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ACTIVATION OF N, N-DIMETHYLAMINOAZOBENZENE CATALYZED BY PEROXIDASE

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by

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ABSTRACT

The mechanism for the metabolic activation of N,N-dimethylaminoazobenzene is unknown. Although cytochrome P-450 dependent mixed function oxygenases are important in the activation of arylamines, there are target tissues for arylamines which do not contain these oxygenases. These tissues do contain peroxidases. Therefore a one electron oxidation mechanism was investigated by studying a Horseradish Peroxidase/hydrogen peroxide catalyzed metabolism of aminoazobenzenes [*i.e*; N-methyl-4-aminoazobenzene (MAB), N,N-dimethyl-4-aminoazobenzene (DAB), aminoazobenzene (AB)], and by following the binding of the activated products to exogenous DNA. In order to further explore the mechanism, the effect of biological and phenolic antioxidants on metabolism and binding of the activated products of [^{14}C] MAB was studied. The oxidation products were analyzed by spectrophotometry, high pressure liquid chromatography and thin layer chromatography. The major product obtained from both MAB and DAB at pH 7.4 was a dimer of MAB, *i.e*; N-methyl,N(MAB)-4-aminoazobenzene. The product obtained at pH 5.0 was 4'-(MAB)-N-methyl-4-aminoazobenzene. It is suggested that DAB is first N-demethylated, and then follows the same metabolic pathway as does MAB. Evidence suggests that AB is also oxidized to a dimer with N-N linkage at pH 7.4 and C-N linkage at pH 5.0.

The binding of the activated products to calf thymus DNA was observed spectrophotometrically. [^{14}C] MAB was used to determine the quantitative binding by liquid scintillation counting. DNA is attacked by a free radical of MAB, and also by other radicals formed in chain propagating reactions with the MAB radical. The maximum binding of [^{14}C] MAB to homo polyriboguanylic acid among the four homo polyribonucleotides suggests the preferential binding of the activated products occurs at the guanine residues in DNA.

The phenolic and biological antioxidants inhibit the DNA binding either by inhibiting the total oxidation of MAB or by converting the reactive metabolites to detoxication products.

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AB	4-Aminoazobenzene
AB dimer	Product of oxidative coupling two molecules of AB.
BHA	Butylated-hydroxyanisole
DAB	N,N-dimethyl-4-aminoazobenzene
HPLC	High pressure liquid chromatography
HRP	Horse radish peroxidase
MAB	N-methyl-4-aminoazobenzene
MAB dimer	Product of oxidative coupling of two molecules of MAB.
m/z	mass/charge
MS	Mass spectrometry
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NMR	Nuclear magnetic resonance
N-OH-AAF	N-hydroxy-2-acetylaminofluorene
PAPS	3-phosphoadenosine-5-phosphate
TLC	Thin layer chromatography

INTRODUCTION

1.Chemical Carcinogenesis:

The chemical carcinogens constitute a large group of synthetic and naturally occuring compounds. The carcinogenic nature of most of these compounds was discovered as a result of incidences of cancer in humans exposed to them, *e.g*; skin cancer in chimney sweeps in Britain led Pott in 1775 to recognize that soot particles were the cause of cancer (reviewed in Heidelberger, 1975).

The diverse nature of carcinogenic compounds renders it impossible to pinpoint a single molecular feature as cancer producing. However, some common features can be identified with regard to their chemical reactivity. Thus, it is now well accepted that in order to manifest their carcinogenicity the chemical carcinogens have to be activated through metabolism. The reactive species are electrophilic and react with the nucleophilic groups of intermediary metabolites, proteins and (to induce cancer) nucleic acids. The carcinogenic potential of a chemical carcinogen can therefore be evaluated by detecting;

(i) the reactive electrophiles in the metabolic profile,

(ii) its binding to the cellular informational macromolecules (Miller & Miller, 1977).

There are however some exceptions, for example, nitrosamides and some alkylating agents are chemically reactive themselves (Heidelberger, 1975). Actinomycin D binds non-covalently to DNA and has been shown to cause mesotheliomas upon intraperitoneal injections (Svoboda *et al.*, 1970).

2.Activation by Monooxygenase System:

The metabolic studies of chemical carcinogens have received great attention during the past four decades. The most extensively studied system has been the cytochrome P-450 containing monooxygenase system of the endoplasmic reticulum. The actual function of this system is the detoxication of foreign compounds and drugs by converting them into water soluble and excretable products but the reactive intermediates are sometimes noxious and lead to carcinogenesis.

The oxidation by the mixed function oxidase system is considered to be a two electron oxidation (reviewed in O'Brien, 1984). Polycyclic aromatic hydrocarbons, aflatoxins and some chlorinated compounds are converted to epoxides. Aromatic amines such as N-acetylaminofluorene are N-hydroxylated. Alkylating agents are formed by the N-dealkylation of nitrosamines and dialkylhydrazines (Magee *et al.*, 1975)

3.Other Activating Systems:

In spite of extensive studies of the monooxygenase system, the possible importance of other systems cannot be overlooked. The activation of arylamines by the cytochrome P-450 system involves the formation of an N-hydroxy derivative which is acetylated or sulphated. These conjugates then lose acetate or sulphate ion producing a reactive electrophile, the nitrenium ion (discussed later). Without the action of acetyl or sulphotransferase these conjugates and the nitrenium ion cannot be formed. These enzymes are not found either in the Zymbal gland or in the mammary gland, but these glands are target tissues

(Irving *et al.*, 1971 & Osborn *et al.*, 1980). Other tissues may also contain an activating system in addition to cytochrome P-450. Brain and kidney medulla contain prostaglandin synthetase. Hepatocytes and Kupffer cells, mammary gland and bone marrow contain peroxidase and uterine tissue contain both peroxidase and prostaglandin synthetase (reviewed in O'Brien, 1984). The ultimate carcinogenic molecules are very unstable and react with the nucleophiles indiscriminately. Therefore they must be produced close to the informational molecules. The presence of peroxidase in the Zymbal gland (Osborne *et al.*, 1980) indicates the role of peroxidase in the activation of carcinogens. In fact, peroxidase and prostaglandin synthetase have been reported to mediate the covalent binding of carcinogens to DNA (Vasdev *et al.*, 1981).

Anthanthrene (a polycyclic aromatic hydrocarbon) does not have a bay region which is required for the formation of diol epoxides by the cytochrome P-450 containing monoxygenase system. None the less it is moderately carcinogenic (Floyd, 1982). The N-demethylation of some tertiary amines leads directly to reactive species when catalyzed by prostaglandin synthetase (Lasker *et al.*, 1981 & Sivarajah *et al.*, 1981) and by hematin and hemoproteins (reviewed by O'Brien, 1984).

Finally it appears that cytochrome P-450 in the presence of organic peroxides activates carcinogens by a route other than two electron oxidation (Rahimtula *et al.*, 1974; Rahimtula & O'Brien, 1975; Nordblom *et al.*, 1976). This is a peroxidase activity, similar to that shown by peroxidase itself with hydrogen peroxide. Takanaka *et al.*, (1982) have reported the hydrogen peroxide dependent activation by leukocyte myeloperoxidase of carcinogenic arylamines

Peroxidase and prostaglandin synthetase mediate the activation of carcinogens by a one electron oxidation mechanism. The radical formed as a result can either bind directly to DNA or bind after further oxidation to an electrophile (O'Brien, 1984)

4.Free Radical Intermediates:

The free radical intermediates of xenobiotics were considered to be toxic intermediates as early as 1940s. This view was supported because the physical carcinogens such as ionizing radiations are known to produce free radicals. The presence of a large number of free radicals in tobacco smoke has also strengthened this concept (Mason *et al.*, 1982).

The toxic effects of free radical metabolites of amines were discussed first by Heubner in 1948 (in Saunders *et al.*, 1964). Nitroxide radical adducts with microsomal membranes have recently been demonstrated (Stier *et al.*, 1980). The cytochrome P-450 of rabbit liver microsomes has been found to catalyze the oxidation of aromatic N-containing compounds with the generation of nitroxide free radicals (Stier *et al.*, 1982).

The formation of free radicals from the oxidation of N-hydroxy-2-acetylaminofluorene catalyzed by horse radish peroxidase and hydrogen peroxide was first shown by Bartsch & Hecker in 1971 & 1972. (Fig.1) A similar reaction was observed with bovine lactoperoxidase or human myeloperoxidase in the presence of hydrogen peroxide (Bartsch *et al.*, 1972). Two potent carcinogens, 2-nitrosofluorene and N-acetoxy-2-aminofluorene are generated from the intermediate nitroxy free radicals (Bartsch *et al.*, 1971). Floyd (1980) showed that

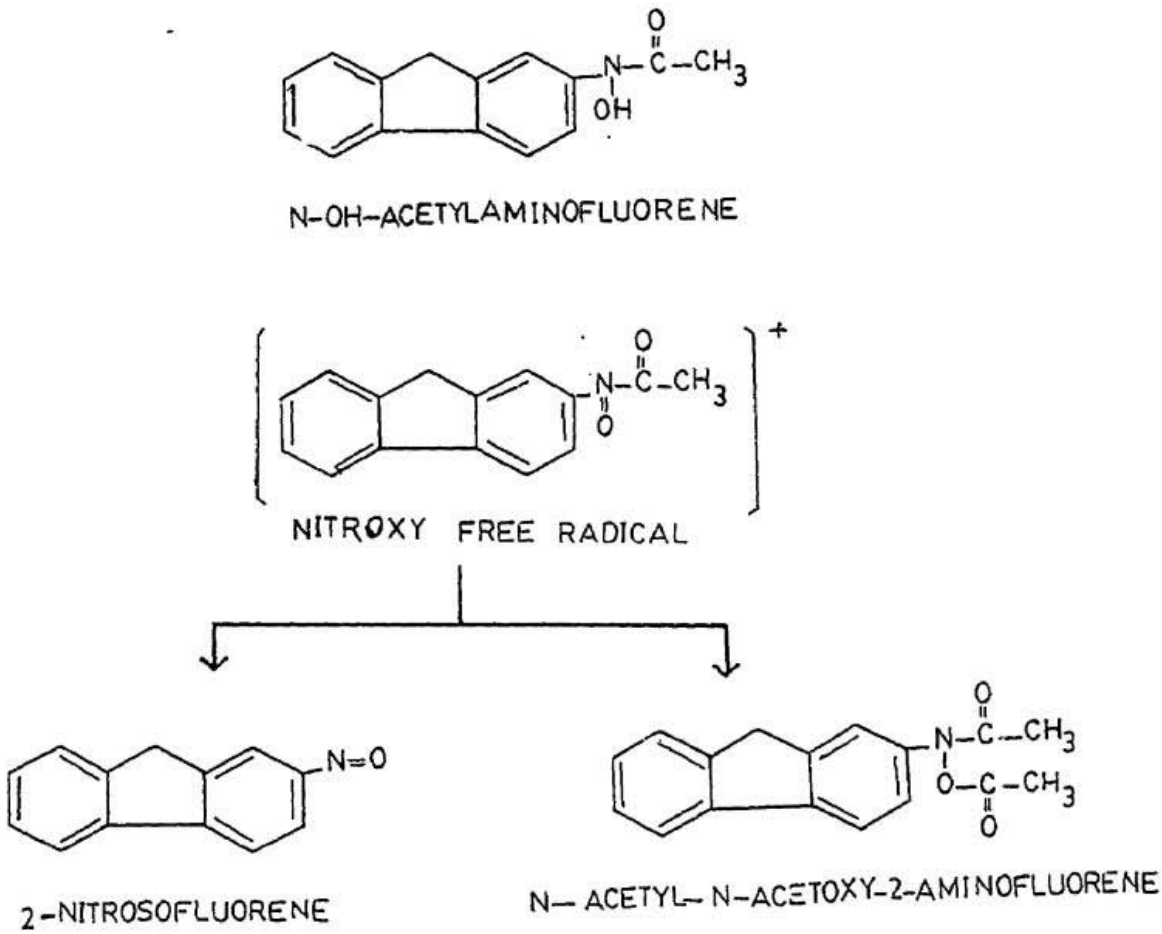


Figure 1: HRP/H_2O_2 catalyzed oxidation of N-OH-AAF.

free radical formation occurred when N-hydroxy-2-acetylaminofluorene (N-OH-AAF) was oxidized by rat mammary gland parenchymal cells. These observations explain the induction of tumours by 2-acetylaminofluorene (AAF) in rat mammary glands.

Mason *et al.*, (1982) have suggested that to implicate free radicals as chemical carcinogens, at least three conditions must be met;

(i) the existence of free radical metabolites must be demonstrated,

(ii) a comprehensive knowledge of free radical chemistry under physiological conditions is required,

(iii) the activation of chemical carcinogens into the free radical must take place at the location of the tumour.

The free radicals formed during the metabolism of azo dyes, quinone anticancer drugs and gentian violet have been detected by electron spin resonance (esr) spectrometry (Mason *et al.*, 1982) Chemical carcinogens including benzo(a)pyrene (Nagata *et al.*, 1974; Kimura *et al.*, 1980), aminoazo dyes, 2-acetylaminofluorene, naphthylamines and 4-hydroxy-aminoquinoline-1-oxide were found to generate free radicals on metabolism (Floyd *et al.*, 1982). The *in vitro* binding of these free radicals to DNA plays a role in carcinogenesis (Stier *et al.*, 1972). A correlation has been demonstrated between radical formation and the carcinogenicity of aromatic amines (Stier *et al.*, 1980).

5.Mechanism of Action of Horseradish Peroxidase:

The horse radish peroxidase/hydrogen peroxide system has often been assumed to be a model for all peroxidase/peroxide systems. Saunders *et al.* (1964) have proposed a cyclic enzymic mechanism (Fig.2). This scheme clearly indicates that HRP first reacts with hydrogen peroxide and is converted into Compound I. The substrate to be oxidized acts as an electron donor and reacts with Compound I. As a result Compound I is converted into Compound II and the substrate is oxidized. The Compound II reacts with a second molecule of the substrate (electron donor) and is converted back to the original peroxidase. In the presence of the excess peroxide Compound II is inactivated to Compound III and IV.(Fig.2)

Bartsch & Hecker (1971) proposed the same scheme. One enzymic cycle oxidizes two molecules of the substrate. The free radical generated as a result then undergoes disproportionation after forming a very unstable dimer.

6.Arylamines as Carcinogens:

The arylamines were recognized as carcinogens in the late nineteenth century, when the products and by-products of the aniline dye industry were observed to cause bladder cancer in the workers (Mason *et al.*, 1982). N,N-Dimethyl-4-aminoazobenzene (DAB) is known to induce liver and bladder cancer in rats and dogs respectively. Dogs have also been reported to develop pulmonary and hepatic tumours. However, it was not found to be carcinogenic in guinea pigs and hamsters (Magee *et al.*, 1982).

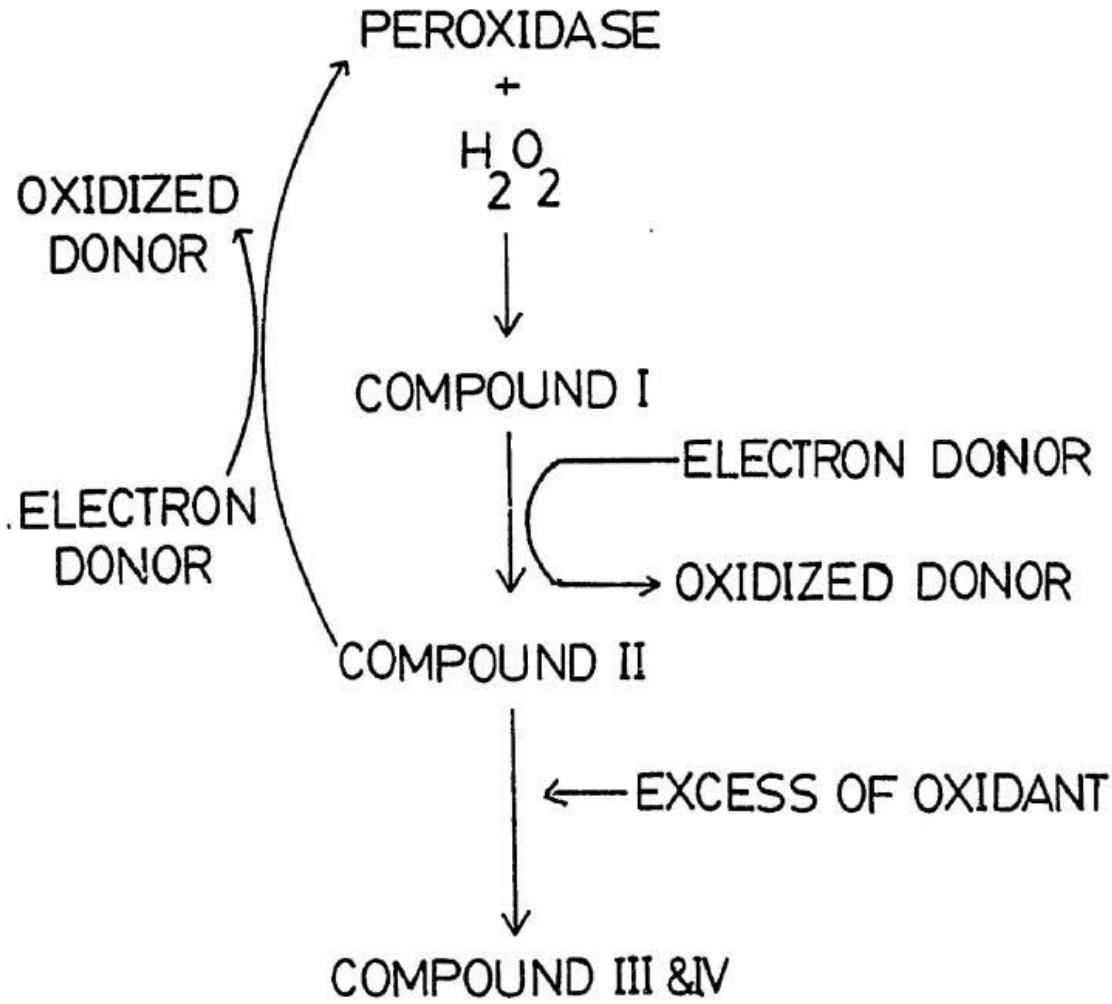


Figure 2: Mechanism of peroxidase catalyzed oxidations.
(after Saunders et al., 1964)

7.Mechanism of Cytochrome P-450 Catalyzed Activation of Arylamines:

Extensive studies of arylamines and in particular DAB with microsomal systems have led to the conclusion that three sequential steps are required for the metabolic activation of aminoazo dyes such as N,N-dimethyl-4-aminoazobenzene (DAB), N-methyl-4-aminoazobenzene (MAB), and methylated DAB. Microsomal cytochrome P-450 containing monoxygenase N-demethylates DAB to MAB (Guengerich, 1977). A microsomal flavoprotein, amine oxidase then N-hydroxylates this secondary amine. The N-hydroxylated aminoazobenzene is further acted upon by the cytosolic sulphotransferase and is converted into the sulphate conjugate (Kadlubar *et al.*, 1976). It is assumed that the decomposition of this sulphate conjugate gives rise to the nitrenium ion (Fig.3). Then the binding of the nitrenium ion to the cellular macromolecules leads to the initiation of neoplasia (Heidelberger, 1975; Kadlubar *et al.*, 1976; Vasdev *et al.*, 1981). A correlation has been demonstrated between the carcinogenic activity of DAB and the rate of N-hydroxylation of DAB and its derivatives (Kimura *et al.*, 1982). The increased hepatocarcinogenicity of 3'-methyl,N,N-dimethyl-4-aminoazobenzene in rats whose diet has been supplemented by sodium sulphate supports the role of sulphotransferase in the activation of aminoazo dyes (Blunck & Growther, 1975).

Poirier *et al.* (1967) synthesized N-benzoyloxy-MAB as a model ultimate carcinogen (the sulphuric acid ester of this dye is extremely unstable), and the non-enzymic *in vitro* binding of this compound to proteins, amino acids and nucleic acids was observed. Wislocki *et al.*, in 1975 also reported the non-enzymic binding of the synthetic esters to guanine, deoxyguanosine and amino acids. The

structure of the protein bound dye was found to be comparable to the protein bound dye from the liver of a rat treated with MAB. Moreover the dye caused sarcomas in rats at the injection site (Poirier *et al.*, 1967; Lin *et al.*, 1969; Lin *et al.*, 1975).

Kimura and coworkers (1982) discovered that inhibitors of cytochrome P-450 such as carbon monoxide, metyrapone and SKF 525A did not inhibit the N-hydroxylation of MAB in the rat liver microsomal system. However, the N-hydroxylation was found to be inhibited by α-naphthoflavone, a specific inhibitor of arylhydrocarbon hydroxylase activity in methylcholanthrene induced hepatic microsomes. Therefore, it was concluded that cytochrome P-448 and not cytochrome P-450 is involved in the N-hydroxylation of MAB. The mutagenic activation was reported to be inhibited with the antibody to cytochrome P-448, but the inefficient inhibition of the N-hydroxylation of MAB derivatives by carbon monoxide was not explained.

The interaction of the ultimate carcinogen with the macromolecules results in the initiation of neoplasia. It has been shown that DAB after activation with the microsomes caused an increased acceptance of the initiator tRNA for L-methionine and this effect was observed with other amino acids such as L-alanine, L-lysine and L-glycine with their corresponding tRNAs (Stivborova *et al.*, 1980).

[^{3}H]-3'-Methyl DAB is metabolized by rat liver microsomes and cytosol to at least two electrophiles. One of these electrophiles showed preferential binding to yeast RNA whilst another bound to the tissue proteins. NADPH/NADH and

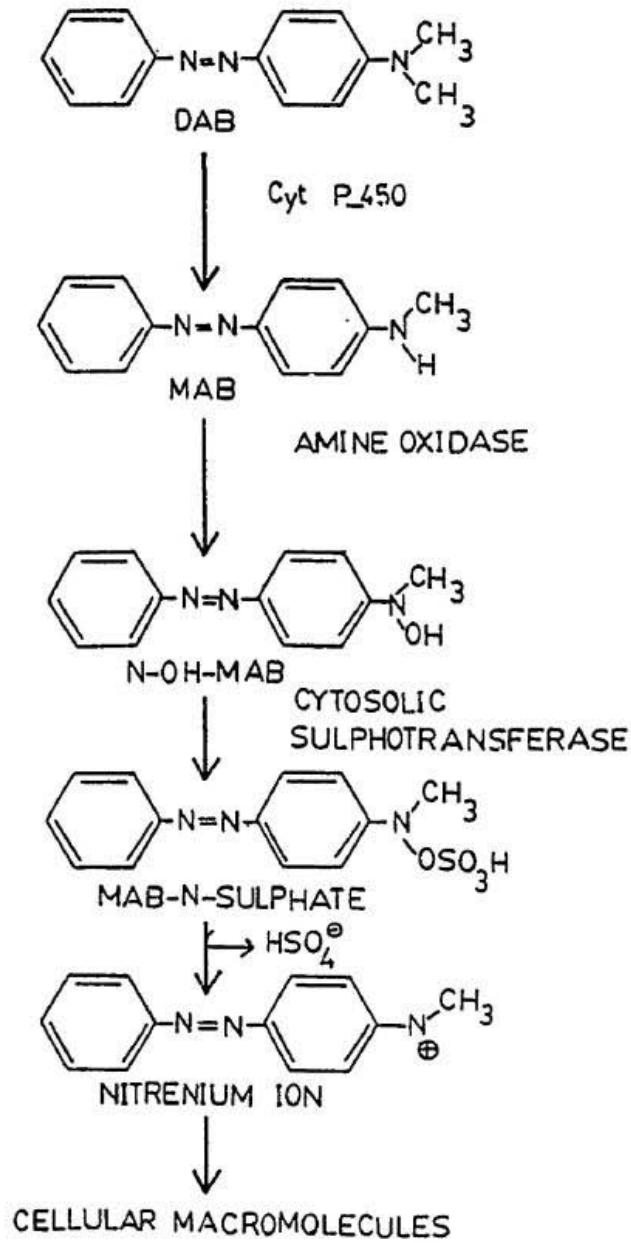


Figure 3: Activation of DAB catalyzed by monoxygenase system.

cytosolic 3-phosphoadenosine-5-phosphosulphate (PAPS) were required as cofactors for the RNA binding but not for the protein binding (Lebuc & Blunck, 1979). The rats were treated with [^{3}H] MAB and the major adduct isolated from the liver DNA was observed to co-chromatograph with N-(deoxyguanosine-8-yl)-MAB. This adduct contained about 45% of the total [^{3}H] bound to the DNA (Tarpley *et al.*, 1980). Beland (1980) showed that 3-(deoxyguanosine-N^2-yl) MAB was the second major adduct (10% of the total [^{3}H] bound to DNA) and 0.5-5% of the [^{3}H] was found in the other adducts (Tarpley *et al.*, 1980).

Poirier *et al.* (1967) used [^{14}C] labelled N-benzoyloxy-MAB to study binding of the activated MAB to DNA. Using thin layer chromatography, binding to deoxyadenosine was not detected but analysis by HPLC (High Pressure Liquid Chromatography), showed that 0.14% of the total bound radioactivity was due to a MAB derivative of deoxyadenosine. Under similar conditions 5% of the bound radioactivity was attached to deoxyguanosine (Tarpley *et al.*, 1980). These results were again consistent with *in vivo* hepatic DNA binding (Lin *et al.*, 1975).

Beland and coworkers (1980) showed that synthetic N-benzoyloxy-MAB reacted *in vitro* with DNA. Binding to an extent of 1 molecule/1000 nucleotide residues of DNA was found. After HPLC 6 adducts were detected and two of these were found to be the same as those isolated from liver DNA following MAB administration to rats. These adducts were identified by mass, ultra violet, and nuclear magnetic resonance spectrometry (NMR). N-(deoxyguanosine-8-yl)-MAB was not detected after the seventh day of treatment whereas 3-(deoxyguanosine-N^2-yl)-MAB was persistent at a constant level for 14 days.

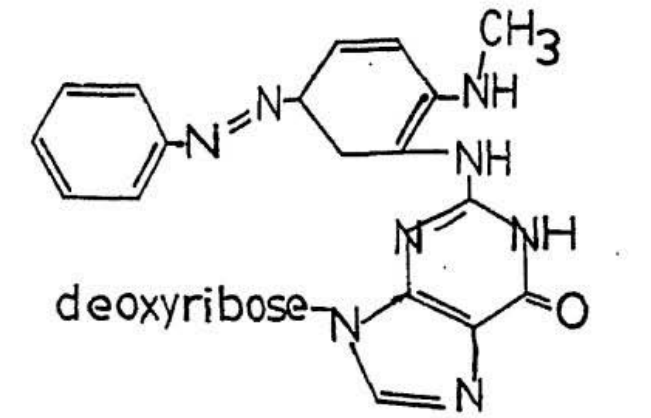
However the former adduct was found to be predominant in the beginning. This finding is in complete agreement with Warwick and Roberts' report (1967) that only 40% of the total bound radioactivity was found to remain permanently bound whereas 60% was lost within one week after treatment.

One of the adducts was shown to have a covalent bond through the amine nitrogen of MAB (Lin *et al.*, 1975). The pH partitioning experiments showed that substitution did not occur through the N^1 or O^6 position of the purine residue (Moore & Koreeda, 1976). NMR spectrometry indicated that the N-methyl moiety was a singlet and that there was an absence of the MAB amine proton. This suggested that the substitution occurred through the amine nitrogen of MAB. The absence of the C_8 proton of deoxyguanosine suggested that the adduct was N-(deoxyguanosine-8-yl)-MAB (Beland *et al.*, 1980). The structures of the known deoxyguanosine MAB adducts formed in hepatic DNA in rats and mice are given in Fig. 4.

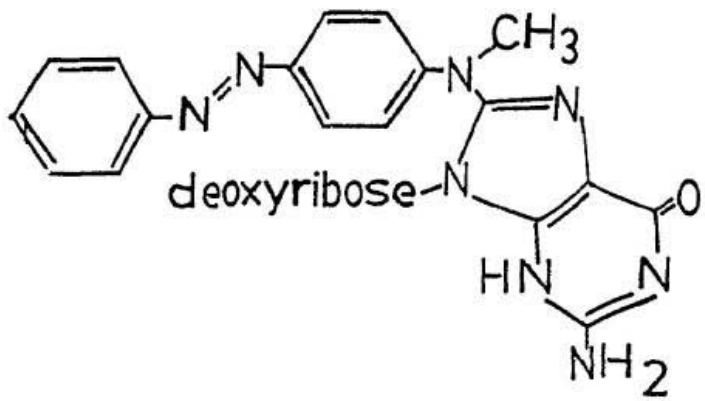
Thus, both metabolic and binding studies clearly indicate that the ultimate species binding to DNA is a nitrenium ion of MAB and its derivatives. However, despite extensive work, the mechanism of activation is still not well understood. Furthemore very little if any attention has been paid to the activation mechanism by the peroxidases.

8.Peroxidase Catalyzed Oxidation of Aromatic Amines:

Saunders *et al.*, (1964) reviewed the peroxidase catalyzed oxidation of different aromatic chemical compounds. It was suggested that the oxidation of aromatic amines involves the formation of free radicals either by the abstraction



3-(deoxyguanosine-N^2-yl)-MAB



(N-deoxyguanosine-o-yl)-MAB

Figure 4: The *in vivo* formed deoxyguanosine-MAB adducts.

of an electron and then of a proton, or by the direct removal of a hydrogen atom. The free radical formation is then followed by the pairing of these radicals in different possible ways. As an example the scheme for aniline oxidation is given in Fig. 5.

The oxidation of N,N-dimethyl-aniline at pH 4.5 by peroxidase and hydrogen peroxide led to the formation of N,N,N',N',tetramethyl-benzidine with the elimination of two H atoms. It has therefore been suggested that some of the free radical intermediates dimerize to form benzidine (Fig.6), while others are further oxidized. Other possible dimerization reactions could lead to a quinone diimine (Fig.7). Hey and coworkers (in Saunders *et al.*, 1964) have demonstrated that radical substitution could lead to a mixture of all isomers, the meta isomers being the least.

The following mechanism of N-dealkylation of N-alkylamines by HRP/H_2O_2 has been proposed (Saunders *et al.*, 1964);

$$R\text{-}NH.CH_3 + H_2O_2 \text{ ----------> } R.NH.CH_2OH + H_2O.$$

$$R.NH.CH_2OH \text{ ----------> } R.NH_2 + HCHO.$$

Griffin and Ting (1978) proposed the following mechanism for the N-demethylation of aminopyrines by the peroxidase system;

$$R_2N\text{-}CH_3 \text{ ----------> } R_2\dot{N}^+\text{-}CH_3$$

$$R_2N^+\text{-}\overset{\curvearrowleft}{CH_2}\text{-}H \text{ ----------> } R_2N^+{=}CH_2 + H^{\cdot}$$

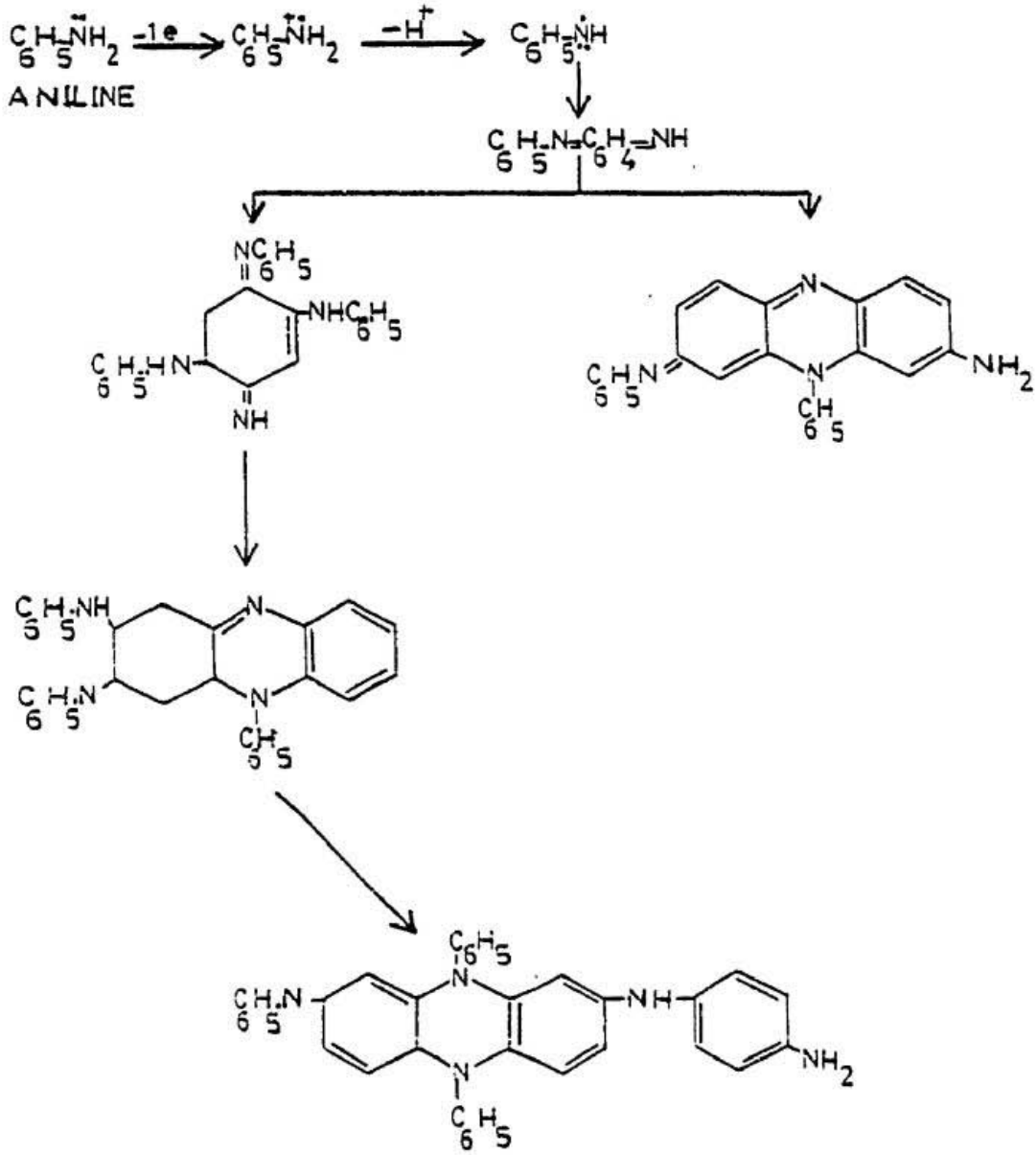


Figure 5: Scheme of aniline oxidation catalyzed by horse radish peroxidase/hydrogen peroxide.

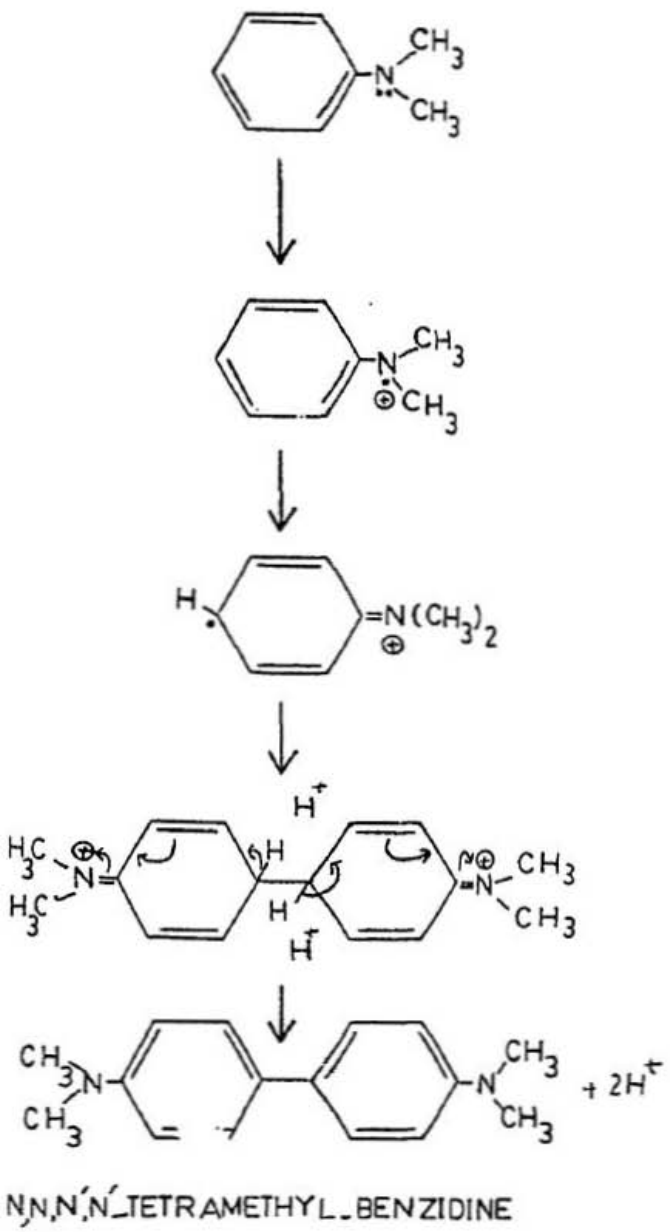


Figure 6: Oxidation of N,N-dimethyl-aniline Catalyzed by HRP/H_2O_2

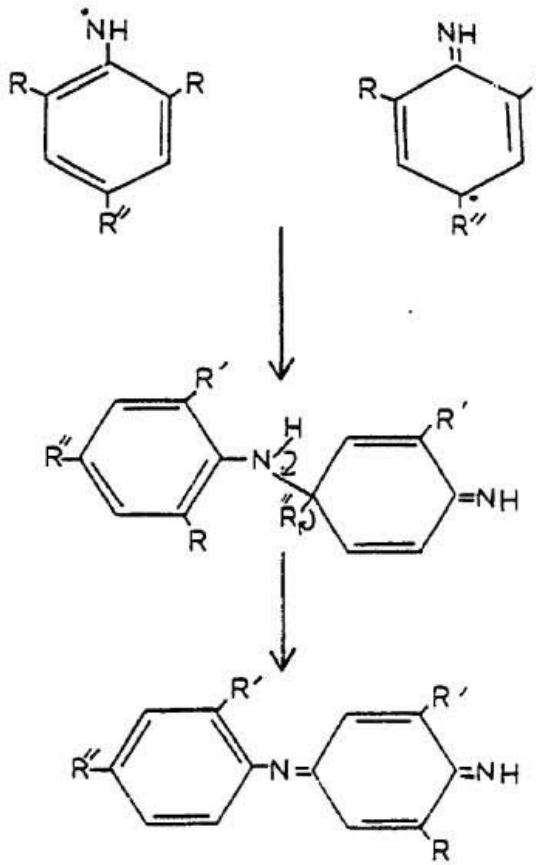


Figure 7: Dimerization of the Free Radicals to Quinone diimine.

Nordblom *et al.*, (1976) suggested that the peroxidase/ peroxide system also catalyzes the hydroxylation of the substrate and compared the stoichiometry of the reaction with that of cytochrome P-450;

(i) $RH + O_2 + NADPH + H^+ \longrightarrow ROH + H_2O + NADP^+$.

(cytochrome P-450 system)

(ii) $RH + XOOH \longrightarrow ROH + XOH$. (peroxidase system)

RH here represents the substrate and it was suggested by Nordblom *et al.* that an N-methyl substrate is N-hydroxylated first and then spontaneously converted into demethylated amine with the liberation of formaldehyde.

9.Protection From Carcinogenic Metabolites:

The requirement of metabolic oxidation and macromolecular binding for the chemical carcinogen to exhibit its carcinogenic activity, makes it obvious that the factors inhibiting the activation and/or binding will also reduce the carcinogenicity of the chemical. It has also been reported that chemical carcinogens bind to a wide variety of physiological nucleophilic compounds (Heidelberger, 1975; Miller & Miller, 1977). Thus, these nucleophilic compounds may also serve as protective agents.

Wattenberg (1978), has indicated that many antioxidants can reduce the induction of cancer by interacting with the chemical carcinogens. Kliehus *et al.* (1975) demonstrated that the pretreatment of rats with the radioprotective agent cystamine inhibited hepatic protein synthesis induced by a nitrosamine and also inhibited the methylation of DNA. It has been proposed that cystamine either reacts with the carbonium ion, the presumed active product of the nitrosamine, or it might inhibit the metabolism of dimethyl nitrosamine. Kliehus *et al.* (1975) strongly suggested that the protective effect was due to metabolic inhibition. Ascorbic acid has also been reported to act as a scavenger as the alkylating intermediates of nitrosamines were found to alkylate ascorbic acid in mammalian cells (Edgar, 1974). Ascorbate concentration of 5 mM to 20 mM showed a 36% inhibition of DNA methylation.

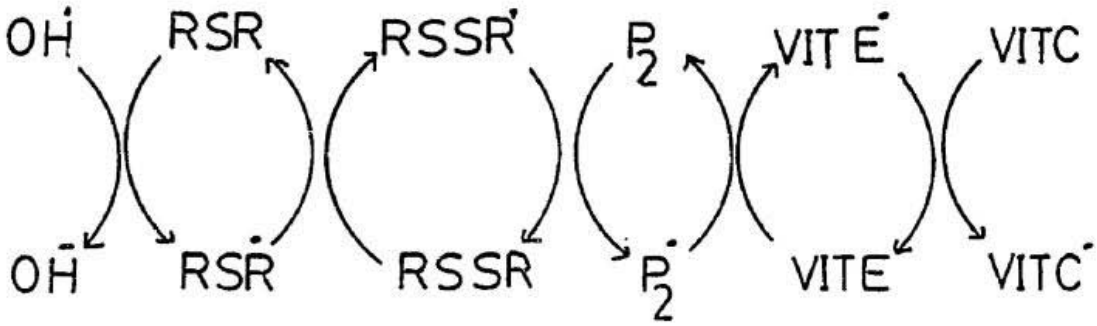
The benzo(a)pyrene induced mutagenicity in *Salmonella typhimurium* strain TA 98 decreased to 54% in the presence of cysteine (Wattenberg, 1981). The amino acids tryptophan and methionine have been shown to have a protective role against electrophilic radicals (Wilson, 1982).

It has been shown that phenols prevented the binding of benzidine to DNA by forming adducts with the oxidized active benzidine molecules.(Josephy *et.al.*,1982) The formation of the coloured adducts of benzidine with butylated hydroxyanisole (BHA), guaicol, epinephrine and serotonin in a peroxidase/peroxide system has also been reported. (Josephy *et al.*, 1982). The inhibition of peroxidase catalyzed *in vitro* binding of benzidine to DNA in the presence of phenolic and biological donors has been reported by Tsuruta *et al.* (1982).

It has also been proposed that appropriate free radical scavengers can be effectively used to control the radical reactions in biological systems. For example a radical can be repaired by accepting an electron from a reducing agent,

$$T^{\cdot} + RSH \text{ ----------} > TH + RS^{\cdot}.$$

The electron donating scavenger is oxidized as a result. The sequential electron transfer reactions of radicals have also been shown.



Thus the net reaction can be written as:

$$OH^{\cdot} + \text{Vit C} \text{ ----------} > \text{Vit C}^{\cdot} + OH^{-}$$

In discussing these processes, Wilson (1982) has suggested that such a protective cocktail can also overcome steric and solubility barriers.

Clearly, the above review shows that DAB and its derivatives are potent carcinogens and are oxidized by the cellular enzymes to reactive molecules. The binding of these activated molecules to DNA then initiates the carcinogenicity. Cytochrome P-450 containing monoxygenase system is well known for carrying out the two electron oxidation of the carcinogens. However, other activating systems are also equally important. The role of ionizing radiations in free radical mediated carcinogenic processes directed the attention of many workers towards free radical intermediates. The detection of the free radicals in the metabolic pathways of many chemical carcinogens was subsequently recognized. Furthermore, peroxidases in the presence of peroxides have been discovered to catalyze the one electron oxidations of their substrate to free radicals. The

occurrence of peroxidases in many target tissues, particularly those not known to contain the monoxygenase system, or sulphotransferases *e.g*; the Zymbal gland of the sebaceous ear duct , mammary gland, and bone marrow indicates the importance of peroxidases in carcinogenesis. Furthermore, peroxidases in the presence of peroxides have been discovered to catalyze the one electron oxidations of their substrates to free radicals. Although the ultimate carcinogen of DAB and its derivatives has been found to be a free radical of MAB, the activation of the aminoazo dyes by the peroxidase system has not been explored.

Therefore, the *in vitro* metabolism of dimethyl aminoazobenzene (DAB), monomethyl-aminoazobenzene (MAB) and aminoazobenzene (AB) was studied. The major products obtained have been identified. The binding of the activated intermediates to calf thymus DNA has also been measured quantitatively. Furthermore, the effect of various phenolic and biological reducing agents on the metabolic profile, total oxidation of MAB and binding of the oxidation products of MAB to DNA has been investigated. It is known that the antioxidants exhibit their inhibitory effect by inhibiting the oxidation of the potential carcinogens. Moreover antioxidants are also considered to be the free radical scavengers. A study of their effect on the peroxidase catalyzed oxidation of a chemical and the detection of concomitantly bound radioactivity to DNA could certainly provide some valuable explanation about the carcinogenic mechanism of that chemical. For example, the absence of a metabolite from the metabolic profile in the presence of an antioxidant, especially when it is associated with the reduction or inhibition in DNA binding can help in determining the nature of the activated

metabolite and then further work could be carried out for the identification of that particular product.

MATERIALS AND METHODS:

Materials:

The following chemicals were obtained from Sigma Chemical Co; Horse-Radish Peroxidase (HRP type IV), calf thymus DNA (grade VII), polyriboguanylic acid, polyriboadenylic acid, polyribocytidylic acid, polyribouridylic acid, uric acid (2,6,8 trioxypurine), xanthine (2,6, dihydroxypurine), guanine, adenine, L-cysteine, N-acetyl-L-cysteine, nicotinamide adenine dinucleotide phosphate reduced form (NADPH), glutathione (GSH), Butylated hydroxyanisole, xylenol (2,6 dimethyl phenol). Hydrogen peroxide, ACS grade, ethyl acetate, ethanol, benzene, methanol (HPLC grade), ether, chloroform, were provided by Fisher Chemical Co. Sodium chloride, tris, sodium acetate, hydrochloric acid, acetic acid and TLC plate coated with silica gel were also purchased from Fisher Chemical Co. L-ascorbic acid (grade Analar) was purchased from BDH and 1-naphthol from Eastman Organic Chemicals. ACS (aqueous counting solution) was obtained from Amersham Chemical CO.

METHODS:

1.Synthesis of [^{14}C] MAB:

[^{14}C]-MAB was synthesized by Tsuruta in our laboratory following the method of Maurier and Chauvier (1970) with modifications. [^{14}C-Aniline (mol.wt 194 and specific radioactivity 102mCi/mmole) was diluted with cold aniline, *i.e*; 0.475 mg of [^{14}C]-aniline hydrogen sulphate was mixed with 47 mg of cold aniline. 256 μl of 6N HCl (hydrochloric acid) were added to the above mixture. 0.5 g of ice was mixed thoroughly. To this an aqueous solution of sodium nitrite (0.35 g in 80 μl water) was added dropwise with shaking. After five minutes, a solution of N-

methylaniline hydrochloride (0.073 g in 0.15 ml) was added with shaking. An aqueous solution of sodium acetate (0.136 g in 160 μl of water) was added after 15 minutes and allowed to stand for 30 minutes. Ethanol (2.0 ml) was added followed glacial acetic acid until a clear solution was obtained.

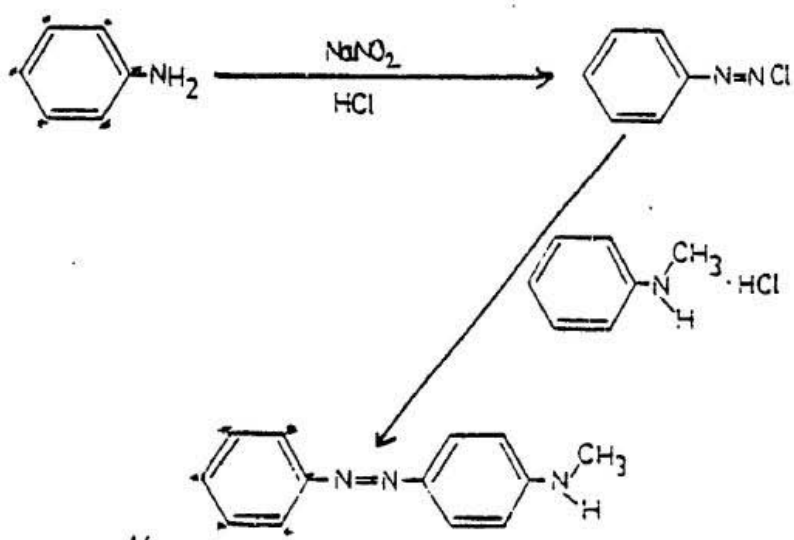


Fig. 3: [^{14}C-N METHYLAMINOAZOBENZENE

[^{14}C]-MAB was precipitated with the addition of 40 ml of H_2O, filtered, washed with 10% acetic acid solution and dried (yield.ca.50 mg). The yellow needles of [^{14}C-MAB were dissolved in heptane and recrystallyzed using charcoal powder (yield 27.5 mg). The melting point was 87°C and the MAB had a specific radioactivity of 0.75 μCi/μmole. A single spot (R_F=0.75) was obtained on silica gel thin layer chromatography plate which was developed in toluene:methanol (14:1 by volume). The radioactivity on the entire chromatogram was measured and 98% of the total radioactivity was detected in the [^{14}C] MAB spot.

2.Metabolism of Aminoazobenzenes:

The activation of arylamine azo dyes (MAB, DAB and AB) catalyzed by HRP/H_2O_2 was carried out in a reaction mixture (total volume 2.0 ml) The complete reaction mixture contained 0.05 M Tris-HCl buffer, pH 7.4 or 0.1 M sodium acetate-acetic acid buffer, pH 5.0, MAB, DAB or AB (50 μM), and H_2O_2 (100 μM). The reaction was initiated by adding 1 μg of HRP and incubating at 37°C in a shaking water bath. After a total incubation of 30 minutes, the reaction was stopped with 3.0 ml of ethyl acetate : acetone (2:1 by volume), shaken well and centrifuged for 2 minutes. The yellow colour was extracted completely into organic layer and comprised 95-96% of the total radioactivity (Results) originally added to the reaction mixture. The remaining aqueous layer was extracted two more times with the same solvent. The pooled organic extract was then evaporated and redissolved in 200 μl of methanol for HPLC or TLC analysis.

3.HPLC:

A Water Associates' High Pressure Liquid Chromatograph with a μBondapak C_{18}, reverse phase column was used to separate and identify the organic soluble metabolites. A volume of 10 μl or 20 μl of the above mentioned sample was injected into the column with the automatic injecting system of the instrument. A linear gradient of water:methanol (35:65 by volume) to 100% methanol was employed for 30 minutes with a flow rate of 1 ml/minute. The metabolites were detected from the absorbance peaks measured at 405 nm and were identified by comparing their retention times with the retention times of the authentic standards separated under similar conditions. The quantity of unmetabolized aminoazobenzene was measured by comparing the peak height

with that of the known amount of the standard product chromatographed under similar conditions. The total amount of the oxidized dye was determined by subtracting the amount of unoxidized dye from the total amount originally added to the reaction mixture.

4.TLC:

The organic soluble metabolites obtained from the pH 7.4 reaction mixtures were dissolved in methanol after evaporating the ethyl acetate:acetone (2:1) layer. The products from the pH 5.0 reaction mixtures were dissolved in methanol:benzene (1:1) since they were not soluble in either of the solvent separately. The total volume of the organic soluble metabolites i.e; 30-50 μl was applied to the 20 x 20 cm TLC plate coated with 60 silica gel. The plate was then developed in benzene:ethyl acetate (9:1 by volume) and after drying the coloured products were recorded.

5.Mass Spectral Analysis:

The metabolic products were separated by silica gel thin layer chromatography and extracted into ethanol. The solvent was evaporated under the N_2 stream and the dried residue was assayed by mass spectrometry. Analyses were carried out on a VG 7070HS double focusing mass spectrometer with a 2035 data system. The samples were introduced using a direct insertion probe which was heated if necessary to obtain a spectrum. The temperature of the ionization chamber was 200°C and ions were generated by electron impact using 70ev electrons. The calibration file was created from perfluorokerosene MS data which was then used to mass calibrate from subsequent samples. Whenever possible a series of consecutive scans was averaged using the data system. Fragments of $<$ 2% intensity were ignored (Gregory,B. personal communications).

6.Binding of the activated MAB metabolites to DNA:

The reaction mixtures (final volume 2.0 ml) contained 3 mg of calf thymus DNA in addition to the other constituents mentioned above in "Metabolism". DNA was added before starting the reaction. (i) The incubations were carried out in a shaking water bath for a period of 30 minutes. (ii) The organic soluble unbound MAB and its metabolites were extracted three times with 3.0 ml of ethyl acetate:acetone (2:1 by volume). (iii) The residual ethyl acetate:acetone was evaporated from the aqueous phase under N_2 stream. (iv) Sodium dodecyl sulphate (SDS) was added to a final concentration of 1% and 0.5 mg of protease (*E.coli*) were added to the aqueous phase and incubated at 37°C for 30-60 minutes (until the solution was completly clear indicating the digestion of protein). (v) The dissolved protein was extracted with one volume of water saturated phenol:chloroform (1:1 by volume). (vi) Sodium chloride was added to the aqueous phase to a final concentration of 0.5 M and subsequently DNA was precipitated with 2 volumes of ice cold ethanol. In order to obtain a better recovery, it was stored at -20°C for two hours and was centrifuged for 10 minutes. (vii) The supernatant was discarded, the precipitates were redissolved in water and reprecipitated as before. (viii) The precipitates were washed twice with ethanol and finally with ether. (ix) The residual ether was evaporated under N_2 stream. (x) The DNA precipitates were dissolved in 1 ml of distilled water. (xi) The absorption spectra (from 700 nm to 330 nm) of the DNA bound dye were recorded by a Shimadzu recording spectrophotometer, model UV 240.

[^{14}C]-MAB was used for the quantitative determination of the binding of its

metabolites to DNA. The procedure was exactly the same as described above except after step (xi). An aliquot of 0.5 ml was taken from step(x), mixed with 10 ml of the liquid scintillation counting solution (ACS) and the radioactivity was counted by Liquid Scintillation Counting. An aliquot of 30 μl (taken from step (x)) was diluted with of water (final volume 3.0 ml) and the absorbance was recorded at 260 nm so as to measure the recovery of DNA.

7.Time Course of the Binding of the Activated Metabolites of [^{14}C]-MAB to DNA:

Again the same procedure (described above) was followed except that the DNA was added to the reaction mixture either before or after a measured interval of starting the reaction. The incubations were carried out for a total of 30 minutes period after adding the DNA.

8.Measurement of Absorbance Spectra:

The absorption spectra were recorded (from 700nm to 330nm) by a Shimadzu recording spectrophotometer, model UV-240. The reaction mixtures consisting of partial and complete system were incubated at 37°C for 30 minutes before recording the spectra. The isolated DNA from the complete and partial reaction mixtures was dissolved in water and the spectra were recorded. The major metabolic products of aminoazobenzenes were scraped from the TLC plates, extracted in ethanol and the spectra were recorded.

9.Effect of the Reducing Agents:

The composition of the complete reaction mixture was the same as mentioned above, the known quantity of the reducing agent was added to the reaction mixture before starting the reaction unless otherwise mentioned. After a

total incubation of 30 minutes at 37°C, the reaction mixture was extracted with 3 X 3 ml of ethyl acetate : acetone (2:1 by volume). The pooled organic extract was evaporated under a stream of N_2 and the contents were dissolved in 200 μl of methanol. An aliquot of 10 μl was injected into the HPLC column to determine the amount of unoxidized MAB as described above. The rest of the extract was applied to the silica gel TLC plate and the radioactivity was measured in the unoxidized [^{14}C] MAB spot.

DNA was precipitated from the aqueous phase of the reaction mixture as before and the radioactivity due to the bound metabolites of [^{14}C] MAB was measured.

10.Measurement of the Oxidation of the Reducing Agents by the MAB/HRP/H_2O_2 Reaction Mixture:

Ascorbic acid: The reaction mixture contained 0.05 M Tris-HCl buffer pH 7.4, 100 μM H_2O_2, 100 μM ascorbic acid and the absorbance was recorded at 265 nm for 1 minute. 25 μM MAB was added and the change in absorbance at 265 nm was recorded for 3 minutes. The control consisted of the buffer, 100 μM ascorbic acid and 25 μM MAB.

NADPH:-The reaction mixture consisted of 0.05 M Tris-HCl buffer pH 7.4, 10 μM H_2O_2, 100 μM NADPH. The absorbance at 340 nm was recorded for one minute, 5 μM of MAB was added and the change in absorbance was recorded for 3 minutes. The control consisted of 5 μM MAB and 100 μM NADPH in the buffer.

RESULTS

A.The Oxidation of Aminoazobenzenes:

DAB and MAB were oxidized by H_2O_2 in a reaction catalyzed by horseradish peroxidase. The absorption spectra of the reaction mixtures were measured, and the reaction products were extracted and examined by High Presuure liquid Chromatography (HPLC), Thin Layer Chromatography (TLC) and mass spectrometry.

1.Absorption Spectra:

The absorption spectra of partial and complete reaction mixtures of MAB and DAB were measured and are shown in Fig.9 and Fig.10. MAB and DAB absorbed maximally at 400 and 440 nm respectively at pH 7.4, and after oxidation both reaction mixtures absorbed at 390 nm.

2.High Presure Liquid Chromatography:

Metabolites were extracted from reaction mixtures with ethyl acetate : acetone (2:1). The aqueous phase remaining after the extraction of a reaction mixture originally containing MAB was colourless, and in experiments with [^{14}C] MAB the aqueous phase retained only between 4 and 5% of the total radioactivity (Table 7 and Table 8).

Extracts from reaction mixtures containing MAB (Fig.11a) and DAB (Fig.11b) contained absorbing material with a retention time of the starting compound, but both extracts also contained a major product with the same retention time. The minor products were present.

The extract from the MAB reaction contained a compound with the same

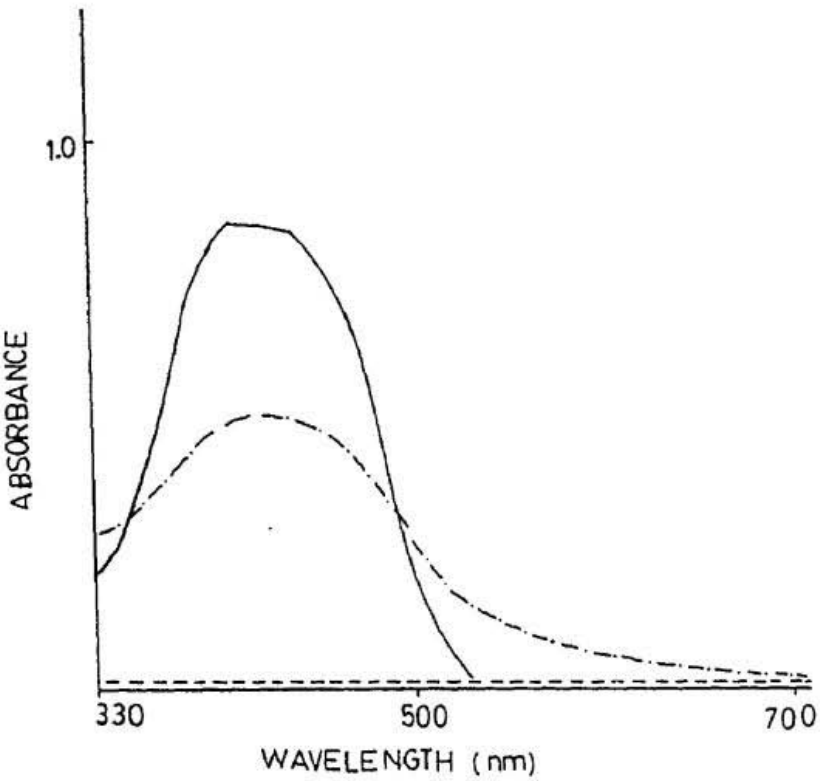


Fig.9:

Figure 9: Absorption spectrum of MAB and the products formed by HRP/H_2O_2 catalyzed oxidation at pH 7.4:

The complete reaction mixture (total volume 2.0 ml) consisted of 0.05 M Tris-HCl buffer (pH 7.4), 50 μM MAB, 100 μM H_2O_2, 1 μg HRP and the absorption spectrum was measured after 30 minutes. The absorption spectrum did not change after this time.

---- HRP + H_2O_2 in buffer.

—— MAB in buffer.

—·—·— MAB + HRP + H_2O_2.

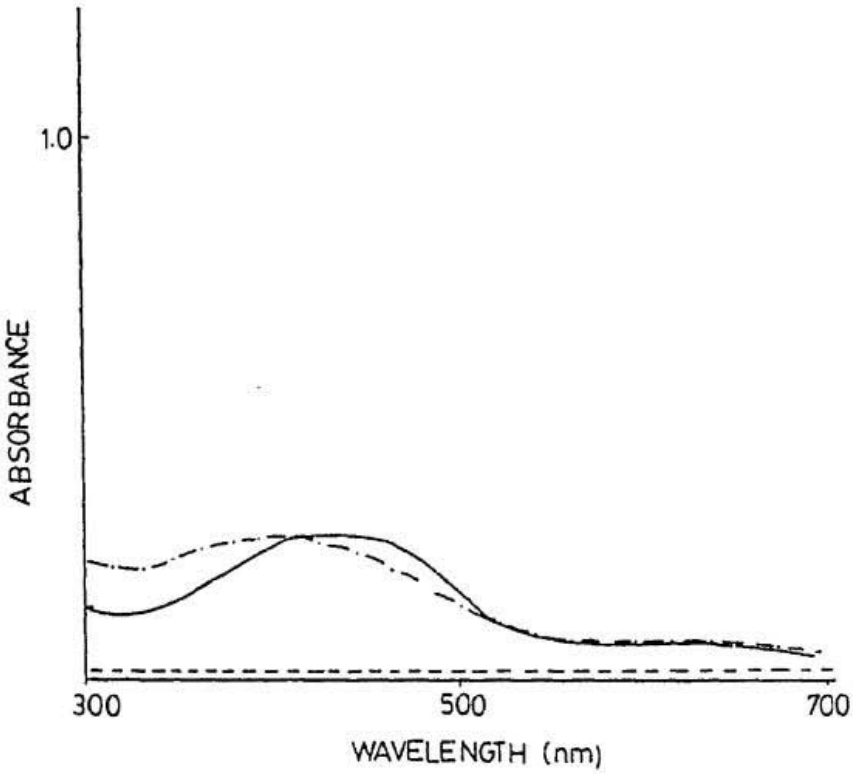


Fig.10:

Figure10: The absorption spectrum of DAB and products formed by HRP/H_2O_2 catalyzed oxidation at pH 7.4:

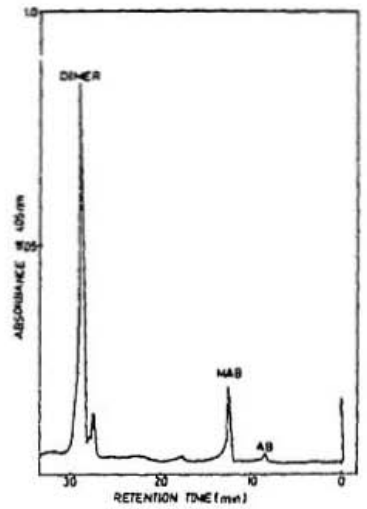
The complete reaction mixture (2.0 ml) consisted of 0.05 M Tris-HCl buffer (pH 7.4), 50 μM DAB, 100 μM H_2O_2, 1 μg HRP and the absorption spectrum was measured after 30 minutes. The absorption spectrum did not change after this time.

--- HRP + H_2O_2 in buffer.

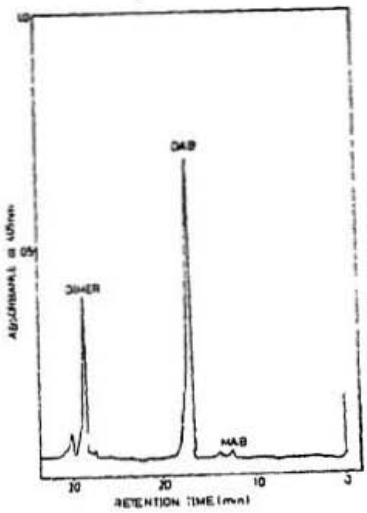
—— DAB in buffer.

—·— DAB + HRP + H_2O_2.

a


DIMER
MAB
AB
ABSORBANCE at 405nm
RETENTION TIME (mn)

b


DAB
DIMER
MAB
RETENTION TIME (mn)

c

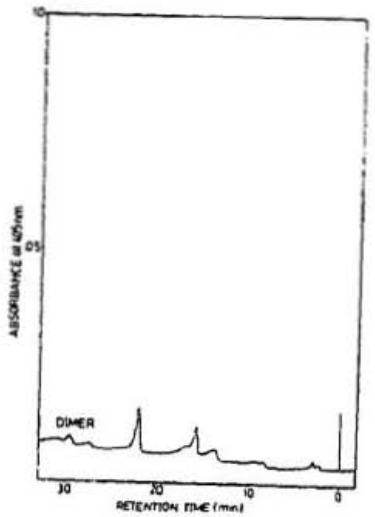

DIMER
ABSORBANCE at 405nm
RETENTION TIME (mn)

Fig.11 :

Figure11: Aminoazobenzenes and their oxidation products separated by HPLC: (a; MAB. b;DAB. c;AB.

The reaction mixtures (2.0 ml) contained 0.05 M Tris-HCl buffer (pH 7.4), 50 μM DAB, 100 μM H_2O_2 and

1 μg of HRP. After incubating at 37°C for 30 minutes, the reactions were stopped and extracted with ethyl acetate : acetone. The ethyl acetate : acetone extracts were evaporated and redissolved in 200 μl of methanol and a volume of 20 μl was injected into the HPLC system. The column used was a C_{18} μBondapak Reverse Phase. The gradient was allowed to change linearly from 65% methanol in water (by volume) to 100% methanol over a period of 30 minutes. The flow rate was 1 ml/minute and the products were detected by absorbance at 405 nm.

retention time as aminoazobenzene (AB) (Fig.11a), and that from the DAB reaction contained one with the retention time of MAB (Fig.11b) but AB was not detected in this profile. However, a small peak at 30 minutes retention time was observed which was common to that of AB profile. The ethyl acetate : acetone extractable metabolites formed from the oxidation of AB by H_2O_2 catalyzed by peroxidase are shown in Fig.11c.

3.Time Course of the Oxidation of MAB:

Table 2 shows that 89% of MAB was oxidized in 10 seconds and the reaction was complete in less than 1 minute. The amount of the major product formed at 10 seconds was the same as formed at 1 minute *i.e*; about 80%. Therefore it can be assumed that this product is stable towards further oxidation and is formed from some unstable intermediate and/or intermediates.

Table 3 illustrates the comparison of the total oxidation of MAB, DAB and AB catalyzed by HRP/H_2O_2 under similar conditions. It has been observed that AB was oxidized completely. The oxidation of MAB was also close to completion, *i.e;* 97%, but DAB was oxidized the least, *i.e;* 15%.

4.Thin Layer Chromatography;

Four products were detected in extracts of the reaction mixture containing DAB. One had an R_F identical to that of authentic DAB, and one identical to that MAB. There were numerous products in extracts of reaction mixtures containing MAB and AB.

These included compounds with an R_F identical with that of the starting compound. The reaction products of MAB and DAB also contained a common

Table 1: The peak heights of the oxidation products of MAB, DAB and AB separated by HPLC.

	Peak Heights								
	Retention Time (min)								
	8	12	14	16	17	22	27	28	29
	AB	MAB		DAB					
MAB		258							
MAB + HRP + H_2O_2	2.0	6.0			1.0			108	
DAB				154					
DAB + HRP + H_2O_2		4.0	3.0	77	3.0		3.0	47	6.0
AB	70								
AB + HRP + H_2O_2	30		3.0			9.0	2.0		14

The ethyl acetate : acetone extracts of the reaction mixtures were evaporated, redissolved in methanol and injected into the HPLC system. The gradient was applied linearly from 65% methanol/water to 100% methanol for 30 minutes. The flow rate was 1ml/minute and the products were monitored by absorbance at 405 nm. The column was a C_{18} μBondapak Reverse Phase.

Table 2: Measurement of MAB oxidation by HPLC: Time Curve.

	Peak Heights		
	Unoxidized MAB	Dimer	Oxidized MAB
No HRP	225 mm = 100%	0	0
10 sec.	24.5 = 10.9%	37.0 mm	89.5%
30 sec.	19.0 = 8.5	38	91.5
1.0 min	6.0 = 2.7	42.5	97.2
2.0 min	6.0 = 2.7	38.0	97.2

The ethyl acetate : acetone extracts of the reaction mixtures were evaporated, redissolved in 200 μl of methanol and a volume of 5 μl was injected into the HPLC system. The gradient was applied linearly from 65% methanol/water to 100% methanol for 30 minutes. The flow rate was 1 ml/minute and the products were monitored by the absorbance at 405 nm.

Table 3: Total oxidation of MAB, DAB and AB catalyzed by HRP/H_2O_2.

	Total	Unoxidized Substrate	Oxidized Substrate	% Oxidized Substrate
MAB	100 nmole	3.0 nmole	97 nmole	97%
DAB	100 nmole	85 nmole	15 nmole	15%
AB	100 nmole	o.2 nmole	99.8 nmole	99.8%

The reactions were carried out in a total volume of 2.0 ml containing 0.05 M Tris-HCl buffer pH 7.4, 50 μM MAB, 100 μM H_2O_2, 1 μg of HRP. After 30 minutes of incubation at 37°C, the mixtures were extracted with ethyl acetate : acetone. The extracts were evaporated, redissolved in 200 μl of methanol and a volume of 5 μl was injected into the HPLC system (Materials and Methods). The peak heights of unmetabolized substrates were compared with the peak heights of the known quantities of the standard compounds. The quantity of the substrate oxidized was calculated by substracting the unoxidized amount from the total amount taken originally.

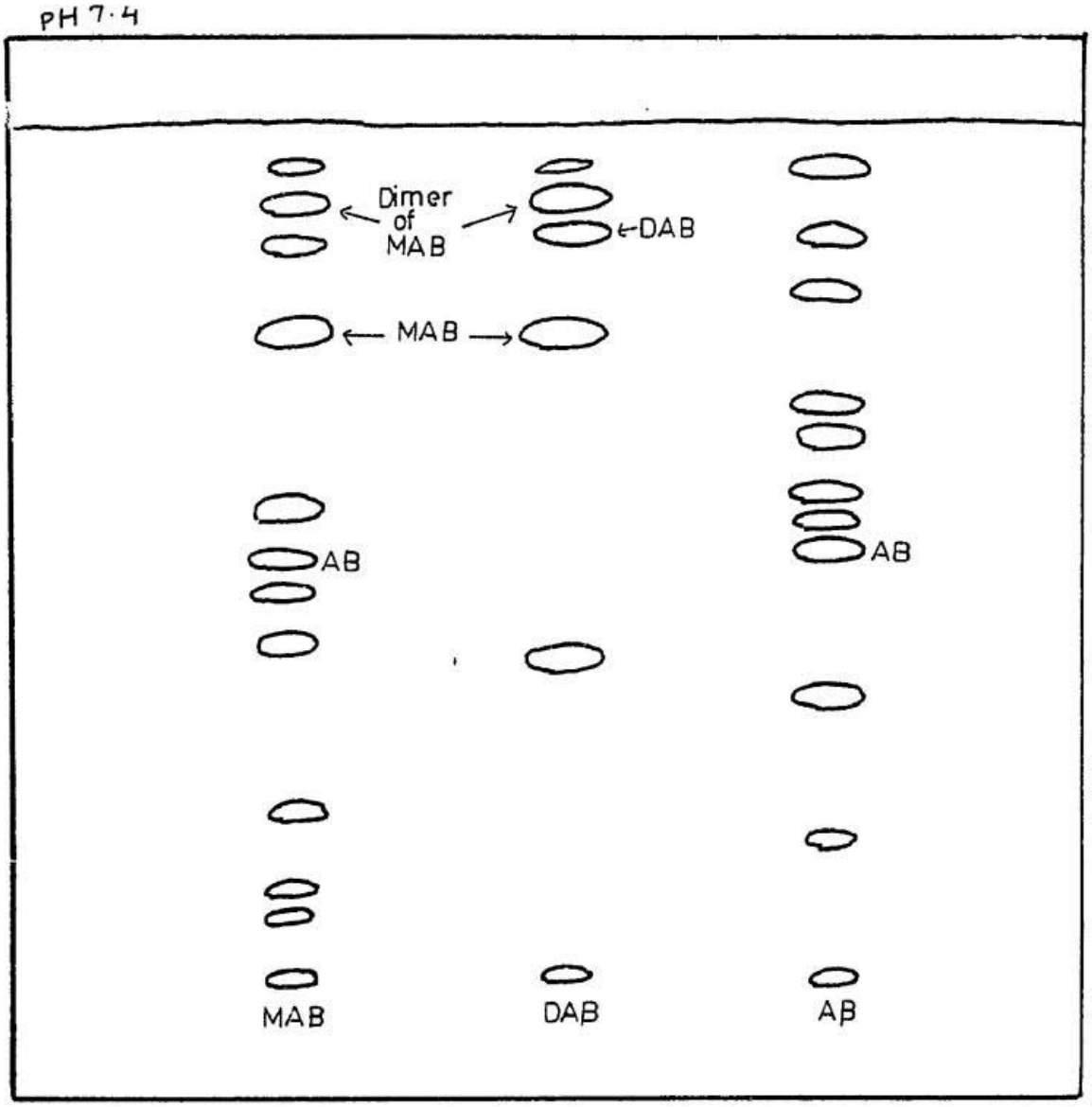


Fig.12:

Figure12: Thin layer chromatogram of the oxidation products of DAB, MAB and AB (catalyzed by HRP/H_2O_2 at pH 7.4):

The reaction mixtures (2.0 ml) contained 0.05 M Tris-HCl buffer pH 7.4, 50 μM MAB, 100 μM H_2O_2 and

1 μg of HRP. After 30 minutes of incubation at 37°C. the reaction mixtures were extracted with ethyl acetate: acetone, the solvent was evaporated and the contents were dissolved in a small volume of methanol and applied to the silica gel plates. The chromatograms were developed in benzene : ethyl acetate (9:1).

metabolite. This was a dominant spot on the chromatograms with R_F 0.87. However, in the case of AB all the bands had almost the same intensity of colour and no band was common to any band in MAB or DAB profile. This supports the HPLC results and suggests that MAB and DAB follow the same metabolic route which is different from that of AB.

5.Oxidation of MAB at pH 5.0:

The preliminary studies carried out in our laboratory showed that the products formed at pH 5.0 bind twice as effectively to DNA as the products formed at pH 7.4. Much of the published work on HRP has been done at a slightly acidic pH rather than at pH 7.4. The oxidation pathways of arylamino compounds have been determined primarily at pH 4.5 (Saunders *et al*;1964).

The absorption spectra of partial and complete reaction mixtures at pH 5.0 are shown in Fig.13. Although the absorption spectrum of MAB is similar to that at pH 7.4, the absorption spectrum of the complete reaction mixture is different.

After extraction of a reaction mixture with ethyl acetate : acetone, and evaporation of the solvents, the residue (unlike that from reactions at pH 7.4) was not completely soluble in methanol. Products were not, therefore separated and measured by HPLC. Instead they were examined by TLC (Fig.14). The predominant product had the same R_F as that formed at pH 7.4, but its colour was orange whereas the latter was yellow. More products were obtained from DAB at pH 5.0 than at pH 7.4.

The major products were extracted from TLC plates with ethanol, and the

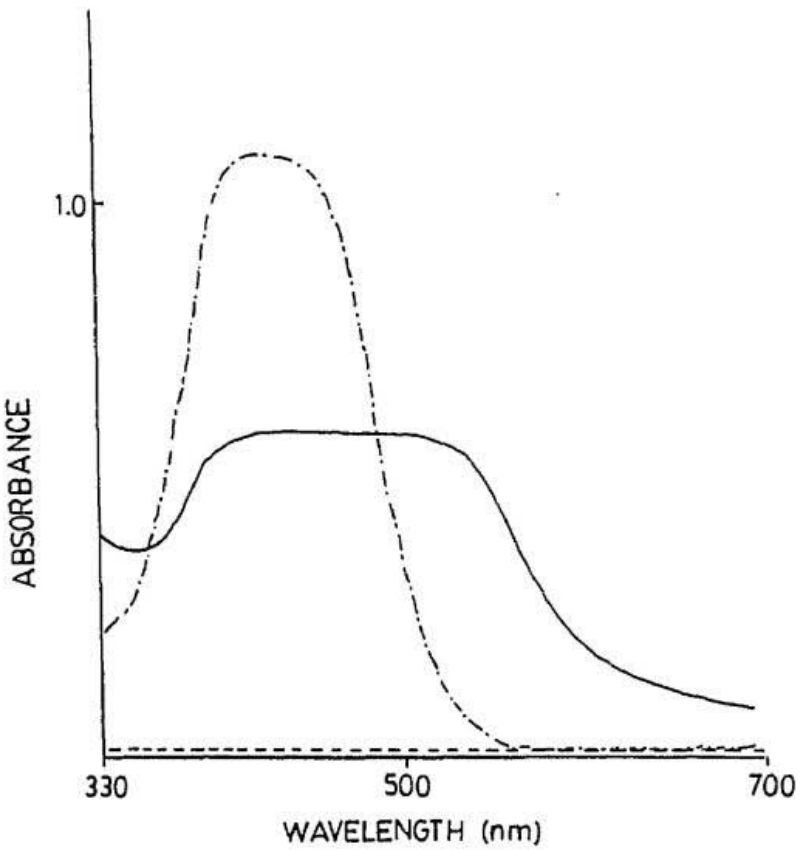


Fig.13:

Figure13: The absorption spectrum of MAB and the products formed by HRP/H_2O_2 catalyzed oxidation at pH 5.0:

The complete reaction mixture consisted of 0.1 M Sodium acetate-acetic acid buffer pH 5.0, 50 μM MAB, 100 μM H_2O_2, and 1 μg of HRP. The absorption spectrum was recorded after 30 minutes of incubation at 37°C. The spectrum did not change after this time.

--- HRP + H_2O_2 in buffer.

—·— MAB in buffer.

—— Complete reaction mixture.

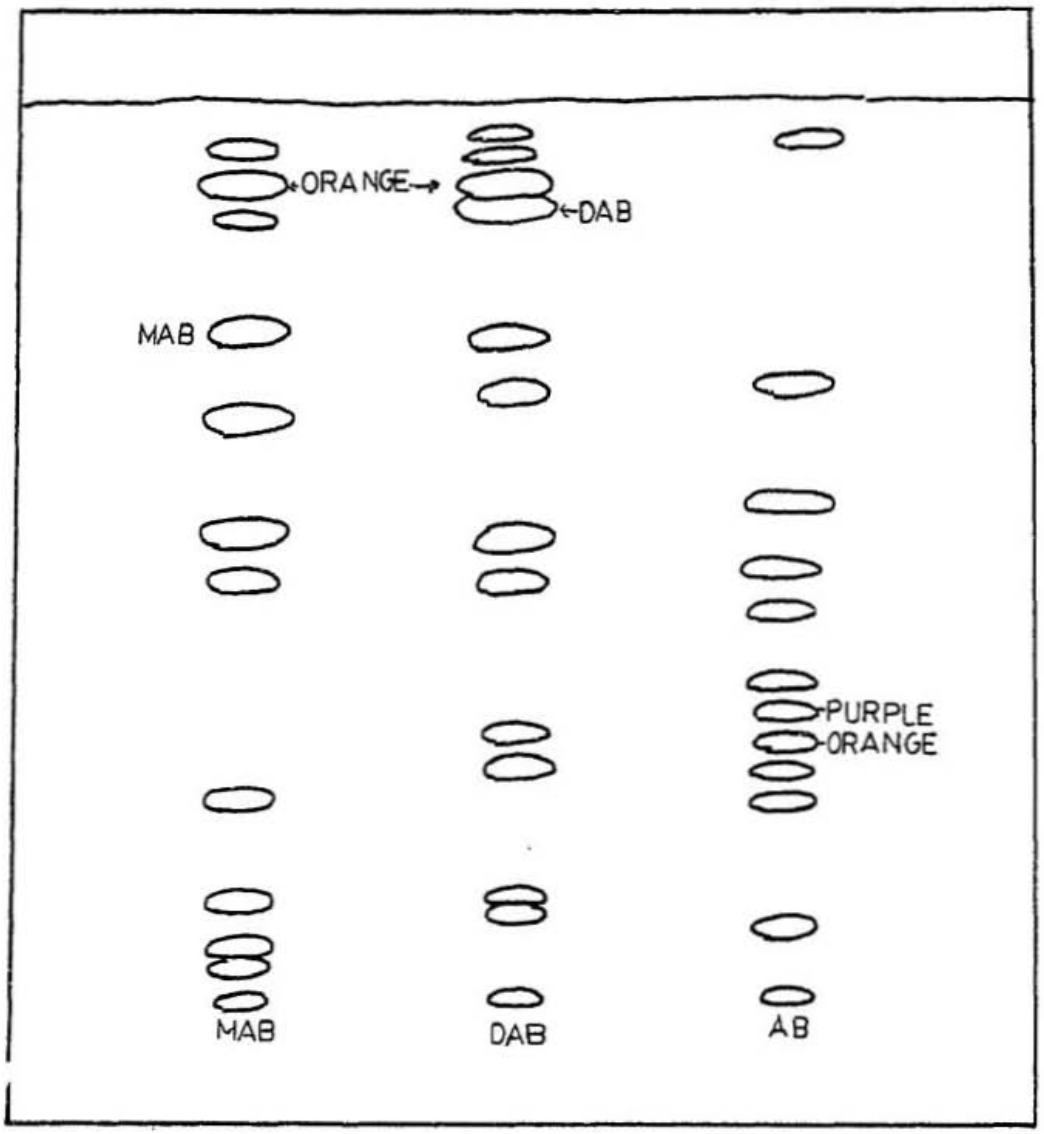


Fig.14:

Figure14: Thin layer chromatogram of the oxidation products of MAB, DAB and AB (Catalyzed by HRP/H_2O_2 at pH 5.0:

The complete reaction mixtures (final volume 2.0 ml) consisting of pH 5.0 buffer (0.1 M sodium acetate-acetic acid), 50 μM of the substrate, 100 μM H_2O_2 and 1 μg of HRP were extracted with ethyl acetate : acetone after 30 minutes of incubation at 37°C. The extracts were evaporated and redissolved in a small volume of benzene and methanol and applied on silica gel plates. The chromatograms were developed in benzene : ethyl acetate (9:1).

absorption spectra were recorded (Fig.15). The pH 5.0 product absorbed maximally over a broad range of wavelength i.e; from 390 nm to 500 nm.; whereas the pH 7.4 product absorbed maximally at 390 nm. These spectra were however, comparable to the spectra of their respective reaction mixtures.

6.Mass Spectral Analysis:

The major products obtained from MAB and DAB oxidation were isolated and purified by thin layer chromatography, and analyzed by mass spectrometry (Materials and Methods). It was observed that the molecular ions of the products obtained from both MAB and DAB at pH 7.4 had the same mass number (420) and their fragmentation patterns were the same. Fig.17a and 17b indicate that the next fragment to the parent ion had the same mass number as MAB (211) and the smaller fragments had the same pattern as that of standard MAB (at 134,106,77 and 28) (Fig.16a).

The mass number 420 indicates that the product is a "dimer" of MAB as it has twice the molecular weight of MAB less two.

The maior product of MAB formed in a reaction mixture at pH 5.0 was also analyzed. Fig.18 shows that it had the same mass number as that formed at pH 7.4, *i.e*; 420, but the fragmentation pattern was distinct from that of the pH 7.4 product.

Examination by HPLC and TLC of the oxidation products of AB does not reveal any predominant product. The most non-polar product obtained in a reaction mixture at pH 7.4 (retention time 30 minutes by HPLC; R_F 0.93 on TLC) was examined by mass spectrometry. The mass spectrum (Fig.19) indicates that

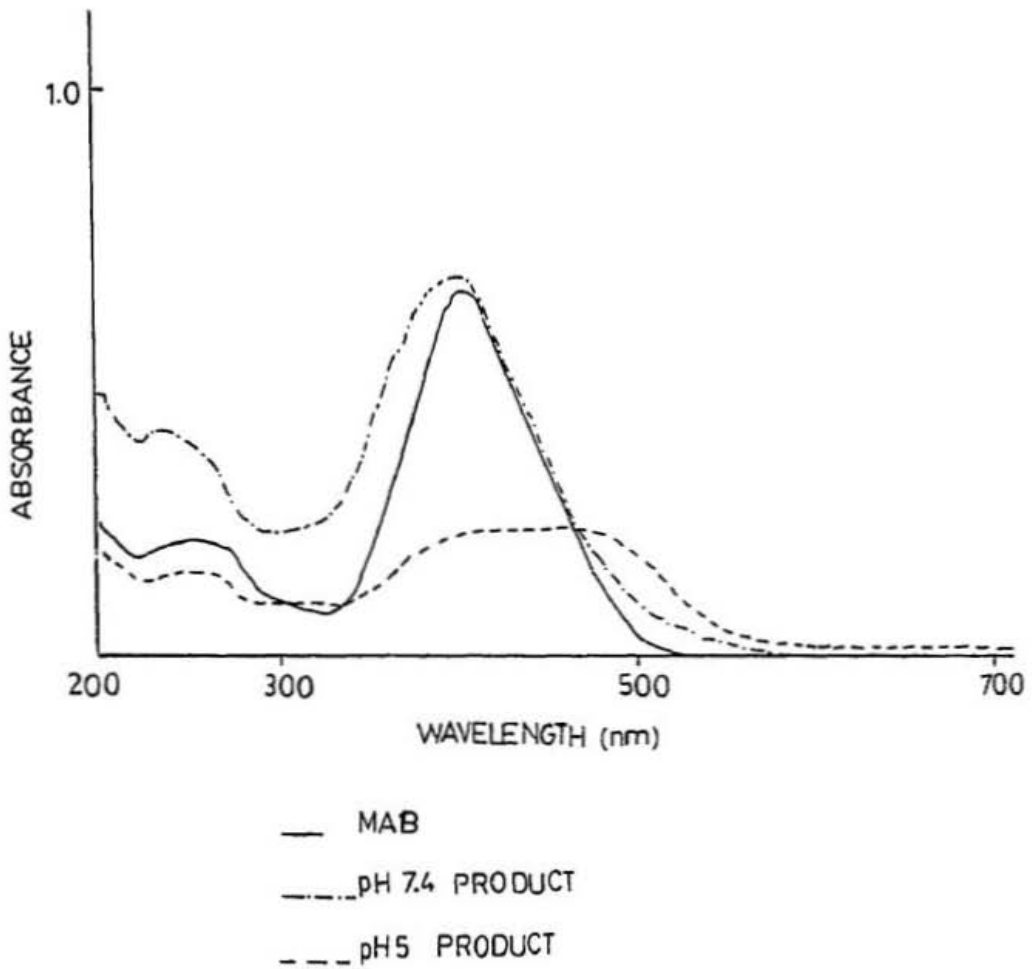

1.0
ABSORBANCE
200
300
500
700
WAVELENGTH (nm)
MAB
pH 7.4 PRODUCT
pH 5 PRODUCT

Fig.15:

Figure 15: The absorption spectra of the major products formed at pH 7.4 and pH 5.0 from MAB:

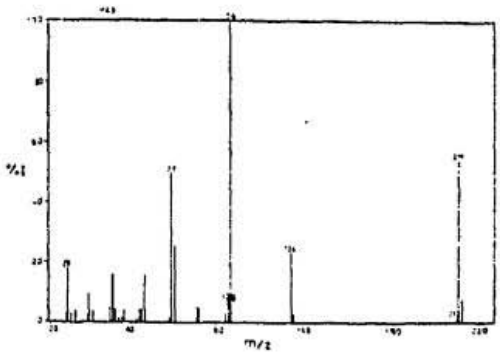
The major oxidation products of MAB obtained from pH 7.4 and pH 5.0 reaction mixtures were scraped from the silica gel TLC plate, extracted in ethanol and spectra were recorded.

—·— Major Product of MAB formed at pH 7.4.

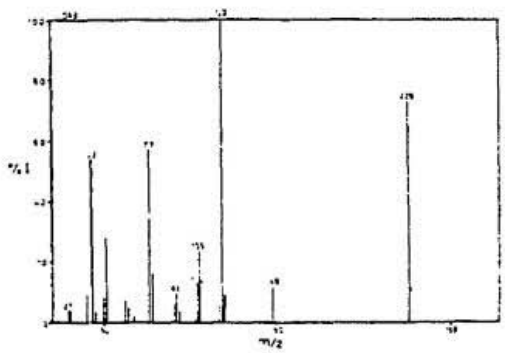
----- Major Product of MAB formed at pH 5.0.

—— MAB.

a



b



c

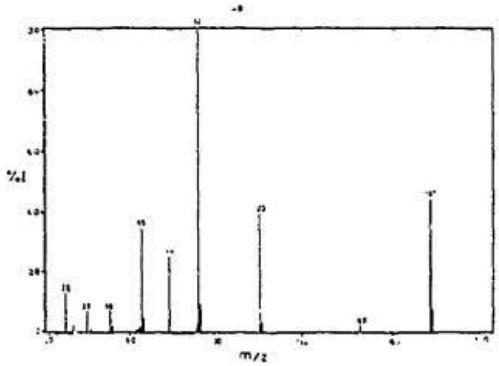
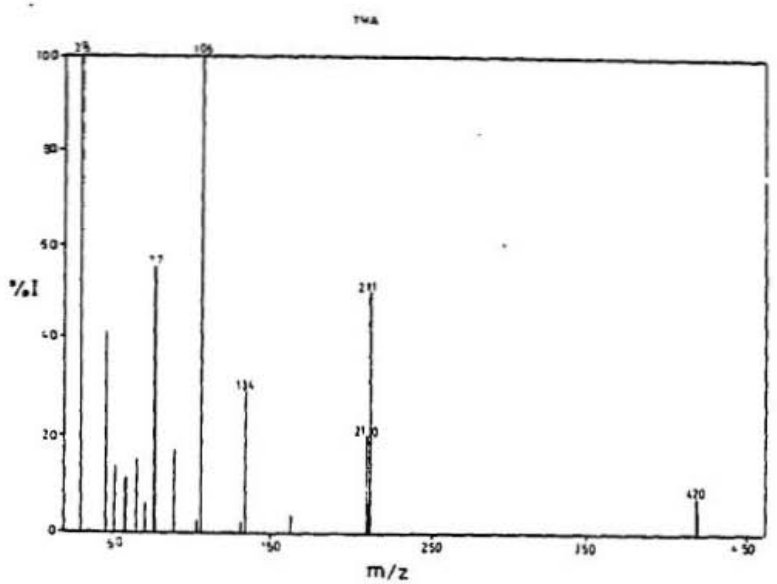


Fig.16:

Figure16: Mass spectra of standard (a) MAB, (b) DAB and (c) AB:

a



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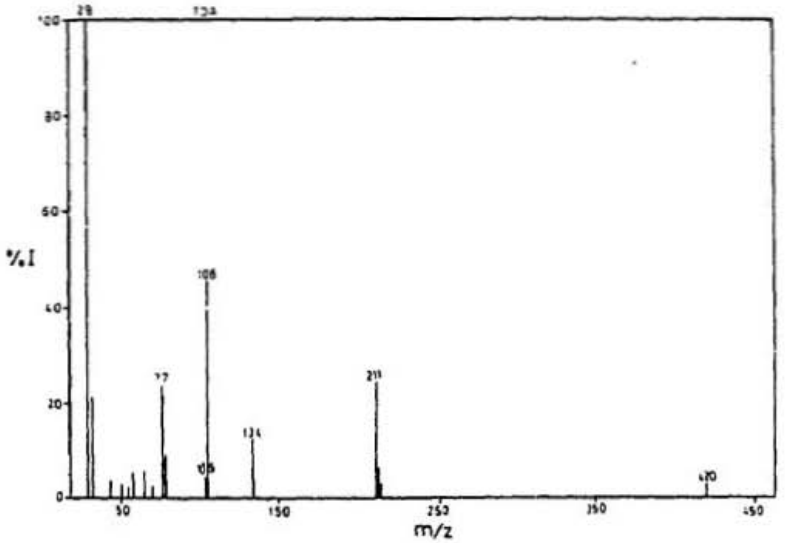


Fig.17:

Figure 7: Mass spectra of the major products of (a) MAB and (b) DAB formed by oxidation at pH 7.4:

The bands of major products were scraped from silica gel TLC plates, extracted into ethanol and the mass spectra were recorded after evaporating the ethanol.

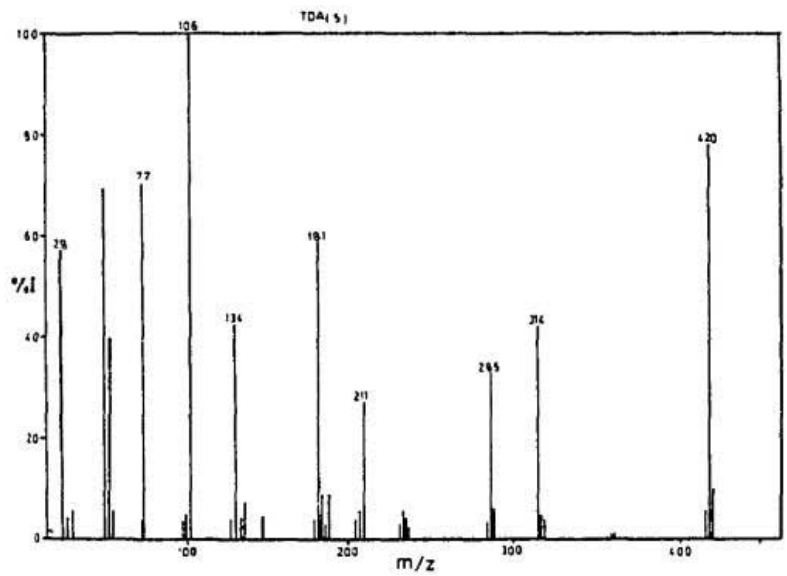


Fig. 18:

Figure 18: Mass spectrum of the major product formed by oxidation of MAB at pH 5.0:

The major product formed by a HRP/H_2O_2 catalyzed oxidation was isolated and purified by thin layer chromatography and the mass spectrum was recorded.

the mass number of this product is 390 which is equivalent to two molecules of AB less four hydrogens. This information and the fragmentation pattern indicates that the molecule was formed due to N-N coupling of the amino groups resulting into the formation of an azo bond between the two molecules of AB. A small peak of this metabolite was also observed in the HPLC profile of metabolites of DAB (Fig.11b).

7.Effect of Ascorbic Acid on the Oxidation of Aminoazobenzenes:

Reaction mixtures (2.0ml) contained buffer, MAB (50 μM) H_2O_2 (100 μM), and HRP were (1 μg) added in that order at 37°C. Ascorbic acid was added either immediately before the HRP, or after a measured interval after initiating the reaction with HRP. After total incubation period of 30 minutes, the reaction mixture was extracted with ethyl acetate : acetone, and after evaporation of the solvent the extract was chromatographed on silica gel TLC plates as described in "Materials and Methods". The results are shown in Fig.20, 21, and 22.

When ascorbic acid was added before HRP, the oxidation of MAB, DAB and AB was almost completely inhibited. When ascorbic acid was added after 10 seconds to the reaction mixtures containing MAB and DAB, it had no effect on the generation of oxidation products (results not given). In contrast when ascorbic acid was added after 2 minutes to a reaction mixture containing AB, one of the products normally formed (purple in colour) disappeared and another (orange in colour) was increased (Fig.22). The products were extracted from the TLC plates with ethanol, and their mass spectra were determined (Fig.23, 24 and 25).

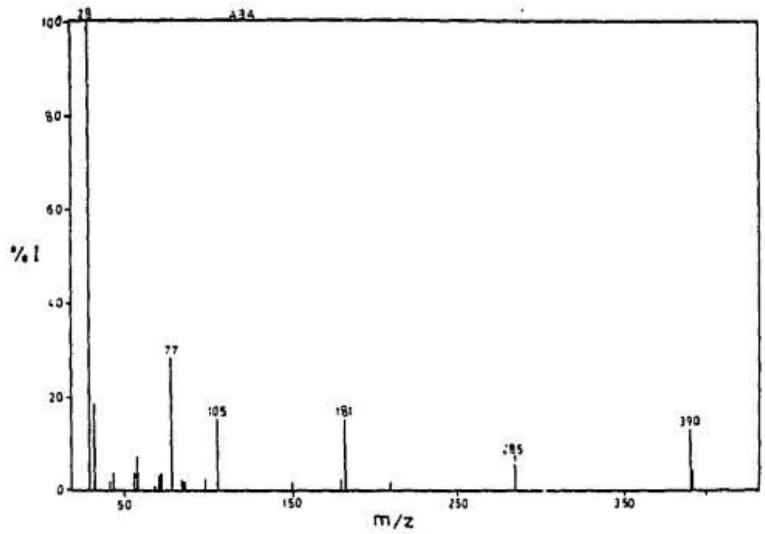


Figure 19: Mass spectrum of the oxidation product of AB formed by HRP/H_2O_2 catalyzed oxidation at pH 7.4:

The oxidation product of AB with R_F value 0.93 was isolated from the TLC plate and mass spectrum was recorded.

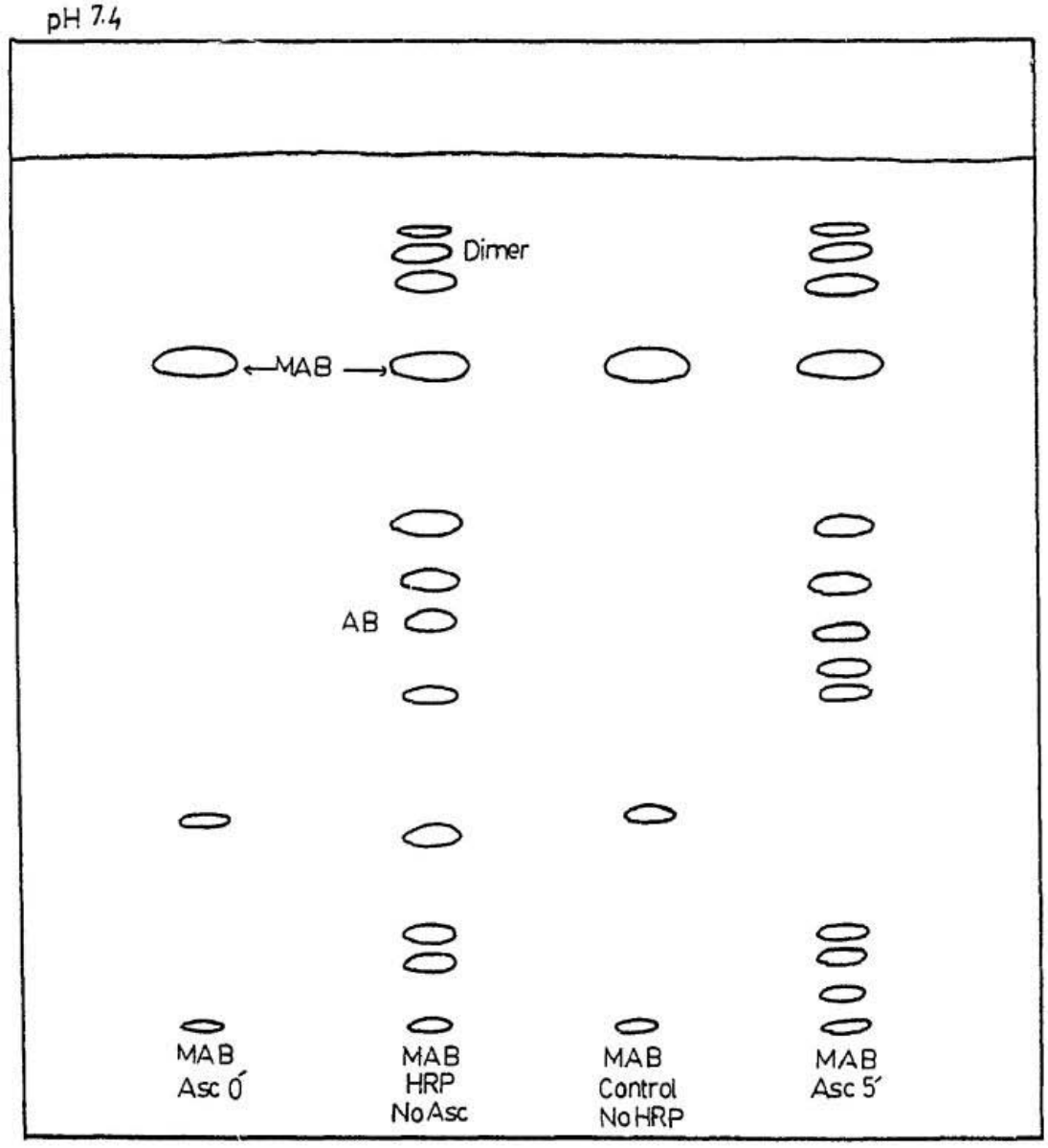

pH 7.4
Dimer
MAB
AB
MAB
Asc 0'
MAB
HRP
No Asc
MAB
Control
No HRP
MAB
Asc 5'

Fig. 20:

Figure 20: Effect of ascorbic acid on the HRP/H_2O_2 catalyzed oxidation of MAB at pH 7.4:

The complete reaction mixture contained 0.05 M Tris-HCl buffer pH 7.4, 50 μM MAB, 100 μM H_2O_2. Ascorbic acid was added either before starting the reaction with 1 μg of HRP or after 5 minutes of starting the reaction. The reaction mixtures were extracted with ethyl acetate : acetone after 30 minutes of total incubation at 37°C. The solvent was evaporated, the contents were dissolved in methanol and applied on a silica gel TLC plate. The chromatogram was developed in benzene : ethyl acetate (9:1).

pH 7.4

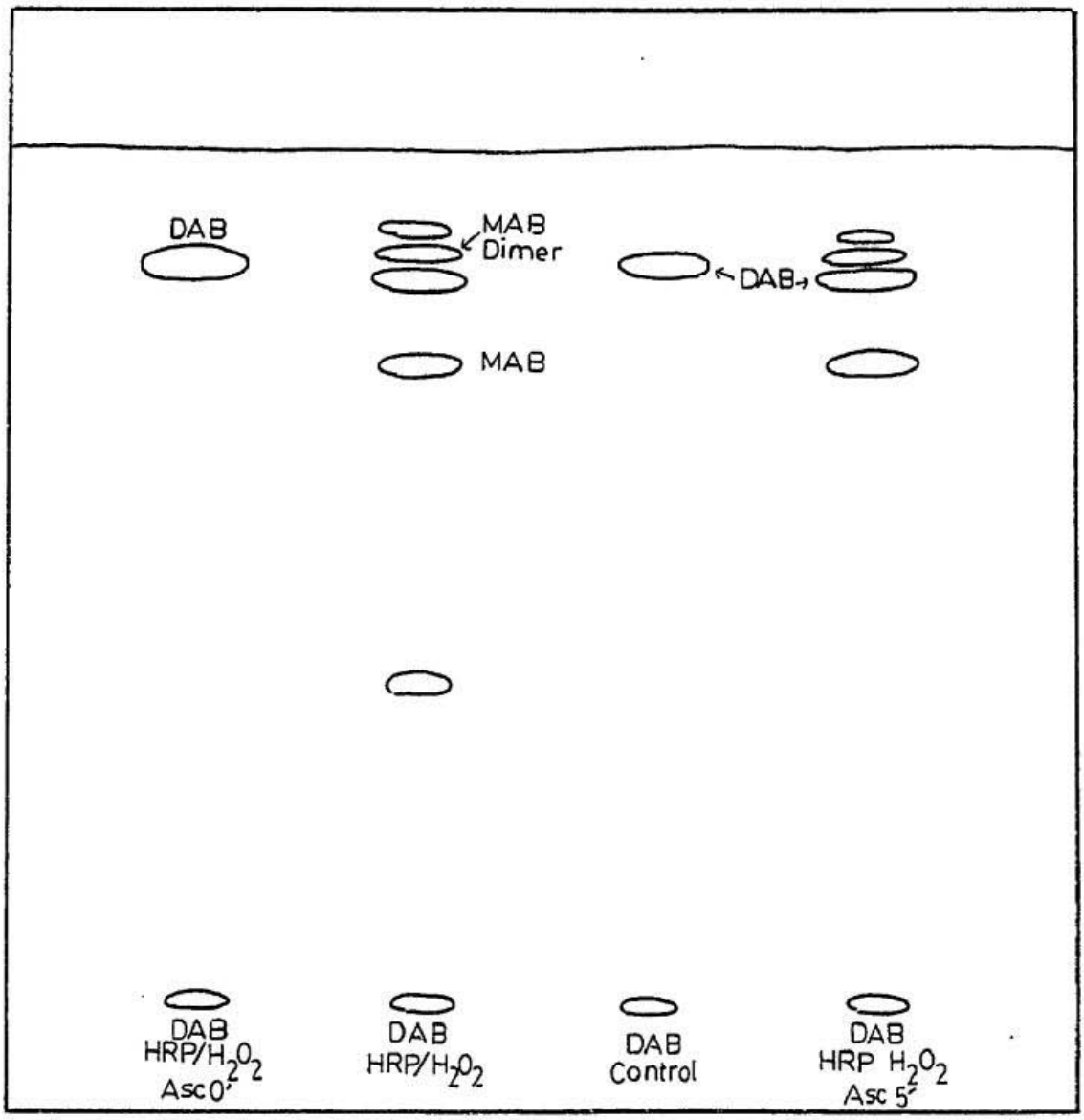

DAB
MAB
Dimer
DAB
MAB
DAB
HRP/H2O2
Asc 0'
DAB
HRP/H2O2
DAB
Control
DAB
HRP H2O2
Asc 5'

Fig. 21 :

Figure 21: Effect of ascorbic acid on the oxidation of DAB catalyzed by HRP/H_2O_2 at pH 7.4:

The complete reaction mixture contained 0.05 M Tris-HCl buffer pH 7.4, 50 μM DAB, 100 μM H_2O_2. Ascorbic acid was added either before starting the reaction with 1 μg of HRP or after 5 minutes of starting the reaction. The reaction mixtures were extracted with ethyl acetate : acetone after 30 minutes of total incubation at 37°C. The solvent was evaporated, the contents were dissolved in methanol and applied on silica gel TLC plate. The chromatogram was developed in benzene : ethyl acetate (9:1).

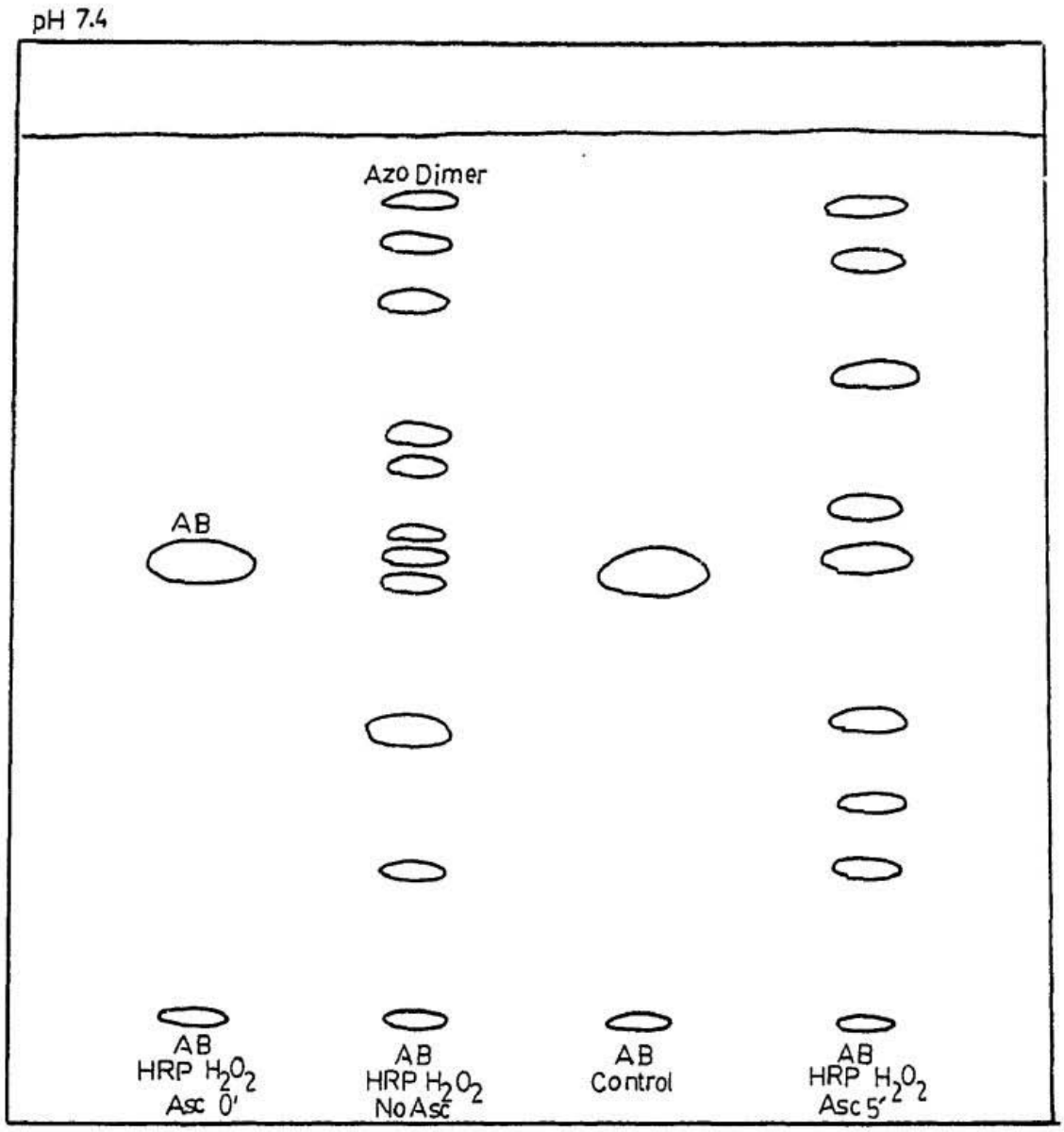

pH 7.4
Azo Dimer
AB
AB
HRP H_2O_2
Asc 0'
AB
HRP H_2O_2
No Asc
AB
Control
AB
HRP H_2O_2
Asc 5'

Fig.22:

Figure 22: Effect of ascorbic acid on the oxidation of AB catalyzed by HRP/H_2O_2 at pH 7.4:

The complete reaction mixture contained 0.05 MTris-HCl buffer pH 7.4, 50 μM AB, 100 μM H_2O_2. Ascorbic acid was added either before starting the reaction with 1 μg of HRP or after 5 minutes of starting the reaction. The reaction mixtures were extracted with ethyl acetate : acetone after 30 minutes of total incubation at 37°C. The solvent was evaporated, the contents were dissolved in methanol and applied on a silica gel TLC plate. The chromatogram was developed in benzene : ethyl acetate (9:1).

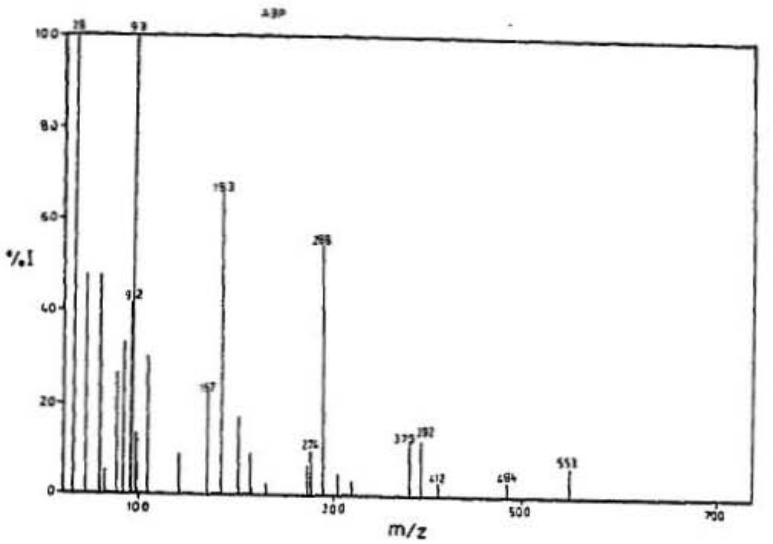


Figure 23: Mass spectrum of the purple product of AB formed in the absence of ascorbic acid:

The reaction was carried out under the standard conditions described in "Materials and Methods". The organic soluble metabolites were chromatographed on silica gel TLC plate and the purple band (R_F 0.33) was isolated and the mass spectrum was recorded.

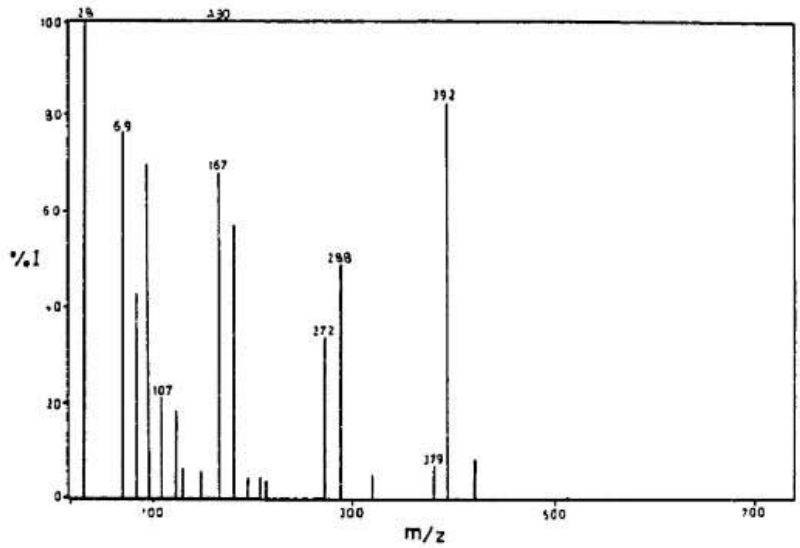


Figure 24: Mass spectrum of the orange product of AB formed in the absence of ascorbic acid:

The reaction was carried out under the standard conditions described in "Materials and Methods". The organic soluble metabolites were chromatographed on silica gel TLC plate and the orange band (R_F 0.25) was isolated and the mass spectrum was recorded.

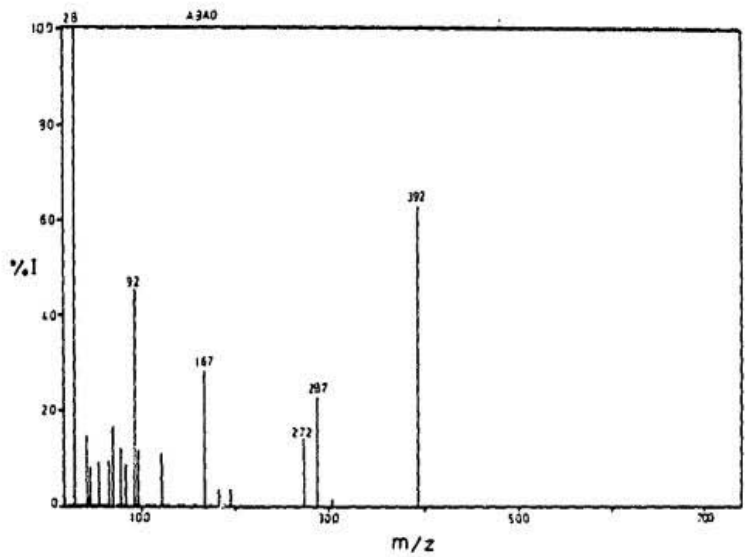


Figure 25: Mass spectrum of the orange product of AB formed in the presence of ascorbic acid:

The reaction was carried out under the standard conditions described in "Materials and Methods". The organic soluble metabolites were chromatographed on silica gel TLC plate and the orange band (R_F 0.25) was isolated and the mass spectrum was recorded.

B.Binding of Activated Molecules to DNA & Other Macromolecules:

The DNA binding was carried out at pH 7.4 and pH 5.0 under standard conditions as described in "Materials & Methods". Assuming identical extinction coefficients, it is evident from Fig.26 that the oxidation products of AB bind less to DNA than those of MAB and DAB (Fig.26 & 27).

It was assumed that the MAB product/s binding to DNA were very reactive and therefore it was important to find out if they were stable or not. To determine this, DNA was added to the reaction mixture at different time intervals after starting the reaction. Fig.28 shows that the maximum binding took place when DNA was added to the reaction mixture before and immediately after starting the reaction.

The results are shown in Fig.28. It is indicated that binding to DNA occurred even when DNA was added 30 minutes after starting the reaction. This indicates the possibility of at least two types of products that were responsible for binding to DNA. The most reactive product bound early, and a less reactive or slowly generated species continued to react as late as 30 minutes after starting the reaction. These conclusions have to be reviewed with caution because the amount of the product bound was only 2-5%.

Similar results were obtained using homo polyribonucleotides to bind oxidation products of MAB. The results are shown in Table 4. Polyriboguanylic acid showed the highest binding. Polyribocytidylic acid and polyribouridylic acid showed the least binding. Therefore it can be suggested that the oxidation products of MAB bind preferentially to guanine residue in DNA. The purines

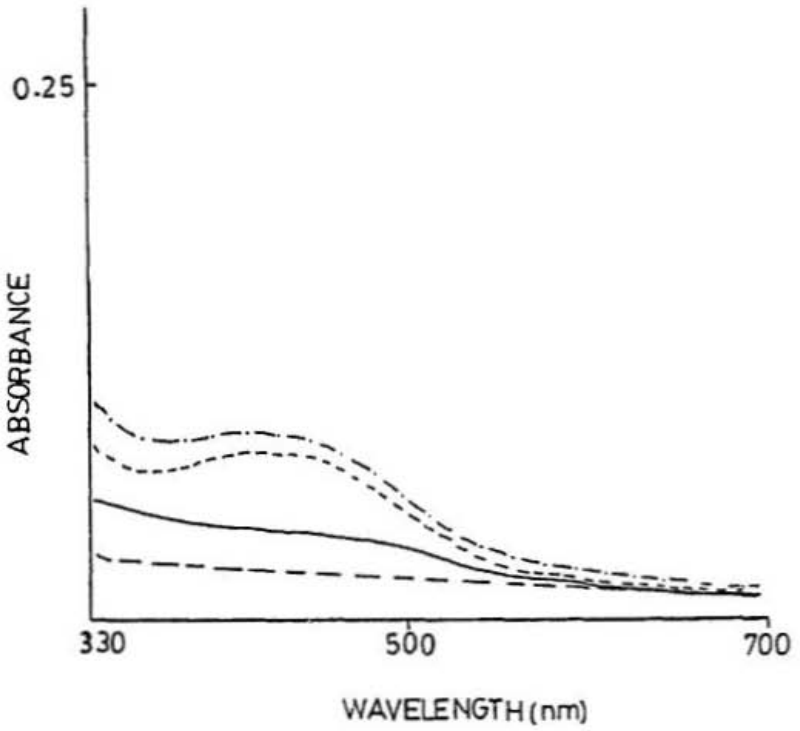


Fig.26 :

Figure 26: Binding of the oxidation products of MAB, DAB and AB to calf thymus DNA at pH 7.4:

The reaction mixtures (2.0 ml) contained 0.05 M Tris-HCl buffer pH 7.4, 3 mg of calf thymus DNA, 50 μM MAB, DAB or AB, 100 μM H_2O_2 and 1 μg of HRP and .

were incubated at 37°C for 30 minutes. Then DNA was isolated by the standard procedure as described in "Materials and Methods", dissolved in water and the spectrum was recorded.

--- DNA.

—·— MAB + DNA.

----- DAB + DNA.

—— AB + DNA.

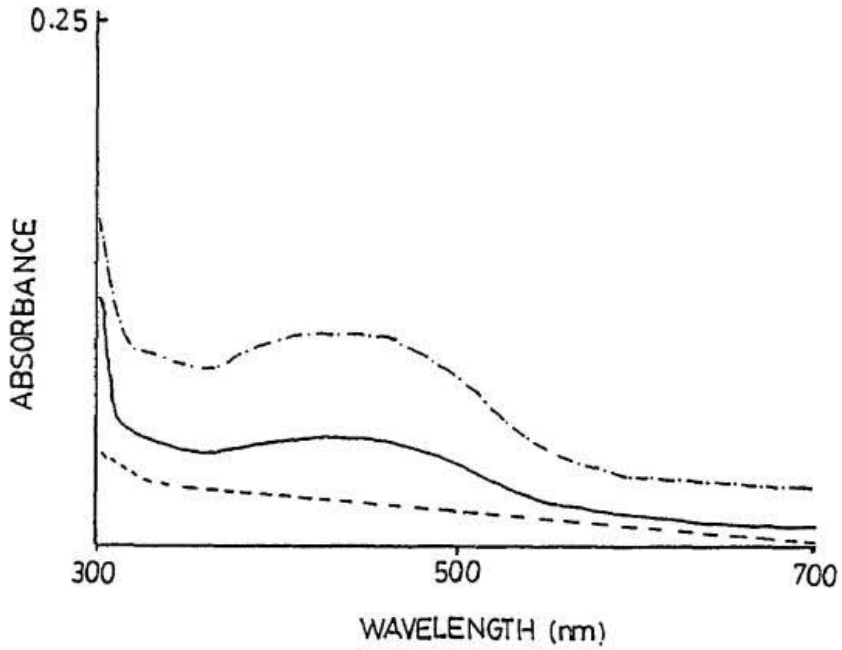


Fig. 27:

Figure27: The binding of the oxidation products of MAB and DAB to calf thymus DNA at pH 5.0:

The reaction mixtures (2.0 ml) contained Sodium acetate-acetic acid buffer pH 5.0, 3 mg of calf thymus DNA, 50 μM MAB or DAB 100 μM H_2O_2 and 1 μg of HRP and were incubated at 37°C for 30 minutes. Then DNA was isolated by the standard procedure as described in "Materials and Methods", dissolved in water and the spectrum was recorded.

--- DNA.

—·— MAB + DNA.

—— DAB + DNA.

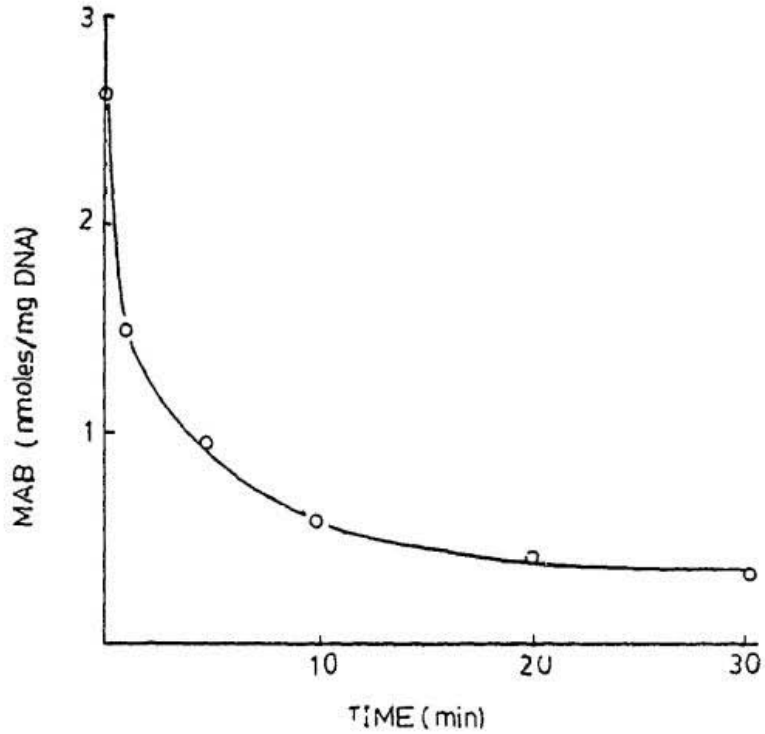


Fig . 28 :

Figure28: Time Course of Covalent Binding of Oxidation Products of MAB to Calf Thymus DNA:

The reaction mixtures (2.0 ml) contained 0.05 M Tris-HCl buffer pH 7.4, 50 μM [^{14}C] MAB, 100 μM H_2O_2 and 1 μg of HRP at 37°C. 3 mg of DNA was added at different times after starting the reaction. After a total incubation of 30 minutes of DNA in each tube the reaction was stopped and DNA was isolated as described in "Materials and Methods" and counted for the bound radioactivity.

Table 4: Peroxidase catalyzed binding of MAB products to macromolecules.

	MAB Binding (nmoles/mg DNA)
DNA	1.5
DNA (denatured)	1.3
Polyriboguanylic acid	2.2
Polyriboadenylic acid	0.7
polyribocytidylic acid	0.2
polyribouridylic acid	0.6

The reaction mixture (2.0 ml) containing 0.05 M Tris-HCl buffer (pH 7.4), 50 μM of ^{14}C MAB, 100 μM of H_2O_2, 3.0 mg of the macromolecule and 1 μg of HRP was incubated at 37°C for 30 minutes. DNA was isolated, dissolved in 1 ml of water and an aliquot of 0.5 ml was counted for the bound radioactivity.

(guanine, adenine, xanthine and purinol) did not inhibit or enhance the binding of oxidation products of MAB to DNA (Table 5).

C.Effect of Biological Reducing Agents:

The results are given in Table 6. Ascorbic acid strongly inhibited MAB oxidation and its binding to DNA. Cysteine did not inhibit the oxidation of MAB even at the concentration of 500 μM but did inhibit binding to DNA. N-acetyl-L-cysteine showed inhibition of both oxidation and DNA binding (Fig.26 & 27). Glutathione(reduced form, *i.e*; GSH) inhibited DNA binding more than it reduced the oxidation of MAB. Tyrosine decreased both MAB oxidation and binding to DNA. NADPH did not inhibit MAB oxidation but decreased DNA binding. Uric acid was the most effective at inhibiting MAB oxidation as well as DNA binding.

The effect of MAB on NADPH oxidation by H_2O_2 catalyzed by horseradish peroxidase was determined by measuring the decrease in absorbance at 340 nm over a period of time (Fig.29). It was observed that as soon as MAB was added to the reaction mixture the absorbance at 340 nm decreased sharply. In the absence of NADPH the absorbance at 340 nm was constant. Therefore NADPH was oxidized by the oxidation products formed from MAB.

The overall oxidation of MAB was not inhibited (Table 6) and it is likely that the oxidation products of MAB are not reduced back to MAB but to some other products which do not react with DNA.

Table 5: Effect of purines on the binding of the oxidation products of MAB to DNA by peroxidase System.

	Radioactivity bound (expressed in nmoles MAB/mg DNA)	Non-extractable Products after precipitating DNA
No Purines	2.1	3.7
Guanine	2.3	3.5
Xanthine	1.7	5.5
Hypoxanthine	2.4	4.0
Allopurinol	2.3	3.9

The reaction mixtures (2.0 ml) contained 0.05 M Tris-HCl buffer (pH 7.4), 50 μM MAB, 100 μM H_2O_2, 3.0 mg of calf thymus DNA, 200 μM of the purine and 1 μg of HRP were incubated at 37°C for 30 minutes. The aqueous phase after precipitating the DNA was measured for the radioactivity.

Table 6: Effect of biological donors on peroxidase/H_2O_2 catalyzed binding of MAB products to DNA.

Inhibitor		% Binding to DNA	% MAB Oxi- tion
None		100	100
L-ascorbate	50 μM	30	47
	100 μM	8	7
Cysteine	100 μM	68	97
	200 μM	34	96
	500 μM	36	92
N-acetyl-L-cysteine	50 μM	72	96
	100 μM	11	41
Glutathione	50 μM	100	95
	100 μM	41	86
Tyrosine	100 μM	41	63
NADPH	100 μM	59	99
	200 μM	60	99
	500 μM	50	96
Uric acid	50 μM	100	96
	100 μM	8	25
	200 μM	2	12
	500 μM	2	11

The reaction mixtures (2.0 ml) containing 0.05 M Tris-HCl buffer (pH 7.4), 50 μM [^{14}C] MAB, 100 μM H_2O_2, 3.0 mg of calf thymus DNA , 1 μg HRP and the inhibitor were incubated at 37°C for 30 minutes.

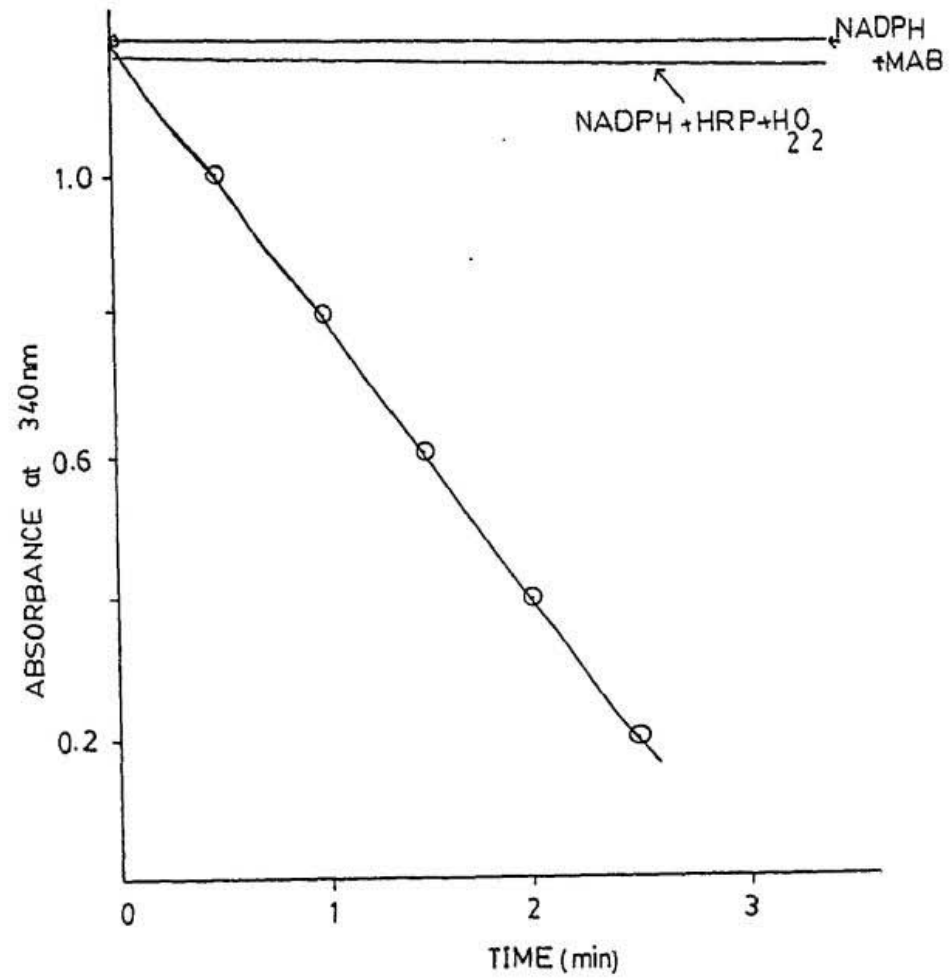
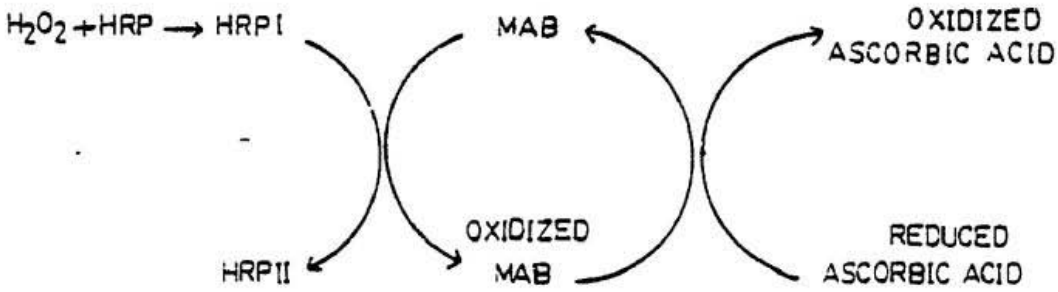

NADPH
+MAB
NADPH + HRP + H_2O_2
1.0
0.6
0.2
ABSORBANCE at 340nm
0
1
2
3
TIME (min)

Fig. 29.

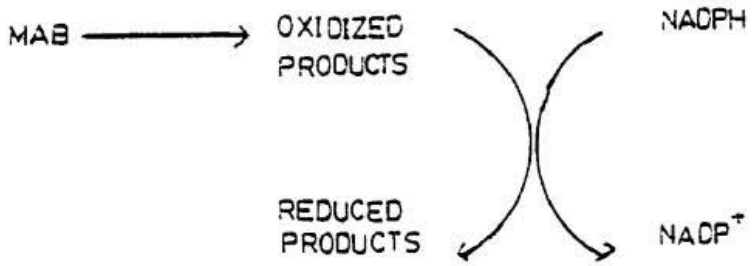
Figure 29: Decrease in A_{340} due to NADPH oxidation by MAB/HRP/H_2O_2 reaction mixture:

The complete reaction mixture (final volume 3 ml) contained 0.05 M Tris-HCl buffer (pH 7.4), 100 μM NADPH, 10 μM H_2O_2, 1.5 μg HRP and 5 μM MAB. The controls contained, (i) NADPH and MAB and (ii) NADPH, HRP and H_2O_2.



Ascorbic acid was also oxidized rapidly by MAB horseradish peroxidase and hydrogen peroxide at pH 7.4. This was observed as the decrease in absorbance at 265nm. In the absence of either ascorbic acid or MAB the absorbance at 265 nm was constant (Fig.30).

As the total oxidation of MAB was inhibited by ascorbic acid (Table 6), it seems that the primary oxidation product of MAB (probably the MAB radical cation) was reduced back to MAB with resulting ascorbic acid oxidation.



Uric acid showed a similar effect, and inhibited MAB oxidation and DNA binding probably by reducing the primary oxidation product back to MAB.

N-acetyl-L-cysteine exhibited a more inhibitory effect on DNA binding than on MAB oxidation. Therefore the possibility of MAB adduct formation with N-acetyl-L-cysteine was explored. The results in Table 7 do not suggest the formation of water soluble adducts. Another experiment was carried out to detect adduct formation with GSH, N-acetyl-L-cysteine, and cysteine. After the

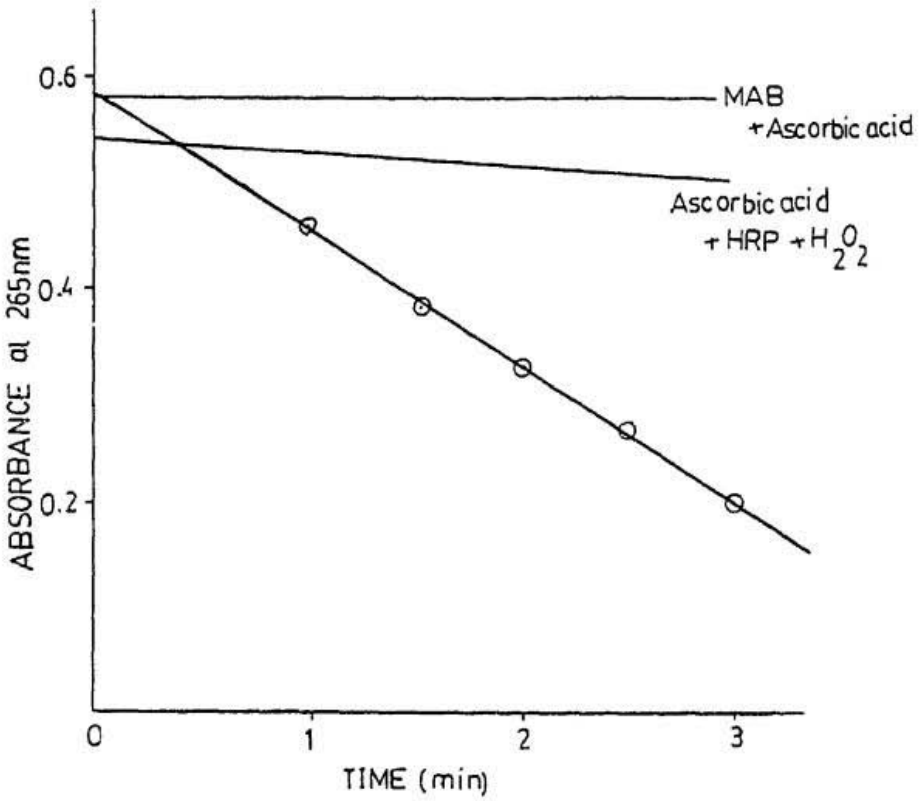

0.6
0.4
0.2
ABSORBANCE at 265nm
MAB
+Ascorbic acid
Ascorbic acid
+HRP +H_2O_2
0
1
2
3
TIME (min)

Fig. 30:

Figure 30: Decrease in A_{265} due to ascorbic acid oxidation by MAB/HRP/H_2O_2 reaction mixture:

The complete reaction mixture (3 ml) contained 0.05 M Tris _HCl buffer pH 7.4, ascorbic acid (100 μM), HRP (1.5 μg) and MAB (25 μM. The controls were (i) ascorbic acid and MAB and (ii) ascorbic acid, HRP and H_2O_2.

Table 1: Effect of N-acetyl-L-cysteine on HRP/H_2O_2 catalyzed oxidation of MAB and its distribution into water and organic phases.

	No DNA			With DNA		
	Organic Soluble		Aqueous	Organic Soluble		Aqueous
	Unoxidized MAB nmoles	Oxidized MAB nmoles	Phase nmoles	Unoxidized MAB nmoles	Oxidized MAB nmoles	Phase nmoles
Control	4.8	95.2	5.7	2.1	97.9	25.3
50 μM	40.5	59.5	5.1	53.6	46.4	8.4
100 μM	53.4	46.6	3.4	45.5	54.5	4.1

The reaction mixtuers (2.0 ml) contained 0.05 M Tris-HCl buffer (pH 7.4), 50 μM [^{14}C] MAB, 100 μM H_2O_2, and 1 μg 0f HRP. The incubation period was 30 minutes at 37°C. The organic soluble metabolites were extracted with ethyl acetate : acetone (2:1), evaporated, redissolved in methanol and applied to the silica gel plate. After developing in benzene : ethyl acetate (9:1), the unoxidized MAB was scraped and radioctivity was counted. The aqueous phase left after the organic extracts was counted for water soluble radioactivity.)
Control=No N-acetyl-L-cysteine.
50 and 100 μM correspond to N-acetyl-L-cysteine.

extraction of organic soluble MAB and its oxidation products, the aqueous phase was extracted with water saturated butanol, and radioactivity was measured in both the butanol extract and the remaining water phase (Table 8). The detectable radioactivity was not greater than that in the control. The inhibitory effect on DNA binding is therefore principally due to reduction of the oxidation products (radical cations and dimeric products). However at higher peroxidase concentrations and at pH 6.5, water soluble adducts were formed (Rahimtula,M and O'Brien,J.P. personal communications).

D.The Effect of Phenolic Compounds:

These experiments were carried out as described for biological reducing agents. The phenolic antioxidant butylated hydroxyanisoi (BHA) inhibited MAB oxidation and decreased DNA binding concomitantly (Table 9). Other phenolic compounds had a similar effect. α-Naphthol inhibited very strongly both DNA binding and oxidation of MAB. Xylenol(2,6,dimethyl phenol) decreased the DNA binding to 58% at a 100 μM concentration but MAB oxidation was not inhibited significantly. In the case of 4-methyl catechol however, both binding and oxidation were inhibited to the same extent and the inhibition was dose dependent. Presumably α-naphthol reduces the oxidized dimers and radical cation whereas xylenol reduced the oxidized dimers and BHA reduced the radical cations.

Table 8: The effect of the reducing agents with thiol groups on the aqueous soluble MAB metabolites.

	Water Phase	Butanol Phase	Total Aqueous Soluble
	nmoles	nmoles	nmoles
Control (No HRP)	0.02	0.03	0.03
Control (No reducing agent)	2.5	1.3	3.8
Glutathione (500 μM)	2.2	0.4	2.6
Cysteine (500 μM)	2.1	1.0	3.0
N-acetyl-L -cysteine (500 μM)	2.8	0.7	3.5

The reaction mixtures (2.0 ml) containing 0.05 M Tris-HCl bufferb (pH 7.4), 50 μM [^{14}C] MAB, 100 μM H_2O_2, 1 μg HRP and 500 μM of the reducing agent were incubated at 37°C. After 30 minutes, they were extracted with ethyl acetate : acetone and ether. The remaining aqueous phase was extracted with water saturated butanol and the two phases were separately counted for radioctivity.

Table 9: The effect of phenolic compounds on peroxidase/H_2O_2 catalyzed binding of MAB products to DNA.

Inhibitor		% Binding to DNA	% MAB Oxidation
None		100	100
Butylated hydroxyanisole	50 μM	12	11
2,6-dimethyl phenol	50 μM	100	93
	100 μM	58	93
α-naphthol	50 μM	3.0	43
	100 μM	1.0	47
4-methyl catechol	50 μM	73	99
	100 μM	51	88
	200 μM	4.0	58

The reaction mixtures (2.0 ml) contained 0.05 M Tris-HCl buffer (pH 7.4), 50 μM [^{14}C]-MAB, 100 μM H_2O_2, the inhibitor and 1 μg of HRP. After incubating at 37°C for 30 minutes, organic soluble metabolites and unoxidized MAB were extracted with ethyl acetate : acetone for HPLC. DNA was isolated from the aqueous phase and the bound radioactivity was counted.

DISCUSSION

The electrophilic nature of ultimate chemical carcinogens and their binding to the macromolecules to initiate neoplasia is now well accepted. The role of monoxygenase system in this activation by a two electron oxidation mechanism has been extensively studied. Activation of chemical carcinogens by alternate mechanisms is evident from various observations discussed in the "Introduction" (Vasdev & O'Brien, 1982; Floyd, 1982; Mason *et al.*, 1982; O'Brien, 1984).

The detection of free radicals in the metabolic pathways of a number of chemicals (*e.g*; benzo(a)pyrene, aminoazo-dyes, 2-acetylaminofluorene, naphthylamines and nitrosoquinoline) has been pointed out. A direct correlation between the formation of free radicals and carcinogenesis of dimethyl and monomethyl aminoazobenzene derivatives has been demonstrated (Stier *et al.*, 1980; Kimura *et al.*, 1982)

The cisternae of the endoplasmic reticulum and the nuclear envelope appear to be the principal intracellular sites for peroxidases. The peroxidase activity in the target organs for various chemicals strengthens the importance of one electron oxidation in carcinogenesis. Thus, leucocytes, eosinophils, peritoneal macrophages, bone marrow cells, liver Kupffer cells, thyroid follicular epithelial cells, salivary gland, uterine endometrium, colon crypts , Lieberkuhn mucous secretory cells and Zymbal gland have been reported to contain peroxidase activity. Lactoperoxidase activity in the salivary glands and mammary glands (Anderson *et al.*, 1975) and peroxidase activity of prostaglandin synthetase have also been known to catalyze the activation of xenobiotics (Sivarajah *et al.*, 1981).

Information about the mechanisms of carcinogenicity of dimethyl aminoazobenzene and its derivatives, especially in the target organs in which peroxidases are known to be active (*e.g*; the Zymbal gland of external ear duct, leucocytes, and mammary glands) is scarce. Therefore the present work was carried out to study the mechanism of activation of arylaminoazobenzenes catalyzed by HRP/H_2O_2 and the binding of their activated products to the calf thymus DNA. The metabolites obtained were identified in order to understand the metabolic pathways and recognize the reactive intermediates responsible for binding to DNA. The relationship between the oxidation of MAB and its binding to DNA in the presence of different biological and phenolic antioxidants was also explored.

1.Oxidation of MAB, DAB and AB:

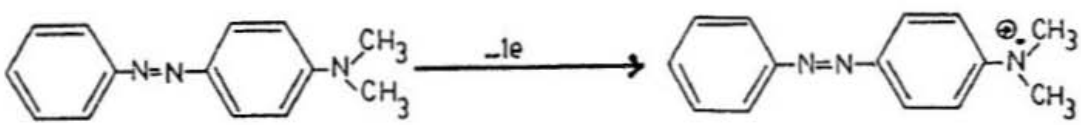
(i) Oxidation at pH 7.4: The yellow coloured metabolites and unmetabolized MAB and DAB were almost completely extractable from reaction mixtures by ethyl acetate:acetone (2:1). The remaining aqueous phase was found to be colourless. From [^{14}C] MAB, 4% to 5% of the total radioactivity was detected in the aqueous phase after extraction (Table.5 & Table.8). These results show that more than 95% of MAB and its metabolites are extracted in the organic solvent and it is likely that this also applies to DAB and its metabolites.

The major products obtained from the HRP/H_2O_2 catalyzed oxidation of DAB and MAB at pH 7.4 were identified by thin layer chromatography, high pressure liquid chromatography and mass spectrometry. It was observed that these products had the same retention time on HPLC (Fig.11a & 11b) and the

same R_F value on TLC (Fig.12). The mass spectra (Fig.17a & 17b) further supported this observation. It can be suggested from these results that the predominant product from both MAB and DAB is a dimer of MAB. The molecular ion had mass 420 which suggests it is derived from two molecules of MAB with the abstraction of two atoms of hydrogen. The fragmentation pattern (Fig.17a & b) shows a peak with mass 211 below the parent ion . This corresponds to MAB itself, and the peaks with lower mass correspond to those observed in the mass spectrum of MAB (Fig.16a).

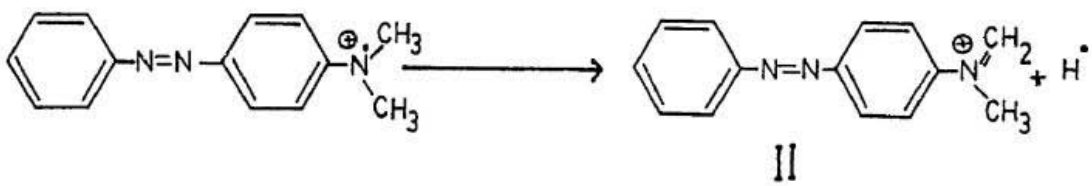
The known reactions catalyzed by peroxidases are radical reactions (Introduction). Radical mechanism can explain the structure of the major products identified.

One electron oxidation of DAB could give a radical cation (I),



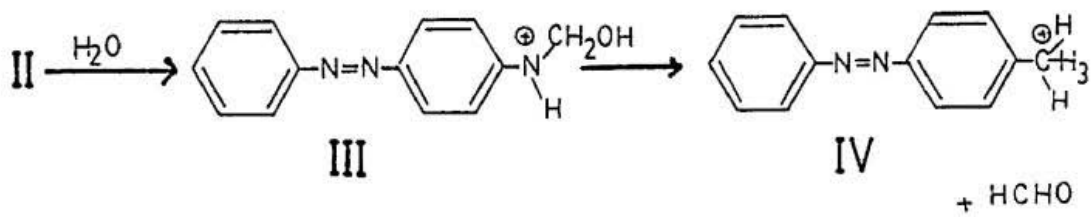
I

The radical cation disproportionates to give an iminium ion II and a hydrogen radical,



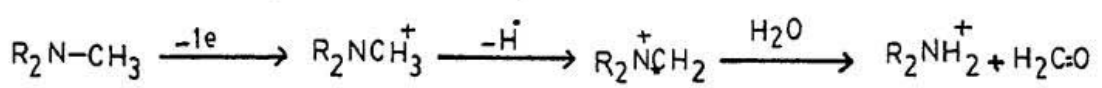
II

The iminium ion is then hydrolyzed to the secondary amine IV,



III IV

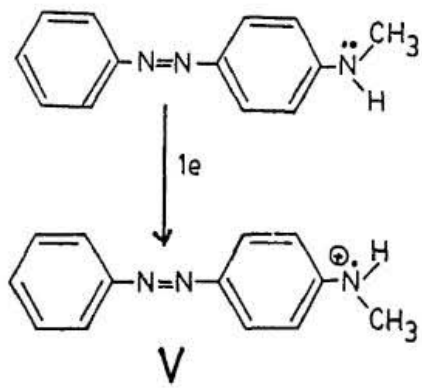
These reactions follow proposed mechanism for the demethylation of aminopyrine by HRP/H_2O_2 first reported by Gillette *et al.* (1958). Kadlubar (1973) demonstrated that cytochrome P-450 catalyzed the N-demethylation reaction in the presence of various organic hydroperoxides. However, Griffin in 1977 observed that HRP/H_2O_2 system catalyzed the N-demethylation of aminopyrine with a much greater rate than the cytochrome P-450/cumene hydroperoxide system. In 1978 the same investigator proposed a reaction mechanism showing that the oxygen atom of formaldehyde comes from water and not directly from the oxidant *i.e*; the peroxide.



The free radical formed by the abstraction of one electron undergoes further oxidation. The iminium cation is then hydrolyzed to amine and formaldehyde.

Thus if DAB is demethylated it is not surprising to find the same final product as that obtained from MAB. It should be noted that under the standard reactions 85% of the DAB remained at the end of the reaction whereas only 3 to 5% of the MAB remained unmetabolized (Table.3). The reaction was however complete. This indicates that an aminoazobenzene is not a much preferred substrate for the peroxidase when its amino nitrogen is fully saturated with the alkyl groups. It has been reported that the hydrolysis of the iminium cation of aminopyrine is much slower than its formation (Griffin, 1977). Therefore it could be possible that the hydrolysis of the iminium cation of DAB to MAB and formaldehyde is rate limiting.

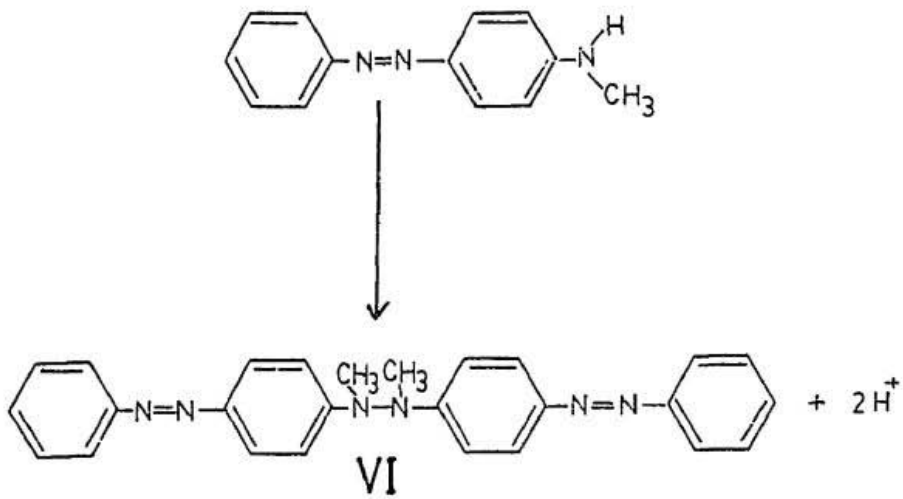
The one electron oxidation of MAB gives rise to radicals (V),



V

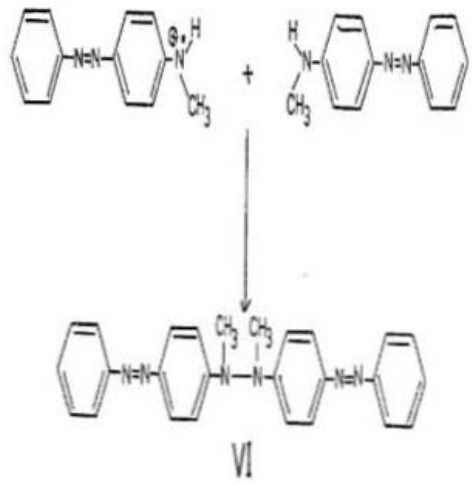
Free radicals formed from MAB could couple in three ways leading to three types of bonds between the two radicals, *i.e*; C-C, C-N or N-N. Among these the N-N bond is believed to be the least stable in the m ss spectrometer, and the C-C bond the most stable. (Attah Poku, personal communications). The mass spectra

(Fig.17) shows that the molecular ion peak (420 m/z) is followed by a MAB ion peak (211 m/z). Then the fragmentation follows the same pattern as that of the standard MAB (Fig.16a). The relative intensity of the molecular ion was about four times greater than that of MAB ion. Clearly it is indicated that the MAB molecules in the dimer are linked through a bond which is immediately cleaved into two MAB ions in the mass spectrometer. Therefore it can be suggested that the product (VI) arises by a chain terminating mechanism involving N-N linkage between the two radicals,



VI

Because radicals occur in low concentrations, the probability of collision of two rare species is extremely low. The radicals must in general interact with an abundant (stable) species (J.orr; personal communications) It is possible if not probable, that the major product of MAB oxidation is formed by the reaction,



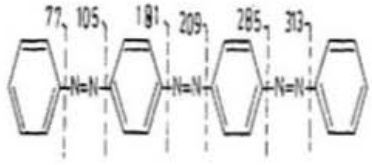
VI

The presence of MAB and MAB dimer in the metabolic profile of DAB suggests that DAB is first demethylated to MAB and then follows the same metabolic pathway as does MAB.

The formation of a larger number of products from AB and MAB than DAB probably indicates that the number of methyl groups on amino nitrogen not only affects the rate of oxidation of these compounds but also affects the number of oxidation products obtained. Since AB does not have any methyl group on its amino nitrogen, it is oxidized to a greater variety of products than MAB and in the same way MAB is oxidized to a greater extent than DAB.

The absence of any predominant product in the metabolic profile of AB suggests that the chain propagation reactions are more favourable than chain termination reactions. The most nonpolar product, *i.e*; R_f value, 0.93 and retention time, 30 minutes, was analyzed mass spectrally (Fig.19). The mass

number 390 indicates that it consists of two molecules of AB less four hydrogens, *i.e.* 197 x 2 = 394 - 4 = 390. The fragmentation pattern reflects that the coupling was head to head and it led to the formation of an azo bond between the two AB molecules (VIII).



VII

A small peak of this product was also observed in the metabolic profile of DAB (Fig.11b). This could be due to either of the following scheme:

(i) DAB ---> MAB ---> AB ---> [AB-AB]

or

(ii) DAB ---> MAB ---> [MAB-MAB] ---> [AB-AB]

Fig.2 depicts the mechanism of action of HRP and H_2O_2 system on electron donor substrate, where it is proposed that the substrate is oxidized to its free radical by donating one electron to the Peroxidase I or Peroxidase II compound. (Saunders *et al.*, 1964 & Bartsch & Hecker, 1971).

Fig.5 shows the oxidation of aniline by HRP/H_2O_2 system. This pathway has been suggested by Saunders *et.al.*, (1964). The free radical formed by the elimination of one electron is shown to dimerize and then polymerize leading to a complex known as Aniline Black.

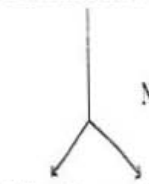
Bartsch & Hecker in 1971 proposed the peroxidase catalyzed metabolic pathway of N-Hydroxy-2-acetylaminofluorene (N-OH-AAF) as follows:

$$HRP + H_2O_2 \longrightarrow HRPI + H_2O$$

HRPI + N-OH-AAF ----------> HRPII + Nitroxide radical of N-OH-AAF

HRPII + N-OH-AAF ----------> HRP + Nitroxide radical of N-OH-AAF

Two nitroxide radicals ----------> An Intermediate Dimer of N-OH-AAF



2-Nitrosofluorene + N-OAc-AAF

The dimer intermediate was said to be formed and disproportionate very rapidly and therefore was not detectable.

The large amount of MAB dimer obtained from MAB oxidation (i.e; 40% of the total radioactivity of MAB was detected in the dimer) indicates the product is quite stable. Table 2 shows that it is formed very rapidly, *i.e.*, 89 % was formed within the first minute of the reaction. This indicates that probably a highly reactive and unstable intermediate leads to the formation of the dimer by chain termination reaction.

The formation of some of the minor products was not reproducible. Probably their highly reactive and therefore unstable nature did not allow their detection consistently.

The detection of dimer in the metabolites of all the three aminoazobenzenes and the available information in the literature suggests that MAB is oxidized to a free radical first and then the free radical dimerizes. However unlike the unstable N-OH-AAF dimer intermediate this dimer is fairly stable.

(ii) Oxidation at pH 5.0: The previous work which proposes the model of HRP/H_2O_2 mediated oxidation of aromatic amines (Fig.5) was carried out at an acidic pH (Saunders *et al.*, (1964). Griffin *et al.* (1979) reported that the decay rate of free radicals at acidic pH was much lower than at alkaline pH. Moreover, Tsuruta *et.al.*, (unpublished work) have reported that at acidic pH, the HRP/H_2O_2 catalyzed activated products of aromatic amino compounds showed at least two times more binding to DNA than at neutral pH. Therefore some of the work was carried out at pH 5.0.

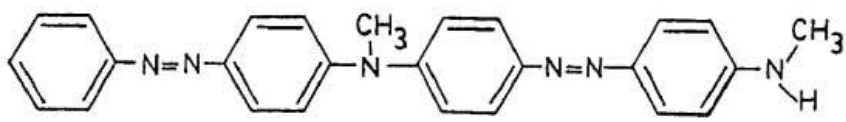
After oxidation of MAB catalyzed by HRP/H_2O_2 at pH 5.0, the number of products (especially of DAB on TLC plate) (Fig.14) was found to be larger than that obtained at pH 7.4 (Fig.12). None of the products seemed to be comparable with the products formed at pH 7.4. The major MAB and DAB product was orange in colour (the corresponding pH 7.4 product was yellow) though it had the same R_F value, *i.e*; 0.87. AB did not show any major product. The colour and R_F values of these products were different from those obtained at pH 7.4. The

separation of these products was not possible by HPLC since they were not soluble in methanol.

The complete reaction mixture had a different absorption spectrum (Fig.21) than that of pH 7.4 (Fig.17). Each of these spectra was similar to that of the major metabolite purified from it (Fig.23). Clearly different products are formed from aminoazobenzenes at the two pH values.

The mass spectrum (Fig.18) showed a molecular ion with mass 420, again suggesting that it was formed from two molecules of MAB with the elimination of two atoms of Hydrogen. But the fragmentation pattern clearly shows the difference.

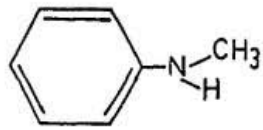
The sequential breakdown of the parent molecule from one end to the other suggests the structure IX.



VIII

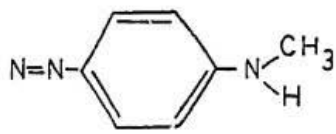
The relative intensity of the molecular ion was comparable with that of many fragments. There were fragments with masses greater than that of MAB (314, 285).

The fragment with mass 314 could be formed from the product by cleavage of the group with mass 106 (X),



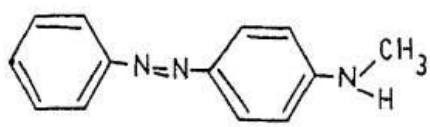
IX

The fragment with mass 285 could be formed by cleavage of the group with mass 134 (XI) and the addition of 1 H ,



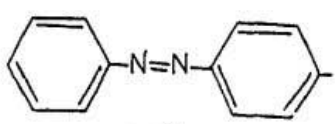
X

The fragment with mass 211 could be formed by the loss of the group (XII) and the addition of H.



XI

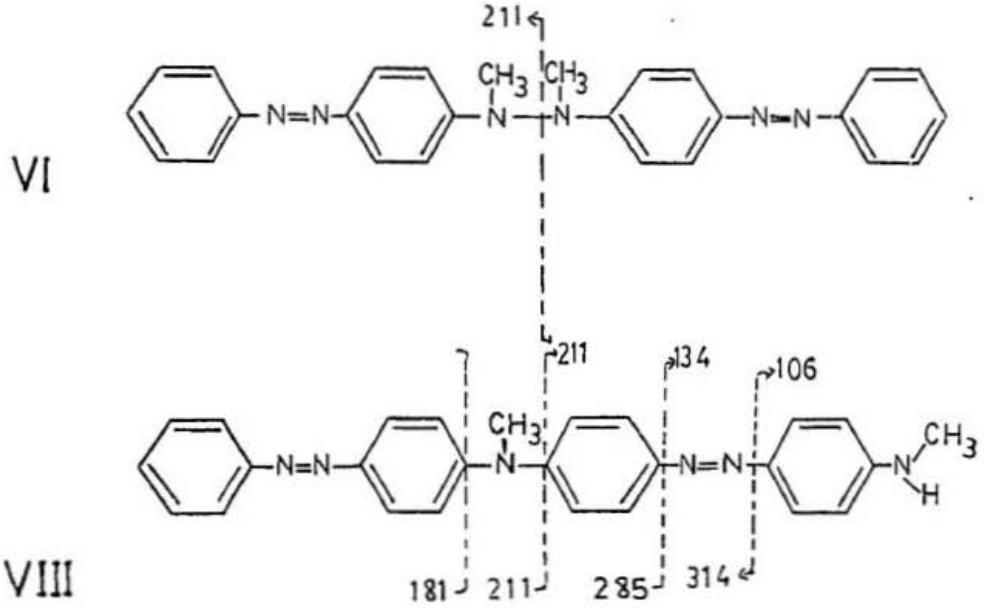
Furthermore the fragment with mass 181 corresponds to the structure,



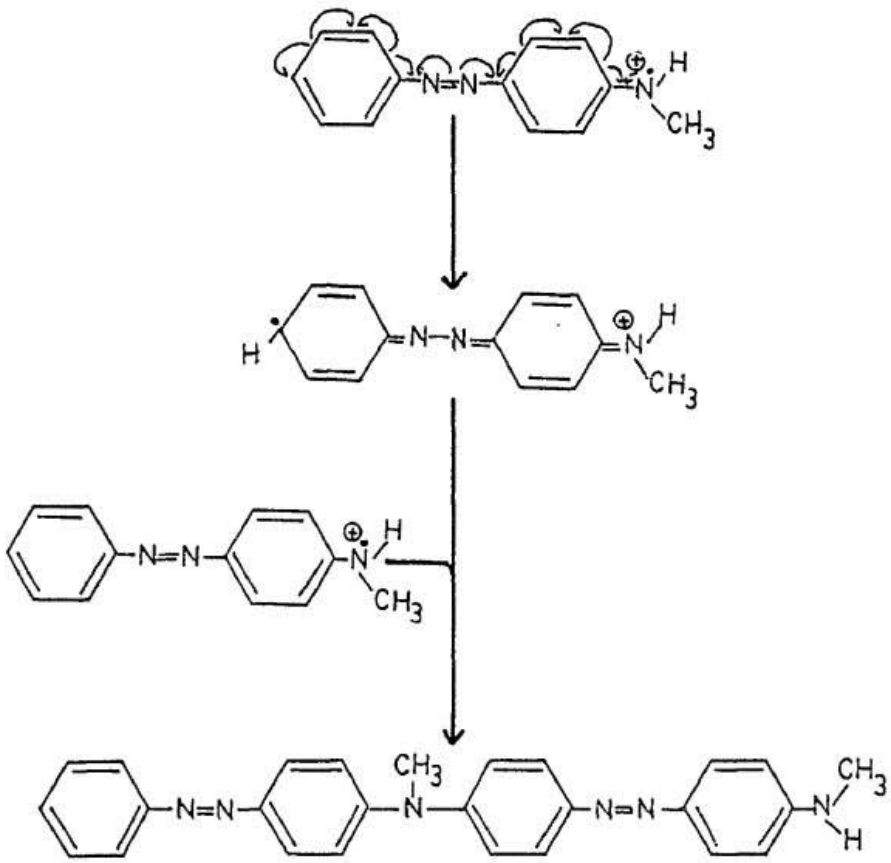
XII

Because the fragment with 211 does not have a strong peak, the fragmentation

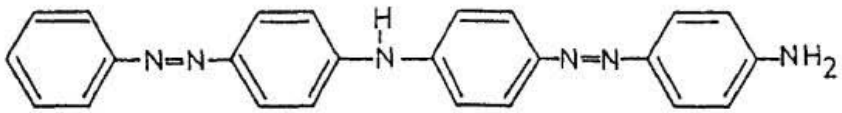
described above presumably occurred sequentially. The C-N bond being more stable than the N-N bond in the mass spectrometer, causes this type of fragmentation. Consequently the probable structure of the product (IX) could be formed by linking two molecules of MAB by a C to N bond, (rather than by an N-N or a C-C bond).



The reaction could occur after the transfer of the radical electron to an aromatic ring,



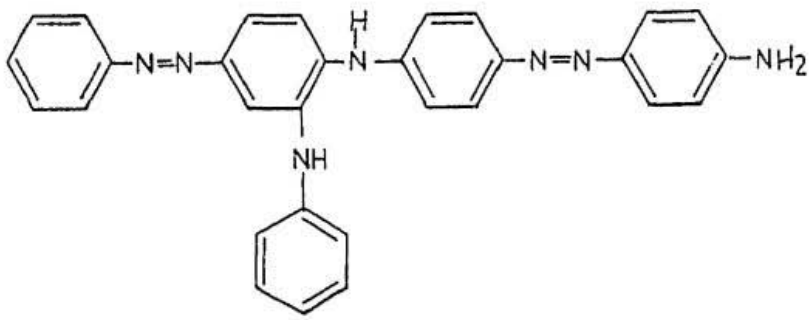
The mass spectrum of one of the oxidation product of AB isolated from the TLC plate (R_F 0.25) of the ethyl acetate:acetone extract of the reaction mixture at pH 5.0 (Fig.24) suggests that the product is a dimer of AB formed by C-N coupling,



XIII

(The mass number was 392, *i.e.*, two ABs less two hydrogens.) The head to tail

coupling caused it to lose two hydrogens unlike the pH 7.4 product which loses four hydrogens due to head to head coupling. An adjacent band (R_F 0.33) was also analyzed mass spectrally. The mass number of the molecular ion (Fig.23) suggests that the molecular weight is 484 The structure XIV is probably a fragmentation product of a trimer or tetramer and has been derived from the fragmentation pattern (Fig.23).



XIV

The structure XIV suggests that its precursor (XIII) is not a stable product like the MAB dimer. Rather it is further polymerized either by being attacked by a free radical or by an enzymatic reaction. The HRP/H_2O_2 catalyzed oxidation scheme of aromatic amino compounds (Fig.5)by Saunders et al. (1964) also suggests this possibility. The polymer, presumably a tetramer, seems to be very unstable and probably fragments into two molecules, one of them having a molecular weight 484, the isolated compound. This product was completely inhibited when ascorbic acid was added to the reaction mixture after two minutes of starting the reaction. The inhibition was associated with an increase in the

adjacent dimer (XIII) as the colour of that band was intensified with the disappearance of that of the (XIV). This suggests that ascorbic acid prevents further polymerization into the tetramer.

2.Binding of Oxidation Products to DNA:

It has been hypothesized that peroxidase catalyzed one electron oxidation converts chemical carcinogens into their reactive free radical forms which in turn bind covalently to the cellular DNA. Lasker *et al.* (1969) proposed that free radicals of 3,4 benzo(a)pyrene are involved in the iodine induced chemical binding to DNA. Then Len & Folk (1973) speculated the binding of MAB to DNA in the same system. Tarpley *et al.* (1980) discovered the structures of the adducts of the reactive MAB intermediates with DNA bases. As discussed earlier in "Introduction", Fig.4 shows that both N-(deoxyguanosine-8-yl)-MAB and 3-(deoxyguanosine-^{2}N-yl)-MAB are basically adducts of MAB and the guanine residue of DNA. These adducts were formed by the rat liver DNA when MAB was administered to the rats in the diet. This observation suggests that the reactive intermediate binding to DNA was a nitrenium ion of MAB as similar adducts were formed with N-benzoyloxy-MAB and DNA *in vitro*. Fig.28 shows that at least two products were responsible for binding to DNA, one was very reactive and therefore showed maximum binding in the first minute of the reaction, the other seemed to be comparatively stable and hence the binding was observed even when DNA was added to the reaction mixture at 15 to 30 minutes after starting the reaction.

O'Brien (1984) has suggested that the imine derivatives of p-

phenylenediamine trimers are oxidized at pH 4.0 and bind to DNA. Although from the present results the MAB dimer seems to be quite stable, it is possible that it is further oxidized (at a very low rate) to iminium form. And probably it is this product that binds to DNA when DNA is added to the reaction mixture at 15 to 30 minutes after starting the reaction.

Table 4 shows that the most preferred homo polyribonucleotide for MAB oxidation products was found to be homo polyriboguanylic acid. The minimum binding was shown by homo polyribocytidylic and homo polyribouridylic acid. Thus, it can be proposed that the ultimate carcinogenic forms of MAB preferentially bind to guanine residues in DNA. However, purines when tested for DNA binding did not show any binding (Table.5).

3.Effect of Reducing Agents on Binding to DNA:

The inhibitory effect of antioxidants on chemical carcinogenesis has been reported previously (Wattenberg, 1978). Kleihus *et al.* (1975) have suggested that the radioprotective agent cystamine inhibits the metabolic activation of nitrosamines and hence the nitrosamine induced methylation of hepatic DNA. Ascorbic acid has also been reported to exihibit the same effect.

The carcinogenesis inhibiting property of the trapping agents can be used to trace and understand the mechanism of activation of the chemical carcinogens. The inhibition in DNA binding in the presence of a reducing agent when compared with the metabolic profile in the presence and absence of the same agent can provide quite valuable information, *e.g*; the disappearance of a product and/or products if associated with the inhibition in DNA binding could help to

recognize the reactive intermediate in the metabolic profile of a certain carcinogen.

The reducing agents can achieve the inhibition in DNA binding and consequently the carcinogenicity of a compound in three possible ways;

(i) by reducing the reactive intermediates back to the original compound,

(ii) by reducing the intermediate to a an unreactive product,

(iii) by forming an adduct with the reactive intermediate and preventing DNA binding.

Though this is a promising area for understanding the mechanism of activation, very little if any attention has been paid to this field. As the reducing agents are known to reduce the free radicals (Floyd, 1980), their effect for understanding peroxidase catalyzed oxidation could be particularly valuable.

Laishes & Stich (1973), while studying the DNA damage in the human skin fibroblasts induced by 2-acetylaminofluorene (AAF), suggested that cysteine could probably decrease the damage by enhancing the repair of DNA. However, no evidence is available to support this proposal. Rosin & Stich (1973) reported that the N-hydroxy-AAF and N-acetoxy-AAF induced mutagenesis in *Salmonella typhimurium* was inhibited by cysteine. Two possible suggestions were extended;

(i) Cysteine prevents DNA by interacting with the reactive nitrenium ion intermediate of N-acetoxy-AAF,

(ii) the inhibition of DNA replication by cysteine could also prevent the cell cycle specific action of N-acetoxy-AAF (if N-acetoxy-AAF only affects the replicating DNA).

Ascorbic acid was found to inhibit strongly the oxidation as well as the DNA binding by MAB (Table 6). 100 μM added to the reaction mixture reduced the DNA binding from 100% to 8% and the total oxidation was also reduced to the same extent. 50 μM concentration however showed lesser inhibition. The rapid oxidation of ascorbic acid by HRP/H_2O_2 on the addition of MAB to the reaction mixture (Fig.29) can explain that the oxidation products of MAB (MAB free radicals) are reduced back to MAB by oxidizing the ascorbic acid and as a result the total oxidation of MAB appears to be inhibited. The DNA binding is then inhibited indirectly due to the unavailability of the reactive electrophilic intermediates.

The observation that ascorbic acid did not show its inhibitory effect on the oxidation of MAB and binding of its products to DNA when it was added to the reaction mixture after one minute of starting the reaction (results not included) illustrates the rapidity of the reaction and indicates that the free radical and/or other reactive intermediates are highly unstable and therefore are converted into the stable metabolites within the first minute of the reaction and consequently ascorbic acid does not reduce them back to MAB.

NADPH reduced the DNA binding of MAB from 100% to about 50-60% but the total oxidation of MAB was not found to be inhibited even with 5 nmoles of

NADPH. The rapid oxidation of NADPH with MAB/HRP/H_2O_2 in the reaction mixture was demonstrated by measuring the decrease in the NADPH absorbance at 340 nm. Thus NADPH does not reduce the MAB free radicals back to MAB but reduces the reactive intermediates to the inactive reduced forms. Therefore the total oxidation remains unaffected but the DNA binding is reduced to about 50 to 60%.

Cysteine showed a similar effect, *i.e*; no inhibition of the total oxidation but a 32% decrease in DNA binding. At higher concentrations the binding of activated MAB to DNA was further decreased without affecting the total oxidation (Table 6). N-acetyl-L-cysteine was found to be better than cysteine and NADPH at inhibiting both the total oxidation of MAB and binding of its products to DNA. However the total oxidation was inhibited to a lesser extent than the DNA binding. Glutathione showed more or less similar effects. These results with the sulpher containing amino acids and glutathione suggested the possibility of the formation of adducts of the reactive electrophilic intermediates with these reducing agents. One of the important functions of these compounds in the body is considered to be the excretion of toxic products by forming the water soluble adducts with them and rendering them excretable. Therefore, the aqueous phase of the reaction mixture after the extraction of the organic soluble metabolites was tested for the adducts. The results shown in Table 7 and Table 8 ruled out this possibility. However pH 6.3 at a higher peroxidase concentration showed the formation of water soluble glutathione-MAB adducts (Rahimtula,M & O'Brien,J.P., personal communications)

Uric acid inhibited both the total MAB oxidation and DNA binding very effectively at a concentration of 100 μM. Its rapid oxidation was noted by the HRP/H_2O_2 and MAB by observing the decrease in uric acid absorbance at 300 nm.

The phenolic antioxidant butylated-hydroxyanisole (BHA) and α-naphthol exhibited a strong inhibition of MAB oxidation as well as DNA binding. It can be assumed again that like ascorbic acid and uric acid they reduce the free radicals formed from MAB immediately back to MAB and are themselves oxidized. 4-methyl catechol was found to have the same effect. However, xylenol (2,6-dimethyl phenol) did not inhibit the total oxidation but reduced DNA binding to 58%, when it was added at the concentration of 50 μM.

However, in order to understand the whole mechanism of the initiation of methyl aminoazobenzenes's carcinogenicity completely, further work is required to identify;

(i) the different reactive intermediates formed during MAB oxidation,

(ii) effect of the antioxidants, individually and in different appropriate combinations on the metabolic profiles and DNA binding,

(iii) adducts formed as a result of *in vivo* and *in vitro* bindings,

(iv) adducts of the reactive intermediates and the antioxidants.

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