with HRP (10 μg) and H_2O_2 (0.5 mM) in the presence or in the absence of DNA in 3.0 ml. of 0.1 M Tris-HCl buffer pH 7.4 for 15 minutes at 37 °C.

Binding to DNA and protein was determined as described in Materials and Methods (Sections 2.2.2.3 and 2.2.3.2).

Mean ± S.E.M. for 3 experiments are given.

* Boiled Microsomes were used as a source of protein to determine the protein binding.

dize various xenobiotics (O'Brien, 1982). Table 4.1 shows that cumene hydroperoxide in the presence of rat liver microsomes mediates the binding of ^{14}C-phenol oxidation products to protein. The binding is four fold higher as compared to that of in the presence of NADPH. However, again the products did not bind to DNA under the reaction conditions studied.

Oxidation of ^{14}C-phenol by horseradish peroxidase/H_2O_2 resulted in approximately 65% of the radioactivity binding to exogenously added calf-thymus DNA. The radioactivity was also bound to protein if heat denatured rat liver microsomes were substituted for DNA. No binding to DNA or negligible binding to protein occurred in the absence of HRP or H_2O_2, showing that the process is mediated by HRP and that the binding is dependent upon H_2O_2. These results indicate that phenol can be activated to reactive products that bind to DNA.

Oxidation of phenol with tyrosinase is also studied for a comparison since this enzyme specifically hydroxylates at the *ortho* position to form catechol (Sanada et al., 1972). Catechol is further oxidized by tyrosinase to o-benzoquinone. As shown in table 4.1 the oxidation of phenol by tyrosinase resulted in products that can bind to protein but not to DNA.

Horseradish peroxidase/H_2O_2-mediated ^{14}C-phenol oxidation to products that bind to DNA was investigated. Several variables were examined in order

to find the optimal conditions. Figure 4.1 shows that the rate of binding to DNA was dependent upon the peroxidase concentration. In the presence of 10 μg peroxidase the binding was linear for 1 minute and reached a plateau within 3 minutes. In the presence of only 0.1 μg peroxidase the binding was linear for 30 minutes before reaching a plateau. Figure 4.2 shows that phenol binds optimally at an equimolar concentration of H_2O_2. Any further increase in hydrogen peroxide concentration did not affect the binding. Figure 4.3 shows the effect of pH on phenol oxidation products binding to DNA. As the figure shows, the binding of phenol oxidation products to DNA was not significantly affected over a wide range of pH.

Initial observations showed that most of the phenol oxidized by the peroxidase/H_2O_2 system precipitates into dark-brown coloured, granular specks in the absence of DNA. These precipitates are considered to be polymers (Joschek and Miller, 1966; Klibanov, Tsuman-Tu and Scott, 1983; Saunders, Holmer-Siedle and Stark, 1964). Upon extraction with ethyl acetate, these precipitates remained at the interphase of the organic and aqueous layers. More than 98% of the radioactivity was removed from the aqueous layer just by a single extraction. Figure 4.4 shows the distribution of radioactivity in the precipitate and the organic layer, when the incubations were performed at 20°C in the absence of DNA and the mixtures were extracted with ethyl acetate at different times of incubations. As shown in the figure, the formation of precip-

Figure 4.1

Effect of peroxidase concentration and incubation time on peroxidase/H_2O_2 mediated [U-^{14}C]Phenol binding to DNA:

Incubations were carried out in a total volume of 3 ml 0.1 M Tris-HCl buffer pH 7.4 at 37 °C. The following additions were made: phenol (0.2 mM), HRP (concentrations as indicated in the figure), and DNA (3 mg). Reactions were started by the addition of H_2O_2 (0.5 mM). Note: The added phenol had a specific activity of 0.0015 μCi/nmole. The same specific activity is used consistently.

● ● ● : HRP (10 μg); X X X : HRP (1 μg); ■ ■ ■ : HRP (0.1 μg).

Quantitative measurement of irreversible binding to DNA was performed as described in Materials and Methods (section 2.2.2.3).

Results are expressed as Means ± S.D. n=3.

FIGURE 4.1

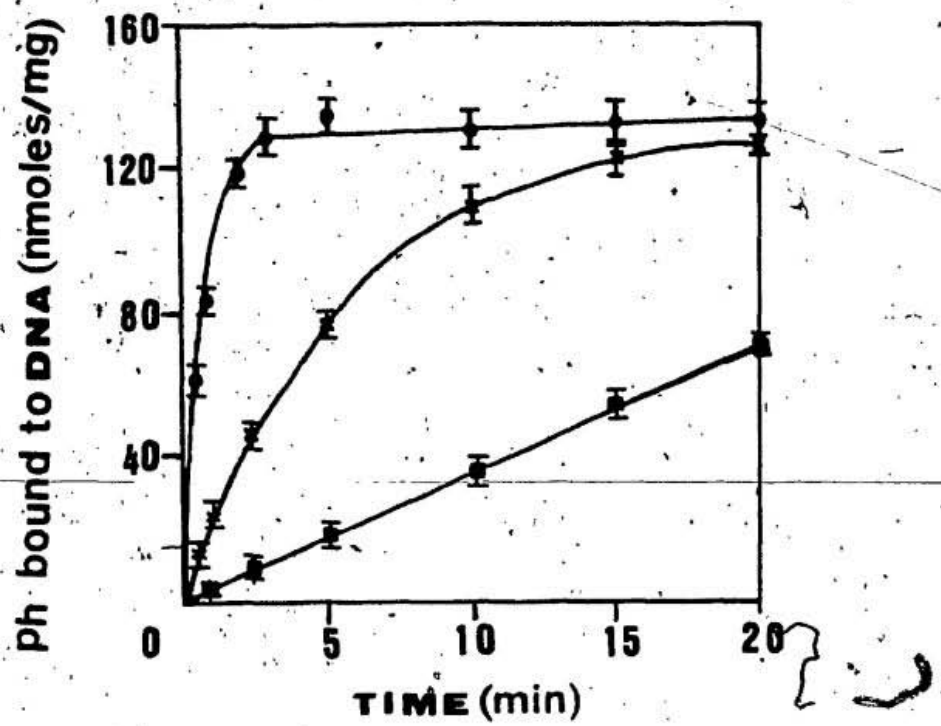


Figure 4.2

Effect of H_2O_2 concentration on [U- ^{14}C]Phenol binding to DNA catalyzed by peroxidase:

Incubation mixtures contained phenol, HRP (10 μg), and DNA (3 mg). in 3 ml of 0.1 M Tris-HCl buffer pH 7.4. Reactions were started by the addition of H_2O_2.

●●●: Phenol (0.2 mM); ▲▲▲: Phenol (0.1 mM).

Results are expressed as Means ± S.D. n=3.

FIGURE 4.2

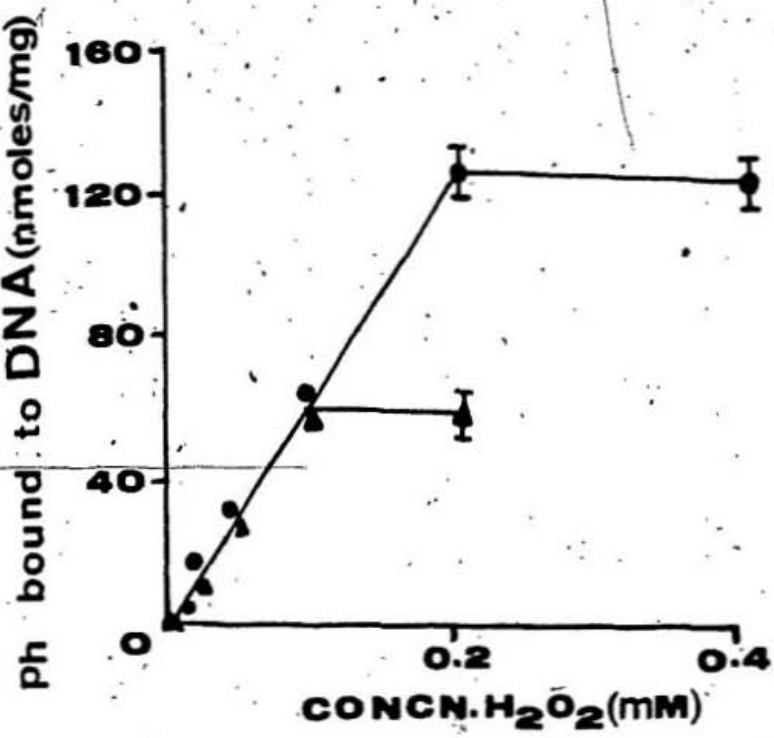


Figure 4.3

Effect of pH on the [U- ^{14}C]Phenol binding to DNA catalyzed by peroxidase/H_2O_2:

The reaction mixtures contained phenol (200 μM), HRP (10 μg), H_2O_2 (220 μM) and DNA (3 mg) in 3 ml of 0.1 M Tris-HCl buffer (pH range 7.0 - 10.0) or in 3 ml of 0.1 M Sodium acetate buffer (pH range 3.0 - 6.0). Reactions were started by the addition of H_2O_2 and incubated for 15 minutes at 37 °C.

FIGURE 4.3

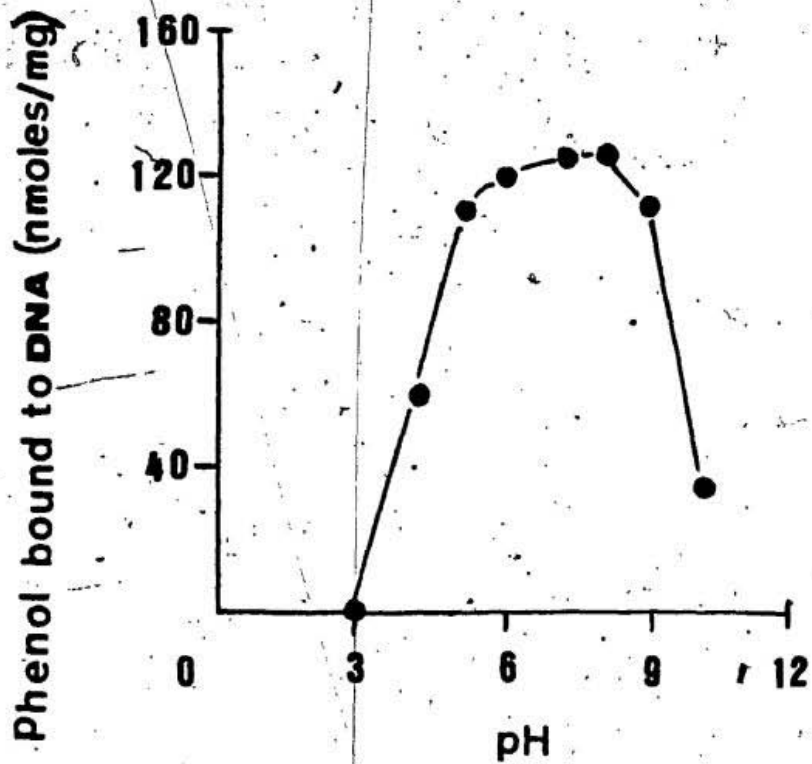


Figure 4.4

Effect of time on distribution of radioactivity in aqueous and organic layers of peroxidase/H_2O_2-oxidized phenol extracted with ethyl acetate:

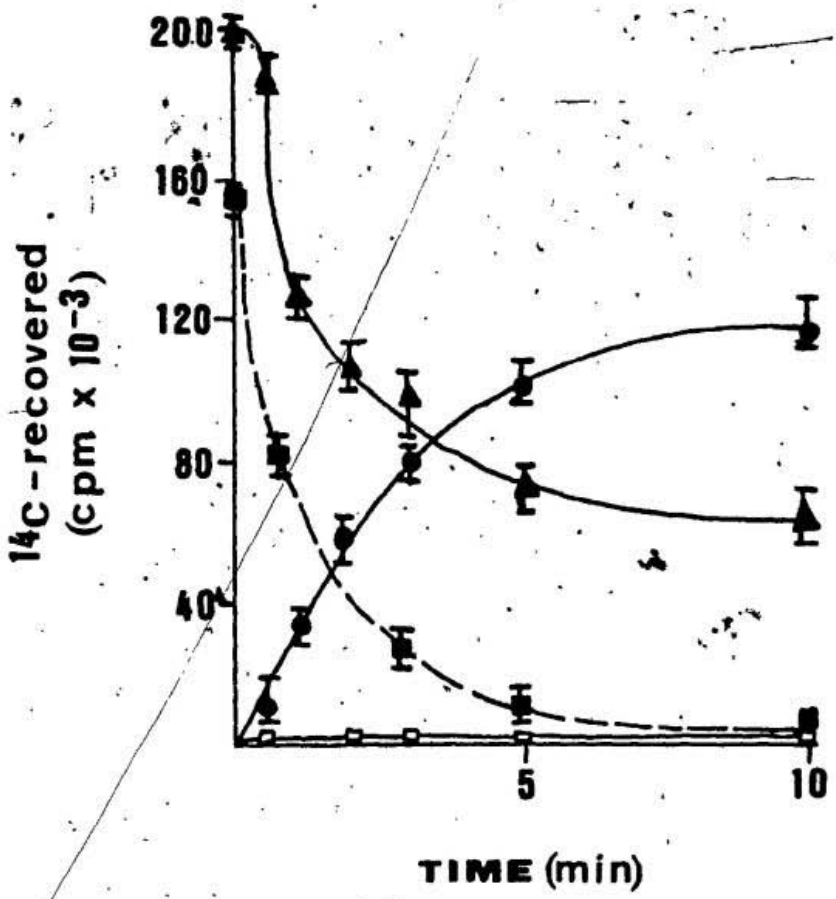
Reaction mixtures contained phenol (0.2 mM), HRP (10 μg) and H_2O_2 (0.2 mM) in a 3 ml of 0.1 M Tris-HCl buffer pH 7.4. Incubations were carried out at 20 ° C for different times before extraction with ethyl acetate.

▲ ▲ ▲ : Radioactivity in the organic layer; ● ● ● : Radioactivity in the insoluble polymer at the interphase of organic and aqueous layers and recovered by filtration; □ □ □ : Radioactivity in the aqueous layer;

Some incubations were carried out with DNA added at different times of incubation: ■ ■ ■ : Radioactivity recovered in DNA if DNA was added at different times of incubation.

Results are expressed as Means ± S.D. n=3.

FIGURE 4.4



itate was linear up to 3 minutes with the maximum amount being formed at around 5 minutes. Up to 60-70% of the phenol could be recovered as precipitate. However, if the incubations were performed at either 30°C or at 37°C, the maximum precipitate formation occurred only within 3 minutes. The aqueous reaction mixtures, after extraction with ethyl acetate (to remove unchanged phenol and biphenols), were assayed quantitatively for peroxidase activity using 3,3',5,5',-tetramethylbenzidine and following the rate of oxidation at 655 nm in the presence of H_2O_2 (Andrews and Krinsky, 1982). It was found that 80-90% of peroxidase was inactivated when incubations were performed at 20°C. However, little or no inactivation of peroxidase occurred at either 30°C or 37°C. This indicated that the rate of precipitation is dependent upon both the concentration of enzyme and the temperature. At higher temperatures, the rate of precipitation was faster than the inactivation of the enzyme and at low temperatures the rate of inactivation of the enzyme is higher than the rate of precipitation. Consequently, the decrease of the radioactivity in the organic layer was faster at 30°C or at 37°C than at 20°C. The broken line in Figure 4.4 shows the radioactivity recovered in DNA if the DNA was added at different times of incubation. As can been seen, less than 10% of the added radioactivity was recovered in DNA if the DNA was added after 5 minutes of incubation. This indicates that the reactive intermediates responsible for binding are highly unstable and short-lived. This result also shows that the unbound phenol products are effectively

removed during the isolation procedure. The life-time of the reactive intermediates closely matched the rate of precipitate formation, indicating that the intermediates in precipitate formation bind to DNA.

Figure 4.5 shows the distribution of radioactivity as a function of time when the incubations were performed in the presence of DNA at 20° C and extracted with ethyl acetate at different times of incubation. As shown in the figure, in contrast to that in the absence of DNA, the radioactivity in the aqueous layer after extractions was found to increase with time reaching a maximum within 2 to 3 minutes. The remaining radioactivity was recovered in the organic layer. It appears that DNA prevents some step(s) in precipitate formation, i.e., the reactive metabolites formed from phenol bind to DNA and thus are being prevented from precipitation. Precipitating the DNA in the aqueous layer resulted in the recovery of all the radioactivity in the DNA. Incubations performed at 30° C or 37° C, however, showed no effect on the distribution of radioactivity in the aqueous and organic layers probably because the DNA protects the peroxidase from inactivation.

Figure 4.6 shows the results of chromatography of the DNA bound to ^{14}C-phenol products on a Sephacryl-300 column (40 x 2.5 cm) eluted with 0.2 M phosphate buffer pH 7.4. Peroxidase activity was measured quantitatively using 3,3',5,5'-tetramethylbenzidine as a substrate and following the rate of oxidation at 655 nm (Andrews and Krinsky, 1982). As can be seen from

Figure 4.5

Effect of DNA on the distribution of radioactivity in the aqueous and organic layers of peroxidase/H_2O_2-oxidized [U-^{14}C]Phenol extracted with ethyl acetate :

Incubation mixtures contained phenol (0.2 mM), HRP (10 μg), H_2O_2 (0.22 mM) and DNA (3 mg) in 3 ml of 0.1 M Tris-HCl buffer pH 7.4. Incubations were carried out at 20 °C for different times before extraction with ethyl acetate.

□ □ □ : Radioactivity in aqueous layer; ▲ ▲ ▲ : Radioactivity in organic layer; ● ● ● : Radioactivity in insoluble polymer recovered by filtration.

Results are expressed as Means ± S.D. n=3.

FIGURE 4.5

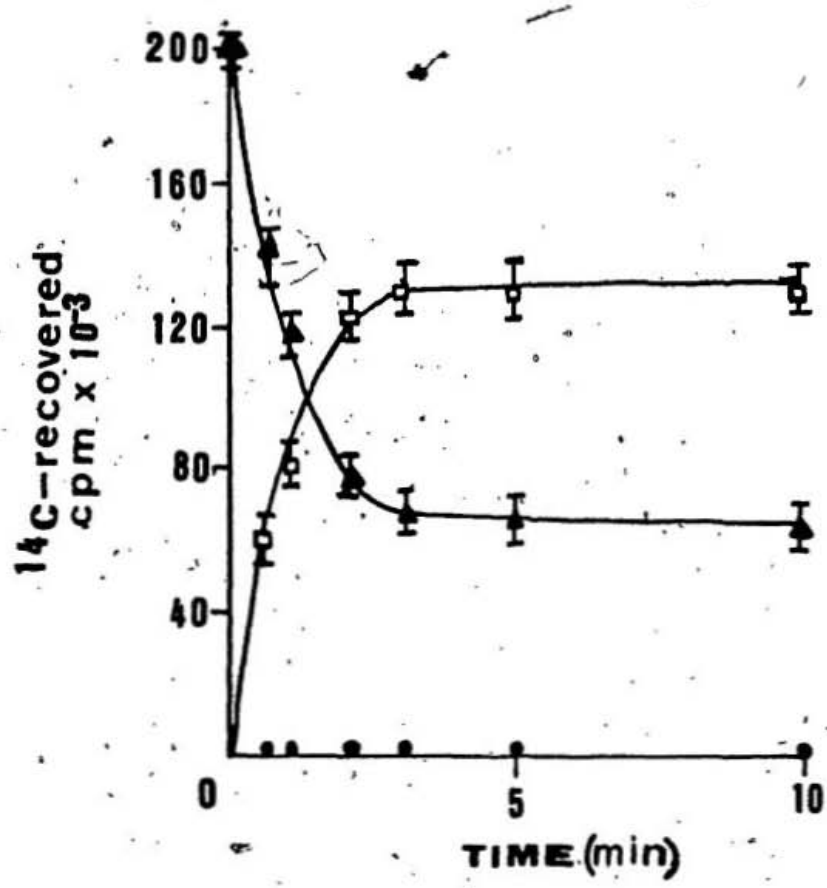


Figure 4.6

Elution profile from Sephacryl-300 column of DNA and peroxidase mixture with bound [U-^{14}C] Phenol products :

Phenol (0.2 mM) was incubated with HRP (10 μg), H_2O_2 (0.22 mM) and DNA (3 mg) at 20°C for 15 minutes in 3 ml Tris-HCl buffer pH 7.4. After ethyl acetate extractions and precipitation with ethanol, DNA was redissolved in 1 ml distilled water and applied on to the Sephacryl-300 column. The samples were eluted with 0.2 M phosphate buffer pH 7.4 containing 0.1 M sodium chloride. The eluted fractions (3.5 ml each) were measured for DNA content at 260 nm and for peroxidase activity. Aliquots of the fractions were used for the measurement of radioactivity.

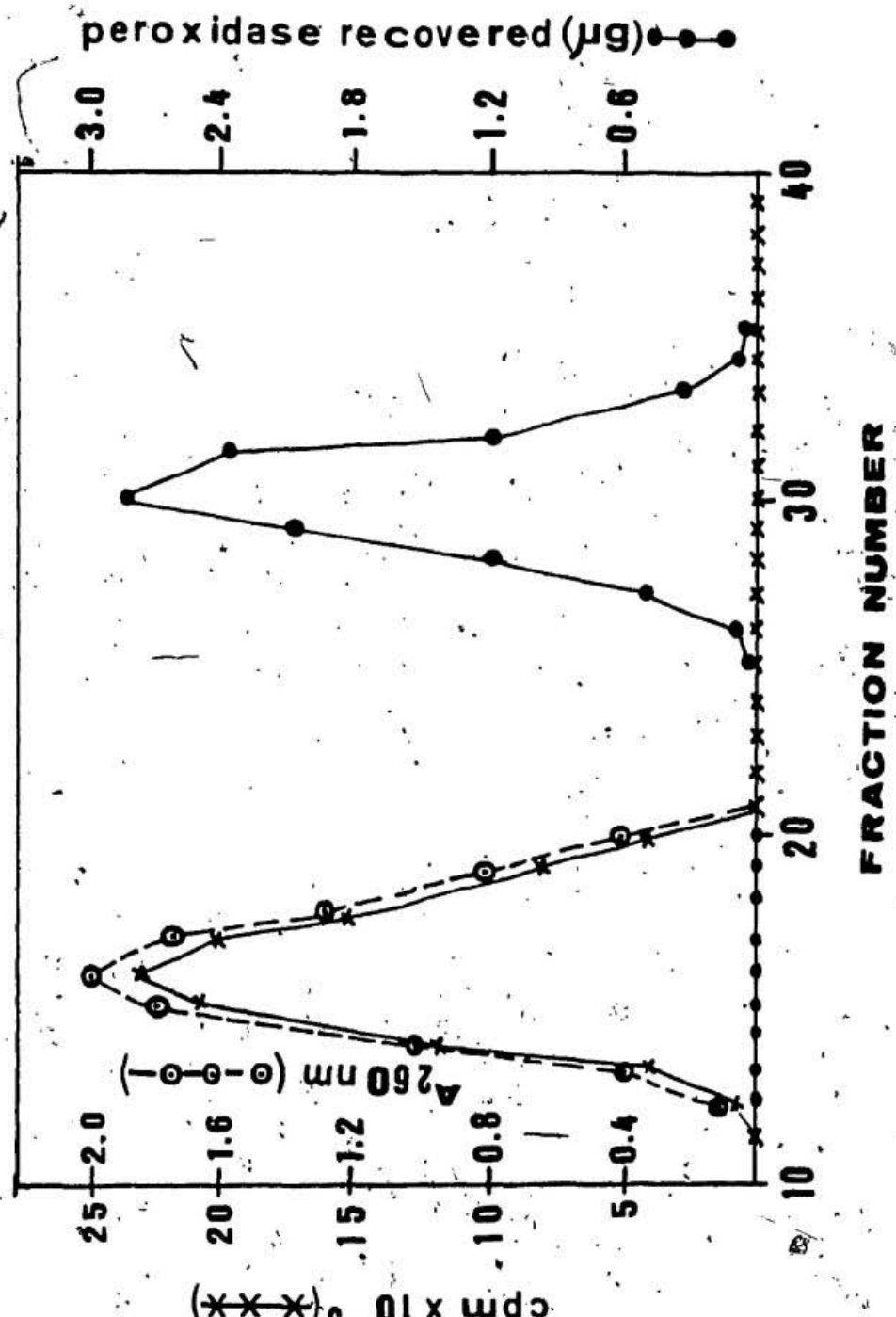

peroxidase recovered (μg)
3.0
2.4
1.8
1.2
0.6
40
30
20
10
FRACTION NUMBER
$A_{280\,nm}$ (o-o-o-)
2.0
1.6
1.2
0.8
0.4
cpm x 10^{-3} (x-x-x)
25
20
15
10
5

FIGURE 4.8

Figure 4.6, the ^{14}C-phenol radioactivity co-eluted with DNA and not with the peroxidase fraction. More than 90% of the peroxidase activity was recovered in the peroxidase fraction and was not bound to the DNA. This shows that all the radioactivity was bound only to DNA and that the observed binding to DNA is not due to phenol oxidation products binding to peroxidase. This result also shows that no inactivation of peroxidase occurred when incubations were carried out at 20°C in the presence of DNA.

The reactivities of t-RNA and various homopolymers of ribonucleotides were also compared (Table 4.2). Binding to various homopolyribonucleotides and t-RNA readily occurred indicating that the reactive intermediates bind to all ribonucleotide bases.

As can be seen from Table 4.3, ascorbate, N-acetylcysteine and glutathione prevented phenol oxidation products binding to DNA. The reaction mixture became colourless on addition of ascorbic acid, but was not affected by glutathione. Glutathione did not inhibit the binding of phenol oxidation products to DNA as a result of being a competitive donor for peroxidase, since it did not affect the amount of phenol oxidized. Fifty per cent of the radioactivity remained in the aqueous phase following ethyl acetate extraction, indicating conjugate formation with the reactive phenol intermediate. With DNA in the reaction mixture, much less DNA conjugate was formed, indicating that the reactive species binding to DNA also forms the GSH conjugate. Similar

Table 4.2
Binding of ^{14}C-phenol oxidation products to macromolecules

Macromolecule	Binding to macromolecule (nmol/mg)*
polyadenylic acid	69.5±7.1
polyguanylic acid	72.0±6.6
polycytidylic acid	43.0±2.5
polyuridylic acid	47.0±3.3
t-RNA	120.0±10.2
DNA	130.0±10.5
single stranded DNA	125.0±12.2
serum albumin	60.0±6.0
polylysine	130.0±12.0

Incubation conditions: ^{14}C-phenol (200 μM) were incubated with peroxidase (10 μg), macromolecule (3 mg) and H_2O_2 (220 μM) for 15 minutes at 20°C in 3 ml Tris HCl buffer pH 7.4. Methodology was described in Materials and Methods (Section 2.2.2.3). The Mean±S.E.M. for 3 experiments are given.

* refers to the amount of radioactive material associated with the DNA expressed in terms of the amount of original labelled compound added to the system.

Table 4.3

Effect of biological donors on horseradish peroxidase/H_2O_2 catalysed phenol binding to DNA

Donor		In the presence of DNA		In the absence of DNA	
		%Phenol [a] oxidized	%Binding to [b] DNA	%Phenol [a] oxidized	%retained in [c] the aqueous layer
None		100	68	100	1±0.5
Ascorbate	0.2 mM	100	65±5.8	100	1±0.5
	0.4 mM	20±2.2	5±2.0	80±5.0	1±0.5
	0.8 mM	0	0	20±2.5	1±0.5
Glutathione	0.2 mM	100	68±5.6	100	25±2.5
	0.4 mM	90±6.0	30±2.9	100	40±4.2
	0.8 mM	80±5.0	20±2.0	75±5.6	50±5.2
N-Acetyl-cysteine	0.2 mM	100	53±4.6	100	30±2.8
	0.4 mM	50±4.8	1±0.5	100	40±4.0
	0.8 mM	30±2.6	0	75±5.8	50±4.6
Proline	2.0 mM	100	68±5.6	100	1±0.5
Lysine	2.0 mM	100	66±5.5	100	1±0.5

a: Analyzed by high pressure liquid chromatography as described in Materials and Methods (Section 2.2.4.1).

b: Binding was performed as described in Materials and Methods(Section 2.2.2.3) using 0.2 mM phenol except that 0.4 mM H_2O_2 was used.

c: After extraction thrice with ethyl acetate, radioactivity in the aqueous layers was measured by scintillation counting.

Mean±S.E.M. for 3 experiments are given.

results were obtained with N-acetylcysteine. Ascorbate also prevented phenol oxidation products binding to DNA; however, all the radioactivity was extracted into ethyl acetate and phenol oxidation was completely inhibited. Little oxidation of ascorbate (measured at 265 nm) was seen at pH 7.4 by the peroxidase/H_2O_2 system but rapid oxidation occurred in the presence of phenol. Ascorbate therefore does not inhibit the phenol oxidation and binding to DNA by being a competitive donor for peroxidase, but does so by reducing the reactive oxidized phenol intermediates back to phenol. Glutathione and N-acetylcysteine also partly inhibited the rate of phenol oxidation so that this protection of binding to DNA is due to reduction of the reactive phenol intermediates and conjugate formation. Lysine and proline did not inhibit the binding of phenol oxidation products to DNA even though they readily form conjugates with quinones (Mason and Peterson 1965; Rees and Pirie, 1967). This suggests that quinones are not the reactive phenolic intermediates involved in binding to DNA.

4.3) Discussion :

The above results indicate that phenol can be oxidized by peroxidase/H_2O_2 to products that bind irreversibly to DNA. As shown in Table 4.3, approximately 65% of the phenol oxidation products bound to DNA. This works out to a binding level of 150 nmols per mg DNA, which

corresponds to 1 phenol derivative bound per 25 nucleotides. Thus, extensive *in vitro* binding to DNA of a metabolite of benzene, a weak carcinogen, is reported for the first time. Binding to various homopolyribonucleotides and t-RNA also readily occurred (Table 4.2) indicating that the reactive intermediates could bind to all ribonucleotide bases. The reactive metabolites also seem to form thiol conjugates (Table 4.3). An analysis of one or more of these conjugates should reveal the reactive phenol metabolites involved. In chapter 8 the structure of a GSH-conjugate formed with a p,p'-biphenol oxidation product is identified. Sawahata and Neal (1982) proposed that p,p'-biphenoquinone, the further oxidation product of p,p'-biphenol, could be one of the reactive metabolites responsible for benzene induced bone marrow toxicity. However, as shown in table 4.3 lysine and proline did not inhibit the phenol oxidation products binding to DNA, although they readily form conjugates with quinones (Mason and Peterson 1965; Rees and Pirie, 1967). This suggests that quinones may not be the reactive products that bind to the DNA during peroxidase/H_2O_2-mediated oxidation of phenol in the presence of DNA.

Activation of phenol with rat liver microsomes and NADPH, however, did not result in phenol oxidation products binding to DNA, although protein binding occurs. Similar levels of protein binding were reported by other investigators (Sawahata and Neal 1983; Tunek et al., 1980). They found that phenol was initially hydroxylated by liver cytochrome P-450 monooxygenase to

hydroquinone and catechol in a 20:1 ratio. Further oxidation of hydroquinone and catechol by cytochrome P-450 monooxygenase formed benzosemiquinones and/or benzoquinones which bound to microsomal protein and formed a glutathione conjugate. Incubation of rat liver microsomes with benzene and NADPH also formed the same reactive products (Tunek et al., 1980).

The present study also showed that rat liver microsomes in the presence of cumene hydroperoxide activate phenol to reactive products which bind to protein. However, the products did not bind to the DNA under the conditions studied. Highly purified rat liver cytochrome P-450 (kindly provided by Dr. Anver D. Rahimtula) in the presence of cumene hydroperoxide, did not catalyze the oxidation of phenol to products that bind to DNA although extensive binding to P-450 protein occurred (results not shown). Analysis of the products formed (concentrated ethyl acetate extracts were analysed by thin layer chromatography using the solvent system benzene:methanol:acetic acid 70:8:4) showed that hydroquinone and catechol were formed in this system. Hydroquinone was formed in approximately ten times higher amounts than catechol (results not shown). The presence of ascorbate in the incubation mixture was required for maximal hydroquinone and catechol formation. Presumably, hydroquinone and catechol are rapidly oxidized peroxidatively as soon as they are formed. Ascorbate also inhibited ^{14}C-phenol binding to protein, probably by reducing the reactive benzoquinones back to hydroquinone and

catechol. Benzene, however, is not hydroxylated by rat liver microsomes and cumene hydroperoxide (McCarthy and White, 1983). Benzene also did not bind to microsomal proteins or DNA in the presence of rat liver microsomes and cumene hydroperoxide (results not shown). Oxidation of ^{14}C-phenol with tyrosinase also suggested that o-benzoquinone may not bind to DNA.

Phenol is also cooxidized by prostaglandin H synthase during arachidonic acid biotransformation to prostaglandins (Egan et al., 1981; Egan, Gale and Keuhl, 1979; Egan, Paxton and Keuhl, 1976; Smith and Lands, 1971). However, there are no reports on the detailed analysis of the products formed. Egan, Gale and Keuhl (1979) reported that a product with similar properties to a polymer was formed as the major product.

In conclusion, the present study demonstrates for the first time *in vitro* binding of phenol oxidation products to DNA mediated by a peroxidase system. The presence of myeloperoxidase in bone marrow makes this reaction interesting.

CHAPTER 5

PROPERTIES OF PHENOL OXIDATION PRODUCT(S) THAT BIND TO DNA.

5.1) Introduction :

In the previous chapter it was shown that when ^{14}C-phenol was incubated with horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2) in the presence of DNA, approximately 65% of the radioactivity was bound to DNA. The DNA bound products were not extracted into ethyl acetate and were not released from DNA by repeated precipitations. Addition of DNA at different times of incubation however resulted in a decreased radioactivity association with DNA. No radioactivity association with DNA occurred if the DNA was added after 5 minutes of incubation indicating that very highly reactive and short lived products were formed by phenol oxidation with peroxidase and H_2O_2. In the absence of DNA approximately 65% of the radioactivity resulted in precipitate formation, indicating that intermediates involved in the precipitate formation bind to the DNA. Adduct formation occurred also with various homopolyribonucleotides, t-RNA, protein and glutathione. In this chapter the results regarding the nature of the reactive products formed from phenol by peroxidase/H_2O_2 oxidation and the mechanism of their formation, have been

presented.

5.2) Results :

The DNA isolated from the reaction mixture is uniformly dark brown coloured and had an absorbance maximum at 418 nm with a shoulder at 550 nm (Figure 5.1) and a peak at 291 nm (Figure 5.2). Reduction with dithionite before extraction and purification of DNA, resulted in the disappearance of the band at 418 nm (including the shoulder at 550 nm) and a shift in the ultra-violet absorbance maxima to 287 nm with a shoulder at 274 nm (Figure 5.3). Various phenol derivatives were also tested for the ability to bind to DNA following peroxidase/H_2O_2-catalyzed oxidation. The DNA, however, isolated from reaction mixtures containing o-cresol, 2,6-dimethyl phenol, 2,6-dimethoxy phenol, guaiacol, 2-tert-butylphenol, were not coloured in spite of the fact that the reaction mixtures were deeply coloured and formation of precipitates has occurred. Furthermore, the presence of DNA in the reaction mixture did not affect the formation of coloured products or the formation of precipitates. The coloured oxidized products of these substituted phenols therefore do not bind to DNA and DNA does not prevent their formation.

o,o'-Biphenol and p,p'-biphenol have been identified among the products formed during the peroxidase-catalyzed oxidation of phenol (Sawahata and

Figure 5.1

Spectral properties of DNA-bound products from peroxidase-oxidised phenol, p,p'-biphenol and o,o'-biphenol (in the visible region).

——: Phenol (200 μM)/HRP (10 μg)/ H_2O_2 (210 μM)/DNA (3 mg), incubated for 3 minutes before DNA pptn; - - - : o,o'-Biphenol (200 μM)/ HRP (10 μg)/ H_2O_2 (210 μM)/DNA (3 mg), incubated for 30 minutes before DNA pptn;——: p,p'-Biphenol (200μM)/HRP (10 μg)/ H_2O_2 (210 μM)/DNA (3 mg), incubated for 30 minutes before DNA pptn.

FIGURE 5.1

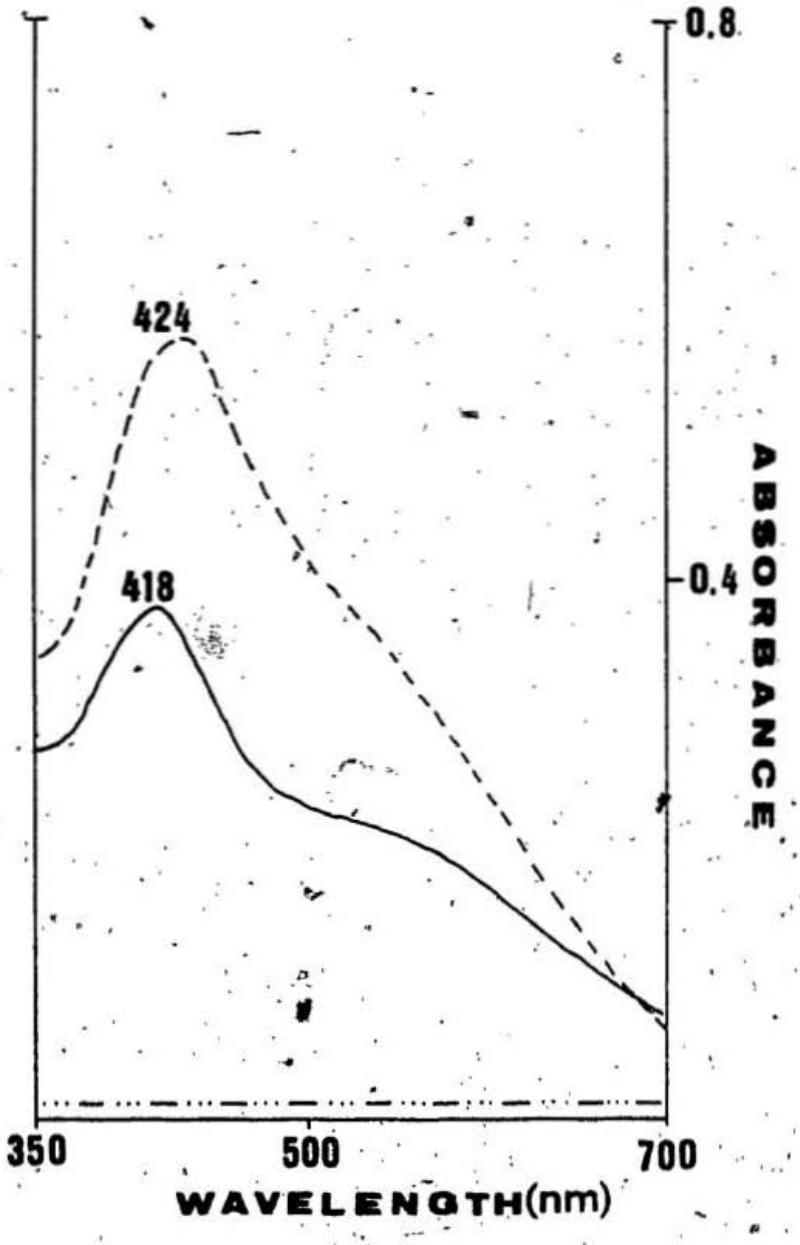
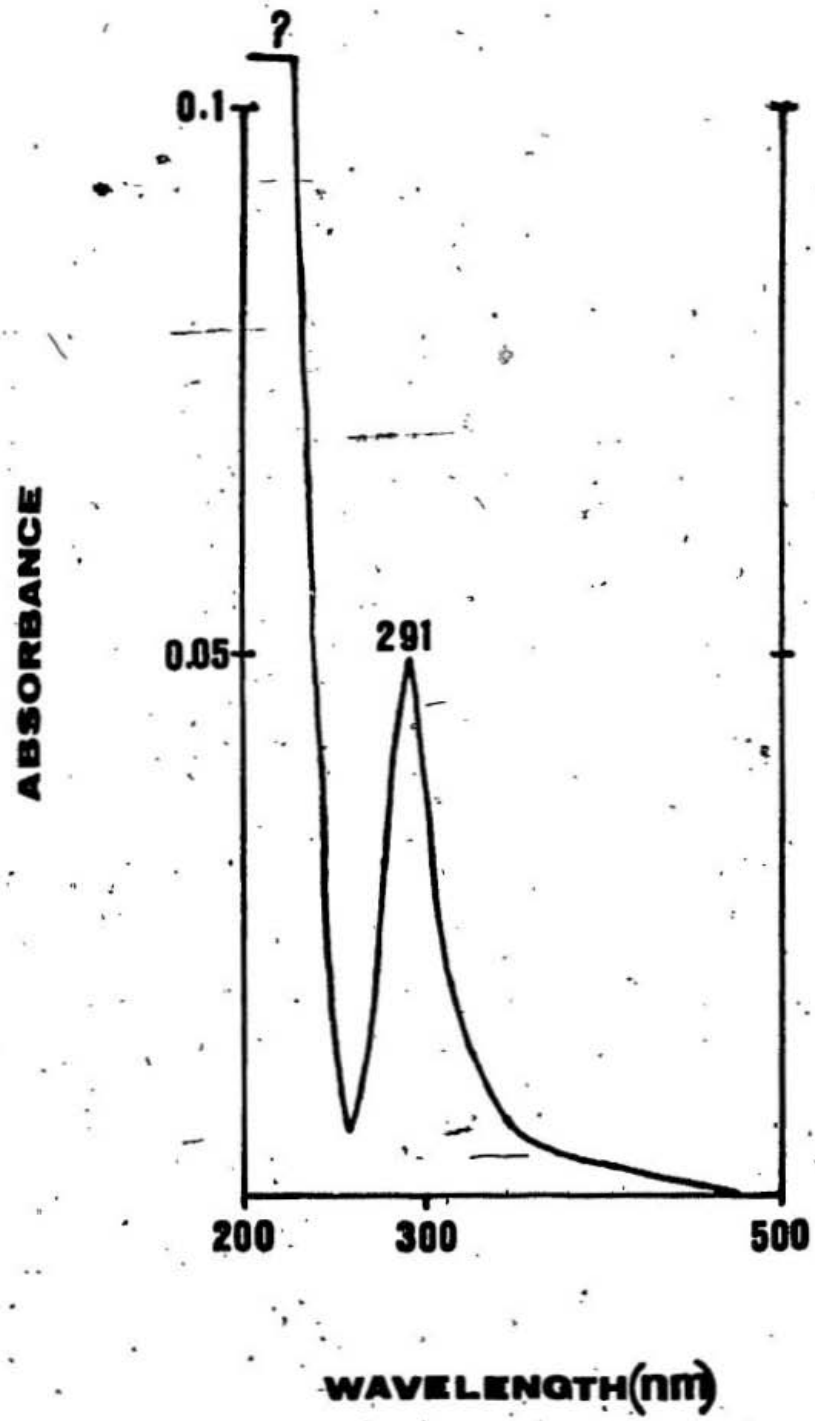


Figure 5.2

Difference spectrum for the DNA-bound oxidized products of phenol.

Reaction conditions for incubation are same as for Figure 5.1. Final solution, after redissolving the DNA, contained 75μg/ml of DNA and scanned against reference solution containing an equal amount of unbound (not bound with phenol oxidation products) DNA.

FIGURE 5.2



Neal, 1982). o,o'-Biphenol also formed brown coloured DNA following a peroxidase oxidation. The o,o'-biphenol:DNA products (Figure 5.1) had a similar absorption to the phenol:DNA product with maxima at 423 nm (with a shoulder at 550 nm) and 287 nm (with a shoulder at 274 nm). Reduction with dithionite resulted in a DNA adduct identical to that formed with phenol (Figure 5.3). On the other hand, ^{14}C-p,p'-biphenol, another phenol oxidation product was ineffective in binding to DNA in the peroxidase system and there was also no change in the spectrum of the DNA (Figure 5.1). These results suggest that the phenol oxidation products binding to DNA are either derived from o,o'-biphenol or are identical to the products formed and binding to DNA from o,o'-biphenol. However, addition of DNA at the end of the reaction to the o,o'-biphenol reaction mixture resulted in no binding of phenol oxidation products to DNA, indicating that DNA must be present during the oxidation before binding occurs.

The nature of the products in the reaction mixture was further investigated. Initial observations showed that yellow and red products were formed during phenol oxidation by HRP/H_2O_2. The yellow coloured product was reduced easily by ascorbate and was extracted into ethyl acetate both in the presence and in the absence of DNA. However, the red coloured product was not easily reduced by ascorbate and was not extracted into ethyl acetate when phenol was incubated in the presence of DNA. o,o'-Biphenol gave similar

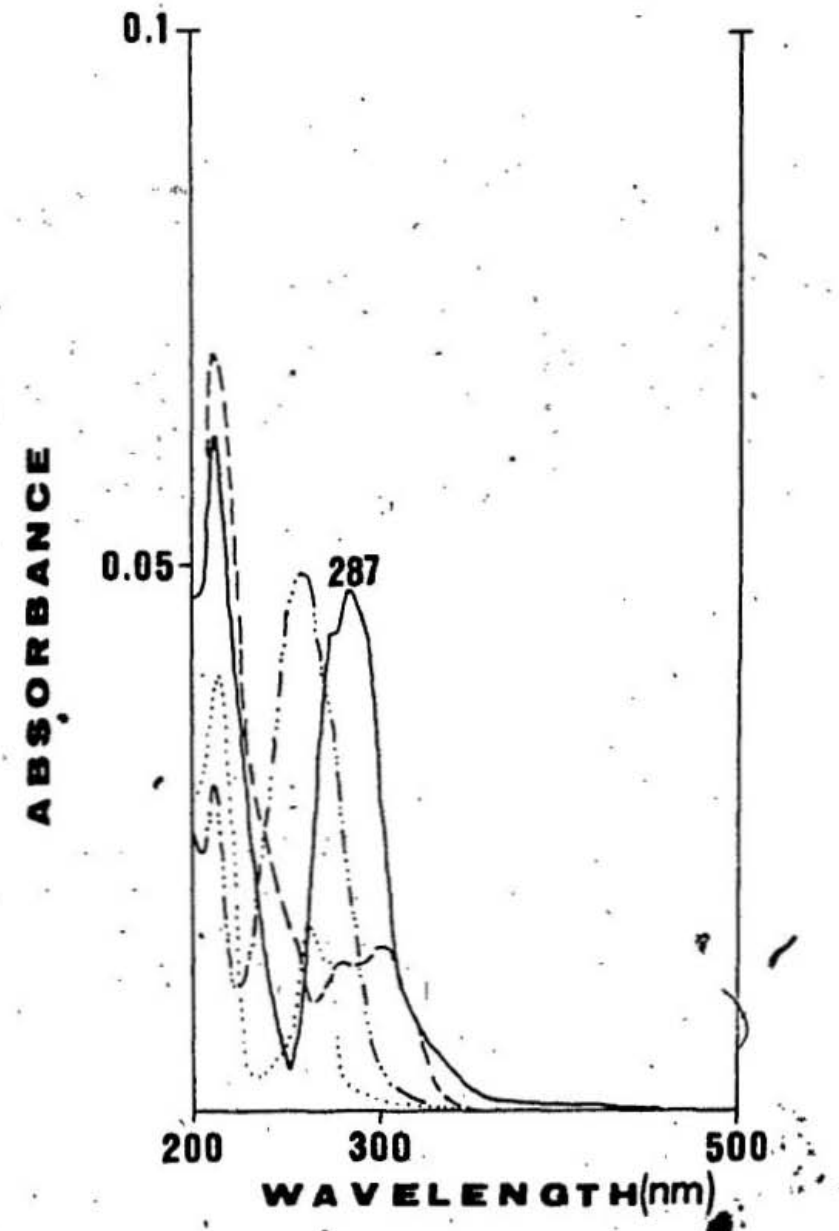
Figure 5.3

Difference spectra for the dithionite-reduced DNA bound phenol or o,o′-biphenol oxidation products.

—— : Phenol or o,o′-biphenol (DNA bound products); ..... : Phenol standard (10 μM); - - - : o,o′-Biphenol standard (2 μM); -·-·-·- : p,p′-Biphenol standard (2 μM).

All the incubations were carried out in 3 ml of 0.1 M Tris-HCl buffer pH 7.4 at 37 °C.

FIGURE 5.3



findings except that the rate of formation of coloured products was slower than that of phenol. p,p'-Biphenol was rapidly oxidized but formed only a yellow coloured product which was easily reduced by ascorbate and was also extracted into ethyl acetate both in the presence and in the absence of DNA.

Table 5.1 shows the percentage of radioactivity incorporated into the products of phenol oxidation extracted by ethyl acetate and separated by thin layer chromatography. As shown in the table phenol was completely oxidized within 3 minutes under the conditions studied. It appears that the red material at the origin (unknown 1, R_F 0.00) and a minor product (unknown 4, R_F 0.62) disappeared when DNA was present in the reaction mixture. The red material remained at the origin could not be eluted into ethyl acetate from the silica gel and is presumed to be polymeric in nature. Precipitate formation was also inhibited in the presence of DNA. These precipitates were not extracted into ethyl acetate from the reaction mixture and remained at the interphase of the aqueous and organic layers. It is concluded that either DNA binds the precipitates formed from phenol, or DNA prevents the formation of the precipitate by binding an intermediate(s) involved in its formation. Similar results were obtained with o,o'-biphenol. However, none of the p,p'-biphenol products (mainly p,p'-biphenoquinone) were affected by the presence of DNA.

Table 5.1
Time course for ^{14}C-Phenol Metabolism by Peroxidase

Product Formed	Without DNA			With DNA		
	30 Sec	1 Min	3 Min	30 Sec	1Min	3 Min
Polymer(insoluble)	20±2.2	40±3.5	65±5.0	-	-	-
o,o'-biphenol	5±0.0	5±0.0	2±0.0	5±0.0	5±0.0	2±0.0
p,p'-biphenol (a)	5±0.0	5±0.0	5±0.0	5±0.0	5±0.0	5±0.0
p,p'-biphenol (b)	10±2.0	15±1.0	20±2.0	13±1.0	15±2.0	22±2.0
p,p'-biphenoquinone (a)	5±1.4	10±2.2	10±2.4	5±1.8	10±2.8	10±2.5
p,p'-biphenoquinone (b)	0	0	0	0	0	0
Unknown 1	10±2.4	4±1.2	2±0.8	2±1.0	1±0.5	0.5±0.5
Unknown 2	5±0.5	5±0.5	5±0.5	5±0.5	5±0.5	5±0.5
Unknown 3	5±0.5	5±0.5	5±0.5	5±0.5	5±0.5	5±0.5
Unknown 4	1±0.2	1±0.4	1±0.4	0	0	0
Phenol oxidized	60±2.5	75±3.4	100±0.0	62±1.4	74±3.0	100±0.0

o,o'-Biphenol and p,p'-biphenol were quantitated by h.p.l.c. (section 2.2.4.1) and t.l.c. (section 2.2.4.2) p,p'-Biphenoquinone was determined by spectrophotometry and t.l.c. Unknowns 1,2,3 and 4 were quantitated by their radioactivity. The radioactivity of the insoluble polymer was measured by filtration.

Incubation conditions: Phenol (0.2 mM) was incubated with HRP (10 μg) and H_2O_2 (0.2 mM) with and without DNA (3 mg) in 3 ml Tris-HCl buffer pH 7.4 for appropriate times as indicated at 30° C. The incubation mixtures were extracted with the ethyl acetate at the end of the incubation period and the ethyl acetate extracts were analyzed for metabolites.

(a) and (b) (for p,p'-biphenol and p,p'-biphenoquinone) indicate the experiments in the absence of ascorbate and in the presence of ascorbate respectively.

Mean ± S.E.M. for 3 experiments expressed as % of total radioactivity incubated.

Table 5.1 also showed that from the original phenol 5% was converted to p,p′-biphenol and 5% was recovered as o,o′-biphenol. The maximum amount of o,o′-biphenol appeared within 30 seconds both in the presence and in the absence of DNA and disappeared slowly on further incubation. The maximum amount of p,p′-biphenol was also formed within 30 seconds of incubation with no change on further incubation. p,p′-Biphenoquinone was formed maximally at 1 minute of incubation and started decreasing slowly after 3 minutes. Addition of ascorbate at different times of incubation before extraction increased p,p′-biphenol formation approximately two to three times as a result of reducing the p,p′-biphenoquinone in the reaction mixture. o,o′-Biphenol formation was not affected by ascorbate treatment before extraction. It is very likely that much more o,o′-biphenol than p,p′-biphenol is formed since the formed o,o′-biphenol disappeared completely within 5 minutes. DNA present in the incubation mixtures, however, had no effect on the o,o′-biphenol, p,p′-biphenol and p,p′-biphenoquinone formation under the conditions studied.

Figure 5.4 shows the spectra for the peroxidase-oxidized phenol, p,p′-biphenol and o,o′-biphenol. It appears that approximately 20% of the phenol is oxidized to p,p′-biphenoquinone as determined from the extinction coefficient at 399 nm. However, the absorbance at 500 nm for the products formed with 100 μM phenol was similar to that formed with 50 μM o,o′-biphenol.

Figure 5.4

Spectral properties of oxidation products of phenol, o,o′-biphenol and p,p′-biphenol in aqueous reaction mixture :

Incubations were carried out in 3 ml of 0.1 M Tris-HCl buffer pH 7.4. ——: Phenol (100 μM)/HRP (10 μg)/H_2O_2 (120 μM) scanned at three minutes of incubation; - - - : p,p′-Biphenol (10 μM)/HRP (10 μg)/ H_2O_2 (60 μM), scanned at one minute of incubation; ..... : o,o′-Biphenol (50 μM)/HRP (10 μg)/H_2O_2 (60 μM), scanned at 15 minutes of incubation.

FIGURE 5.4

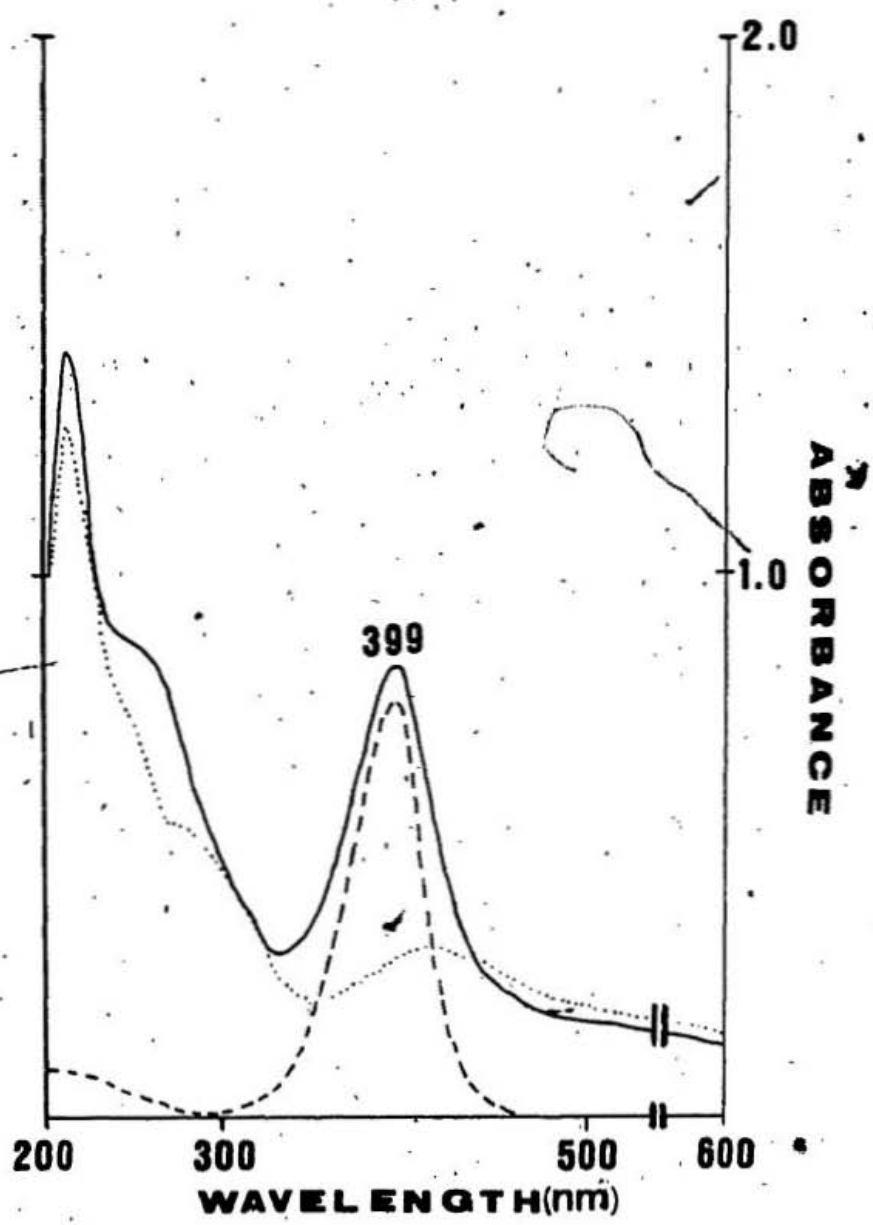


Figure 5.5 shows the spectra for the ethyl acetate extracts of o,o′-biphenol oxidation products with and without DNA in the reaction mixture. The red product(s) had absorbance maxima at 403 and 500 nm. The spectra of the unextracted reaction mixture was the same whether DNA was present or not indicating that DNA did not affect the formation of these products in the aqueous reaction mixture. However, in the presence of DNA only 45% of the product(s) was extracted into ethyl acetate whereas in the absence of DNA 95% of the product(s) was extracted indicating the red product was strongly bound to DNA. The maximal effect of DNA was found at 30 minutes of incubation (as observed from the difference in absorbance at 500 nm). If equimolar phenol was present to speed up the oxidation of o,o′-biphenol, the maximal effect of DNA was found at 3 minutes of incubation. The products of o,o′-biphenol reaction mixture were run on t.l.c. but most of the product remained as a red band on the origin. This band was markedly decreased if DNA was present in the reaction mixture as a result of DNA binding.

The ethyl acetate extracts of either phenol oxidation products or o,o′-biphenol oxidation products, dried under N_2, were analyzed by mass spectroscopy. The mass spectrum for phenol oxidation products (Figure 5.6) had peaks at 186, 278, 370 and 462 corresponding to the molecular ions of biphenol, triphenol, tetraphenol or pentaphenol respectively, with relative intensities in decreasing order. However, o,o′-biphenol oxidation products (Fig-

Figure 5.5

Oxidation of o,o′-biphenol in the presence of DNA.

The incubation was carried out in a total volume of 3 ml of 0.1 M Tris-HCl buffer, pH 7.4 at room temp. for 30 minutes. Reaction mixtures contained o,o′-biphenol (100 μM), HRP (10 μg), H_2O_2 (120 μM) with and without DNA (3 mg). The ethyl acetate extracts were scanned spectrally.

FIGURE 5.5

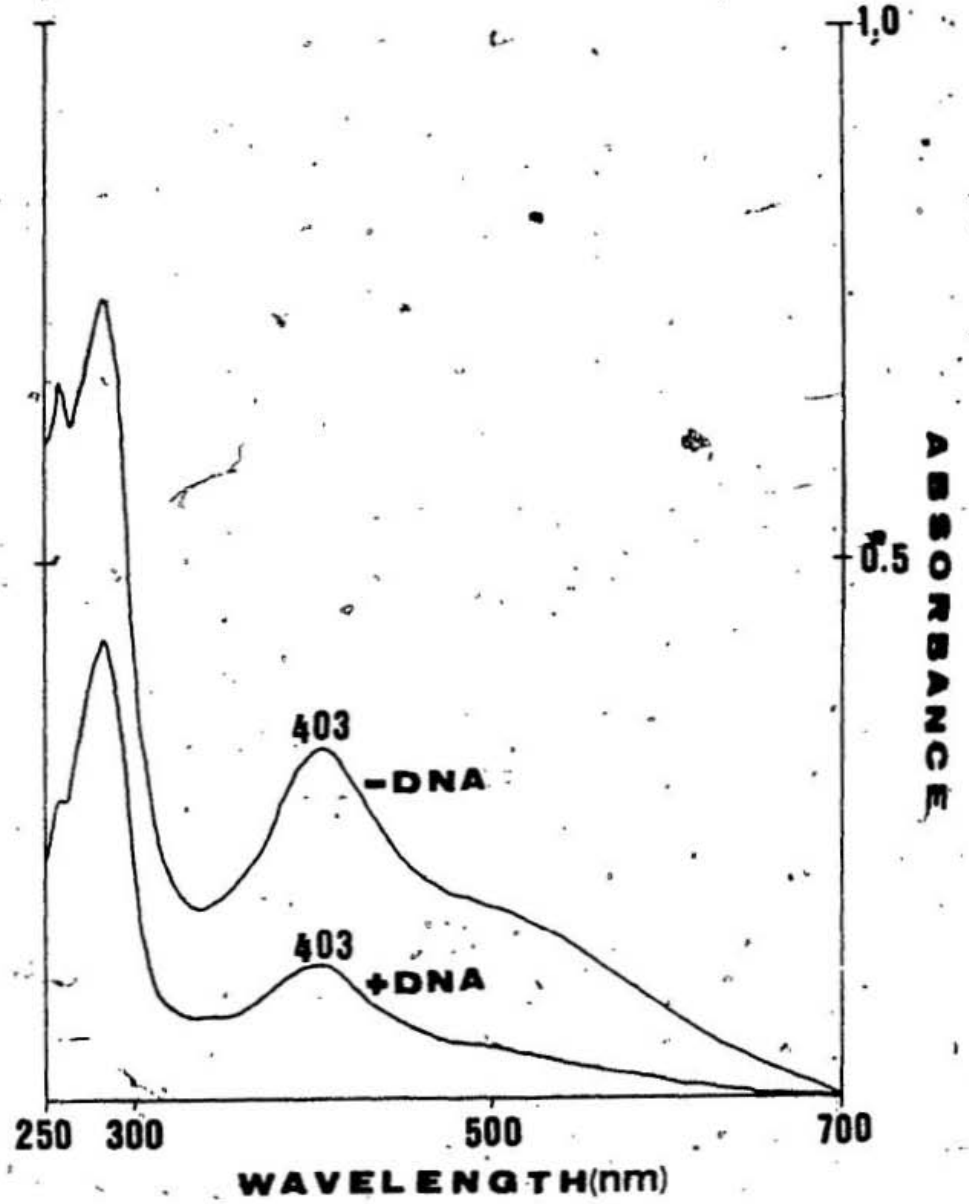

1.0
0.5
ABSORBANCE
403
-DNA
403
+DNA
250
300
500
700
WAVELENGTH(nm)

FIGURE 5.6

MASS SPECTRUM FOR PHENOL OXIDATION PRODUCTS

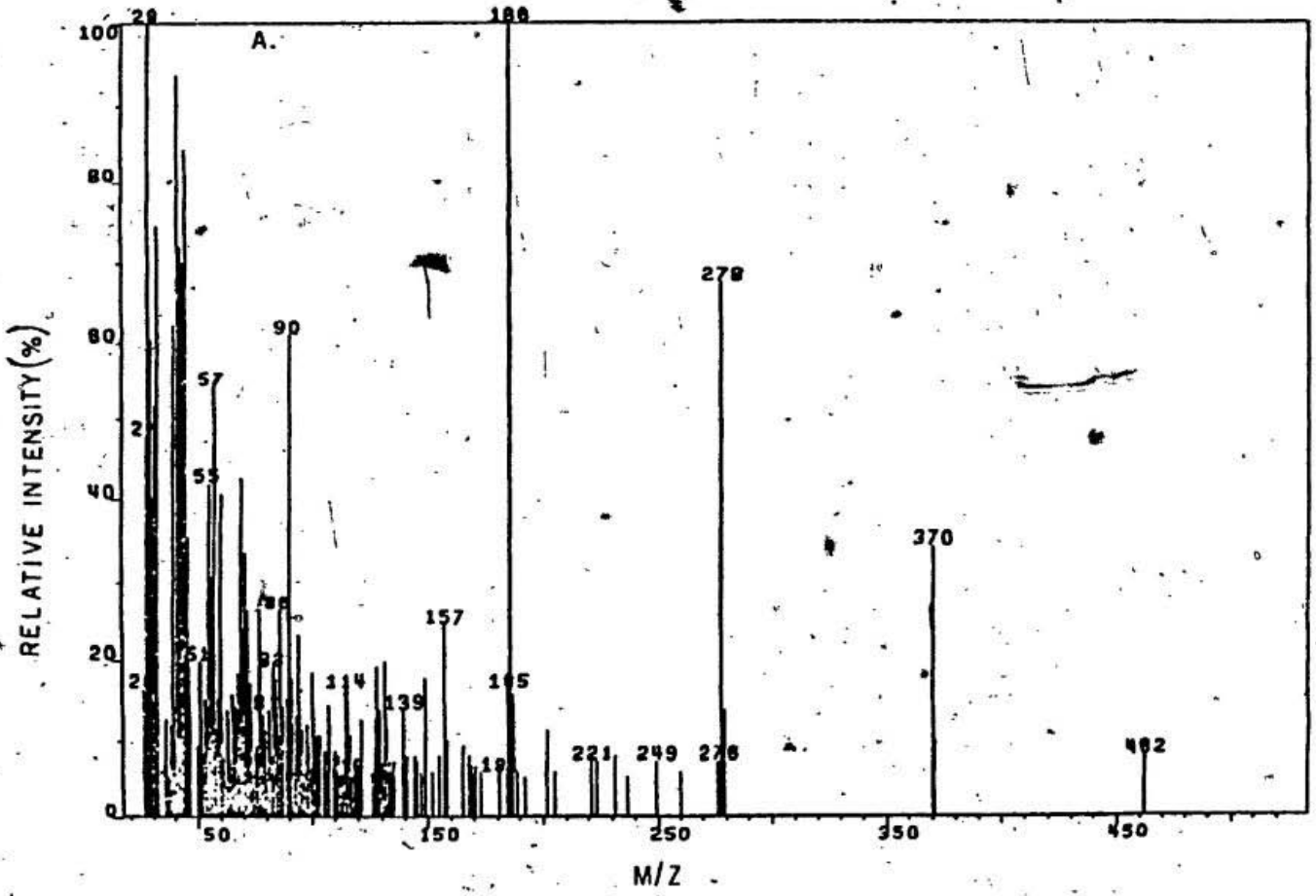
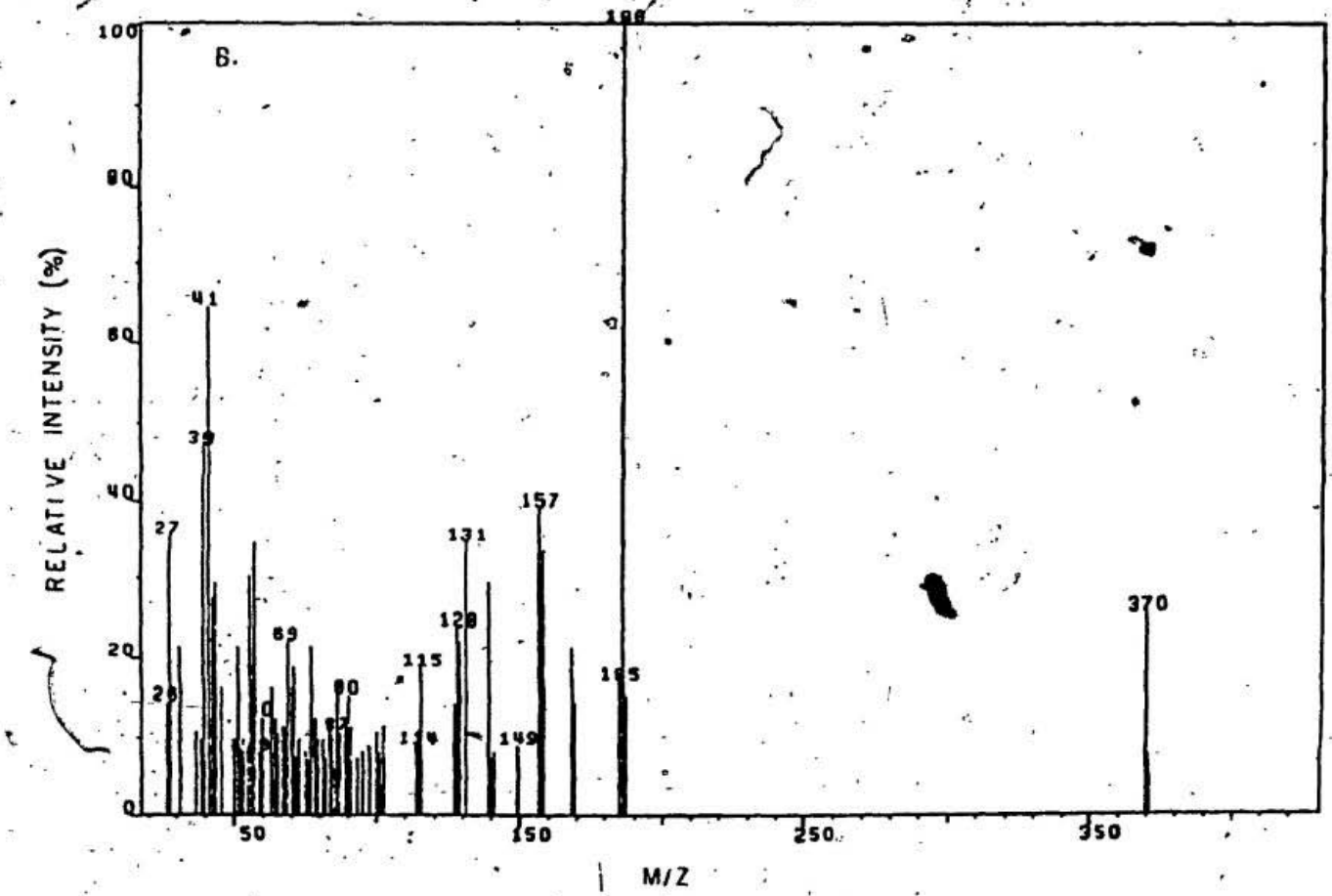


FIGURE 5.7

MASS SPECTRUM FOR O,O'-BIPHENOL OXIDATION PRODUCTS



ure 5.7) had only two peaks at 186 and 370 corresponding to molecular ions of biphenol and a dimer of biphenol (tetramer of phenol); peaks at 278 and 462 were absent. This could indicate that the peak at 278 present in Figure 5.6 may not be a fragmentation product of 370. Furthermore, when o,o'-biphenol oxidation products were run on t.l.c. all the o,o'-biphenol appeared to be oxidized, indicating that the peak at 186 may not have been a fragmentation product of 370. However, whether the peak at 278 is a fragment of 462 is not known.

Partial or complete hydrolysis of DNA to oligonucleotides or nucleosides revealed the nature of the products binding to DNA. The enzymic hydrolysis of phenol oxidation products bound-DNA, isolated and purified as described in materials and methods, by deoxyribonuclease, alkaline phosphatase and phosphodiesterase according to the method of Baird and Brookes (1973) (section 2.2.2.4) results in the release of brown specks (presumably polymer) into the medium. Table 5.2 shows that butanol extraction removed almost all the radioactivity from the aqueous layer. However, little radioactivity extracted into butanol layer. The remaining radioactivity in the aqueous layer was recovered as unhydrolyzed DNA in the first peak and had an absorbance maximum at 420 nm when the aqueous layer was passed through a Sephadex LH-20 column. This suggests that some of the bound products prevent complete digestion of DNA. The nucleotides released by digestion eluted as several

Table 5.2

Effects of n-butanol extractions on enzymatically hydrolyzed ^{14}C-phenol products bound DNA or polyribonucleotides

		% ^{14}C-phenol extracted into butanol			% ^{14}C-phenol at interphase			% ^{14}C-remaining in the aqueous layer.		
	HRP :	0.02μg	0.2μg	10μg	0.02μg	0.2μg	10μg	0.02μg	0.2μg	10μg
DNA	pH 7.4:	4±0.8	4±1.1	4±1.2	17±2.2	52±3.5	65±4.2	16±1.1	10±0.8	10±0.8
	pH 5.0:	2±0.4	2±0.6	2±0.8	10±1.8	36±2.6	66±4.4	2±0.4	8±0.5	2±0.2
Poly A	pH 7.4:	2±0.6	5±2.2	2±1.1	12±1.5	30±2.6	38±3.6	5±0.3	5±0.2	5±0.3
	pH 5.0:	1±0.2	1±0.4	1±0.4	2±0.8	15±2.6	38±3.2	2±0.4	2±0.2	2±0.5
Poly G	pH 7.4:	2±0.5	2±0.5	2±0.5	5±1.2	32±2.8	40±4.2	1±0.2	1±0.5	1±0.4
	pH 5.0:	2±0.2	2±0.3	2±0.4	5±1.5	22±2.8	38±3.8	1±0.3	2±0.5	1±0.6
Poly C	pH 7.4:	2±0.4	2±0.3	2±0.2	10±2.2	18±2.6	20±3.2	5±0.4	5±1.2	5±0.8
	pH 5.0:	1±0.2	1±0.4	1±0.2	2±1.0	10±1.2	15±2.3	2±0.6	2±0.5	5±1.2
Poly U	pH 7.4:	2±0.6	2±0.8	2±0.8	18±2.1	25±2.4	15±2.2	5±0.8	5±0.6	8±0.8
	pH 5.0:	1±0.5	1±0.2	1±0.2	5±1.1	10±2.2	15±2.4	2±0.6	2±0.8	5±1.2

Incubations Conditions: - Phenol (0.2 mM) was incubated with HRP (0.02 or 0.2 or 10 μg) and H_2O_2 (0.2 mM) for 15 min in the presence of DNA (3 mg) or polyribonucleotides (3 mg each) in 3 ml of 0.1 M Tris-HCl buffer pH 7.4 or in 3 ml of 0.1 M Sodium Acetate buffer pH 5.0. At the end of incubation the reaction mixtures were extracted with ethyl acetate (2 times). The macromolecules in the aqueous layers were hydrolyzed and further extracted with n-butanol. The radioactivity in organic layer, aqueous layer and at the interphase was measured by scintillation counting. Results are expressed as per cent of the radioactivity of the original ^{14}C-phenol added to the incubation mixture. For DNA or polyribonucleotide isolation and hydrolysis see Materials and Methods (section 2.2.2.4).

Mean ± S.E.M. for 3 experiments are given.

peaks but did not contain any radioactivity.

Table 5.2 also shows that enzymic hydrolysis of the ^{14}C-phenol oxidation products bound-polyribonucleotides with ribonucleases also resulted in the release of ^{14}C-products at the interphase. o,o'-Biphenol products bound to DNA or polyribonucleotides gave similar results upon hydrolysis. All of the released brown specks settled at the interphase upon butanol extraction. However, the amount of unhydrolyzed DNA was higher compared to that of phenol bound DNA. Acid hydrolysis (in 1 M HCl at 100 °C for 1 hour) of DNA bound with phenol or o,o'-biphenol oxidation products also resulted in the removal of the brown specks from DNA.

Mg^{2+} had no effect on the ^{14}C-phenol binding to DNA or polyribonucleotides indicating that phosphate groups of the DNA may not be involved in the binding. Furthermore, sodium dodecyl sulfate (10%) or an equal volume of saturated phenol at 60 °C could not remove the ^{14}C-phenol products from the DNA or polyribonucleotides.

Figure 5.8 compares the ^{14}C-phenol products binding to DNA with the absorbance of the bound DNA. It can be seen that the maximal amount of the ^{14}C-phenol bound to DNA within 3 minutes. However, the maximal absorbance of the bound DNA was not reached until 10 min (Figure 5.8). Figure 5.9 shows the influence of H_2O_2 on ^{14}C-phenol binding and the absorbance of

Figure 5.8

Comparison of ^{14}C-phenol products binding to DNA with colour formation on DNA; Time course for phenol binding: Colour formation on DNA was measured by absorbance at 418 nm.

^{14}C-Phenol (0.2 mM) was incubated with HRP and H_2O_2 (0.22 mM) in the presence of DNA (3 mg) in 3 ml of 0.1 M Tris-HCl buffer pH 7.4, at 37 °C.

● ● ●: HRP (10 [illegible]); * * : HRP (1 μg).

FIGURE 5.8

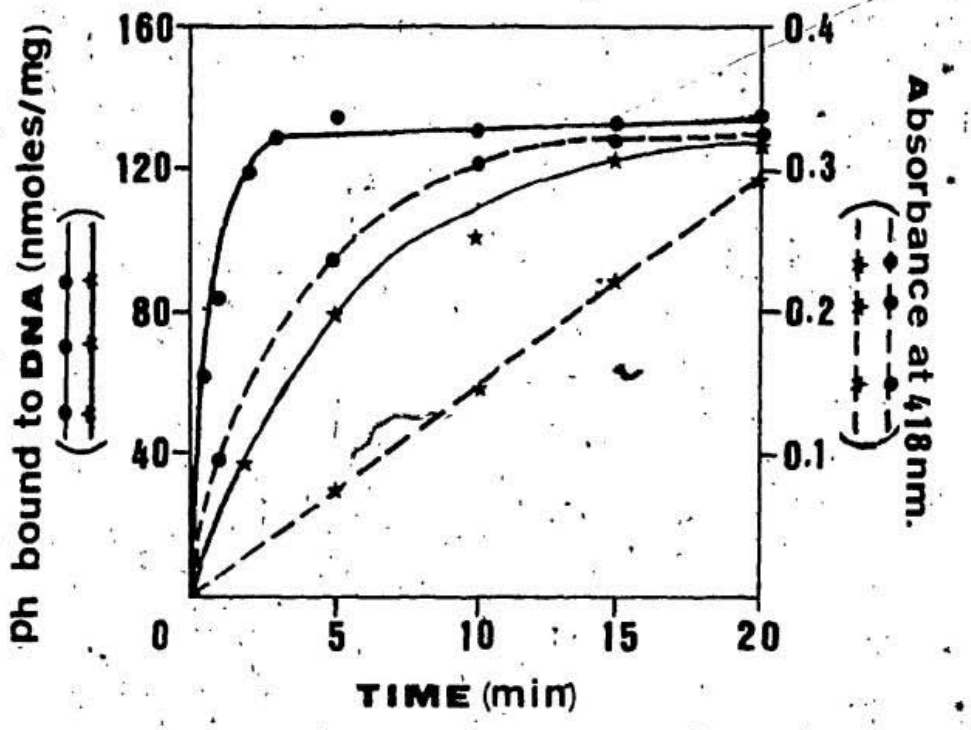
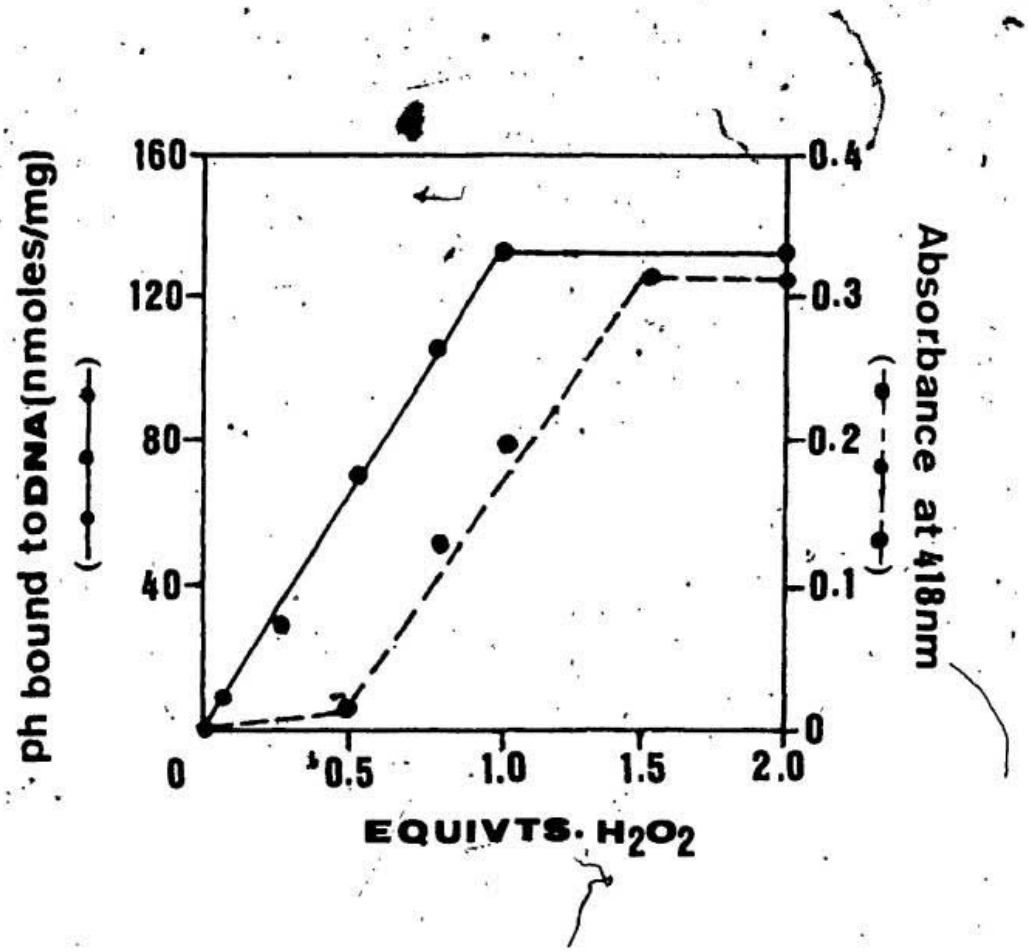

160
120
80
40
0
5
10
15
20
0.4
0.3
0.2
0.1
Ph bound to DNA (nmoles/mg)
Absorbance at 418nm.
TIME (min)

Figure 5.9

Comparison of ^{14}C-phenol products binding to DNA with colour formation on DNA; Effect of H_2O_2 concentration on DNA binding:

Phenol (0.2 mM) was incubated with HRP (10 μg) and H_2O_2 in the presence of DNA (3 mg) for 20 minutes in 3 ml of 0.1 M Tris-HCl buffer pH 7.4 at 37 ° C.

FIGURE 5.9


160
120
80
40
0
0.5
1.0
1.5
2.0
0.4
0.3
0.2
0.1
0
ph bound toDNA(nmoles/mg)
Absorbance at 418nm
EQUIVTS. H_2O_2

the bound DNA. Although the maximal amount of ^{14}C-phenol was bound at 1 equiv. concentration of H_2O_2, the maximal absorbance of the bound DNA was formed at 1.5 equiv. concentration of H_2O_2. It is concluded that some of the phenolic products bound to DNA are colourless and can be further oxidized to bound coloured products.

Figure 5.10 shows the rate of o,o′-biphenol oxidation (as measured by increase in the 500 nm product) in the presence of phenol and p,p′-biphenol. The increase in the rate of oxidation of o,o′-biphenol was directly proportional to the concentration of phenol. With a concentration of phenol equimolar to o,o′-biphenol, the rate of o,o′-biphenol oxidation was increased by a factor of 12. p,p′-Biphenol was much less effective at increasing the o,o′-biphenol oxidation. Furthermore, Figure 5.11 shows that o,o′-biphenol even at very low concentration (0.1 molar equiv.) completely inhibits p,p′-biphenoquinone formation from phenol although o,o′-biphenol did not inhibit the rate and amount of p,p′-biphenoquinone formation from p,p′-biphenol.

Since phenol and its oxidation products (biphenols) can influence the oxidation of each other during peroxidase-catalyzed oxidation, it may be worth determining the rate constants for individual substrates, during peroxidase-catalyzed oxidation, which may further improve the understanding of the mechanism of phenol oxidation to polymers by peroxidase. In table 5.3 the rate constants for the reduction of peroxidase compound II by phenol and its

Figure 5.10

Effect of phenol and p,p′-biphenol on the rate of 500 nm product formation from o,o′-biphenol:

Reaction mixtures contained o,o′-biphenol (50 μM), HRP (10 μg) and varying concentrations of either phenol or p,p′-biphenol. Reactions were started by the addition of H_2O_2 (120 μM) and the increase in absorbance was followed at 500 nm using a Shimadzu UV-240 spectrophotometer.

● ● ● : in the presence of phenol; X X X : in the presence of p,p′-biphenol.

FIGURE 5.10

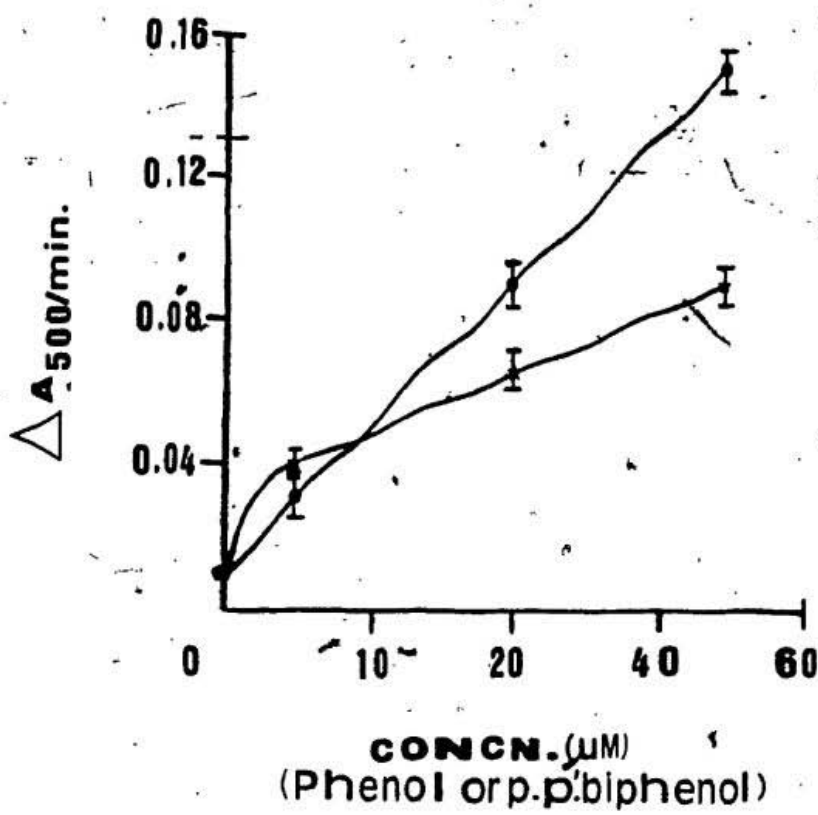

0.16
0.12
0.08
0.04
ΔA_{500}/min.
0
10
20
40
60
CONCN.(μM)
(Phenol or p.p'biphenol)

Figure 5.11

Effect of o,o'-biphenol on the rate of p,p'-biphenoquinone formation from phenol:

Reaction mixtures contained phenol (50 μM), HRP (1 μg) and varying concentrations of o,o'-biphenol. Reactions were started by adding H_2O_2 (120 μM) and followed at 400 nm using Shimadzu UV-240 spectrophotometer.

FIGURE 5.11

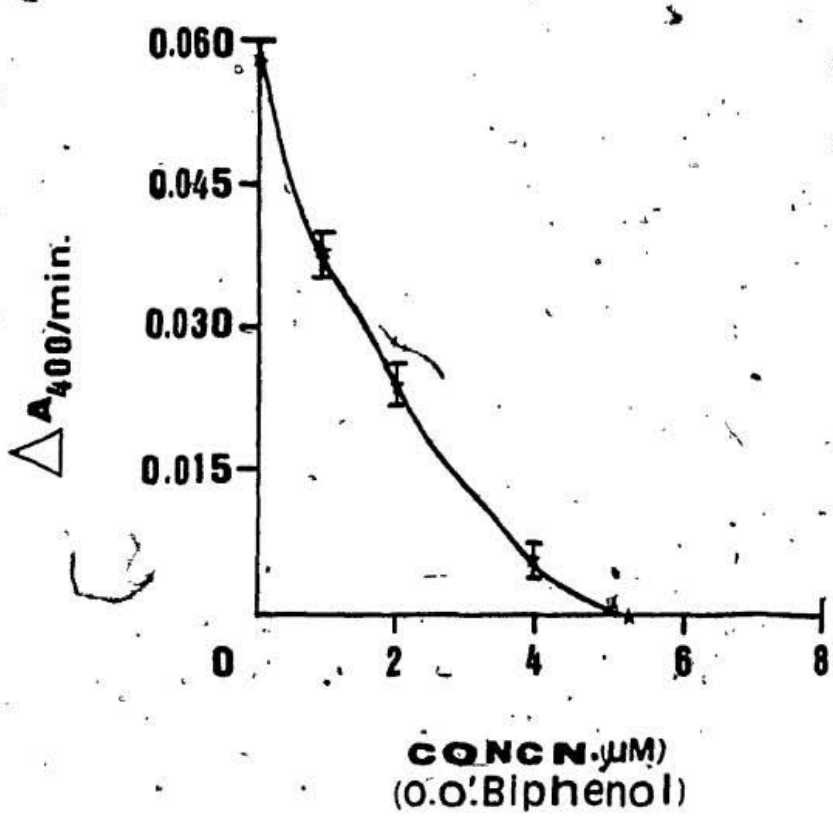

0.060
0.045
0.030
0.015
$\Delta A_{400/min.}$
0
2
4
6
8
CONCN.(μM)
(o.o'Biphenol)

Table 5.3
Reduction Rate constants for peroxidase compound-II(K_3) by phenol and its oxidation products

Substrate	$K_3(M^{-1}S^{-1})$
Phenol	$5.5\pm0.3\times10^4$
p,p′-Biphenol	$2.2\pm0.1\times10^7$
o,o′-Biphenol	$7.1\pm0.4\times10^3$
p.Phenylphenol	$6.6\pm0.3\times10^6$
o.Phenylphenol	$2.4\pm0.1\times10^4$

Reduction rate constants were determined as described in Materials and methods (Section 2.2.2.4). Mean ± S.E.M. for 3 experiments are given.

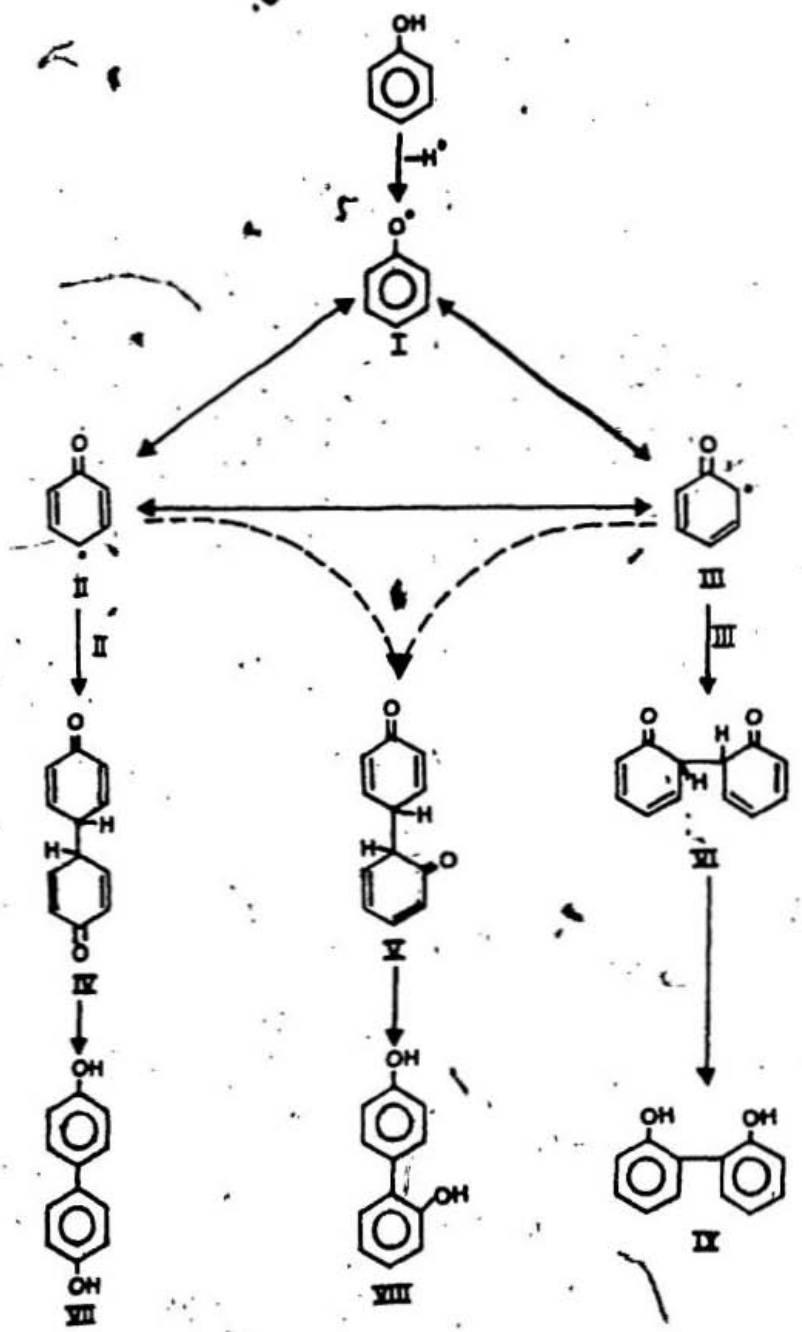
oxidation products have been determined. It can be seen that the rates of oxidation differed widely and were in decreasing order of effectiveness:- p,p'-biphenol, phenol and o,o'-biphenol. p-Phenylphenol was also a far more active substrate than o-phenylphenol.

5.3) Discussion :

Enzymic hydrolysis of DNA bound with ^{14}C-phenol oxidation products indicates that DNA bound products could be polymers. Phenol is initially oxidized (Figure 5.12) to phenoxy radicals (I or II or III) and these radicals dimerize to form dienones (IV, V and VI) which eventually tautomerize into biphenols (VII, VIII and IX) in protic solvents (Harborne, 1964; Taylor and Battersby, 1967). In addition dimers with C-O bond also may be formed. o,o'-Biphenol and p,p'-biphenol were formed in a peroxidase system (Sawahata and Neal, 1982). Biphenols are also substrates for peroxidases (Sawahata and Neal, 1982). Mass spectral results indicated that trimers, tetramers and pentamers are also formed by peroxidase oxidation of phenol (Figure 5.6). In conclusion, complex polymeric products could result from phenol oxidation. Enzymic hydrolysis of DNA bound with o,o'-biphenol oxidation products also resulted in the release of bound products. However, none of the p,p'-biphenol products were bound to DNA.

FIGURE 5.1.2

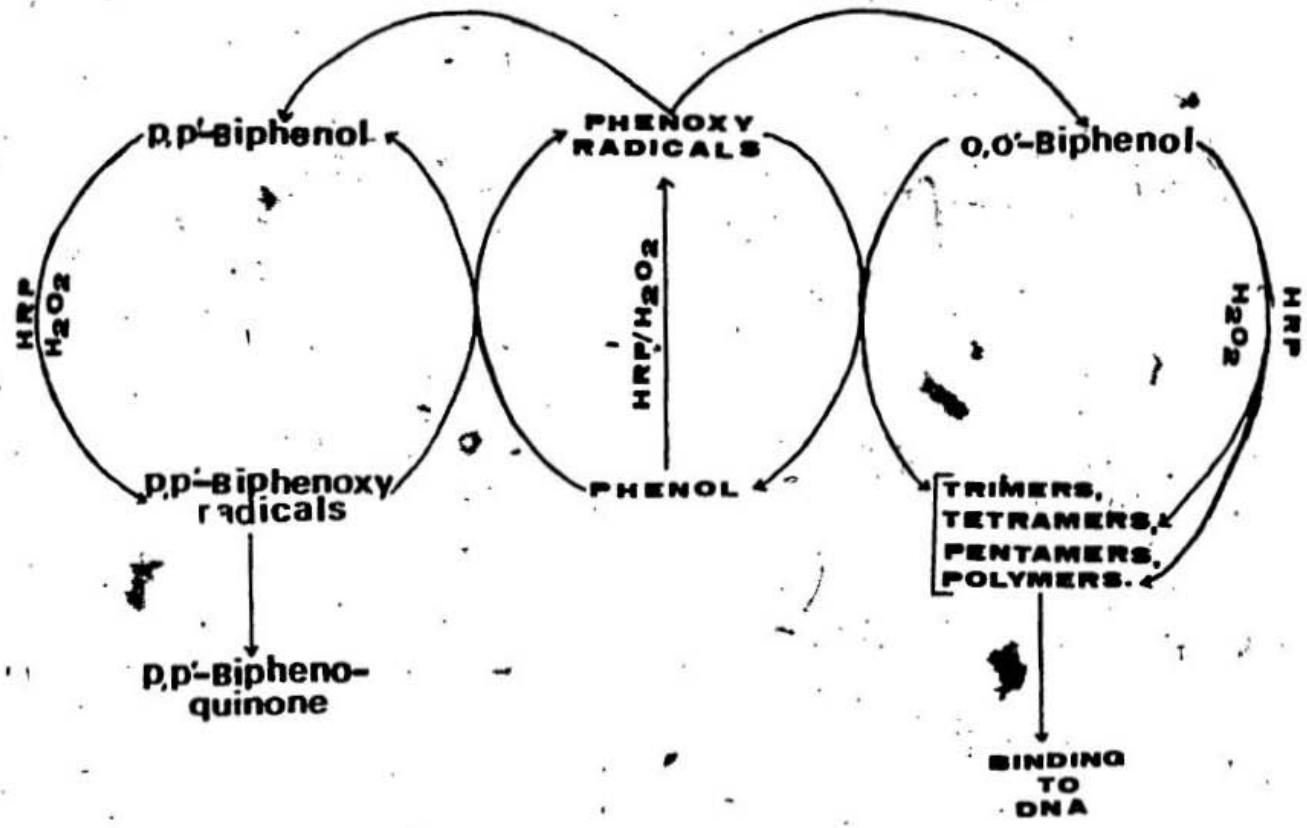
OXIDATIVE COUPLING OF PHENOLS : SCHEME - I
(Taylor and Battersby, 1967)



Peroxidase catalyzes the oxidation of many hydrogen donors (Chance, 1949). The hydrogen donors act by converting peroxidase compound I to II and converting compound II back to peroxidase. The latter is usually the slowest step (Chance, 1949). The reduction rate constants (Table 5.3) indicate that p,p′-biphenol was 400 times more effective than phenol. The above results showed that phenol even at very low concentrations greatly enhanced the oxidation of o,o′-biphenol (Figure 5.10). Furthermore, p,p′-biphenoquinone formation from phenol was inhibited in the presence of even very low concentrations of o,o′-biphenol (Figure 5.11). On the other hand o,o′-biphenol had no effect on the rate and amount of formation of p,p′-biphenoquinone from p,p′-biphenol. These results suggest that the p,p′-biphenosemiquinone radicals formed from p,p′-biphenol may oxidize phenol non-enzymically. The results also suggest that phenoxy radicals formed from phenol either enzymically or non-enzymically may also oxidize o,o′-biphenol to the polymeric product. The proposed scheme for the interaction of these phenoxy radicals in the oxidation of phenol and subsequent DNA binding is presented in Figure 5.13. When comparing the amount of red product formed (Figure 5.4) as a result of o,o′-biphenol or phenol oxidation it is clear that most of the phenol is initially oxidized to o,o′-biphenol. Only 20% of the phenol is initially oxidized to p,p′-biphenoquinone. Figure 5.13 shows that p,p′-biphenosemiquinone radicals formed from p,p′-biphenol can oxidize phenol non-enzymically to phenoxy radicals and phenoxy radicals in turn oxidize o,o′-biphenol to the red polymeric

FIGURE 5.13

PROPOSED SCHEME FOR PEROXIDASE OXIDATION OF PHENOL LEADING TO DNA BINDING



product. However, the possibility of p,p'-biphenosemiquinone radicals directly interacting with o,o'-biphenol metabolism cannot be excluded. Using fluorimetry, Danner et al. (1973) also showed that o,o'-biphenol is the principal product formed from phenol by peroxidase oxidation.

Melanin or lignin-like polymeric products can be generated by the oxidation of various phenols *in vitro* (Harborne, 1964; Sealy et al., 1980; Taylor and Battersby, 1967). These polymers differ in structure and reactivity. Melanins are considered to be redox polymers containing quinone, semiquinone and hydroquinone type units in equilibrium (Sealy et al., 1980). They also contain free radicals under all known conditions (Sealy et al., 1980). They also readily bind to metal ions, certain drugs and quaternary bipyridium salts. Although, the nature of the binding is not known, it is believed that they form charge transfer complexes in which the drug acts as an electron donor and melanin acts as an electron acceptor. Such an interaction is possible here between DNA and the phenol polymer. Anionic detergents like sodium dodecyl sulfate were ineffective in removing the bound phenol polymers from the DNA. An equal volume of saturated phenol at 60°C also failed to remove the phenol although this treatment removes adriamycin from DNA (Eliott, Gianni and Myers, 1984).

The formation of the products found in phenol coupling (during the oxidation of phenols with chemical agents) have been explained by the following

mechanisms:

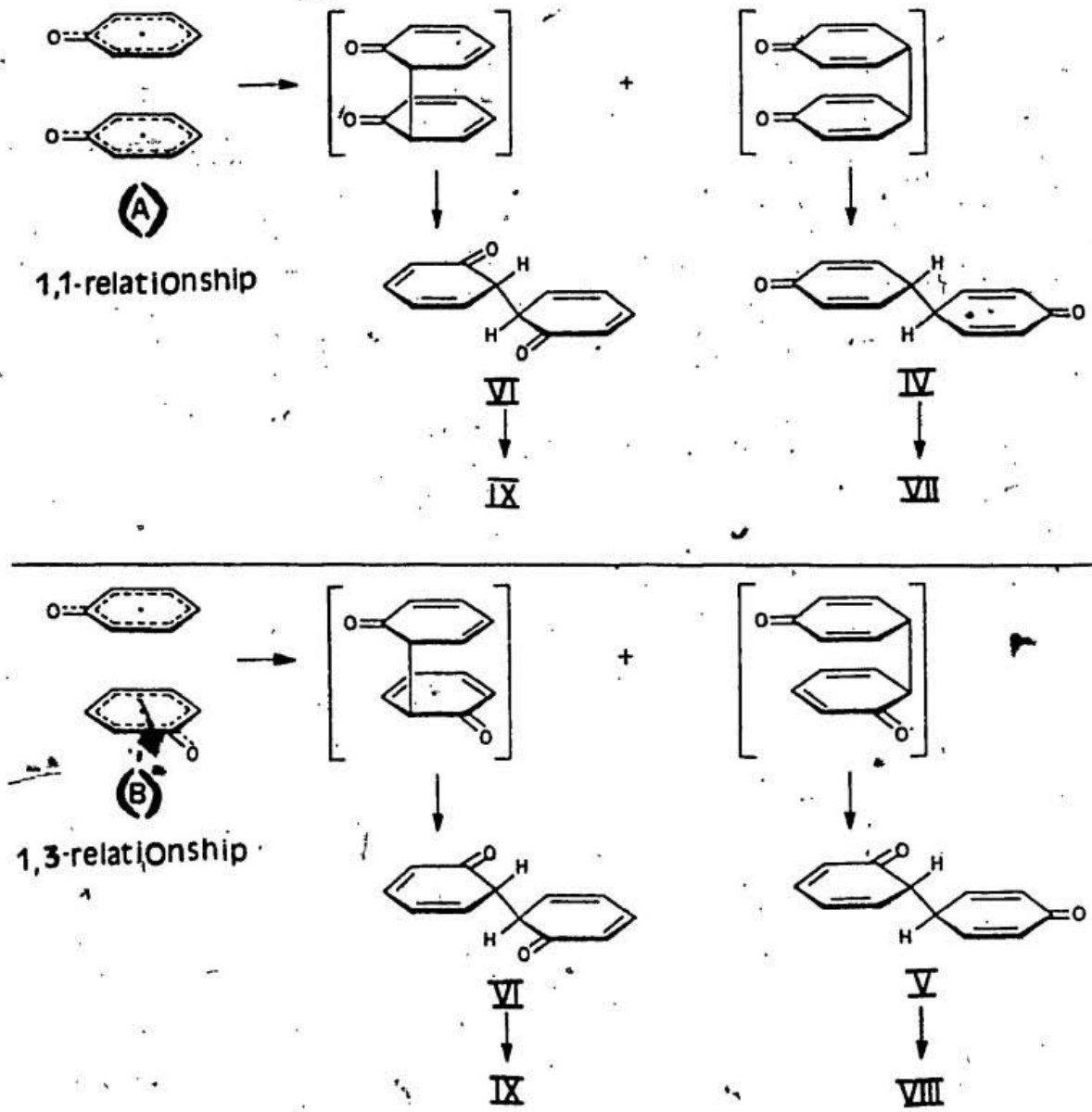
1) Phenoxy radicals appear to be stabilized by resonance, and the odd electron is distributed over the oxygen atom and all carbon atoms of the aromatic ring (Figure 5.12). The observed coupling positions (*ortho* and *para* but not *meta* show that a free phenoxy radical is reactive only on the oxygen and the *ortho*- and *para*-carbon atoms (Taylor and Battersby, 1967). It is originally believed that spin density factors play a major role in the coupling of phenoxy radicals. The high spin density is found on oxygen and at the *para* position, whereas both *ortho* positions show coupling constants of half this magnitude. But the coupling constants at 1, 3 and 5 positions appear to be negative. It is believed that coupling of phenoxy radicals should occur preferentially at the site of highest spin density (Fleming, 1976). Oxidation of 2,6-dimethylphenol under a variety of conditions gives the *para-para*-coupled dimer as the major product with relatively small amounts of the *ortho-para*-coupled product (Dalgleish, Nonhebel and Pauson, 1977). By contrast, oxidation of p-cresol with a variety of metal agents, gives rather more *ortho-para*-coupled product (Pummerer's ketone) than the *ortho-ortho*-coupled product (Anderson et al., 1977). All these data were consistent with the idea that the spin density distribution in the intermediate phenoxyl radicals playing a significant part in determining the ratios of products.

2) Armstrong et al. (1983) have critically evaluated the role of spin density factors in phenol coupling reactions. They showed that phenol and 3,5-dimethyl phenol, both of which have highest spin density at the *para* position, upon oxidation with various chemicals, give predominantly *ortho-ortho*-product. However, under certain conditions (see Armstrong et al., 1983) *ortho-para*-product is the major product formed at the expense of *ortho-ortho* product. They (Armstrong et al., 1983) have suggested that two phenoxy radicals favourably approach each other in 1,3-relation rather than 1,1-relationship (Figure 5.14). The two-most probable 'transition states' are (A) and (B). The interaction between phenoxy radicals would be greater in (A) than (B) as the sites of highest spin density (the *para* position) lie directly above each other. However, electrostatic repulsion between the two electronegative oxygens would also be more powerful in (A) than (B). The 'transition state' (A) would lead to the *para-para* dimer and also the *ortho-ortho* dimer, *via* (IV) and (VI) respectively, while the transition state (B) could give rise to both the *ortho-ortho*- and *ortho-para*-dimers, *via* (VIII) and (IX) respectively. That these latter are the major products suggests that the most-favourable coupling route proceeds *via* the 'transition state' (B).

The results presented in this chapter also suggest that o,o'-biphenol may be the major product formed from phenol upon oxidation with HRP and H_2O_2. p,p'-Biphenol is also formed but appears to be in minor amounts. Whether

FIGURE 5.14

OXIDATIVE COUPLING OF PHENOLS : SCHEME - II
(Armstrong et al., 1983)



o,p′-biphenol is formed or not, is not known. Sawahata and Neal (1982) have reported that no significant formation of Pummerer's ketone (rearrangement product of o,p′-biphenol) occurred, under their conditions of study.

Another interesting point that could be raised at this stage is the mechanism of p,p′-biphenol oxidation to form p,p′-biphenoquinone. It is postulated in the scheme presented in Figure 5.13 that p,p′-biphenol oxidation to form p,p′-biphenoquinone proceeds *via* intermediary formation of p,p′-biphenosemiquinone radicals, based on the results presented in Figure 5.10 and Figure 5.11. However, no definitive evidence has been presented regarding the mechanism of p,p′-biphenol oxidation. Electron paramagnetic resonance studies may result in a definitive answer. Thus, there are still many questions to be answered. What I have described in this chapter is a speculative pathway which may stimulate further research in this area.

CHAPTER 6

PEROXIDASE-H_2O_2 OR BONE MARROW HOMOGENATE-H_2O_2-MEDIATED ACTIVATION OF PHENOL AND BINDING TO PROTEIN

6.1) Introduction :

While the phenol activation studies in the previous two chapters were performed with horseradish peroxidase, other investigators (Sawahata and Neal, 1982; Sawahata, Rickert and Greenlee, 1985) have attempted to study the peroxidase/ H_2O_2-mediated activation of phenol in bone marrow homogenate. Their results showed that 85% of the oxidized phenol was bound to the bone marrow protein. Only 5% of the oxidized phenol accounted for the total biphenols formed. Both o,o′-biphenol and p,p′-biphenol were detected in approximately equal amounts. No other metabolites were detected. However, p,p′-biphenoquinone was also shown to be formed when partially purified myeloperoxidase was substituted for bone marrow homogenate. Sawahata, Rickert and Greenlee (1985) have concluded that p,p′-biphenoquinone is the major reactive metabolite binding to proteins.

In this chapter, (1) results are presented on the possible mechanism(s) of activation of phenol by bone marrow homogenate (see section 2.2.1.2 of

Materials and Methods) and H_2O_2. In contrast to the results obtained by Sawahata, Rickert and Greenlee (1985) the present study shows that o,o'-biphenol is the major metabolite formed initially from phenol and further oxidation of o,o'-biphenol accounts for the majority of the protein binding; (2) evidence is also presented on the nature of binding between phenol oxidation products and BSA, when phenol is oxidized by HRP and H_2O_2.

6.2) Results :

Figure 6.1 shows the effect of bone marrow protein concentration on the rate of phenol oxidation. As shown in the figure, the rate of phenol oxidation increased with increasing protein concentration. Figure 6.2 shows that ^{14}C-phenol activation by bone marrow homogenate can result in reactive products which bind to protein. The amount of phenol bound is proportional to the concentration of H_2O_2 (up to 10 mM). The need for a large H_2O_2 excess suggests that most of the H_2O_2 may be decomposed by catalase in the bone marrow homogenate. Figure 6.2 also shows that pre-incubation, for 5 minutes, of bone marrow homogenate with H_2O_2 results in the inhibition of protein binding, presumably by inactivating the enzyme(s) involved in phenol activation.

Table 6.1 shows the time-course for the metabolism and protein-binding of ^{14}C-phenol activated by bone marrow homogenate and H_2O_2. The effect

Figure 6.1

Effect of different concentrations of bone marrow protein on the rate of phenol oxidation catalyzed by bone marrow homogenate and H_2O_2 :

^{14}C-phenol (200 nmoles) was incubated with bone marrow homogenate (concentrations as indicated in the figure) and H_2O_2 (10 mM) in 1 ml Tris-HCl buffer pH 7.4 for 1 minute at 37 °C. (Note: Added phenol contained a specific activity of 0.0015 μCi/nmole).

After incubations for appropriate times, the reaction mixtures were extracted with ethyl acetate, and the phenol oxidation was measured by radioactivity on t.l.c. (section 2.2.4.2) or by h.p.l.c. (section 2.2.4.1) as described in Materials and Methods.

FIGURE 6.1

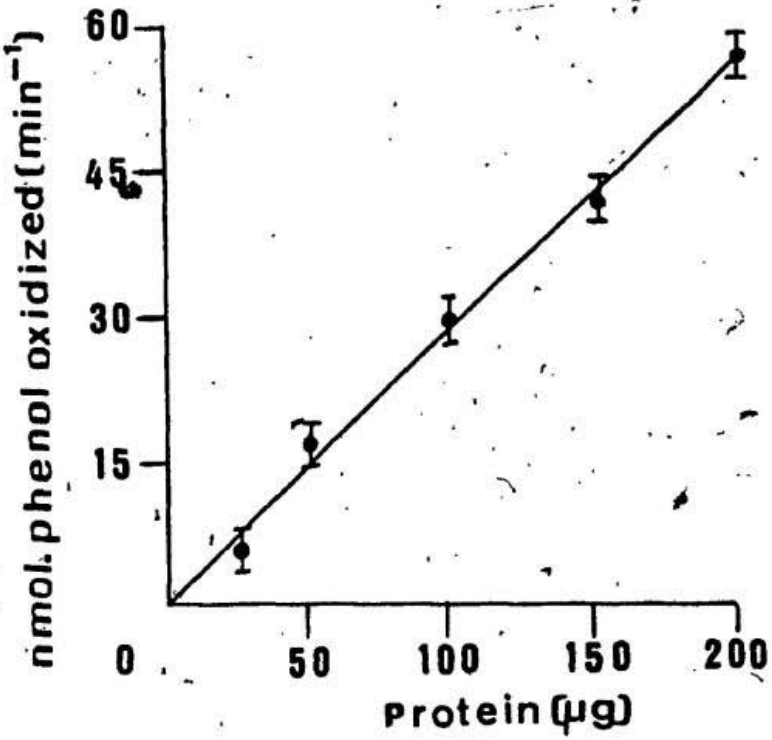

60
45
30
15
0
50
100
150
200
nmol. phenol oxidized (min⁻¹)
Protein (µg)

Figure 6.2

Effect of different concentrations of H_2O_2 on protein-binding of ^{14}C-phenol catalysed by bone marrow homogenate and H_2O_2 :

Phenol (0.2 mM) was incubated with bone marrow homogenate (200 μg) and H_2O_2 (concentrations as indicated in the figure) in 1 ml of 0.1 M tris-HCl buffer pH 7.4 at 37 °C.

-O-O-O-: as described above; -A-A-A-: pre-incubated for 5 minutes with 1 mM H_2O_2 before the addition of phenol and H_2O_2; -[]-[]-[]-: pre-incubated for 5 minutes with 10 mM H_2O_2 before the addition of phenol and H_2O_2.

FIGURE 6.2

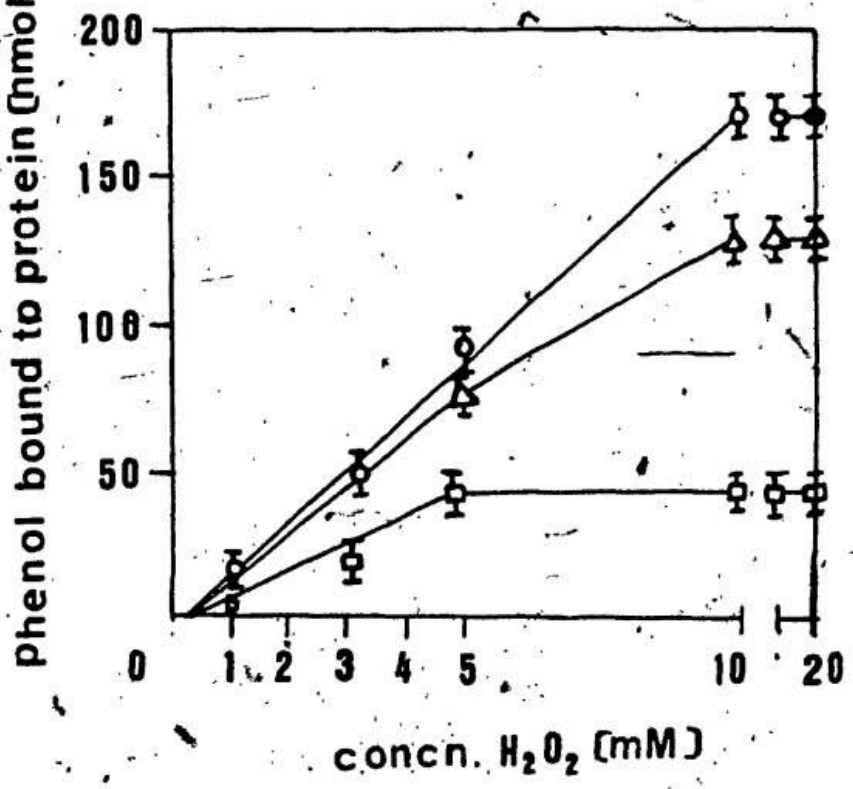

Phenol bound to protein (nmoles)
200
150
100
50
0
1
2
3
4
5
10
20
concn. H_2O_2 (mM)

Table 6.1

Time course for ^{14}C-phenol metabolism by bone marrow homogenate (Percentage of the radioactivity incorporated into products)

Product Formed	15 sec		30 sec		1 min		2 min		5 min	
	- Asc	+ Asc	- Asc	+ Asc	- Asc	+ Asc	- Asc	+ Asc	- Asc	+ Asc
o,o'-Biphenol	2±0	2±0	2±0	3±0	2±0	4±0	3±0	6±0	4±0	16±0
p,p'-Biphenol	1±0	1±0	1±0	1±0	1±0	1±0	1±0	2±0	2±0	4±0
Unknown 1	0	0	0	0	0	0	0	0	1±0.5	1±0.5
Protein bound	0	0	4±2	4±	22±4	20±4	61±6	58±6	84±8	78±7
Phenol oxidized	6±2	6±2	15±3	15±3	27±3	26±4	68±5	67±5	100	100

o,o'-Biphenol and p,p'-biphenol were quantitated by h.p.l.c. (section 2.2.4.1) and t.l.c.(section 2.2.4.2) described in Materials and Methods. Unknown 1 was quantitated by its radioactivity on t.l.c. Protein binding was determined as described in Materials and Methods (section 2.2.3.2).

Incubation conditions : Phenol (0.2 mM) was incubated with 200 μg bone marrow homogenate and 10 mM H_2O_2 at 37°C in 1 ml of 0.1 M Tris-HCl buffer pH 7.4.

Note : Ascorbic acid (1.0 mM) was added at the end of various incubation times, before extraction with ethyl acetate.

Mean ± S.E.M. for three experiments are given.

of ascorbate, added at different times of incubation, was also studied in order to understand the mechanism of activation. As shown in the table, 100% of the phenol was oxidized within 5 minutes. Approximately 75-85% of the phenol was bound to bone marrow protein. Both o,o'-biphenol and p,p'-biphenol were formed from phenol. However, they accounted only for less than 20% of the total phenol oxidized. It is possible that both the biphenols are further oxidized to products which bind the bone marrow protein. No hydroquinone or catechol formation was detected under these conditions.

Addition of ascorbate one minute before the extraction with ethyl acetate resulted in increased amounts of extractable o,o'-biphenol and p,p'-biphenol (Table 6.1). A maximum of four-fold increase in o,o'-biphenol formation was observed. Although, p,p'-biphenol formation was also increased, the total p,p'-biphenol formed was at least four-fold lower than the total o,o'-biphenol formed. Thus, o,o'-biphenol appears to be the major metabolite formed from phenol during activation by bone marrow homogenate and H_2O_2. The results in table 6.1 also show no decrease in protein-bound radioactivity in the presence of ascorbate suggesting that ascorbate is unable to remove the products already bound to protein. A minor unknown product with an $R_F = 0.00$ was detected (both in the presence and in the absence of ascorbate), only at 5 minutes of incubation. However, the amount formed appears to be unchanged compared to the amount formed in the absence of ascorbate. This compound

was not eluted from the silica gel with ethyl acetate. A compound with similar properties, presumably polymer, was formed in previous incubations when HRP was used instead of bone marrow homogenate (see Chapter 5).

Pre-incubation of bone marrow homogenate with N-ethylmaleimide [(1.0 mM)(further dialyzed to remove excess N-ethylmaleimide)] decreased protein thiols by 90% but decreased the protein binding of ^{14}C-phenol oxidation products by only 10-20%, suggesting that the majority of the binding of phenol oxidation products to protein may not involve sulfuhydryl groups.

Figure 6.3 (a) shows the protein profile of a bone marrow homogenate after the protein has been separated into different bands by SDS-polyacrylamide gel electrophoresis. Addition of phenol to the bone marrow homogenate had no effect on the separation of these proteins (Figure 6.3 (b)). Addition of H_2O_2, resulted in the disappearance of some low molecular weight proteins and the appearance of three high molecular weight - proteins which remained at the origin and/or close to the origin (the high molecular-weight proteins are marked with arrows) (Figure 6.3 (c)), which may suggest that H_2O_2 some how cross-linked these low molecular weight proteins to higher molecular weight proteins. When ^{14}C-phenol and H_2O_2 were incubated with the bone marrow homogenate the low molecular weight proteins disappeared again, as in (c); and a decreased Commassie blue staining intensity of the protein bands with molecular weights 80,000, 76,000, 66,000, 58,000 and 36,000

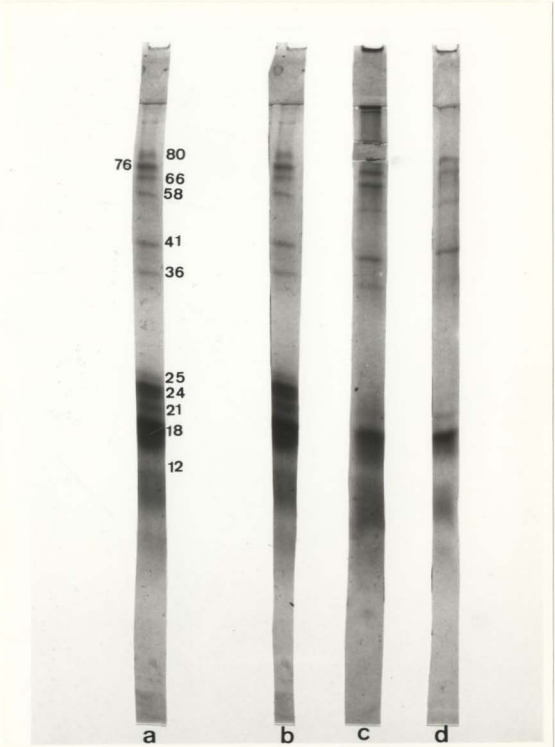
Figure 6.3

SDS-Polyacrylamide gel electrophoresis for bone marrow homogenate protein:

a) 1 a) incubated with none; b) incubated with 0.2 mM ^{14}C-phenol; c) incubated with 10 mM H_2O_2; d) incubated with 0.2 mM ^{14}C phenol and 10 mM H_2O_2.

This experiment was performed only once at Dr. R.K. Murray's laboratory, Department of Biochemistry, University of Toronto).

FIGURE 6.3



also occurred. No stain was detected, at the origin or near the origin, indicating that no high molecular weight proteins appeared. However, an autoradiograph showed that most of the radioactivity remained at the origin. This may suggest that the phenol oxidation products may have cross-linked some proteins to a high molecular weight protein which stayed at the origin; and the absence of Commassie blue stain at the origin may indicate that bound phenol oxidation products may have prevented the staining of the high molecular weight protein, with Commassie blue. Alternatively, it is possible that the binding of ^{14}C-phenol oxidation products to proteins may involve a non-covalent interaction with a phenolic polymer which separates from the protein on electrophoresis and remains at the origin.

When bone marrow homogenate proteins were separated by SDS-polyacrylamide gel electrophoresis, the proteins with molecular weights 80,000, 76,000, 66,000 and 58,000 also specifically stained with 3,3'-diaminobenzidine-H_2O_2 a stain used to detect peroxidases (Desser et al., 1972; Graham and Karnovsky, 1966). However, these proteins disappeared (as determined by staining with 3,3'-diaminobenzidine) when the bone marrow homogenate was pre-incubated with H_2O_2 and suggesting that H_2O_2 inactivated the peroxidase activity of these proteins. Furthermore, results from Figure 6.2 showed that, pre-incubation of bone marrow homogenate for 5 minutes with H_2O_2 alone and further incubation with ^{14}C-phenol and H_2O_2 resulted in inhibition of

protein binding by 75%. This indicated that pre-incubation with H_2O_2 actually inactivated the proteins involved in the activation of phenol and thus may have inhibited phenol oxidation, which is reflected in decreased binding to protein. The above mentioned proteins also disappeared (determined with 3,3'-diaminobenzidine) when phenol and H_2O_2 were incubated with bone marrow homogenate. Furthermore, the disappearance of these proteins and the appearance of a high molecular weight protein at the origin (see above paragraph) when the bone marrow homogenate was incubated with phenol and H_2O_2, also suggests that one or more of these peroxidases may be involved in phenol activation.

To further investigate how strongly phenol oxidation products are bound to protein, oxidation of phenol by HRP and H_2O_2 in the presence of BSA was investigated.

Figure 6.4 shows the spectral properties of phenol oxidation products bound to BSA. As shown in the figure, in the absence of BSA, phenol oxidation product(s) had absorbance maxima at 210 nm and 399 nm and a shoulder in the 240-250 nm region. In the presence of BSA, phenol oxidation product(s) had absorbance maxima at 242 nm and 294 nm. The reaction mixtures were extracted with ethyl acetate, after which the protein in the aqueous layer was precipitated with TCA (final concentration 10%). The protein pellet was redissolved in 3.0 ml of 0.1 M Tris-HCl buffer pH 7.4 and re-scanned. The

Figure 6.4

Spectral properties of phenol or o,o'-biphenol oxidation products bound BSA.

..... : Phenol (100 μM)/HRP (10 μg)/ H_2O_2 (100 μM)

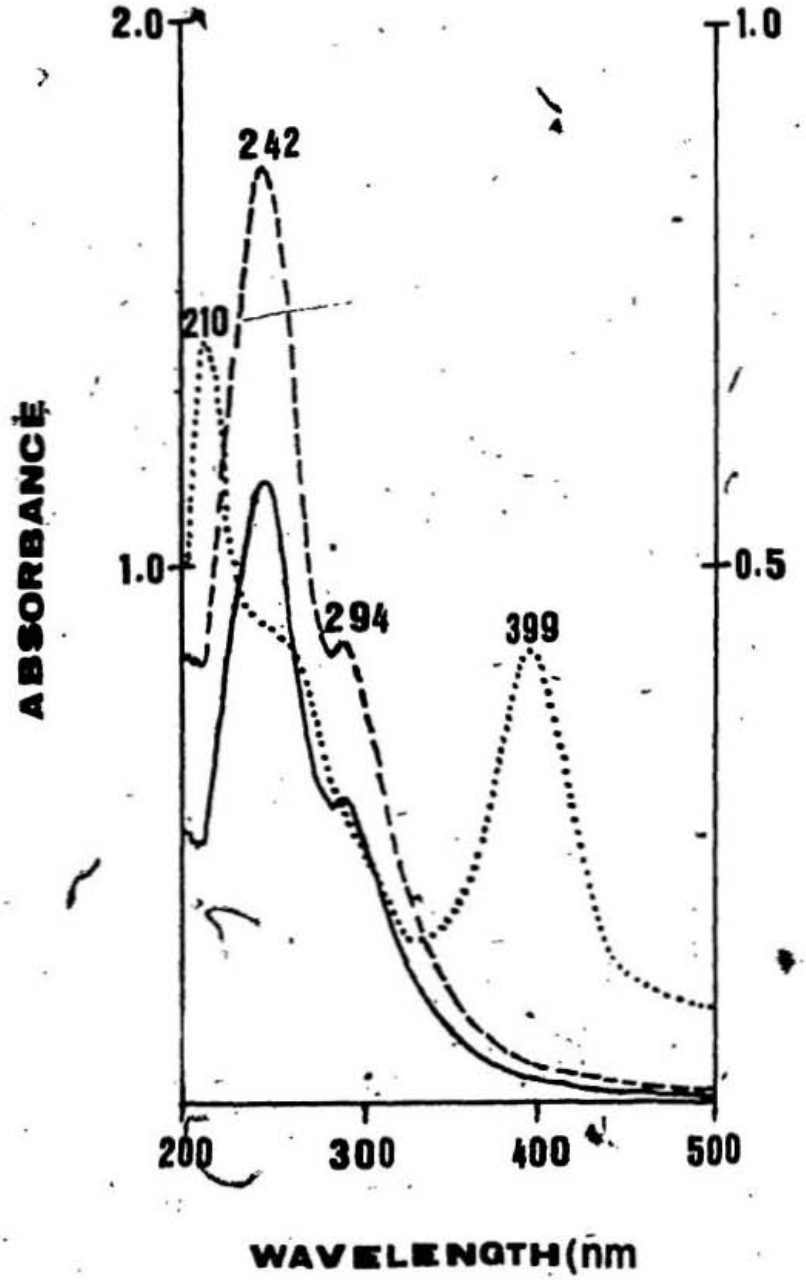
——: Phenol (100 μM)/HRP (10 μg)/ H_2O_2 (100 μM)/BSA (5 mg)

- - - : o,o'-Biphenol (100 μM)/HRP (10 μg)/ H_2O_2 (100 μM)/BSA (5 mg)

Reactions were performed in 3 ml of 0.1 M Tris-HCl buffer pH 7.4 at room temperature. After incubation for 30 minutes, reaction mixtures containing BSA were extracted with ethyl acetate (two-times) and the BSA was precipitated with TCA (10% final). BSA was redissolved in 3 ml of 0.1 M Tris-HCl buffer pH 7.4 and scanned using Shimadzu UV-240 spectrophotometer. (Reference cuvettes contained equal amount of BSA).

Reaction mixture containing no BSA, was scanned after 30 minutes of incubation.

FIGURE 6.4


2.0
1.0
242
210
294
399
1.0
0.5
ABSORBANCE
ABSORBANCE
200
300
400
500
WAVELENGTH (nm

products bound to protein were not removed during extraction and precipitation as evidenced by the presence of the peaks at 242 nm and 294 nm. Figure 6.4 also shows the spectrum for o,o'-biphenol oxidation products bound to BSA. The spectrum shows absorbance maxima at 242 nm and 294 nm suggesting that the products binding to protein by o,o'-biphenol oxidation are similar to the phenol oxidation products binding to protein. On the other hand, p,p'-biphenol oxidation products bound to BSA have an absorbance maximum at 259 nm (see Chapter 8, Figure 8.2).

Table 6.2 shows the effects of protease digestion and dinitrofluorobenzene derivatization of the resulting amino acids, on the ^{14}C-phenol oxidation products bound to BSA. Before protease digestion (column 'A'), approximately 75% of the radioactivity remained in the aqueous layer after ethyl acetate extractions, if phenol was incubated with 10 μg HRP and equimolar H_2O_2; only 30% of the radioactivity remained in the aqueous layer, after ethyl acetate extractions, if phenol was incubated with 0.2 μg HRP and equimolar H_2O_2. The remaining of the radioactivity was recovered in the ethyl acetate extracts. Digestion of the BSA bound phenol oxidation products with protease (column 'B') and extraction with ethyl acetate removed 50-70% of the radioactivity bound to BSA (the radioactivity removed was recovered in the ethyl acetate extracts). This suggests that the majority of the products bound to BSA may be non-covalent in nature. However, it could also be possible that the

Table 6.2

Effects of protease treatment and protease/DNFB treatment on BSA containing bound phenol oxidation products.

Incubation condition	% Radioactivity[a] remaining in aqueous phase after extractions with ethyl acetate[b].			
	A	B	C	
			pH 9.0	pH 1.0
Phenol	0.3±0.2	0.3±0.2	0.3±0.2	0.3±0.2
+10μg HRP	0.3±0.2	0.3±0.2	0.3±0.2	0.3±0.2
+0.2μg HRP + H_2O_2	29.5±2.5	17.6±2.4	16.6±3.2	1.2±0.6
+10μg HRP +H_2O_2	74.1±5.8	24.2±3.4	24.4±6.0	2.1±0.8

Phenol (200 μM) was incubated with 0.2 μg HRP or 10 μg HRP and H_2O_2 (200 μM) in 3.0 ml Tris-HCl buffer pH 7.4 for 30 minutes. Controls in the absence of H_2O_2 or in the absence of H_2O_2 and HRP were included.

a: 100% radioactivity represents the total radioactivity added to the incubation mixture.
b: The rest of the radioactivity was recovered in the ethyl acetate layers.

A: undigested reaction mixtures; B: reaction mixtures digested with pronase; C: reaction mixtures digested with pronase and treated with DNFB.

For detailed experimental procedures see Materials and Methods, Sections 2.2.3.2 and 2.2.3.3.

Mean ± S.E.M. for three experiments are given.

products are covalently bound to released amino acids or peptides and are extracted into ethyl acetate.

Amino acids from an aqueous solution can be extracted into an organic solvent following derivatization with dinitrofluorobenzene (DNFB) under acidic conditions (see Materials and Methods section 2.2.3.3). Thus, amino acids covalently bound to drug metabolites can be effectively isolated by this procedure (Jollow et al. 1973). Column 'C' in table 6.2 shows that more than 95% of the radioactivity from the aqueous layer was extracted, from the acidic aqueous reaction mixtures containing BSA bound with phenol oxidation products, after digestion with protease and treatment with DNFB. However, DNFB treatment under alkaline conditions did not increase the amount of radioactivity extracted from the aqueous layers, compared to that of the reaction mixtures not treated with DNFB. This suggests that the binding of some fraction of the phenol oxidation products to BSA may be covalent in nature.

The presence of GSH in the reaction mixture prevented the binding of ^{14}C-phenol oxidation products to protein. GSH also prevented the peroxidase catalyzed phenol oxidation to dimers (data not shown). However, GSH was rapidly and completely oxidized to GSSG (see Chapter 7; figure 7.2). In the absence of phenol, no GSH oxidation occurred in the peroxidase/ H_2O_2 reaction mixture.

Table 6.3 compares the effectiveness of phenol, o,o′-biphenol or p,p′-biphenol oxidation products in reacting with thiol groups on BSA. As can be seen in the Table, the thiol group of bovine serum albumin reacted with p,p′-biphenol oxidation products as effectively as with N-ethylmaleimide. However, the o,o′-biphenol oxidation products or H_2O_2 were ineffective in reducing the number of thiol groups. The small reduction in thiol groups, observed with phenol oxidation products, therefore, may be best attributed to the reaction with p,p′-biphenoquinone formed from phenol oxidation.

6.3) Discussion :

Sawahata and coworkers (1985) have proposed that p,p′-biphenoquinone may be the major reactive product binding to protein when phenol was activated with bone marrow homogenate and H_2O_2. However, the present results indicate that p,p′-biphenoquinone may be one of the reactive metabolites binding to protein but not the major reactive metabolite. The three-fold increase in o,o′-biphenol formation, in the presence of ascorbate, suggests that the majority of the binding may be due to o,o′-biphenol-derived reactive products. The possibility that phenoxy radicals may be directly involved in protein binding appears to be unlikely for the following reasons; (1) if the phenoxy radicals are directly binding to proteins, there should have been a lag period observed in the formation of biphenols. However, both o,o′-biphenol and p,p′-biphenol were detected as early as 15 seconds of incubation, at which time no

Table 6.3

Reaction of phenol, o,o′-biphenol or p,p′-biphenol oxidation products with thiol groups of bovine serum albumin

Treatment	nmol-SH/mg protein
None	9.8±0.5
+Phenol + H_2O_2	7.4±0.3
+o,o′-Biphenol + H_2O_2	8.8±0.5
+p,p′-Biphenol + H_2O_2	1.4±0.3
+H_2O_2[a]	9.5±0.6
+N-Ethylmaleimide[b]	0.3±0.2

Reaction mixtures of 3.0 ml of 0.1 M Tris-HCl buffer pH 7.4 contained : 5 mg bovine serum albumin, 50 μM phenol (or 25 μM p,p′-biphenol or 25 μM o,o′-biphenol), 1 equivalent of H_2O_2 and 10 μg peroxidase. The reaction mixtures were incubated for 30 minutes at 22 °C. Protein thiols were assayed by the procedure of Sedlak and Lindsay (1968).

a: H_2O_2 (50 μM)
b: 3.0 ml of 0.1 M Tris-HCl buffer pH 7.4, 5 mg bovine serum albumin and 20 μM N-ethylmaleimide were incubated as above.

Mean ± S.E.M. for three experiments are given.

protein binding was observed; (2) Sawahata, Rickert and Greenlee (1985) reported that p,p'-biphenoquinone formation was detected when phenol was incubated with partially purified myeloperoxidase but not with bone marrow homogenate- H_2O_2. But p,p'-biphenol formation was detected in both the cases. Furthermore, the results from table 6.1 suggests that p,p'-biphenol and o,o'-biphenol are further oxidized by bone marrow homogenate.

Results from figure 6.3 show that when bone marrow homogenate is incubated with phenol and H_2O_2, protein bands of molecular weight approximately, 80,000, 76,000, 66,000, 58,000 and 36,000 had a decrease in intensity of commassie blue stain (Figure 6.3(d)) in comparison to the intensity of commassie blue stain, of these protein bands, in Figure 6.3 (a), (b) and (c). However, the intensity of commassie blue stain of protein band with molecular weight 41,000 appears to be not affected by any of the treatments (see Figure 6.3 (a) to (d)), mentioned above, which suggests that the decrease in the intensity of the proteins bands - 80,000, 76,000, 66,000 58,000 and 36,000 - may not be due to the low protein content, in the sample applied on the gel. Interestingly, all these proteins (except the proteins of molecular weight 36,000 and 41,000) specifically stained with 3,3'-diaminobenzidine- H_2O_2 indicating that these bands may be peroxidases. It appears that bone marrow from different sources contain at least two different types of peroxidases, namely eosinophil and heterophil (myelo) peroxidases (Desser et al., 1972). The molecular weights of

these enzymes (holoenzymes) were reported to be around 140,000-160,000 (Anderson, Atkin and Eyre, 1982; Desser et al., 1972; Zgliczynski et al., 1968). Upon SDS-polyacrylamide gel electrophoresis they were shown to be separated into subunits which were reported to have different molecular weights (approximately 50,000-80,000) from different laboratories. Recently, it was reported that myeloperoxidase may also contain subunits of molecular weight approximately 15,000 (Anderson, Atkin and Eyre, 1982). The present results show that some low molecular weight proteins (18,000-24,000) disappeared when bone marrow homogenate was incubated with H_2O_2 (Figure 6.3 (c)) or phenol and H_2O_2 (Figure 6.3 (d)). However, whether these proteins are the subunits of peroxidases or not, needs further investigation. Recently, Ohno and Gallin (1985) reported that H_2O_2 alone can inactivate myeloperoxidase. Inactivation of myeloperoxidase may occur by an oxidative destruction of the heme moiety. On the other hand, incubation of bone marrow homogenate with phenol and H_2O_2 results in rapid activation of phenol, and the inactivation of myeloperoxidase may occur by phenol metabolites. Phenol metabolites may also attack other proteins in bone marrow homogenate, which may result in the covalent or non-covalent protein cross-linking.

N-Ethylmaleimide (1 mM), a sulfhydryl reagent, appears to decrease the binding of phenol oxidation product(s) to proteins in bone marrow homogenate, by approximately 10%, suggesting that groups other than thiols are

involved or that the binding may not be covalent. It may be suggested from the results of Figure 6.3 (d) that Commassie blue and phenol products bind to the same functional groups of proteins. Commassie blue appears to interact electrostatically with positive charges on basic amino acids in proteins, which is further enhanced by hydrophobic bonding (Tal, Silberstein and Nusser, 1985). In the previous chapter it was proposed that phenol oxidation products (presumably polymers) bind to DNA by forming charge-transfer complexes with DNA. A similar interaction could also explain much of the protein binding with certain amino acids acting as electron donors and phenol polymer acting as electron acceptor.

In order to understand the nature of interaction between phenol metabolites and proteins in more detail, oxidation of phenol by HRP and H_2O_2, in the presence of BSA was investigated (Figure 6.4, Tables 6.1 and 6.2). In support of a non-covalent interaction it was found that a majority of the product(s) bound to BSA was released when BSA (containing bound phenol oxidation products) was digested with protease, and the released product(s) was extracted into ethyl acetate. This implies that majority of the binding of phenol oxidation products to BSA may be through non-covalent interactions. However, it could also be possible that the products are covalently bound to amino acids and peptides and are extracted into ethyl acetate. It was proposed earlier in chapter 5 that binding of phenol oxidation products to DNA may be non-

covalent and that the binding between phenol oxidation products and DNA may occur through charge-transfer complexes. The released product(s), upon DNA hydrolysis, however, were not extracted into butanol (or ethyl acetate) but stayed at the interphase between the organic and aqueous layers. Interestingly, it was indicated by the results of this chapter that the radioactivity was present in the organic layer after extraction with ethyl acetate, of the protease digested BSA. This may indicate that BSA prevents the precipitation of the phenol oxidation products.

Some fraction of the bound products were not released from BSA when BSA was digested with protease. However, they were extracted into ethyl acetate when the digested BSA was treated with DNFB and acidified. This implies that these metabolites were covalently linked to amino acid residues.

Table 6.3 shows that p,p′-biphenol oxidation product(s) can react well with thiol groups on BSA. However, o,o′-biphenol oxidation product(s) or phenol oxidation product(s) reacted poorly with thiol groups on BSA. Reaction of phenol oxidation product(s), with thiol groups on BSA, appears to be greater than the reaction of o,o′-biphenol oxidation product(s). Since, (1) phenoxy radicals appear not to bind to protein (table 6.1); (2) p,p′-biphenol is a minor product of phenol oxidation and (3) o,o′-biphenol oxidation product(s) appear not to react with thiol groups on BSA, it is tempting to postulate that p,p′-biphenoquinone (further oxidation product of p,p′-biphenol) formed from

phenol could be reacting with thiol groups on BSA. A similar reaction may explain why p,p′-biphenoquinone was not detected when phenol was oxidized by bone marrow homogenate, but was detected when oxidized by partially purified myeloperoxidase (Sawahata, Rickert and Greenlee, 1985). Furthermore, results from chapter 8, show that oxidation of p,p′-biphenol in the presence of GSH resulted in the formation of covalent conjugates with thiol group of GSH. The major conjugate formed is identified as 3-S-(glutathion-yl)-p,p′-biphenol, and the results are consistent with the suggestion that the conjugate was formed from the reaction of p,p′-biphenoquinone with the thiol group of GSH. However, further studies are necessary to determine the structure of covalent conjugate between BSA and oxidized phenol product(s). Preliminary studies on protease digested BSA (bound with phenol products), after extraction with ethyl acetate, showed a peak at 257 nm, upon spectral analysis (characteristic of p,p′-biphenol-GSH conjugate; Chapter 8; figure 8.2), which suggests that the covalent interaction with BSA may involve reaction of p,p′-biphenoquinone with a cysteine residue. If this is the case, whether p,p′-biphenoquinone could cross-link the proteins or not should be investigated, in comparison with o,o′-biphenol oxidation products, which may give further information on the mechanism of interactions of phenol oxidation products with proteins.

In conclusion, the results presented in this chapter suggests that o,o′-

biphenol was the major product formed, when phenol was oxidized by bone marrow homogenate and H_2O_2. The data is also consistent with the suggestion that further oxidation product(s) of o,o'-biphenol may account for the majority of the products binding to protein. However, further studies are necessary to delineate the mechanism of interaction of phenol oxidation products with proteins.

CHAPTER 7

PEROXIDASE-CATALYZED OXYGEN ACTIVATION BY ARYLAMINE CARCINOGENS AND PHENOL.

7.1) Introduction :

Reactions of reactive metabolites of various carcinogens with glutathione have become an interesting subject of discussion with regard to the role of glutathione in detoxifying the reactive metabolites involved (Moldeus and Jernstrom, 1983; Smith et al., 1983; Reed, 1985). The majority of the evidence to date, obtained from various studies, indicates that glutathione can conjugate with some electrophilic metabolites of various carcinogens either in the presence or in the absence of glutathione transferases. It is believed that these conjugation reactions are involved in detoxification of these electrophilic metabolites and that the depletion of cellular glutathione can modify the toxicity and carcinogenicity of those xenobiotics. However, aromatic hydroxylamines and alkylating metabolites of nitrosamines may react with DNA much more effectively than they do with GSH (Mulder et al., 1984; Pearson and Songstad, 1967).

Recent studies from various laboratories indicate that glutathione can also

interact with free-radical metabolites of xenobiotics (Moldeus and Jernstrom, 1983; Smith et al. 1983; Reed, 1985). Thiyl radicals were shown to be formed initially which dimerize to form GSSG. In the process, reactive oxygen products such as superoxy radicals are formed. Whether the reactions of glutathione with xenobiotic free-radicals result in detoxification or toxicity is not known.

In the present chapter, results are presented on the reaction mechanism by which GSH or NADH, interact with free-radicals derived from various arylamine carcinogens and phenol, during oxidation with peroxidase and H_2O_2 in the presence of GSH or NADH. Reactions with NADH were performed for a comparison so as to understand the mechanisms in detail. The reactions of peroxidase/H_2O_2-catalyzed oxidation of non-carcinogenic arylamines such as mesidine, aniline, 1-naphthylamine and phenols such as hydroquinone, catechol, p,p'-biphenol and o,o'-biphenol, in the presence of NADH or GSH are also compared.

7.2) Results :

Table 7.1 shows that the oxidation of the arylamines 1-naphthylamine, 2-naphthylamine or N,N'-dimethyl-p-toluidine (N,N'-DMPT) by HRP and H_2O_2 can result in some oxygen uptake. Much more rapid oxygen uptake occurred

Table 7.1

PEROXIDASE MEDIATED OXYGEN ACTIVATION BY ARYLAMINE CARCINOGENS AND PHENOL.

Substrate	Oxygen consumed(μM)		
	with NADH	with GSH	minus NADH/GSH
none	0.5±0.5	0.5±0.5	0.5±0.5
2-Naphthylamine (0.01 mM)	142.8±14.6	137.5±15.7	16.5±3.5
1-Naphthylamine (0.01 mM)	32.4±4.8	30.8±6.2	28.8±4.4
4-Aminobiphenyl (0.01 mM)	140.8±16.4	137.8±14.1	11.5±4.4
MAB (0.01 mM)	106.6±10.2	76.2±12.2	8.5±4.5
N,N'-DMPT (0.01 mM)	108.4±10.6	86.5±13.4	34.6±6.6
2-Aminofluorene (0.01 mM)	62.4±8.2	69.8±5.2	5.5±5.5
Mesidine (0.1 mM)	0.5±0.5	0.5±0.5	0.5±0.5
Aniline (0.1 mM)	0.5±0.5	0.5±0.5	0.5±0.5
Phenol (0.1 mM)	114.2±14.4	119.1±13.2	0.5±0.5
o,o'-Biphenol (0.1 mM)	105.2±15.4	112.1±14.1	0.5±0.5
p,p'-Biphenol (0.1 mM)	0.5±0.5	0.5±0.5	0.5±0.5
Catechol (0.1 mM)	0.5±0.5	0.5±0.5	0.5±0.5
Hydroquinone (0.1 mM)	0.5±0.5	0.5±0.5	0.5±0.5

Incubation Conditions :- The reaction mixtures contained in 2 ml of 0.1M Tris-HCl, 1.0 mM EDTA buffer pH 7.4, phenol or arylamine substrate (concentrations as indicated), HRP (10 μg), NADH (200 μM) or GSH (400 μM). Reactions were started by the addition of H_2O_2 (10 μM) and followed until complete (usually 1-2 minutes). See Materials and Methods for the measurement of oxygen consumption(Section 2.2.6.2).

Mean ± S.E.M for 3 experiments are given.

when NADH or GSH are included in the reaction mixture (except with 1-naphthylamine). Little oxygen consumption was observed in the absence of HRP or H_2O_2. Phenol and o,o'-biphenol were active only in the presence of NADH or GSH. 2-Aminofluorene, 4-aminobiphenyl or methylaminoazobenzene (MAB) resulted in only little oxygen consumption in the absence of NADH or GSH under the conditions studied, but resulted in extensive oxygen consumption in the presence of NADH or GSH. p,p'-Biphenol, hydroquinone, mesidine or aniline were not active even in the presence of NADH or GSH.

Figure 7.1 and 7.2 shows the effect of varying H_2O_2 concentration (Figure 7.1) or SOD (Figure 7.2) on the total oxygen consumption catalyzed by peroxidase in a reaction mixture containing phenol or 2-aminofluorene, NADH, H_2O_2 and HRP. Oxygen consumption was proportional to the NADH oxidized. Approximately 0.80 moles of oxygen were consumed for the oxidation of 1 mole of NADH. Similar results were obtained when GSH was used instead of NADH. GSSG was formed in stoichiometric amounts from the GSH and approximately 0.40 moles of oxygen were consumed for the oxidation of 1 mole of GSH. The disappearance of NADH with time in the reaction mixtures was followed at 340 nm. In the absence of arylamines or phenols the oxidation of NADH by the HRP/H_2O_2 reaction mixture was very slow at this pH. However, in the presence of trace amounts of arylamines or phenols the oxidation of NADH was rapid. Figure 7.2 shows the effect of SOD on the amount of

Figure 7.1

Oxidation of NADH and GSH by HRP-H_2O_2 in the presence of phenol and 2-aminofluorene; Dependence on H_2O_2 concentration:

2 ml reaction mixtures of 0.1 M Tris-HCl, 1.0 mM EDTA buffer pH 7.4 contained : phenol (200 μM) or 2-aminofluorene (20 μM), HRP (1 μg). and NADH (200 μM) or GSH (400 μM). Reactions were started by the addition of H_2O_2.

X X X : In the presence of 2-aminofluorene; ●●● : In the presence of phenol.

Note: ● or X represent the average values of oxygen consumption from three experiments. Error bars represent the largest standard deviation obtained from average values of NADH oxidized or GSSG formed (from three experiments). (Standard deviation for oxygen consumption was not greater than the error bars represented).

(The total oxygen consumed, NADH oxidized or GSSG formed (from 2 moles of GSH in the reaction mixture was measured as described in Materials and Methods (Sections 2.2.7 and 2.2.9).

FIGURE 7.1

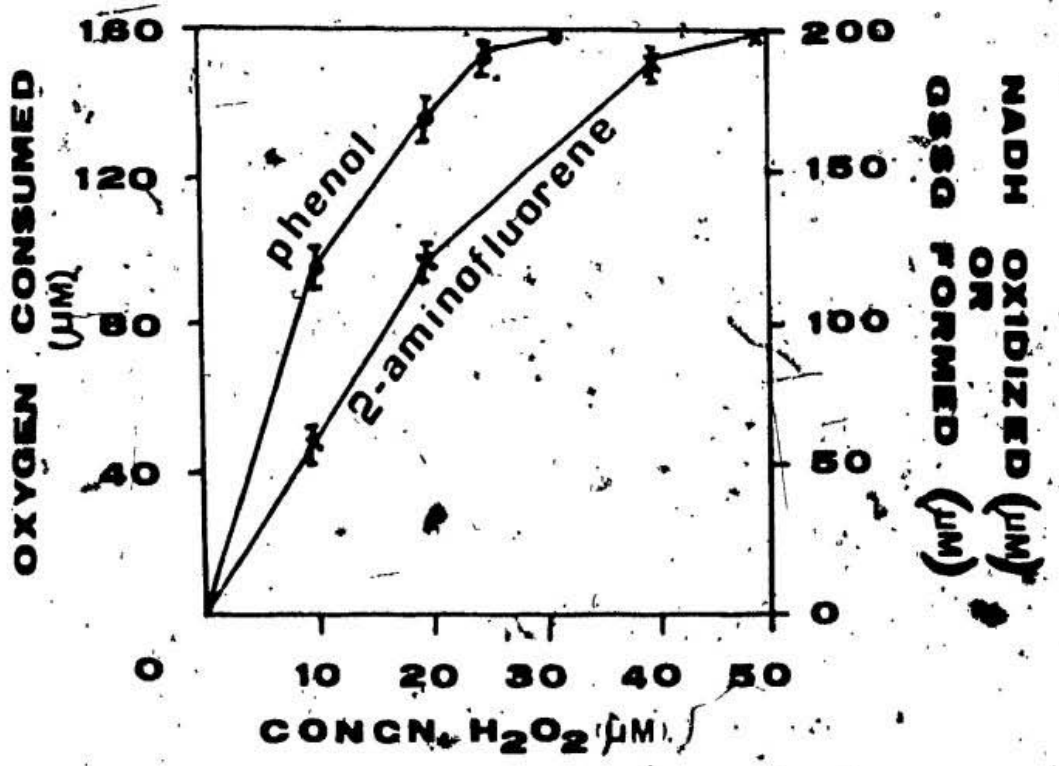

OXYGEN CONSUMED (μM)
NADH OXIDIZED (μM) OR OSBD FORMED (μM)
phenol
2-aminofluorene
CONCN. H_2O_2 (μM)
0
10
20
30
40
50
40
80
120
160
0
50
100
150
200

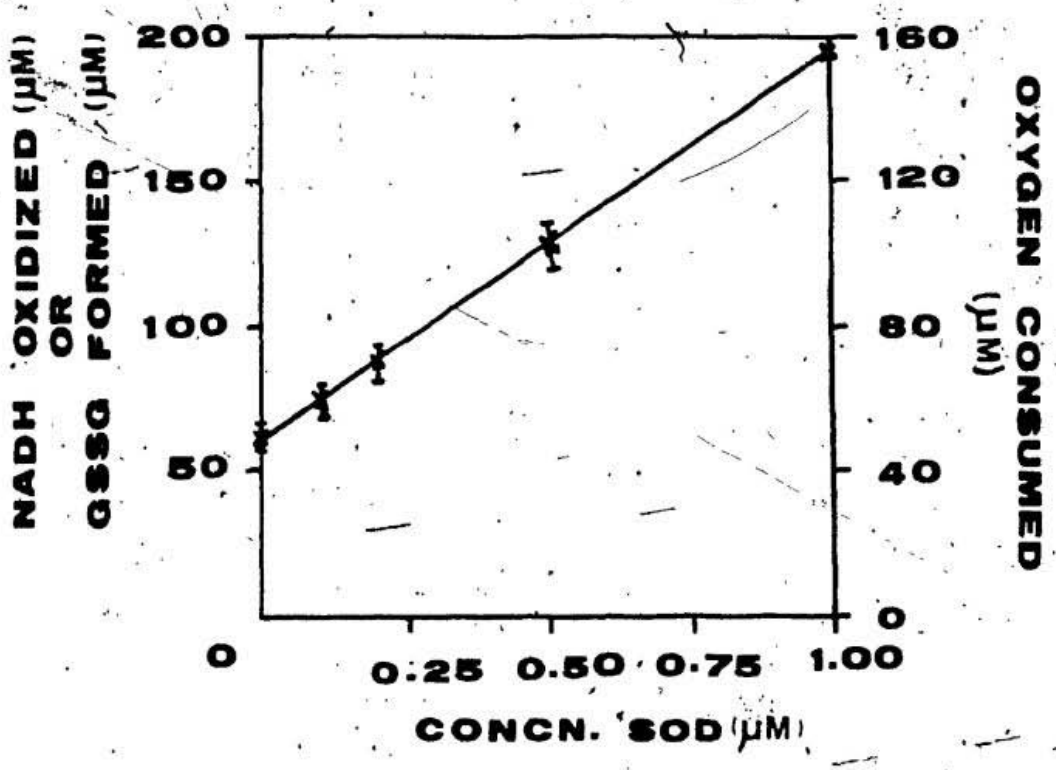
Figure 7.2

Oxidation of NADH and GSH by HRP/H_2O_2 in the presence of 2-aminofluorene; Dependence on SOD concentration :

2 ml reaction mixtures of 0.1 M Tris-HCl, 1.0 mM EDTA buffer pH 7.4 contained : aminofluorene (20 μM), HRP (1 μg) and NADH (200 μM) or GSH (400 μM), SOD (concentrations as indicated in the figure) and the reactions were started by the addition of H_2O_2 (5 μM).

Note: The X points represent the average values of oxygen consumption from three experiments. Error bars represent the standard deviations obtained from the average values of NADH oxidized or GSSG formed (from three experiments). (Standard deviation for oxygen consumption was not greater than the error bars represented).

FIGURE 7.2


NADH OXIDIZED (μM) OR GSSG FORMED (μM)
200
150
100
50
0
160
120
80
40
0
OXYGEN CONSUMED (μM)
0·25
0·50
0·75
1·00
CONCN. SOD (μM)

NADH oxidized or GSSG formed in relation to oxygen consumed. As the figure shows, addition of 1 μM SOD resulted in the complete oxidation of NADH and a two-fold increase in the accompanying oxygen uptake. The addition of SOD also resulted in the complete oxidation of GSH and a two-fold increase in the accompanying oxygen uptake.

As shown in figure 7.3, phenol enhanced the initial reduction of ferricytochrome *c* in the H_2O_2/HRP/NADH system. Increasing the concentration of phenol increased the initial rate of reduction of ferricytochrome *c*. Above 50 μM concentration of phenol the rate was too fast to measure. No reduction of ferricytochrome *c* occurred with phenol alone in the absence of H_2O_2. The enhancement by phenol of the reduction rate was completely prevented by superoxide dismutase (1 μM), suggesting that superoxide was responsible for cytochrome *c* reduction.

The extent of superoxide formation by the peroxidase/H_2O_2/thyroxine system was previously estimated from the inhibition of O_2 uptake by ferricytochrome *c* and its prevention by superoxide dismutase (Takayama and Nakano, 1977). Figure 7.4 shows the oxygen consumption by phenol in the HRP/H_2O_2/NADH system. High H_2O_2 concentration was also used so as to oxidize all the NADH in the system. As shown in the figure, ferricytochrome *c* inhibited oxygen consumption indicating that superoxy radical formation was

Figure 7.3

Reduction of ferricytochromec during phenol mediated NADH oxidation by HRP/H_2O_2 :

See Materials and Methods (section 2.2.10) for reaction conditions.

FIGURE 7.3

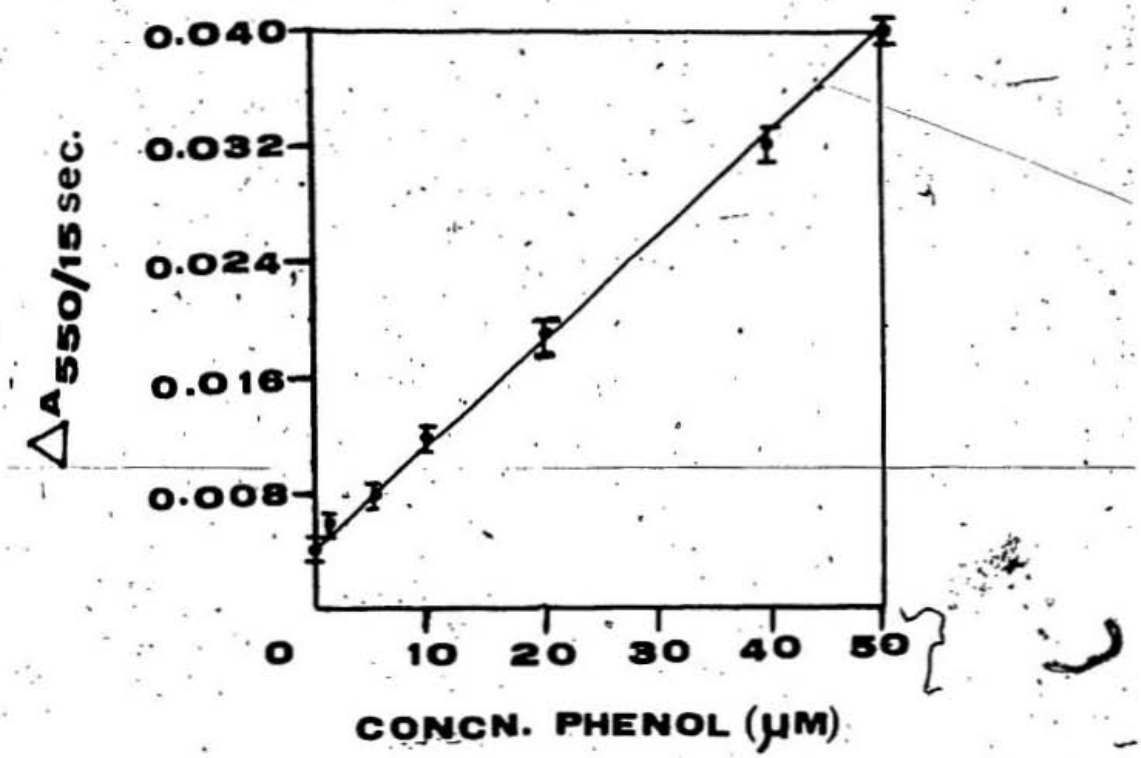

0.040
0.032
0.024
0.016
0.008
$\Delta A_{550/15\,sec.}$
0
10
20
30
40
50
CONCN. PHENOL (μM)

Figure 7.4

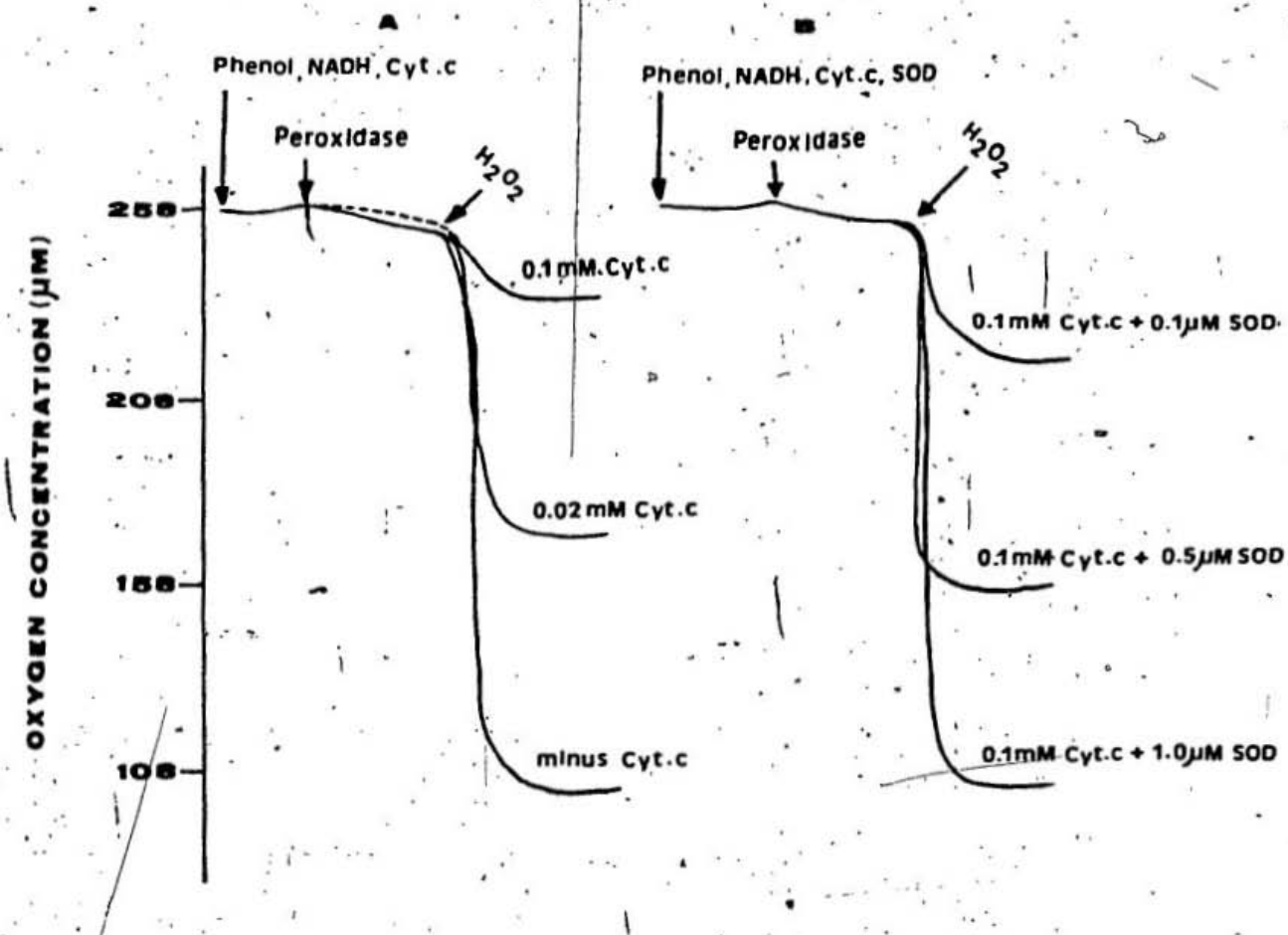
A) Effect of ferricytochrome *c* and SOD on oxygen consumption by arylamine or phenol/NADH/HRP/H_2O_2 system :

2 ml of 0.1 M Tris-HCl, 1.0 mM EDTA buffer pH 7.4 contained : NADH (200 μM), phenol (400 μM), HRP (10 μg) and ferricytochrome *c* (concentrations as indicated in the figure). Reactions were started by the addition of H_2O_2 (100 μM).

B) Effect of SOD on inhibition by ferricytochrome *c*:

Reaction conditions are as described in (A).

FIGURE 7.4


A
Phenol, NADH, Cyt.c
Peroxidase
H_2O_2
0.1mM.Cyt.c
0.02mM Cyt.c
minus Cyt.c
B
Phenol, NADH, Cyt.c, SOD
Peroxidase
H_2O_2
0.1mM Cyt.c + 0.1μM SOD
0.1mM Cyt.c + 0.5μM SOD
0.1mM Cyt.c + 1.0μM SOD
OXYGEN CONCENTRATION (μM)
250
200
150
100

required for oxygen consumption. Approximately 0.15 mM ferricytochrome *c* was required to inhibit the oxygen consumption completely. However, addition of only 0.02 mM ferricytochrome *c* resulted in a 50% inhibition of oxygen consumption, probably because reduced cytochrome *c* can also be oxidized by superoxy radicals back to oxidized cytochrome *c* (ferricytochrome *c*) (Takayama and Nakano, 1977). Superoxide dismutase (1 μM) prevented the inhibition by ferricytochrome *c*. Similar results were obtained when phenol was replaced by aminofluorene or other arylamine substrates. Ferricytochrome *c*, however, did not affect the amount of NADH oxidized in this system indicating that superoxy radicals do not oxidize NADH.

Figure 7.5 shows that oxidation of NADH occurred and is of first-order with respect to phenol concentration when fixed concentrations of other reactants were used. The rate can be calculated from

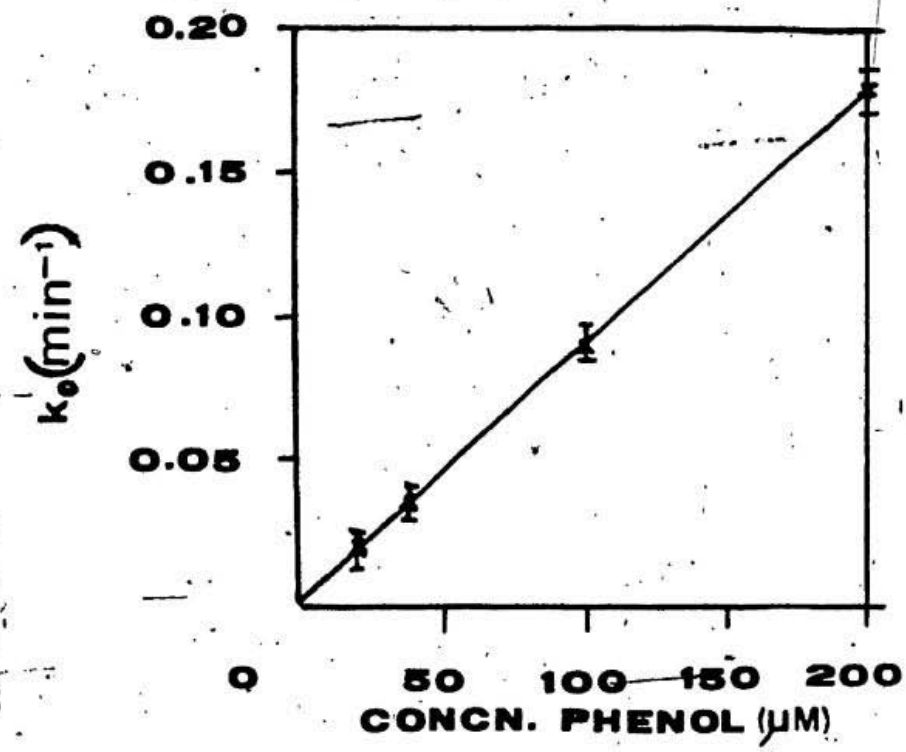
$$-\left(\frac{d[NADH]}{dt}\right) = k_0[Ph][NADH] = k_1[Ph]$$

where $k_0[NADH] = k_1$ is the apparent first-order rate constant (min^{-1}) in which [Ph] is the initial concentration of phenol. The rate constants, however, were independent of H_2O_2 concentration provided the H_2O_2 concentration was above 30 μM. Below these concentrations NADH oxidation was not complete (see Figure 7.1); however, the initial rates for NADH oxidation were not affected. NADH oxidation measured with other compounds were also

Figure 7.5
First-order rate constants (k_0) were plotted against concentration of phenol :

2 ml reaction mixtures of 0.1 M Tris-HCl, 1.0 mM EDTA buffer pH 7.4 contained, phenol (concentrations as indicated in the figure), HRP (1 μg), NADH (200 μM). The reactions were started by the addition of H_2O_2 (20 μM).

FIGURE 7.5



apparently first-order reactions.

Table 7.2 shows the apparent first-order rate constants of all the compounds tested under the conditions used for following oxygen uptake. It can be seen that compounds such as aniline, mesidine and p,p'-biphenol which did not activate oxygen were effective at catalyzing NADH oxidation. However, hydroquinone did not catalyze NADH oxidation indicating that the semiquinone or quinone products did not oxidize NADH. 2-Naphthylamine was found to be the most potent compound followed by 2-aminofluorene and 4-aminobiphenyl. At these peroxidase concentrations the rate of NADH oxidation was dependent on peroxidase concentration indicating that the rate of peroxidase/H_2O_2-catalyzed oxidation of the arylamine or phenolic compounds was rate limiting. In the case of p,p'-biphenol, however, the rate of NADH oxidation was not affected over the range of 0.1 μg to 500 μg peroxidase. p,p'-Biphenol appears to be the most effective peroxidase substrate known (see Table 5.3; Chapter 5) and therefore the rate of p,p'-biphenol oxidation was not rate limiting. Table 7.2 also shows the rate constants for NADH oxidation at high peroxidase concentrations. Under these conditions the rate was independent of peroxidase concentrations and therefore reflects the ability of the various arylamine oxidation products to oxidize NADH.

Table 7.2
First-Order Rate Constants in Arylamine and Phenol mediated oxidation of NADH by HRP-H_2O_2.

Substrate	k_1(apparent)		k_1
	HRP[a]	LP[a]	HRP[b]
none	0.00	0.00	0.00
2-Aminofluorene	0.32±0.08	0.44±0.10	N.M.
1-Naphthylamine	0.1±0.024	0.28±0.06	N.M.
2-Naphthylamine	0.8±0.2	0.8±0.2	N.M.
4-Aminobiphenyl	0.14±0.04	0.12±0.03	N.M.
N,N'-DMPT	0.10±0.03	0.12±0.03	0.42±0.06
MAB	0.06±0.02	0.04±0.01	0.34±0.05
AB	0.04±0.01	0.03±0.01	0.31±0.04
Mesidine	0.08±0.02	0.16±0.03	0.14±0.03
Aniline	0.04±0.01	0.10±0.02	0.10±0.02
Phenol	0.02±0.01	0.42±0.08	0.03±0.01
o,o'-Biphenol	0.005±0.001	0.08±0.02	0.03±0.01
p,p'-Biphenol	0.03±0.01	0.06±0.02	0.04±0.01
Hydroquinone	0.00	0.00	0.00
Catechol	0.18±0.05	0.95±0.10	0.04±0.01

a. Reaction mixtures of 2 ml 0.1 M Tris HCl, 1.0 mM EDTA buffer pH 7.4 contained : NADH (100 μM) substrate(10 μM), HRP (1 μg), or LP (10 μg). Reactions were started by the addition of H_2O_2(10 μM).
* All the substrates used were of 10 μM concentrations except for p.p'.biphenol which was used at the concentrations of 5 μM.

b. Reaction mixtures contained NADH (50 μM), substrate (0.5 μM), HRP (100 μg). Reactions were started by the addition of H_2O_2 (50 μM).
N.M. : Too fast to measure. Mean ± S.E.M. for 3 experiments are given.

7.3) Discussion :

Figure 7.1 and 7.2 showed that trace amounts of phenol or arylamines can catalyze the oxidation of NADH in a reaction mixture containing peroxidase and H_2O_2. Other investigators have shown that some phenols can act as catalysts in the oxidation of NADH by the hydrogen peroxide and peroxidase (Takayama and Nakano, 1977). The mechanism is believed to be due to the initial oxidation of phenol to phenoxy radicals by peroxidase/H_2O_2 and the reaction of phenoxy radicals with NADH to form NAD radicals. The reaction of NAD radicals with oxygen forms O_2^- (Takayama and Nakano, 1977). The above results (Figure 7.1) showed that 0.80 moles of oxygen were consumed per mole of NADH oxidized, which is close to the value of 0.83 moles of oxygen consumed per mole of NADH oxidation reported by Takayama and Nakano, (1977) where thyroxine was used as catalyst. They also reported that 54% of the total flux of electrons from NADH to oxygen resulted in superoxy radical production. The mechanism in the presence of GSH also appears to be similar, except that 0.40 moles of oxygen were consumed per mole of GSH oxidized.

Figure 7.1 showed that a small amount of H_2O_2 in the presence of peroxidase and a small amount of arylamine or phenolic donor catalyzed the oxidation of a large excess of NADH and GSH. Since superoxide radicals do not oxidize NADH (Takayama and Nakano, 1977), they presumably dismutate to H_2O_2, thus allowing the reaction to proceed. It is also known that O_2^- can

inactivate peroxidase by converting it to compound III (Yamazaki and Piette, 1963). The addition of superoxide dismutase to the reaction mixture resulted in a two-fold increase in the total NADH or GSH oxidized. This is probably as a result of the increased H_2O_2 levels. Superoxy dismutase also prevents the inactivation of the peroxidase as a result of compound III formation by superoxy radicals (Yamazaki and Piette, 1963).

A mechanism for the oxygen activation can be described by the following equations :

$$2ArNH_2 + H_2O_2 \rightarrow 2ArNH + 2H_2O \qquad (1)$$

With some arylamines, the radical formed is further oxidized by oxygen and superoxide radicals may be formed. Oxygen uptake was previously reported for the peroxidase-catalyzed oxidation of N,N'-DMPT (Ashley, Davis and Griffin, 1980). Superoxide radicals are formed during the peroxidase catalyzed oxidation of halogenated N,N'-dimethylanilines (Galliani and Rindone, 1979).

In the presence of NADH the arylamine radicals could react with NADH forming NAD :-

$$ArNH + NADH \rightarrow ArNH_2 + NAD \qquad (2)$$

Willson (1970) has reported a second-order rate constant of $1.9 \times 10^9\ M^{-1}\ s^{-1}$ for the following reaction.

$$NAD + O_2 + H^+ \rightarrow NAD^+ + \dot{O}_2^- \qquad (3)$$

The requirement for only catalytic (Figure 7.1) amounts of H_2O_2 could be explained if the H_2O_2 formed by dismutation of the superoxide radicals (reaction [4]) participated in reaction (1).

$$2\dot{O}_2^- \rightleftharpoons H_2O_2 + O_2 \qquad (4)$$

A different mechanism for superoxy radical formation in the presence of GSH is likely as the thiyl radicals react with O_2 to form a peroxy sulfenyl radical which reacted further to form higher oxidation states of GSH (Wefers and Sies, 1983) including GSSG.

$$ArNH + GS^- \rightarrow ArNH_2 + GS \qquad (5)$$

The thiyl radical formed can react with GS^- to form a disulfide radical anion which is autoxidisable (Harman, Mottley and Mason 1984; Saez et al., 1982). Other investigators have found a cysteine oxidase activity for peroxidase (Olsen and Davies, 1976) and a similar mechanism for the H_2O_2 formation has been proposed (Harman, Mottley and Mason, 1984). Barton and Packer (1970) have reported second-order rate constants of $3 \times 10^9\ M^{-1}\ s^{-1}$ and $4 \times$

10^8 M^{-1} s^{-1} respectively for the following reactions. However, Quintiliani (1976) has reported 6.6 x 10^8 M^{-1} s^{-1} and 1.6 x 10^8 M^{-1} s^{-1} respectively for these reactions:-

$$GS + GS^- \rightarrow GSSG^- \quad (6)$$

$$GSSG^- + O_2 \rightarrow GSSG + O_2^- \quad (7)$$

At low catalytic concentrations, the non-carcinogenic arylamines mesidine, aniline, or catechol or p,p′-biphenol were ineffective in catalyzing oxygen activation although they were effective in mediating the oxidation of NADH. With these compounds, the following very rapid reaction could prevent NAD accumulation and the above chain reaction but result in NADH oxidation by redox cycling :

$$NAD + ArNH \rightarrow NAD^+ + ArNH_2 \quad (8)$$

However, the quinone products of catechol and p,p′-biphenol rapidly oxidized NADH. Furthermore, during the p,p′-biphenol/peroxidase/NADH/H_2O_2-catalyzed oxidation, p,p′-biphenol existed as p,p′-biphenoquinone (see Chapter 8). Quinones oxidize NADH by hydride transfer rather than electron transfer (Carlson and Miller, 1985). It is therefore unlikely that p,p′-biphenoxy radicals are involved in NADH oxidation.

Recently oxygen uptake and thiyl radical formation has been reported with peroxidase, GSH and much higher non-catalytic concentrations of H_2O_2 and p-phenetidine than those used here (Ross et al., 1985). This oxygen uptake was ascribed to the following reactions :-

$$GS + O_2 \rightarrow GSO_2^{\cdot} \qquad (9)$$

$$2GSO_2^{\cdot} + H_2O \rightarrow GSO_2H + GSO_3H \qquad (10)$$

The lack of superoxide radical and H_2O_2 formation would then explain why little oxygen uptake occurs at catalytic concentrations of p-phenetidine and H_2O_2. However, no GSO_2H or GSO_3H formation occurred (determined by the H.p.l.c. method of Reed et al., 1980) in the arylamine radical catalyzed GSH oxidation reported in this chapter.

The reaction described in this chapter shows that, for every mole of NADH oxidized, 0.8 moles of O_2 was consumed and for every mole of GSH oxidized 0.4 moles of O_2 was consumed. Takayama and Nakano (1977) obtained 0.83 moles of oxygen consumption for every mole of NADH oxidized, when thyroxine was used as a substrate.

According to the reaction sequence presented [reaction(1) to reaction (4)]: one mole of H_2O_2 converts two moles of $ArNH_2$ to two moles of $ArNH^{\cdot}$ and two moles of H_2O. The two moles of $ArNH^{\cdot}$ would oxidize two moles of

NADH to two moles of NAD· and two moles of O_2 to yield two moles of NAD and two moles of O_2^-. Then the dismutation of two moles of O_2^- generate one mole of O_2 and one mole of H_2O_2. The net result is two moles of NADH oxidized and one mole of O_2 consumed and two moles of NAD and two moles of H_2O are formed as shown below:-

$$2ArNH_2 + H_2O_2 \rightarrow 2ArNH + 2H_2O \quad (1)$$

$$2ArNH + 2NADH \rightarrow 2ArNH_2 + 2NAD \quad (2)$$

$$2NAD + 2O_2 + H^+ \rightarrow 2NAD^+ + 2O_2^- \quad (3)$$

$$2O_2^- \rightleftharpoons H_2O_2 + O_2 \quad (4)$$

$$2NADH + O_2 + 2H^+ \rightarrow 2NAD^+ + 2H_2O \quad (11)$$

Thus, according to reaction (11), 0.5 moles of O_2 consumption is expected for every mole of NADH oxidized. However, the results show that 0.8 moles of O_2 consumption for every mole of NADH oxidized. This suggests that reactions of molecular oxygen, other than with NAD· may also be occurring. In Table 1 it is shown that oxygen consumption by arylamines, even in the absence of NADH, occurs to some extent. Oxygen uptake was previously

reported for the peroxidase-catalyzed oxidation of N,N'-DMPT (Ashley, Davis and Griffin, 1980). Peroxidase-catalyzed oxidation of N,N'-dimethylanilines also results in oxygen consumption and superoxide radicals are formed (Galliani and Rindone, 1979).

$$ArNH + O_2 + H^+ \rightarrow ArNH_2 + O_2^- \qquad (12)'$$

This reaction in addition to the reactions (1) - (4) may explain the additional oxygen consumption and the observed stoichiometry of 0.8 moles of oxygen consumption per mole of NADH oxidation.

Reactions of phenoxy radicals of 2,4,6-tri-*tert*-butylphenol with oxygen result in the formation of peroxides (Taylor and Battersby, eds., 1967). However, the phenoxy radicals formed from the oxidation of 2,6-di-*tert*-butylphenol dimerize much faster than the reaction with oxygen. A similar reaction may explain why phenoxy radicals did not react with oxygen (Table 1). However, in the presence of NADH, due to the reactions of phenoxy radicals with NADH, the dimerization reactions may be prevented. Under these conditions some of the oxygen uptake may be due to addition reactions with phenoxy radicals which would be favoured over dimerization when the steady state concentration of phenoxy radicals is low. Addition reactions of oxygen with arylamine radicals may also occur.

A similar kind of reaction may explain the stoichiometry of 0.4 moles of oxygen consumption for 1 mole of GSH oxidation.

CHAPTER 8

PEROXIDASE/H_2O_2-MEDIATED FORMATION OF 3-S-(GLUTATHION-yl)-P,P'-BIPHENOL FROM P,P'-BIPHENOL AND GLUTATHIONE

8.1) Introduction :

In the previous chapter it was shown that oxidation of phenol or o,o'-biphenol (but not p,p'-biphenol or hydroquinone or catechol) peroxidase/ H_2O_2 in the presence of GSH results in superoxy radical formation. Hydroquinone and catechol were known to be oxidized to the corresponding electrophilic quinones. (p-benzoquinone and o-benzoquinone respectively) *via* the intermediate semiquinone-radical formation. p-Benzoquinone and o-benzoquinone readily form covalent conjugates with GSH (Tunek et al., 1980; Sawahata and Neal, 1983; Sanada et al., 1972).

In this chapter results are presented on the products formed when p,p'-biphenoquinone reacts with GSH.

8.2) Results :

Oxidation of p,p′-biphenol with horseradish peroxidase and H_2O_2 resulted in the instant formation of a yellow product (399 nm). It is relatively stable with little change in the absorbance over 30 minutes (extractable with ethyl acetate). Figure 8.1 shows the mass spectrum of p,p′-biphenol oxidation product. The reaction mixture after oxidation of p,p′-biphenol with HRP and H_2O_2, was extracted with ethyl acetate. The organic layer was evaporated under N_2 and subjected to mass spectroscopy. As shown in the Figure 8.1 the major molecular ion found is 186, which suggests that it is a dimer of phenol. However, no p,p′-biphenol was detected if an aliquot of concentrated ethyl acetate extract was subjected to h.p.l.c. or t.l.c. This may suggest that p,p′-biphenoquinone could have been reduced to p,p′-biphenol under the conditions studied for mass spectroscopy.

In the presence of bovine serum albumin the absorbance at 399 nm (yellow colour) disappeared completely within 15 minutes (Figure 8.2). Little p,p′-biphenol or yellow colour was extractable into ethyl acetate suggesting that the yellow coloured product was bound to the protein. Similar results were obtained when p,p′-biphenol was oxidized with bone marrow homogenate and H_2O_2. Figure 8.3 shows the difference spectrum of the bovine serum albumin bound p,p′-biphenol oxidation products which have an absorbance maxima at 259 nm. If equimolar GSH is present in the incubation mixture the protein binding is readily inhibited and none or little absorbance at 259 nm

FIGURE 8.1

MASS SPECTRUM FOR P,P'-BIPHENOL OXIDATION PRODUCTS

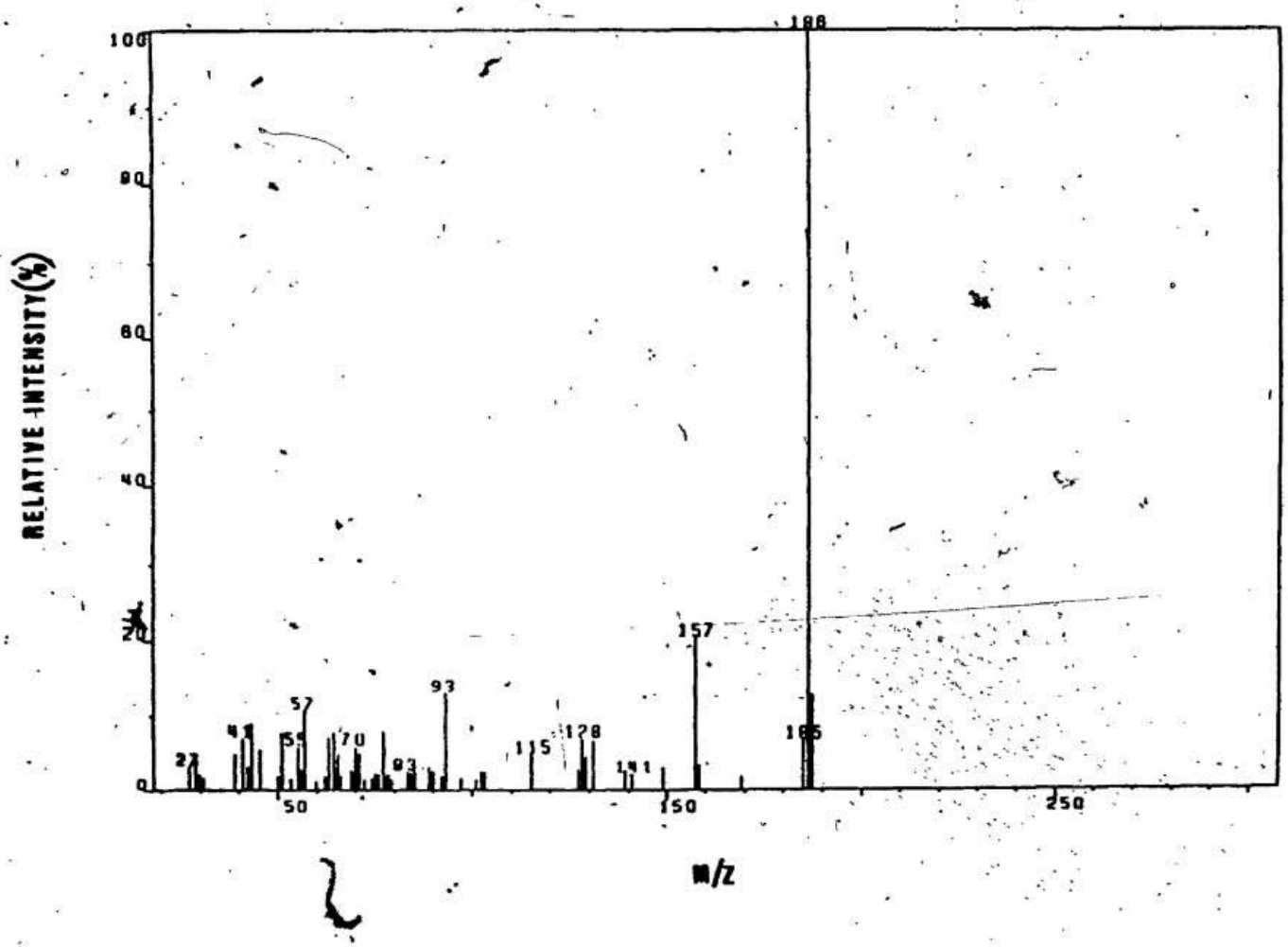


Figure 8:2

Spectral properties of p,p′-biphenol oxidation products in the presence of bovine serum albumin:

p,p′-Biphenol (50 μM) was incubated with HRP (1 μg) and H_2O_2 (60 μM) in the presence of serum albumin (3 mg) in 3 ml of 0.1 M Tris-HCl buffer pH 7.4. The reaction was started by the addition of H_2O_2 and the reaction mixture was scanned for about 15 minutes using Shimadzu UV-240 Spectrophotometer.

Maximal p,p′-biphenoquinone was formed in the first scan. Subsequent scans indicate that serum albumin causes a rapid decrease in the p,p′-biphenoquinone. Each scan represents 40 seconds.

FIGURE 8.2

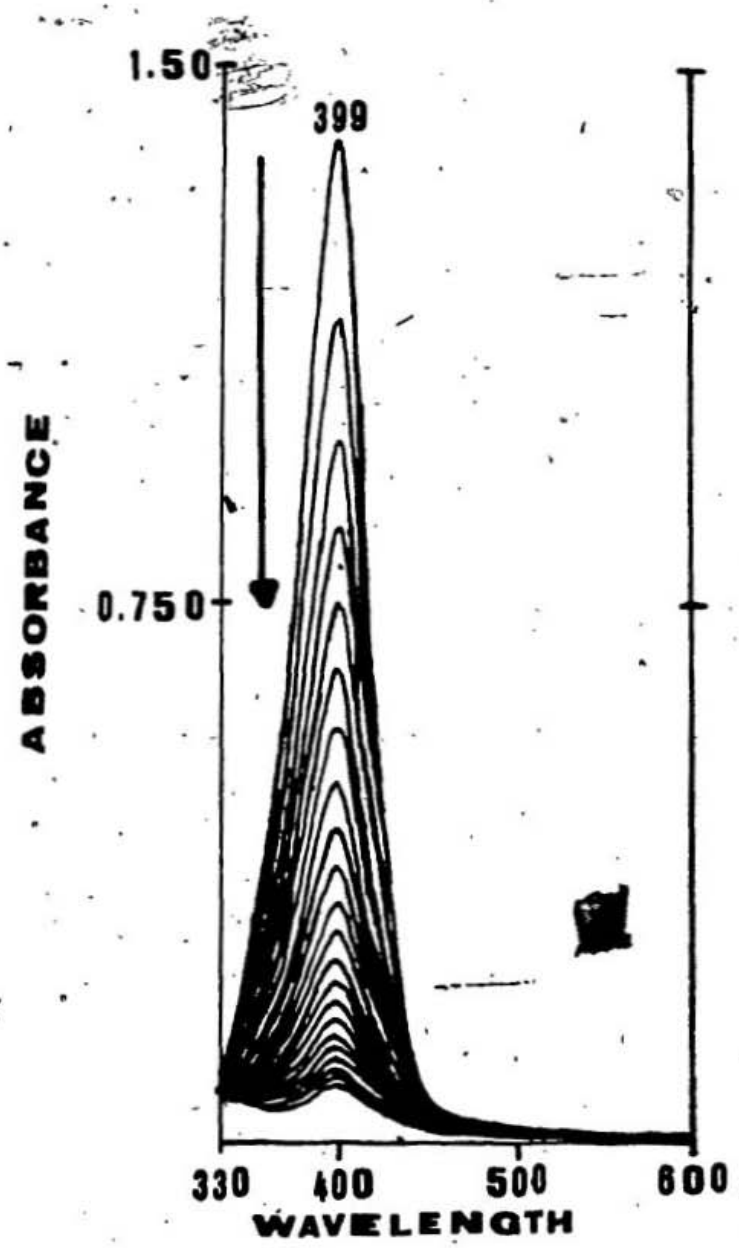


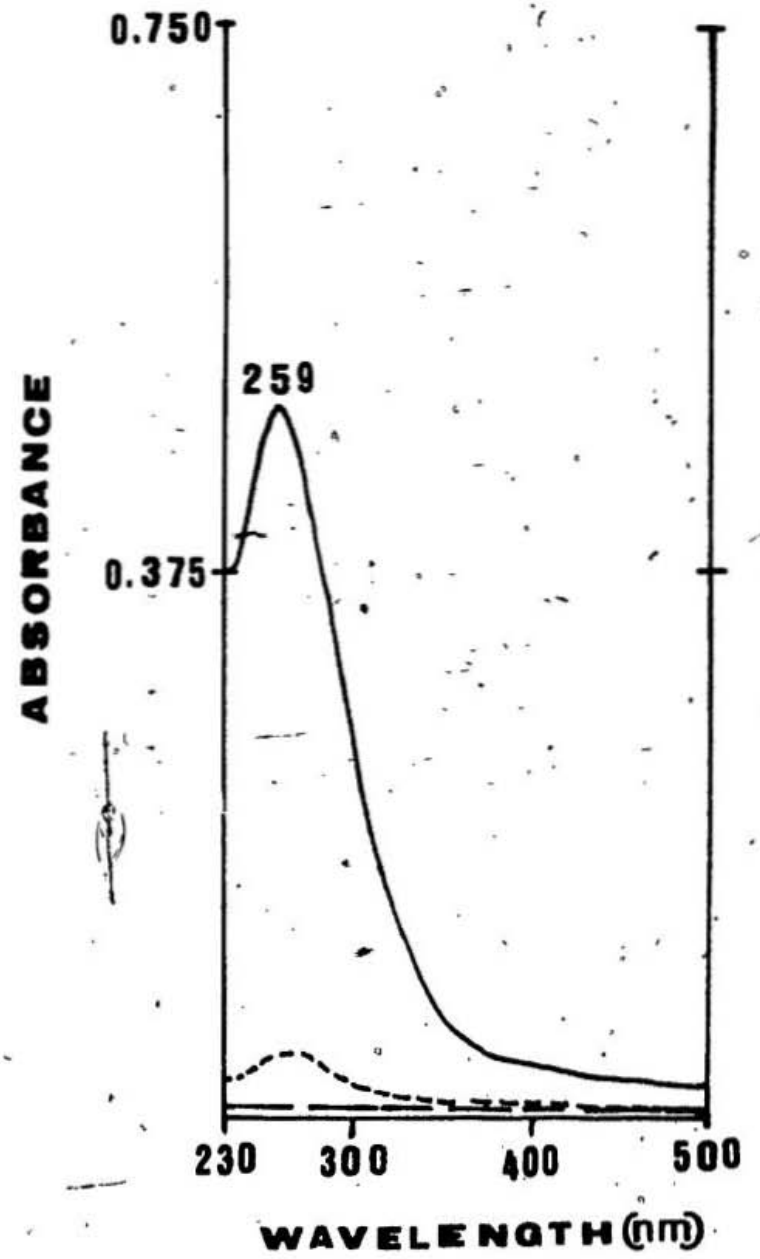
Figure 8.3

Difference spectra for the p,p'-biphenol oxidation products bound bovine serum albumin:

p,p'-Biphenol (50 μM) was incubated with HRP (1 μg) and H_2O_2 (60 μM) in the presence of serum albumin (250 μg) for 15 minutes in 3 ml of 0.1 M Tris-HCl buffer pH 7.4 after which the protein was precipitated with the addition of ice-cold 10% TCA (300 μl) and redissolved in 0.1 M Tris-HCl buffer pH 7.4 and the difference spectrum was obtained by scanning in the UV-region using Shimadzu UV-240 Spectrophotometer.

——: Protein bound products; ----- : Protein bound products in the presence of glutathione (50 μM).

FIGURE 8.3



can be obtained. 3,3′,5,5′-(methoxy)$_4$4,4′-Biphenoquinone or 3,3′,5,5′-(methyl)$_4$4,4′-biphenoquinone, formed from 2,6-dimethoxyphenol and 2,6-dimethylphenol, did not bind to protein. This suggests that the *ortho* position of p,p′-biphenoquinone may be required for protein binding.

Figure 8.4 shows the spectral studies on the oxidation of p,p′-biphenol by horseradish peroxidase and H_2O_2 in the presence of GSH. p,p′-Biphenol has an absorbance maxima at 257 nm and 216 nm. Upon oxidation with HRP and equimolar H_2O_2, most of the p,p′-biphenol (95-100%) was oxidized to the p,p′-biphenoquinone. As shown in figure 8.4, addition of 1/2 eqvt (eqvts. of p,p′-biphenol) of GSH resulted in a decrease of p,p′-biphenoquinone (~25%) with a slight increase in the absorbance around 500 nm (orange-red). Addition of another 1/2 equivalent of GSH resulted in a 50% decrease in p,p′-biphenoquinone formation with an increase in the absorbance at 495 nm (purple colour), 257 nm and 216 nm. Addition of 2 eqvts. of GSH, however, resulted in the complete disappearance of p,p′-biphenoquinone and a further increase in the *uv* absorbance at 257 nm and 216 nm. The absorbance at 216 nm was now much higher than that of originally added p,p′-biphenol. Extraction with ethyl acetate resulted in the recovery of 50% of originally added p,p′-biphenol (see section 8.3; Discussion).

Examination of the water-soluble metabolites by h.p.l.c. (Figure 8.5) formed in the above incubations in the presence of GSH resulted in the separation of

Figure 8.4

Effect of glutathione on spectral properties of p,p′-biphenol oxidation products formed by oxidation with HRP/H_2O_2.

- - - : p,p′-Biphenol (50 μM); ..... : p,p′-Biphenol (50 μM)/ HRP (10 μg)/ H_2O_2 (60 μM); ——: p,p′-Biphenol (50 μM)/GSH (25 μM)/HRP (10 μg)/ H_2O_2 (60 μM); - - - - - : p,p′-biphenol (50 μM)/GSH (50 μM)/HRP (10 μM)/ H_2O_2 (60 μM); — — — : p,p′-Biphenol (50 μM)/GSH (100 μM)/HRP (10 μM)/ H_2O_2 (60 μM).

FIGURE 8.4

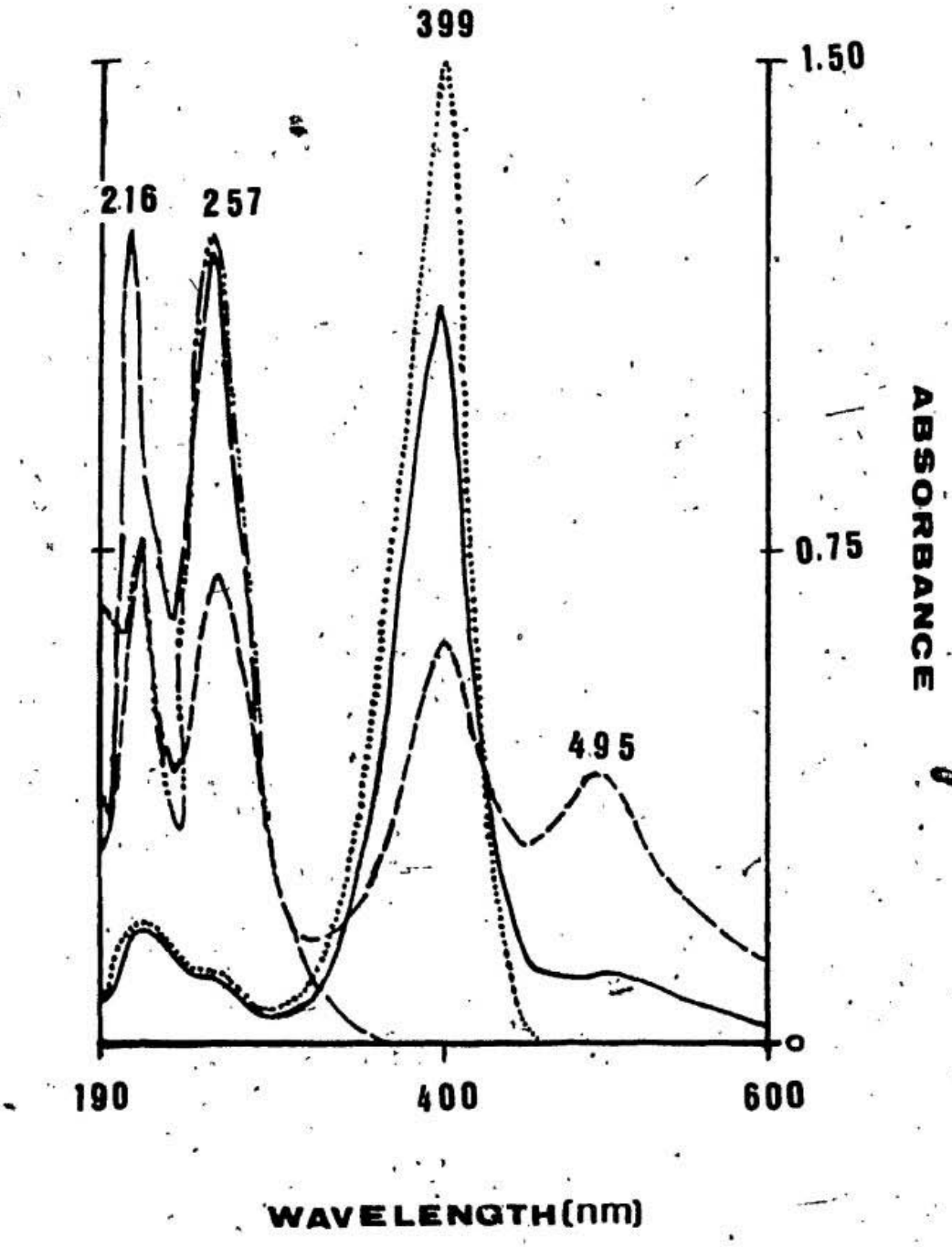
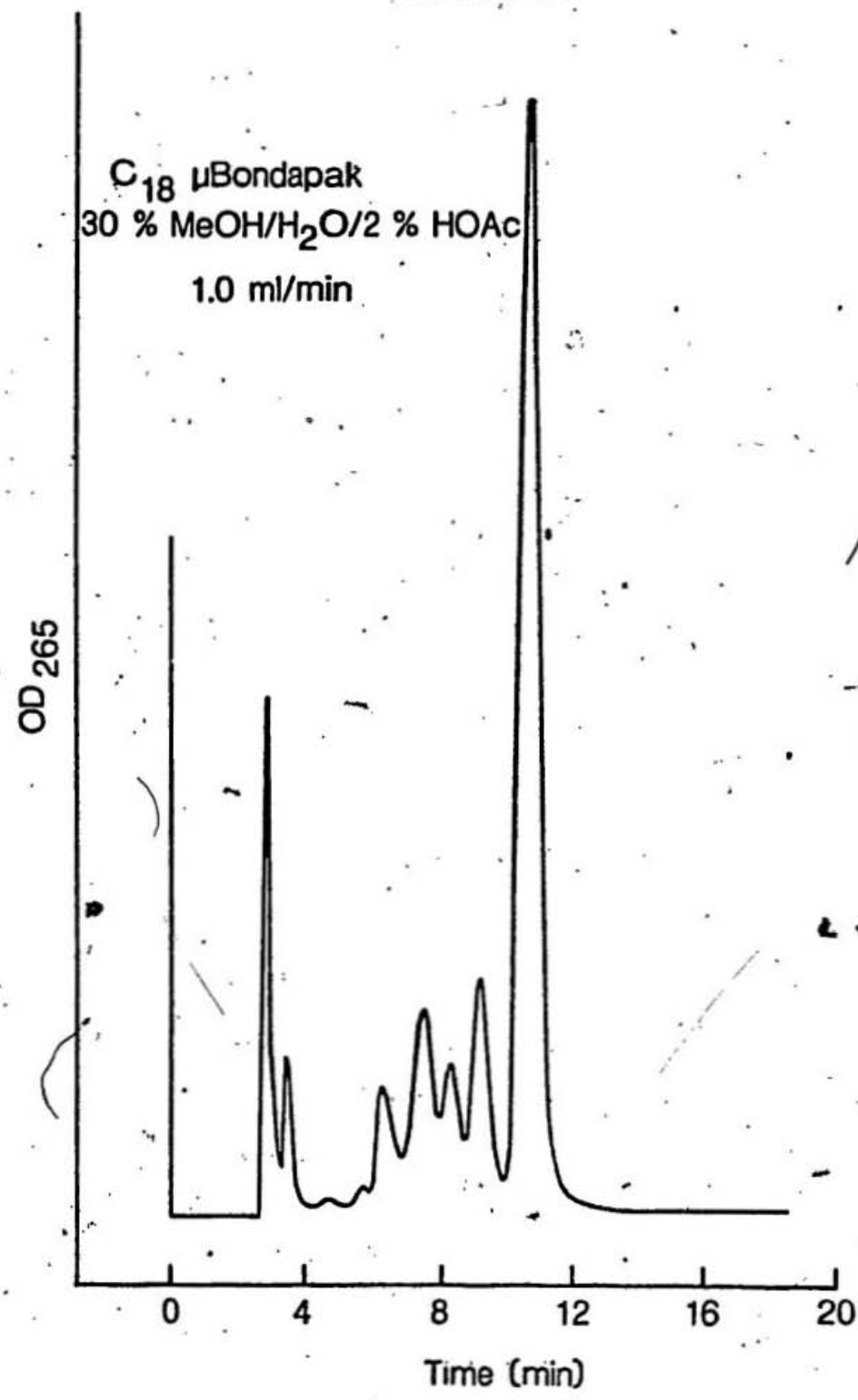

399
1.50
216
257
0.75
ABSORBANCE
495
0
190
400
600
WAVELENGTH(nm)

Figure 8.5

HPLC tracing of water-soluble products from incubation of p,p'-biphenol with HRP/H_2O_2 and glutathione:

See Materials and Methods (section 2.2.5.2) for procedures.

FIGURE 8.5


C_{18} μBondapak
30 % MeOH/H_2O/2 % HOAc
1.0 ml/min
OD_{265}
0
4
8
12
16
20
Time (min)

several compounds, one of which predominates (retention time 10.7 minutes). This material was isolated from a large-scale incubations (at pH 5.5 which gave increased yields of the predominant peak) from numerous h.p.l.c. runs.

The presence of GSH in this water-soluble metabolite was indicated by acid hydrolysis and identification of glutamic acid (R_F 0.37) and glycine (R_F 0.33) by thin-layer chromatography as described in Materials and Methods (section 2.2.5.5).

Figure 8.6 shows the FAB-mass spectrum of the above isolated water-soluble metabolite. The presence of $(M+1)^+$ ion of 492 is consistent with a monosubstituted conjugate of GSH and p,p'-biphenoquinone. Presence of the fragment ion at 307 indicated the presence of glutathione in the conjugate.

Figure 8.7 shows the proton-NMR spectrum of p,p'-biphenol (Figure 8.7a) and of the water-soluble metabolite (Figure 8.7b). The spectrum of p,p'-biphenol shows two-doublets centered at 7.38 ppm and 6.80 ppm (J=8.5 cps). The down-field resonances are a result of the *meta* protons while the upfield resonances are a result of the *ortho* protons shielded by the hydroxyl groups. The spectrum of the metabolite gives an additional doublet at 7.43 ppm (J=2.2 cps), which is shifted down-field indicating that this proton is next to an S-alkyl group. The 2', 6' and 3', 5' protons of p,p'-biphenol are unaffected, whereas the 5 and 6 protons show a slight down-field and upfield shift

Figure 8.6

FAB-Mass spectroscopy tracing of isolated water-soluble product from the incubation of p,p'-biphenol with HRP/H_2O_2 and glutathione:

See Materials and Methods (section 2.2.5.3) for instrumentation.

FIGURE 8.6

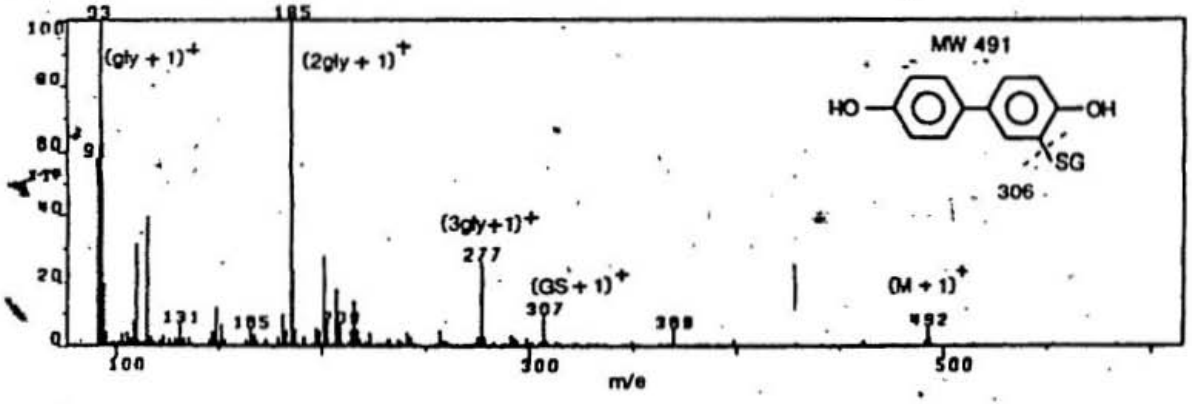

100
80
60
40
20
0
%
(gly + 1)+
(2gly + 1)+
(3gly+1)+
277
(GS + 1)+
307
(M + 1)+
492
121
185
308
100
300
500
m/e
MW 491
HO
OH
SG
306

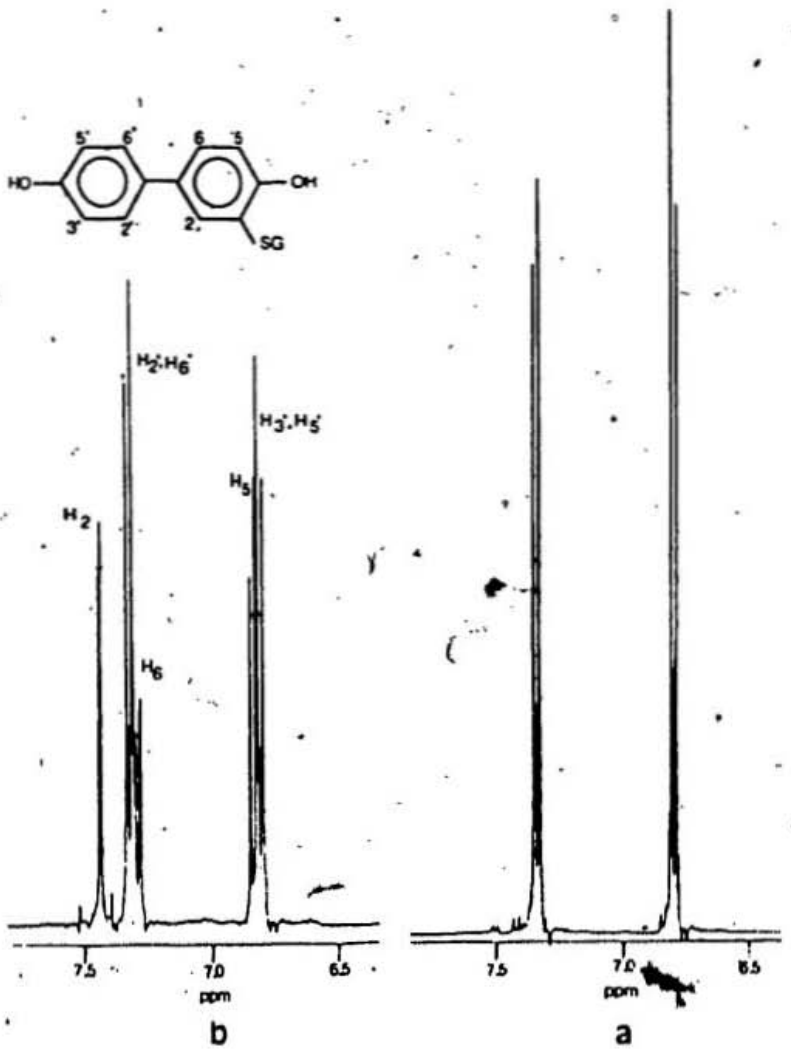
Figure 8.7

Proton-NMR [400MHz] tracings of the aromatic region (a): p,p′-biphenol [D_2O/ CD_3OD] and (b): isolated water-soluble product [D_2O] from incubation of p,p′-biphenol with HRP/H_2O_2:

The chemical shifts (ppm) are those down-field from the external standard Trimethylsilane.

See Materials and Methods (section 2.2.5.4) for instrumentation.

FIGURE 8.7

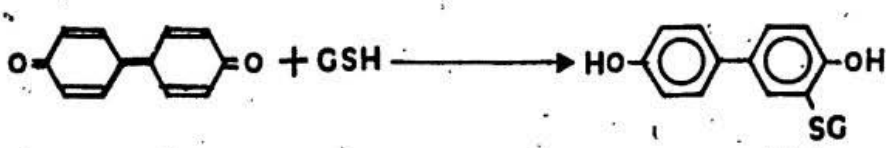


respectively. Other assignments are summarized in Table 8.1. These data are consistent with the structure, 3-S-(glutathion-yl)-p,p′-biphenol.

Oxidation of 3-S-(glutathion-yl)-p,p′-biphenol with HRP (1 μg) and one-equivalent of H_2O_2 resulted in the formation of orange-red pigment (409 nm, 510 nm) which presumably is the p,p′-biphenoquinone of the conjugate. The addition of GSH (one-eqvt) led to the formation of the purple pigment originally seen in the oxidation of p,p′-biphenol. H.p.l.c. analysis of the incubation mixture showed the formation of more polar products presumably conjugates which are multi-substituted with glutathione.

8.3) Discussion :

The results presented above indicate that p,p′-biphenoquinone can readily form covalent conjugates with sulfhydryl group of GSH. The following reaction mechanism may be proposed for the conjugation reaction of p,p′-biphenoquinone with GSH.



In the presence of excess of H_2O_2, peroxidase may also oxidize the conjugate further.

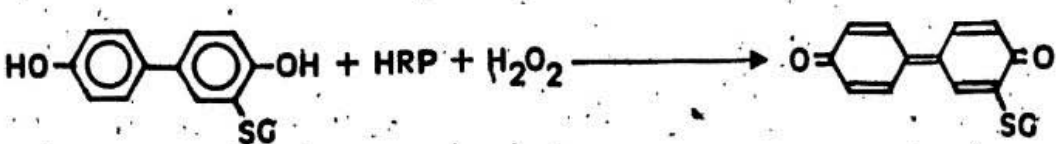


Table 8.1

Summary of proton-NMR assignments for p,p'-biphenol and 3-(glutathion-s-yl)-p,p'-biphenol

Assignments	Chemical Shifts (ppm) from TMS as external standard[a]		
	glutathione[b]	p,p'-biphenol[c]	3-(glutathion-S-yl)-p,p'-biphenol[d]
H_2	———	7.36 (d,4)	7.43 (d,1)
$H_{2'}$	———		7.31 (d,2)
$H_{6'}$	———		
H_6	———		7.21 (dd,1)
H_3	———	6.80 (d,4)	
$H_{3'}$	———		6.80 (d,2)
$H_{5'}$	———		
H_5	———		6.83 (d,1)
gly α,α'	3.81 (s,2)	———	3.53 (m,3)
cys α	4.40 (t,1)	———	4.29 (dd,1)
cys β	2.79 (d,1)	———	3.20 (dd,1)
cys β'	2.77 (d,1)	———	3.08 (dd,1)
glu α	3.66 (t,1)	———	3.53 (m,3)
glu β,β'	2.01 (m,2)	———	1.87 (m,2)
glu γ,γ'	2.39 (m,2)	———	2.24 (m,2)

a: Following the chemical shift, the splitting and the number of protons are in parantheses.

b: Glutathione spectra were recorded at room temperature in D_2O. Coupling constants are: $J_{cys\alpha,\beta} = 6.4Hz$, $J_{cys\beta,\beta} = 1.8Hz$, $J_{gly\alpha,\beta} = 6.4Hz$

c: p,p'-Biphenol spectra were recorded at room temperature in D_2O/CD_3OD. Coupling constants are: $J_{5,6} = 8.5Hz, J_{2',3'} = 8.5Hz, J_{2,3} = 8.5Hz; J_{2',3'} = 8.5Hz$.

d: 3-(Glutathion-S-yl)-p,p'-biphenol spectra were recorded at room temperature in D_2O. Coupling constants are: $J_{5,6} = 8.4Hz, J_{2',3'} = 8.7Hz, J_{2',3'} = 8.7Hz, J_{2,5} = 2.2Hz, J_{cys\alpha,\beta} = 4.7Hz, J_{cys\alpha,\beta} = 8.1Hz, J_{cys\beta,\beta} = 14.4Hz$.

Other abbreviations:
TMS: Trimethylsilane; ppm: parts per million; s: singlet; d: doublet; dd: doublet of doublets; t: triplet; m: multiplet.

The results from figure 8.4 suggested that some p,p′-biphenoquinone is also reduced to p,p′-biphenol by GSH. The detailed mechanism of reduction is still under investigation, however, it appears that glutathione conjugate [3-S-(glutathion-yl)-p,p′-biphenol] is responsible for the reduction of some of the p,p′-biphenoquinone. Minor amounts of GSSG are also formed during this reaction, but only when GSH was used above 2 eqvts. (eqvts. of p,p′-biphenol). Direct addition of 3-S-(glutathion-yl)-p,p′-biphenol (1.8 eqvts.) to p,p′-biphenoquinone led to the recovery of 83%-100% of the theoretical amount of p,p′-biphenol. The reaction mixture showed an orange-red colour which may be the quinone of the conjugate.

In chapter 4 (Table 4.3) it was shown that some water-soluble products can be formed from phenol during peroxidase- H_2O_2 oxidation in the presence of GSH; however, only upon longer incubation times and with high H_2O_2 concentration. H.p.l.c. analysis showed that 3-S-(glutathion-yl)-p,p′-biphenol was not formed in such incubations. Thus, the products binding to GSH during phenol oxidation may be similar to those phenol oxidation products interacting with DNA (see results in Chapter 5).

CHAPTER 9

GENERAL DISCUSSION AND CONCLUSIONS

Although the results presented in this thesis suggest a role for H_2O_2-dependent activation of carcinogens by peroxidases, and thus in the initiation of chemical carcinogenesis by chemicals, a number of questions still arise. Thus, the first and immediate question that may be raised is the site of intracellular location of these peroxidases and the ability of these activated products to reach the DNA in the nucleus. Secondly, the nature of the damage to DNA and the efficiency with which the bound products inhibit DNA replication or cause mutagenesis. Thirdly, the source of H_2O_2 in the cell is in question. At present these questions cannot be answered completely but at least some suggestions may be made with the available evidence, which may be useful in designing new experiments or in developing new techniques which may expedite the issue of understanding the processes involved in the initiation and promotion phases of chemical carcinogenesis.

9.1) Intracellular activation and DNA damage of xenobiotic chemicals by peroxidase and H_2O_2 :- Histochemical evidence shows that most of the peroxidases are located in the endoplasmic reticulum and in the nuclear membrane of the cells (reviewed by O'Brien, 1984). The presence of

this enzyme in the outer envelope of the nuclear membrane indicates that the activating system is in close proximity to the nuclear DNA. However, activation of the carcinogen at a site away from the nucleus may pose the question of the stability of the reactive products and their ability to reach the nuclear DNA. Myeloperoxidase of polymorphonuclear leukocytes appear to be located in the azurophilic granules in the cell, a site which is away from the nucleus. However, activation of various arylamine carcinogens and phenol by myeloperoxidase in polymorphonuclear leukocytes (Chapter 3) resulted in products which are bound to the nuclear DNA, clearly indicating that the reactive products can reach the nuclear DNA.

9.2) Nature of DNA damage: The nature of the damage to DNA that can lead to the oncogenesis at present is not known. It is also not known whether the reaction with DNA alone is sufficient, or whether other mechanisms also participate in the process of oncogenesis. Nevertheless, binding *in vivo* to DNA by a variety of carcinogens appear to correlate with carcinogenesis more than the binding either with protein or RNA (Bresnick and Eastman, 1982).

It is believed that covalent interaction of electrophilic metabolites of xenobiotics with DNA may play a major role in the initiation and promotion stages of chemical carcinogenesis (Miller and Miller, 1985). On the other hand, noncovalent interaction with DNA of some anti-cancer drugs like proflavine and

acridine orange analogues (Baguley et al., 1981 a and b; Muller and Crother, 1975; Muller and Gautier, 1975) and adriamycin (Youngman and Elstner, 1984) have been related to their ability to cause mutagenesis (Muller, Crother and Waring, 1973; Wright et al., 1980) or carcinogenicity (Sieber and Adamson, 1975; Waring, 1981). The results presented in chapter 5 indicate that phenol polymers may be non-covalently interacting with DNA. Benzidine also rapidly polymerized on oxidation with peroxidase/H_2O_2 and these polymers were shown to cause DNA binding through non-covalent interactions (O'Brien et al., 1985). Similar results have been reported also for diethylstilbestrol (Epe and Metzler, 1985), phenylenediamine (O'Brien 1984, 1985) and methylaminoazobenzene (O'Brien, 1985). Very recently, acetaminophen was also shown to form polymers upon oxidation with HRP and H_2O_2 (Potter, Miller and Hinson, 1985 and 1986). However, the interactions of polymers with DNA and their ability to cause irrepairable mutagenic changes remain to be established. However, the binding of these polymers to DNA is so strong that normal procedures for establishing covalent binding to DNA were not effective in removing all of these polymers from DNA (Chapter 4 and 5). Thus, if this type of binding occurs in the cells, repair may be difficult and the cell may die very readily. However, cell proliferation may be initiated in neighbouring less damaged cells.

Interestingly, upon enzymic hydrolysis of benzidine oxidation products

bound to DNA, a small fraction of a covalent nucleotide adduct with similar properties to the N-deoxyguanosin-8-yl)-benzidine (O'Brien et al., 1985), was found to be the major *in vivo* adduct in the dog bladder (Kadlubar et al., 1985).

9.2.1) Oxidative DNA damage : DNA damage may not necessarily be mediated by the reactive metabolites of carcinogens alone. Reduced oxygen species like the hydroxyl radicals were shown to hydroxylate the guanine residues in DNA (Kasai and Nishimura, 1984) and cause DNA single strand breaks (Scholes, Willson and Ebert, 1969). These reactions of reactive oxygen species have been related to mutagenicity (Kasai and Nishimura, 1984), carcinogenicity (Kasai and Nishimura, 1984), chromosomal aberrations (Emerit et al., 1982) and tumor promotion (Zimmerman and Cerutti, 1984). Moreover, these oxygen radicals or other radicals may also attack macromolecules or lipids and change membrane permeability. In the process other reactive radicals can be generated which can also cause damage to DNA (Ames, 1984).

Thus, the type of peroxidase-catalyzed oxygen activation reported in chapter 7 involving intracellular GSH and NADH may also be of significance in chemical carcinogenesis. Thus, autoxidizing thiols have been shown to be mutagenic (Glatt, Protic-Sabljic and Oesch, 1983) and cytotoxic to isolated rat hepatocytes (Saez et al., 1982; Vina et al., 1983). Hydroxyl radical formation has been shown to occur with autoxidizing thiols and has been given as an

explanation for their cytotoxicity (Vina et al., 1983). The following reaction mechanism has been suggested by those authors (Vina et al., 1983).:

$$RSSR^{\cdot-} + H_2O_2 \rightarrow OH^{\cdot} + RSSR + OH^{-} \quad (1)$$

Moreover, reaction of H_2O_2 with ferrous ions (reaction 2) or with superoxide radicals (catalyzed by ferric ions)(reaction 3) could also result in hydroxyl radicals as shown below:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{-} + OH^{\cdot} \quad (2)$$

$$O_2^{\cdot-} + H_2O_2 \xrightarrow{Fe^{3+}} OH^{\cdot} + OH^{-} + O_2 \quad (3)$$

Another cytotoxicity mechanism could be the oxidative stress ensuing as a result of the peroxidase-arylamine or phenol-mediated oxidation of GSH to GSSG. As the phenol or arylamine effectively redox cycles (via its free radical), the GSSG reductase, particularly if inactivated by the oxidation products, may not be effective at preventing the GSSG formation. The GSSG readily forms mixed-disulfide with protein which leads to enzyme inactivation (Offermann et al., 1984), activation of glucose-6-phosphate dehydrogenase (Egglestone and Krebs, 1974), inhibition of membrane Ca^{2+} pump (Brigelius, 1985) and results in cytotoxicity (Ziegler, 1985).

These effects suggest that glutathione under certain circumstances, may

also play a role in the in the initiation and promotion of carcinogenesis in addition to its role in detoxification.

9.3) Source(s) of H_2O_2 :- Intracellular steady-state H_2O_2 concentrations are very low, generally in the range of 0.1 - 0.001 mM (Sies et al., 1973). Thus, the intracellular activation of xenobiotics by peroxidase(s) may become limited by the availability of H_2O_2.

Results from chapter 7 indicate that free radicals formed by the peroxidase- catalyzed oxidation of some arylamines can directly interact with molecular oxygen to form superoxide radicals and H_2O_2. More interestingly, the formation of superoxide radicals is enhanced several fold in the presence of NADH or GSH. Intracellular concentrations of NADH are very low but many tissues are rich in reduced glutathione (0.5 - 10.0 mM). This suggests that some H_2O_2 can be generated in the cell by the oxidase activity of peroxidase and may not require additional sources of H_2O_2. Interestingly, the ability of arylamine carcinogens to activate oxygen in the presence of NADH or glutathione appears to correlate with the carcinogenic potency of the arylamines. Thus, the non-carcinogenic arylamines mesidine and aniline were not effective in catalyzing the oxygen activation although they were effective in mediating oxidation of NADH.

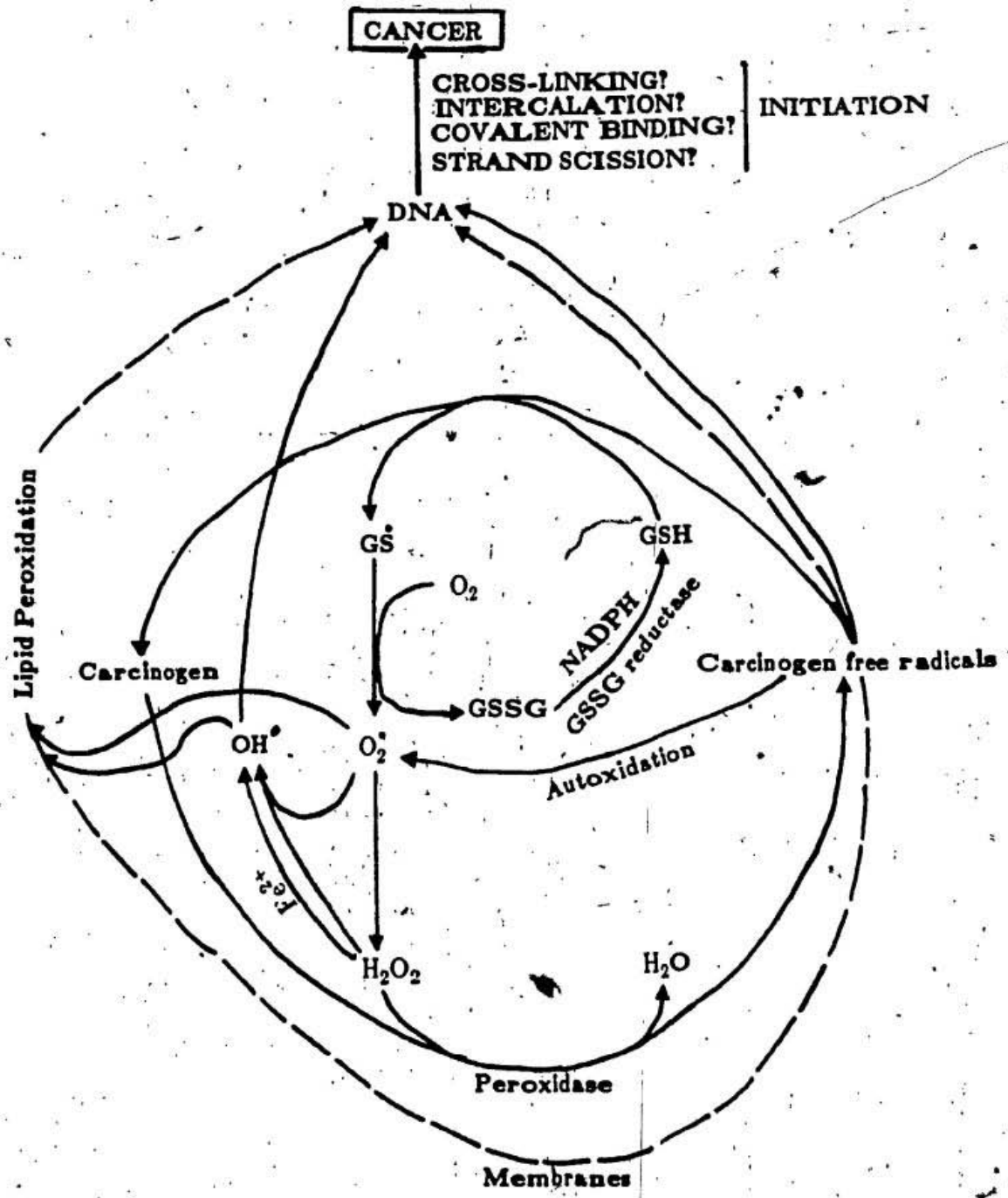
Plasma membranes of a variety of cells eg: liver, erythrocytes and Hela cells and the plant cell wall contain superoxide forming NAD(P)H oxidase (Ramasarma, 1982). Neutrophils also possess transmembrane NAD(P)H oxidases which are believed to form H_2O_2 for the antibacterial function of this cell (Baehner, Boxer and Ingraham, 1982). A thiol (GSH) oxidase is also associated with the plasma membrane of renal tubular epithelial cells and intestinal epithelial cells (Lash, Jones and Orrenius, 1984). In view of the role superoxide radicals may play in cytotoxicity (Borg and Schaich, 1984) or carcinogenesis (Zimmerman and Cerutti, 1984), activation of these oxidase systems by catalytic concentrations of carcinogenic arylamines or phenol could have biological consequences. However, it should be noted that the cells are also equipped with various defense mechanisms such as catalases and glutathione peroxidases to keep the H_2O_2 concentration at a minimum (Reed, 1985). It remains to be seen whether any of these suggested activation mechanisms can occur *in vivo* despite the defense mechanisms.

Figure 9.1 summarizes the postulated scheme for the possible mechanisms by which peroxidase/H_2O_2-mediated activation of carcinogens may initiate the carcinogenesis by chemicals.

Stier et al. (1980) have proposed that the redox cycling of stable nitroxide radicals formed from carcinogenic arylamines may be of importance in arylamine induced carcinogenesis. Nakayama et al. (1983) recently reported that

FIGURE 9.1

POSTULATED SCHEME FOR THE ROLE OF A PEROXIDASE IN THE INITIATION OF CHEMICAL CARCINOGENESIS



superoxy radicals were formed during the autoxidation of N-hydroxy and nitroxy radical metabolites of carcinogenic arylamines including 1- and 2-naphthylamine. The results presented in table 7.1 (Chapter 7) showed that oxygen consumption also occurred during the oxidation of 1- and 2-naphthylamine presumably due to the autoxidation of cation radicals formed from these substrates. But, in the presence of NADH or GSH, extensive superoxy radical formation and redox cycling of H_2O_2 occurred with carcinogenic 2-naphthylamine, whereas no enhancement in superoxy radical formation occurred with the non-carcinogenic 1-naphthylamine.

9.4) Benzene and leukemia :

Benzene has been known to be a powerful bone marrow or blood poison since before the turn of the century. The earliest of the reports on benzene-induced hematopoietic toxicity appeared in 1897 (reviewed by Snyder et al., 1977). In 1928, the first report establishing the relationship of benzene exposure to leukemia was published. Since then, numerous cases of leukemia among individuals exposed to benzene have been reported in several countries.

Although the toxicity of benzene was noted as early as 1897, only recently have the toxic effects of benzene gained attention. This is primarily because of extensive commercial use of benzene in industries (Gerarde, 1956; Greenberg, Mayers and Goldwater, 1939; Hardy and Elkins, 1948; Tammers, 1976; Wilson,

1942; Wintobe, Lee and Boggs, 1974) : leather industry, both artificial and natural; the manufacture of artificial rubber; in gilding; bronzing and silvering; varnishes, shellac and paint removing; in the printing industry; and in dry cleaning. Benzene is a constituent of high octane gasoline used in airplane and automobile engines. In addition benzene has been used in the manufacture of explosives, pesticides, plastics, synthetic detergents, conditioners, antioxidants and medicinals. Benzene is also used as a solvent for rubber gums, resins, celluloid, fats and alkaloids.

The primary means of entrance of benzene into the body is *via* inhalation. A study of humans exposed to benzene revealed that it rapidly reaches equilibrium with the alveolar air and that saturation could be achieved within minutes (Hunter and Blair, 1972). In experiments carried out in rats, the concentration of benzene in the blood was demonstrated to be parallel to the concentration in the inspired air (Deichmann, Macdonald and Bernal, 1963). The human experiments also showed that benzene is retained in the body for many hours after the exposure has been terminated and that elimination of benzene *via* the lungs accounted for only 12 % of the excretion during the first six hours after exposure.

The principal metabolites of benzene *in vivo* are the phenol, catechol and hydroquinone, of which phenol is the major metabolite. These phenols are then further processed by sulfation or glucuronidation to give the conjugated

end products (reviewed by Snyder and Kocsis, 1975).

The mechanism of benzene-induced hematopoietic toxicity and leukemia is thought to involve initial hydroxylation *via* an epoxide intermediate to phenol by the liver microsomal cytochrome P-450 monooxygenase system. Although in the beginning it was proposed that benzene epoxide could be the reactive metabolite (Snyder and Kocsis, 1975), studies by Tunek et al. (1980) indicate that the further activation of phenol is necessary to generate the reactive metabolites. Moreover, benzene epoxide is not mutagenic in the *Salmonella* assay system for mutagenesis (Jung et al., 1981). It was shown that phenol is further hydroxylated to hydroquinone and catechol (Tunek et al., 1980; Sawahata and Neal, 1983), the former as the major metabolite (Sawahata and Neal, 1983). The semiquinone and/or benzoquinone products of hydroquinone and catechol bind to protein and glutathione. However, phenol did not bind to DNA following activation by liver microsomes and NADPH or following activation with tyrosinase (see Chapter 4; Table 4.1). Sawahata and coworkers (Sawahata and Neal, 1982; Sawahata, Greenlee and Irons, 1985) reported that bone marrow peroxidase can activate phenol to form biphenols and it was proposed that p,p'-biphenoquinone was the major reactive species involved in benzene toxicity. However, the results in chapter 6 showed that the o,o'-biphenol is the major metabolite formed during the oxidation of phenol by bone marrow homogenate and H_2O_2 and that the further oxidation

of o,o′-biphenol accounted for the majority of the protein binding which occurred. Results from chapter 4 (Table 4.1) showed that extensive binding of phenol oxidation products to DNA could occur when phenol was activated with peroxidase and H_2O_2. However, it is unlikely that p,p′-biphenoquinone binds to DNA (Chapter 4). Furthermore, peroxidase/H_2O_2-mediated activation of phenol or o,o′-biphenol in the presence of NADH or glutathione, was proposed to result in extensive superoxy radical and H_2O_2 formation (Chapter 7). In the process GSSG was formed through thiyl radicals. However, hydroquinone, catechol and p,p′-biphenol did not result in superoxy radical formation (see Table 7.1; Chapter 7). Instead they form quinones which could oxidize NADH (except hydroquinone) by hydride transfer (see Discussion of Chapter 7) or covalent conjugates with sulfhydryl group of glutathione (see Discussion of Chapter 8). However, minor amounts of GSSG formation also occurred when p,p′-biphenoquinone was reacted with GSH (see Discussion, Chapter 8). *ortho* and *para* Benzoquinones, i.e., the oxidation products of hydroquinone and catechol, did not form GSSG with GSH (up to 2 eqvts). However, some GSSG (10% of the added GSH) formation occurred with these quinones in the presence of large excess of GSH (5.0 mM) (results not shown). Detailed studies are required in order to understand the mechanism. p,p′-Biphenoquinone has a very high redox potential (954 mV) (Brown and Todd, 1954) of the quinones. On the other hand *ortho and para* benzoquinones have redox potentials of 792 mV and 715 mV respectively. The formation of

sulfhydryl conjugates by all these quinones suggest that sulfhydryl conjugation of quinones is independent redox potentials.

Table 9.1 summarizes the present knowledge on the reactions of benzene metabolites with various biomolecules. Thus, one-electron oxidation of phenol readily results in phenoxy radicals that react with NADH or GSH to form superoxy radicals and products that bind to DNA or protein or GSH. Melanin-like polymers appear to be binding non-covalently to DNA (Chapter 5). Reaction of phenoxy radicals with GSH also resulted in GSSG formation. Peroxidase oxidation of p,p'-biphenol, hydroquinone and catechol resulted in covalent conjugation with protein and glutathione. GSSG is also formed to some extent but comparatively at higher amounts of GSH. 1,2,4 $(OH)_3$-Benzene, a minor metabolite of benzene *in vivo* (Snyder and Kocsis, 1977), also forms superoxy radicals during autoxidation.

Recently, Hinson et al. (1985) suggested that microsomal metabolism of benzene to phenol may involve another intermediate cyclohexadienone. They suggested that the cyclohexadienone can be formed through an enzyme-substrate complex or by the rearrangement of benzene epoxide intermediate. Further isomerization of cyclohexadienone may result in the formation of phenol. It is possible that metabolism of benzene in bone marrow may result in direct formation of cyclohexadienone rather than through an epoxide intermediate. However, the reactivity of this metabolite towards cellular

Table 9.1

Summary : Metabolic activation of benzene metabolites

	AFTER -1e OXIDATION					
	GSH CONJUGATE	PROTEIN BINDING	DNA BINDING	O_2^- (NADH OR GSH)	O_2^- (AUTOXID.)	GSSG
1. PHENOL	Polymer?	Polymer?	Polymer?	+++	(-)	++++
2. o,o'-BIPHENOL	Polymer?	Polymer?	Polymer?	++	-	++++
3. p,p'-BIPHENOL	2GS-	++++	-	-	-	++
4. HYDROQUINONE	2GS-	+++	(+)	-	+	+
5. CATECHOL	4GS-	++	-	-	++	+
6. 1,2,4$(OH)_3$-BENZENE	5GS?	+	(-)	-	++++	

See text for explanation.

The numerical value for GS represents the probable site of GSH conjugation on the aromatic ring.

nucleophiles is not known.

It is also interesting that pathways other than hydroxylation of benzene may also exist *in vivo*. *trans, trans*-Muconic acid, an open-chain metabolite was originally shown to be formed by Parke and Williams (1954) and recently confirmed by Gad-el Karim, Ramanujam and Legator (1985). However, the mechanism of ring opening and the enzymes involved in this pathway have not been explored. This metabolite (*trans, trans*-muconic acid) was not formed, when phenol or catechol or hydroquinone were administered instead of benzene. Gad-el Karim, Ramanujam and Legator (1985) proposed that mucondialdehyde formed from benzene may be the reactive metabolite which causes benzene toxicity. At present, it is a pure conjecture to suspect that this metabolite might induce benzene toxicity or carcinogenicity, but the data suggest that other possibilities exist for the study of benzene-induced carcinogenicity and toxicity. Thus, there are still many questions to be answered.

Phenol is a non-carcinogen under the conditions of NIH bioassay (U.S. National Cancer Institute Bioassay Technical Report, 203, 1980). However, chronic or acute exposure of phenol causes nephrotoxicity in humans and animals (Coan, Baggs and Bossman, 1982). Phenol is also an hemolytic agent (Machleidt, Roth and Seeman, 1972), and intravenous administration of phenol to several species caused hemoglobinuria (Oehme and Davis, 1970). These hematopoietic effects of phenol may not be due to its effects in bone marrow,

but possibly due to its effects in circulating blood, since phenol appears not to reach bone marrow in experimental animals (reviewed by Snyder et al., 1977). Nephrotoxicity of phenol may be explained by its ability to reach kidney through the circulation (Coan, Baggs and Bossman, 1982). Thus, phenol may also mimic the leukemogenic actions of benzene, if administered *in situ* into bone marrow.

Another debatable point in the extrapolation of peroxidation of phenol to benzene induced bone marrow toxicity and leukemia, is its relative importance in relation to its hydroxylation reaction followed by the formation of reactive semiquinones or benzoquinones. Although, the results from the chapter 4 suggests that benzoquinones may not bind to DNA, Rushmore et al. (1983) have demonstrated the formation of guanine nucleoside adducts in mitochondrial DNA after the metabolism of benzene by rabbit bone marrow cell mitoplasts. Incubation of mitoplasts with labelled benzene and unlabelled benzoquinone or hydroquinone reduced the yield of labelled benzene adducts in mitochondrial DNA suggesting that these compounds are the metabolites that covalently bind to guanine residues in DNA. At present it is difficult to answer this question, however, a suggestion can be made. Results presented in chapter 8 showed that p,p'-biphenoquinone can form glutathione conjugate. Since, *ortho*- and *para*-benzoquinones, metabolites of catechol and hydroquinone respectively, also form sulfhydryl conjugates (Lunte and Kissinger, 1983;

Sawahata and Neal, 1983; Tunek et al., 1980), then, determining the various glutathione conjugates found in the bone marrow following the *in vivo* administration of benzene or phenol may distinguish the relative importance of proposed activation pathways.

9.5) Conclusion :-

In conclusion, this thesis reports on the mechanisms by which hydrogen peroxide-mediated formation of reactive radicals formed during activation of arylamine carcinogens and phenol by peroxidases interacts with various biomolecules which could have consequences in potentiating the toxic or carcinogenic effects of these agents. In the preceding chapters it was shown that **(a)** the peroxidase-mediated activation of arylamine carcinogens and phenol in a cell can result in the irreversible binding of the carcinogens to nuclear DNA., **(b)** Polymers of phenol appears to be the reactive products binding to DNA during the *in vitro* peroxidase/H_2O_2-catalyzed oxidation of phenol. A free radical chain mechanism is proposed for the formation of polymers. The relevance in benzene-induced leukemia and bone marrow toxicity was discussed., **(c)** It was also shown that peroxidase-mediated activation of arylamine carcinogens and phenol in the presence of NADH or GSH can result in redox cycling and lead to hydrogen peroxide formation *via* superoxy radicals. It was suggested that this could be an important source of hydrogen peroxide *in vivo*.

Further studies are necessary to see the relative importance of this activation pathway with regard to the other activation pathways namely, NADPH-dependent mixed-function oxidase or arachidonate-dependent prostaglandin synthetase mediated activation of carcinogens and toxins. Further investigations along these lines would aid in elucidation of mysteries underlying the molecular mechanisms of initiation and promotion of carcinogenesis. Thus, the intracellular consequences of free-radicals generated by a one-electron oxidation of chemical carcinogens should be compared with the more widely studied intracellular consequences of electrophiles generated by a two-electron oxidation.

PUBLICATIONS

1. C.Kandaswami, V.V.Subrahmanyam and P.J.O'Brien, (1982): The effects of asbestos on the metabolism of benzo(a)pyrene, Polynuclear Aromatic Hydrocarbons,(A.J.Dennis and M.Cooke eds.) pp. 389-403.

2. Y. Tsuruta, V.V.Subrahmanyam, W. Marshall and P.J.O'Brien, (1985): Peroxidase-mediated irreversible binding of Arylamine Carcinogens to DNA in intact polymorphonuclear leukocytes activated by a tumor promoter, Chem. Biol. Interacns., **53**, 25-35.

3. V.V.Subrahmanyam and P.J.O'Brien, (1985): Peroxidase-catalysed phenol binding to DNA, Xenobiotica, **15**, 859-871.

4. V.V.Subrahmanyam and P.J.O'Brien, (1985): Phenol oxidation products formed by a peroxidase reaction that bind to DNA, Xenobiotica, **15**, 873-885.

5. P.J.O'Brien, V.V.Subrahmanyam, B.Gregory, R.Fourney, W.S.Davidson, A.D.Rahimtula and Y.Tsuruta (1985): One-electron oxidation in cell toxicity, In Microsomes and Drug Oxidations (A.R.Boobis, J.Caldwell, F.Dematties, and C.R.Elcombe eds.) pp. 284-295.

6. V.V.Subrahmanyam and P.J.O'Brien, (1985): Peroxidase-catalyzed oxygen activation by arylamine carcinogens and phenol, Chem. Biol. Interacns., **56**, 185-199.

REFERENCES

Ali, A.E., Barrett, J.C. and Eling, T.E. (1980) : Prostaglandin and thromboxane production by fibroblasts and vascular endothelial cells., Prostaglandins, **20**, 667-688.

Ames, B.N., Hollstein, M.C. and Cathcart, R. (1982). : Lipid peroxidation and oxidative damage to DNA., In Lipid Peroxides in Biology and Medicine, edited by Yagi, K., (Academic Press : New York) pp. 339-351.

Anderson, W.A., Trantalis, J. and Kang, Y.H. (1975) : Ultra-structural localization of endogenous mammary gland peroxidase during lactogenesis in the rat : Results after tannic acid-formaldehyde- glutaraldehyde fixation., J. Histochem. Cytochem., **23**, 295-302.

Anderson, M.R., Atkin, C.L. and Eyre, H.J. (1982) : Intact form of myeloperoxidase from normal human neutrophils., Arch. Biochem. Biophys., **214**, 273-283.

Anderson, R.A., Dalgleish, D.T., Nonhebel, D.C. and Pauson, P.L. (1977) : Oxidation of phenols, Part I. A study of the oxidation of p-cresol and other p-alkylphenols., J. Chem. Res., (S) 12-13 (M) 0201-0231.

Andersson, B., Larsson, R., Rahimtula, A. and Moldeus, P. (1984) : Prosta-

glandin synthase and horseradish peroxidase catalyzed DNA binding of p-phenetidine., Carcinogenesis, **5**, 161-165.

Andrews, L.S., Sasame, H.A. and Gillette, J.R. (1979) : [^{3}H]Benzene metabolism in rabbit bone marrow., Life Sci., **25**, 567-572.

Andrews, P.C. and Krinsky, N.I. (1981) : The reductive cleavage of myeloperoxidase in half, producing enzymically active hemi-myeloperoxidase., J. Biol. Chem., **256**, 4211-4218.

Andrews, P.C. and Krinsky, N.I. (1982) : Quantitative determination of myeloperoxidase using tetramethyl benzidine as substrate., Analyt. Biochem., **127**, 346-350.

Arfellini, G., Grill, S., Colacci, A., Mazzullo, M. and Prodi, G. (1985) : *in vivo* and *in vitro* binding of benzene to nucleic acids and proteins of various rat and mouse organs., Cancer lett., **28**, 159-168.

Armstrong, D.R., Cameron, C., Nonhebel, D.C. and Perkins, P.G. (1983) : Oxidative coupling of phenols. Part 6. A study of the role of spin density factors on the product compositions in the oxidations of 3,5-Dimethyl phenol and phenol., J. Chem. Soc. Perkin Trans II. 563-568.

Ashley, P.L., Davis, D.K. and Griffin, B.W. (1980) : Electron acceptor function of O_2 in radical N-demethylation reactions catalyzed by hemoproteins.

Biochem. Biophys. Res. Commun., **97**, 660-666.

Badwey, J.A. and Karnovsky, M.L. (1980) : Active oxygen species and the functions of phagocytic leukocytes., Ann. Rev. Biochem., **49**, 695-726.

Baehner, R.L., Boxer, A.L. and Ingraham, L.M. (1982) : Reduced oxygen by-products and white blood cells., In Free Radicals in Biology, edited by Pryor, W. (Academic Press : New York) pp. 91-113.

Baguley, B.C., Denny, W.A., Atwell, G.A. and Cain, B.F. (1981a) : Potential antitumor agents. 34: Quantitative relationships between DNA binding and molecular structure for 9-anilinoacridines substituted in the anilino ring., J. Med. Chem., **24**, 170-177.

Baguley, B.C., Denny, W.A., Atwell, G.J. and Cain, B.F. (1981b) : Potential antitumor agents. 35. Quantitative relationships between antitumor (L1210) potency and DNA binding for 4'-(9-acrydinylamino) methanesulfon-m-anisidide analogues., J. Med. Chem., **24**, 520-525.

Baird, W.M. and Brookes, P. (1973) : Isolation of the hydrocarbon-deoxyribonucleoside product from the DNA of mouse embryo cells treated in culture with 7-methylbenz(a)anthracene- ^{3}H., Cancer Res., **33**, 2378-2385.

Barton, J.P. and Packer, J.E. (1970) : Radiolysis of oxygenated cysteine solutions at neutral pH: The role of RSSR and O_2^{-}., Int. J. Radiat. Phys.

Chem. **2**, 159-166.

Beland, F.A., Beranek, D.T., Dooley, K.L., Heflich, R.H. and Kadlubar, F.F. (1983) : Arylamine-DNA adducts *in vitro* and *in vivo*: Their role in bacterial mutagenesis and urinary bladder carcinogenesis., Environ. Hlth. Perspect., **49**, 125-134.

Bennett, S., Marshall, W. and O'Brien, P.J. (1982) : Metabolic activation of diethylstilbestrol by prostaglandin synthetase as a mechanism for its carcinogenicity., In Prostaglandins and Cancer, edited by Powles, T., Bockman, R.S., Honn, K.V. and Ramwell, P. (Alan. R. Liss, Inc. : New York), pp. 143-148.

Bentfeld, M.E., Nichols, B.A. and Bainton, D.F. (1977) : Ultrastructural localization of peroxidase in leukocytes of rat bone marrow and blood., Anat. Rec., **187**, 219-240.

Beswick, P.H. and Slater, T.F. (1978) : The 'metabolic burst' in bovine blood polymorphonuclear leukocytes when stimulated by phagocytosis., Biochem. Soc. Transacns., **5**, 1299-1301.

Bills, T.K., Smith, J.B. and Silver, M.J. (1976) : Metabolism of [^{14}C] arachidonic acid by human platelets., Biochem. Biophys. Acta., **424**, 303-314.

Birnboim, H.C. (1982) : DNA strand breakage in human leukocytes exposed

to tumor promotor, phorbol myristate acetate., Science, **215**, 1247-1249.

Bjorkhem, I. (1982) : Rate limiting step in microsomal cytochrome P-450 catalyzed hydroxylations., In Pharmacol. Therap., edited by Schenkman, J.B., and Kupffer, D., (Pergamon Press : New York), pp. 645-666.

Borg, D.C. and Schaich, K.M. (1984) : Cytotoxicity from coupled redox cycling of autoxidizing xenobiotics and metals., Isr. J. Chem., **24**, 38-53.

Boyd, J.A., Harvan, D.J. and Eling, T.E. (1983) : The oxidation of 2-aminofluorene by prostaglandin endoperoxide synthetase : comparison with other peroxidases., J. Biol. Chem., **258**, 8246-8254.

Boyd, M.R. (1980a) : Biochemical mechanisms in chemical induced lung injury : Role of metabolic activation., CRC Crit. Rev. Toxicol. **7**, 103-176.

Boyd, M.R. (1980b) : Biochemical mechanisms of pulmonary toxicity of furan derivatives., In Rev. In Biochem. Toxicol., Vol. 2., edited by Hodgson, E., Bend, J.R. and Philpot, R.M. (Elsevier-North Holland : Amsterdam), pp. 71-102.

Bresnick, E. and Eastman, A. (1982) : Alkylation of mammalian cell DNA, persistence of adducts and relationship to carcinogenesis., Drug Metab. Rev., **13**, 189-205.

Brigelius, R. (1985) : Mixed disulfides: Biological functions and increase in oxidative stress., In Oxidative Stress, edited by H. Sies, (Academic Press: London), pp. 243-272.

Brokelman, J. and Fawcett, D.W. (1969) : The localization of endogenous peroxidase in the rat uterus and its induction by estradiol., Biol. Reprod., 1, 59-71.

Brown, S.A. (1964) : Lignin and Tannin biosynthesis. In Biochemistry of phenolic compounds, edited by Harborne, J.B. (Academic Press : London) pp. 361-398.

Brown, B.R. and Todd, A.R. (1954) : Colouring matters of the aphididae; Part. IX, Some reactions of extended quinones., J. Chem. Soc., 1280-1285.

Bryant, R.W., Simon, T.C. and Bailey, J.M. (1982) : Role of glutathione peroxidase and hexose monophosphate shunt in the platelet lipoxygenase pathway., J. Biol. Chem., **257**, 14937-14943.

Bryant, R.W., Simon, T.C. and Bailey, J.M. (1983) : Hydroperoxy fatty-acid formation in selenium deficient rat platelets: coupling of glutathione peroxidase to the lipoxygenase pathway., Biochem. Biophys. Res. Commun., **117**, 183-189.

Burke, M.D. and Orrenius, S. (1982) : Isolation and comparison of endo-

plasmic reticulum membranes and their mixed-function oxidase activities from mammalian extrahepatic tissues., In Pharmacol. Therap., edited by Schenkman, J.B. and Kupffer, D., (Pergamon Press : New York), pp. 47-97.

Busbee, D.L., Shaw, C.R. and Cantrell, E.T. (1972) : Aryl hydrocarbon hydroxylase induction in leukocytes., Science, **178**, 315-316.

Cairns, J. (1981) : The origin of human cancers., Nature (London), **289**, 353-357.

Capdevilla, J., Estabrook, R.W. and Prough, R.A. (1980) : Differences in the mechanism of NADPH and cumene-hydroperoxide supported reactions of cytochrome P-450., Arch. Biochem. Biophys., **200**, 186-195.

Carlson, B.W. and Miller, L.L. (1985) : NADH oxidation by quinones., J. Amer. Chem. Soc., **107**, 479-485.

Cavalieri, E.L. and Rogan, E.G. (1984) : One-electron and two-electron oxidation in aromatic hydrocarbon carcinogenesis., Free Radicals in Biology, Vol. VI, edited by Pryor, W., (Academic Press : New York) pp. 323-369.

Cederbaum, A.I. and Cohen, G. (1980) : Inhibition of the microsomal oxidation of ethanol and 1- butanol by the free-radical spin-trapping agent 5,5-dimethyl-1-pyrroline-1-oxide., Arch. Biochem. Biophys., **204**, 397-403.

Chance, B. (1949) : The enzyme-substrate compounds of horseradish peroxidase and peroxidase, II, Kinetics of formation and decomposition of the primary and secondary complexes., Arch. Biochem. Biophys., **22**, 224-252.

Clayson, D.B. and Garner, R.C. (1976) : Carcinogenic aromatic amines and related compounds., In Chemical Carcinogenesis, edited by Searle, E.C., (ACS Monographs 173), pp. 366-461.

Coan, M.L., Baggs, R.B. and Bossman, H.B. (1982) : Demonstration of direct toxicity of phenol on kidney., Res. Commun. Chem. Pathol. Pharmacol., **36**, 229-239.

Cohen, H.S., Freedman, M.L. and Goldstein, B.D. (1978) : Invited Review - The problem of benzene in our environment: clinical and molecular considerations., Amer. J. Med. Sc., **275**, 125-136.

Cohen, S.M., Zenser, T.V., Murasaki, G., Fukushima, S., Mattammal, M.B., Rapp, N.S. and Davis, B.B. (1981) : Aspirin inhibition of N-[4-(5-Nitro-2-furyl)-2-thiazolyl] formamide-induced lesions of the urinary bladder correlated with inhibition of metabolism by bladder prostaglandin endoperoxide synthetase., Cancer Res., **41**, 3355-3359.

Conney, A.H. (1982) : Induction of microsomal enzymes by foreign chemicals and carcinogenesis by polycyclic aromatic hydrocarbons: G.H.A. Clowes Memorial Lecturer., Cancer Res., **42**, 4875-4917.

Craddock, M.V. (1976) : Cell proliferation and experimental liver cancer., In Liver Cell Cancer, edited by Cameron,H.M., Linsell, D.A. and Warwick, G.P. (Elsevier-Amsterdam), pp. 153-201.

Cronkite, E.P., Bullis, J.E., Inoue, T. and Drew, R.T. (1984) : Benzene inhalation produces leukemia in mice., Toxicol. Appl. Pharmacol., **75**, 358-361.

Dalgleish, D.T., Nonhebel, D.C. and Pauson, P.L. (1977) : Oxidation of phenols, Part II. A study of oxidation of 2,6-dimethyl and 2,6-di-t-butylphenols., J. Chem. Res., (S) 14 (M) 0232-0242.

Danner, D.J., Brignac, P.J. Jr., Arceneaux, D. and Patel, V. (1973) : The oxidation of phenol and its reaction product by horseradish peroxidase and hydrogen peroxide., Arch. Biochem. Biophys., **156**, 759-763.

Dao, T.L. (1969) : Studies on mechanism of carcinogenesis in the mammary gland. Progr. Exp. Tumor Res., **11**, 235-261.

Davidson, D.G.D. and Eastham, W.N. (1966) : Acute liver necrosis following overdose of paracetamol., Br. Med. J. **2**, 497-499.

Diguiseppi, J. and Fridovich, I. (1984) : The toxicology of molecular oxygen. CRC Crit. Rev. Toxicol., **12**, 315-342.

Dean, B.J. (1985) : Recent findings on the genetic toxicology of benzene,

toluene, xylenes and phenols., Mutat. Res., **154**, 153-181.

Dechatelet, L.R.l, McCall, C.E., Cooper, M.R. and Shirley, P.S. (1974) : Ascorbic acid levels in phagocytic cells., Proc. Soc. Exp. Biol. Med., **145**, 1170-1174.

Degen, G.H., Eling, T.E. and Mclahlan, J.A. (1982) : Involvement of prostaglandin synthetase in the peroxidative metabolism of diethylstilbestrol in Syrian Hamster Embryo Fibroblast cell cultures., Cancer Res., **42**, 919-923.

Deichmann, W.B., MacDonald, W.E., Bernal, E. (1963) : The hematopoietic tissue toxicity of benzene vapors., Toxicol. Appl. Pharmacol., **5**, 201-224.

Desser, R.K., Himmelhoch, S.R., Evans, W.H., Januska, M. and Mage, M. and Shelton, E. (1972) : Guinea pig heterophil and oesonophil peroxidase., Arch. Biochem. Biophys., **148**, 452-465.

Doll, R. and Peto, R. (1981) : The causes of cancer: quantitative estimates of avoidable risks of cancer in the united states today., J. Natl. Cancer. Inst., **66**, 1193-1308.

Drake, J.W. and Baltz, R.H. (1976) : The biochemistry of mutagenesis., Ann. Rev. Biochem., 11-37.

Dybing, E., Nelson, S.D., Mitchell, J.R., Sasame, H.A. and Gillette, J.R.

(1976) : Oxidation of α-methyl dopa and other catechols by cytochrome P-450 generated superoxide anion: possible mechanism of methyl dopa hepatitis., Mol. Pharmacol., **12**, 911-920.

Egan, R.W., Paxton, J. and Keuhl, F.A.Jr. (1976) : Mechanism for irreversible self-deactivation of prostaglandin synthetase., J. Bio. Chem., **251**, 7329-7335.

Egan, R.W., Gale, P.H. and Keuhl, F.A.Jr. (1979) : Reduction of hydroperoxides in the prostaglandin biosynthetic pathway by a microsomal peroxidase., J. Biol. Chem., **254**, 3295-3302.

Egan, R.W., Gale, P.H., Baptista, E.M., Kennicott, K.L., VanderHeuvel, W.J.A., Walker, R.W., Fagerness, P.E. and Keuhl, F.A.Jr. (1981) : Oxidation reactions by prostaglandin cyclooxygenase-hydroperoxidase., J. Biol. Chem., **256**, 7352-7361.

Eling, T.E., Boyd, J.A., Reed, G., Mason, R.P. and Sivarajah, K. (1983) : Xenobiotic metabolism by prostaglandin endoperoxide synthetase., Drug Metab. Rev. **14**, 1023-1053.

Eliott, H., Gianni, L. and Myers, C. (1984) : Oxidative destruction of DNA by the adriamycin-iron complex., Biochemistry, **23**, 928-936.

Emerit, I., Keck, M., Levy, A., Feingold, J. and Michelson, A.M. (1982) :

Activated oxygen species at the origin of chromosome breakage and sister-chromatid exchange., Mut. Res., **103**, 165-172.

Epe, B. and Metzler, E. (1985) : Nature of the macromolecular binding of diethylstilbestrol to DNA and protein following oxidation by peroxidase/hydrogen peroxide., Chem. Biol. Interacns. (In press).

Erslev, A.J. (1974) : In Drugs and Hematologic Reactions, edited by Dimitrov, N.V. and Nodine, J.H. (Grunne and Stratton : New York) pp. 65-78.

Fahimi, H.D. (1970) : The fine structural localization of endogenous and exogenous peroxidase activity in kupffer cells of rat liver., J. Cell Biol., **47**, 247-262.

Floyd, R.A., Soong, L.M., Walker, R.N. and Stuart, M. (1976) : Lipid hydroperoxide activation of N-hydroxy-N-acetylaminofluorene via a free radical route., Cancer Res., **36**, 2761-2767.

Floyd, R.A., Soong, L.M. and Culver, P.C. (1976) : Horseradish peroxidase/hydrogen peroxide-catalyzed oxidation of the carcinogen N-Hydroxy-N-acetyl-2-aminofluorene as effected by cyanide and ascorbate., Cancer Res., **36**, 1510-1519.

Gabig. T.G., Schervish, E.W. and Santiga, J.T. (1982) : Functional relationship of the cytochrome b to the superoxide generating oxidase of human neu-

trophils., J. Biol. Chem., **257**, 4114-4119.

Gad-el Karim, M.M., Ramanujam, V.M.S., Legator, M.S. (1985) : *trans, trans*-Muconic acid, an open chain urinary metabolite of benzene in mice. Quantification by high-pressure liquid chromatography., Xenobiotica, **15**, 211-220.

Galliani, G. and Rindone, B. (1979) : Formation of superoxide radical anion in the peroxidase catalyzed oxidation of three aromatic tertiary amines with hydrogen peroxide., J. Chem. Soc. Perkin Trans. II, 1-3.

Gelboin, H.V. (1980) : Benzo[a]pyrene metabolism, activation and carcinogenesis: Role and regulation of mixed-function oxidases and related enzymes., Physiol. Rev., **60**, 1107-1166.

George, P. (1953) : The chemical nature of the second hydrogen peroxide compound formed by cytochrome *c* peroxidase and horseradish peroxidase: Titration with reducing agents., Biochem. J., **54**, 267-276.

Gerarde, H.W. (1956) : Toxicological studies on hydrocarbons II: A comparative study of the effect of benzene and certain mono-n-alkylbenzenes on hemopoisis and bone marrow metabolism in rats, AMA Arch. Indust. Health., **13**, 468-473.

Gill, D.P. and Ahmed, A.E. (1981) : Covalent binding of [^{14}C] benzene to

cellular organelles and bone marrow nucleic nucleic acids., Biochem. Pharmacol., **30**, 1127-1131.

Gillette, J.R., Mitchell, J.R. and Brodie, B.B. (1974) : Biochemical mechanisms of drug toxicity., Ann. Rev. Pharmacol., **14**, 271-288.

Glatt, H., Protic-Sabljic, M. and Oesch, F. (1983) : Mutagenicity of glutathione and cysteine in the Ames test., Science, **220**, 961-963.

Goldstein, B.D. and Snyder, C.A. (1982) : Benzene leukemogenesis., Environ. Sci. Res., **25**, 2778b1289.

Goldstein, B.D., Witz, G., Amoniso, M., Stone, D.D. and Troll, W. (1981) : Stimulation of human polymorphonuclear leukocyte superoxide anion radical production by tumor promoters., Cancer Lett., **11**, 257-262.

Gollmer, L., Graf, H. and Ullrich, V. (1984) : Characterization of the benzene monooxygenase system in rabbit bone marrow., Biochem. Pharmacol., **33**, 3597-3602.

Gonasun, L.M., Witmer, C.M., Kocsis, J.J. and Snyder, R. (1973) : Benzene metabolism in mouse liver microsomes., Toxicol. Appl. Pharmacol., **26**, 398-406.

Graham, R.C. and Karnovsky, M.J. (1966) : The early stages of absorption

of injected horseradish peroxidase in the proximal tubules of mouse kidney: Ultrastructural cytochemistry by a new technique., The J. Histochem. Cytochem., **14**, 291-302.

Greenberg, L., Mayers, M.R., and Goldwater, L. (1939) : Benzene (benzol) poisoning in the rotogravure printing industry in New York City., J. Indust. Hyg. Toxicol., **30**, 196-200.

Griffin, B.W., Marth, C., Yasukochi, Y. and Masters, B.S.S. (1980) : Radical mechanism of aminopyrine oxidation by cumene hydroperoxide-catalyzed by purified liver microsomal cytochrome P-450., Arch. Biochem. Biophys., **205**, 543-553.

Gronow, M. (1980) : Nuclear proteins and chemical carcinogenesis. Chemico-Biol. Interacns., **29**, 1-30.

Groves, J.T., Akinbote, O.F. and Avaria, G.E. (1980) : On the mechanism of oygen insertion catalyzed by cytochrome P-450: Hydroxylation with partial allylic rearrangement., In Microsomes, Drug Oxidations and Chemical Carcinogenesis, edited by Coon, M.J., Conney.A.H., Estabrook. R.W., Gelboin, H.V., Gillette, J.R. and O'Brien, P.J. (Academic Press : New York), pp. 253-261.

Haley, T.J. (1975) : Benzidine revisited: a review of the literature and problems associated with the use of benzidine and its congeners., Clin. Toxicol.,

8, 13-42.

Hankin, J.M. (1964) : Lignin-A natural polymeric product of phenol oxidation., In Oxidative coupling of phenols, edited by Taylor, W.I. and Battersby, A.R. (Marcel Dekker : New York) pp. 361-398.

Harborne, J.B. (ed.) (1964) : Biochemistry of phenolic compounds, (Academic Press : London)r.

Hardy, H.L. and Elkins, H.B. (1948) : Medical aspects of maximum allowable concentrations : Benzene., J. Indust. Hyg. Toxicol., **30**, 196-200.

Harper, C., Drew, R.T. and Fouts, J.R. (1975) : Species differences in benzene hydroxylation to phenol by pulmonary and hepatic microsomes., Drug Metab. Dispos., **3**. 381-388.

Harman, S.L., Mottley, C. and Mason, R.P. (1984) : Free-radical metabolites of L-cysteine oxidation., J. Biol. Chem., **259**, 5606-5611.

Harrison, J.E. and Schultz, J. (1976) : Studies on chlorinating activity of myeloperoxidase., J. Biol. Chem. **251**, 1371-1374.

Hesse, S., Mezger, M. and Wolff, T. (1978) : Activation of [^{14}C]chlorobiphenyls to protein-binding metabolites by rat liver microsomes., Chem. Biol. Interacns., **20**, 355-365.

Hewson, W.D. and Dunford, H.B. (1976) : Stoichiometry of the reaction between horseradish peroxidase and p-cresol., J. Biol. Chem., **251**., 6036-6042.

Hinson, J.A., Freeman, J.P., Potter, D.W., Mitchum, R.K. and Evans, F.E. (1985) : Mechanism of microsomal metabolism of benzene to phenol., Mol. Pharmacol., **27**, 574-577.

Hong, S.L. (1980) : Effect of bradykinin and thrombin on prostacyclin synthesis in endothelial cells from calf and pig aorta and human umbilical cord vein., Thromb. Res. **18**, 787-795.

Hrycay, E.G. and O'Brien, P.J. (1971) : Cytochrome P-450 as a microsomal peroxidase utilizing a lipid peroxide substrate., Arch. Biochem. Biophys., **147**, 14-28.

Hrycay, E.G. and O'Brien, P.J. (1973) : Microsomal electron transport: 1. Reduced Nicotinamide adenine dinucleotide phosphate-cytochrome *c* reductase and cytochrome P-450 as electron carriers in microsomal NADPH-peroxidase activity., Arch. Biochem. Biophys., **157**, 7-22.

Hunter, C.G., Blair, D. (1972) : Pharmacokinetic studies in man., Ann. Occup. Hyg., **15**, 193-199.

Infante, P.F. (1978) : Leukemia among workers exposed to benzene., Texas

Reports on Biology and Medicine, **37**, 153-160.

Irons, R.D., Dent, J.G., Baker, T.S. and Rickert, D.E. (1980) : Benzene is metabolized and covalently bound in bone marrow *in situ*., Chem. Biol. Interacns., **30**, 241-245.

Irving, C.C., Janss, D.H. and Russell, L.T. (1971) : Lack of N-hydroxy-2-acetylaminofluorene sulfotransferase activity in the mammary gland and zymbal's gland of the rat., Cancer Res., **31**, 387-391.

Janzen, E.G. (1980) : A critical review of spin-trapping in biological systems. In Free Radicals in Biology, Vol. IV, edited by Pryor, W. (Academic Press : New York) pp. 116-154.

Jellinck, P.H. and Fletcher, R. (1971) : Interaction of [4- ^{14}C]estradiol and its metabolites with polynucleotides in the presence of peroxidase., Can. J. Biochem., **49**, 885-890.

Jollow, D.J., Mitchell, J.R., Potter, W.Z., Davis, D.C., Gillette, J.R. and Brodie, B.B. (1973) : Acetaminophen-induced hepatic necrosis. II - Role of covalent binding *in vivo*., J. Pharmacol. Exper. Therap., **187**, 195-202.

Joschek, H.I. and Miller, D.E. (1966) : Photooxidation of phenols, cresols cresols and dihydroxybenzenes., J. Amer. Chem. Soc. **88**, 3273-3281.

Josephy, P.D., Eling, T.E. and Mason, R.P. (1983) : Co-oxidation of benzidine by prostaglandin synthetase and comparison with the action of horseradish peroxidase., J. Biol. Chem., **258**, 5561-5569.

Jung, R., Bermann, D., Glatt, H.R. and Oesch, F. (1981) : Mutagenicity of structurally related oxiranes: derivatives of benzene and its hydrogenated congeners., Mutat. Res., **81**, 11-19.

Kadlubar, F.F., Miller, J.A. and Miller, E.C. (1977) : Hepatic microsomal N-glucuronidation and nucleic acid binding of N-hydroxy arylamines in relation to urinary bladder carcinogenesis., Cancer Res., **37**, 805-814.

Kadlubar, F.F., Frederick, C.B., Weiss, C.C. and Zenser, T.V. (1982) : Prostaglandin endoperoxide synthetase-mediated metabolism of carcinogenic aromatic amines and their binding to DNA and protein., Biochem. Biophys. Res. Commun., **108**, 253-258.

Kadlubar, F.F. (1985) : The role of arylamine-DNA adducts in benzidine carcinogenesis and mutagenesis., In Third Intern. Symp. Biol. React. Interm., Abstract 14.

Kasai, H. and Nishimura, S. (1984) : Hydroxylation of deoxyguanosine at the C-8 position by ascorbic acid and other reducing agents., Nucleic Acids Res., **12**, 2137-2145.

Kimura, S., Elce, J.S. and Jellinck, P.J. (1983) : Immunological relationship between peroxidases in oesonophils, uterus and other tissues of the rat., Biochem. J., **213**, 165-170.

King, C.M. (1982) : N-Substituted aromatic compounds., In Chemical Carcinogenesis, edited by Nicolini, C. (Plenum Press : New York) pp. 25-46.

Kirby, K.S. (1965) : Isolation and characterization of ribosomal ribonucleic acid., Biochem. J., **96**, 266-269.

Kinsella, A.R., Gainer, H. St. C. and Butler, J. (1983) : Investigation of a possible role for superoxide anion production in tumor promotion., Carcinogenesis, **4**, 717-719.

Klibanov, A.M., Tsuman-Tu and Scott, K.P. (1983) : Peroxidase-catalyzed removal of phenols from coal-conversion waste waters., Science, **221**, 259-261.

Korbut, R., Boyd, J.A. and Eling, T.E. (1981) : Respiratory movements alter the generation of prostacyclin and thromboxane A_2 in isolated rat lungs: the influence of arachidonic acid-pathway inhibitors on the ratio between pulmonary prostacyclin and thromboxane A_2., Prostaglandins, **21**, 491-503.

Krauss, R.S. and Eling, T.E. (1984) : Arachidonic acid-dependent co-

oxidation: A potential pathway for the activation of chemical carcinogens *in vivo*., Biochem. Pharmacol., **33**, 3319-3324.

Kreig, T., Goerz, G., Lissner, R., Bolsen, K. and Ullrich, V. (1978) : Drug monooxygenase activity in the Harderian gland., Biochem. Pharmacol., **27**, 575-577.

Kulmacz, R.J. and Lands, W.E. (1983) : Characteristics of prostaglandin H synthase., In Advances in Prostaglandin, Thromboxane and Leukotriene Research, edited by Samuelsson, B., Paoletti, R. and Ramwell, P., (Raven Press : New York) Vol. 11, pp. 93-98.

Kuthan, H., Ullrich, V. and Estabrook, R.W. (1982) : A quantitative test for superoxide radicals produced in biological systems., Biochem. J., **203**, 551-558.

Lash, L.H., Jones, D.P. and Orrenius, S. (1984) : The renal thiol (GSH) oxidase., Biochem. Biophys. Acta., **770**, 191-200.

Laskin, S. and Goldstein B.D. (eds.), (1977) : Benzene toxicity : A critical evaluation., J. Toxicol. Environ. Hlth. Suppl. 2.

Levine, L. (1981) : Arachidonic acid transformation and tumor promotion., Adv. Cancer Res., **35**, 49-79.

Leihr, J.G., Dague, B.B., Ballatore, A.M. and Henkin, J. (1983) : Diethylstilbestron (DES) Quinone: A reactive intermediate in DES metabolism., Biochem. Pharmacol., *32*, 3711-3718.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) : Protein measurement with the folin-phenol reagent., J. Biol. Chem., **193**, 265-275.

Lunte, S.M. and Kissinger, P.T. (1983) : Detection and identification of sulfhydryl conjugates of p-benzoquinone in microsomal incubations of benzene and phenol., Chem. Biol. Interacns., **47**, 195-202.

Lutz, W.K. and Schlatter, C.H. (1977) : Mechanism of the carcinogenic action of benzene: Irreversible binding to rat liver DNA., Chem. Biol. Interacns., **18**, 241-245.

Machleidt, H., Roth, S. and Seeman, P. (1972) : The hydrophobic expansion of erythrocyte membranes by the phenol anesthetics., Biochem. Biophys. Acta., **255**, 178-189.

Malejka-Giganti, D., Gutmann, H.R. and Rydell, R.E. (1973) : Mammary carcinogenesis in the rat by topical application of fluorenylhydroxamic acids., Cancer Res., **33**, 2489-2497.

Maltoni, C., Cotti, G., Valgimigli, L. and Mandrioli, A. (1982) : Zymbal

gland carcinomas in rats following exposure to benzene by inhalation., Amer. J. Indust. Med., **3**, 11-16.

Maltoni, C., Conti, B. and Cotti, G. (1983) : Benzene: A multipotential carcinogen: Results of long term bioassays performed at the Bologna Institute of Oncology., Amer. J. Indust. Med., **4**, 589-630.

Marcus, A.J. (1978) : The role of lipids in platelet function : with particular reference to the arachidonic acid pathway., J. Lipid Res., **19**, 793-826.

Marnett, L.J., Wloodawer, P. and Samuelsson, B. (1975) : Co-oxygenation of organic substrates by the prostaglandin synthetase of sheep vesicular gland., J. Biol. Chem., **250**, 8510-8517.

Marnett, L.J., Reed, G.A. and Johnson, J.T. (1977) : Prostaglandin synthetase dependent benzo(a)pyrene oxidation: Products of the oxidation and inhibition of their formation by antioxidants., Biochem. Biophys. Res. Commun., **79**, 569-576.

Marnett, L.J., Johnson, J.T. and Bienkowski, M.J. (1979) : Arachindonic acid-dependent metabolism of 7,8-Dihydroxy-7,8-Dihydrobenzo(a)pyrene by ram seminal vesicles., FEBS Lett., **106**, 13-16.

Marnett, L.J., Dix, T.A., Sachs, R.J. and Siedlik, P.H. (1983) : Oxidations by fatty acid hydroperoxides and prostaglandin synthase., In Advances in

Prostaglandins, Thromboxanes and Leukotriene Research, edited by Samuelsson, B., Paoletti, R. and Ramwell, P., (Raven Press : New York), pp. 79-85.

Marshall, W. and O'Brien, P.J. (1984) : Multiple mechanisms of the metabolic activation of the carcinogen acetylaminofluorene., In Icosanoids and Cancer, edited by Thaler-Dao et al., (Raven Press: New York), pp. 49-63.

Mason, H.S. and Peterson, E.W. (1965) : Melanoproteins: I. Reactions between enzyme generated quinones and amino acids., Biochem. Biophys. Acta., III, 134-146.

Mason, R.P. (1982) : Free-radical metabolites in the metabolism of toxic chemicals., In Free Radicals in Biology, Vol. V, edited by Pryor, W., (Academic Press : New York) pp. 161-221.

Massey, V. (1959) : The microestimation of succinate and the extinction coefficient of cytochrome *c*., Biochem. Biophys. Acta., 34, 255-256.

McCarthy, M.B. and White, R.E. (1983) : Functional differences between peroxidase compound I and the cytochrome P-450 reactive oxygen intermediate., J. Biol. Chem., 258, 9153-9158.

McPhail, L.C., and Snyderman, R. (1983) : Activation of the respiratory

burst enzyme in human polymorphonuclear leukocytes by chemoattractants and other soluble stimuli: Evidence that same oxidase is activated by different transductional mechanisms., J. Clin. Invest., 72, 192-200.

Meister, A. and Anderson, M.E. (1983) : Glutathione., Ann. Rev. Biochem., 52, 711-760.

Mendelson, D.S., Metz, E.N. and Sagone, A.L. (1977) : Effects of phagocytosis on the reduced soluble sulfhydryl content of human granulocytes., Blood, 50, 1023-1031.

Metzler, M. and Mclahlan, J.A. (1978) : Peroxidase-mediated oxidation a possible pathway for metabolic activation of diethylstilbestrol., Biochem. Biophys. Res. Commun., 85, 874-884.

Metzler, M. and Epe, B. (1984) : Peroxidase-mediated binding of diethylstilbestrol anologs to DNA., *in vitro*: A possible role for a phenoxy radical. Chem. Biol. Interacns., 50, 351-360.

Michaelis, L., (1939) : Free-radicals as intermediate steps of oxidation-reduction., Cold Spring Harbor Symp. Quant. Biol., VII, 33-49.

Miller, E.C. and Miller, J.A. (1985) : Some historical perspectives on the metabolism of xenobiotic chemicals to reactive electrophiles., In Bioactivation of Foreign Compounds, edited by Anders, M.W., Biochemical Phar-

macology and toxicology; (Academic Press : New York) series of monographs; pp. 3-28.

Mitchell, J.R., McMurty, R.J., Statham, C.N. and Nelson, S.D. (1977) : Molecular basis for several drug induced nephropathies., Amer. J. Med., **62**, 518-526.

Mohandas, J., Duggin, J.G., Harnath, J.S. and Tiller, D.J. (1981) : Regional differences in peroxidatic activation of paracetamol (acetaminophen) mediated by cytochrome P-450 and prostaglandin endoperoxide synthetase in rabbit kidney., Res. Commun. Chem. Pathol. Pharmacol., **34**, 69-80.

Moldeus, P. and Jernstrom, B. (1983) : Interaction of glutathione with reactive intermediates., In Functions of Glutathione: Biochemical, Physiological, Toxicological and Clinical aspects., edited by Larrson, A., (Raven Press : NewYork), pp. 99-108.

Morrison, M. and Allen, P.Z. (1967) : Lactoperoxidase: Identification and isolation from harderian and lacrimal glands., Science, **152**, 1626-1628.

Morton, K.C., King, C.M., Vaught, J.B., Want, C.Y., Lee, H.S. and Marnett, L.J. (1983) : Prostaglandin H synthase mediated reaction of carcinogenic arylamines with tRNA and homopolyribonucleotides., Biochem. Biophys. Res. Commun., **111**, 96-103.

Mulder, G.J., Kadlubar, F.F., Mays, J.B. and Hinson, J.A. (1984) : Reaction of mutagenic phenacetin metabolites with glutathione and DNA., Mol. Pharmacol., **26**, 342-347.

Muller, W. and Gautier, F. (1975) : Interactions of heteroaromatic compounds with nucleic acids: A. T. Specific non-intercalating DNA ligands., Eur. J. Biochem., **54**, 385-394.

Muller, W. Crothers, D.M. and Waring, M.J. (1973) : A non-intercalating proflavine derivative., Eur. J. Biochem., **39**, 223-234.

Muller, W. and Crothers, D.M. (1975) : Interactions of heteroaromatic compounds with nucleic acids : 1. The influence of heteroatoms and polarizability on the base specificity of intercalating ligands., Eur. J. Biochem. **54**, 267-277.

Munier, M. and Chauveau, J. (1970) : Binding of dimethylaminoazobenzene metabolites to DNBA and proteins: *in vitro* studies on a microsomal dependent system., Int. J. Cancer, **6**, 463-469.

Murphy, P. (1976) : The Neutrophil., (Plenum Medical Book Company : New York and London)

Nagoa, M., and Sugimura, T. (1978) : Environmental mutagens and carcinogens., Ann. Rev. Genet., **12**, 117-159.

Nagata, C., Kodama, M., Ioki, Y., and Nagata, T. (1982) : Free-radicals produced from chemical carcinogens and their significance in carcinogenesis., In Free Radicals and Cancer, edited by Floyd, R.A., (Dekker : New York), pp. 1-62.

Nakayama, T., Kimura, T., Kodama, M. and Nagata, C. (1982) : Electron spin resonance study on the metabolism of 2-naphthylamine and 1-naphthylamine in rat liver microsomes., Gann, **73**, 382-390.

Nakayama, T., Kimura, T., Kodama, M. and Nagata, C. (1983) : Generation of hydrogen peroxide and superoxide anion from active metabolites of naphthylamines and aminoazo dyes: its possible role in carcinogenesis., Carcinogenesis, **4**, 765-769.

National Cancer Institute Carcinogenesis Technical Report, Number 203, National Institutes of Health, Bethesda, Md. (1980) : Bioassay of phenol for possible carcinogenicity

Nauseef, W.M., Metcalf, J.A. and Root, R.K. (1983) : Role of myeloperoxidase in the respiratory burst of human neutrophils., Blood, **61**, 483-495.

Nelson, S.D., Mitchell, J.R., Dybing, E. and Sasame, H.A. (1976) : Cytochrome P-450 mediated oxidation of 2-hydroxyestrogens to reactive intermediates., Biochem. Biophs. Res. Commun., **70**, 1157-1165.

Norymberski, J.K. (1977) : Polymerization of oestradiol by potassium ferricyanide and by horseradish peroxidase., FEBS Lett., 76, 231-234.

NTP Technical Report 289 (1985) : NTP Technical Report on the toxicology and carcinogenesis studies of benzene in F 244/N rats and B6C3F1 mice (gavage studies). NIH publication No. 84-2545, U.S. Department of Health and Human Services

O'Brien, P.J. (1982) : Hydroperoxides and superoxides in microsomal oxidations. In Pharmacol. Therap., edited by Schenkman, J.B. and Kupfer, D., (Pergamon Press : New York), pp. 567-586.

O'Brien, P.J. (1984) : Multiple mechanisms of metabolic activation of aromatic amine carcinogens. In Free Radicals in Biology, Vol. VI, edited by Pryor, W. (Academic Press : New York), pp. 289-322.

O'Brien, P.J. (1985) : Free radical-mediated DNA binding., Environ. Hlth. Perspect., 64, 219-232.

O'Brien, P.J. and Rahimtula, A.D. (1980) : The peroxidase function of cytochromeP-450 with possible implications in carcinogenesis., In Cytochrome P-450, edited by Gustaffson, J.A., (Elsevier, North-Holland - Amsterdam), pp. 273-281.

O'Brien, P.J., Subrahmanyam, V.V., Gregory, B., Fourney, R., Davidson,

W.S., Rahimtula, A.D., and Tsuruta, Y. (1985) : One-electron oxidation in cell toxicity. In Microsomes and Drug Oxidations, edited by A.R. Boobis, J. Caldwell, F. DeMatties, C.R. Elcombe., (Taylor and Francis : London and Philadelphia) pp. 284-295.

Oehme, F.W. and Davis, L.E. (1970) : The comparative toxicity and biotransformation of phenol., Toxicol. Appl. Pharmacol., **5**, 36-48.

Offermann, M.K., McKay, M.J., Mash, M.W. and Bond, J.S. (1984) : Glutathione disulfide inactivates, destabilizes and enhances proteolytic susceptibility of fructose-1,6-bisphosphate aldolase., J. Biol. Chem., **259**, 8886-8891.

Oglesby, L.A., Flammang, T.J., Tullis, D.L. and Kadlubar, F.F. (1981) : Rapid absorption, distributin and excretion of carcinogenic N-hydroxyarylamines after direct urethral instillation int the rat urinary bladder., Carcingenesis, **2**, 15-20.

Ohki, S., Ogino, N., Yamamato, S. and Hayaishi, O. (1979) : Prostaglandin hydroperoxidase, an integral part of prostaglanding endoperoxide synthetase from bovine vesicular gland microsomes., J. Biol. Chem., **254**, 829-836.

Ohno, Y. and Gallin, J.I. (1985) : Diffusion of extracellular hydrogen peroxide into intracellular compartments of human neutrophils: Studies utilizing the inactivation of myelopeoxidase by hydrogen peroxide and azide., J. Biol. Chem., **260**, 8438-8446.

Oliver, J.M., Berlin, R.D., Baehner, R.L. and Boxer, L.A. (1977) : Mechanisms of microfublule disassembly : *in vivo* studies in normal and chronic granulomatous disease leukocytes., Br. J. Hematol., **37**, 311-322.

Olsen, J. and Davis, L. (1976) : The oxidation of dithiothreitol by peroxidases and oxygen., Biochem. Biophys. Acta., **445**, 324-329.

Osborne, J.C., Metzler, M. and Neumann, H.G. (1980) : Peroxidase activity in the rat Zymbal gland and its possible role in the metabolic activation of aminostilbenes in the target tissue., Cancer Lett., **8**, 221-226.

Panthananickal, A., Weller, P. and Marnett, L.J. (1983) : Stereoselectivity of the epoxidation of 7,8-dihydrobenzo(a)pyrene by prostaglandin H synthase and cytochrome P-450 determined by the identification of polyguanylic acid adducts., J. Biol. Chem., **258**, 4411-4418.

Parke, D.V. and Williams, R.T. (1953) : Studies in Detoxication - 49 - The metabolism of benzene containing ^{14}C-benzene., Biochem. J., **54**, 231-238.

Patriarca, P., Cramer, R., Marussi, M., Rossi, F. and Romeo, D. (1971) : Mode of activation of granule-bound NADPH oxidase in leukocytes during phagocytosis., Biochem. Biophys. Acta. **237**, 335-338.

Patriarca, P., Dri, P., Kakinuma, K., Tedesco, F. and Rossi, F. (1975) : Studies on the mechanism of metabolic stimulation in polymorphonuclear leuko-

cytes during phagocytosis I : Evidence for superoxide anion involvement in the oxidation of NADPH., Biochem. Biophys. Acta., **385**, 380-386.

Pearson, R.G. and Songstad, J. (1967) : Application of the principle of hard and soft acids and bases to organic chemistry., J. Amer. Chem. Soc., **89**, 1827-1836.

Pelkonen, O. and Nebert, D.W. (1982) : Metabolism of polycyclic aromatic hydrocarbons: Etiologic role in carcinogenesis., Pharmacol. Rev., **34**, 189-222.

Piper, P.J. and Vane, J.R. (1971) : The release of prostaglandins from lung and other tissues., Ann. N.Y. Acad. Sci., **180**, 363-385.

Phillips, D.H. (1983) : Fifty years of benzo(a)pyrene., Nature, **303**, 468-472.

Pohl, R.J. and Fouts, J.R. (1983) : Cytochrome P-450 dependent xenobiotic metabolizing activity in Zymbal's gland a specialized sebaceous gland of rodents., Cancer Res., **43**, 3660-3662.

Potter, D.W., Miller, D.W. and Hinson, J.A. (1985) : Identification of acetaminophen polymerization products catalyzed by horseradish peroxidase., J. Biol. Chem., **260**, 12174-12180.

Potter, D.W., Miller, D.W., and Hinson, J.A. (1986) : Horseradhsi

peroxidase-catalyzed oxidation of acetaminophen to intermediates that form polymers or conjugate with glutathione., Mol. Pharmacol., **29**, 155-162.

Pound, A.W. (1968) : Carcinogenesis and cell proliferation, New Zealand Med. J. Suppl., 88-99.

Quintiliani, M., Badiello, R., Tamba, R. and Gorin, G. (1976) : Radiation chemical basis for the role of glutathione in cellular radiation sensitivity., In Modification of Radiosensitivity of Biological Systems, IAEA, Vienna pp. 29-37.

Radomski, J.L., Brill, E., Deichmann, W.B. and Glass, E.M. (1971) : Carcinogenicity testing of N-hydroxy and other oxidation and decomposition products of 1- and 2-naphthylamine., Cancer Res. **31**, 1461-1467.

Radomski, J.L., Hearn, W.L., Radomski, T., Moreno, H. and Scott, W.E. (1977) : Isolation of the glucuronic acid conjugate of N-hydroxy-4-aminobiphenyl from dog urine and its mutagenic activity., Cancer Res., **37**, 1756-1762.

Ramasarma, T. (1982) : Generation of H_2O_2 in biomembranes. Biochem. Biophys. Acta., **694**, 69-93.

Rauckman, E.J., Rosen, G.M. and Cavaguaro, J. (1982) : Norcocaine nitroxide: A potential hepatotoxic metabolite of cocaine., Mol. Pharmacol., **21**,

458-463.

Reed, D.J. (1985) : Cellular defense mechanisms against reactive metabolites., In Bioactivation of foreign compounds, edited by Anders, M.W., : Biochemical Pharmacology and Toxicology: A series of monographs., (Academic Press : New York), pp. 71-108.

Reed, D.J., Babson, J.R., Beatty, P.W., Brodie, A.E., Ellis, W.W. and Potter, D.W. (1980) : High pressure liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide and related thiols and disulfides., Analyt. Biochem., **106**, 55-62.

Reed, G.A. and Marnett, L.J. (1979) : Peroxidatic oxidation of benzo(a)pyrene and prostaglandin biosynthesis., Biochemistry, **18**, 2923-2929.

Rees, R.J. and Pirie, A. (1967) : Possible reactions of 1,2-naphthoquinone in the eye., Biochem. J., **102**, 853-863.

Renneberg, R., Capdevilla, J., Chacos, N., Estabrook, R.W.and Prough, R.A. (1981) : Hydrogen peroxide-supported oxidation of benzo(a)pyrene by rat liver microsomal fractions., Biochem. Pharmacol., **30**, 843-848.

Rickert, D.E., Baker, T.S., Bus, J.S., Barrow, C.S. and Irons, R.D. (1979) : Benzene disposition in the rat after exposure by inhalation., Toxicol. Appl.

Pharmacol. 49, 417-423.

Riva, S.C. (1966) : Interactions of methylated acridines with DNA., Biochem. Biophys. Res. Commun., 23, 606-611.

Robertson, I.G.C., Sivarajah, K., Eling, T.E. and Zeiger, E. (1981) : Activation of some aromatic amines to mutagenic products by prostaglandin endoperoxide synthetase., Cancer Res., 43, 476-480.

Roerig, D.L., Reak, C.J. and Wang, R.I.H. (1976) : Enzymatic conversion of morphine to pseudomorphine., Biochem. Pharmacol., 25, 1075-1080.

Rogan, E.G., Patricia, A., Katomski, P.A., Roth, R.W. and Cavalieri, E.L. (1979) : Horseradish peroxidase/hydrogen peroxide-catalyzed binding of aromatic hydrocarbons to DNA., J. Biol. Chem., 254, 7055-7059.

Rollins, T.E. and Smith, W.L. (1980) : Subcellular localization of prostaglandin-forming cyclooxygenase in Swiss Mouse 3T3 Fibroblasts by electron microscopic immunocytochemistry., J. Biol. Chem., 255, 4872-4875.

Ross, D., Larrson, R., Anderson, B., Nilsson, U., Lindquist, T., Lindeke, B. and Moldeus, P. (1985) : The oxidation of p-phenetidine by horseradish peroxidase and prostaglandin synthetase and the fate of glutathione during such oxidations., Biochem. Pharmacol., 34, 343-351.

Rossi, F., Della-Bianca, V., and Bellavite, P. (1981) : Inhibition of the respiratory burst and of phagocytosis by nordihydroguairatic acid in neutrophils., FEBS Lett., **127**, 183-187.

Rossi, F. and Zatti, M. (1968) : Mechanism of the respiratory stimulation in sapponine-treated leukocytes: The KCN-insensitive oxidation of NADH., Biochem. Biophys. Acta., **153** 296-299.

Rushmore, T.H., Kalf, G.K. and Snyder, R. (1983) : Covalent binding of benzene metabolites to mitochondrial DNA inhibits macromolecular synthesis., Fed. Proc., **42**, 1041.

Saez, G., Thornalley, P.J., Hill, H.A.O., Hems, R. and Bannister, J.V. (1982) : The production of free-radicals during the autoxidation of cysteine and their effect on isolated rat hepatocytes., Biochem. Biophys. Acta., **719**, 24-31.

Samuelsson, B. (1983) : Leukotrienes: Mediators of immediate hypersensitivity reactions and inflammation., Science, **220**, 568-575.

Sanada, H., Suzue, R., Nakashima, Y. and Kawada, S. (1972) : Effect of thiol compounds on melanin formation by tyrosinase., Biochem. Biophys. Acta., **261**, 258-266.

Sato, R. and Omura, T. (eds.), (1978) : Cytochrome P-450, (Academic Press :

New York).

Saunders, B.C., Holmer-Siedle and Stark, B.P. (eds.) (1964) : Peroxidase, (Butterworth : Washington).

Sawahata, T. and Neal, R.A. (1982) : Horseradish peroxidase-mediated oxidation of phenol., Biochem. Biophys. Res. Commun., **109**, 988-994.

Sawahata, T. and Neal, R.A. (1983) : Biotransformation of phenol to hydroquinone and catechol by rat liver microsomes., Mol. Pharmacol., **23**, 453-460.

Sawahata, T., Rickert, D.E. and Greenlee, W.F. (1985) : Metabolism of benzene and its metabolites in bone marrow., In toxicology of the blood and bone marrow. edited by Irons, R.D., (Raven Press : New York), pp. 141-148.

Scholes, G., Willson, R.L. and Ebert, M. (1969) : Pulse radiolysis of aqueous solutions of deoxyribonucleotides and of DNA: Reaction with hydroxyl radicals., J. Chem. Soc, Chem. Commun., 17-18.

Schrenk, H.H., Yant, W.P. and Pearce, S.J. (1941) : Absorption, distribution and elimination of benzene by body tissues and fluids of dogs exposed to benzene vapor., J. Indust. Hyg. Toxicol., **23**, 20-34.

Schut, H.A.J. and Castonguay, A. (1984) Metabolism of carcinogenic amino derivatives in various species and DNA alkylation by their metabolites.,

Drug Metab. Rev., 15, 753-839.

Schwartz, C.S., Snyder, R. and Kalf, G.F. (1985) : The inhibition of mitochondrial DNA replication *in vitro* by the metabolites of benzene, hydroquinone and p-benzoquinone., Chem. Biol. Interacns., 53, 327-350.

Sealy, R.C., Felix, C.C., Hyde, J.S. and Swartz, H.M. (1980) : Structure and reactivity of melanins: Influence of free-radicals and metal ions., In Free Radicals in Biology, Vol. IV., edited by Pryor, W., (Academic Press, Inc.), pp. 209-259.

Sedlak, J. and Lindslay, R.H. (1968) : Estimation of total, protein-bound and nonprotein sulfhydryl groups in tissue with Ellman reagent., Analyt. Biochem., 25, 192-205.

Sieber, S.M. and Adamson, H.R. (1975) : Toxicity of antineoplastic agents., Adv. Cancer Res., 22, 57-155.

Sies, H., Bucher, T., Oshino, N. and Chance, B. (1973) : Heme occupancy of catalase in hemoglobin-free perfused rat liver and of isolated rat liver catalase., Arch. Biochem. Biophys., 154, 106-116.

Sietz, J.F. (1969) : In the Biochemistry of cells of blood and bone marrow, edited by Thomas, C.C. (Springfield : Illinois).

Sivarajah, K., Lasker, J.M. and Eling, T.E. (1981) : Prostaglandin synthetase-dependent co-oxidation of (±)benzo(a)pyrene-7-8-dihydrodiol by human lung and other mammalian tissues, Cancer Res., **41**, 1834-1839.

Sivarajah, K., Anderson, M.W. and Eling, T.E. (1978) : Metabolism of benzo(a)pyrene to reactive intermediates via prostaglandin biosynthesis. Life Sci., **23**, 2571-2578.

Sivarajah, K., Mukhtar, H. and Eling, T.E. (1979) : Arachidonic acid-dependent metabolism of (±)Trans-7,8-Dihydroxy-7,8-dihydrobenzo(a)pyrene (BP-7,8 diol) to 7,10/8,9 Tetrols., FEBS Lett., **106**, 17-20.

Slater, T.F. (1984) : Free-radical mechanisms in tissue injury., Biochem. J., **222**, 1-15.

Smart, R.C. and Zannoni, V.G. (1984) : DT-diaphorase and peroxidase influence the covalent binding of the metabolites of phenol, the major metabolite of benzene., Mol. Pharmacol. **26**, 105-111.

Smart, R.C. and Zannoni, V.G. (1985) : Effect of ascorbate on covalent binding of benzene and phenol metabolites to isolated tissue preparations., Toxicol. Appl. Pharmacol., **77**, 334-343.

Smith, W.L. and Lands, W.E.M. (1971) : Stimulation and blockade of prostaglandin biosynthesis., J. Biol. Chem., **246**, 6700-6702.

Smith, C.V., Hughes, H., Lauterberg, B.H. and Mitchell, J.R. (1983) : Chemical Nature of Reactive Metabolites determines their biological interactions with glutathione., In Functions of glutathione : Biochemical, Physiological, Toxicological and Clinical aspects, edited by Larrson, A., (Raven Press : New York), pp. 125-137.

Snyder, R. and Kocsis, J.J. (1975) : Current concepts of chronic benzene toxicity., CRC Crit. Rev. Toxicol. **3**, 265-288.

Snyder, R., Lee, E.W., Kocsis, J.J. and Witmer, C.M. (1977) : Bone marrow depressant and leukemogenic actions of benzene., Life Sci., **21**, 1709-1721.

Snyder, R., Lee, E.W. and Kocsis, J.J. (1978) : Binding of labelled benzene metabolites to mouse liver and bone marrow., Res. Commun. Chem. Pathol. Pharmacol., **20**, 191-194.

Snyder, R., Longacre, S.L., Witmer, C.M., Kocsis, J.J., Andrews, L.S. and Lee, E.W. (1980) : Biochemical toxicology of benzene., In Rev. in Biochem. Toxicol., Vol. 3., edited by Hodgson, E., Bend, J.R. and Philpot, R.M., (Elsevier, North-Holland), pp. 123-153.

Sontum, S.F. and Case, D.A, (1985) : Electron structures of active site models for compounds I and II of peroxidase., J. Amer. Chem. Soc., **107**, 4013-4015.

Srivastava, S.K. and Beutler, E. (1967) : Permeability of normal and glucose-6-phosphate dehydrogenase deficient erythrocytes to glutathione., **28**, 659-664.

Stier, A., Clauss, R., Lucke, A. and Rietz, I. (1980) : Redox cycle of stable mixed nitroxides formed from carcinogenic aromatic amines., Xenobiotica, **10**, 661-673.

Strum, J.M. and Karnovsky, M.J. (1970) : Cytochemical localization of endogenous peroxidase in thyroid follicular cells., J. Cell. Biol., **44**, 655-666.

Susan, S.M. and Adamson, R.H. (1975) : Toxicity of antineoplastic agents in man: Chromosomal aberrations, antifertility effects, congenital malformations and carcinogenic potential., Adv. Cancer Res., **22**, 57-155.

Takanaka, K. and O'Brien, P.J. (1975 a) : Mechanisms of H_2O_2 formation by leukocytes: (1) Evidence for a plasma membrane location., Arch. Biochem. Biophys., **169**, 428-435.

Takanaka, K. and O'Brien, P.J. (1975 b) : Mechanisms of H_2O_2 formation by leukocytes: (2) Properties of the NAD(P)H oxidase activity of intact leukocytes., Arch. Biochem. Biophys., **169**, 436-442.

Takanaka, K., and O'Brien, P.J. (1980) : Generation of activated oxygen

species by polymorphonuclear leukocytes., FEBS Lett., **110**, 283-286.

Takanaka, K., O'Brien, P.J., Tsuruta, Y. and Rahimtula, A.D. (1982) : Tumor promoter stimulated irreversible binding of N-methylaminoazobenzene to polymorphonuclear leukocyte DNA., Cancer Lett., **15**, 311-315.

Takayama, K. and Nakano, M. (1977) : Mechanism of thyroxine-mediated oxidation of reduced nicotinamide adenine dinucleotide in peroxidase-H_2O_2 system., Biochemistry, **16**, 1921-1926.

Tal, M., Silberstein, A. and Nusser, E. (1985) : Why does coomassie brilliant blue R interact differently with different proteins?: A partial answer., J. Biol. Chem., **260**, 9976-9980.

Tammers, M.A. (1976) : Total synthesis of benzene and its derivatives as major gasoline extenders., Science, **193**, 231-232.

Taylor, W.I. and Battersby, A.R. (eds.) (1967) : Oxidative coupling of phenols., (Marcel and Dekker : New York).

Trush, M.A., Mimnaugh, E.G. and Gram, T.E. (1982) : Activation of pharmacological agents to radical intermediates: Implications for the role of free-radicals in drug action and toxicity., Biochem. Pharmacol. **31**, 3335-3346.

Tsuruta, Y., Josephy, P.D., Rahimtula, A.D. and O'Brien, P.J. (1985) : Peroxidase-catalyzed benzidine binding to DNA and other macromolecules., Chem. Biol. Interacns., **54**, 143-158.

Tunek, A., Platt, K.L., Bentley, P. and Oesch, F. (1979) : Microsomal metabolism of benzene to species irreversibly binding to microsomal proteins and effects of modifications of this metabolism., Mol. Pharmacol., **14**, 920-929.

Tunek, A., Platt, K.L., Przybylski, M. and Oesch, F. (1980) : Multi-step metabolic activation of benzene: Effect of superoxide dismutase on covalent binding to microsomal macromolecules and identification of glutathione conjugates using high pressure liquid chromatography and field desorption mass spectrometry., Chem. Biol. Interacns., **33**, 1-17.

Venkatachalam, M.A., Saltoni, M.H. and Fahimi, H.D. (1970) Fine structural localization of peroxidase activity in the epithelium of the large intestine of rat., J. Cell Biol., **46**, 168-173.

Vigliani, E.C. (1976) : Leukemia associated with benzene exposure., Ann. N.Y. Acad. Sci., **271**, 143-153.

Vina, J., Saez, G.T., Wiggins, D., Roberts, A.F.C., Hems, R. and Krebs, H.A. (1983) : The effect of cysteine oxidation on isolated hepatocytes., Biochem. J. **212**, 39-44.

Wagai, N. and Hosoya, T. (1982) : Partial purification of estrogen-dependent peroxidase of rat uterus and comparison of the properties with those of other animal peroxidases., J. Biochem., **91**, 1931-1942.

Walsch, C.E., Waite, B.M., Thomas, M.J. and DeChatelet, L.R. (1981) : Release and metabolism of arachidonic acid in human neutrophils., J. Biol. Chem., **256**, 7228-7234.

Waring, M.J. (1981) : DNA modification and cancer., Ann. Rev. Biochem., **50**, 159-192.

Wattenberg, L.W. (1981) : Inhibitors of chemical carcinogenesis., Adv. Cancer Res., **28**, 197-226.

Wattenberg, L.W. and Leong, J.L. (1971) : Tissue distribution studies of polycyclic hydrocarbon hydroxylase activity., In Handbook of Experimental Pharmacology, edited by Brodie, B.B. and Gillette, J.R., (27, Springer-Verlag: Berlin), 422-430.

Wefers, H. and Sies, H. (1983) : Oxidation of glutathione by the superoxide radical to the disulfide and the sulfonate yielding singlet oxygen., Eur. J. Biochem. **137**, 29-36.

West, P.R., Harman, L.S., Josephy, P.D. and Mason, R.P. (1984) : Acetaminophen: Enzymatic formation of a transient phenoxyl free-radical.,

Biochem. Pharmacol., **33**, 2933-2936.

White, R.E. and Coon, M.J. (1980) : Oxygen activation by cytochrome P-450., Ann. Rev. Biochem., **49**, 315-356.

Willson, R.L. (1970) : Pulse radiolysis studies of electron transfer in aerobic solutions., J. Chem. Soc. Chem. Commun., 1005.

Wilson, R.H. (1942) : Benzene poisoning in industry., J. Lab. Clin. Med., **27**, 1517-1521.

Wintobe, M.M., Lee, G.R. and Bogs, D.R. (eds.) (1974) : Clinical hematology, (7th edition : Philadelphia, Lea and Febiger), p. 1745.

Wise, R.W., Zenser, T.V., Kadlubar, F.F. and Davis, B.B. (1984) : Metabolic activation of carcinogenic aromatic amines by dog bladder and kidney prostaglandin H synthase., Cancer Res., **44**, 1893-1897.

Wong, P.K., Hampton, M.J. and Floyd, R.A. (1982) : Evidence for lipoxygenase peroxidase activation of N-hydroxy-2-acetyl-aminofluorene by mammary gland cells., In Prostaglandins and Cancer, edited by Powles, T.J., Bockman, R.S., Honn, K.V. and Ramwell, P., (Alan R.Liss, Inc., : New York) pp. 167-179.

Wright, R.G.Mc.R., Wakelin, L.P.G., Fieldes, A., Acheson, R.M. and War-

ing, M.J. (1980) : Effects of ring substituents and linker chains on the bifunctional intercalation of diacridines into deoxyribonucleic acid., Biochemistry, **19**, 5825-5836.

Yamazaki, I. and Piette, L. (1963) : The mechanism of aerobic oxidase reaction catalyzed by peroxidase., Biochem. Biophys. Acta., **77**, 47-63.

Yang, S.K., Deutsch, J. and Gelboin, H.V. (1978) : Benzo[a]pyrene metabolism: Activation and detoxification., In Polycyclic Hydrocarbons and Cancer, Vol.1, edited by Gelboin, H.V. and P.O.P. Tso's, (Academic Press: New York) pp. 205-231.

Youngman, R.J. and Elstner, E.F. (1984) : On the interaction of adriamycin with DNA : Investigations of spectral changes., Arch. biochem. Biophys., **231**, 424-429.

Zenser, T.V., Mattammal, M.B. and Davis, B.B. (1979) : Co-oxidation of benzidine by renal medullary prostaglandin cyclo-oxygenase., J. Pharmacol. Exp. Therap. **211**, 460-464.

Ziegler, D.M. (1985) : Role of reversible oxidation -reduction of enzyme thiols-disulfide in metabolic regulation., Ann. Rev. Biochem., **54**, **54**, 305-329.

Zimmerman, R. and Cerutti, P. (1984) : Active oxygen acts as a promotor

of transformation in mouse embryo C3H/ $10T^{1/2}$/C-18 fibroblasts., Proc. Natl. Acad. Sci., **81**, 2085-2087.

Zgliczynski, J.M., Stelmaszynska, T., Ostrowski, W., Naskalski, J. and Sznajd, J. (1968) : Myeloperoxidase of human leukaemic leukocytes: Oxidation of amino acids in the presence of hydrogen peroxide., Eur. J. Biochem., **4**, 540-547.