STIMULATION OF LIPID PEROXIDATION BY DIHYDROXYFUMARATE: THE ACTION OF ANTIOXIDANTS AND THE ROLE OF FREE RADICALS

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by

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ABSTRACT

The absolute rate constants for the reactions of free radicals $OH^{\cdot}$, e^{-}_{aq}, $Br_2^{\cdot -}$, $I_2^{\cdot -}$, $(SCN)_2^{\cdot -}$, $N_3^{\cdot -}$, $CPZ^{\cdot +}$, NO_2 with dihydroxyfumaric acid have been determined by pulse radiolysis. Reactions of the isopropanol radical ($CH_3\dot{C}OHCH_3$) with dihydroxyfumaric acid, dithiothreitol (DTT), α-methyl propionyl glycine, cysteine, cysteamine, glutathione and D-penicillamine, the iron complexes Fe^{3+}/EDTA and Fe^{3+}/DTPA and 5,5-dimethyl-1-pyrroline-1-oxide (DMPO), and the stable free radical 4-hydroxy:-2,2,6,6-tetramethyl piperidino-1 oxyl (TMPN) have been measured by competition using p-nitroacetophenone (PNAP) as a reference. The rate constants of reaction of the halogenated peroxy radicals derived from carbon tetrachloride, halothane and chloroform with β-carotene have been also meansured. The yields of destruction of dihydroxyfumaric acid exposed to ^{60}Co γ-rays in aqueous solutions and the oxygen consumption at different doses rates have been studied.

At the biochemical level, the peroxidation of lipids of rat liver microsomes stimulated by additions of ferrous sulphate/dihydroxyfumaric acid have been studied as function of concentration, pH, temperature and incubation time. The inhibitory effects of propyl gallate, promethazine, 6 hydroxyl 1,4 dimethyl carbazole (HDC), the chelating agents desferal, EDTA, DTPA, 1:10 phenanthroline, 8-hydroxyquinoline have been assessed by measuring the ED_{50} for malondialdehyde (MDA) formation using thiobarbituric acid (TBA) as an indicator. The individual stimulatory or inhibitory effect on MDA production of the organic ions Ni^{2+}, Mg^{2+}, Ca^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+} and Mn^{2+} were evaluated.

SUMMARY

1. Using the pulse radiolysis technique, the absolute rate constants for the reactions of free radicals $OH^{\cdot}$, e^{-}_{aq}, $Br^{\cdot}_{2}{}^{-}$, $I^{\cdot}_{2}{}^{-}$, $(SCN)^{\cdot}_{2}{}^{-}$, $N^{\cdot}_{3}$, NO_2, $CPZ^{\cdot+}$ with dihydroxyfumaric acid have been determined.

2. The rate constants of the reaction of the isopropanol radical ($CH_3\dot{C}OHCH_3$) with dihydroxyfumaric acid, the thiol compounds dithiothreitol (DTT), α-methyl propionyl glycine (thiola), cysteine, cysteamine, glutathione, and D-penicillamine and the iron complexes chelated with EDTA, DTPA, desferal have been determined.

3. The rate constants of the methyl peroxy radicals derived from CCl_4, halothane and chloroform with β-carotene have also been determined.

4. The yields of destruction of dihydroxyfumaric acid exposed to ^{60}Co γ-rays at different dose rates, in aqueous solution of different pH values have been measured.

5. The stable free radicals such as triacetonemine-N-oxyl (TAN), and TMPN and the antioxidant propyl gallate had protective effect.

6. Rat liver microsomal lipid peroxidation was stimulated <u>in vitro</u> by Fe^{2+}/dihydroxyfumaric acid; the increase in malonaldehyde (MDA) was found to be dependent on time, ferrous concentration, pH and temperature.

7. Pre-heating of microsomes at 100°C for 15 minutes still permitted substantial peroxidation by Fe^{2+}/ dihydroxyfumaric acid.

8. Peroxidation was inhibited by 6-hydroxy 1,4 dimethyl carbazole (HDC), propyl gallate, promethazine, chlorpromazine, the chelating agents desferal, EDTA, DTPA, 1:10 phenanthroline, 8-hydroxyquinoline, their ED_{50} values were obtained for MDA production using TBA as an indicator.

9. Fe^{2+}/dihydroxyfumaric acid - mediated lipid peroxidation was inhibited by SKF 525A, metyrapone, and slightly affected by 2,5 diphenylfuran, β-carotene, superoxide dismutase (SOD), p-chloromercuribenzoate (pCMB) and was unaffected by diazobicyclooctane (DABCO), catalase, tryptophan, thiourea, picolinic acid and sodium salicylate.

10. Additions to the microsomal fraction of the inorganic ions Ni^{2+}, Mg^{2+}, Ca^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+} and Mn^{2+} were useful to evaluate the individual stimulatory or inhibitory effect on MDA production.

11. Apart from the significance of the results obtained in elucidating the mode of action of Fe^{2+}/dihydroxyfumaric acid, important points have been made concerning the pitfalls of the experimental approach adopted here; this approach has been investigated, the antioxidant action of compounds of biological interest.

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

a) General aspects

The term "radical" has a long history. The concept of "radicals" was introduced by Lavoisier in his theory of acids, which he believed to be compounds of oxygen. He designated the element or group of elements which combined with oxygen in the acid a "radical". The oxygen theory of acids soon passed out of favour, but the word "radical" was still frequently used to signify a group of elements which retained their identity through a series of reactions, for example, the ethyl groups in diethyl sulfide. The distinction between "free" and "bound" radicals, was not possible until the development of methods by which free radicals could be characterised directly.

The modern interest in free radical reactions perhaps began in the 1930's with the studies of Kharasch and Mayo, who demonstrated that the anomalous addition of HBr to propene involves radicals, and the studies of Waters and Hey on the mechanism of homolytic aromatic substitution. (Pryor, 1976.)

A free radical may be defined as any atom, group of atoms or molecules in a particular state with one unpaired electron occupying an outer orbital. A few common molecules, nitric oxide (NO) and nitrogen dioxide (NO_2), contain an unpaired electron in an outer orbital in their normal state and by definition, are free radicals.

A biradical is a species containing two unpaired electrons in outer orbitals and molecular oxygen (O_2) is an example. A "radical ion" is a free radical with a positive or negative charge, e.g. H_3N^+, the protonated amine radical and $O_2^{\cdot -}$, the superoxide anion radical.

b) The importance of free radicals in Biology

Free radicals have now been identified in many biological systems. There is good evidence that endogenous free radical reactions play essential roles in numerous biochemical pathways, e.g. hydroxylation reactions by the endoplasmic reticulum (McCay and Poyer, 1976); phagocytosis by macrophages and leukocytes (Babior et al., 1973; and McCord, 1974); lipid peroxide catabolism (Chow and Tappel, 1972) and photosynthesis (Sogo et al., 1957).

Such reactions, however, are generally well controlled, but the possibility of unintended damaging reactions of free radicals with essential biomolecules is always possible. This apparent paradox is one of the difficulties inherent in the study of free radical mechanisms in medicine and biology. A specific free radical species may be normal or pathological in a highly variable fashion, depending on the milieu in which it is found. The same species which is normally innocuous can cause lethal damage in different circumstances, the term

"normal" or "pathologic" become relevant only when taken in the context of the system or process being examined and are not inherent properties of free radicals.

The key issue that differentiates normal and pathological free radical process is the control and containment of free radical reactions by various mechanisms. All biological systems exist in a state of balance such that vital free radical reactions may continue in the midst of susceptible biomolecules. This homeostatic condition may be unbalanced by the introduction of free radicals in such numbers or types so that the normally existing control mechanisms are no longer adequate to cope effectively with this added burden. Alternatively, a failure of some or all of the endogenous control mechanisms can upset the normally balanced system so that the previously controlled radical species can no longer be restrained. A crude anology is a nuclear reactor pile in that the conditions which allow an explosion may be created by the introduction of excess reactive fuel or the failure of the controlling elements; in either case the system can no longer contain the reaction.

Some toxic agents that involve free radical reactions are listed (Table 1).

Table 1 : Toxic Agents that involve Free Radical Reactions:

Class	Agents	References
1.- Alkyl polyhalides	CCl_4	Di Luzio (1973)
	DDT, PBB, Freon	Walling and Huyser (1963)
	Carcinogens	Seligman and Demopoulos (1973)
2.- Alcohols and their metabolites	Ethanol/acetaldehyde	Di Luzio (1973); Flamm et al., (1977)
	Methanol/formaldehyde	Walling and Huyser (1963)
3.- Oxidant gases	Hyperbaric oxygen	Kimball et al., (1976); Takala and Niinikoski (1974).
	Singlet oxygen	Krinsky (1974)
	Superoxide	Fridovich (1974)
	Ozone	Goldstein et al., (1970)

Table 1 - continued

Class	Agents	References
4.- Free radical gases	NO, NO_2	Stacey and Harris (1963)
5.- Extravasated blood	Platelets Polymorphonuclear Leukocytes	Kaufman et al., (1973); Hamberg and Samuelsson (1974) McCord (1974)
6.- Ischemia/cellular hypoxia	Uncontrolled electron transport free radicals	Ruzicka et al., (1975; Flamm et al., (1979)
7.- Biogenic amines	6-OH DOPA and 6-OH DOPA amine	Sachs and Jonsson (1975); Cohen and Heikkila (1974)
8.- Quinones	Alloxan/Dialuric acid	Cohen and Heikkila (1974)

Table 1 - continued

Class	Agents	References
9.- Metals and other elements	Iron Platinum Copper Phosphorus	Tappel (1961) McGinnes et al., (1978) Yu et al., (1974) Stacey and Harris (1963)
10.- Polymers	Polyvinyl chloride residues of monomers	Walling and Huyser (1963)
11.- Xanthines	Xanthine oxidase and Superoxide radical	Kellogg and Fridovich (1977)
12.- Deficiency states	α- tocopherol Selenium	Williams et al., (1975); Yu et al., (1974) Serfass and Ganther (1976)

Table 1 - continued

Class	Agents	References
13.- Radiation	Ultraviolet	Seligman and Demopoulos (1973)
	Ionizing	Alper and Hodgkins (1969)

c) The toxicity of superoxide anion

Much of the current interest in the role of free radicals in disease stems from the finding that the copper-containing protein erythrocuprein catalyses the dismutation of the superoxide radicals $O_2^{\cdot -}$.

$$O_2^{\cdot -} + O_2^{\cdot -} \xrightarrow[2H^+]{\text{enzyme}} H_2O_2 + O_2 \qquad (1)$$

The protein has since been renamed superoxide dismutase and has been credited with an important function of protecting the cell from the potentially damaging reactions of the superoxide radical anion. Although the true damaging nature of this radical is matter of debate, it has been demonstrated that reactions of $O_2^{\cdot -}$, formed in the autooxidation of menadione or dialuric acid or from the autooxidation of dihydroxyfumaric acid can lead to the haemolysis of erythrocytes (Goldberg and Stern, 1976; Leipzing et al., 1975; Cohen and Heikkila, 1974; and Goldberg and Stern, 1977a, 1977b). Recent results, however, suggest that the true haemolytic agent is likely to be the hydroxyl radical $OH^{\cdot}$, the singlet oxygen $O_2(^1S)$ or hydrogen peroxide H_2O_2, formed as the result of secondary reactions of the superoxide radical anion. (Haber and Weiss, 1934; Michelson and Durosay, 1977; Lynch and Fridovich, 1978).

$$2H^+ + O_2^{\cdot -} + O_2^{\cdot -} \longrightarrow H_2O_2 + {}^1O_2 \qquad (2)$$

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

$$OH^{\cdot} + H_2O_2 \longrightarrow O_2^{\cdot -} + H_2O + H^+ \quad (4)$$

$$OH^{\cdot} + O_2^{\cdot -} \longrightarrow {}^1O_2 + OH^- \quad (5)$$

The production of free hydroxyl radicals may also occur by the breakdown of H_2O_2 catalysed by metals such as iron, the Fenton reaction (Haber and Weiss, 1934).

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH^{\cdot} \quad (6)$$

$$OH^{\cdot} + H_2O_2 \longrightarrow H_2O + H^+ + O_2^{\cdot -} \quad (4)$$

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

$$Fe^{3+} + H_2O_2 \longrightarrow Fe^{2+} + 2H^+ + O_2^{\cdot -} \quad (7)$$

$$Fe^{3+} + O_2^{\cdot -} \longrightarrow Fe^{2+} + O_2 \quad (8)$$

These reactions do not represent a complete list of those taking place in Fenton's reagent, but they do show catalytic amounts of iron salts can cause decomposition of H_2O_2 by way of $O_2^{\cdot -}$ and $OH^{\cdot}$ reactions (3 and 4). Indeed, reaction 3 shows that $O_2^{\cdot -}$ could itself cause a free radical chain decomposition of H_2O_2. The hydroxyl radical is a powerful oxidant. It is generated by the radiolysis of water and accounts for a large fraction of the effects of such radiation in biological systems.

For this reason the chemistry of $OH^{\cdot}$ has been extensively studied.

In some works, it has been suggested that $O_2^{\cdot -}$ or singlet oxygen formed as a result of its dismutation are involved in the peroxidation of lipids (Richter et al., 1975; Fee and Teitelbaum, 1972).

d) Lipid peroxidation

The role of membrane lipid peroxidation as a primary event in the toxicity of xenobiotics has been subject of intense interest and controversy in recent years (Bus and Gibson, 1979).

Peroxidation of unsaturated membrane lipids can lead to:

(a) the perturbation of the membrane microarchitecture due to the introduction of hydrophilic functions, with a resultant alteration in membrane permeability.

(b) inhibition of enzyme activity by toxic degradation products.

(c) cross-linking and polymerisation of proteins and nucleotides by free radical intermediates in the peroxidizing lipids.

Lipid peroxidation has been broadly defined (Tappel,

1973), as the oxidative deterioration of polyunsaturated lipids. Peroxidation of lipids involves the reaction of oxygen with polyunsaturated lipids and the formation of lipid free radicals and semi-stable hydroperoxides, which then promote free radical chain oxidations (Tappel, 1973; Barber and Bernheim, 1967).

Like other free radical chain reactions, lipid peroxidation can be considered to occur in three steps (Pryor, 1973). The first is the "initiation" process by which the lipid radicals are generated. The second is a series of "propagation" reactions in which the number of free radicals is conserved as the peroxidation reactions proceed. Finally, there is a series of "termination" reactions by which free radicals are destroyed.

Initiation

An initiation reaction is a reaction in which free radicals are formed, reaction (9).

$$x{:}y \longrightarrow x^{\cdot} + y^{\cdot} \qquad (9)$$

A two-electron bond is broken homolytically to produce two fragments, each of which contains a lone electron. This occurs spontaneously with some substances, like peroxides, $RO{:}OR \longrightarrow 2RO^{\cdot}$ and is facilitated by heat and heavy metals. Physical agents like ultraviolet light or X-irradiation can also cleave chemical bonds

e.g.,

$$Cl_2 \longrightarrow 2Cl^{\cdot} \qquad (10)$$

Alternatively, initiation can occur through the reaction of a radical which does not subsequently participate in the chain reaction, for example, the hydroxyl radical from the Fenton reaction mentioned above or the trichloromethyl peroxy radicals formed from the one electron reduction of carbon tetrachloride in the presence of oxygen.

$$e^-_{aq} + CCl_4 \longrightarrow CCl_3^{\cdot} + Cl^- \qquad (11)$$

$$O_2 + CCl_3^{\cdot} \longrightarrow CCl_3O_2^{\cdot} \qquad (12)$$

$$CCl_3O_2^{\cdot} + RH \longrightarrow CCl_3O_2H + R^{\cdot} \qquad (13)$$

Propagation

These reactions are the single most important event, biological speaking, because of the fact that minute quantities of radical species are capable of producing widespread chemical damage. To mention briefly, the propagation steps which perpetuate the free radical centre in a chain reaction are of four types:

1. Atom transfer; often involving hydrogen,

$$R^{\cdot} + R'H \longrightarrow RH + R'^{\cdot} \qquad (14)$$

or

$$RO_2^{\bullet} + R'H \longrightarrow RO_2H + R'^{\bullet} \qquad (15)$$

Such hydrogen abstractions are basic to lipid radical reactions.

2. Addition reactions;

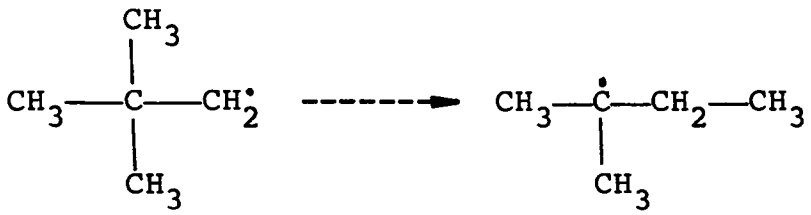
$$CH_3^{\bullet} + RCH{=}CH_2 \longrightarrow R\dot{C}HCH_2CH_3 \qquad (16)$$

3. Fragmentation reactions; this class of reactions occur because of the tendency for radical chain reactions to "run-down", energetically speaking. Thus a large alkyl (carbon-centred) radical will fragment into smaller radicals (methyl, ethyl, or propyl) when possible. Alkoxy (oxygen-centred) radicals will often fragment to liberate a β-cleavage product with the formation of a carbonyl structure, (aldehyde, ketonic and acidic functions are produced in this way). Likewise, an alkyl radical can fragment releasing a smaller radical species, forming a trans double bond. Whether the double bond is formed between two carbons, a carbon and oxygen, or a carbon and nitrogen, this process of cleavage of bonds with the release of a smaller radical fragment is called β-scission, e.g.

$$R{-}\overset{|}{\underset{|}{C}}{-}\overset{|}{\underset{|}{C}}{\cdot} \longrightarrow R^{\bullet} + \overset{|}{\underset{|}{C}}{=}\overset{|}{\underset{|}{C}} \qquad (17)$$

4. Rearrangement reactions; this occurs when the radical centre shifts from one atom within a single molecule to

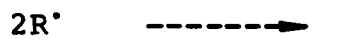
another, resulting in a structural rearrangement of the molecule. This can be considered as an intramolecular atom transfer, e.g.



(18)

Termination

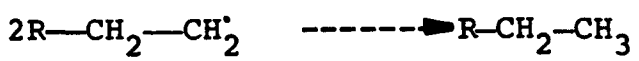
1. radical-radical combination: two radicals can combine to form a new covalent bond



(19)

These termination are important from the point of view of creating anomalous molecular structures in strategic locations, for instance among the membrane lipids and proteins, nucleic acids, or the oligoglycans of glycoproteins.

2. disproportionation: two free radicals can react so that one is reduced and the other forms a double bond.

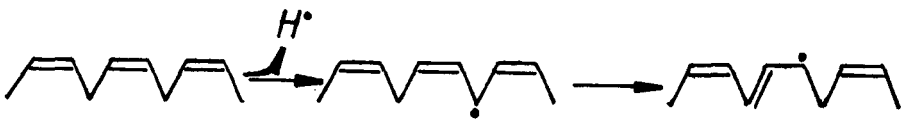


(20)

This is the second type of reaction for the formation of trans double bonds found in peroxidatively damaged lipids.

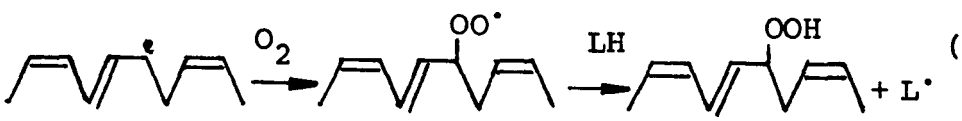
Briefly, the three steps of lipids peroxidation can be simplified as follows:

1.- Initiation:



(21)

2.- Propagation:



(22)

3.- Termination:

$$L^{\bullet} + L^{\bullet} \longrightarrow \text{nonradical products} \quad (23)$$

$$L^{\bullet} + LO_2^{\bullet} \longrightarrow \text{nonradical products} \quad (24)$$

$$LO_2^{\bullet} + LO_2^{\bullet} \longrightarrow \text{nonradical products} \quad (25)$$

Where:

LH = polyunsaturated lipid;

$L^{\bullet}$ = lipid radical;

$LO_2^{\bullet}$ = lipid hydroperoxy radical.

As shown above, unsaturated fatty acids are susceptible to peroxidation since the presence of a double bond weakens the carbon-hydrogen bond on the carbon atom

adjacent to the unsaturated carbon-carbon bond (Swern, 1961; Demopoulos, 1973). Thus, allylic hydrogens are partially "activated" and are susceptible to abstraction by small amounts of initiators (initiation reaction). The initiation of lipid peroxidation in biological tissues may be accomplished by singlet oxygen (a higher state of oxygen), hydroxyl radicals, and a number of other radicals produced from a variety of environmental chemicals.

The lipid hydroperoxides (LOOH) that are generated in the various initiation reactions are unstable and decompose to form additional radical products. The decomposition of lipid hydroperoxides is catalysed by trace amounts of transition metal ions (Holman, 1954; Barber and Bernheim, 1967; and Heaton and Uri, 1961).

$$LOOH + M^{n+} \longrightarrow LO^{\cdot} + OH^{\cdot} + M^{(n+1)+} \quad (26)$$

$$LOOH + M^{(n+1)+} \longrightarrow LOO^{\cdot} + H^{+} + M^{n+} \quad (27)$$

The catalytic decomposition of lipid hydroperoxides along with the propagation reactions previously described are, therefore, autocatalytic, as more free radicals are the products of the reactions. The autocatalytic reactions continue until interventions by antioxidants, which react with the free radicals and thereby interrupt the chain reactions process occur (Demopoulos, 1973). The exact nature of metal chelates and their distribution

in the extracellular and intracellular space is not well understood: a variety of chelated metals particularly iron and copper chelated to substances, such as ADP and citrate, may be important (Del Maestro, 1980). Since the chelated metals at physiological pH would be in the Me^{n+} form in equilibrium with a much smaller amount in the reduced form, $Me^{(n-1)+}$, $O_2^{\cdot -}$ generation may exert a controlling influence on the generation of OH$^{\cdot}$ by reducing the Me^{n+} present. The dismutation product of $O_2^{\cdot -}$, H_2O_2, then reacts with $Me^{(n-1)+}$, as follows:

$$Me^{(n-1)+}\text{chelate} + H_2O_2 \longrightarrow Me^{n+}\text{chelate} + OH^{\cdot} + OH^{-} \quad (28)$$

to regenerate Me^{n+} and therefore this cycling can continue even in the presence of minute amounts of metal catalyst. It has been suggested (Czapski and Ilan, 1978) that the formation of a $Fe^{3+}O_2^{-\cdot}$ complex may be one mechanism for increasing the lifetime of $O_2^{\cdot -}$ and thereby contribute to its toxicity. Superoxide-metal chelated complexes may also be intimately involved in the peroxidation of lipids (Del Maestro, 1979). Clearly therefore, the presence of metal catalysts in any model or biological system studied as in the living organism will exert a profound influence on subsequent OH$^{\cdot}$ formation and the biochemical and cellular injury related to OH$^{\cdot}$ dependent processes.

Membranous organelles such as mitochondria and endoplasmic reticula are readily peroxidized because of their high content of polyunsaturated fatty acids (Rouser, et al.,

1968). Malonaldehyde (MDA) is one of the final degradation products and its presence can be detected by adding thiobarbituric acid to an acid extract of the peroxidized lipid. After suitable incubations the resulting chromophore can be read spectrophotometrically at λ=535nm. This method is sensitive, simple and convenient to use, correlating well with other means of detecting peroxidation (Barber and Bernheim, 1967; Bus and Gibson, 1979; and Esterbauer and Slater, 1981).

e) Inhibition of lipid peroxidation by antioxidants

Clearly, lipid peroxidation is an extremely destructive process because of the perpetuating free-radical chain reaction. If it is left unchecked, all polyunsaturated fatty acids in the biomembrane may be consumed. Some of these fatty acids radicals, however, may undergo cross-linkages with one another. This free radical quenching reaction in arresting the process of lipid peroxidation remains a topic of research.

Nucleophiles which inhibit the free radical process involved in the peroxidation of polyunsaturated fatty acids are collectively termed "antioxidants". Most antioxidants suppress lipid peroxidation by furnishing electrons or hydrogen atoms to quench the free radicals. Other substances may exert their inhibitory action in

different ways. For instance, EDTA can inhibit lipid peroxidation by binding ferrous iron involved in initiation and propagation reactions. This last type is not a free radical scavenger, but could be considered as an antioxidant.

In normal circumstances, organisms must be able to deal with highly damaging radical species. In order to reduce the numbers of chemical species which are capable of initiating free radical reactions, cellular defence systems have been developed to give protection, i.e. superoxide radicals are removed by the superoxide dismutase (Fridovich, 1978), hydrogen peroxide and organic hydroperoxides are reduced by catalase and glutathione peroxidase, respectively (Chance et al., 1979; Mills, 1957). In addition to the enzymatic protection, mammalian tissues also possess several low molecular weight compounds capable of scavenging free radicals, notably the tocopherols and ascorbic acid (Slater, 1972).

However, the situation with ascorbic acid is complicated. The vitamin itself can undergo auto-oxidation in the presence of metal ions and can stimulate as well as inhibit lipid peroxidation, depending on the conditions (Dianzani and Ugazio, 1978; Römer et al., 1981). The related compound dihydroxyfumaric acid also undergoes autooxidation in the presence of metal ions. A commercial application of dihydroxyfumaric acid is found in a patent developed by Nelson Research and Development

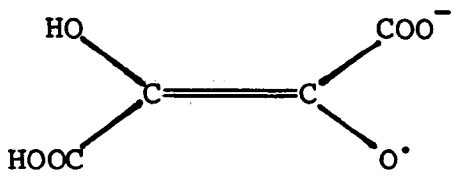
Co.,(1979). The invention consisted in a physiologic solution containing dihydroxyfumaric acid, employed for sterilizing contact lenses submerged in cultures of Staphylococcus or Pseudomonas at concentration of 10^8 organisms/ml. After 30 minutes of treatment in the dihydroxyfumaric acid solution, the contact lenses contained only 10^2 organisms/ml. Since, the participation and formation of free radicals in biological systems has been involved. It is the aim of the present thesis to study the free radical properties of dihydroxyfumaric acid.

f) Biochemical studies with dihydroxyfumaric acid (DHF)

Although dihydroxymaleic acid was prepared as long ago as by (Burgoin, 1874) and later studied extensively by (Fenton, 1894; 1895; 1896; 1898) and (Fenton and Wilkis, 1912), for many years, no satisfactory proof of its configuration has been given, until Hartree (1953), and Gupta (1953) showed evidences that the so-called dihydroxymaleic acid was dihydroxyfumaric acid.

Yamazaki, et al., (1960), in one of the first studies of transient radicals in solution, reported the observation of direct physical evidence of free radicals generated during the peroxidatic oxidations of hydroquinone, ascorbic acid and dihydroxyfumaric acid. The

signals monitored for dihydroxyfumaric acid were single assymetric lines, and were tentatively assigned to the following free radical.



Halliwell and Ahluwalia (1976), demonstrated that in the presence of dihydroxyfumaric acid, horseradish peroxidase catalyses the conversion of p-coumaric acid into caffeic acid at pH = 6. This hydroxylation was completely inhibited by superoxide dismutase as well as by low concentrations of Mn^{2+} or Cu^{2+}. Again, Halliwell and De Rycker (1978), studying the system dihydroxyfumaric acid/peroxidase found that O_2 uptake was completely inhibited by superoxide dismutase added at any time during the reaction, whereas heat denatured enzyme had no effect.

Goldberg and Stern (1977a) investigated the auto-oxidation of dihydroxyfumaric acid and found that in human red cells exposed to this compound a rapid breakdown of the cellular haemoglobin to methemoglobin and other green pigments occurred. This haemoglobin breakdown was inhibited by superoxide dismutase or catalase and was accelerated by lactoperoxidase (LP) added externally to the red cell medium.

Autor et al., (1980), showed that at physiologic pH pulmonary macrophages incubated with concentrations of dihydroxyfumaric acid ranging from 180 to 720 µg/ml, suffered a significant loss of viability after two hours, the extent depending upon dihydroxyfumaric acid concentration. The toxic effect was preventable by superoxide dismutase and catalase additions to the incubation system before the dihydroxyfumaric acid was added. Their results strongly suggested that the hydroxyl radicals generated could be the radical species toxic in the system under analysis.

Recently, Fischer and Hamburger, (1981) exposed isolated rat pancreatic islets for 5 minutes to 0.4 mg/ml of dihydroxyfumaric acid in an oxygenated buffer solution, and as a result of that exposure, the islets exhibited a 60% reduction in glucose-stimulated insulin release. The presence of superoxide dismutase, catalase or diethylenetriamine-pentaacetic acid, during the exposure period protected the islets. They suggested that superoxide anions, hydrogen peroxide and hydroxyl radicals play a role in the insulin inhibitory effects caused by dihydroxyfumaric acid exposure.

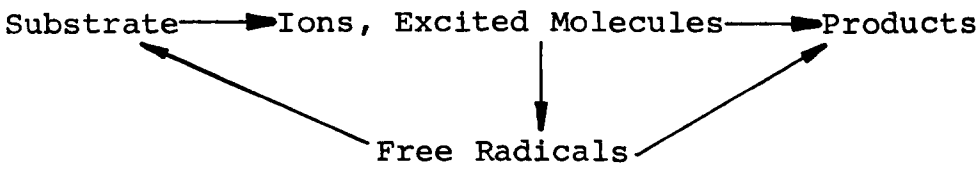
In view of the above interest in dihydroxyfumaric acid and the uncertainty concerning its mode of biological action, it was considered useful to investigate further the chemical and biochemical properties of the compound using stationary state and pulse radiolysis methods.

Using these techniques information concerning the free radical properties of the compound which might be useful in characterising further its mechanisms of autooxidation might be obtained. With this information a greater understanding of the manner by which the compound can stimulate peroxidation on liver microsomes might be forthcoming.

g) Application of radiation techniques to free radical chemistry generally

Much of the recent significant progress in our understanding of free radical reactions pertinent to biology has come from the application of radiation chemical techniques particularly pulse radiolysis. It is still a common misconception that when systems are irradiated a vast array of reactions take place which cannot possibly be identified. However, it is becoming increasingly realised that by the careful design of experimental systems and the use of selective free radical scavengers, very useful information concerning a particular free radical reaction can often be obtained.

Deposition of energy by high energy radiations (hard X- and γ-rays and fast electron) lead to the formation of a large spectrum of ionized and excited species, which fairly rapidly are either deactivated to the starting substrate or result in other products, usually via free radical formation, as follows:



The overall process of producing chemical changes by irradiation of dilute aqueous solutions starts with the absorption of the radiation by the water and terminates with the reestablishment of chemical equilibrium.

It is known that the first steps in the interaction between ionizing radiation and any system is the production of ions, electrons and excited species. Since radiation interacts primarily with the target electrons, in a system consisting of several components, each will be initially affected in proportion to the contribution it makes to the total number of electrons present. It follows that in systems approximating biological situations of dilute aqueous solutions of biochemicals, it is the water that absorbs nearly all of the energy deposited.

The nature and yields of the species initially produced are now well established. The so-called primary products form within about 10^{-9} sec. after the passage of the ionizing ray and they are agents which bring about chemical changes in aqueous solutions. Written in the conventional way, the scheme shown in equation (29):

$$H_2O \longrightarrow H^{\cdot}, OH^{\cdot}, e^-_{aq}; H_2, H_2O_2, H_3O^+ \qquad (29)$$

The radical yields expressed as the number of the radical species formed per 100 eV absorbed by solution are as follows:

1) Radical yields		2) Molecular yields	
a). $G(e^-_{aq})$	= 2.7	d). $G(H_2)$	= 0.45
b). $G(OH^\cdot)$	= 2.7	e). $G(H_2O_2)$	= 0.7
c). $G(H^\cdot)$	= 0.55		

The primary reactive intermediate radicals (a,b,c) can be converted to the other chemical species by well established techniques. Thus, in acid solution, hydrated electrons react with hydronium ions forming H atoms according to reaction (30):

$$e^-_{aq} + H_3O^+ \longrightarrow H^\cdot \qquad (30)$$

$k_{30} = 2 \times 10^{10} M^{-1}s^{-1}$, Hart and Anbar, (1970)

Conversely, in alkali solutions the hydrogen atom can be deprotonated according to equilibrium (31), producing hydrated electrons:

$$OH^- + H \rightleftharpoons e^-_{aq} \qquad (31)$$

pK = 9.7 Thomas et al., (1966)

Thus, in acid or alkali solution one can have the reducing intermediate a or c.

Such conversions can be done also through Brønsted-catalysis (Stein, 1971) as shown in reaction (32), its rate depending on ionic strength:

$$e^-_{aq} + H_2PO_4^- \longrightarrow H + HPO_4^{2-} \qquad (32)$$

$$k_{32} = (6\text{-}10) \times 10^7 M^{-1}s^{-1}, \text{ Anbar } \underline{et\ al.}, (1973)$$

It is also possible to convert the oxidizing hydroxyl radical to reducing radicals, H or e^-_{aq} depending on the pH, by radiolyzing solutions at high pressures of H_2.

$$H_2 + OH^{\cdot} \longrightarrow H + H_2O \qquad (33)$$

$$k_{33} = (3.5\text{-}6) \times 10^7 M^{-1}s^{-1}, \text{ Dorfman and Adams, (1973)}$$

Under such conditions all primary radicals can be converted to one of the reducing forms.

On the other hand, the reducing radical e^-_{aq}, in the presence of nitrous oxide, reacts at a near diffusion controlled rate to give OH$^{\cdot}$ (Anbar <u>et al.</u>, 1973).

$$e^-_{aq} + N_2O \longrightarrow OH^- + N_2 + OH^{\cdot} \qquad (34)$$

$$k_{34} = (5 \text{ to } 8) \times 10^9 M^{-1}s^{-1}$$

Under these conditions the solution becomes almost an uniradical system, with a 90% yield of hydroxyl radicals.

Thus, by irradiating solutions containing dihydroxyfumaric acid in the presence of nitrous oxide information concerning the reaction of $OH^{\cdot}$ with the compound might be obtained provided the rate of reaction of e^-_{aq} at concentrations of the compound is not sufficient to allow the reaction

$$e^-_{aq} + DHF \dashrightarrow \text{products} \qquad (35)$$

to occur significantly.

Fortunately, with the advent of pulse radiolysis a large number of rate constants of reactions of the e^-_{aq} are now known. The technique has also enabled the rates of reactions of the other radicals to be determined accurately.

Pulse radiolysis can be considered as the high energy radiation analogue of flash photolysis. It can provide accurate information concerning the rates of reaction and redox properties of a wide variety of radicals in solution in times very short compared to the time scale of the biochemical process to be investigated.

The source of high energy radiation can be a linear accelerator or a Van de Graaff generator producing pulses

of electrons of 2 ns - 2 μs length.

If an electron pulse of dose 1 Krad (10 J/kg) is absorbed by a dilute aqueous solution, 2.7 μM $OH^{\cdot}$; 2.7 μM e^{-}_{aq}; and o.6 μM $H^{\cdot}$ are produced. The rate of reaction of radicals can be measured directly by observing the formation of an absorbing products or the decay of absorbing reactant as in the case of the reactions of e^{-}_{aq}. When neither the products nor the reacting radical or molecules absorb significantly, competition studies can be employed, for example the determination of the rates of reaction of $OH^{\cdot}$ with alcohols (Adams et al., 1965b).

As will be seen later, several other scavenger systems, in addition to nitrous oxide, are now widely used on pulse radiolysis studies, for example, t-butanol, isopropanol - acetone and halide ions.

h) Aims of the present work

In the present work the above pulse radiolysis systems have been used to determine the rate of reactions of a selection of oxidizing radicals with dihydroxyfumaric acid. With this information γ-radiation studies have been undertaken in an attempt to throw further light on the mechanism of autooxidation of dihydroxyfumaric acid. Finally, the ability of dihydroxyfumaric acid to initiate peroxidation on rat liver microsomes and the effect of pH, concentration, metal ions and inhibitors has been examined in an attempt to characterize the system more closely and assess its relevance as a model of lipid peroxidation *in vivo*.

CHAPTER 2

IONIZING RADIATION STUDIES

2a) Introduction

The research of dynamic process in biological systems necessitates the use of methods by which times scales comparable to those of natural biochemical features can be resolved. Information from static structure, e.g. X-ray diffraction of crystalline biomolecular mechanism can yield detailed and quantitative structural information on entire bio-molecules. However, deductions from static structure alone on the dynamic behaviour in solution or *in vivo* may lead to incomplete conclusions. So, the technique of pulse radiolysis, developed during the 1960's is now widely applied to the study of free radicals, excited state and ionic reactions. Basically, this technique monitors the change in optical absorption produced in samples irradiated by a well-defined short pulse of ionizing radiation.

Despite the considerable volume of work done on the radiation chemistry of organic compounds, as far as is known no studies of dihydroxyfumaric acid have been reported. Considering the use of dihydroxyfumaric acid on biological models as is described earlier, pulse radiolysis and irradiation studies have been undertaken to gain further information on the compound's free radicals.

2b) Experimental procedure

i) Pulse radiolysis equipment

Free radicals reactions were studied using the Brunel University 4 MeV 200 ns linear accelerator (Vickers Ltd; England) and associated equipment for kinetic spectroscopy and computer analysis of the data (Willson, 1970; 1978). The solutions were irradiated in a quartz cell of 1.5 cm. The cell was filled or emptied through an automatic system using a syringe pump or solenoid valve. The chemical changes resulting from the 0.2 μs electron pulse were detected as changes of absorbance at a wavelength selected by a monochromator and converted into an electrical signal by a photomultiplier tube. The signal was sent through cables to the oscilloscope and electronic equipment within a Faraday cage, for analysis. (Figure 1)

The trace obtained on the oscilloscope could be:

a) Stored on a magnetic disc.

b) Sent onto a graphic display screen, or

c) Printed on a hard copy printer.

Undesirable wavelengths of light (such as the ultra-violet) were prevented from the studied sample by the use of selected filters. In order to get a better statistical analysis in some cases or to avoid the presence of noise on traces produced, sometimes single traces were not

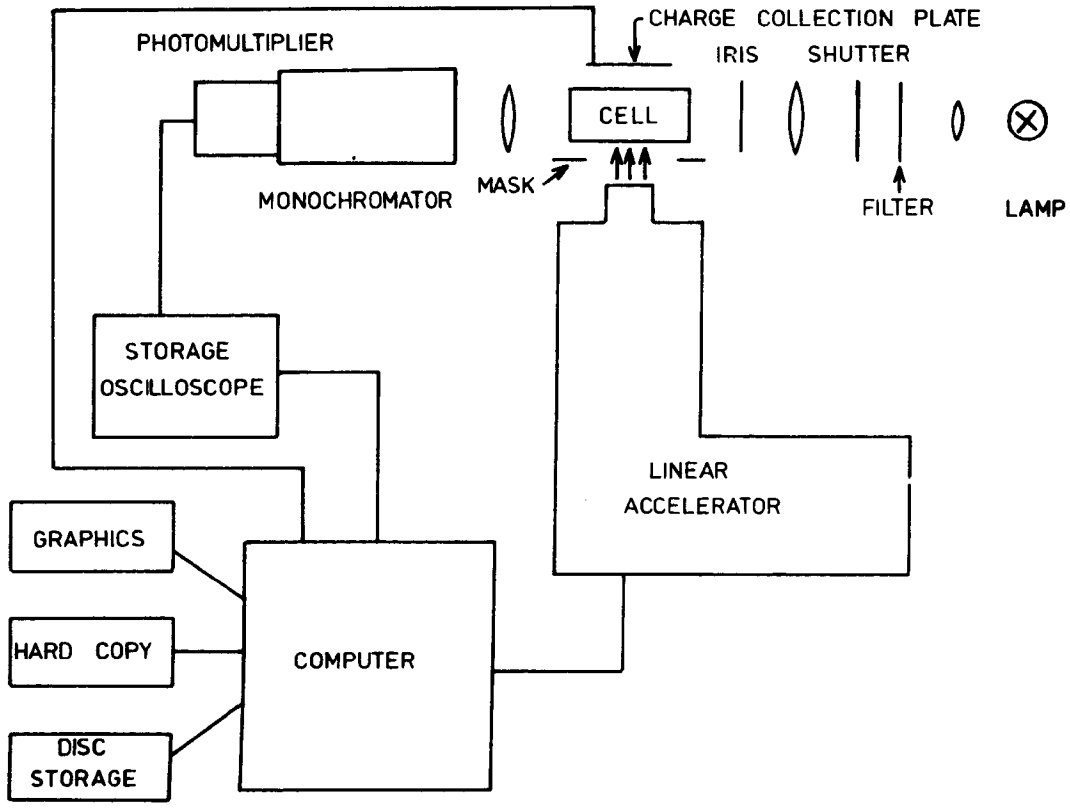


Figure 1: Pulse radiolysis equipment at Brunel University

(Reprinted with permission from Searle, (1981))

enough. Consequently, the average of 5 to 9 traces were taken.

ii) Kinetic analysis

First order rate constants

The simplest processes are first-order reactions, whose rates are proportional to the first power of a single concentration term. For instance, consider a reaction of the form

where: $A + S \xrightarrow{k} P$ (36)

The rate of change of the compounds is given by the differential equation:

$$\frac{-d[A]}{dt} = \frac{d[P]}{dt} = k[S][A] \quad (37)$$

where k is the bimolecular or absolute rate constant and [A] and [S] are the concentrations of the appropriate substance. It is assumed that the volume, temperature, and other factors which may affect k are unchanged during the reaction. If S is in excess such that the concentration of [S] can be considered constant, then k[S] will be a constant = k', the first order rate constant. Then substituting in (37), gives us:

$$\frac{-d[A]}{dt} = k'[A] \qquad (38)$$

Integrating $\quad -\ln[A] = k't + c \qquad (39)$

where c is a constant

At $t = 0$; let $[A] = [A_0] \qquad (40)$

thus, $\quad c = -\ln[A_0] \qquad (41)$

$\therefore \quad \ln[A] = -k't + \ln[A_0] \qquad (42)$

or $\quad \frac{\ln[A]}{[A_0]} = -k't \qquad (43)$

or, in the exponential form,

$$A = A_0 e^{-k't} \qquad (44)$$

also, $\quad \underline{\ln A = -k't + \ln A_0} \qquad (45)$

The units of k are sec^{-1}.

A useful expression can be derived by examining the time required for the concentration of A to fall by 50% from any chosen value. Then $[A] = \frac{1}{2}[A_0]$ gives:

$$t_{\frac{1}{2}} = \frac{\ln 2}{k'} = \frac{0.693}{k'} \qquad (46)$$

The absorbance of compound A will thus follow an exponential decay and the slope of the plot of lnA against t enables k' to be obtained. The absolute rate constant for the reaction, the bimolecular rate constant, k, can

then be found from

$$k = \frac{k'}{[S]} \qquad (47)$$

and can be derived from the slope of the graph of k' against [S].

Second order kinetics

Second-order reaction kinetics apply when a reactant dimerizes or disproportionates. This happens frequently with free radicals which are unstable and often more reactive toward each other than towards more stable species.

The equation:

$$A + A \xrightarrow{k} P \qquad (48)$$

represents a second order process. The rate of change of the compound A will be given by:

$$\frac{-d[A]}{dt} = 2k[A]^2 \qquad (49)$$

Integrating and applying Beer-Lambert's law produces the following relationship for the variation in absorbance of compound A:

$$\frac{1}{[A]} = \frac{2kt}{\varepsilon \ell} + \frac{1}{[A_o]} \qquad (50)$$

$$2kt = \varepsilon \ell \left[\frac{1}{[A]} - \frac{1}{[A_o]} \right] \qquad (51)$$

where: ε = extinction coefficient

ℓ = optical length of the cell

The reaction constant is written as 2k to allow for the disappearance of two reactant molecules per event. To test whether the investigated process follows these kinetics, values of $1/[A]$ are plotted as a function of time for different initial concentrations of A. The value of 2k can then be found from the slope of the line providing the extinction coefficient of compound A and the optical length of the cell are known.

Competition kinetics

Rate constants cannot always be obtained directly by following the growth in absorption of a reaction product or the loss of the absorption of the reactant. An indirect method by competition with a reference solute which yields a strongly absorbing species on reaction must then be used. The unknown compound must have a different absorption spectrum to the reference solute; and must present little or no absorption at a convenient wavelength where the reference solute absorbs. The reference

compound and unknown solutes must not react. The rate constant of the reaction of the radical under study with the reference solute must be known. For example, for a simple system in which A and B compete for the hydroxyl radical ($OH^{\bullet}$), consider the reactions:

$$OH^{\bullet} + A \xrightarrow{k_A} P_A \qquad (52)$$

$$OH^{\bullet} + B \xrightarrow{k_B} P_B \qquad (53)$$

A reacts efficiently with the hydroxyl radical with known $k_{(OH + A)}$ to give a product P_A, having a suitable absorption spectrum. Its concentration is then

$$(OD)_o \propto [P_A]_o \qquad (54)$$

where $(OD)_o$ denotes the absorption measured under strictly defined conditions. If one now adds to the solution a substance B whose rate constant $k_{(OH + B)}$ is to be determined, then it will scavenge a certain fraction of the $OH^{\bullet}$ radicals. As a consequence, the light absorption due to P_A will be decreased, and the new value of the optical density (OD) can be expressed by

$$(OD) = [P_A]_B = \frac{[P_A]_o}{1 + \frac{k_{(OH + B)}[B]}{k_{(OH + A)}[A]}} \qquad (55)$$

From these relations, the expression (56) is obtained, which is convenient for the construction of the competition

diagram.

$$\frac{(OD)_o}{(OD)} = 1 + \left[\frac{k_{(OH + B)}}{k_{(OH + A)}}\right] \frac{[B]}{[A]} \qquad (56)$$

By plotting on the x-axis different ratios of concentrations of scavengers $[B]/[A]$, and plotting on the y-axis $(OD)_o/(OD)$, a straight line relationship with a y-axis intercept of 1 is obtained. The slope of the line gives the relative rate constant. Since the rate constant for $OH^{\cdot}$ + A is known, then by substitution the rate constant $OH^{\cdot}$ + B can be obtained.

iii) Pulse radiolysis dosimetry

In order to know the radiation dose received by the irradiated sample, and to make comparisons of radical yields or to compare spectra obtained, it was necessary every day to normalise the data found from one trace to a standard radiation dose (1 Krad). The dosimetry calibration curve was done, using a stock solution (10^{-2}M) of thiocyanate. On pulse radiolysis of an aqueous air saturated thiocyanate solution presents a transient absorption in the range (480 - 500) nm. In the case of dosimetry the λ = 500 wavelength was used, with a value $(G\varepsilon)_{SCN} = 2.1 \times 10^4 M^{-1}s^{-1}$. (Adams et al., 1965a; Willson, 1978). If the change in absorbance observed at 500 nm for a pulse of dose D_{SCN} corresponding to a monitor reading m_{SCN} is A_{SCN}, then the next formula can be applied.

$$D_{SCN} = \frac{9.6 \times 10^8 \, A_{SCN} \text{ rads}}{2.1 \times 10^4 \, \ell} \quad (57)$$

$$D_{SCN} = \frac{4.6 \times 10^4 \, A_{SCN} \text{ rads}}{\ell} \quad (58)$$

Thus the dose of a pulse of monitor value m is given by

$$D = \frac{m \, D_{SCN}}{m_{SCN}} \quad (59)$$

where D_{SCN} is obtained from the absorption of $(SCN)_2^{\cdot -}$ at 500 nm.

iv) Irradiation source

A cobalt - 60 γ-ray source is the most convenient and easiest to use of the high-energy radiation sources to research the steady-state reactions. Depending only on spontaneous emission of rays from a radioactive source, it need have no moving parts, requires little maintenance, and can be left to do its work for any length of time without attention. With its half life of 5.27 years, it seldom requires replacement. A great many designs of ^{60}Co sources are in use at present. The problem in design is to keep the cobalt adequately shielded on all sides while providing means for inserting the sample to

be irradiated.

There are mechanisms that allow precise control of the length of exposure and position of the sample under study, reproducibility being an important factor in this type of study. Experiments were carried out using the 2000 Curie cobalt source at Brunel University.

v) *γ*-irradiation dosimetry

For most radiation-chemical purposes it is useful to compare the amount of chemical reaction in the solution being studied with the amount of change in a standard system, the specific radiation yield of which has been carefully determined by the use of primary physical standards. A great many solutions have been proposed for use as "chemical dosimeters" of this kind. Only one has found wide acceptance. This is the air-saturated solution of 0.01M ferrous sulphate in 0.8N H_2SO_4 dissolved in double glass distilled water. This preparation (the Fricke dosimeter) is very stable and can be kept at room temperature for several weeks without deterioration. (Fricke and Hart, 1966). In this system the ferrous iron is oxidized to the ferric state. Originally Fricke determined the amount of ferrous iron disappearing colorimetrically by means of the o-phenanthroline complex. A notable advance in convenience of use occurred when

Hardwick (1952) pointed out that the quantity of ferric iron formed could be determined very sensitively by direct reading of the optical absorbancy of the solution with an ultraviolet spectrophotometer at the wavelength 304 nm. At this wavelength the absorption due to ferrous iron is negligible. To determine the concentration of ferric iron from the spectrophotometer reading, one has merely to know the molar extinction coefficient. This particular quantity has been found to increase unusually rapidly with temperature 0.7% per degree centigrade, so that the temperature of the solution during the optical measurement must be known. Values of the extinction coefficient as determined at various laboratories are shown in Table 2.

Table 2: Reported values of ferric ion molar extinction coefficient in 0.8 H_2SO_4 at 25°C.

Institution	Name	Value
Argonne National Lab.....	Hart	2,225
Cambridge University.....	Swallow	2,167
Brookhaven National Lab.....	Allen	2,195
Edinburgh University.....	Miller	2,201
Oak Ridge National Lab.....	Hochanadel	2,240
Sloan-Kettering Institute.....	Laughlin	2,172
Paris.....	Lefort	2,205
Average		2,201
Standard deviation		0.4%

(taken from Allen, 1961)

Its one disadvantage is its sensitivity to organic impurities, which increase the yield over the standard value. Dewhurst (1952) discovered that the addition of chloride ion could largely inhibit the action of the impurities and ensure the correct yield value.

When irradiated, ferrous ions present in Fricke solutions are oxidised to ferric ions, as follows:

$$Fe^{2+} + OH^{\cdot} \longrightarrow Fe^{3+} + OH^{-} \quad (60)$$

$$H^{\cdot} + O_2 \longrightarrow HO_2^{\cdot} \quad (61)$$

$$Fe^{2+} + HO_2^{\cdot} \longrightarrow Fe^{3+} + HO_2^{-} \quad (62)$$

$$HO_2^{-} + H^{+} \longrightarrow H_2O_2 \quad (63)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\cdot} + OH^{-} \quad (64)$$

Overall, the yield for the production of ferric ions can be given by $G(Fe^{3+}) = 2G_{H_2O_2} + 3G_H + G_{OH}$ (where G is the number of species produced per 100 eV of radiation deposited).

For ^{60}Co γ-rays and energetic X-rays $G_H = 3.70$; $G_{OH} = 2.92$; $G_{H_2O_2} = 0.78$, therefore substituting these values, a $G(Fe^{3+}) = 15.6$ is obtained.

For the ^{60}Co irradiation, 10 ml aliquots of Fricke solution were placed at 10, 16, 20, 25, 30, 35, 40, 50 and 60 cm distances respectively from the source and irradiated for a given period such as 10 minutes. Due to the radiation, ferrous ions were converted to ferric ions according to reactions (60, 62 and 64). The ferric ions were detected and measured at 304 nm, using a Pye Unicam SP 500 spectrophotometer.

The dose in rads was calculated, applying the following formula:

$$D \text{ rads} = \frac{0.944 \times 10^9 \times \Delta(O.D.)}{\Delta\varepsilon \times G(Fe^{3+})} \qquad (65)$$

where:

$\Delta(O.D.)$ is the measured extinction of Fe^{3+} at 304 nm.

$\Delta\varepsilon$ is the difference in molar extinction coefficients ($M^{-1}cm^{-1}$) between Fe^{2+} and Fe^{3+} at 304 nm.

$G(Fe^{3+})$ = 15.6 (a similar value was assumed for ^{60}Co γ-irradiation, in this work)

vi) <u>Preparation of dihydroxyfumaric acid solutions for irradiation experiments</u>

For the study of dihydroxyfumaric acid irradiated solutions with γ-rays; fresh stock solutions (1 - 10 mM)

containing a determined amount of diethylenetriaminepenta-acetic acid (DTPA) to chelate possible metal impurities present and to prevent further autoxidations were prepared not more than 10 min. before that the experiment was properly conducted. 10 ml aliquots were taken and dispensed in stoppered glass tubes. Later, the samples were irradiated during different intervals of time, in minutes. After irradiation, the samples were read in the spectrophotometer at 290 nm in order to determine the amount of dihydroxyfumaric acid present. Controls not irradiated were left at room temperature and also checked in their concentration of dihydroxyfumaric acid. The results obtained were done in triplicate and the error standard assessed and deduced from controls and samples studied. The employed time to perform each individual experiment was averaged at 30 min. The pH and absorbance values of each solution were also monitored before each experiment was performed. Sometimes it was necessary to introduce a dilution factor to obtain results in the suitable range of Optical Density (OD). Doubly distilled water was used as solvent.

vii) Measurements of oxygen consumption

The oxygen consumption in 10 ml of unirradiated and irradiated solutions containing dihydroxyfumaric acid in the range 0.5 to 10 mM and in presence of diethylene-triaminepenta-acetic acid (100 µM) at pH 3.2 and room temperature was measured using a YSI Model 5331 (Oxygen electrode).

2c) Materials

i) Chemicals

Propan-2-ol, acetone, potassium bromide, potassium iodide, potassium hydroxide, potassium thiocyanate, sodium azide, sodium nitrate, ascorbic acid (Analar), were supplied by BDH Ltd.

The diammonium salt of 2,2' - azinobis - (3 - ethyl-benzthiazoline - 6 - sulphonic acid) (ABTS) was purchased from Boehringer Ltd., in the form of a green-blue powder.

Dihydroxyfumaric acid, DL - dithiothreitol (DTT), cysteine (hydrochloride), cysteamine (hydrochloride), D-penicillamine, glutathione, diethylenetriaminepenta-acetic acid (DTPA), n-propyl gallate were obtained from Sigma U.K. Ltd., and were of the purest grade available.

2,2,6,6,-tetramethyl-4-oxo piperidinooxy (TAN), 4-hydroxy-2,2,6,6,-tetra-methyl piperidino-1 oxyl (TMPN) and 2,2,5,5,-tetramethyl-3-pyrroline 3-carboxamide were obtained from Eastman Kodak Co., Rochester, N.Y. 14650.

The following compounds were gifts: paranitroaceto-phenone ("PNAP",Gray Laboratory, Mount Vernon Hospital, U.K.); 2-mercaptopropionyl glycine ("thiola", 2-MPG; Santen Pharmaceutical Co. Tokyo, Japan). chlorpromazine and promethazine, supplied as the hydrochlorides (May and Baker Ltd., Dagenham, Essex).

t-butanol was purified by fraction crystallisation. Solutions were saturated with oxygen-free nitrogen or nitrous oxide (British Oxygen Ltd.) using the syringe-bubbling technique (Willson, 1970).

Reagents other than those specified were of the highest purity available and were obtained from BDH Chemicals, Sigma Chemical Co. or Aldrich Chemical Co.

All solutions were freshly prepared in doubly distilled or Millipore filtered water and exposed to the minimum of light. Adjustment of the pH of the solutions was carried out by titrating with potassium hydroxide or hydrochloric acid as required.

Between runs, all glassware used was washed with tap water then rinsed with doubly distilled and Millipore filtered water and left to drain and dried. Periodically, all glassware was soaked in chromic acid solution, rinsed throughly with doubly distilled water and dried in a clean oven for 1 to ½ hour.

2d) Results

i) Absorption spectrum of unirradiated controls of dihydroxyfumaric acid

Before solutions of dihydroxyfumaric acid were irradiated, it was important to know the rate of change of its absorption due to spontaneous autooxidation. The absorption spectrum of a solution containing dihydroxyfumaric acid (200 μM) and in presence of DTPA (40 μM) at pH 3 and room temperature was undertaken. Dihydroxyfumaric acid absorbs strongly in the ultraviolet at 290 nm immediately after preparation, and after 30 minutes as is shown (Figure 2). When similar experiments were undertaken in the presence of phosphate buffer (2 mM) pH 7.4 (Figure 3), without doubt no destruction of dihydroxyfumaric acid was observed. The absorption maximum corresponds to ε = 9000 $M^{-1}cm^{-1}$.

As dihydroxyfumaric acid showed a destruction of 25% in 30 minutes in the absence of phosphate buffer, it was of interest to measure the oxygen consumption over a similar period for different concentrations of dihydroxyfumaric acid in the concentration range 0.5 to 10 mM in presence of DTPA (100 μM) at pH 3.2 and room temperature (16.3 to 17)^{o}C. The results obtained are shown in Figure 4. The depletion of oxygen for each dihydroxyfumaric acid solution studied was monitored for 10 minutes and in that time it can be seen that the oxygen consumption reached was 23.7, 37, 74.8 and 100% respectively. The extent of destruction of

dihydroxyfumaric acid occurring with these different concentrations (0.5 to 10) mM were also measured. After 10 minutes, solutions were diluted down in phosphate buffer pH 7.4 corresponding to an initial concentration of 100 μM dihydroxyfumaric acid at zero time. Phosphate buffer pH 7.4 was chosen as the dilutent because of the fact that the previous experiments had shown protection on the oxidation rate of dihydroxyfumaric acid. The results obtained are in Figure 5 .

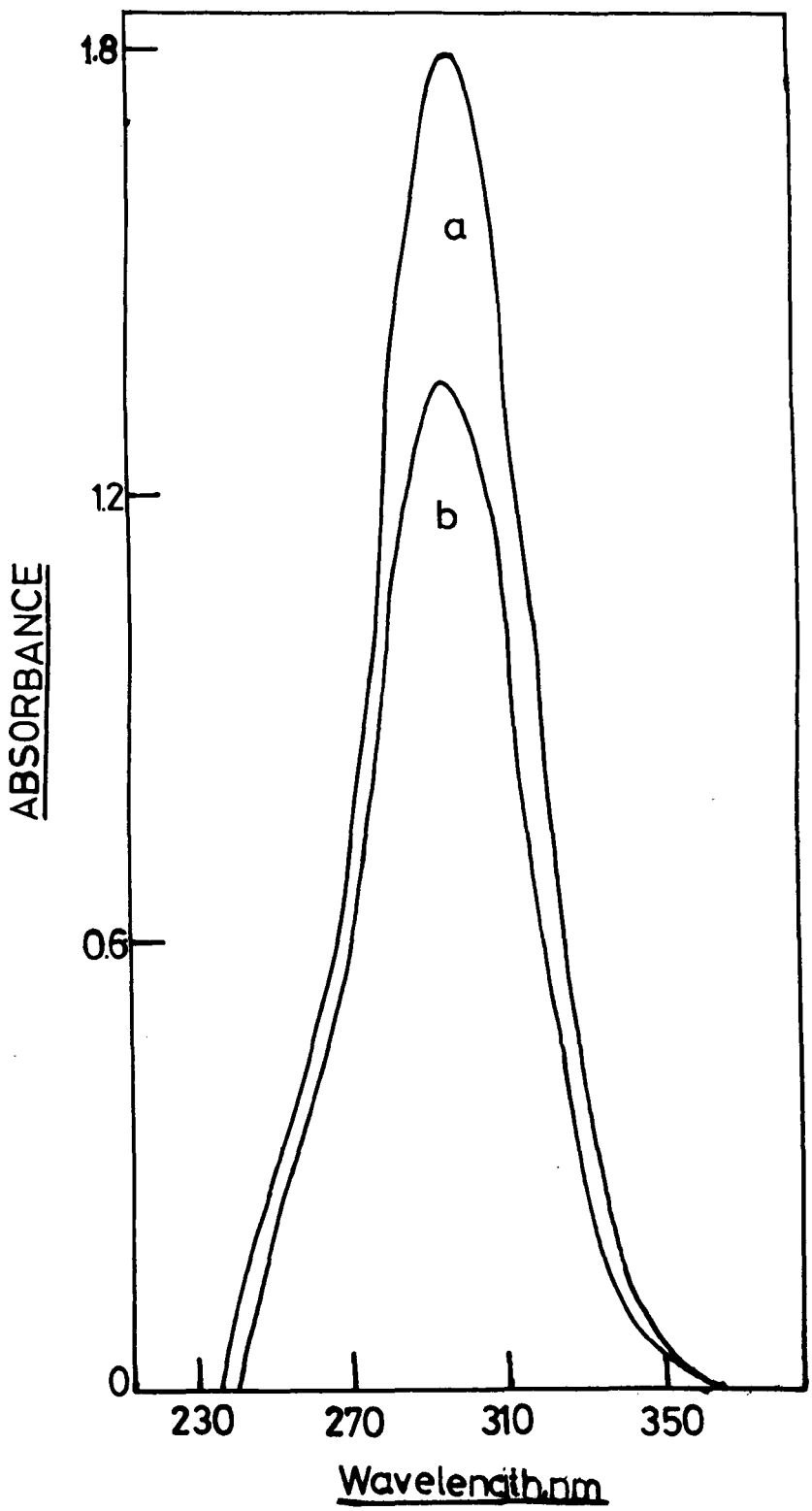


Figure 2: Absorption spectrum for a solution containing DHF (200μM) and DTPA (40μM) at pH 3.2 ± 0.1.
(a) spectrum recorded 5 minutes after preparing the solution.
(b) spectrum recorded 30 minutes later.

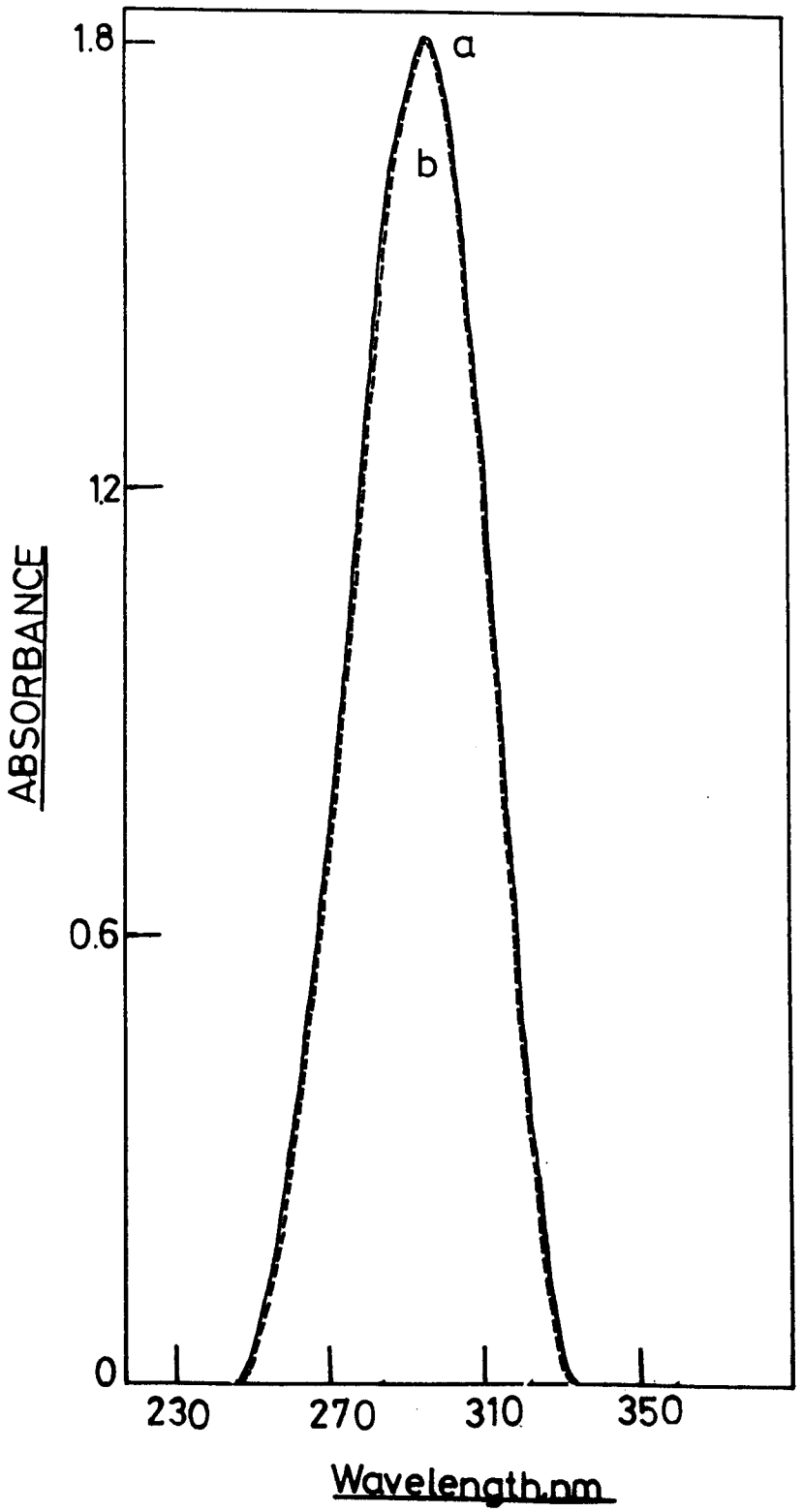


Figure 3: Absorption spectrum for a solution containing DHF (200μM) and DTPA (40μM) in phosphate buffer pH 7.4. The spectrum obtained was recorded at (a) 5 minutes and (b) 60 minutes after preparing the solution.

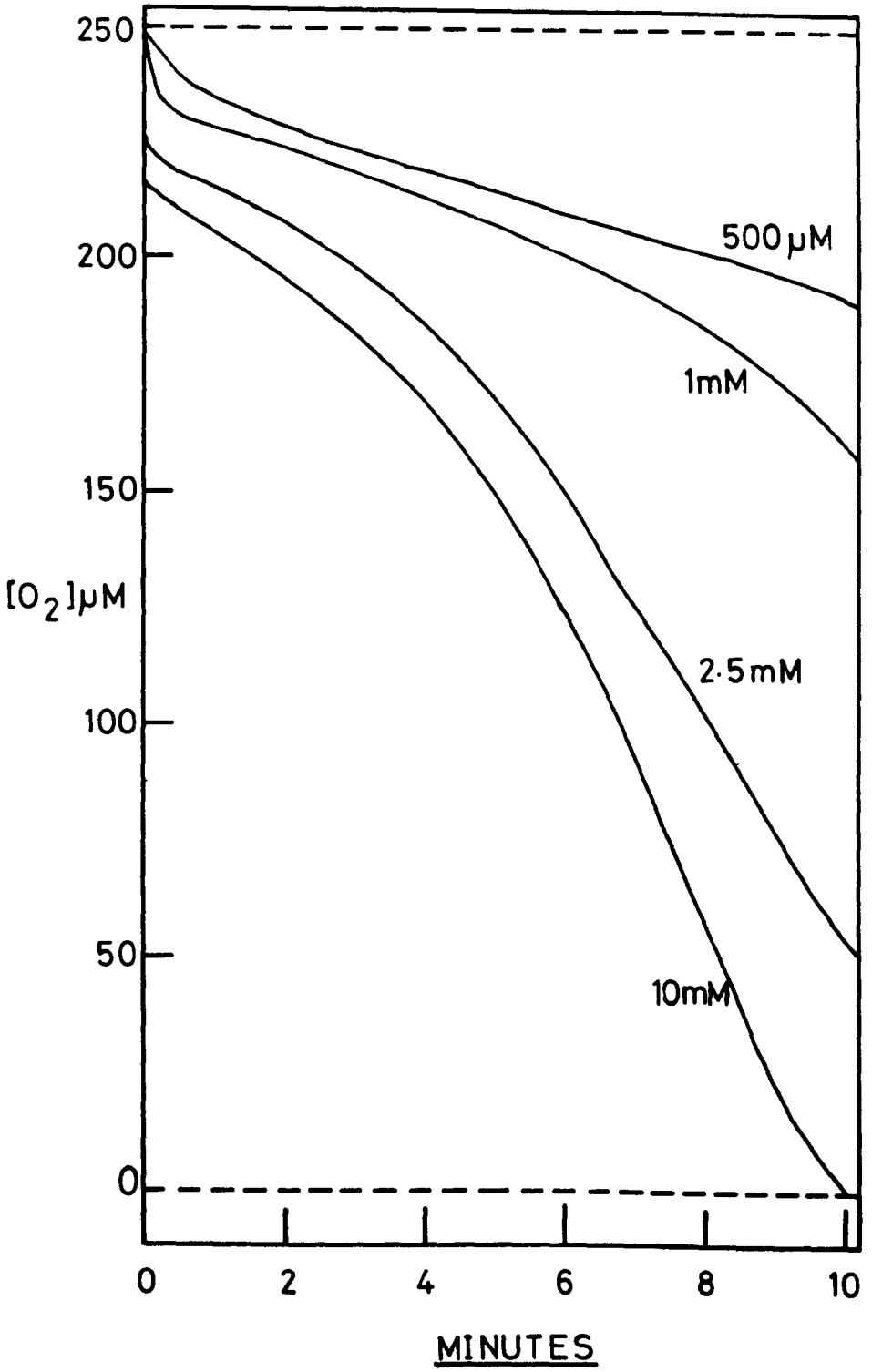


Figure 4 : Effect of dihydroxyfumaric acid concentration on oxygen consumption in unirradiated solutions.

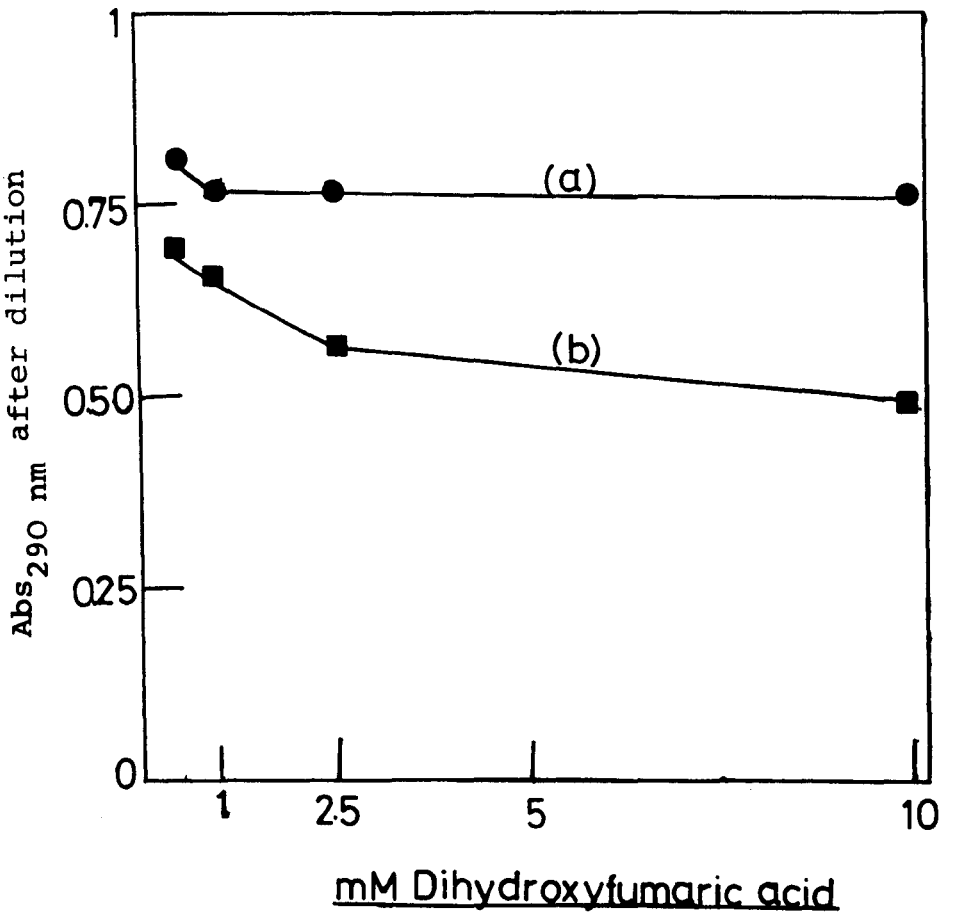


Figure 5: Oxidation rate of unirradiated dihydroxyfumaric acid.

(a) Absorbance of fresh solutions at 0 time after dilution in phosphate buffer pH 7.4 to a concentration of 100 µM.

(b) Absorbance recorded after 10 minutes.

ii) Yields of destruction of dihydroxyfumaric acid by ^{60}Co - γ- rays

The following experiments were an attempt to determine the G value for the destruction of dihydroxyfumaric acid (200 μM) in the presence of DTPA (40 μM) at pH 3, room temperature (15.7 to 16.4)°C. Solutions were irradiated with doses of 30 rads/min and 121 rads/min, for 8 minutes. The yield-dose curves obtained are shown in Figure 6 . They are not linear, but G(-DHF) was calculated taking the initial portion of the curve over the first two minutes and the equation (65a):

$$G = \frac{A \times 9.6 \times 10^8}{\varepsilon \times \ell \times \text{Dose (rads)}} \qquad (65a)$$

where: A is the difference in absorption between irradiated and unirradiated samples after 2 minutes.

ε is the molar extinction coefficient ($M^{-1}cm^{-1}$), at 290 nm in water: ($\varepsilon_{290\ nm} = 9000\ M^{-1}cm^{-1}$).

ℓ = optical length of the cell.

Similar experiments were undertaken for solutions containing dihydroxyfumaric acid (300 μM) in the presence of DTPA (20 μM) at pH 3, and irradiated with 30 rads/min and 121 rads/min, respectively. The results obtained are summarised in Table 3.

Table 3: G values obtained for the destruction of DHF irradiated

System	30 rads/min	121 rads/min
200 µM DHF / 40 µM DTPA	945	269
300 µM DHF / 20 µM DTPA	891	226

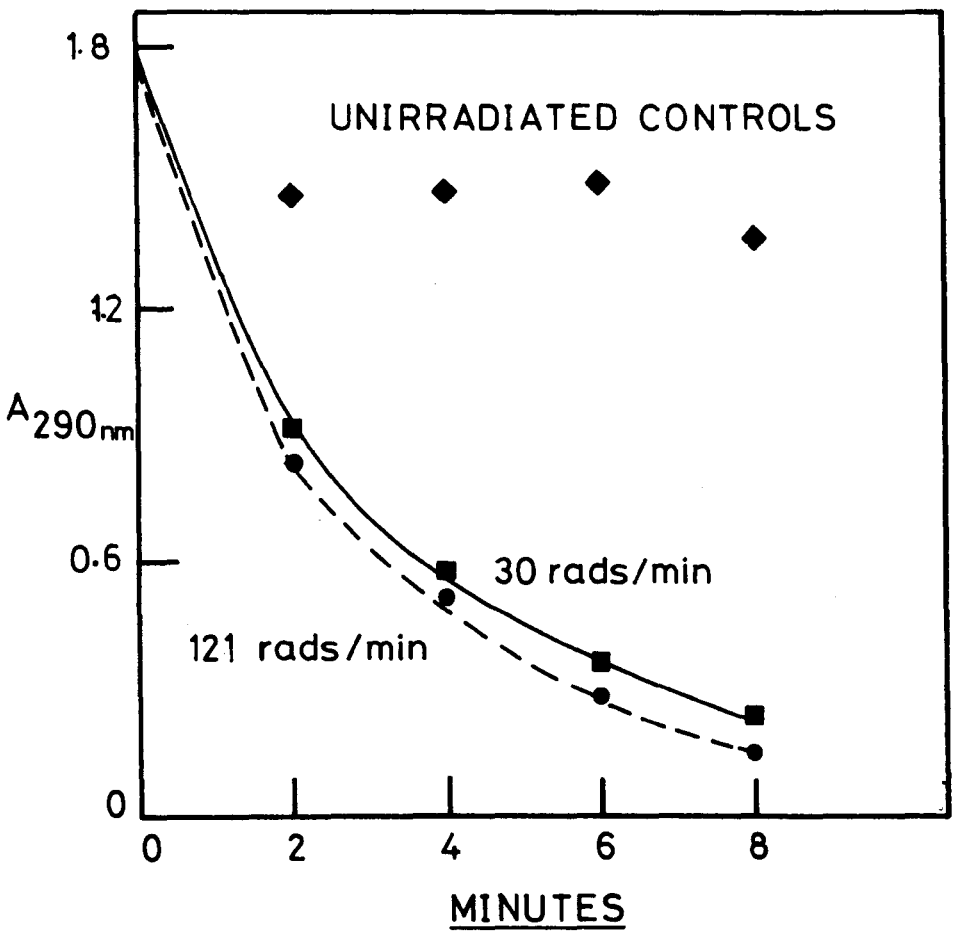


Figure 6 : Yield of destruction of dihydroxyfumaric acid by γ-rays.

(*) The readings absorbance for unirradiated controls were taken after 20 minutes.

iii) pH effect on irradiation of dihydroxyfumaric acid in solution

Solutions containing dihydroxyfumaric acid (200 μM) and DTPA (40 μM) in the pH range (3 to 10) were irradiated at room temperature (16.5°C) at the dose rate of 30 rads/min and 121 rads/min during 8 minutes of irradiation time. The results obtained show that the oxidation rate for dihydroxyfumaric acid was faster in the acid region than in the alkaline region (Figure 7).

iv) Oxygen consumption in irradiated solutions of dihydroxyfumaric acid

The rate of oxygen consumption was monitored for solutions containing dihydroxyfumaric acid at the concentrations of 0.5, 1, and 10 mM in the presence of DTPA (100 μM) at pH 3.2, room temperature (15 to 17)°C, irradiated at the dose rate of 166 rads/min. (Figure 8).

Similarly, the rate of oxygen consumption was determined for solutions of dihydroxyfumaric acid 10 mM and DTPA (100 μM) at pH 3, room temperature and irradiated with the dose rate of 30 rads/min, 166 rads/min and 1000 rads/min, respectively. (Figure 9).

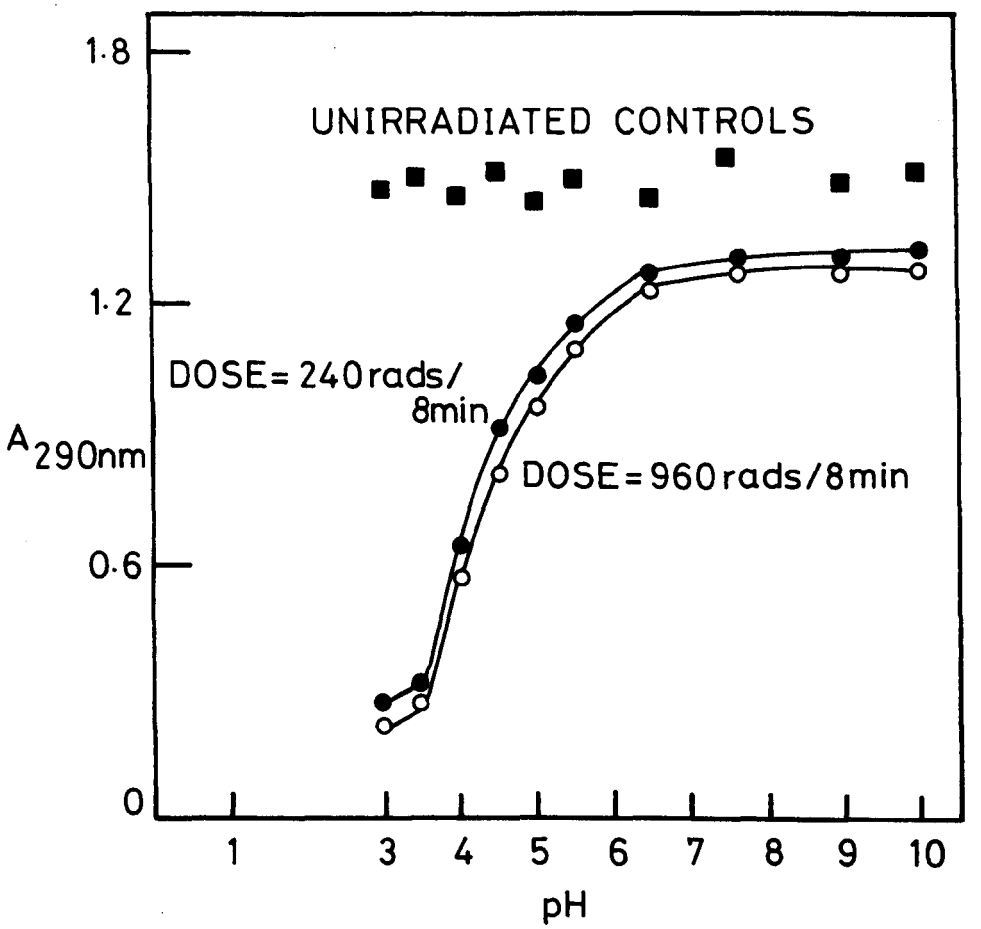


Figure 7 : pH effect and yield of dihydroxyfumaric acid destruction.

(*) The readings absorbance for unirradiated controls were taken after 20 minutes.

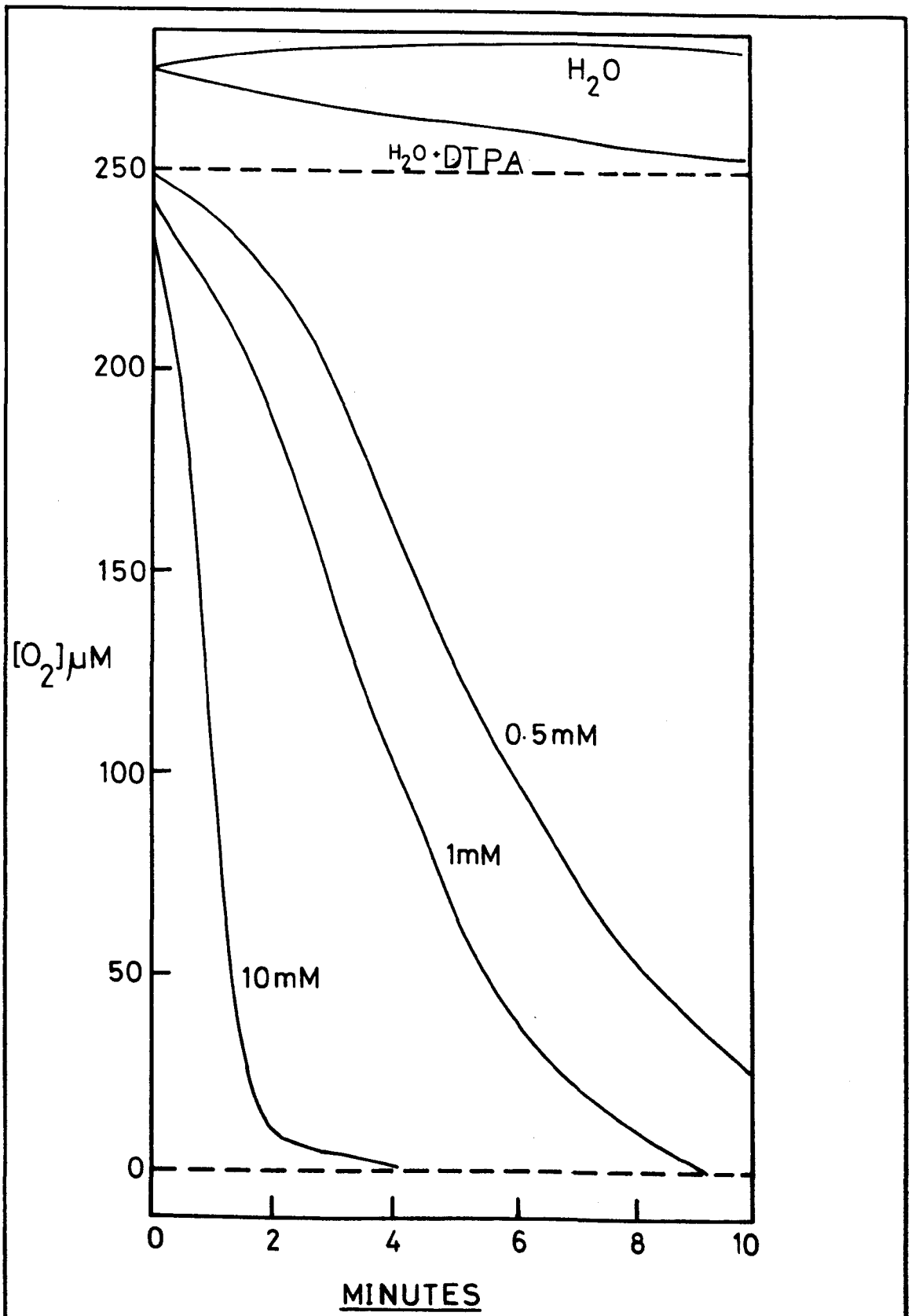


Figure 8 : Effect of dihydroxyfumaric acid concentration on oxygen consumption in irradiated solutions. Dose = 166 rads/min.

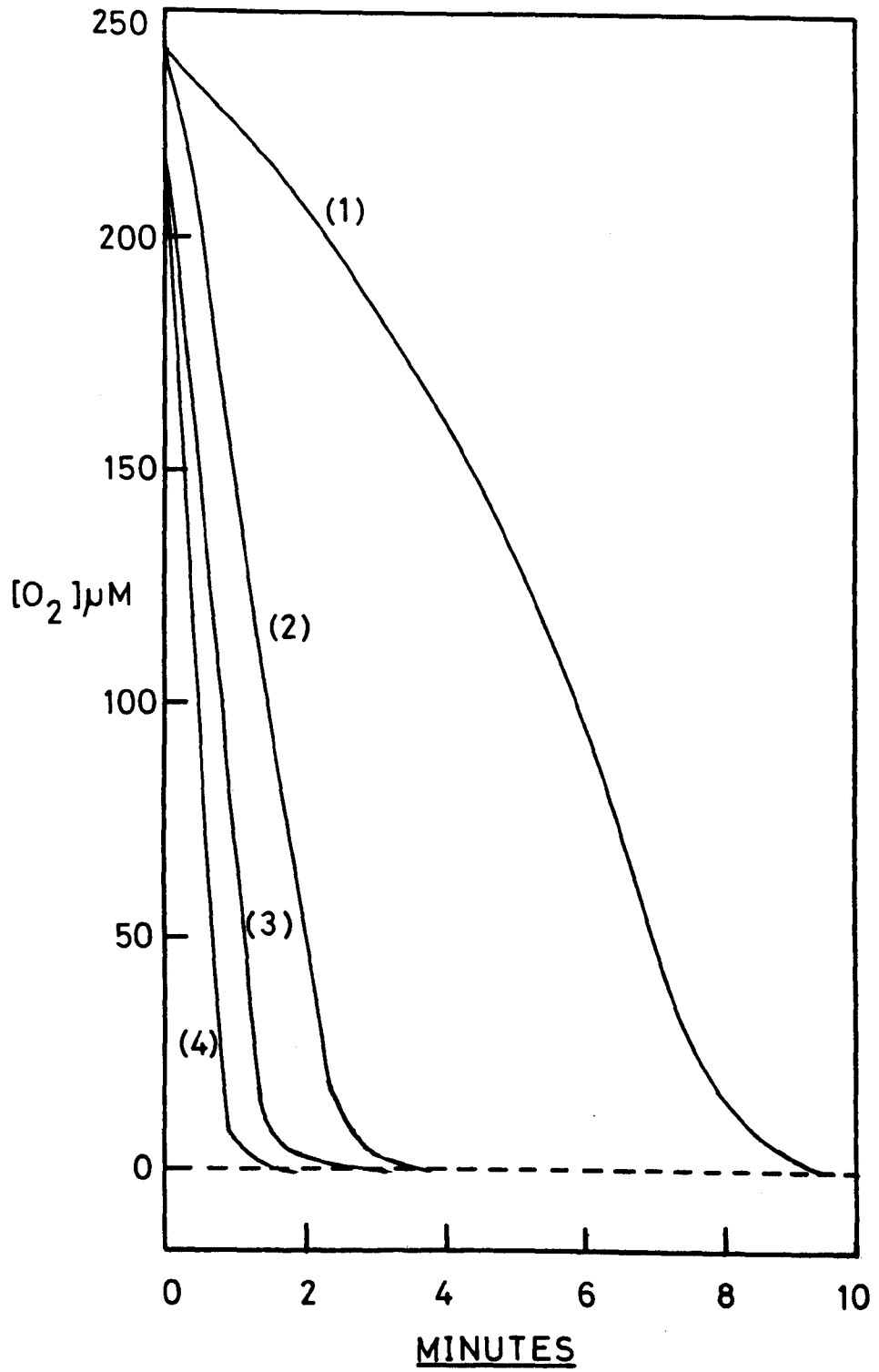


Figure 9 : Effect of dose rate on oxygen consumption on irradiated solutions of dihydroxyfumaric acid (10 mM) and DTPA (100 μM).

(1) Control not irradiated;

(2) Dose rate = 30 rads/min;

(3) Dose rate = 166 rads/min;

(4) Dose rate = 1000 rads/min.

v) Effect of Radical Scavengers on the oxidation rate of dihydroxyfumaric acid

The use of nitroxide compounds in the experimental work with dihydroxyfumaric acid was considered, in order to determine the effect of antioxidants on the system irradiated.

Solutions containing dihydroxyfumaric acid (100 μM) in presence of DTPA (20 μM) at pH 3.4 and additions of 2,2,6,6,-tetramethyl-4-oxo-piperidinooxy (TAN) in the concentrations (0.5, 2.5, and 5) μM, 4-hydroxy-2,2,6,6, -tetramethyl piperidino-1-oxy (TMPN) in the concentrations of (0.5 to 5) μM and 2,2,5,5,-tetramethyl 3-pyrroline 3-carboxamide at similar concentrations were irradiated with the dose rate of 30 rads/min during the intervals of 3, 6 and 9 minutes, respectively. Subsequently, the absorption at 290 nm for dihydroxyfumaric acid was read spectrophotometrically. The results obtained are summarised in Table 4.

Similar experiments were conducted for solutions containing dihydroxyfumaric acid (100 μM), DTPA (20 μM) at pH 3.2 but replacing the nitroxide compounds by ethanol (0.5 to 5) μM or the antioxidant propyl gallate in the concentration range of (0.5 to 25) μM and irradiated with 30 rads/min during 3, 6 or 9 minutes, respectively. The results obtained are shown in Table 4.

Table 4 : EFFECT OF DIFFERENT COMPOUNDS ON THE OXIDATION RATE OF DIHYDROXYFUMARIC ACID (100 µM) IN PRESENCE OF DETAPAC (20 µM)

Compound	µM/	Unirradiated controls		Irradiated samples/Dose=30 rads/min		
		Before experiment 0 min.	After experiment 30 min.	3 min.	6 min.	9 min.
				Abs 290 nm	Abs 290 nm	Abs 290 nm
2,2,6,6,- Tetramethyl - 4 - oxo piperidinooxy	0	0.84 *	0.67	0.35	0.20	0.10
	0.5	0.87	0.76	0.45	0.30	0.16
	2.5	0.87	0.79	0.58	0.45	0.31
	5.0	0.88	0.82	0.63	0.54	0.45
4 - Hydroxy - 2,2,6,6, - Tetra - methyl piperidino - 1 - oxyl.	0	0.84 *	0.61	0.33	0.21	0.11
	0.5	——	——	0.45	0.26	0.15
	2.5	——	——	0.59	0.46	0.30
	5.0	——	——	0.62	0.55	0.45
2,2,5,5,- Tetramethyl - 3 - pyrroline 3 - carboxamide	0	0.84 *	0.64	0.42	0.26	0.19
	0.5	——	0.62	0.33	0.20	0.11
	5.0	——	0.62	0.32	0.20	0.12

Table 4 (Continued)

Compound	µM/	Unirradiated controls		Irradiated samples/Dose=30 rads/min		
		Before experiment 0 min.	After experiment 30 min.	3 min.	6 min.	9 min.
				Abs 290 nm	Abs 290 nm	Abs 290 nm
Ethanol	0	0.84 *	0.65	0.43	0.26	0.20
	0.5	——	0.66	0.34	0.21	0.11
	5.0	——	0.67	0.35	0.21	0.11
Propyl gallate	0	0.84 *	0.66	0.42	0.26	0.26
	0.5	——	0.70	0.38	0.23	0.15
	2.5	——	0.69	0.40	0.26	0.17
	5.0	——	0.69	0.43	0.27	0.19
	10.0	——	0.71	0.48	0.33	0.26
	25.0	——	0.85	0.61	0.46	0.40

Note: * Is the average of 8 values. Each experiment was done in triplicate.

vi) Effect of Mn^{2+} on the oxidation rate of dihydroxyfumaric acid

In view of the protective effect of Mn^{2+} on the rat liver microsomes exposed to peroxidation by Fe^{2+}/ dihydroxyfumaric acid couple as it will be seen later. It was of interest to know how Mn^{2+} could affect the oxidation rate of dihydroxyfumaric acid. A catalytic effect of manganous chloride (10 to 50) μM was observed when added to solutions containing dihydroxyfumaric acid (400 μM) in presence of DTPA (80 μM) or in absence of the chelating agent. Samples at (zero time) were diluted 1:1 in water before the absorption was read at 290 nm in the spectrophotometer. Similar situation was undertaken for the samples after 20 minutes as is shown in Figure 11 .

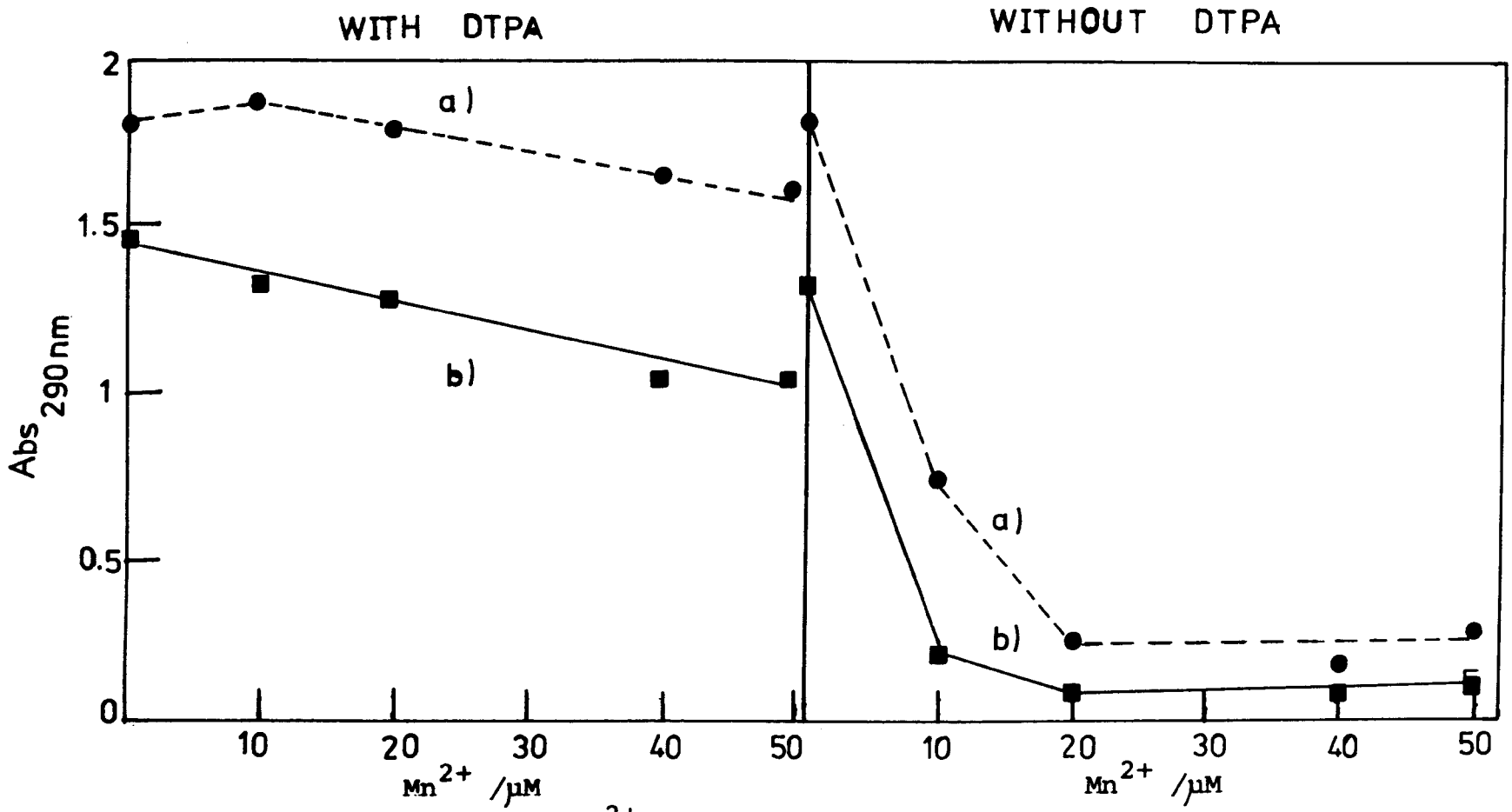


Figure 10: Effect of Mn^{2+} on oxidation of dihydroxyfumaric acid.

a) Absorbance readings taken for controls at (O) time.

b) Absorbance readings taken for samples 20 minutes later.

vii) Reaction with the hydroxyl radical

On pulse radiolysis of N_2O saturated solutions containing dihydroxyfumaric acid (2.5×10^{-3}M), no strong absorption due to the product of the reaction of the hydroxyl radicals was observed. Competition studies for the hydroxyl radical therefore were undertaken using the diammonium salt of 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) as the reference compound. It has been shown previously that the OH$^{\bullet}$ radicals react with ABTS according to:

$$OH^{\bullet} + ABTS \longrightarrow ABTS^{\bullet +} + \text{products} \quad (66)$$

$$k_{66} = 1.2 \times 10^{10} M^{-1} s^{-1}$$

One of the products is the radical cation $ABTS^{\bullet +}$ which has a strong absorption at 415 nm, that can be observed directly. N_2O-saturated solution containing ABTS (10^{-4}M) in the presence of dihydroxyfumaric acid in the range 0.5 to 8×10^{-4}M at pH 3.5 and 7, were pulse irradiated. The maximum absorption of $ABTS^{\bullet +}$ at 415 nm decreased with increasing concentrations of dihydroxyfumaric acid, in agreement with the competing reaction (67) and equation (68) where A_0 and A are the measured

$$OH^{\bullet} + ABTS \longrightarrow \text{Absorption at 415nm} \quad (66)$$

$$OH^{\bullet} + DHF \longrightarrow \text{(No absorption at 415nm)} \quad (67)$$

$$A_o/A = 1 + \frac{k_{67}[DHF]}{k_{66}[ABTS]} \quad (68)$$

absorption at 415 nm in the absence and presence of dihydroxyfumaric acid, respectively. From the slopes of the competition plots (Figure 11), values of k_{67}/k_{66} were derived. Values for k_{67} were subsequently calculated, taking $k_{66} = 1.2 \times 10^{10} M^{-1}s^{-1}$ as was determined by (Wolfenden and Willson, 1982).

Since Fenton in one of his original papers stated that dihydroxymaleic acid (actually dihydroxyfumaric acid) is formed when tartaric acid was incubated with ferrous sulphate and H_2O_2, it was interesting to measure the rate constant of the reaction of hydroxyl radical with tartaric acid in a similar manner. A linear competition plot was again obtained (Figure 11). A general reaction would be written as:

$$OH^{\bullet} + ABTS \longrightarrow ABTS^{\bullet +} + \text{products} \quad (66)$$

$$OH^{\bullet} + \text{tartaric acid} \longrightarrow \text{products} \quad (69)$$

The rate constants of $k_{69}(OH^{\bullet} + \text{tartaric acid}) = 5.9 \times 10^{8} M^{-1}s^{-1}$ at pH 3.5 and 7 were determined.

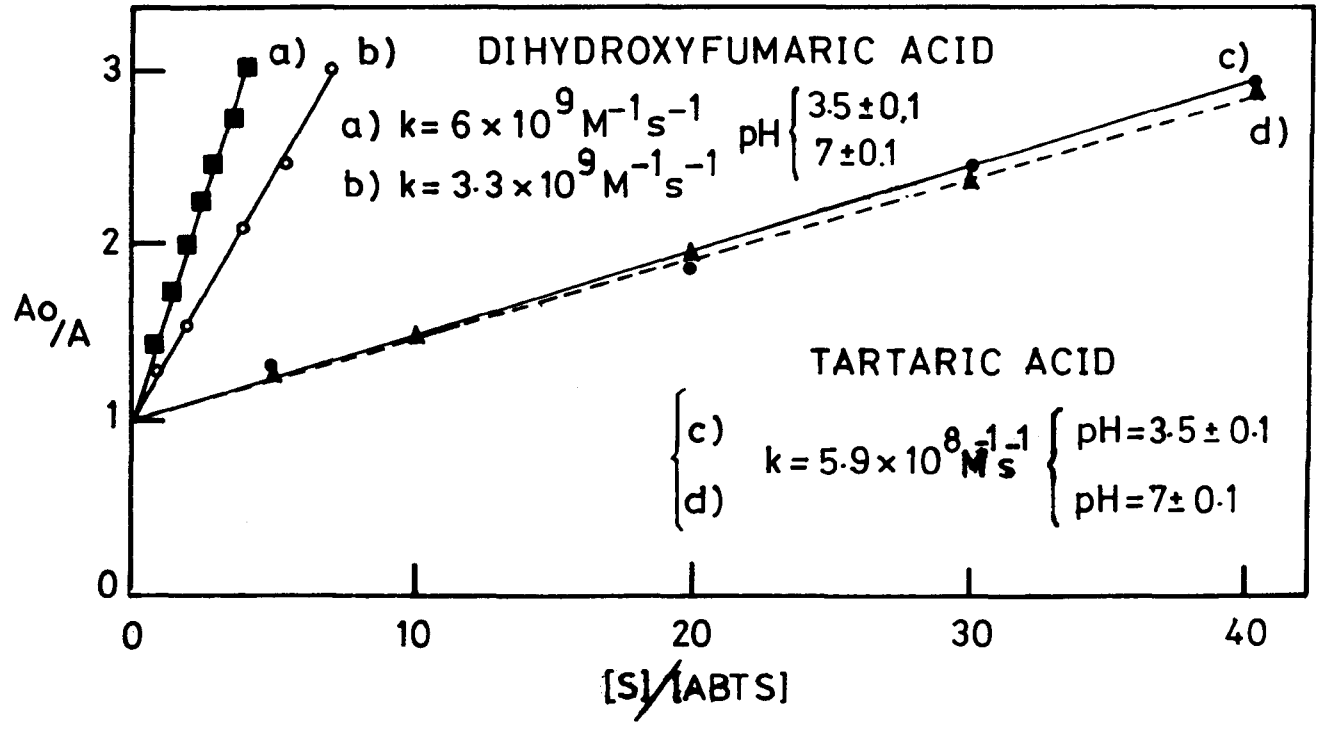


Figure 11: Competition plot for the reaction of OH· with ABTS and a) with dihydroxyfumaric acid, b) with tartaric acid.

viii) Reaction with the solvated electron (e^-_{aq})

On pulse radiolysis of nitrogen-saturated solutions containing t-butanol (5×10^{-2}M) to scavenge hydroxyl radicals, at pH 8, the strong characteristic absorption of the solvated electron was observed immediately after the radiation pulse. In the additional presence of dihydroxyfumaric acid in the range (16 to 80 μM) the absorption at 580 nm decayed exponentially and first order in dihydroxyfumaric acid concentration in agreement with:

$$e^-_{aq} + \text{DHF} \longrightarrow \text{dihydroxyfumaric acid electron adduct} \quad (70)$$

Plot of first order rate constant against dihydroxyfumaric acid was linear for the decrease in absorbance at 580 nm. The slope of the line yielded bimolecular rate constant of $k_{70} = 2.1 \times 10^{10} M^{-1} s^{-1}$ (Figure 12). Examinations at other wavelengths showed that an absorption at 360 nm from DHF subsequently appeared very rapidly. The change in absorbance was small presumably because the electron adduct of dihydroxyfumaric acid has a low molar absorption coefficient. However, the half life for this grow-in appeared to match with the decay of the electron adduct at 580 nm. The rate constant for the formation of the absorption at 360 nm was not determined in this work.

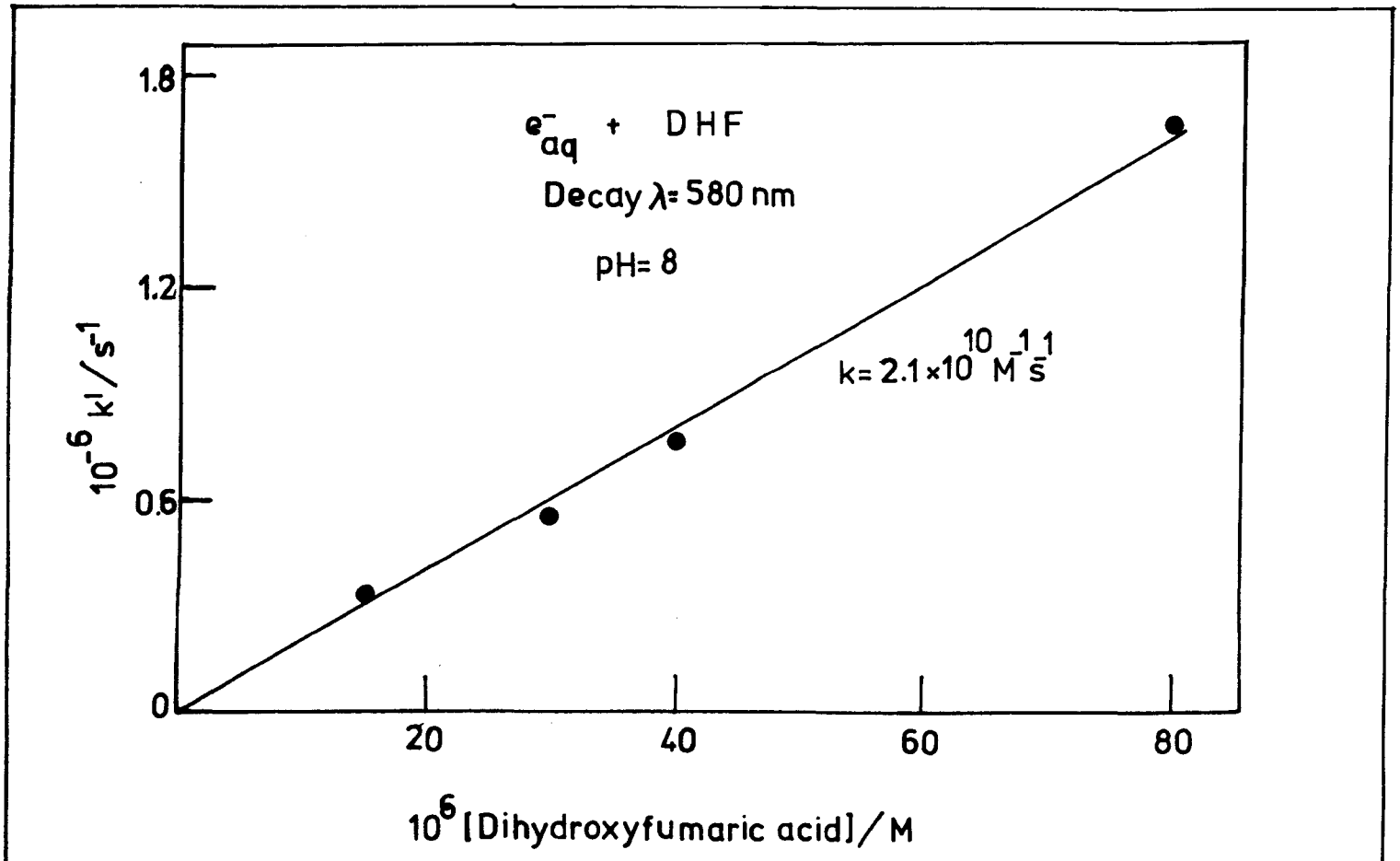


Figure 12: Plot of first-order rate constant against dihydroxyfumaric acid concentration for the decay of e_{aq} absorption at 580 nm.

ix) Reactions with inorganic electrophilic radicals

Halide atoms: $Cl^{\bullet}$; $Br^{\bullet}$; $I^{\bullet}$ can be present in irradiated solutions as the radicals $Cl_2^{\bullet -}$; $Br_2^{\bullet -}$; and $I_2^{\bullet -}$. The radicals absorb strongly in the visible region and their reactions with other compounds can be observed directly. A general equation would be written as:

$$OH^{\bullet} + 2X^{-} \longrightarrow OH^{-} + X_2^{\bullet -} \quad (71)$$

$$X_2^{\bullet -} + S \longrightarrow \text{products} \quad (72)$$

On pulse radiolysis of 0.1M potassium thiocyanate solutions saturated with nitrous oxide at pH 7, the characteristic strong transient absorption of $(SCN)_2^{\bullet -}$ at 480 nm was observed. In the presence of dihydroxyfumaric acid (5 to 20 x 10^{-4}M) the absorption decayed exponentially and first order in dihydroxyfumaric acid concentration (Figure 13), in agreement with:

$$OH^{\bullet} + SCN^{-} \longrightarrow (SCN\text{-}OH)^{\bullet -} \text{ "fast"} \quad (73)$$

$$(SCN\text{-}OH)^{\bullet -} + SCN^{-} \longrightarrow (SCN)_2^{\bullet -} + OH^{-} \text{ "slower"} \quad (74)$$

$$(SCN)_2^{\bullet -} + DHF \longrightarrow \text{products} \quad (75)$$

Replacing the thiocyanate with 0.1M potassium bromide on solutions saturated with nitrous oxide at pH 7 the characteristic strong transient absorption at 360 nm was observed. In presence of dihydroxyfumaric acid (1 to 4 x 10^{-4}M) the absorption decayed exponentially and first order in dihydroxyfumaric acid concentration

(Figure 13), in agreement with.

$$OH^{\cdot} + 2Br^{-} \longrightarrow Br_2^{\cdot -} + OH^{-} \quad (76)$$

$$Br_2^{\cdot -} + DHF \longrightarrow \text{products} \quad (77)$$

Similar studies were undertaken for 0.1M potassium iodide on solutions saturated with nitrous oxide containing dihydroxyfumaric acid in the range (1.5 to 6 x 10^{-3}M) at pH 7. The absorption for the related radical $I_2^{\cdot -}$ at 350 nm was observed which decayed exponentially when dihydroxyfumaric acid was increased (Figure 14) in agreement with.

$$I_2^{\cdot -} + DHF \longrightarrow \text{products} \quad (78)$$

From the slopes of the kinetics plots the values for the reactions of $k_{77}(Br_2^{\cdot -} + DHF) = 2.6 \times 10^{8} M^{-1}s^{-1}$; $k_{75}(SCN)_2^{\cdot -} + DHF) = 4 \times 10^{7} M^{-1}s^{-1}$; and $k_{78}(I_2^{\cdot -} + DHF) = 1.5 \times 10^{6} M^{-1}s^{-1}$ were determined respectively.

For comparison purposes the rate constant of the reaction of $I_2^{\cdot -}$ with ascorbic acid at pH7 also was determined in a similar manner (Figure 15) in accordance with the following reaction.

$$I_2^{\cdot -} + AH^{-} \longrightarrow \text{products} \quad (79)$$

The rate constant for the reaction $k_{79}(I_2^{\cdot -} + \text{ascorbic acid}) = 2 \times 10^{7} M^{-1}s^{-1}$, was measured.

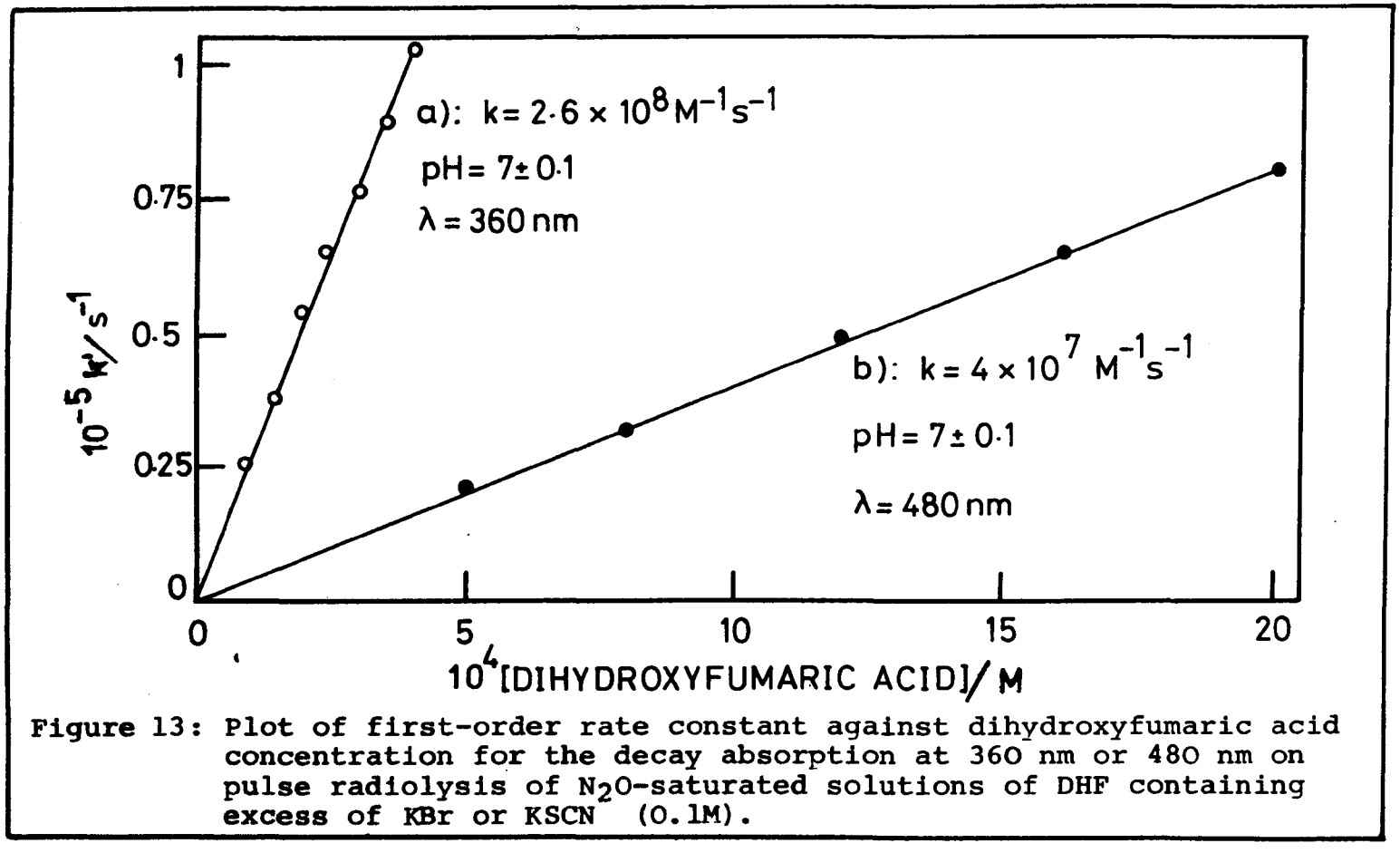


Figure 13: Plot of first-order rate constant against dihydroxyfumaric acid concentration for the decay absorption at 360 nm or 480 nm on pulse radiolysis of N_2O-saturated solutions of DHF containing excess of KBr or KSCN (0.1M).

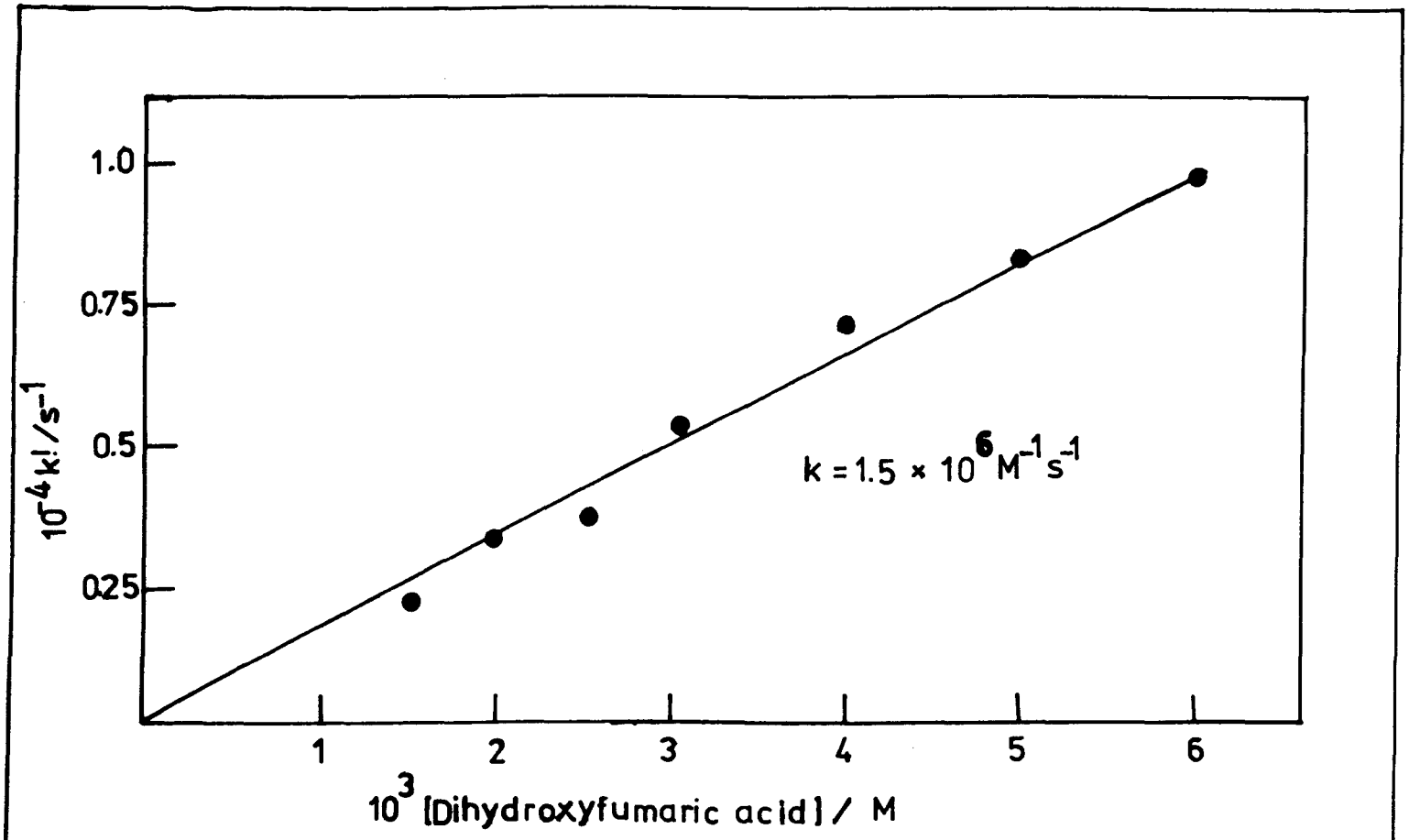


Figure 14: Plot of first-order rate constant against dihydroxyfumaric acid concentration on pulse radiolysis of N_2O-saturated solutions of DHF containing excess of KI (0.1M).

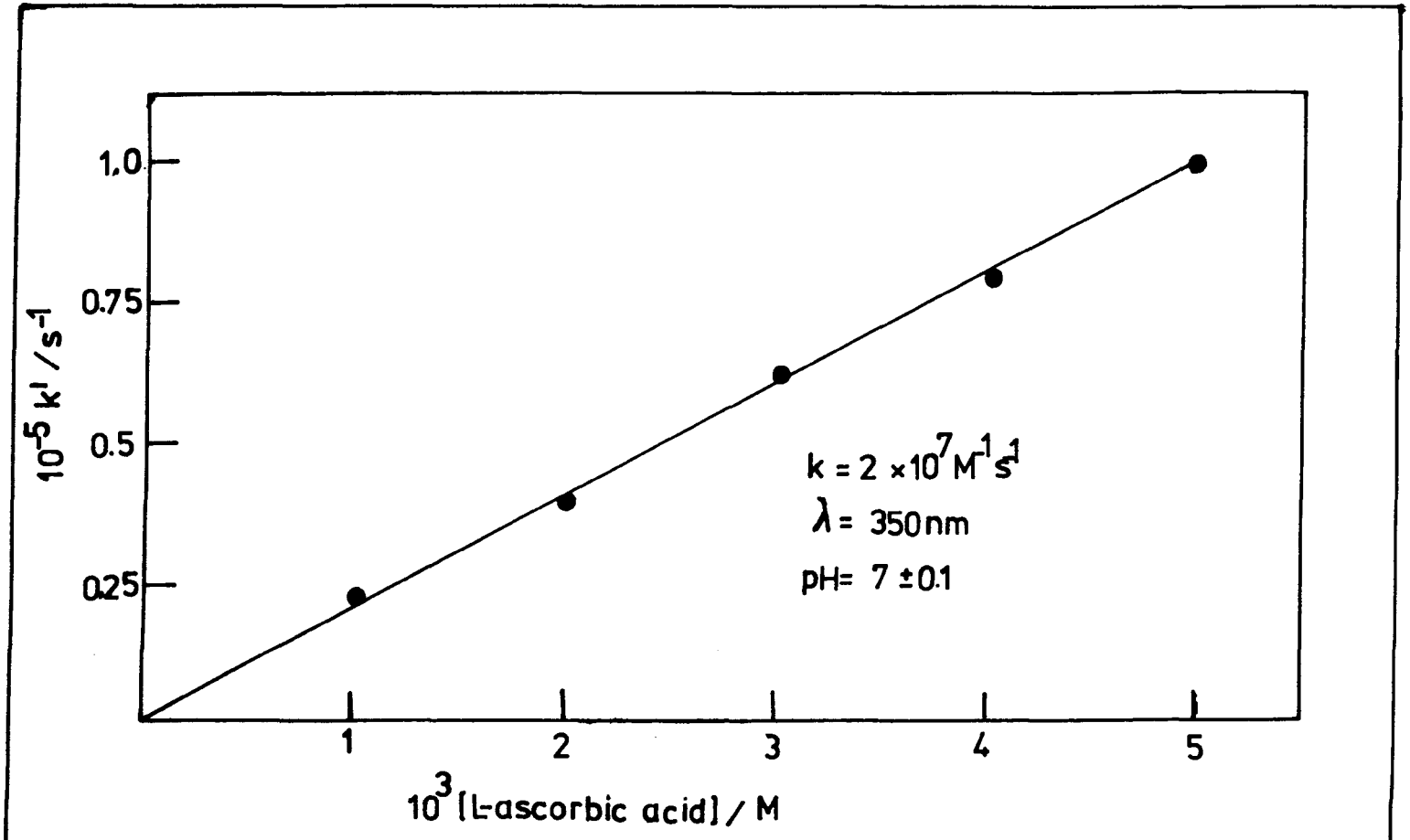


Figure 15: Plot of first-order rate constant against L-ascorbic acid concentration on pulse radiolysis of N_2O-saturated solutions of L-ascorbic acid containing excess of KI(0.1M).

x) Acid Dissociation Constant (pKa) Determination

The pulse radiolysis method can be used to determine both the pKa of a radical species and the pKa of the original solute (Willson, 1978). The former is information of fundamental chemical importance since it determines which radical species is present at a particular pH and these may have greatly differing reactivities with substrates. In the case of the latter, the rate constant of the reaction of a particular radical (for example, $Br_2^{\bullet -}$) with the parent compound can be studied over a pH range. In this work, this type of study was undertaken in order to determine the pKa of dihydroxyfumaric acid.

On N_2O-saturated solutions containing KBr (0.1M), at different pH, the characteristic strong transient absorption at 360 nm was observed. In presence of dihydroxyfumaric acid (2.5×10^{-4}M) at the pH (3 to 12), the absorption decayed exponentially and first order in dihydroxyfumaric acid concentration. By plotting the rate constant k' for ($Br_2^{\bullet -}$ + DHF) against pH, a curve was obtained (Figure 16) from which the pKa value was calculated.

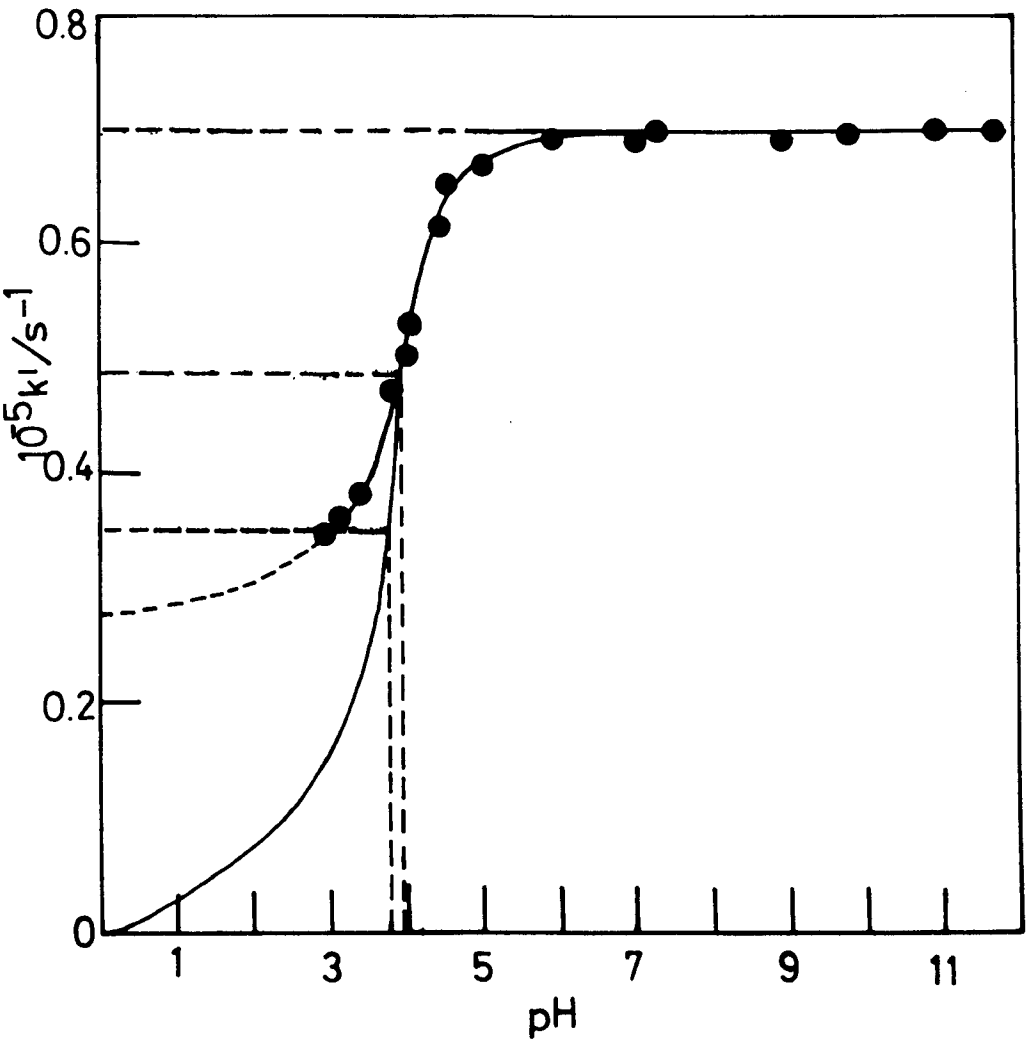


Figure 16: A plot of k($Br_2^{\bullet -}$ + dihydroxyfumaric acid) for different pH values:
Decay of absorbance at λ = 360 nm.
(*) pK value ≈3.8 ± 0.2

xi) Reactions with azide radical $N_3^{\cdot}$

The azide radical $N_3^{\cdot}$ has been found on pulse radiolysis of N_2O-saturated solutions of sodium azide (Hayon and Simic, 1970). The extinction coefficient of $N_3^{\cdot}$ is found to be $\varepsilon_{278} = 2.3 \times 10^3 M^{-1}cm^{-1}$. Azide ions in aqueous solutions undergo an electron oxidation mechanism on reaction with hydroxyl radicals.

$$OH^{\cdot} + N_3^- \longrightarrow N_3^{\cdot} + OH^- \qquad (80)$$

$$k_{80} = 1.2 \times 10^{10} M^{-1}s^{-1}$$

Since dihydroxyfumaric acid absorbs strongly below 300 nm, the direct observation of the decay of $N_3^{\cdot}$ was not possible. It was decided to see whether $N_3^{\cdot}$ reacted rapidly with ABTS to give $ABTS^{\cdot+}$ in the hope that competition studies might be undertaken. On pulse radiolysis of nitrous oxide-saturated solutions containing NaN_3 (10^{-2}M) and ABTS in the concentration range of 1 to 6 x 10^{-5}M at pH = 7, the absorption for the radical cation $ABTS^{\cdot+}$ at 415 nm was observed. The rate of the formation was exponential with first order rate constant increasing with ABTS concentration. The rate constant of k_{81}($N_3^{\cdot}$ + ABTS) was indicated by product build up kinetics (pbk) which gave a value of 6.3 x $10^9 M^{-1}s^{-1}$ (Figure 17) in accordance with

$$N_3^{\cdot} + ABTS \longrightarrow ABTS^{\cdot+} + N_3^- \qquad (81)$$

On pulse radiolysis of N_2O-saturated solutions containing

NaN_3(10^{-2}M), ABTS(10^{-4}M) and dihydroxyfumaric acid in the range 1 to 7 x 10^{-4}M at pH 7, competition kinetics were observed. The maximum absorption of $ABTS^{\cdot+}$ at 415 nm decreased with increasing concentrations of dihydroxyfumaric acid and in agreement with competing reaction (82), and equation (83).

$$N_3^{\cdot} + ABTS \longrightarrow ABTS^{\cdot+} + \text{products} \quad (81)$$

$$N_3^{\cdot} + DHF \longrightarrow \text{products} \quad (82)$$

$$A_o/A = 1 + \frac{k_{82}\,[DHF]}{k_{81}\,[ABTS]} \quad (83)$$

From the slope of the competition plot (Figure 18), the relative rate constant was obtained and taking $k_{81}(N_3^{\cdot} + ABTS) = 6.3 \times 10^9 M^{-1}s^{-1}$, the value for the reaction $k_{82}(N_3^{\cdot} + DHF) = 1.8 \times 10^9 M^{-1}s^{-1}$ was derived.

For comparison purposes, similar competition studies with ascorbic acid were undertaken. The rate constant of the reaction $k(N_3^{\cdot} + \text{Ascorbic acid}) = 4 \times 10^9 M^{-1}s^{-1}$ at pH 7 was determined (Figure 18). A direct method to measure the rate constant of the reaction ($N_3^{\cdot}$ + Ascorbic acid) at 360 nm was also undertaken by pulse radiolysis of nitrous oxide-saturated solutions containing NaN_3(10^{-2}M) and ascorbic acid in the range 1 to 4 x 10^{-5}M at pH 6.9. The absorption of the ascorbic acid radical at 360 nm was observed. The rate of the formation was exponential with first order rate constant increasing with ascorbic acid concentration used in agreement with:

$$N_3^{\cdot} + AH^- \dashrightarrow N_3^- + A^{\cdot -} + H^+ \quad (84)$$

From the product build up kinetics (pbk) a value $k_{84}(N_3^{\cdot} + AH^-) = 3.2 \times 10^9 M^{-1}s^{-1}$ was determined (Figure 19).

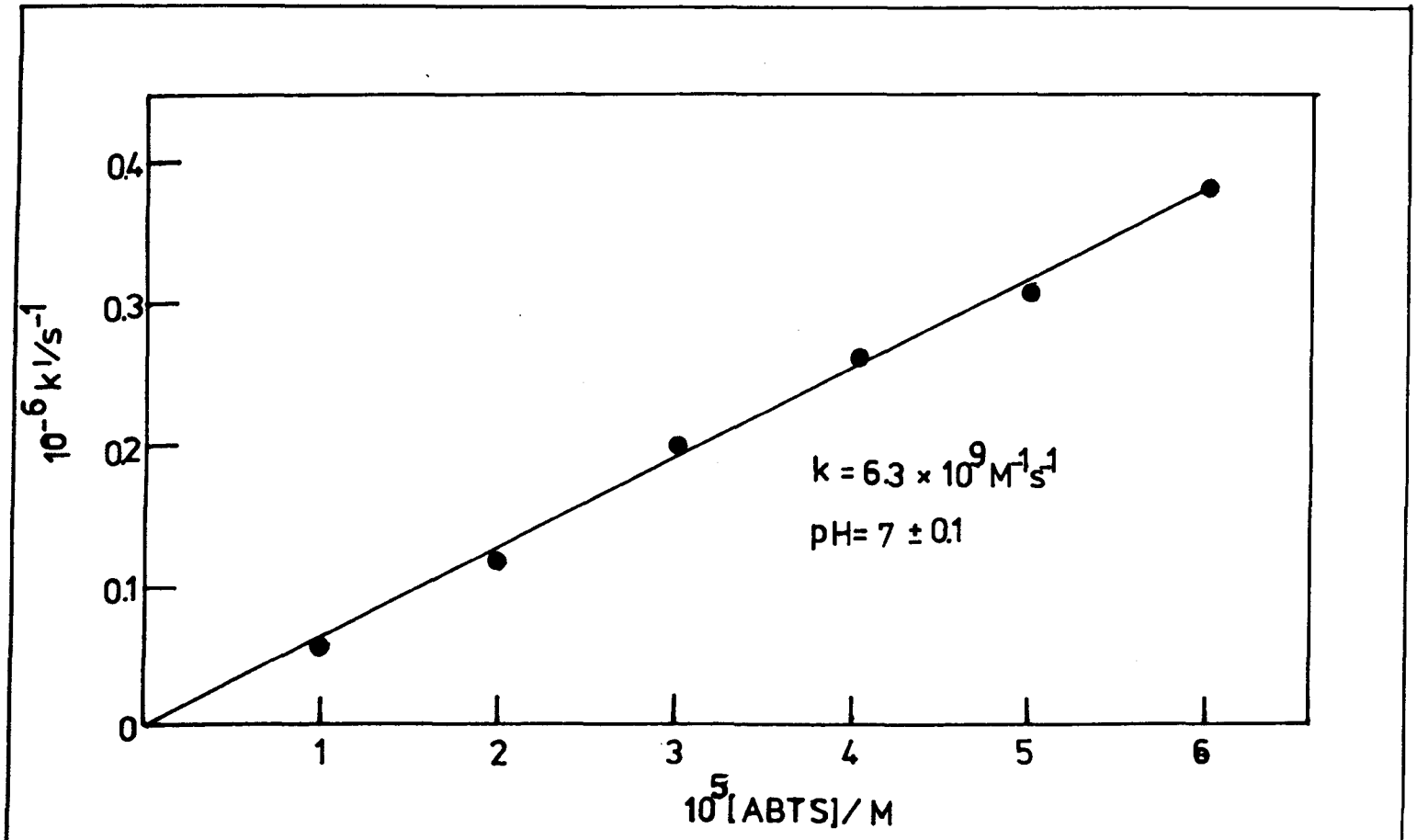


Figure 17: Plot of first-order rate constant against ABTS concentration for formation of absorption at 415 nm on pulse radiolysis of nitrous oxide-saturated solutions containing ABTS and sodium azide (10^{-2}M)

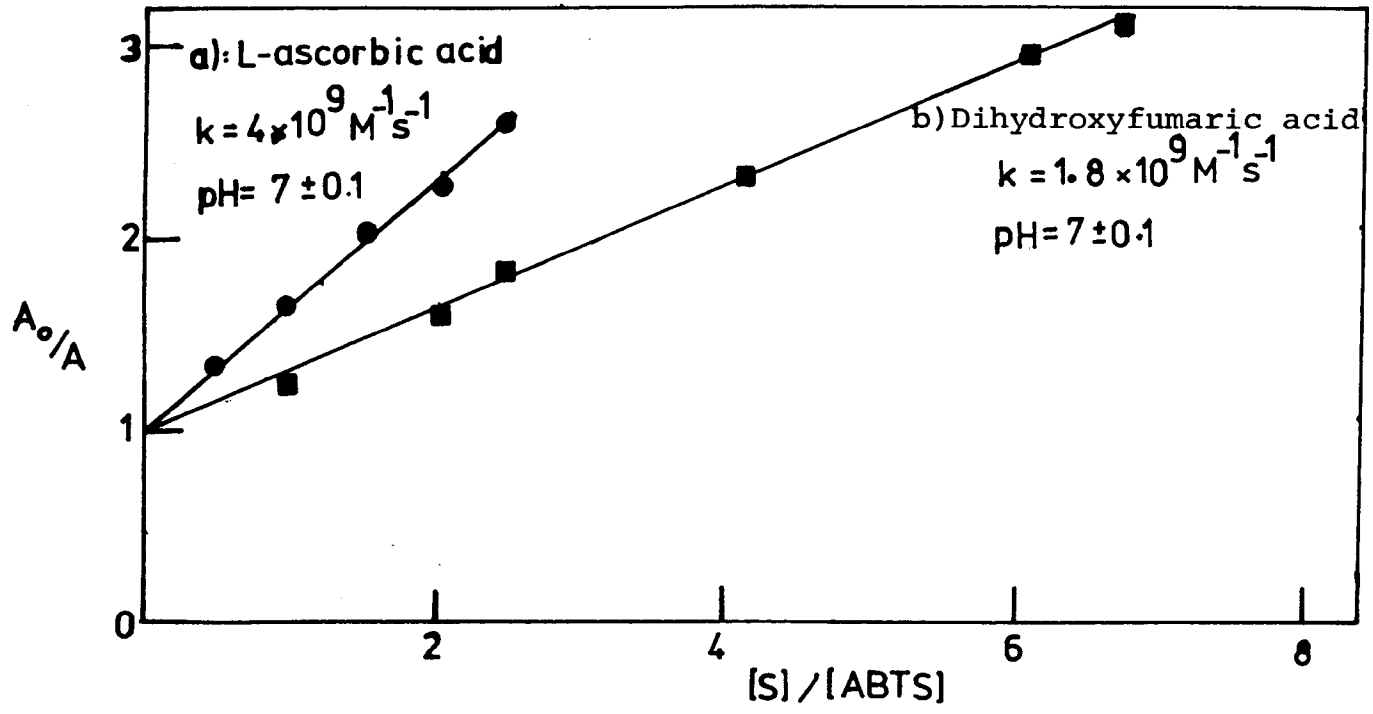


Figure 18: Competition plot for the reaction of $N_3^{\bullet}$ with ABTS and a) with L-ascorbic acid; b) with dihydroxyfumaric acid.

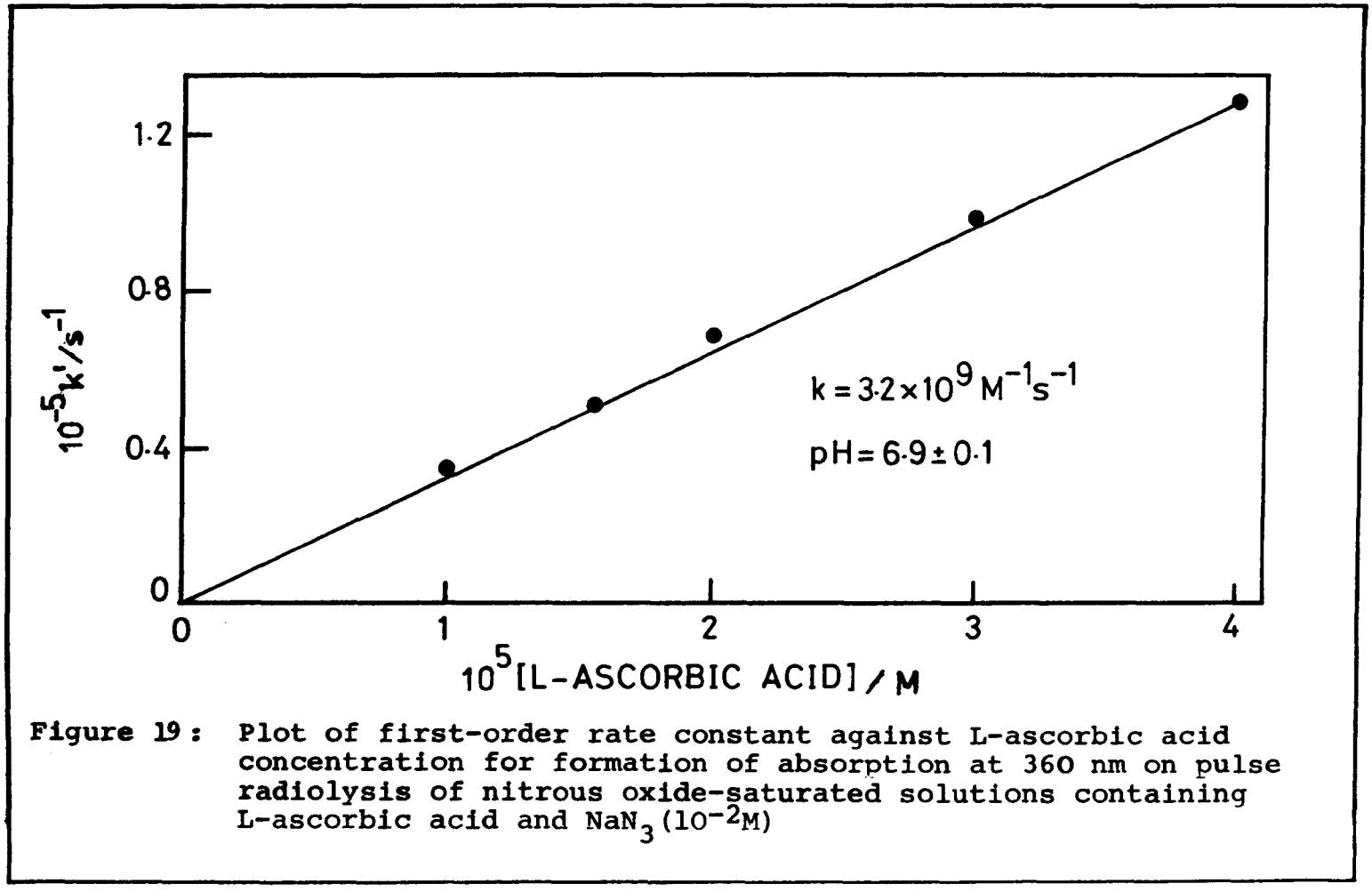


Figure 19 : Plot of first-order rate constant against L-ascorbic acid concentration for formation of absorption at 360 nm on pulse radiolysis of nitrous oxide-saturated solutions containing L-ascorbic acid and NaN_3 (10^{-2}M)

xii) Reactions with nitrogen dioxide (NO_2)

Nitrate ion reacts with the hydrated electron to form $NO_3^{\cdot 2-}$ (Grätzel et al., 1970).

$$e^-_{aq} + NO_3^- \longrightarrow NO_3^{\cdot 2-} \quad (85)$$

$NO_3^{\cdot 2-}$ reacts with water according to:

$$NO_3^{\cdot 2-} + H_2O \longrightarrow NO_2 + 2OH^- \quad (86)$$

with a half life of 12.5 μs, and in acid solution, it picks up a proton according to the equilibria:

$$H_2NO_3 \underset{pKa = 4.8}{\rightleftharpoons} HNO_3^- \underset{pKb = 7.5}{\rightleftharpoons} NO_3^{\cdot 2-} \quad (87)$$

HNO_3^- decays into $NO_2 + OH^-$ with a half life of 3 μs. $NO_3^{\cdot 2-}$ can accept a proton from the $H_2PO_4^-$ ion with $k = 5 \times 10^8 M^{-1}s^{-1}$. The hydroxyl radicals can react with NO_2 to yield pernitrous acid, HO_2NO with $k = 1.3 \times 10^9 M^{-1}s^{-1}$ (pk = 5.3). The undissociated form spontaneously isomerizes to form nitric acid, HNO_3, while the anion is stable. The isomerization with a rate constant of $k_{88} = 1.9 \times 10^3 s^{-1}$ can be written as follows:

$$HO_2NO \longrightarrow HNO_3 \quad (88)$$

Pulse radiolysis studies of nitrogen-saturated solutions containing $NaNO_3$ (0.1M), ABTS in the range 0.5 to 4×10^{-3}M, t-butanol (1M) at pH 6.5 were undertaken. The long-lived radical cation $ABTS^{\cdot +}$ was measured at 415 nm and found to be

formed exponentially with a first order rate constant proportional to ABTS concentration in agreement with

$$e^-_{aq} + NO_3^- \longrightarrow NO_3^{\cdot 2-} \quad (85)$$

$$NO_3^{\cdot 2-} + H_2O \longrightarrow NO_2 + 2OH^- \quad (86)$$

$$NO_2 + ABTS \longrightarrow NO_2^- + ABTS^{\cdot +} \quad (89)$$

Taking the published extinction coefficient for $ABTS^{\cdot +}$, ε_{414} 3.6×10^4 l $mol^{-1}cm^{-1}$, the yield corresponds to 100% of the aquated electrons formed by the pulse. From the slope of (Figure 20), $k_{89} = 3.8 \times 10^7 M^{-1}s^{-1}$ was determined.

Competition method to determine the rate constant of the reaction of NO_2 with dihydroxyfumaric acid was undertaken using ABTS as the reference compound. Nitrogen-saturated solutions containing $NaNO_3$ (5×10^{-2}M), ABTS (5×10^{-4}M) and dihydroxyfumaric acid in the range 0.5 to 2.2×10^{-3}M in t-butanol (1M) at pH 7 were irradiated. The maximum absorption of $ABTS^{\cdot +}$ at 415 nm decreased with increasing concentrations of dihydroxyfumaric acid, in agreement with the competing reaction (90) and the equation (91) as follows:

$$NO_2 + ABTS \longrightarrow NO_2^- + ABTS^{\cdot +} \quad (89)$$

$$NO_2 + DHF \longrightarrow \text{products} \quad (90)$$

$$A_o/A = 1 + \frac{k_{90}\,[DHF]}{k_{89}\,[ABTS]} \quad (91)$$

where A_o and A are the measured extinctions at 415 nm in the absence and presence of dihydroxyfumaric acid, respectively. Taking k_{89} for the reaction (NO_2 + ABTS) = $3.8 \times 10^7 M^{-1}s^{-1}$, the value of the rate constant for the reaction k_{90}(NO_2 + DHF) = $2.2 \times 10^7 M^{-1}s^{-1}$ was derived from the slope of the plot (Figure 21).

For comparison purposes, similar experiments were undertaken with ascorbic acid instead of dihydroxyfumaric acid. The competition plot was again linear, and from the slope (Figure 21), the value for the rate constant of the reaction k_{92}(NO_2 + ascorbic acid) = $3.1 \times 10^7 M^{-1}s^{-1}$ was determined in agreement with the competing reaction (92).

$$NO_2 + ABTS \longrightarrow NO_2^- + ABTS^{\cdot +} \qquad (89)$$

$$NO_2 + AH^- \longrightarrow \text{products (no absorption at 415 nm)} \qquad (92)$$

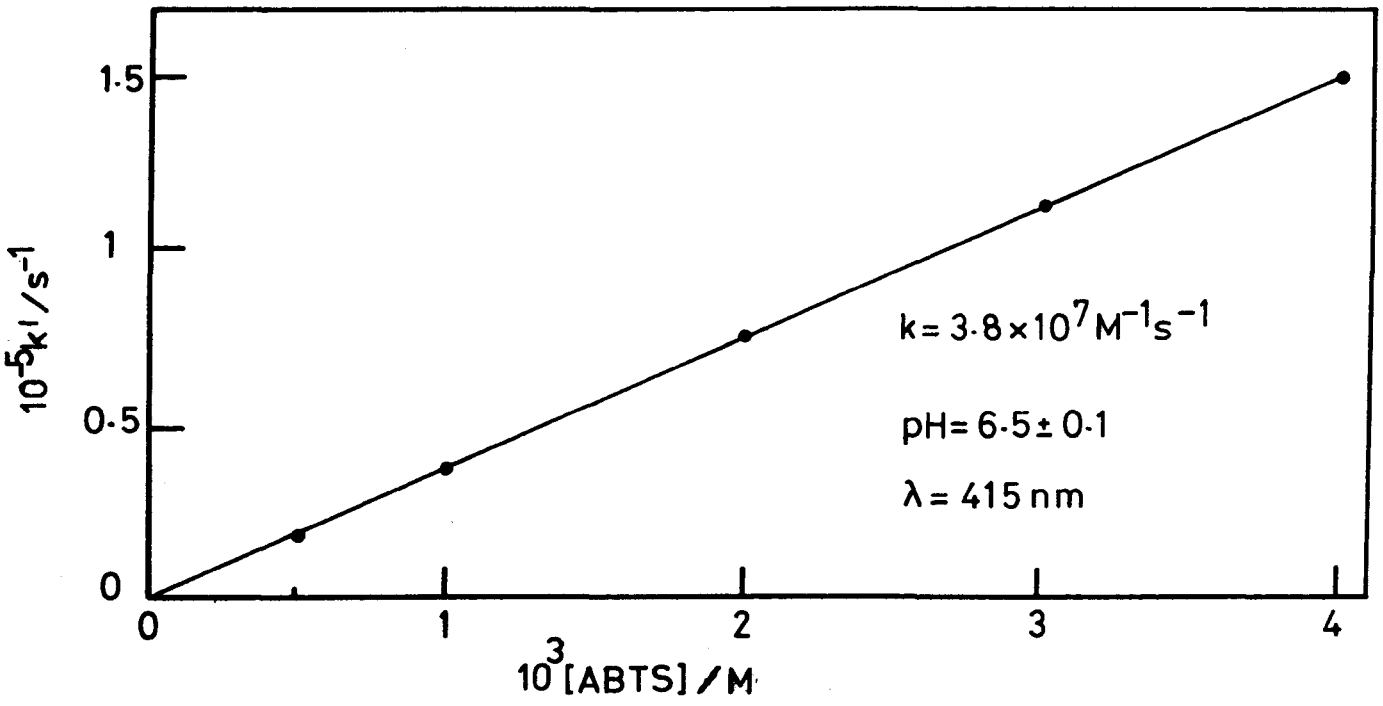


Figure 20: Plot of first-order rate constant against ABTS concentration for formation of absorption at 415 nm on pulse radiolysis of nitrous oxide-saturated solutions containing ABTS and $NaNO_3$

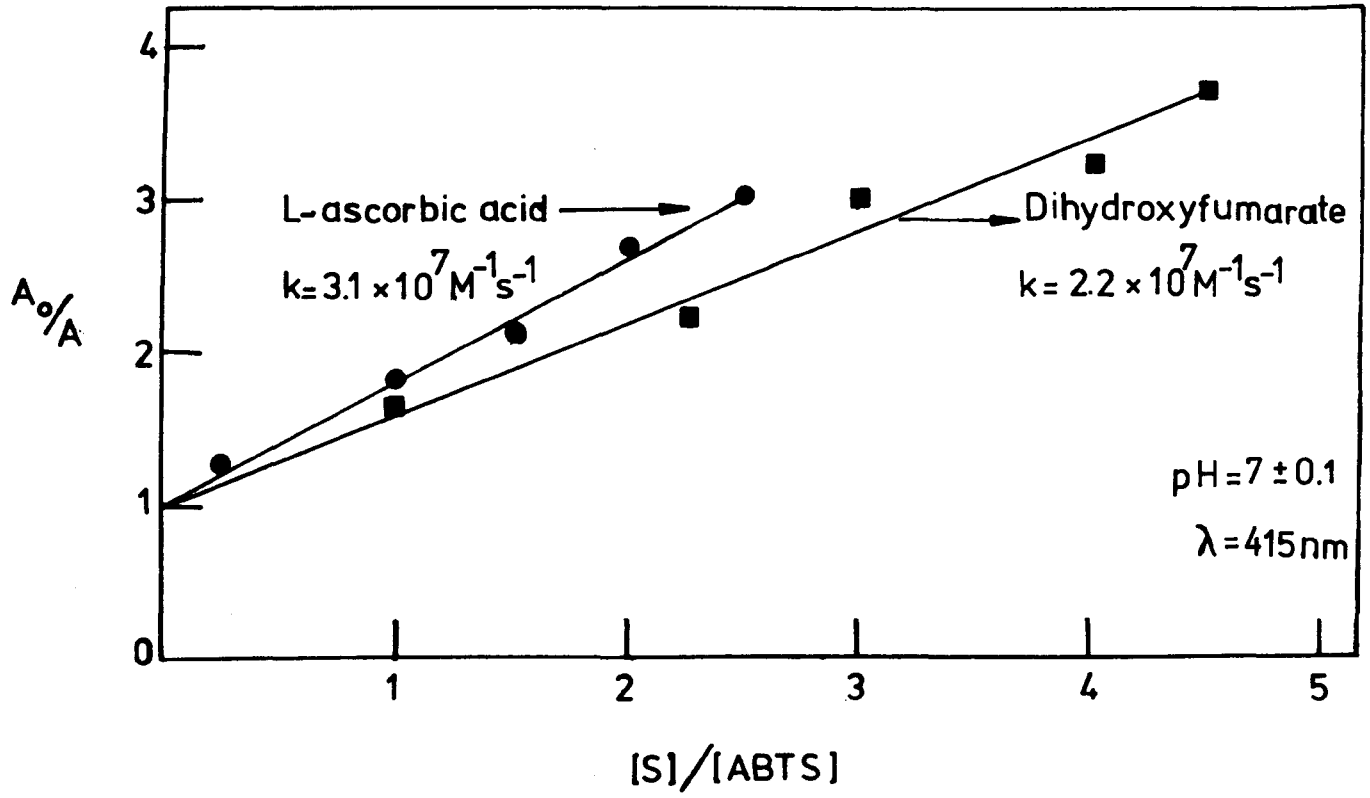


Figure 21: Competition plot for the reaction of NO_2 with ABTS and a) with L-ascorbic acid; b) with dihydroxyfumaric acid.

xiii) Reactions with chlorpromazine radical

Previous studies have showed that the radical-cation formed from the one electron oxidation of phenothiazines can react rapidly with alpha-tocopherol (vitamin E) and ascorbate (vitamin C). (Klein and Toppen, 1978; Pelizzetti et al., 1979; Mahood et al., 1980.)

In view of the similarity between ascorbic acid and dihydroxyfumaric acid, it was considered useful to examine whether dihydroxyfumaric acid could behave similarly. On pulse radiolysis of nitrous oxide-saturated solutions containing potassium bromide (0.1M) and chlorpromazine (10^{-2}M) at pH 6.5, the radical absorption of $CPZ^{\cdot+}$ at 525 nm was observed. In the additional presence of dihydroxyfumaric acid in the concentrations of 1 to 8 x 10^{-4}M, the absorption decayed exponentially and first order in dihydroxyfumaric acid concentration (Figure 22), in agreement with:

$$Br_2^{\cdot-} + CPZ \longrightarrow CPZ^{\cdot+} \qquad (93)$$

$$CPZ^{\cdot+} + DHF \longrightarrow \text{products} \qquad (94)$$

From the slope of the kinetic plot, the value of the reaction of $k_{94}(CPZ^{\cdot+} + DHF) = 3.1 \times 10^8 M^{-1}s^{-1}$ was determined.

Similar experiment was performed replacing chlorpromazine with promethazine. However, when the pH of the solution was adjusted with KOH in the additional

presence of dihydroxyfumaric acid a precipitate was observed. If the pH of the solution was lowering under 5, the experiment was not suitable to get good oscillograms in part probably due to the rapid oxidation of dihydroxyfumaric acid in the acid region.

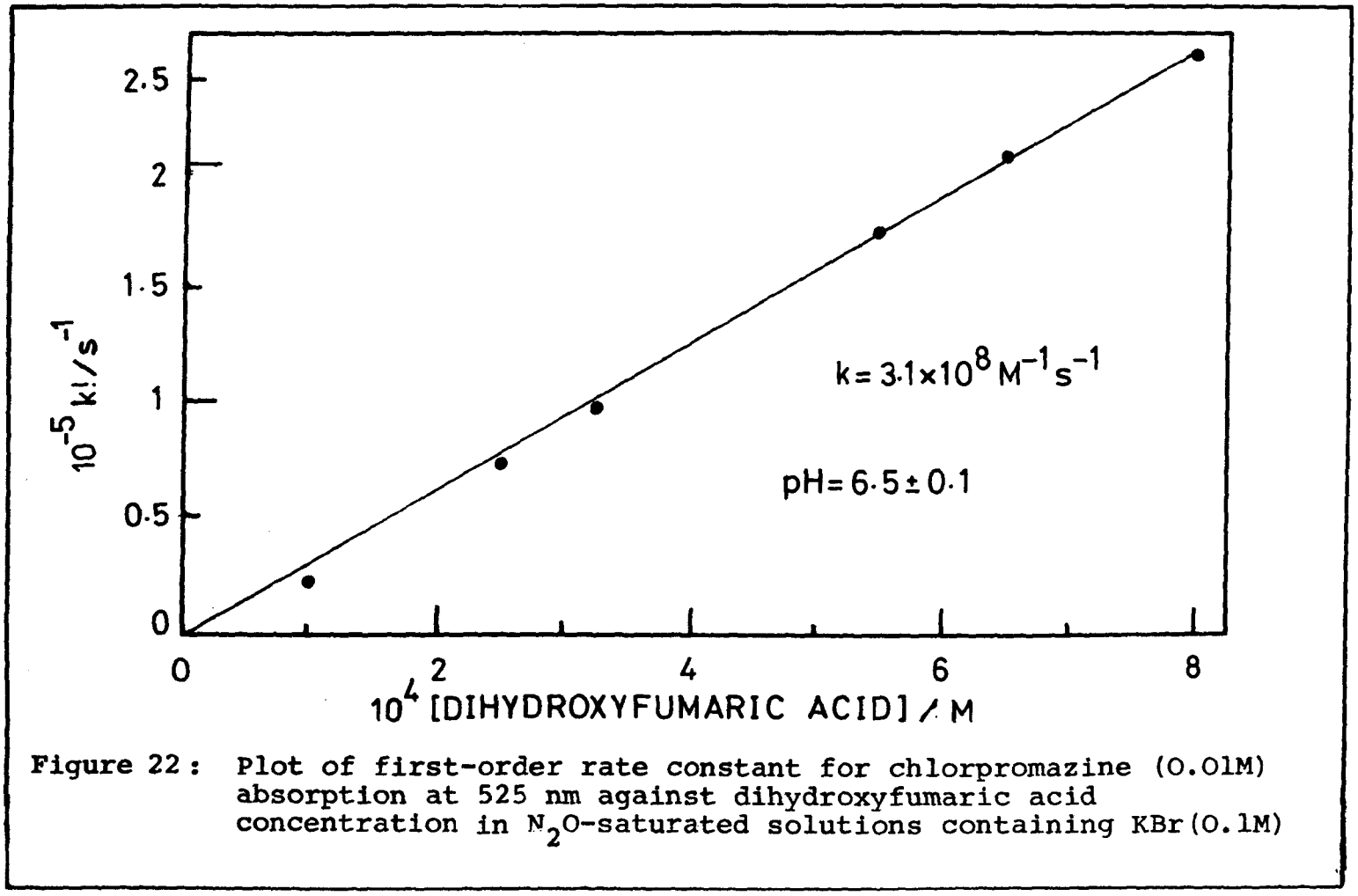


Figure 22 : Plot of first-order rate constant for chlorpromazine (0.01M) absorption at 525 nm against dihydroxyfumaric acid concentration in N_2O-saturated solutions containing KBr(0.1M)

xiv) Reactions with the Isopropanol radical

The isopropanol radical can be readily generated by pulse radiolysis of solutions containing excess acetone and isopropanol in which the following reactions can take place:

$$H_2O \longrightarrow OH^{\cdot}(45\%) + e^-_{(aq)}(45\%) + H^{\cdot}(10\%) \quad (95)$$

$$OH^{\cdot}\ (\text{or } H^{\cdot}) + CH_3CHOHCH_3 \longrightarrow H_2O\ (\text{or } H_2) + CH_3\dot{C}OHCH_3 \quad (96)$$

$$e^-_{(aq)} + CH_3COCH_3 \longrightarrow CH_3COCH_3^{\cdot -} \quad (97)$$

acetone electron adduct

The immediate product is the isopropanol radical or its basic form, the acetone electron adduct, depending on the pH and the equilibrium, as follows:

$$CH_3\dot{C}OHCH_3 + OH^- \underset{pKa = 12.2}{\rightleftharpoons} CH_3COCH_3^{\cdot -} + H_2O \quad (98)$$

Although these radicals can also be formed in N_2O-isopropanol solutions, the acetone system has the advantage that solutions with higher concentrations of additional solutes or acid can be undertaken without the danger of competing reactions taking place.

The isopropanol radical like $H^{\cdot}$ and $OH^{\cdot}$ absorbs in the U.V. and the rates of reaction can only be determined directly when the reactants or products have convenient strong absorptions.

$$CH_3\dot{C}OHCH_3 + RSSR \longrightarrow CH_3COCH_3 + RSSR^{\cdot -} + H^+ \quad (99)$$

lipoate — absorbance increase, λ max 410 nm

When this is not the case, the competition method can be employed using, for example, p-nitroacetophenone (PNAP) as the competing solute which reacts to form the radical-anion $PNAP^{\cdot -}$ (Willson, 1978).

$$CH_3\dot{C}OHCH_3 + \begin{cases} PNAP \longrightarrow PNAP^{\cdot -} + CH_3COCH_3 + H^+ & (100) \\ \quad \lambda max = 360\ nm & \\ CCl_4 \longrightarrow CCl_3^{\cdot} + CH_3COCH_3 + H^+ + Cl^- & (110) \\ \quad \text{no absorption} & \end{cases}$$

Nitrogen-saturated solutions containing PNAP (10^{-4}M), acetone-isopropanol (1M) and different concentrations of dihydroxyfumaric acid, were pulse irradiated. The $PNAP^{\cdot -}$ absorption observed at 360 nm decreased with increasing dihydroxyfumaric acid concentration, in agreement with the competing reaction (101) and equation (102), where A_o and A are the measured:

$$CH_3\dot{C}OHCH_3 + PNAP \longrightarrow PNAP^{\cdot -} + CH_3COCH_3 + H^+ \quad (100)$$

$$CH_3\dot{C}OHCH_3 + DHF \longrightarrow \text{products} \quad (101)$$

$$A_o/A = 1 + \frac{k_{101}[DHF]}{k_{100}[PNAP]} \quad (102)$$

absorptions at 360 nm in the absence and presence of dihydroxyfumaric acid, respectively. From the slope of the competition plot (Figure 23) a value of k_{101}/k_{100} was

derived and, taking $k_{100}(IP^{\cdot} + PNAP) = 3.8 \times 10^{9} M^{-1}s^{-1}$ (Adams and Willson, 1973), the rate constant for the reaction $k_{101}(IP^{\cdot} + DHF) = 5.7 \times 10^{7} M^{-1}s^{-1}$ was derived.

It was also of interest to measure the rate constants of hydrogen transfer to the isopropanol radical using p-nitroacetophenone (PNAP). On pulse radiolysis of nitrous oxide-saturated solutions containing PNAP (10^{-4}M) and excess of isopropanol (1M) or nitrogen-saturated solutions also containing isopropanol and excess of acetone (1M), at pH = 4, the characteristic absorption of the $PNAP^{\cdot -}$ radical-anion at 360 nm was observed. In the additional presence of increasing concentrations of the thiol compounds such as: dithiothreitol (DTT) (1.6 to 8 x 10^{-4}M); 2-mercapto propionyl glycine (thiola) (8 to 24 x 10^{-4}M); glutathione (GSH) (8 to 32 x 10^{-4}M); D-penicillamine (8 to 32 x 10^{-4}M); cysteine (8 to 24 x 10^{-4}M) and cysteamine (8 to 24 x 10^{-4}M), the absorption of $PNAP^{\cdot -}$ was progressively reduced in agreement with the competing reaction (103) and equation (104):

$$CH_3\dot{C}OHCH_3 + PNAP \longrightarrow PNAP^{\cdot -} + CH_3COCH_3 + H^{+} \quad (100)$$

$$CH_3\dot{C}OHCH_3 + RSH \longrightarrow CH_3CHOHCH_3 + RS^{\cdot} \quad (103)$$

$$A_o/A = 1 + \frac{k_{103}[RSH]}{k_{100}[PNAP]} \quad (104)$$

where A_o is the absorption due to $PNAP^{\cdot -}$ in the absence of thiols and A the corresponding absorption in the presence

of a particular compound. From the slopes of the competition plots the relative rate constants k_{103}/k_{100} for each thiol compound studied were obtained and, taking the value of $k_{100}(IP^{\bullet} + PNAP) = 3.8 \times 10^{9} M^{-1}s^{-1}$, the rate constants for the reaction of ($IP^{\bullet}$ + RSH) could be derived. Results are shown in Table 5 and Figure 24.

For comparison purposes, it was also useful to measure the rate of reaction of the isopropanol radical ($IP^{\bullet}$) with the spin trap 5,5-dimethyl - 1 - pyrroline - 1 - oxide (DMPO); the stable free radical (TMPN); and the iron chelates EDTA/Fe^{3+}, DTPA/Fe^{3+}, desferal/Fe^{3+} against PNAP as a reference solute. In all cases good linear competition plots were again obtained (Figures 25 and 26), in agreement with the represented reactions:

$$CH_3\dot{C}OHCH_3 + PNAP \longrightarrow PNAP^{\bullet -} + CH_3COCH_3 + H^{+} \quad (100)$$

$$CH_3\dot{C}OHCH_3 + DMPO \longrightarrow CH_3C(OH)CH_3 - DMP\dot{O} \quad (105)$$

$$CH_3\dot{C}OHCH_3 + TMPN \longrightarrow \text{products} \quad (106)$$

$$CH_3\dot{C}OHCH_3 + EDTA/Fe^{3+} \longrightarrow \text{products} \quad (107)$$

$$CH_3\dot{C}OHCH_3 + DTPA/Fe^{3+} \longrightarrow \text{products} \quad (108)$$

$$CH_3\dot{C}OHCH_3 + Desferal/Fe^{3+} \xrightarrow{\text{"slow"}} \text{products} \quad (109)$$

In an attempt to measure the rate of reaction of the isopropanol radical with the complex glycine/Fe^{3+} against PNAP as a marker, it was not possible to obtain competition due to the formation of a brown precipitate.

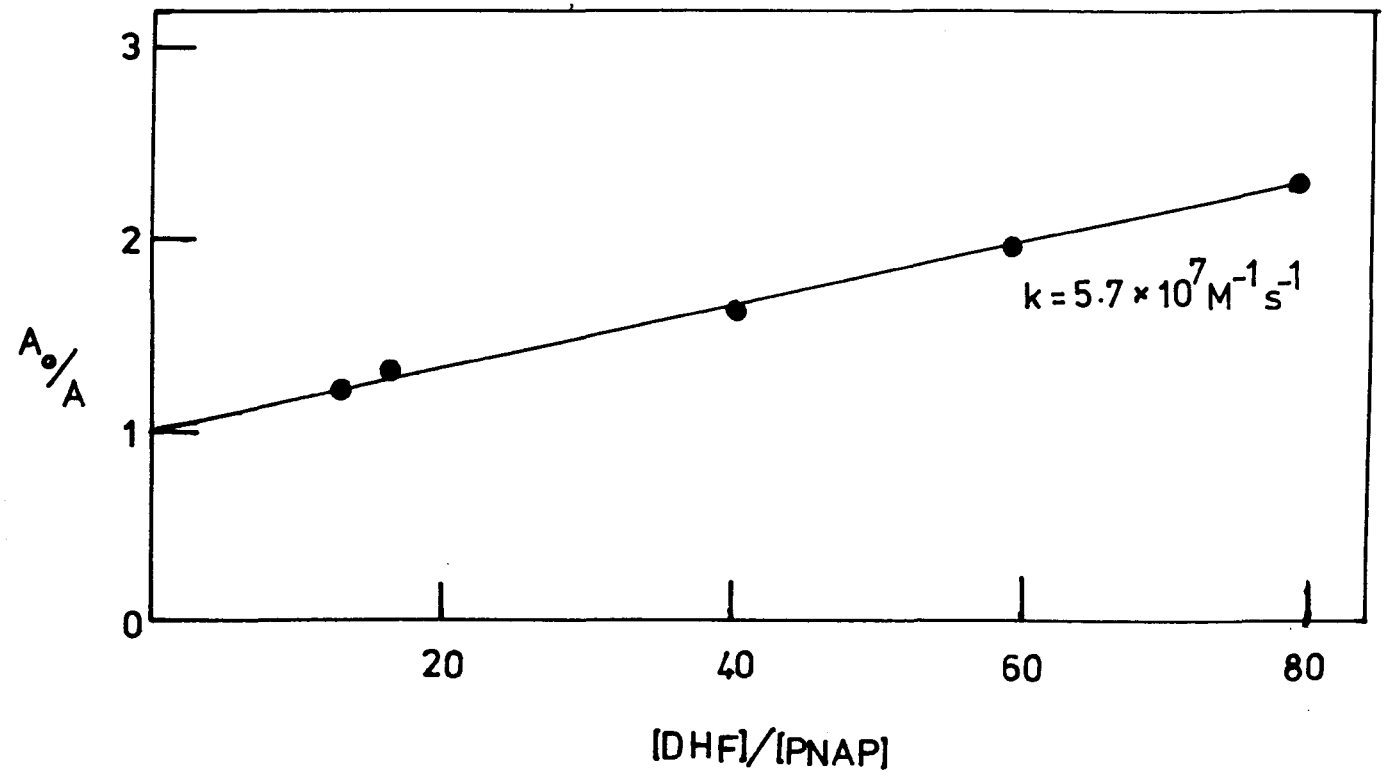


Figure 23 : Competition plot for the reaction of IP$^{\bullet}$ with PNAP and dihydroxyfumaric acid.

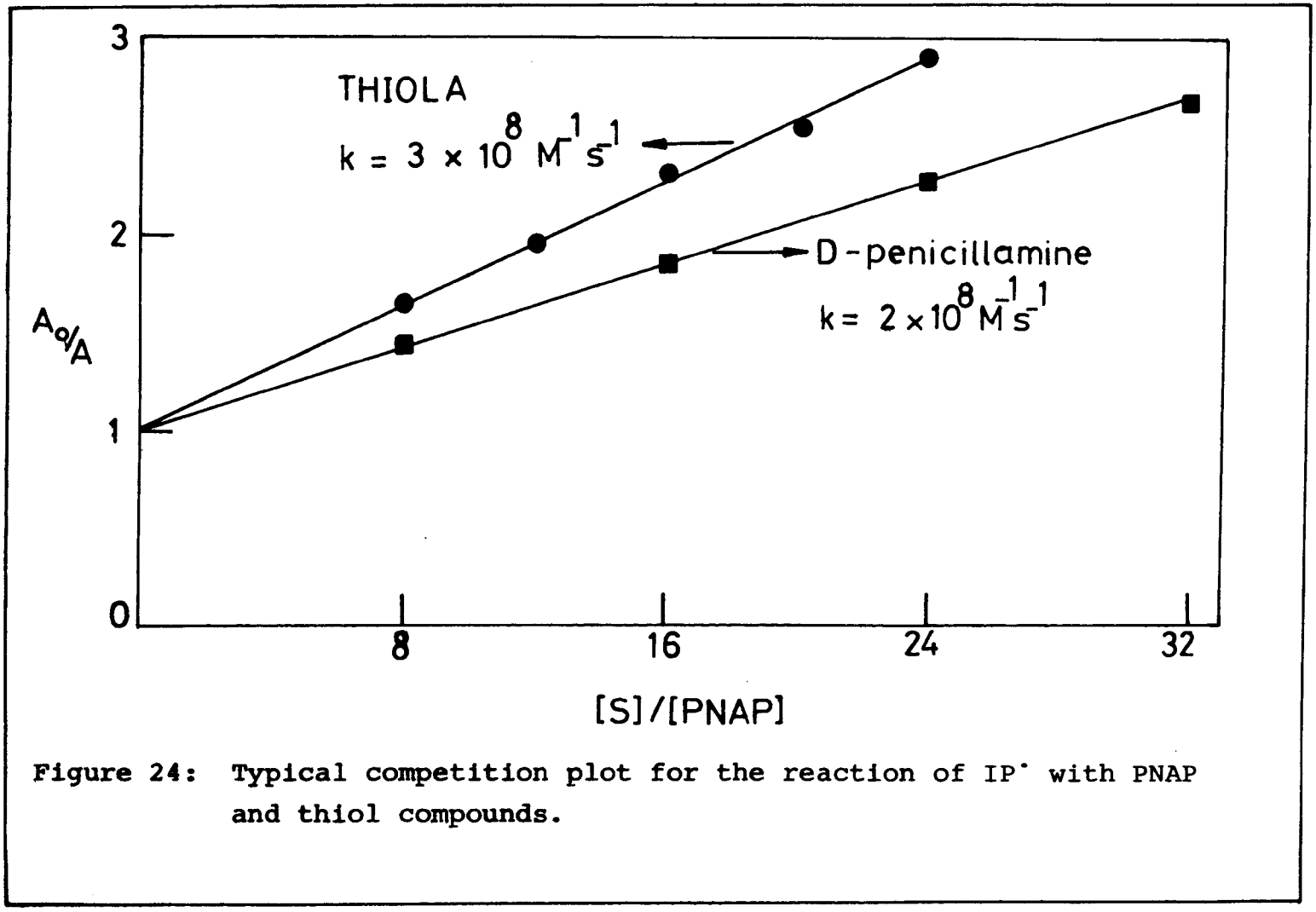


Figure 24: Typical competition plot for the reaction of IP$^{\bullet}$ with PNAP and thiol compounds.

Table 5 : Rate constants for IP$^{\bullet}$ with PNAP and other solutes

Thiol	Radical	System	pH	k x $10^{8}M^{-1}s^{-1}$
Dithiothreitol	isopropanol	ip/Ac/N_2	4.0	10.0
		ip/N_2O	4.0	8.7
Thiola	isopropanol	ip/Ac/N_2	4.0	3.0
		ip/N_2O	4.0	3.2
Cysteamine	isopropanol	ip/Ac/N_2	4.0	2.0
		ip/N_2O	4.0	3.0
Glutathione	isopropanol	ip/Ac/N_2	4.0	2.9
		ip/N_2O	4.0	2.8
Cysteine	isopropanol	ip/Ac/N_2	4.0	2.0
		ip/N_2O	4.0	3.5
Penicillamine	isopropanol	ip/Ac/N_2	4.0	2.0
		ip/N_2O	4.0	2.0

* Key: ip = isopropanol; Ac = acetone; k(IP$^{\bullet}$ + PNAP) = 3.8 x $10^{9}M^{-1}s^{-1}$.

(Taken from Adams and Willson, 1973)

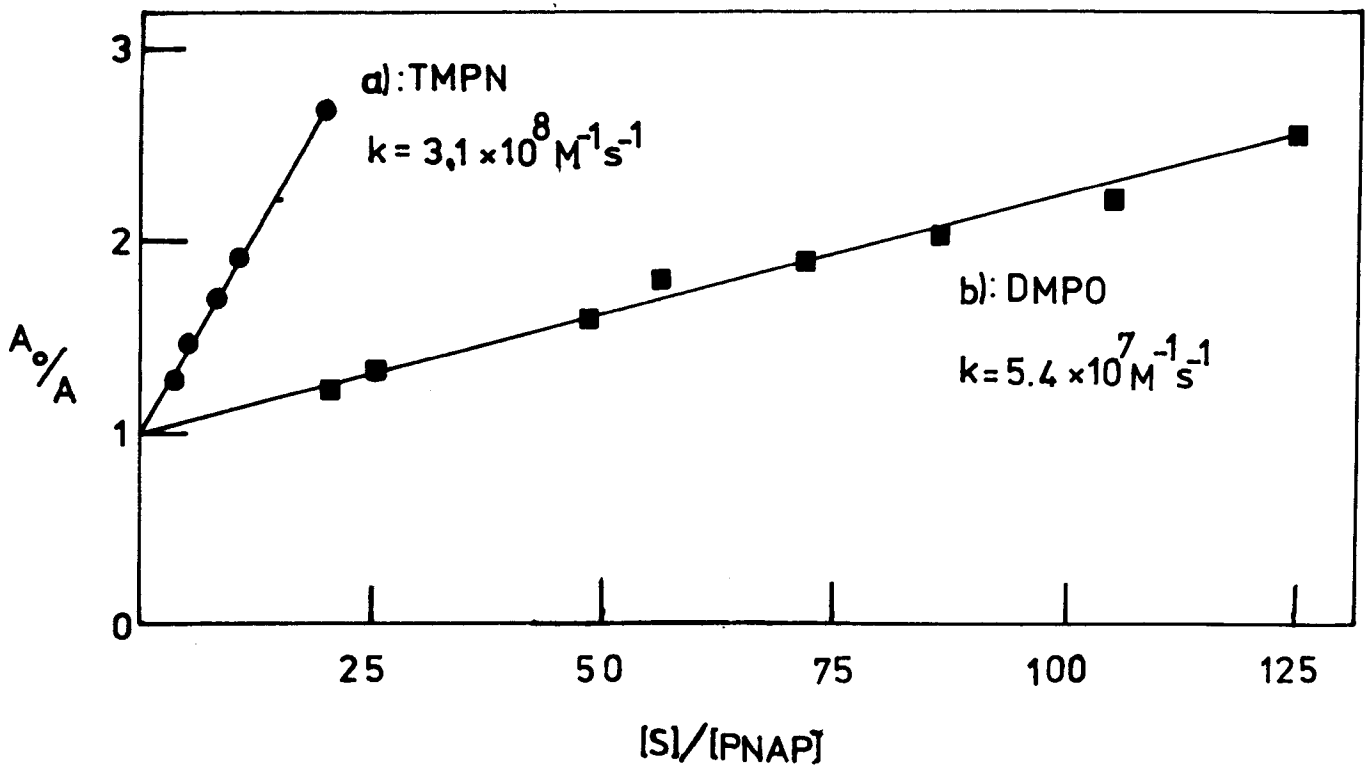


Figure 25: Competition plot for the reaction of IP• with PNAP and other solutes.

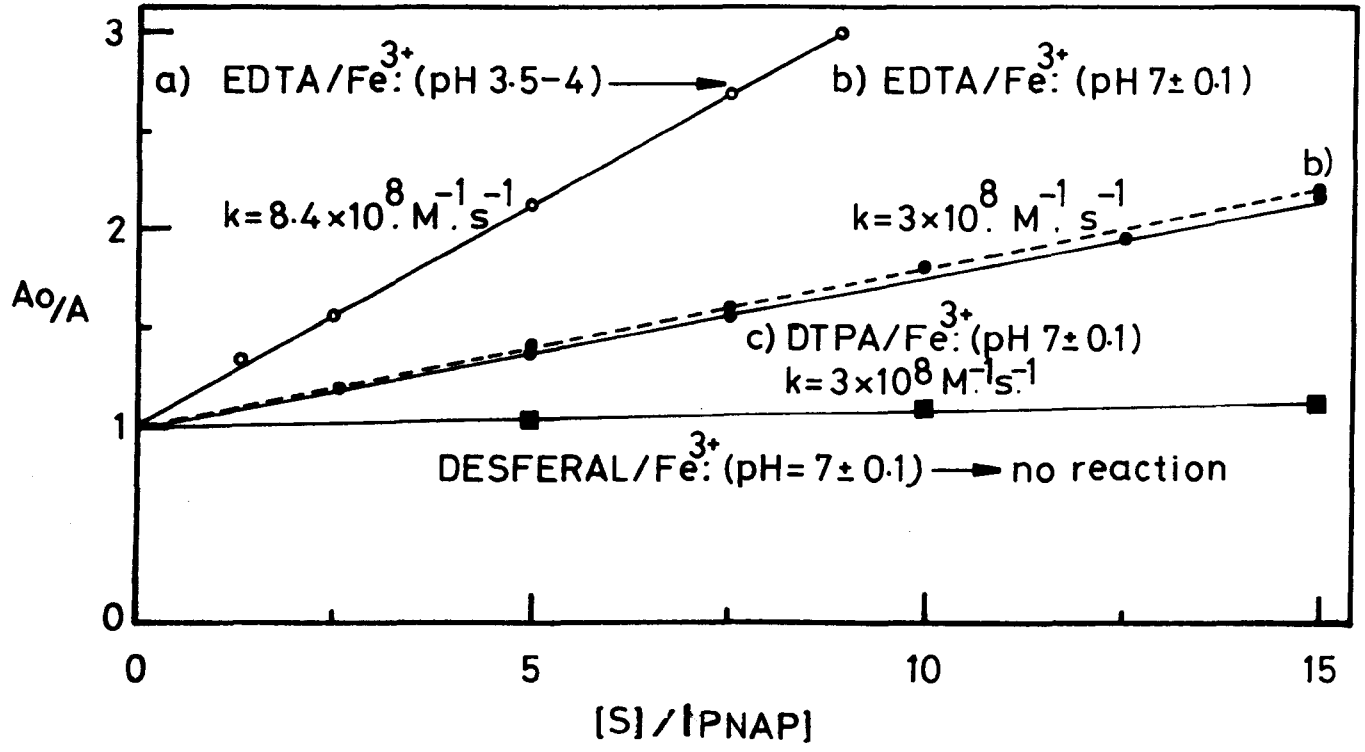


Figure 26: Competition plot for the reaction of IP• with PNAP and some chelating agents.

xv) Reactions with $CCl_3O_2^{\cdot}$ and related methylperoxy radicals

The trichloromethyl peroxy radical can be generated by pulse radiolysis of solutions containing CCl_4, isopropanol-acetone or in solutions containing t-butanol. The general mechanism of reaction is the following:

$$(CH_3)_2\dot{C}OH + CCl_4 \longrightarrow (CH_3)_2CO + \dot{C}Cl_3 + H^+ + Cl^- \quad (110)$$

or

$$e^-_{aq} + CCl_4 \longrightarrow \dot{C}Cl_3 + Cl^- \quad (111)$$

$$OH^{\cdot} + (CH_3)_3COH \longrightarrow H_2O + (CH_3)_2C(OH)\dot{C}H_2 \quad (112)$$

In presence of oxygen, $\dot{C}Cl_3$ reacts rapidly according to:

$$\dot{C}Cl_3 + O_2 \longrightarrow CCl_3O_2^{\cdot} \quad (113)$$

no absorption in the visible region

$CCl_3O_2^{\cdot}$ can act as oxidising agent and its rates of reaction with various nucleophilic compounds have been determined and are in the range $10^7 - 10^8 M^{-1}s^{-1}$ (Packer et al., 1978).

Irradiation of the air saturated solutions containing ABTS, t-butanol and CCl_4 produce a large increase in absorption at 415 nm in agreement with (Wolfenden, 1982):

$$CCl_3O_2^{\cdot} + ABTS \longrightarrow ABTS^{\cdot +} + CCl_3O_2^- \quad (114)$$

$$k_{114} = 1.9 \times 10^9 M^{-1}s^{-1}$$

An attempt to measure the rate of reaction of $CCl_3O_2^{\cdot}$ with dihydroxyfumaric acid by competition method using ABTS as a reference compound was undertaken. Air saturated solutions containing t-butanol (30%), CCl_4 (0.04M), ABTS (2×10^{-4}M) and dihydroxyfumaric acid in the range 0.8 to 3.2×10^{-3}M at pH 6.5 were pulse irradiated. The maximum absorption of $ABTS^{\cdot+}$ at 415 nm decreased with increasing concentrations of dihydroxy-fumaric acid. A linear competition plot was obtained (Figure 27), in agreement with the competing reaction (115):

$$CCl_3O_2^{\cdot} + ABTS \longrightarrow ABTS^{\cdot+} + CCl_3O_2^{-} \qquad (114)$$

$$CCl_3O_2^{\cdot} + DHF \longrightarrow \text{products (no absorption at 415 nm)} \qquad (115)$$

From the slope of the competition plot, the relative rate constant k_{115}/k_{114} was derived and, taking the value for the reaction $k_{114}(CCl_3O_2^{\cdot} + ABTS) = 1.9 \times 10^9 M^{-1}s^{-1}$, the value for the rate constant $k_{115}(CCl_3O_2^{\cdot} + DHF) = 1.4 \times 10^8 M^{-1}s^{-1}$ was subsequently determined.

It was of interest to study the reaction of methylperoxy radicals with β-carotene since, as will be seen later, this has been used as an antioxidant in the lipid peroxidation studies.

On pulse radiolysis of air saturated solutions containing water: t-Butanol (50:50), carbon tetrachloride, halothane and chloroform at 10^{-2}M, respectively, and in

presence of β -carotene in the range 1.5 to 8 x 10^{-6}M, at natural pH, the formation of the absorption of β-carotene$^{\cdot+}$ at λ = 900 nm was observed. The rate of formation was exponential with first order rate constant increasing with β-carotene concentration, in accordance with:

$CCl_3O_2^{\cdot}$ + β-carotene ------► β-carotene$^{\cdot+}$ + products (116)

Halothane-$O_2^{\cdot}$ + β-carotene ------► β-carotene$^{\cdot+}$ + products (117)

$CHCl_2O_2^{\cdot}$ + β-carotene ------► β-carotene$^{\cdot+}$ + products (118)

The bimolecular rate constants determined from the slopes of plots obtained for each compound studied are given in Figure 28.

The characteristic transient absorption spectrum on pulse irradiated solutions containing β-carotene (5 μM), CCl_4(10^{-2}M) at pH 7 in air can be seen in Figure 29. It indicates that in the infra-red region the increase in maximum absorption at 900 nm corresponds to the loss of β-carotene$^{\cdot+}$ absorption observed at 450 to 500 nm.

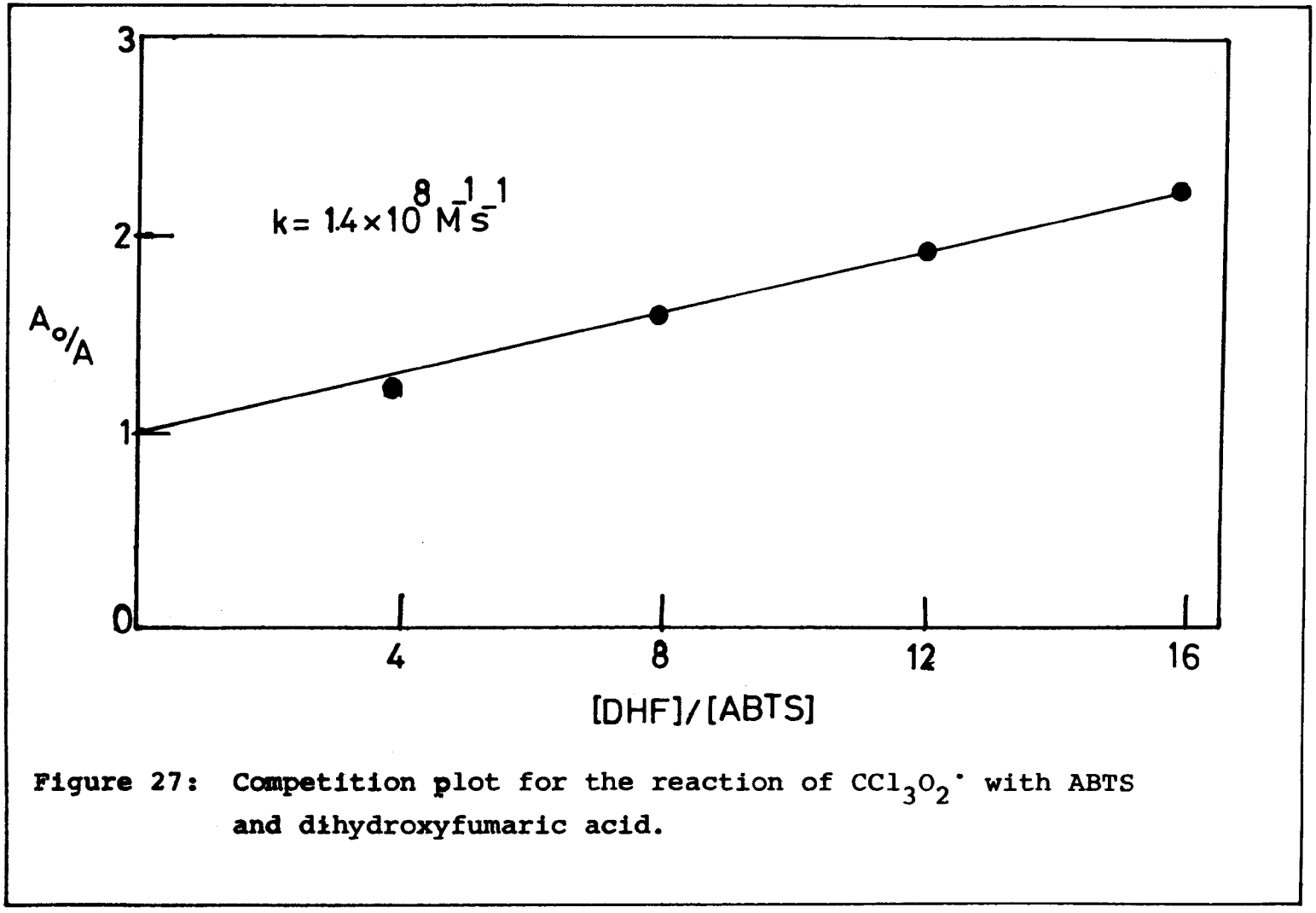


Figure 27: **Competition plot for the reaction of $CCl_3O_2^{\cdot}$ with ABTS and dihydroxyfumaric acid.**

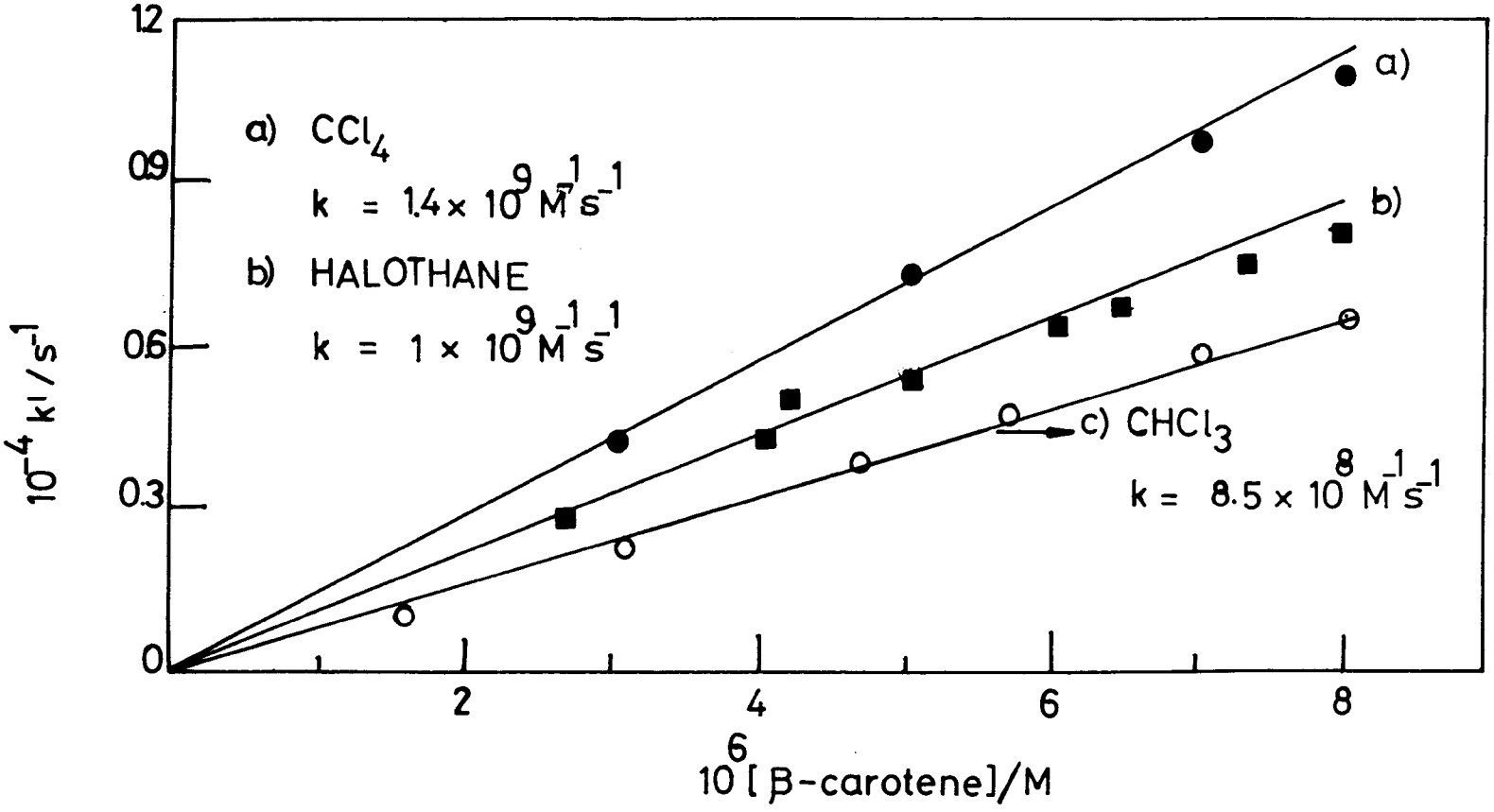


Figure 28: Plots of first order rate constants against β-carotene concentration for the formation of absorption of β-carotene$^{\cdot+}$ at 900 nm on pulse radiolysis of air-saturated solutions containing water: t-butanol(50:50), CCl_4(10^{-2}M), Halothane(10^{-2}M), $CHCl_3$ (10^{-2}M).

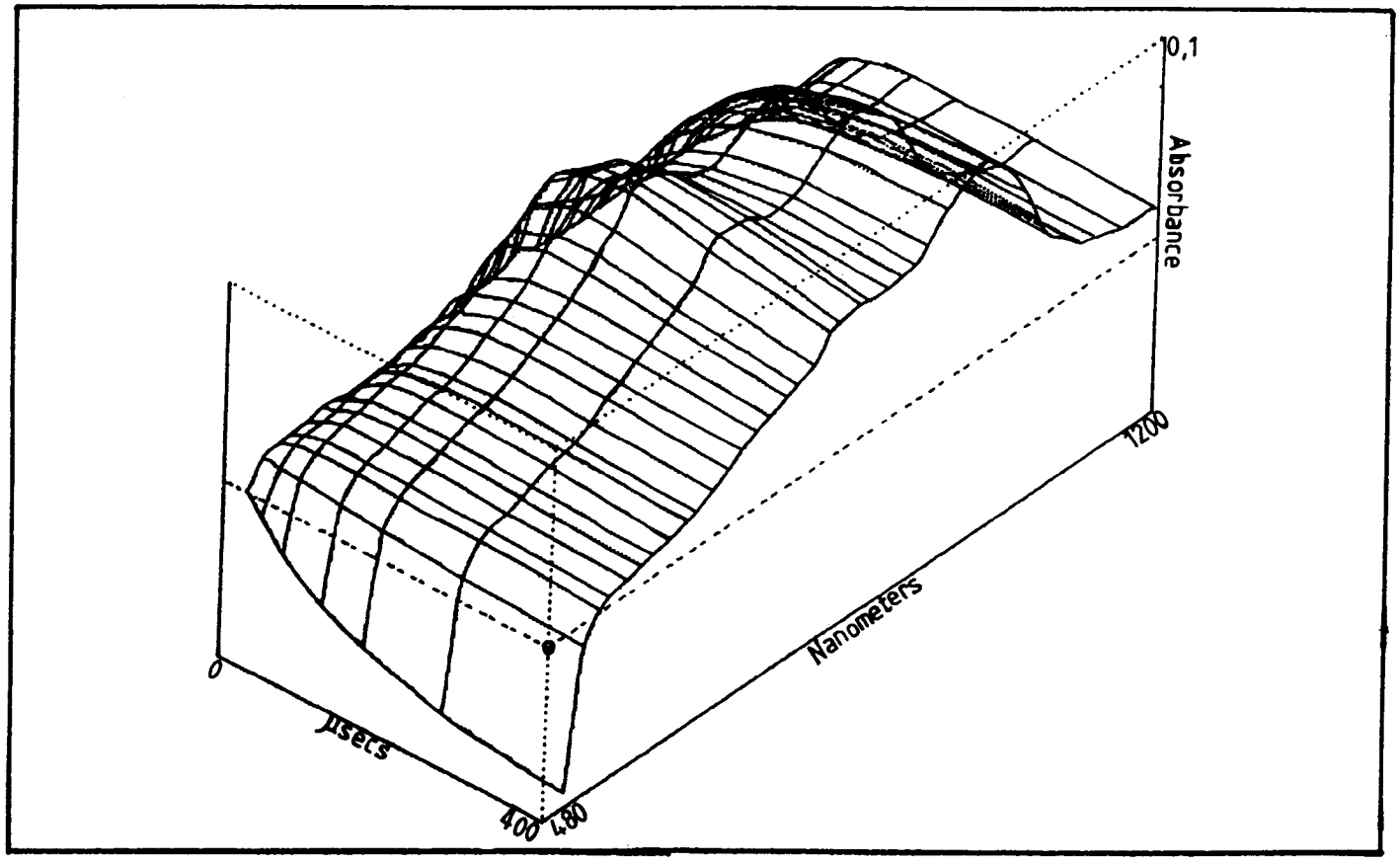


Figure 29: Three dimensional spectrum (time, absorption, wavelength) of the product(s) of the reaction of $CCl_3O_2^{\cdot}$ with β-carotene. The systems containing β-carotene(5 μM), CCl_4(10^{-2}M) in t-butanol: H_2O(50:50) at pH = 7±0.1 in air.

CHAPTER 3

LIPID PEROXIDATION STUDIES

3a) Introduction

Lipid peroxidation *in vivo* has been claimed to be of basic importance in aging processes (Barber and Bernheim, 1967; Packer *et al.*, 1967); in air pollution oxidant damage to cells (Goldstein and Balchum, 1967) and lungs (Thomas *et al.*, 1967); some phases of atherosclerosis (Hartroft, 1965; Perkins *et al.*, 1965); in chlorinated hydrocarbon hepatotoxicity (Recknagel, 1967); in ethanol-induced liver injury (Di Luzio and Hartman, 1967); in oxygen toxicity (Haugaard, 1968) and in the toxicity of a great variety of other xenobiotics. Measurement of lipid peroxidation of homogenates of many different tissues after incubation under suitable conditions of temperature, pH, and the presence of various chemicals can yield valuable information relevant to the study of free radical damage of biological membranes. Free radicals produced during lipid peroxidation are similar to the chemically damaging radical species produced in radiation.

The mechanism of liver microsomal lipid peroxidation has been widely studied (Wills,1969a,1969b; May and McCay, 1968; Pederson *et al.*, 1973; Fong *et al.*, 1973; King *et al.*, 1975). The first step is the abstraction of a hydrogen atom from an allylic methylene group. This leads to the formation of a lipid radical. Subsequently chain reactions result in a breakdown of poly-unsaturated fatty acids and the production of end products including ethane, propane, pentane, and also the production of

malonaldehyde. The liver microsomal fraction has been shown to form large quantities of lipid peroxide, when incubated with iron and ascorbate. In the present thesis special attention has been paid to characterize the damaging effects of dihydroxyfumaric acid added to the rat liver microsomes in the presence of different metal salts especially ferrous sulphate. The effect of antioxidants, chelating agents, free radical scavengers and other chemicals (see Table 8) have been studied to determine their ability to remove free radical species or block reactions involved in the peroxidation system.

3b) Experimental Procedure

i) Preparation of microsomal fractions

Using the technique of differential centrifugation, microsomes were prepared from rat livers. Male Charles River rats (300 - 500 g) which had been starved for 24 hours were killed by cervical dislocation, weighed and then the livers were quickly removed, rinsed with 0.25 M sucrose, weighed and placed in ice-cold beaker with 0.25 M sucrose. All subsequent procedures were performed at about $4^{o}C$. A Potter-Elvehjem homogenizer (teflon-glass) was used for the homogenization. After the preparation was transferred to a measuring cylinder and made up to a known volume 5 ml for every 1g of liver

weighed out (w/v), again using 0.25 M sucrose. The preparation was put into 50ml plastic centrifuge tubes and spun twice at 17.000g for 10 minutes in an MSE 18 centrifuge. This procedure precipitates cell debris, nuclei, mitochondria, lysosomes and also possibly quite a large amount of microsomal membranes. The resulting supernatant was removed, and then transferred as 12ml aliquots into high speed tubes and spun again at 105.000g for 60 minutes on an MSE super-speed centrifuge 65 with the 8 x 14 ml angle rotor. The microsomal fraction was precipitated, the supernatant was discarded and in order to remove the sucrose which can interfere with the determination of lipid peroxidation, the microsomal pellets were rinsed twice with 0.15 M KCl solution. The pellets were finally kept in the centrifuge tubes covered by parafilm in the freezer at -20^{o}C until required. The pellets were considered to have a useful life of only 2 weeks and they were discarded after this period of freezing. To provide a stock of suspensions, each frozen pellet of microsomes was resuspended in 3ml of TRIS:KCl buffer (33 mM TRIS: 100 mM KCl, pH = 7.4). This suspension of microsomes gave a protein value of approximately 15 mg per ml. Protein values were determined colorimetrically by the method of Lowry et al., (1951). Samples of resuspended microsomes were diluted 1 in 200 with distilled water. To determine the value of proteins, the following procedure was used. 0.5ml aliquots of these dilutions were pipetted into test tubes. The stock solutions for the protein estimation

were:

A) 10g sodium carbonate and 2g sodium hydroxide in 500ml of water,

B) 1% copper sulphate solution,

C) 2% potassium tartrate,

D) Solution containing the above stocks in the ratio 1B:1C:100A,

E) Folin and Ciocalteu's (phenol agent) diluted 1:1 with distilled water.

3ml of solution D was added to each 0.5ml sample, mixed and left for 10 minutes. Then 0.3ml of solution E was added, rapidly mixed and the samples left for exactly 30 minutes in order to develop the blue colour which was recorded on a Pye Unicam SP 500 spectrophotometer at 750nm. using a 1cm cell.

The optical density readings obtained were then converted to protein concentration using a calibration curve (see Figure 30). In this way it was possible to get the value of the diluted sample, and in order to know the actual protein content of the stock suspension of microsomes, the dilution factor 1 in 200 was considered.

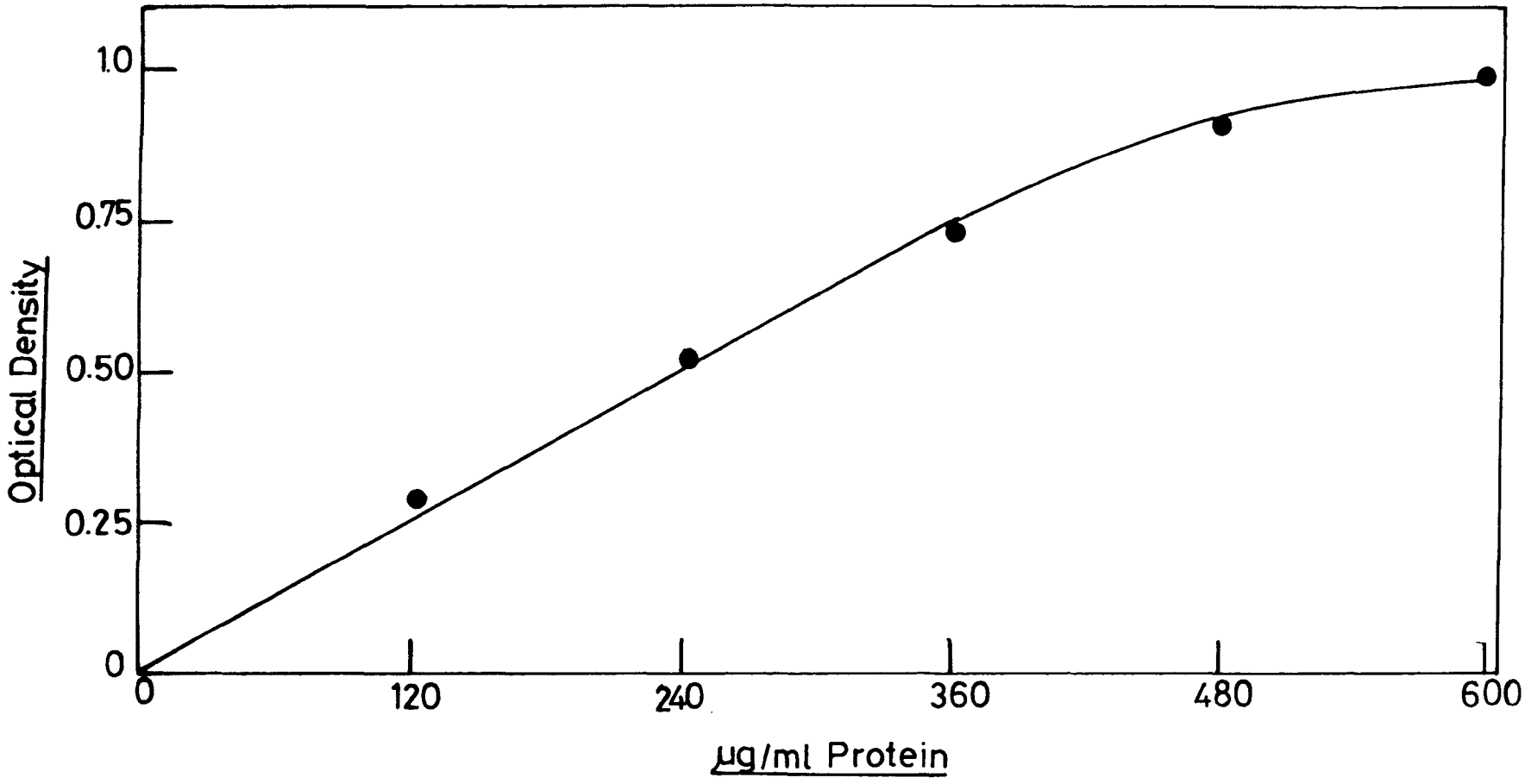


Figure 30: Plot of calibration curve for the Lowry Method to a standard solution of protein (BSA)

3c) Incubation conditions

i) ferrous sulphate/dihydroxyfumaric acid stimulator

Peroxidation of 1 mg protein/ml (final concentration) microsomal suspension was initiated by the addition of ferrous sulphate and dihydroxyfumaric acid to a final concentration of (5 μM and 1 mM) respectively. The start of the reaction was considered to be at the point when dihydroxyfumaric acid was added. Each sample was incubated in stoppered glass tube in a shaking water bath at 37°C. A typical experimental model was composed as shown in Table 6 .

ii) effect of metal salts/dihydroxyfumaric acid

Additions to the sample tube were made as before but different metal salts to final concentrations of (10, 50 or 100) μM were added instead of 5 μM of ferrous sulphate (Table 6).

iii) effect of inhibitors on Fe^{2+}/dihydroxyfumaric acid stimulated lipid peroxidation

A similar method to the system (i) described above was used. Aliquots of 0.15ml of the compound(s) under analysis were included in the microsomes sample (Table 6).

Table 6 : ⟶ ORDER OF ADDITIONS TO EXPERIMENTAL MODEL OF LIPID PEROXIDATION

ml BUFFER pH = 7.4	ml MICROSOMES	ml TEST COMPOUND	ml of ferrous sulphate or another metal salt	ml DIHYDROXYFUMARIC ACID	ml FINAL VOLUME
0.95	0.1	0.15	0.15	0.15	1.5
Final Concentration	≃ 1mg/ml Protein	n µM/	5 µM ; or (10) (50) (100) µM	1 mM	(+) (++)

(+) : Incubation Time = 30 minutes ; (++) : Incubation Temperature = 37°C.

3d) Malonaldehyde detection

The reactions were terminated at 30 minutes by the addition of 3 ml of ice-cold 10% w/v trichloroacetic acid TCA to 1.5 ml of microsomal suspension. After removal of the precipitate by centrifugation at 3000 g in an MSE Super Minor centrifuge for 15 minutes, lipid peroxidation in the samples was determined colorimetrically using the thiobarbituric acid (TBA) method, as described by Wilbur et al., (1949). Following the centrifugation step, 2 ml aliquots of the supernatant were taken, and subsequently, 2 ml of 0.67% w/v TBA was added. The samples were mixed and incubated for 15 minutes in a boiling water bath and then cooled to room temperature. Blanks which included 2 ml of buffer Tris:KCl and 2 ml 10% TCA solution were also treated and measured in the same way as the readings sample. The optical density measurements were carried out in an UNICAM SP 500 (spectrophotometer, model 1 photometer). An extinction coefficient of $A^{1\ cm}_{535\ nm} = 1.56 \times 10^{5}$ mol $^{-1}$. cm^{-1}. was used. The scheme proposed for the TBA reaction was suggested by Sinnhuber and Yu (1958). (Figure 31).

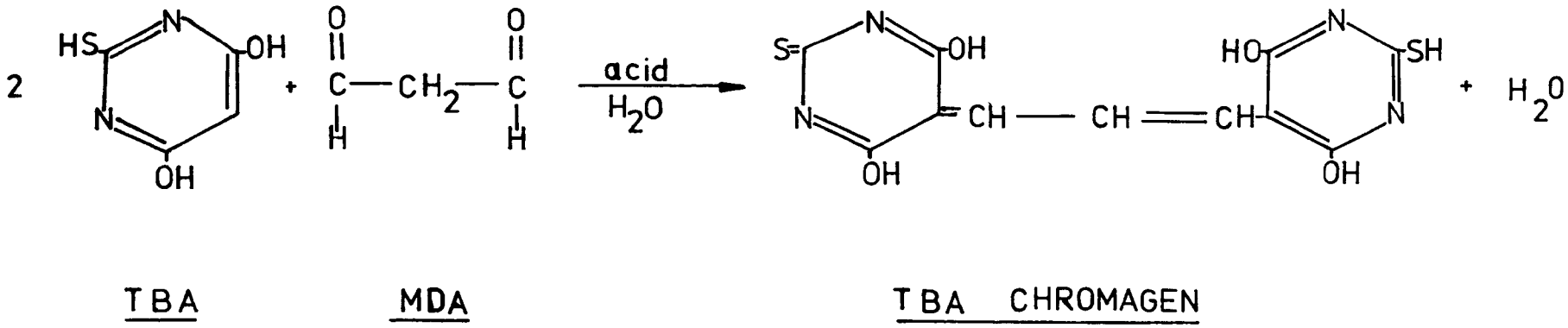


Figure 31 : Scheme proposed for the TBA reaction (Sinnhuber and Yu, 1958)

The reaction involves the condensation of two molecules of thiobarbituric acid with one of malonaldehyde.

3e) Data expression

For simplicity reasons, the peroxidation found in the analysed samples was measured as the amount of TBA reactive material produced (MDA) and expressed in units of optical density. In this thesis statistical methods have been used for the evaluation of measurements of MDA made. Unless otherwise specified the calculations given were performed using a Scientific calculator Casio fx-39, and the basic statistic was available directly from the calculator as will be seen later in the Appendix.

i) ED_{50} determinations

In order to obtain the ED_{50} values, the following formula was applied:

$$I = \frac{(x - y)}{x} \times 100$$

where: I = percentage inhibition value.

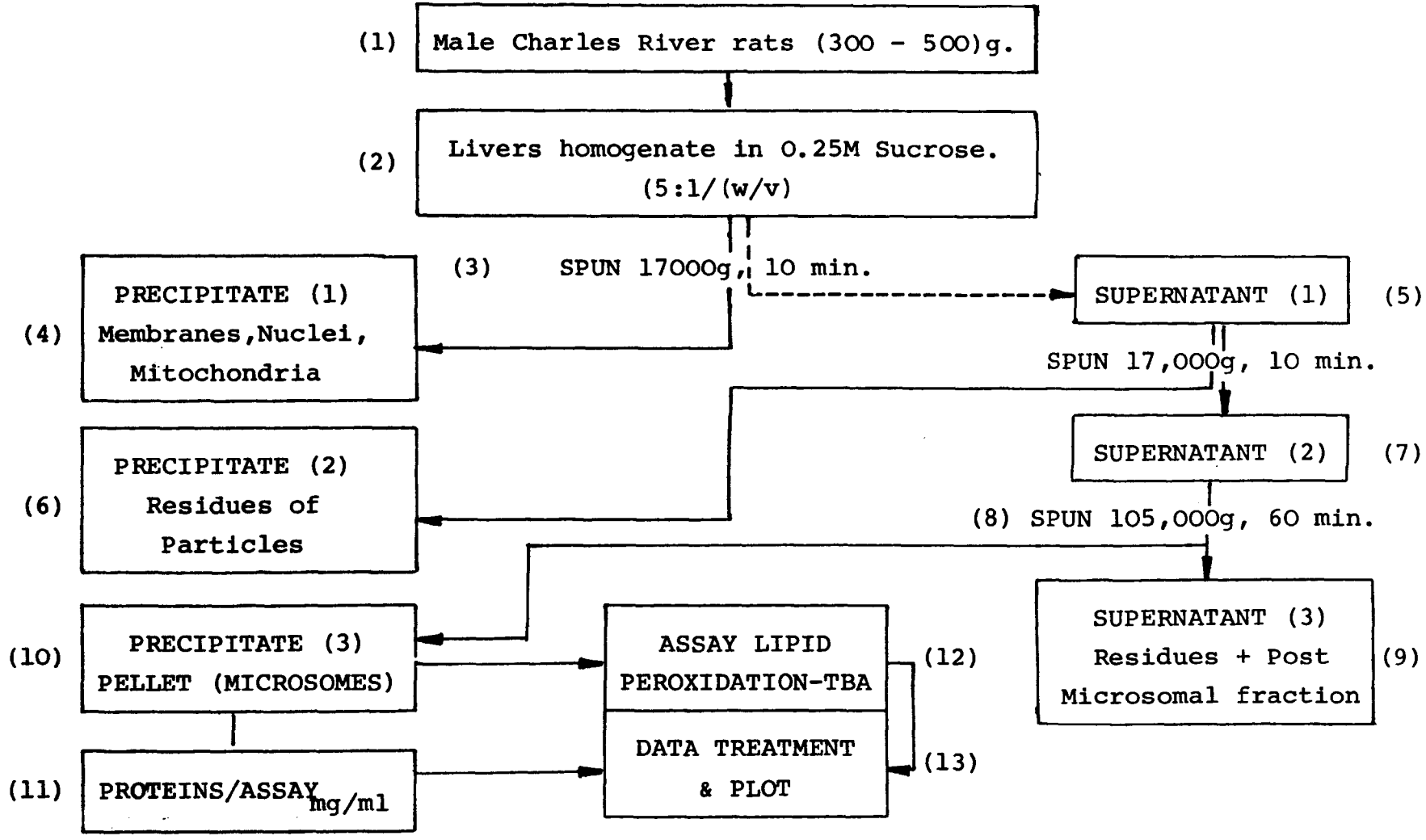
x = mean control (i.e. no test compound) MDA value.

y = mean test MDA value.

Values of I were plotted against the different concentrations of a test compound and the ED_{50} obtained from the curve.

To facilitate a better comprehension, a flow chart (Figure 32) summarizes the experimental work in this part of the thesis.

Figure 32 : A FLOW CHART ILLUSTRATING THE PREPARATION AND ASSAY OF MICROSOMES



3f) Materials

i) Chemicals

β-carotene, superoxide dismutase (SOD), catalase, tryptophan, p-chloromercuribenzoate (pCMB), 2, methyl - 1,2, di - 3 - pyridyl 1 - propanone (metyrapone), 1,10 - phenanthroline, ethylenediaminetetraacetic acid (EDTA), were supplied by Sigma London Chemical Company Ltd., Poole, Dorset.

Diazabicycloctane (DABCO), 2,5 - diphenylfuran, were obtained from the Aldrich Chemical Company Ltd., Gillingham, Dorset.

Thiobarbituric acid (TBA), aminopyrine, Folin and Ciocalteaus phenol reagent were purchased from BDH Chemicals, Poole, Dorset.

Bleomycin was supplied by Lundbeck Ltd., Luton, Beds.

Aminopyrine was purchased from Hopkin and Williams.

SKF 525A (β - dimethylamino ethyl - 3,3' - diphenyl propyl acetate) was supplied by Smith Kline and French, Welwyn Garden City, Herts.

Desferal (desferrioxamine -β) was obtained from CIBA - Geigy, Basle, Switzerland.

Samples of 6 - hydroxy 1,4 - dimethyl carbazole (HDC) were provided by Professor Claude Paoletti, Institut Gustave - Roussy, Villejuif, Paris, France.

Reagents other than those specified were of the highest purity available and were obtained from BDH Chemicals, Sigma Chemicals Co., or Aldrich Chemical Co., or they are described in the section 2c(i).

Doubly distilled water was used to make the solutions.

ii) Animals

Male Wistar rats were supplied by Charles River (U.K.) Limited., Manston Road, Margate, Kent and housed in the Brunel Animal Unit until required. They were fed on Rat and Mouse expanded Breeder Diet N^{O}. 3. (BP. Nutrition Speciality Products Division, Stepfield, Witham, Essex). Rats free from any obvious infection and showing normal weight gain were selected for overnight fasting of 24 hours prior to experiment. Water was available ad libitum.

3g) Results

Before the ferrous sulphate/dihydroxyfumaric acid system could be used to study the effects of antioxidants and inhibitors, it was necessary to characterize the system in order to evaluate how lipid peroxidation in liver microsomes takes place under defined conditions.

i) Effect of dihydroxyfumaric acid concentration

When rat liver microsomes were suspended in Tris:KCl buffer pH = 7.4 and incubated for 30, 60 and 120 minutes respectively in the presence of different concentrations of dihydroxyfumaric acid range 0.25 to 2 mM, the MDA formation was detected. (Figure 33). After two hours of incubation with dihydroxyfumaric acid 2 mM a maximum value optical density = 1.130 was reached. The results clearly confirm that dihydroxyfumaric acid in the course of its oxidation is able to stimulate lipid peroxidation on active microsomes, suggesting a possible role of free radical species in the process.

ii) Effect of ferrous sulphate concentration

Additions of small amounts of iron to peroxidising systems often stimulate lipid peroxidation. It was decided to include ferrous sulphate with dihydroxyfumaric acid in order to observe if the MDA values would increase when both were present together with incubated microsomes.

MDA production (Figure 34) found to be dependent upon the concentration of ferrous sulphate in the range 5 to 50 μM added to 1 mM dihydroxyfumaric acid used, giving at 37°C, during 30 minutes of incubation, values comparable to 2 mM of dihydroxyfumaric acid alone after 2 hours of incubation, see (Figure 33). These results indicate that ferrous sulphate was an important oxidant in this system.

iii) Effect of incubation time

After the addition of 5 μM ferrous sulphate and 1 mM dihydroxyfumaric acid to the microsomal fraction, the reaction was stopped at various times and the MDA measured. A maximum level of peroxidation was shown after 30 minutes (Figure 35).

iv) Effect of microsomal heat treatment

In order to assess the role of enzymes in the lipid peroxidation process, the effect of vigorous heat treatment was investigated. The results illustrated in Table 7 show that both normal and reconstituted microsomes were sensitive to lipid peroxidation, although the better treatment does reduce peroxidation to a greater extent.

Table 7 : Effect of microsomal heat treatment

System	values expressed in Optical Density	
	Heated	Unheated
Only microsomes	0.04	0.03
5 µM ferrous sulphate	0.14	0.15
1 mM dihydroxyfumaric acid	0.21	0.38
ferrous sulphate/ dihydroxyfumaric acid (5 µM : 1 mM)	0.42	0.66

Key: Boiled microsomes for 15 minutes, later cooled down (10 minutes) prior to the experiment being carried out.

v) Effect of temperature

The MDA levels achieved when microsomal fractions were incubated with 5 µM ferrous sulphate and 1 mM dihydroxyfumaric acid at different temperatures can be seen (Figure 36). From 20 to 50°C the peroxidation values were increased progressively, reaching a high point about 48.5°C. However, above this temperature the levels of MDA declined. The fact that a coagulation and precipitation of the microsomal fraction about 50°C may be relevant in this respect.

vi) Effect of pH

The effect of changing the pH on the microsomes incubated with 5 μM ferrous sulphate and 1mM dihydroxy-fumaric acid is shown in Figure 37. The level of peroxidation decreased dramatically on reaching the pH from 5 to 4 or increasing the pH from 6 to 9.

vii) Effect of inhibitors

In order to measure the antioxidant capacity of propyl gallate, HDC, promethazine and chlorpromazine, it was decided to include them in the ferrous sulphate/ dihydroxyfumaric acid system. The results are shown in (Table 8). Because of the effectiveness of HDC, propyl gallate and promethazine in inhibitory peroxidation, a more detailed study of the effect of antioxidant concentration was evaluated and values for ED_{50} determined. The results are shown (Figures 38, 39, 40).

Similar studies were undertaken with the iron chelating agents EDTA, DTPA, 1.10 Phenanthroline, 8-Hydroquinoline, desferal and bleomycin. The results obtained are summarised in Table 8 and Figure 41.

In order to gain information about the responsible species in the lipid peroxidation process, the compounds DABCO, 2,5 diphenylphuran and β-carotene (singlet oxygen scavengers), the enzymes superoxide dismutase and

catalase, tryptophan a scavenger of ($OH^{\cdot}$ + $O_2^{\cdot -}$), thiourea scavenger of $OH^{\cdot}$ also were added to the microsomes prior to initiation of lipid peroxidation stimulated by ferrous sulphate/dihydroxyfumaric acid. As it shown in Table 8 , little effect was observed for these compounds.

The compounds picolinic acid, adamantadine, p-chloromercuribenzoate, aminopyrine and sodium salycilate were studied in order to determine whether some of them could have inhibitory effects on the MDA production stimulated on peroxidised microsomes by the ferrous sulphate/dihydroxyfumaric acid couple. No significative inhibitions were found with these compounds. The results are summarised in Table 8 . Interestingly the P_{450} inhibitors metyrapone and SKF 525A did have considerable inhibitory effect on the MDA production stimulated by Fe^{2+}/dihydroxyfumaric acid. The results obtained are shown in Table 8 .

viii) Effect of different heavy metal salts

On the basis that heavy metal salts are commonly found in living organisms and can undergo a wide variety of reactions, it was decided to investigate the effect of Ni^{2+}, Mg^{2+}, Ca^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+} and Mn^{2+} on incubated microsomes in the presence of dihydroxyfumaric acid (1M). The results are shown in (Table 9). In general, Ni^{2+} and Mg^{2+} did not have any inhibitory action at the concentrations used. Ca^{2+}, Zn^{2+} and Co^{2+} started to show some level of antioxidant action at 100 µM. Cu^{2+} at the concentrations of 50 and 100 µM was quite effective in preventing MDA formation and Mn^{2+} in the range 10 to 100 µM showed a strong antioxidant action.

ix) Inhibitory effects of Mn^{2+} on MDA production by Fe^{2+}/dihydroxyfumaric acid

Because in the previous studies Mn^{2+} showed a strong inhibitory effect on MDA formation and Fe^{2+} stimulates peroxidation, it was considered useful to add both metal ions at different concentrations to the microsomes suspension in addition to dihydroxyfumaric acid (1 mM). The results are shown in Table 10 .

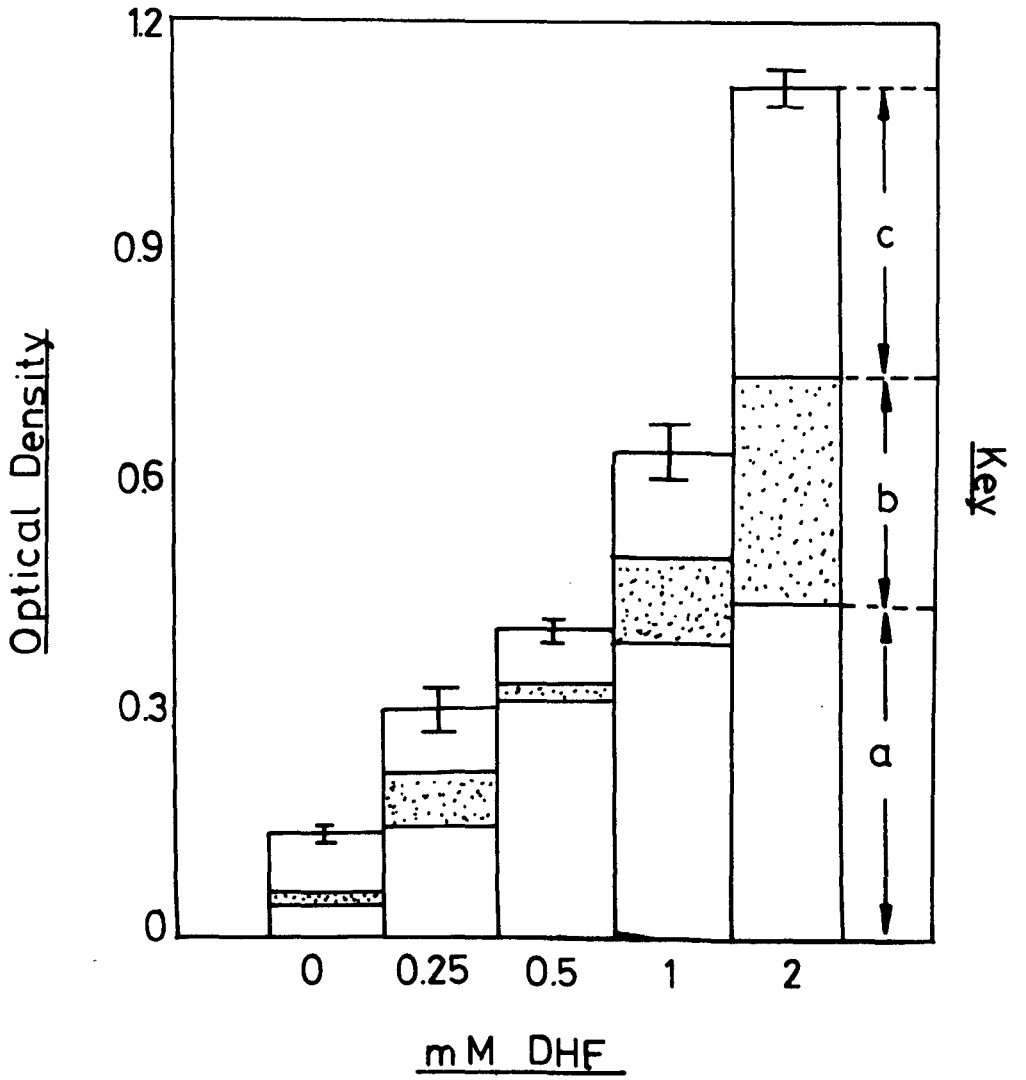


Figure 33: The effect of dihydroxyfumaric acid on MDA production

Final conditions:- 1.15 mg protein/ml microsomes; 37°C;
Tris:KCl buffer pH = 7.4;

Key:- (a) = 30 minutes
(b) = 60 minutes
(c) = 120 minutes
} of incubation respectively

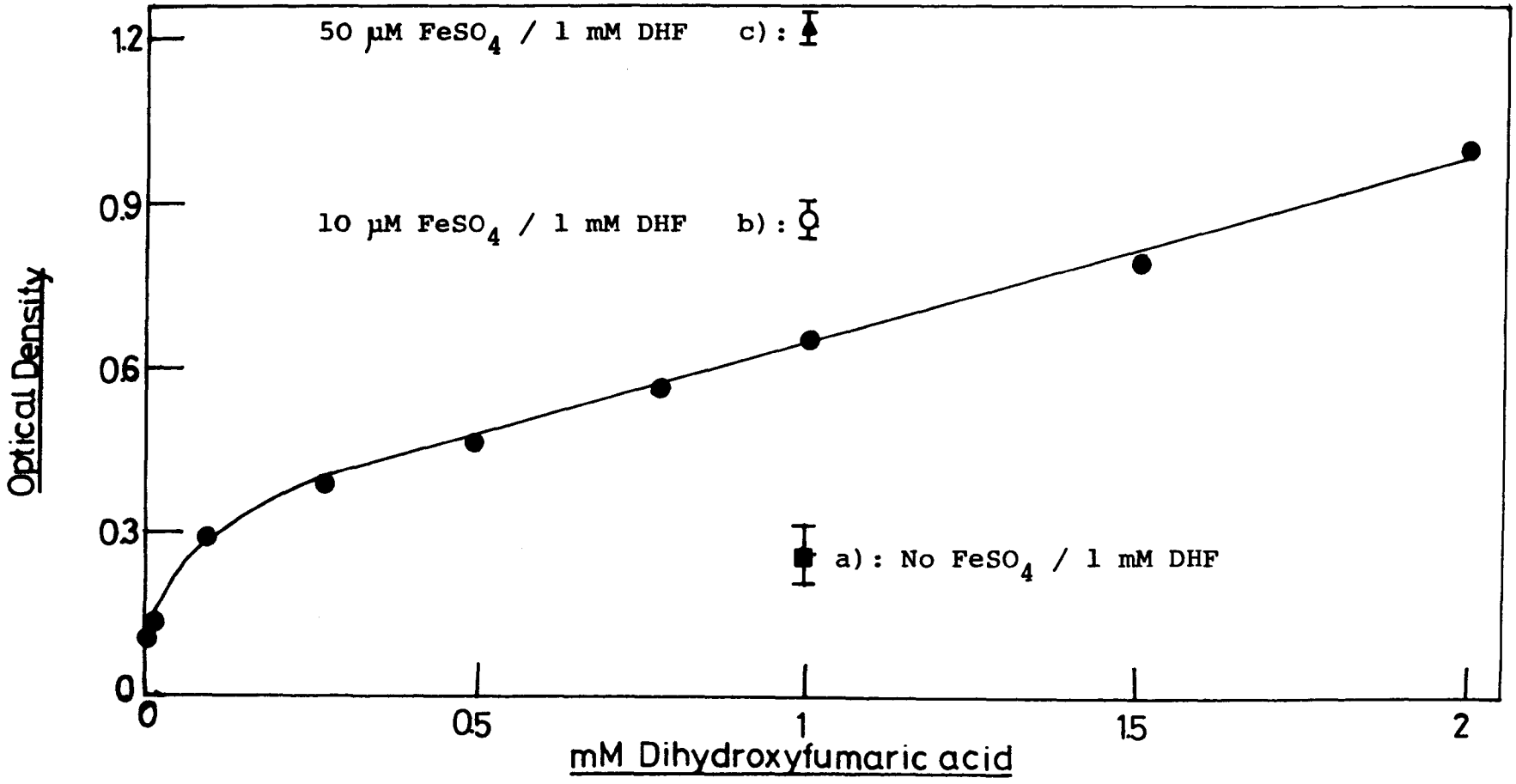


Figure 34: The effect of ferrous sulphate/dihydroxyfumaric acid on MDA production

Final conditions:- 1.4 mg protein/ml microsomes; 37°C; Tris:KCl buffer pH 7.4; = 5 µM $FeSO_4$ / DHF; 30 minutes incubation.

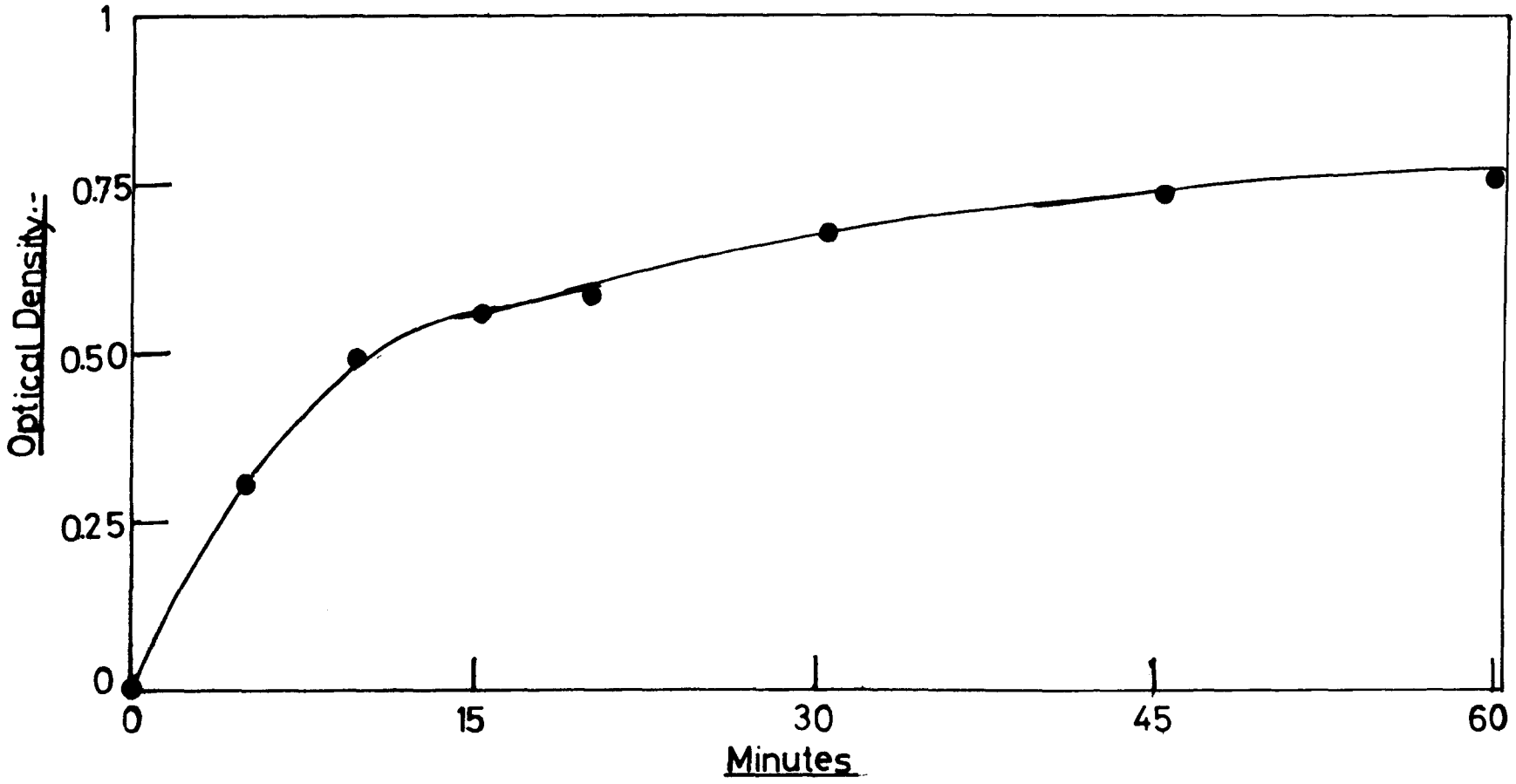


Figure 35: **Time dependent increase in MDA promoted by Fe^{2+}/dihydroxyfumaric acid**

Final conditions:- 1.13 mg protein/ml microsomes; 37 C; Tris:KCl buffer pH 7.4; 5 μM $FeSO_4$ / 1 mM dihydroxyfumaric acid.

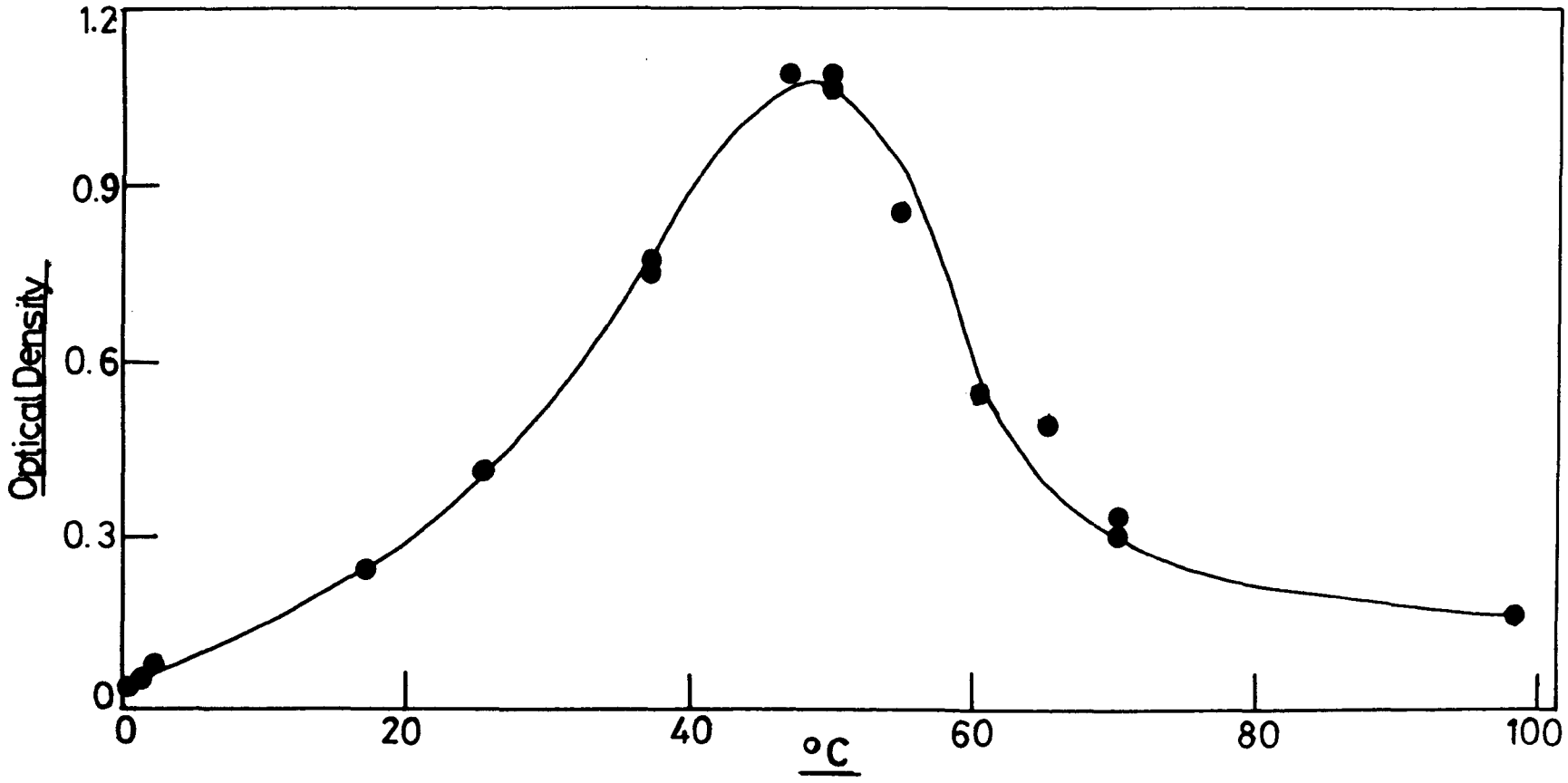


Figure 36: The effect of temperature on MDA production by Fe^{2+}/dihydroxyfumaric acid

Final conditions:- 1.46 mg protein/ml microsomes; 30 minutes incubation; Tris:KCl buffer pH 7.4; 5 µM $FeSO_4$ / 1 mM dihydroxyfumaric acid.

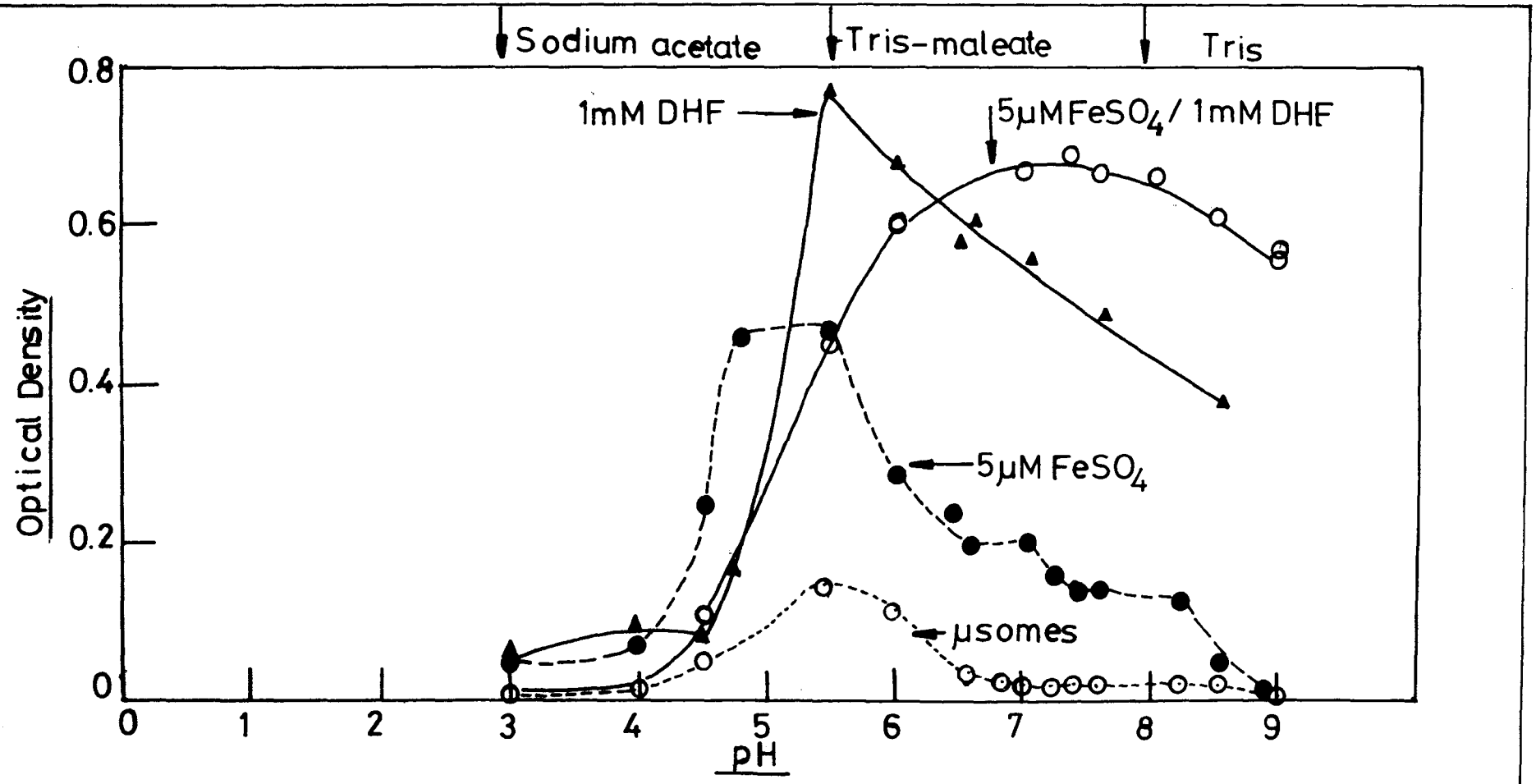


Figure 37: **The effect of pH on MDA production by Fe^{2+}/dihydroxyfumaric acid**
Final conditions:- 1.16 mg protein/ml microsomes; 37°C; 30 minutes incubation.

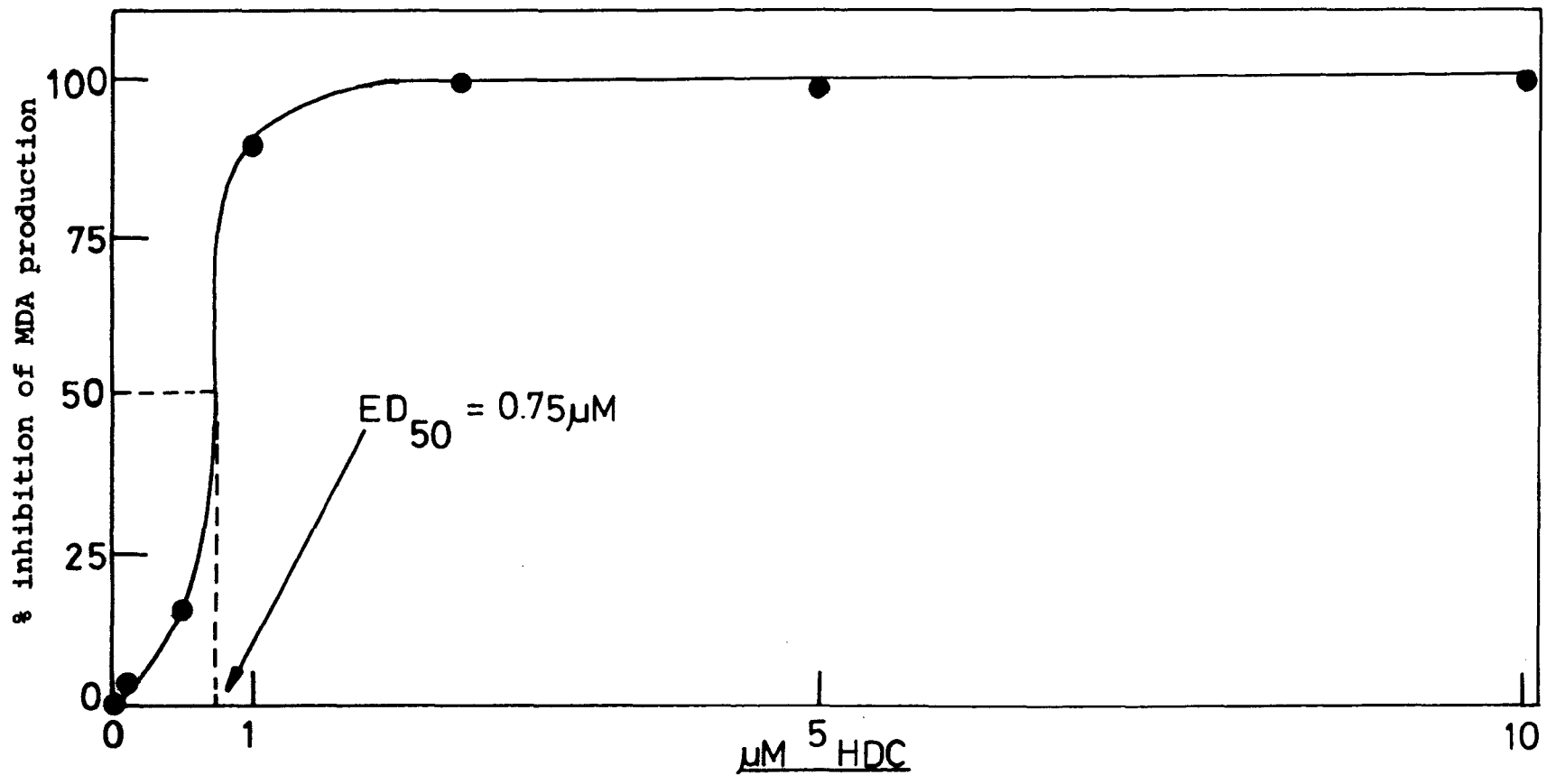


Figure 38: The effect of HDC on MDA production by Fe^{2+}/dihydroxyfumaric acid

Final conditions:- 1.53 mg protein/ml microsomes; 37°C; 30 minutes incubation; Tris:KCl buffer pH 7.4; 5 µM $FeSO_4$ / 1 mM dihydroxyfumaric acid.

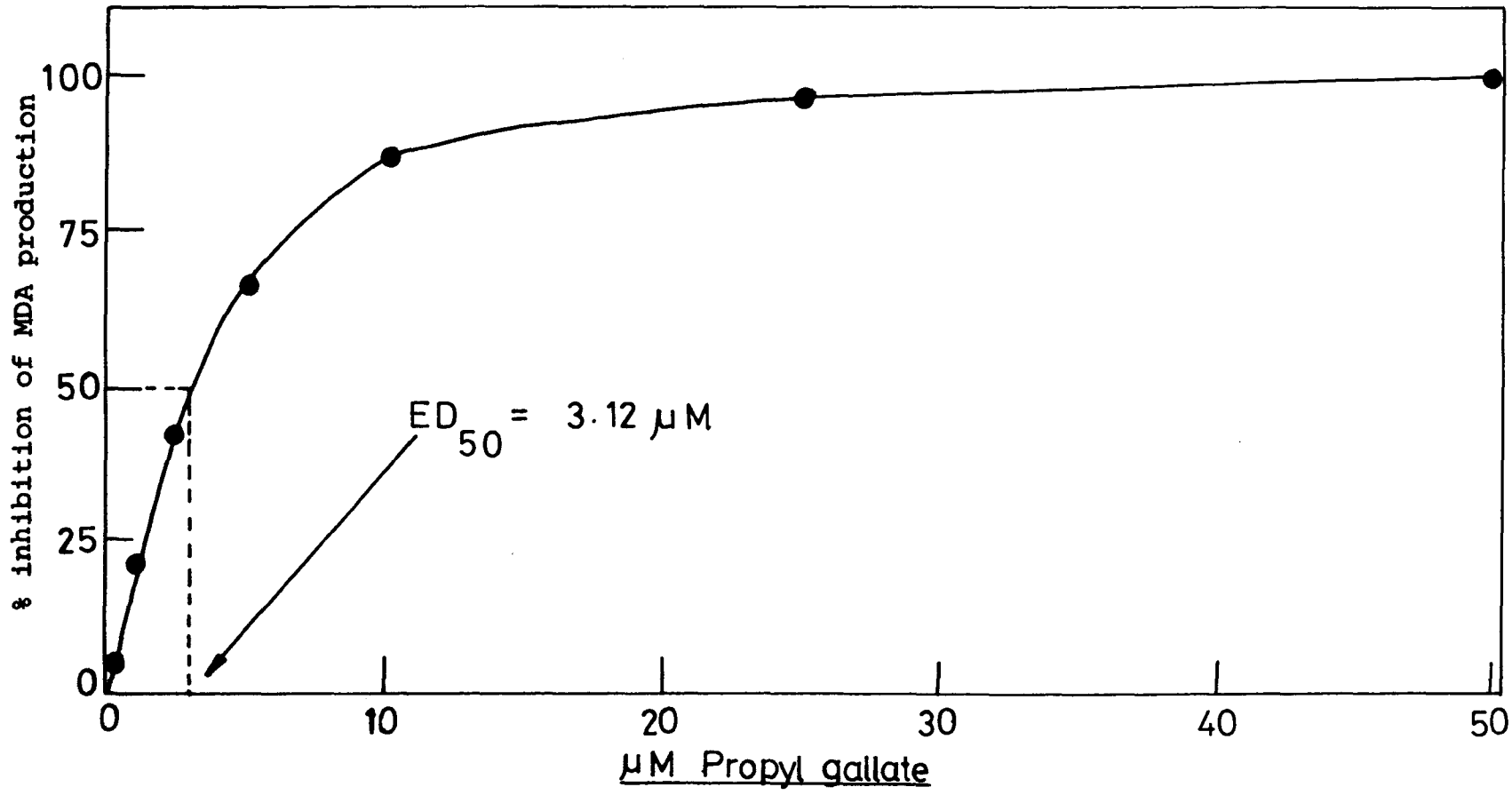


Figure 39: The effect of propyl gallate on MDA production by Fe^{2+}/dihydroxyfumaric acid

Final conditions:- 1.53 mg protein/ml microsomes; 37°C; 30 minutes incubation; Tris:KCl buffer pH 7.4; 5 µM $FeSO_4$ / 1 mM dihydroxyfumaric acid.

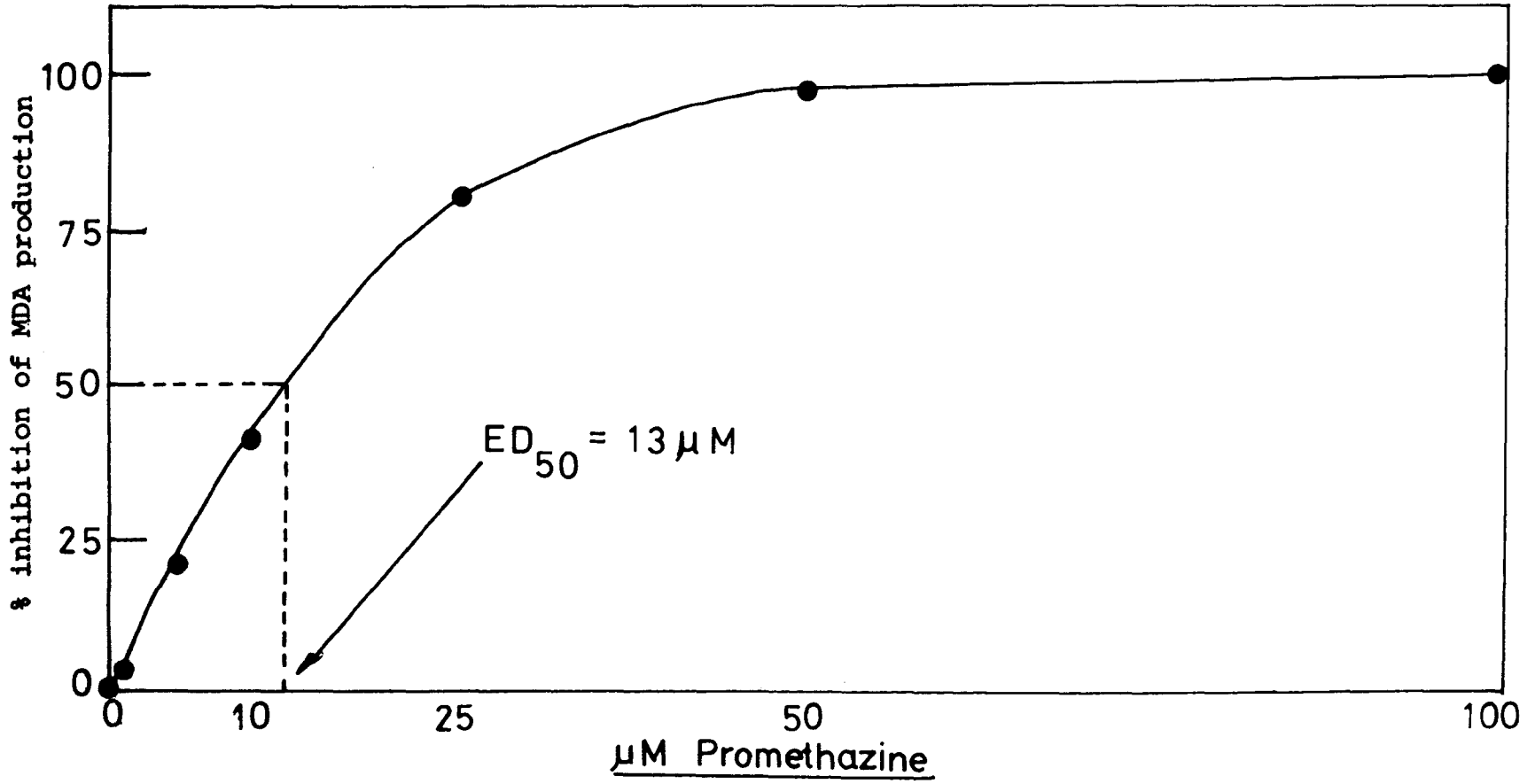


Figure 40: **The effect of promethazine on MDA production by Fe^{2+}/dihydroxyfumaric acid**
Final conditions:- 1.53 mg protein/ml microsomes; 37°C; 30 minutes incubation; Tris:KCl buffer pH 7.4; 5 µM $FeSO_4$ / 1 mM dihydroxyfumaric acid.

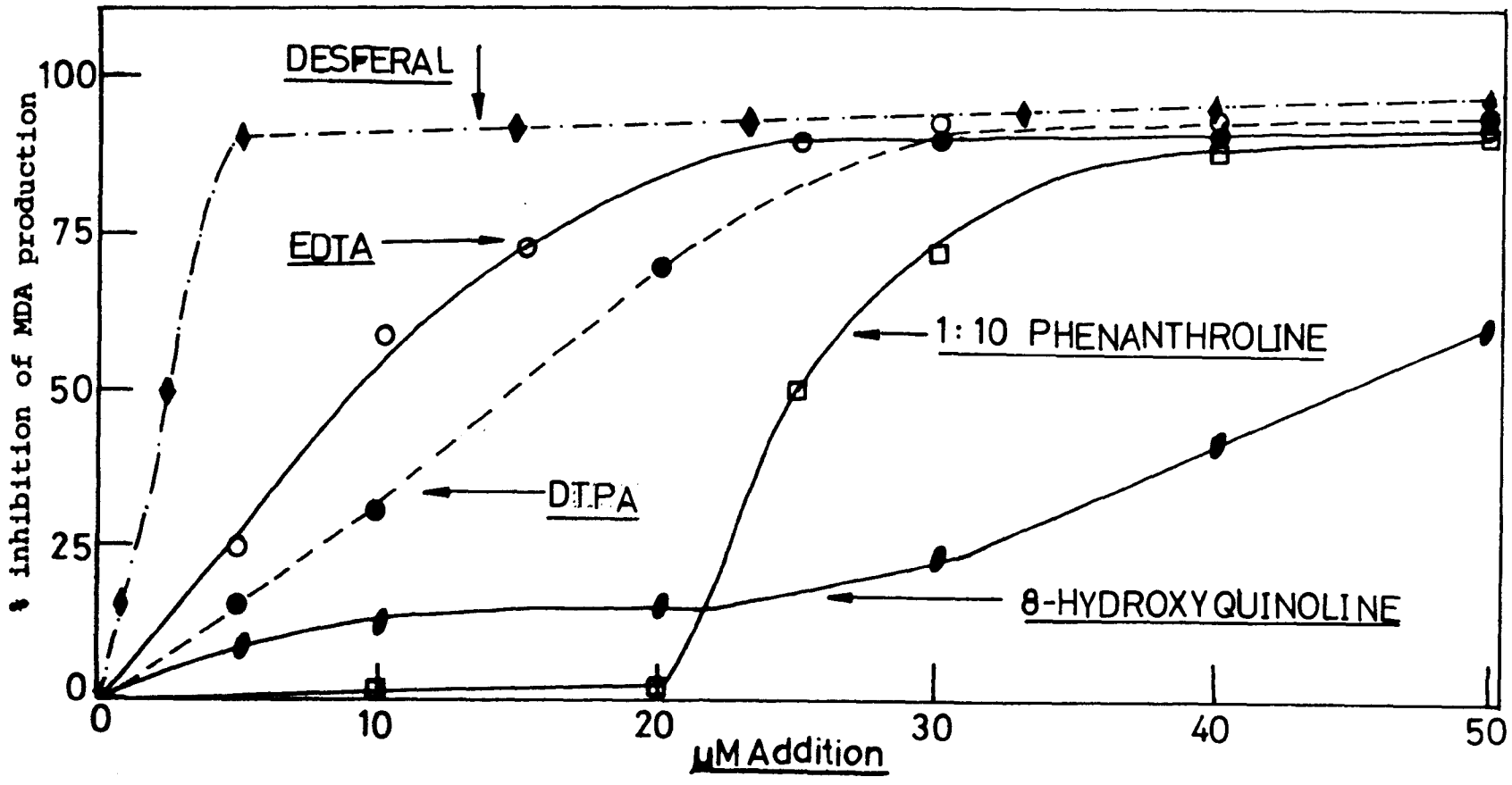


Figure 41: **The effect of chelating agents on MDA production by Fe^{2+}/dihydroxyfumaric acid**

Final conditions:- 1.1 mg protein/ml microsomes; 30 minutes incubation; 37°C; Tris:KCl buffer pH 7.4; 5 µM $FeSO_4$ / 1 mM dihydroxyfumaric acid.

Table 8: THE EFFECT OF INHIBITORS ON Fe^{2+}/DHF STIMULATED LIPID PEROXIDATION:

Final conditions:- 1.59 mg protein/ml microsomes; 37°C;
30 minutes incubation;
Tris:KCl buffer pH = 7.4

Class of Inhibitor	COMPOUND	% MDA production for a given concentration of inhibitor.	
		50 µM	100 µM
Antioxidants	Propyl gallate	0.00	
	HDC	0.00 (*a)	
	Promethazine	1.12	0.00
	Chlorpromazine	59.03	34.33
Iron Chelating Agents	EDTA	7.78	
	DTPA	5.56	
	1:10 Phenanthroline	7.23	
	8-Hydroxyquinoline	38.89	
	Desferal	1.67	
	Bleomycin	84.00	69.00
Singlet oxygen Scavengers	DABCO	100.00	106.00
	2,5 Diphenylfuran	77.00	73.00
	β-carotene	92.00	93.00
$O_2^{\cdot -}$ and H_2O_2 removing Enzymes	SOD	93.00 (*b)	
	Catalase	95.00 (*c)	104.00 (*d)
$OH^{\cdot}$ + $O_2^{\cdot -}$ scavenger	Tryptophan		100.00

Table 8 (continued):

Class of Inhibitor	COMPOUND	% MDA production for a given concentration of inhibitor	
		50 µM	100 µM
$OH^{\bullet}$ scavenger	Thiourea	99.00	98.00
Miscellaneous	Picolinic acid	102.00	99.00
	Adamantadine	91.00	90.00
	p-chloromercuri-benzoate		85.00
	Aminopyrine	100.00	92.00
	Na Salicylate		100.00
P_{450} Inhibitors	Metyrapone		61.00 ($*^{e}$)
	SKF 525A		3.00 ($*^{f}$)

Key: ($*^{a}$): 10 µM of HDC

($*^{b}$): 450 units of SOD. (1 mg/enzyme/3000 units)

($*^{c}$): 250 units of Catalase { (1 mg/enzyme/

($*^{d}$): 450 units of Catalase { 15.000 units)

($*^{e}$): 500 µM of Metyrapone

($*^{f}$): 500 µM of SKF 525A

Table 9 : EFFECTS OF DIFFERENT HEAVY METAL SALTS/ DIHYDROXYFUMARIC ACID ON MDA PRODUCTION:

Final conditions:- 1.2 mg protein/ml microsomes; 37°C; 30 minutes incubation; Tris:KCl buffer = 7.4.

added metal salt	Results are shown as % MDA production for a given concentration of added metal salt + 1 mM DHF.		
		µM	
	10	50	100
Fe^{2+}	322	455	467
Ni^{2+}	110	125	144
Mg^{2+}	101	104	108
Ca^{2+}	96	94	89
Zn^{2+}	87	79	73
Co^{2+}	93	77	53
Cu^{2+}	93	28	20
Mn^{2+}	6	5	4

Note: Controls with a) Only microsomes; b) Only 100 µM of metal salt without DHF; and c) 1 mM DHF were performed with each system. Each point is the average of two different experiments in triplicate.

Table 10: INHIBITORY EFFECTS OF Mn^{2+} ON MDA PRODUCTION BY Fe^{2+}/DHF

Final conditions:- 1.26 mg protein/ml microsomes; 37°C;

30 minutes incubation; Tris:KCl buffer pH = 7.4.

μM Fe^{2+} → / μM Mn^{2+} ↓	% MDA production for a given concentration of metal salt.				
	0	1	5	10	20
0	100	149	227	289	376
1	79	122	177	240	273
5	23	45	98	137	173
10	8	17	50	63	82
20	5	6	7	8	15

Note: Each point is the average of two different experiments in triplicate.

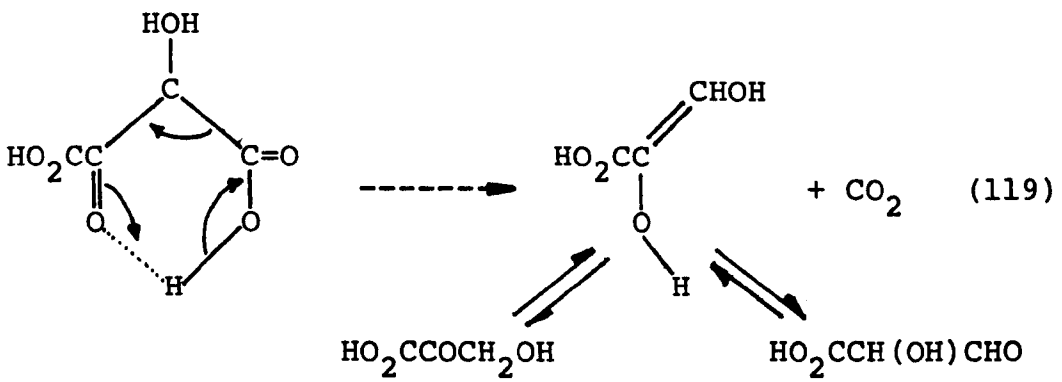
CHAPTER 4

GENERAL DISCUSSION

4a) Discussion

i) Chemistry of dihydroxyfumarate

β-keto acids are known to give an intermediate on decarboxylation (Franke and Brathun, 1931). The formation of this enol intermediate, which can ketonize and give either hydroxypyruvic acid or hydroxymalonic acid hemialdehyde (Hay and Harvie, 1965), has been proposed for dihydroxyfumaric acid.



(119)

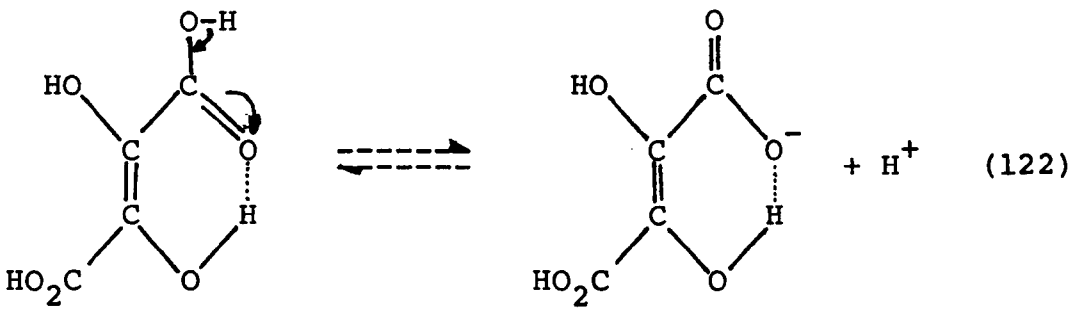
The ionization of dihydroxyfumaric acid can be represented as:

$$H_2A \rightleftharpoons H^+ + HA^- \qquad K_1 = [H^+][HA^-]f_1/[H_2A] \qquad (120)$$

$$HA^- \rightleftharpoons H^+ + A^{2-} \qquad K_2 = [H^+][A^{2-}]f_2/[HA^-]f_1 \qquad (121)$$

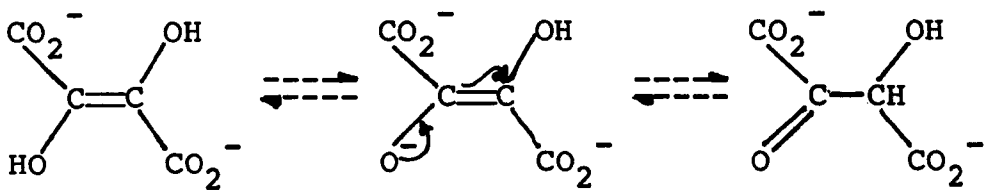
where f_1 and f_2 are the molar activity coefficient for monovalent and divalent ions, and K_1 and K_2 are the thermodynamic ionization constants which are 2.70×10^{-2} and 4.36×10^{-4} corresponding to $pK_1 = 1.6$ and $pK_2 = 3.4$ (Hay and Harvie, 1965).

The low value for the pK_1 is probably due to the intramolecular hydrogen bonding of the type found in maleic acid (reaction 122):



(122)

The strong absorption of dihydroxyfumaric acid in the ultraviolet λ 290 nm has been attributed to the enol tautomer of the acid which is equilibria with the keto-form and can be represented as:



(123)

Hay and Harvie (1965), reported a value of ε_{289} = 9130 $mol^{-1}cm^{-1}$ in water and Hartree (1953) found a value of ε_{290} = 9186 $mol^{-1}cm^{-1}$ in aqueous solution and ε_{308} = 9270 $mol^{-1}cm^{-1}$ in ether. A value of ε_{290} = 9000 $mol^{-1}cm^{-1}$ was derived from the present work.

ii) Oxidation of dihydroxyfumarate

Studies with dihydroxyfumaric acid are complicated by its facile autoxidation. Halliwell (1977), working with dihydroxyfumaric acid, reported that the acid suffers slow autoxidation at pH 6, and that this reaction can be inhibited by superoxide dismutase. He suggested the following mechanism:

$$DHF + O_2 \longrightarrow [X^\cdot] + O_2^{\cdot -} \quad (124)$$

$$2H^+ + O_2^{\cdot -} + DHF \longrightarrow [X^\cdot] + H_2O_2 \quad (125)$$

$$X^\cdot + O_2 \longrightarrow \underset{\text{(Diketosuccinate)}}{DKS} + O_2^{\cdot -} \quad (126)$$

where: $[X^\cdot]$ is a free radical formed by loss of one electron from dihydroxyfumarate. Evidence consistent with these reactions is provided by the observation that the e.s.r. spectrum of $[X^\cdot]$, generated under anaerobic conditions, is immediately lost when O_2 is admitted (Yamazaki and Piette, 1963). Halliwell concluded that a solution of dihydroxyfumaric acid at pH 6 always contains some $O_2^{\cdot -}$ and H_2O_2.

In the present studies, dihydroxyfumaric acid in solution at pH 3, see (Figure 2), suffered a 25% of autoxidation in ½ hour. Although DTPA was used to chelate impurities which might be present in the system, one possibility which might explain these observations could be that although traces of iron were chelated, the following reactions occurred.

$$DTPA(Fe^{2+}) + O_2 \longrightarrow DTPA(Fe^{3+}) + O_2^{\cdot -} \quad (127)$$

$$DTPA(Fe^{3+}) + DHF \longrightarrow DTPA(Fe^{2+}) + DHF(-H)^{\cdot} + H^+ \quad (128)$$

$$DHF(-H)^{\cdot} + O_2 \longrightarrow DKS + O_2^{\cdot -} + H^+ \quad (129)$$

Searle and Tomasi (1982) have recently demonstrated using the spin trap DMPO that hydroxyl radicals can be formed during the iron catalysed oxidation of cysteine according to the following reactions:

$$Fe^{2+} + 2RS^- \longrightarrow Fe^{2+}(RS^-)_2 \quad (130)$$

$$Fe^{2+}(RS^-)_2 + O_2 \rightleftharpoons [Fe^{3+}(RS^-)_2 \ldots O_2^{\cdot -}] \quad (131)$$

$$[Fe^{3+}(RS^-)_2 \ldots O_2^{\cdot -}] + RS^- \longrightarrow Fe^{2+}(RS^-)_2 + O_2^{\cdot -} + RS^{\cdot} \quad (132)$$

$$Fe^{2+}(RS^-)_2 + O_2^{\cdot -} + 2H^+ \longrightarrow Fe^{3+}(RS^-)_2 + H_2O_2 \quad (133)$$

$$Fe^{2+}(RS^-)_2 + H_2O_2 \longrightarrow Fe^{3+}(RS^-)_2 + OH^{\cdot} + OH^- \quad (134)$$

By analogy with the above reactions, in this work, the following reactions resulting in the formation of diketosuccinate (DKS) and hydroxyl radicals may also occur:

$$Fe^{2+} + DHF \longrightarrow [Fe^{2+}(DHF)] \quad (135)$$

$$[Fe^{2+}(DHF)] + O_2 \longrightarrow [Fe^{3+}(DHF) \ldots O_2^{\cdot -}] \quad (136)$$

$$\text{or} \longrightarrow Fe^{3+}(DHF) + O_2^{\cdot -} \quad (137)$$

$$[Fe^{3+}(DHF) \ldots O_2^{\cdot -}] + DHF \longrightarrow DHF(-H)^{\cdot} + H^+ + Fe^{2+}(DHF) + O_2^{\cdot -} \quad (138)$$

$$O_2^{\cdot -} + H^+ \underset{pka=4.9}{\rightleftharpoons} HO_2^{\cdot} \quad (139)$$

$$[Fe^{2+}(DHF)] + HO_2^{\cdot} + H^+ \longrightarrow Fe^{3+}(DHF) + H_2O_2 \quad (140)$$

$$2H^+ + O_2^{\cdot -} + O_2^{\cdot -} \longrightarrow H_2O_2 + O_2 \quad (141)$$

$$H_2O_2 + Fe^{2+}(DHF) \longrightarrow OH^{\cdot} + OH^- + Fe^{3+}(DHF) \quad (142)$$

$$Fe^{3+}(DHF) + DHF \longrightarrow Fe^{2+}(DHF) + DHF(-H)^{\cdot} + H^+ \quad (143)$$

$$DHF(-H)^{\cdot} + O_2 \longrightarrow DKS + O_2^{\cdot -} \quad (144)$$

$$O_2^{\cdot -} + H^+ \rightleftharpoons HO_2^{\cdot} \quad (139)$$

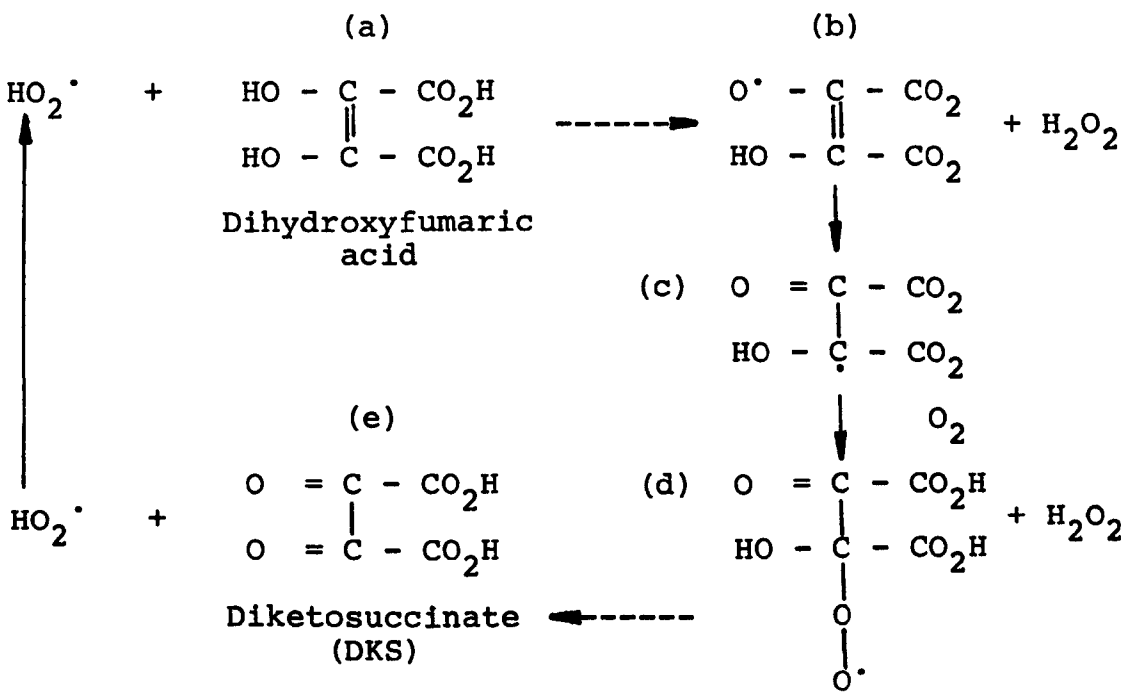
$$HO_2^{\cdot} + DHF \longrightarrow DHF(-H)^{\cdot} + H_2O_2 \quad (145)$$

$$OH^{\cdot} + DHF \longrightarrow DHF(-H)^{\cdot} + H_2O \quad (146)$$

$$HO_2^{\cdot} + O_2^{\cdot -} \longrightarrow H_2O_2 + O_2 \quad (147)$$

$$DHF(-H)^{\cdot} + H_2O_2 \longrightarrow DKS + OH^{\cdot} + H_2O \quad (148)$$

Reactions 144 - 145 can also be written as follows:



$HO_2^{\cdot}$ has a catalytic role in this system as outlined above, i.e. either react with dihydroxyfumaric acid or can recombine to form hydrogen peroxide equation (147).

In the present work, an attempt was made to measure the H_2O_2 formed, using the iodide method (Hochanadel, 1952). However, no amounts of H_2O_2 were detected and it would seem likely that the H_2O_2 formed in the reaction (b) is consumed by further reaction according to reaction (148), this reaction can also be written:

$$\begin{array}{l} O - \underset{|}{C} - CO_2H \\ HO - \underset{:}{C} - CO_2H \end{array} + H_2O_2 \longrightarrow \begin{array}{l} O = \underset{|}{C} - CO_2H \\ O = C - CO_2H \end{array} + OH^{\cdot} + H_2O \qquad (148)$$

The fact that oxidation of dihydroxyfumaric acid was stimulated by radiation and the high G(-DHF) obtained (Table 3) could be attributed to the formation of $OH^{\cdot}$, $O_2^{\cdot -}$, $HO_2^{\cdot}$ and H_2O_2 by the radiolytic decomposition of water according to:

$$H_2O \longrightarrow H_3O^+, OH^{\cdot}, e_{aq}^-, H, H_2O_2, H_2 \qquad (29)$$

Reaction of these species may form other species according to:

$$e_{aq}^- + H_3O^+ \longrightarrow H^{\cdot} + H_2O \qquad (30)$$

$$e_{aq}^- + O_2 \longrightarrow O_2^{\cdot -} \qquad (149)$$

$$H^{\cdot} + O_2 \longrightarrow HO_2^{\cdot} \qquad (150)$$

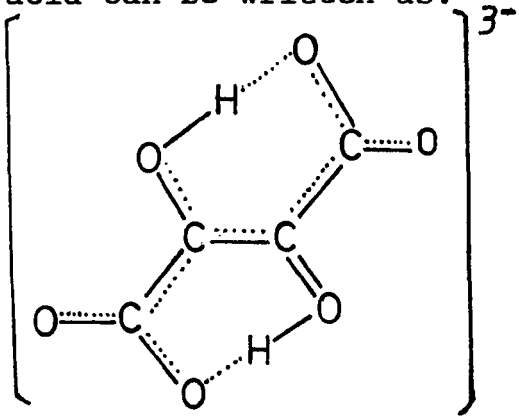
Some e^-_{aq} will react according to:

$$e^-_{aq} + DHF \longrightarrow DHF^{\cdot -} \qquad (151)$$

and some with O_2 to form $O_2^{\cdot -}$

$$e^-_{aq} + O_2 \longrightarrow O_2^{\cdot -} \qquad (149)$$

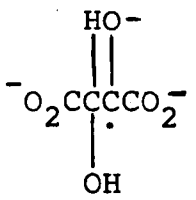
Assuming that the first pathway can take place, in this work it was demonstrated by pulse radiolysis that dihydroxyfumaric acid is very reactive with the e^-_{aq} reaction (70), and (Figure 12) with a rate of reaction $k_{70} = 2.1 \times 10^{10} M^{-1} s^{-1}$. Evidences of the structure of the electron adduct of dihydroxyfumaric acid comes from the work of Neta and Fessenden (1972). They reported that the best possibility of the e^-_{aq} adduct of dihydroxyfumaric acid can be written as:



$g = 2.00390$

$a^H = 0.49$ G (Triplet)

Alternatively, other species produced in dihydroxyfumaric acid in solution have a doublet spectrum with the parameters $g = 2.00413$ and $a^H = 2.77$ G. This splitting seems too large to be that of an OH proton so the e^-_{aq} adduct of the Ketoform may also be considered.



$g = 2.00413$

$a^H = 2.77$ G

This form for the radical was confirmed by the fact that an identical spectrum was obtained by H abstraction from tartrate (Laroff and Fessenden, 1971).

Assuming that the reaction (151) also goes, then the following reactions may also occur:

$$DHF^{\cdot -} + O_2 \longrightarrow DHF + O_2^{\cdot -} \qquad (152)$$

Although in this work no evidence was obtained to determine the rate constant of the reaction (152), in future it could be determined indirectly using a competition method with dihydroxyfumaric acid in excess and a reference solute (X), for example PNAP reacting as follows:

$$DHF^{\cdot -} + PNAP \longrightarrow PNAP^{\cdot -} + DHF \qquad (153)$$

This reaction would occur in competition with reaction (152) and a value for k_{152} might be derived.

On the oxidation of dihydroxyfumaric acid by ^{60}Co-γ-rays the oxygen consumption at least in the first minutes of the reaction decayed rapidly as can be seen on irradiated solutions of dihydroxyfumaric acid (Figures 8,9).

The oxygen consumption in unirradiated solutions of dihydroxyfumaric acid (Figure 4) was decayed also, but at the rate 5 times less compared with the irradiated solutions. The results obtained can be explained using the proposed mechanism (page 149) mentioned above.

From the biochemical point of view, reactions in the presence of molecular oxygen are of particular interest since a marked effect of oxygen on the sensitivity of certain biological systems to ionising radiations has been observed. For example, Johnson, et al., (1951) reported that the presence of oxygen markedly increased the yields of pyruvic acid from α-alanine in aqueous solutions. In this work, the stimulation of oxidation of dihydroxyfumaric acid and the high G(-DHF) values found may be explained by the increase on the rate of production of the initiating radicals in the process. This is perhaps similar to the situation for unsaturated acids which undergo oxidation by chain reactions. Mead, (1952) worked with irradiated solutions of linoleic acid (9,12-octodecadienoic) and obtained high G-values even in the presence of alcohol. Also, Johnson, et al., (1953), studying the action of free radicals produced by X-rays on lactic acid in aqueous solutions with particular reference to the effect of molecular oxygen found that pyruvic acid was produced in yields corresponding to G = 90. Since these yields are considerably in excess of the yields of initial radicals produced by irradiation of aqueous solutions, then results indicate that chain

reactions are occurring as postulated for dihydroxyfumaric acid.

Clearly as it was seen, the recent advances in fast-reactions techniques have given further insight into possible mechanisms of reactions of molecules in solutions, in particular pulse radiolysis is a powerful tool to measure very rapidly the rate constants of primary radicals and other species with a great variety of biological interest.

The absolute rate constants for the reaction of hydroxyl radicals with reducing agents are generally high, approaching those of diffusion-controlled reactions. For ascorbic acid, for example:

$$AH_2 \underset{pk=4.19}{\rightleftharpoons} AH^- + H^+ \qquad (154)$$

Second order rate constants for the reactions:

$$OH^\cdot + AH^- \longrightarrow A^{\cdot -} + H_2O \qquad (155)$$

$$OH^\cdot + AH_2 \longrightarrow AH^\cdot + H_2O \qquad (156)$$

where $k_{155} \sim 10^9 M^{-1}s^{-1}$ (neutral) and $k_{156} = 7.9 \times 10^9 M^{-1}s^{-1}$ (pH = 1) have been reported (Fujimaki and Morita, 1968; Adams, et al., 1965b).

As shown in Figure 11, the rate constant of the reaction (OH$^\cdot$ + DHF) determined by pulse radiolysis at pH 3.5 and 7 was $6 \times 10^9 M^{-1}s^{-1}$ and $3.3 \times 10^9 M^{-1}s^{-1}$, respectively. Unexpectedly, two different values for

that reaction were obtained and moreover the higher value was found at the lower pH. No satisfactory explanation has been found.

In the case of the studies with the halide ions Br^- and I^- and the pseudohalide SCN^-, the reactions between hydroxyl radicals and these ions have been demonstrated to produce the electrophilic radical ions $Br_2^{\cdot -}$, $I_2^{\cdot -}$ and $(SCN)_2^{\cdot -}$, respectively (Baxendale, et al., 1968; Cercek, et al., 1964; and Sutton, et al., 1965). These radicals can oxidise a wide range of compounds (Adams, et al., 1972; Redpath and Willson, 1973). The bimolecular rate constants for $Br_2^{\cdot -}$ and $(SCN)_2^{\cdot -}$ with, for instance, ascorbic acid = $8.7 \times 10^8 M^{-1}s^{-1}$ and $4.8 \times 10^7 M^{-1}s^{-1}$ has been obtained by (Redpath and Willson, 1973); and the rate constants of $I_2^{\cdot -}$ with ascorbic acid = $2 \times 10^7 M^{-1}s^{-1}$ (this work). Comparative values or rate constants for dihydroxyfumaric acid $k_{77} = 2.6 \times 10^8 M^{-1}s^{-1}$; $k_{75} = 4 \times 10^7 M^{-1}s^{-1}$ and $k_{78} = 1.5 \times 10^6 M^{-1}s^{-1}$ (Figures 13, 14), show that the values for ascorbic acid are greater than those for dihydroxyfumaric acid. Possibly the reactivity of the dibasic acids $HOOC(CH_2)_n COOH$ might be increased with increasing values of n. Also, another aspect to point out is that in terms of reactivity, the radical species mentioned above in their oxidative properties must show an order $Br_2^{\cdot -} > (SCN)_2^{\cdot -} > I_2^{\cdot -}$ (Adams, 1972). The results herein reported with ascorbic acid and dihydroxyfumaric acid effectively are confirming this situation.

Regarding the results with nitrate ion (Figure 21) and reactions (90 - 92), it is clearly shown that at the pH at which the experiments were undertaken, both L-ascorbic acid and dihydroxyfumaric acid were present in their anionic forms. The results obtained show that although $NO_3^{\cdot 2-}$ is not an oxidising agent, it reacts with water according to:

$$NO_3^{\cdot 2-} + H_2O \longrightarrow NO_2 + 2OH^- \qquad (86)$$

NO_2 can subsequently react with dihydroxyfumaric acid and ascorbic acid according to:

$$NO_2 + DHF \longrightarrow \text{products} \qquad (90)$$
$$k_{90} = 2.2 \times 10^7 M^{-1}s^{-1}$$

and

$$NO_2 + AH^- \longrightarrow \text{products} \qquad (92)$$
$$k_{92} = 3.1 \times 10^7 M^{-1}s^{-1}$$

Results obtained in this work show that dihydroxyfumaric acid can react rapidly with both $OH^{\cdot}$ radicals and e^-_{aq}. The hydroxyl radical reacts at a near diffusion controlled rate, whereas $(SCN)_2^{\cdot -}$, $I_2^{\cdot -}$ and NO_2 have slower rates. Interestingly, the azide radical $N_3^{\cdot}$ is 6-7 times faster in its rate of reaction than that of $Br_2^{\cdot -}$ and 45 times faster in its rate of reaction with dihydroxyfumaric acid than that obtained with $(SCN)_2^{\cdot -}$. Furthermore in general, L-ascorbic acid has bimolecular rate constants for reactions with these electrophilic radicals greater than those for dihydroxyfumaric acid.

As reported in Table 4 , the additions of TAN and TMPN to irradiated solutions of dihydroxyfumaric acid showed some protective effect on the oxidation rate of the acid. This could be due to competition of TAN and TMPN with dihydroxyfumaric acid for the free radical species generated in aqueous solutions. In fact. (Willson, 1971), using the pulse radiolysis, reported the following:

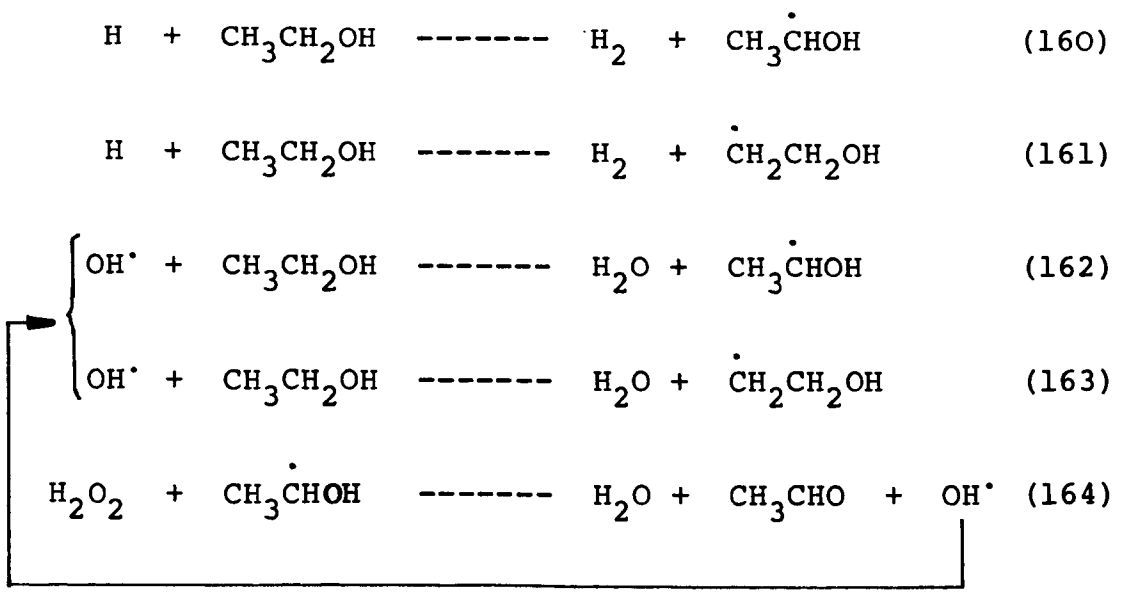
$$e^-_{aq} + TAN \longrightarrow \text{products} \qquad (157)$$
$$k_{157} = 2.2 \pm 0.2 \times 10^{10} M^{-1} s^{-1}$$

$$H + TAN \longrightarrow \text{products} \qquad (158)$$
$$k_{158} = 5.7 \times 10^{9} M^{-1} s^{-1}$$

$$OH^{\cdot} + TAN \longrightarrow \text{products} \qquad (159)$$
$$k_{159} \simeq 10^{8} M^{-1} s^{-1}$$

If the magnitude of the reaction k_{157} is similar to reaction k_{70} and under these circumstances competition between TAN and dihydroxyfumaric acid could be expected and the oxidation of dihydroxyfumaric acid would at least be delayed.

In the experiments with ethanol, no protection was observed in irradiated solutions of dihydroxyfumaric acid. It is known that ethanol can be involved in the following reactions:



Assuming that the last three reactions (162, 163, 164) were also probable, that means an alternative where the $OH^{\bullet}$ and H_2O_2 formed could react as well with dihydroxy-fumaric acid as has been proposed previously.

Willson (1978) has reported a list of absolute rate constants of the reaction of the isopropanol radical with different compounds. In this work, it is interesting to mention the rate constant of the isopropanol radical with dihydroxyfumaric acid $k_{101} = 5.7 \times 10^{7} M^{-1} s^{-1}$ compared with that reported for (IP$^{\bullet}$ + L-ascorbic acid) $= 1.2 \times 10^{6} M^{-1} s^{-1}$ (Redpath and Willson, 1973). This appears to be one exception where dihydroxyfumaric acid is showing a little more reactivity towards a specific free radical specie in comparison to ascorbic acid.

The values of the rate constants of the isopropanol radical with the iron chelated complex (Fe^{3+}/EDTA, Fe^{3+}/DTPA) were obtained in fresh solutions carefully protected from the sunlight, assuming a minimum of

oxidation, however, the values of those rate constants could be influenced in some way by the nature of the ligand complex.

Previous studies have shown that thiyl radicals derived from a number of biologically important sulphur containing compounds can react with ascorbate (Forni, et al., 1983), in accordance with:

$$RS^{\cdot} + AH^{-} \longrightarrow RS^{-} + A^{\cdot -} + H^{+} \quad (165)$$

As was described previously, the rate constant of the isopropanol radical with dihydroxyfumaric acid has been determined. In this work in order to gain information and to compare values, the rate constants of the isopropanol radical with the thiol compounds have also been obtained (Table 5) using a PNAP as a marker, according to the following reactions:

$$CH_3\dot{C}OHCH_3 + PNAP \longrightarrow PNAP^{\cdot -} + CH_3COCH_3 + H^{+} \quad (100)$$

$$CH_3\dot{C}OHCH_3 + RSH \longrightarrow CH_3CHOHCH_3 + RS^{\cdot} \quad (103)$$

The values of the rate constants derived by competition (Table 5) are showing some differences for example in the case of dithiothreitol(DTT), cysteamine and cysteine. Hence the possible subsequent reactions of these radicals probably were present. Since the pK for cysteamine, glutathione, cysteine (Wolfenden, 1982) and dithiothreitol (Redpath, 1973) are 8.35, 8.75, 8.35 and 5.2 respectively, all experiments were carried out at pH 4 to ensure

protonation of the SH group. The interpretation of the results for the organic compounds listed in Table 5 must recognise that the radiolysis of those compounds may produce a large number of products which are formed by a corresponding number of reactions. As it can be seen the values differ from case to case and it is not surprising to find that the occurrence of repair may depend on the chemical structure and these attributes are not related in a simple way. This may be better exemplified by the fact that the difference found between dithiothreitol (DTT) and penicillamine to repair the isopropanol radical is $\sim$5 times fold. The values of the absolute rate constants of the thiols mentioned above to repair the isopropanol radical when the PNAP system is used are in close agreement with the values reported for similar situation when the ABTS system is used as reference (Mora-Arellano et al., 1983). The extensive literature on the radiolysis of SH compounds and disulphides amply illustrates the complex nature of mechanisms involved. Thiols, RSH and oxidised thiols, RSSR have been shown to react quite rapidly with hydroxyl radicals (Adams, et al., 1967) and hydrated electrons (Redpath, 1973), according to:

$$OH^{\bullet} + (RS^{-})RSH \longrightarrow RS^{\bullet} + (OH^{-})H_2O \qquad (166)$$

$$e^{-}_{aq} + RSH \longrightarrow R^{\bullet} + HS^{-} \qquad (167)$$

$$R^{\bullet} + RSH \longrightarrow RS^{\bullet} + RH \qquad (168)$$

$$e^-_{aq} + RSSR \dashrightarrow RSSR^{\cdot -} \quad (169)$$

$$RSSR^{\cdot -} \rightleftharpoons RS^{\cdot} + RS^- \quad (170)$$

Different values found for the rate constants in the systems (ip/ac/N_2 and ip/N_2O) may suggest the possibility of a mimimum fraction of competition between sulphydryl and the organic molecules for the oxidising $OH^{\cdot}$ radicals (reaction 166). Some evidence can be given to endorse this idea. Redpath (1973) found that the rate constant of $k(OH^{\cdot} + DTT) = 1.5 \pm 0.5 \times 10^{10} M^{-1}s^{-1}$ as determined by competition against thiocyanate method, or $k = 1.3 \pm 0.1 \times 10^{10} M^{-1}s^{-1}$ using phenylalanine as a reference solute. Quintiliani, et al., (1977) reported that the value to the reaction:

$$OH^{\cdot} + GSH \dashrightarrow GS^{\cdot} + H_2O \quad (171)$$

$$k_{171} = 1.5 \times 10^{10} M^{-1}s^{-1}$$

For CysSH the quoted information in (Table 11) reveals also the behaviour of the thiol in vitro systems:

Table 11: Solute systems and radical balances at pH 5.3 ($k(M^{-1}s^{-1})$)

Solute system	$k(OH^{\cdot} + Solute)$	Major reacting radical species	$k(CysSH + Radical)$
$N_2O + CH_3OH$	0.9×10^9 (d)	CH_2OH	1×10^8 (e)
$N_2O + CNS^-$	1.1×10^{10} (d)	$(CNS)_2^{\cdot -}$	5×10^7 (f)

Taken from: (d) Willson, et al., (1971)

(e) Adams, et al., (1968)

(f) Adams, et al., (1972)

The studies with CCl_4 in presence of oxygen provide information useful to understanding the reaction of the trichloromethylperoxy radical with dihydroxyfumaric acid, in accordance with:

$$CCl_3O_2^{\cdot} + DHF \longrightarrow \text{products} \qquad (115)$$

$$k_{115} = 1.4 \times 10^8 M^{-1}s^{-1}$$

This observation can be compared with the reactions of alkylperoxyl radical ($XRO_2^{\cdot}$) with ascorbic acid reported by (Packer, et al., 1980), as follows:

$$XR^{\cdot} + O_2 \longrightarrow RO_2^{\cdot} \qquad (172)$$

$$XRO_2^{\cdot} + AH^- \longrightarrow XRO_2H + A^{\cdot -} \qquad (173)$$

In this work, a similar situation has been found with β-carotene. The chlorinated methyl peroxy radicals have been shown to have oxidative powers and their reactions with some biological molecules have been studied (Packer, et al., 1978: Packer, et al., 1980).

In the reaction between $CCl_3O_2^{\cdot}$ and related methyl peroxy radicals with β-carotene, the observed formation of the β-carotene$^{\cdot+}$ at 900 nm occurred exponentially with the rate first order in β-carotene concentration (Figure 28), corresponding to:

$$XRO_2^{\cdot} + \beta\text{-carotene} \longrightarrow \beta\text{-carotene}^{\cdot+} + \text{products} \quad (174)$$

The monitored radical cation β-cation$^{\cdot+}$ at 900 nm is in close agreement with the result reported by (Dawe and Land, 1975) in the same manner as the value for the rate constant $k_{116}(CCl_3O_2^{\cdot} + \beta\text{-carotene}) = 1.4 \times 10^9 M^{-1}s^{-1}$ previously determined by (Packer, et al., 1981).

The results herein reported have some significance if compared to the reactivity of $CCl_3O_2^{\cdot}$ and related methyl peroxyl radicals towards phenothiazines radical cation $PZ^{\cdot+}$; ascorbic radical anion $A^{\cdot-}$; propyl gallate; HDC and the amino acids tyrosine and tryptophan as can be seen in Table 12.

The values of the reactions (116 - 118) show some differences, probably due to the nature of the halogenated-compound in agreement with the formation of different

oxidising radicals. For instance, the reactivity of CCl_4, halothane ($CF_3CHClBr$), and $CHCl_3$ is different and possibly that might be attributed to the electronegative halogen atoms exerting an inductive effect. Also, it can be mentioned that the maximum absorption of the β-carotene$^{\cdot+}$ at 900 nm may be affected by factors such as configuration of the carotene isomer in the solvent, pH and temperature which can play a role in reactions where β-carotene is involved.

The results obtained with the peroxy radicals have special significance when the halogenated compounds are considered from the biochemistry and toxicology point of view, for instance, halothane is used in surgical and other clinical procedures and it is presumed to be metabolized in vivo by the NADPH-dependent drug-metabolism system (Daley, 1972, and Ugazio, 1978).
Although the metabolic formation of a free radical associated with halothane has not been demonstrated at all, it has been postulated (Stier, 1968; Van Dyke and Chenoweth, 1965), that the possible formation of a free radical of halothane itself is responsible for the liver damage observed following the clinical use.

Carbon tetrachloride and Chloroform have been demonstrated to be toxic species to induce pathological phenomena and even to cause death during their use (Ugazio, 1978), and considering the fact that the radicals of these three compounds react quite fast with β-carotene, it would not

be surprising to find that halothane in its metabolism may give a toxic yield for the cell.

Finally, it was demonstrated in this work that β-carotene can enter into reactions with peroxy radicals. Similar reactions may be envisaged in cellular metabolism, however, that does not mean necessarily that similar rate constants must be found in vivo.

Table 12: Reaction rates with halogenated peroxy radicals. ($k = 10^8 M^{-1}s^{-1}$)

(*) rate constants determined for this thesis; (*[a]) Natural pH.

Compound	$CCl_3O_2^\cdot$	$CHCl_2O_2^\cdot$	Halothane-$O_2^\cdot$	Reference
β-carotene	14.1 (*[a]) 15 ± 0.2	8.5 (*[a])	10.0 (*[a])	Mora-Arellano (*) Packer, et al.,(1981)
Propyl gallate	13 pH = 8.5			Searle, (1981)
Hydroxy dimethyl carbazole	8.3 pH = 7.5			Searle, (1981)
Promethazine	6.0 ± 0.4 pH = 6	1.4 pH = 6		Packer, et al.,(1980)
Ascorbic acid	2.0 ± 0.2 pH = 7	2.0 ± 0.4 pH = 7		Packer, et al.,(1980)
Dihydroxyfumaric acid	1.4 pH = 7			Mora-Arellano (*)
Tyrosine	1.3 ± 0.2 pH = 12	1.0 ± 0.1 pH = 12		Packer, et al.,(1980)
Tryptophan	0.85 pH = 7			Searle, (1981)

iii) Lipid peroxidation studies

Most of the studies on lipid peroxidation under conditions in vitro have been done with rat liver microsomes and using the TBA reaction for malonaldehyde (MDA) a three-carbon dicarbonyl compound, $CHOCH_2CHO$. The TBA test has been reviewed at length by (Barber and Bernheim, 1967; and Slater, 1972). Since then there has been almost continuous debate on the validity of this method of analysis, especially as peroxidized lipid can produce more than one compound producing a pink chromogen with TBA (see Saslaw, et al., 1966). Nevertheless, the current consensus of opinion is that TBA test is a reliable and sensitive index of lipid peroxidation in biological samples (Plaa and Witschi, 1976). The latest progress on this point is the report by (Esterbauer and Slater, 1981) that rat liver microsomes peroxidised with ADP/iron produced free MDA, measurable by high pressure lipid chromatography (HPLC) and this correlates well with the "TBA-reactive material" measured by TBA-test.

Although extensive biochemical studies have been undertaken by a variety of investigators, the mechanism(s) resulting in the development of lipid peroxidation in microsomes by different drugs, chemicals and metal ions, is still not resolved. On the basis of the background mentioned in the introduction of this thesis, the discussion of results obtained will be orientated towards possible free radical mechanisms.

With regard to lipid peroxidation with Fe^{2+}/ dihydroxyfumaric acid, it is possible to suggest that the reactions (135 - 148) mentioned before can contribute to explain the results obtained. As it can be seen, hydrogen peroxide could arise by spontaneous dismutation of the superoxide anion equation (141) or formed in the reactions (140, 145), although it is known that the reaction (141) is comparatively slow, $k, \leq 0.3\ M^{-1}s^{-1}$ (Bielski and Allen, 1977) in neutral and alkaline solution. Hydrogen peroxide can also give hydroxyl radicals by a Fenton type reaction (equation 142). The possibility that hydroxyl radicals initiate peroxidation is suggested by the work of (Koster and Slee, 1980), who demonstrated inhibitory effect using catalase and thiourea. However, Svingen, et al., (1979) have suggested that $OH^{\cdot}$, $^1O_2$, H_2O_2 and $O_2^{\cdot -}$ are not responsible species for promoting peroxidation. These authors concluded that some form of the perferril ion such as ($ADP\text{-}Fe^{3+}\text{-}O_2^{\cdot -}$) is responsible for the initiation. However, later the same group proposed that iron plays a role in the breakdown of lipid hydroperoxides formed during peroxidation (Pederson and Aust, 1975). Work with linoleate micelles suggests that superoxide can react with hydroperoxides to form alkoxy radicals, which would be sufficiently reactive to further initiate lipid peroxidation by reaction similar to (175), Sutherland and Gebicki, (1982).

$$O_2^{\cdot -} + LOOH \longrightarrow O_2 + LO^{\cdot} + OH^{-} \qquad (175)$$

However in the present system, roles of O_2, $OH^{\cdot}$ and (Fe^{2+}/DHF) are perhaps more likely as follows:

a) Initiation

$$OH^{\cdot} + LH \longrightarrow L^{\cdot} + H_2O \quad (176)$$

Lipid radical

b) Propagation

$$L^{\cdot} + O_2 \longrightarrow LO_2^{\cdot} \quad (177)$$

$$LO_2^{\cdot} + LH \longrightarrow LO_2H + L^{\cdot} \quad (178)$$

Subsequently, the following reactions can take place:

$$LO_2H + Fe^{2+}(DHF) \longrightarrow LO^{\cdot} + OH^{-} + Fe^{3+}(DHF) \quad (179)$$

$$LO^{\cdot} + Fe^{2+}(DHF) \longrightarrow LO^{-} + Fe^{3+}(DHF) \quad (180)$$

$$LO^{-} + H^{+} \longrightarrow LOH \quad (181)$$

Termination:

$$L^{\cdot} + LO_2^{\cdot} \longrightarrow \text{nonradical products (including MDA)} \quad (24)$$

$CHOCH_2CHO$

or

$$2L^{\cdot} \longrightarrow L\text{-}L \quad (23)$$

$$L^{\cdot} + LOO^{\cdot} \longrightarrow LOOL \quad (24)$$

$$2LOO^{\cdot} \longrightarrow LOOL + O_2 \quad (25)$$

The results shown in (Figure 34) and (Table 9) seem likely to support the idea that peroxy radicals formed on incubated microsomes were stimulated by Fe^{2+}/dihydroxy-fumaric acid, suggested by the increase of MDA measured.

Similarly, high yields of malonaldehyde have been reported in chemically induced peroxidation of liposomes with ferrous sulphate/ascorbic acid system (Fukuzawa, et al., 1981; and Kunimoto, et al., 1981).

An important consequence of these reactions proposed (pages 148-149) is the cycling step in which the iron cycles from $Fe^{II} \longrightarrow Fe^{III} \longrightarrow Fe^{II}$ with the consumption of oxygen and dihydroxyfumaric acid. Also, considering the importance of iron to catalyse reactions, the additions done in presence or in absence of dihydroxy-fumaric acid either in boiled or active microsomes were demonstrating effective effects to stimulate lipid peroxidation measured as MDA formation. Microsomes are very liable to lipid peroxidation and concurrent damage. Iron plus a reducing agent can constitute one of the most powerful catalytic systems to induce peroxidation of microsomes. The results herein obtained are supported with those described by (Hunter, et al., 1964; Wills, 1965; 1966; 1969; O'Brien, 1969; Donovan and Menzel, 1978; Searle, 1981) and in agreement with those reported by (Nakano, 1978; Nakano and Obo, 1979).

The observed effect of temperature on the yields of malonaldehyde (Figure 36) may be due to structural changes in the microsomes. Depending on the physical state of the membrane, the attack of generated free radicals species within system will be facilitated or prevented, e.g. de Kruijff, et al., (1980) reported changes for the

fraction of the endogenous phospholipids of liver microsomes when studying the temperature effect at 37°C and 4°C on biological membranes. More recently, Eichenberger, et al., (1982) observed the liquid-crystalline state for control and rat liver peroxidised microsomes over the temperature range (5 - 37)°C, found a relationship of increasing lipid peroxidation with the order of the microsomal phospholipids acyl chains and such increase in order might be due to the formation of covalent bonds between adjacent lipid radicals. The results shown in Figure 36 would seem to follow a similar pattern of temperature effect with increasing MDA formation until around 55°C following a progressive decreasing. Above 55°C the possibility that intermediates formed as a result of breakdown of poly-unsaturated fatty acids and the production of endproducts suffered trans-formation to volatile products which were not trapped by TBA might be considered.

Nakano and Obo, (1979) reported that dihydroxyfumaric acid induced lipid peroxidation with a maximum pH of 4.5. In this work, dihydroxyfumaric acid alone reached a maximum around pH 5.3, as shown in Figure 37. The extent of lipid peroxidation when Fe^{2+} was added in presence of dihydroxyfumaric acid was effectively enhancing the MDA formation at the neutral pH. To Fe^{2+} alone, the catalysis of lipid peroxidation is pH dependent with the optimum rate occurring at pH 5, which is in close agreement with those reported by (Wills, 1965). The

differences found in those three separate situations, are not surprising at all, because findings in the extent of lipid peroxidation observed in other laboratories with dissimilar incubation systems have demonstrated that variations in the lipid peroxidation can be detected. For example, Wills (1969), working with NADPH and ascorbate as pro-oxidant to stimulate peroxidation on microsomes found a maximum value at pH 7 and 6, respectively. Another aspect to point out would be the buffering capacity of the solutions used (Tris-maleate and Tris-HCl). Kornbrust and Mavis (1980) found optimal rates of MDA formation obtained with (40-80) mM Tris-maleate, at pH 7.4; although, varying the pH between 6.4 and 8.0 had little effect on the rate of lipid peroxidation activity. At lower concentration of or in absence of Tris-maleate, less peroxidation was reported.

Regarding the chelating agents results (Figure 41), desferal resulted to be a very good powerful agent to inhibit the action of Fe^{2+}/dihydroxyfumaric acid, its ED_{50} found about 2.5 μM. Strong inhibitory effects of desferal on a phospholipid system induced lipid peroxidation by Fe^{2+} has been demonstrated by Gutteridge, et al., (1979). They reported that desferal inhibited lipid peroxidation at ED_{50} = 8.5 μM; the iron-catalysed Haber-Weiss reaction and $O_2^{\cdot -}$ induced degradation of hyaluronic acid. Cohen and Sinet (1981), pointed out that the Fenton type reaction between iron salts, iron chelates and H_2O_2 can generate the highly reactive hydroxyl free radical

(Haber-Weiss, 1934; Walling, et al., 1975) as follows:

$$\text{Fe(II)-chelate} + H_2O_2 \longrightarrow \text{Fe(III)-chelate} + OH^{\cdot} + OH^{-} \quad (182)$$

$$\text{Fe(II)-chelate} + OH^{\cdot} \longrightarrow \text{Fe(III)-chelate} + OH^{-} \quad (183)$$

$$2\text{Fe(II)-chelate} + H_2O_2 + 2H^{+} \longrightarrow 2\text{Fe(III)-chelate} + 2H_2O \quad (184)$$

It was unlikely that these last reactions were occurring within the system used, because inhibitions were observed. If the formation of OH· radicals had place, it was probably minimal and the radicals formed probably did not diffuse far from their place of formation, in accordance with the reaction (176). The initiation step was therefore blocked. The difference of results obtained among the chelating agents, desferal, EDTA, DTPA, 1.10-phenanthroline and 8-hydroxyquinoline, may be partly due to different binding forces to chelate iron and to the different oxidation-reduction potential of the corresponding complexes. For instance, Adrien (1973) reported the effect of ligands on reduction-oxidation potentials for iron with EDTA and 8-hydroxyquinoline, E_o = + 0.14 and + 0.27 volts respectively. The effectiveness of the different chelating agents used on microsomes incubated with Fe^{2+}/dihydroxyfumaric acid was compared. ED_{50} values obtained in this work are compared with those found by other workers in different systems, as can be seen in Table 13.

Table 13: ED_{50} values for chelating agents on lipid peroxidised systems

Chelating agent	System	(%) Inhibition	µM	Reference
DESFERAL	Fe^{2+}/DHF	50	2.27 (*)	Mora-Arellano (*[a])
	NADPH-lipid peroxidation	16	1.0	Slater, (1979)
	CCl_4-stimulated lipid p.	41	1.0	Slater, (1979)
	NADPH system	80	0.5 (*[b])	Wills, (1969b)
EDTA	Fe^{2+}/DHF	50	8.40 (*)	Mora-Arellano (*[a])
	Fe^{2+}/cysteine (5:500)µM	50	27.40	Searle, (1981)
	NADPH-lipid peroxidation	51	5.0	Slater, (1979)
	CCl_4-stimulated l.perox.	69	5.0	Slater, (1979)
	NADPH system	95	0.5 (*[b])	Wills, (1969b)
	Ascorbate system	98	0.5 (*[b])	Wills, (1969b)

Table 13 (Continued):

Chelating agent	System	(%) Inhibition	µM	Reference
DTPA	Fe^{2+}/DHF	50	13.0 (*)	Mora-Arellano (*[a])
	Fe^{2+}/DHF (20 µM : 3 mM)	89	1.0	Bindoli and Cavallini, (1980)
1:10 PHENANTHROLINE	Fe^{2+}/DHF	50	22.0 (*)	Mora-Arellano (*[a])
	NADPH system	98	0.5 (*[b])	Wills, (1969b)
	Ascorbate system	94	0.5 (*[b])	Wills, (1969b)
8-HYDROXYQUINOLINE	Fe^{2+}/DHF	50	40.0 (*)	Mora-Arellano (*[a])
	NADPH system	71	0.5 (*[b])	Wills, (1969b)
	Ascorbate system	92	0.5 (*[b])	Wills, (1969b)

Key: (*) corrected values for 1 mg protein/ml; (*[a]) values obtained in this work from(Figure 41)
(*[b]) mM.

It is known that bleomycin can chelate iron forming a complex as follows:

$$BLM + Fe^{2+} \longrightarrow BLM \cdot Fe^{2+} \quad (185)$$

Since some authors, such as Lown and Soo (1977), pointed out that $BLM \cdot Fe^{2+}$ complex may react in accordance with the next mechanisms:

$$BLM \cdot Fe^{2+} + O_2 \longrightarrow BLM \cdot Fe^{3+} + O_2^{\cdot -} \quad (186)$$

$$BLM \cdot Fe^{2+} + O_2^{\cdot -} + 2H^+ \longrightarrow BLM \cdot Fe^{3+} + H_2O_2 \quad (187)$$

$$BLM \cdot Fe^{2+} + H_2O_2 \longrightarrow BLM \cdot Fe^{3+} + OH^{\cdot} + OH^- \quad (188)$$

the results obtained with incubated microsomes (Table 8) show small inhibitions as expected with high concentrations of bleomycin.

During the last several years, there has been heightened interest in the effects produced by antioxidants on a variety of biological systems. The range of these systems extends throughout the entire field of modern biochemistry from the gross effects on the whole organism through the diet to the direct action on isolated chemical pathways. In discussing the antioxidant action of propyl gallate, HDC, promethazine and chlorpromazine, it would be convenient to make a Table to compare the effectiveness of each one of them within the system used, Fe^{2+}/dihydroxy-fumaric acid, but at the same time it is useful to know the inhibitory effects by the same antioxidant compounds on other systems lipid peroxidised. For example, the

antioxidant effect of propyl gallate on MDA production by Fe^{2+}/DHF is shown in Figure 39 , for this compound an ED_{50} = 3.12 μM was derived and a corrected value of 2.03 μM for 1.0 mg protein/ml is given which can be compared with those reported in the literature (Table 14).

Using reactions (176 - 178) to illustrate how lipid and lipid hydroperoxy radicals may be formed, then propyl gallate may prevent lipid peroxidation through a mechanism shown in equations (189 - 191).

$$L^{\cdot} + O_2 \longrightarrow LO_2^{\cdot} \qquad (177)$$

$$LO_2^{\cdot} + LH \longrightarrow LOOH + L^{\cdot} \qquad (178)$$

$$LO_2^{\cdot} + AH_2 \longrightarrow LOOH + AH^{\cdot} \qquad (189)$$

$$LO_2^{\cdot} + AH^{\cdot} \longrightarrow LOOH + A \qquad (190)$$

$$2AH^{\cdot} \longrightarrow AH_2 + A \qquad (191)$$

where: AH_2 = propyl gallate

$L^{\cdot}$ = lipid radical

$LO_2^{\cdot}$ = lipid hydroperoxy radical

In this case, the species $L^{\cdot}$ and $LOO^{\cdot}$ may be scavenged and thereby prevented from further attacking the lipid. Also, it is necessary to consider a binding action of propyl gallate which might be liganding ferric ion through its phenolic groups. If this is the case, then the involvement of iron ions in free radicals reactions would be minimised. Pulse radiolysis has shown for the

Table 14: ED_{50} values for propyl gallate on lipid peroxidised systems

System studied	ED_{50} µM values	Reference
NADPH stimulated lipid peroxidation	$ED_{63} \leq 20$	Slater, (1979)
NADPH/ADP/Fe^{2+}	4	Slater, (1979)
Fe^{2+}/ascorbate	3.30	Malvy, et al., (1980)
Fe^{2+}/cysteine	2.9	Searle, (1981)
CCl_4/NADPH (*)	1 µM $< ED_{50} <$ 20 µM	Slater, (1979)
ROOH	46.7	Malvy, et al., (1980)

(*): ED_{50} corrected to a value for 1.5 mg protein/ml.

reactions of the primary radical species, $OH^{\cdot}$ and the peroxy radical $CCl_3O_2^{\cdot}$ with propyl gallate that the rate constants for these reactions are $1.1 \times 10^{10} M^{-1}s^{-1}$ and $1.3 \times 10^{9} M^{-1}s^{-1}$ (pH 8.5), respectively. (Searle, 1981).

The ED_{50} for propyl gallate in the Fe^{2+}/dihydroxy-fumaric acid (2.03 μM corrected to 1.0 mg protein/ml) compares well with the values obtained for other ferrous containing systems (Table 14). This may reveal a common mechanism of operation.

Regarding the results obtained with the phenothiazine group, promethazine gave an ED_{50} value corrected to 1.0 mg protein/ml of 8.5 μM. A 65.6% inhibition was achieved with 100 μM of chlorpromazine. Searle (1981), working with promethazine in the Fe^{2+}/cysteine system, found an ED_{50} value 11.6 μM and with the Fe^{2+}/ascorbate system the ED_{50} was 13.1 μM. The same author reported for chlorpromazine an ED_{50} = 91.1 μM for 1.0 mg protein/ml using the Fe^{2+}/cysteine system. Also, Slater (1979), studying the NADPH/ADP/Fe^{2+} model of lipid peroxidation reported an ED_{50} = 25 μM for promethazine. If those values obtained are compared, interesting observations can be made from the experimental models mentioned. Firstly, the variability in the results might be explained in terms of dietary antioxidants ingested by the animals. Secondly, the effectiveness of promethazine may be due to its scavenging of free radicals in the first stages of peroxidation. Its action is greater than chlorpromazine

possibly because of difference in affinity, solubility and distribution within membrane components. In fact, if a compound if better solubilised than another within the lipid milieu, it is likely that it will remove or scavenge more efficiently the radicals generated in that domain. Furthermore, it has been determined by pulse radiolysis that there are similar rate constants for ($OH^{\cdot}$ + Promethazine) and ($OH^{\cdot}$ + Chlorpromazine) of $1 \times 10^{10} M^{-1} s^{-1}$ (Bahnemann, 1981). Thus it is quite likely that the difference between promethazine and chlorpromazine may be due to specific interactions at the membrane level.

From the biological point of view, promethazine and chlorpromazine play an important role because they can stabilise membranes at low concentrations (about μM), but at mM concentrations cause lysis (Slater and Riley, 1966; Seeman and Weinstein, 1966).

Whatever type of mechanism is present in vivo, whether radical scavenging or membrane interaction, it has pharmacological importance since both phenothiazines are used clinically. Indeed, these compounds may, due to their amphipathic nature, be found in particular regions of the microsomal membrane. Furthermore, it is likely that their protective action will be particularly dependent on redox potential kinetic parameters. Further knowledge of these aspects will aid interpretation of the results.

Another antioxidant studied was 6 hydroxy 1.4 dimethyl carbazole (HDC), which showed a strong inhibition of MDA production on microsomes stimulated by Fe^{2+}/dihydroxyfumaric acid. An interesting aspect was the very pronounced threshold effect demonstrated in the concentration/inhibition plot. An ED_{50} value = 0.75 µM is given a corrected value of 0.49 µM for 1.0 mg protein/ml (Figure 38). This value is comparable with those ED_{50} values reported by (Searle, 1981), using different lipid peroxidation systems, see (Table 15):

Table 15: ED_{50} values for the inhibition of lipid peroxidation by HDC.

System used	HDC n.moles drug/mg protein
Fe^{II}/ Ascorbate	0.26
Fe^{II}/ Cysteine	0.31
Fe^{II}/ ADP / NADPH	0.30
CCl_4/ NADPH	0.20
Cumene hydroperoxide	12.50

The affinity of HDC for the lipid membrane may be the key to its effectiveness. The arrangement and orientation of HDC within the lipid microsomes may mean than the drug can react rapidly to remove free radicals produced in

the surrounding aqueous medium. Searle (1981) suggested that structure:activity relationships revealed that the hydroxyindole portion of the molecule is largely responsible for the scavenging ability of this carbazole. Also, he pointed out that interactions between the HCD hydroxyl group orientated towards the positively charged head groups of phospholipid membrane may occur. The lipophilicity of HDC and its planar structure may contribute to its efficiency as an antioxidant by permitting it to come into close proximity with radical species. A high rate constant with $OH^{\cdot} = 1.5 \times 10^{10} M^{-1} s^{-1}$ was measured by pulse radiolysis (Searle, 1981). Comparing its effectiveness in the Fe^{2+}/dihydroxyfumaric acid system, HDC was (4.14; 4.63; 17.16; 17.34; 27.44; 46.36; and 82.36) times more potent than propyl gallate; desferal; EDTA; promethazine; DTPA; 1:10 phenanthroline and 8-hydroxyquinoline, respectively.

Normally HDC was added before the initiation of lipid peroxidation, but if it was added 5 or 15 minutes after the start of the experiment, then less inhibition was obtained. This implies that the presence of HDC within the system was necessary in the first stages of lipid peroxidation in order to show its strongest antioxidant properties.

The use of scavengers to detect the presence of $^1O_2$ was employed. 100 µM diphenylfuran gave a 27% inhibition of MDA formation. It is known that diphenylfuran

solubility in water is 5 x 10^{-7}M (Porter and Ingraham, 1974); but when diphenylfuran was given as a suspension in t-butanol it may have been better able to interact with the lipid portion of the microsomal membrane. King, et al., (1975), using 2.5 diphenyl in incubated microsomes in a NADPH generating system found that significant amounts of DPF were converted to dibenzoylethylene during the enzyme reaction and that the peroxidation of lipids in the microsomal membrane was inhibited by only 50 to 60% at saturating concentrations of DPF. The role played by carotenes as antioxidants on biological membranes has been studied. Mathews, (1964) reported that β-carotene was effective in protecting mice against an otherwise lethal photosensitisation due to haemotoporphyrin. Porphyrin photosensitisation is believed to involve a free radical attack on lysosomal membranes in the epidermis. Our system used 100 μM β-carotene which showed little effect at inhibiting the MDA production. This could mean that at the quoted concentrations β-carotene did not efficiently quench the singlet oxygen generated in the medium. Therefore, the first stages of the lipid peroxidation were not blocked efficiently. Consequently there were enough intermediates formed to allow the break-down of end products including MDA. The effect of DABCO was also examined but surprisingly no inhibition was achieved. On the other hand, if the DABCO concentration was increased, stimulation of MDA had apparently taken place. However, Searle (1981), working with high concentrations of DABCO in the Fe^{2+}/cysteine system, reported some inhibition of

lipid peroxidation. Different results with different systems might suggest specificity of attack by some free radical species, in this case the DHF$^{\cdot}$ radical. Another point to consider is that DABCO at physiological pH carries a localised positive charge and it is unlikely that DABCO can penetrate into microsomal membranes to exert any great effect. The reaction of ($OH^{\cdot}$ + DABCO) is quite fast, $k = 1.25 \pm 0.1 \times 10^{9} M^{-1} s^{-1}$ (Anderson and Patel, 1978). This does not necessarily mean a similar mode of action for incubated microsomes, where other factors have to be considered. As these three putative $^{1}O_2$ scavengers did not show much evidence when they removed $^{1}O_2$, the results obtained might suggest that lipid peroxidation by Fe^{2+}/dihydroxyfumaric acid involves other activated oxygen species ($O_2^{\cdot -}$, $OH^{\cdot}$).

The results obtained with SOD and catalase give some information of the roles played by $O_2^{\cdot -}$ and H_2O_2. SOD showed a weak inhibition of lipid peroxidation and CAT was ineffective. Nakano and Obo (1979), described similar results with dihydroxyfumaric acid in rat liver microsomes, where they used 5.6 µM SOD and (470 µg/ml and 235 µg/ml) of CAT to get (100, 115 and 113)% of MDA formation, respectively. Bindoli and Cavallini, (1980), working with brain phospholipid liposomes, reported that DHF autoxidation is sensitive to SOD and CAT, only when $FeSO_4$ is not added; because when $FeSO_4$ is present in the system, SOD and CAT are no longer able to reduce the rate of autoxidation, probably indicating that the reaction proceeds with a

different mechanism in the presence of iron ions. However, under those conditions, the production of superoxide anion could be detected as monitored by the inhibition exerted by SOD on NBT reduction. In rat liver mitochondria, Nakano (1978) reported that SOD and CAT had no effect on the rate of dihydroxyfumaric acid stimulated malonaldehyde formation. Autor, et al., (1980) showed that SOD and CAT maintained the viability of pulmonary macrophages exposed to the action of dihydroxyfumaric acid. It is possible that the action of SOD and CAT against dihydroxyfumaric acid can change depending on the in vitro system used. Therefore, a clear conclusion is difficult to draw. It may be that with Fe^{2+}/dihydroxyfumaric acid couple the free radicals species are generated or are consumed very quickly at the lipid-water interface or the enzymes cannot reach the domains of the microsomal membrane where these radicals react. Under those conditions, the ability of SOD and CAT to remove $O_2^{\cdot -}$ and H_2O_2 and to prevent the lipid peroxidation is limited.

The action of anti-inflamatory agents such as aminopyrine and Na salicylate was studied in incubated microsomes in the presence of Fe^{2+}/dihydroxyfumaric acid. No inhibitory effect was detected with Na salicylate; aminopyrine at 100 µM showed very little effect on MDA formation. Orrenius, et al., (1964) has described that aminopyrine, which is oxidised by microsomes, can cause a significant decrease in the rate of lipid peroxide formation in microsomes incubated in presence of NADPH.

Also, Wills (1969c) pointed out that aminopyrine can regulate the rate of oxygen uptake, diverting it from generation of lipid peroxide to oxidation of the drug. Ashley and Griffin, (1980), have shown in microsomes the cumene hydroperoxide-supported oxidation of aminopyrine, probably through the following mechanism:

$$R_2N{-}{-}CH_3 \xrightarrow{-e^-} R_2N{-}{-}CH_3^+ \xrightarrow{-H^\cdot} R_2N^+{=}{=}CH_2 \xrightarrow{H_2O} R_2NH_2^+ + H_2C{=}O \quad (192)$$

As indicated, aminopyrine can undergo a one-electron oxidation to the free radical species which is further oxidised to a iminium cation. Hydrolysis of the iminium cation yields the amine and formaldehyde. In this work, it would be possible that OH$^\cdot$ could be formed by decomposition of H_2O_2 by Fe^{2+}(DHF) reaction (142). It would be possible that aminopyrine could react with the OH$^\cdot$ generated within the system studied; in fact, indirect evidence has shown that the aminopyrine free radical can be produced in the presence of Fenton's reagent (Griffin, 1977), which is known to generate hydroxyl radicals (Walling, 1975). Also Packer, et al., (1983) unpublished results measured by pulse radiolysis the rate constant $k(OH^\cdot + \text{aminopyrine}) = 1.2 \times 10^{10} M^{-1}s^{-1}$ and for $k(OH^\cdot + \text{antipyrine}) = 2.18 \times 10^{10} M^{-1}s^{-1}$. An alternative explanation may be that the loss of endogenous hemoprotein group caused by dihydroxyfumaric acid is to some extent independent of H_2O_2 formation for example through the production of reactive derivate(s) of dihydroxyfumaric acid which may become bound onto the haem. It may be

also that the aminopyrine free radical once generated can abstract electrons from unsaturated fatty acid in the microsomal membrane, thereby maintaining the chain process of lipid peroxidation.

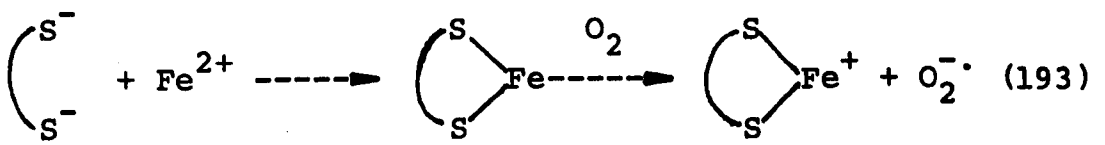
Many papers concerned with lipid peroxidation have considered that cytochrome P_{450} is involved in catalysing the process. Since cyt P_{450} is apparently important in the Fe^{2+}/dihydroxyfumaric acid promoted systems, it was necessary to assess the effects of the known inhibitors Metyrapone and SKF 525A. The effect of SKF 525A in preventing MDA formation could be considered due to a surfactant action on the microsomal membranes. Cheeseman (1982), working with both compounds, reported that SKF 525A inhibits microsomal drug metabolism by blocking cytochrome P_{450}, inhibiting MDA production and also oxygen uptake. Searle (1981), warned that results obtained with SKF 525A must be viewed with caution because the microsomal suspension turned cloudy and white when this compound was added.

The $OH^{\cdot} + O_2^{\cdot -}$ scavengers tryptophan and thiourea did not prevent the lipid peroxidation to any great degree. It is possible that it would require high concentrations of these compounds in order to produce better inhibition, since they must compete successfully with the unsaturated lipids for the hydroxyl radicals generated.

Picolinic acid, although an important cellular chelating agent, was ineffective in preventing MDA formation. Adamantadine and p-chloromercuribenzoate showed only 10% and 15% inhibitions, respectively. However, in contrast Nakano and Obo (1979) used p-CMB (1 mM) on dihydroxyfumaric acid stimulated lipid peroxidation in rat liver mitochondria and microsomes and no inhibition of MDA was achieved. Cheeseman (1982), working with 100 μM p-CMB, detected that the MDA was increased but at higher concentrations of p-CMB a crossover effect occurred and the lipid peroxidation was strongly inhibited. Variability of results with p-CMB seems to indicate that the type of technique employed to study the compound is important.

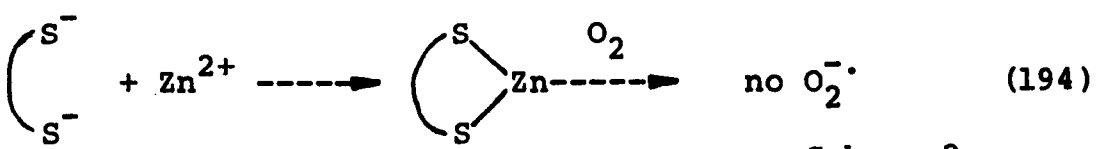
No precise formulation of the mechanism of metal-catalysed oxidation has yet been described, although several alternative mechanisms have been proposed for the Fe^{2+}/ dihydroxyfumaric acid couple. It is likely that heavy metal salts play an important role in the initiation reaction which probably involves the break-down of the low concentrations of lipid peroxide which may be initially present in the microsomal fraction to form free radicals which are essential for the chain reaction to proceed. Regarding the inhibitory effects of Cu^{2+}, a possible explanation is that cupric ions catalyse dihydroxyfumaric acid oxidation by a mechanism that does not involve $O_2^{\cdot -}$. The inhibitory effects herein reported are in close agreement with the results found by Nakano and Obo (1979) on a

similar system. But, on the other hand, it is not surprising to find inhibition when zinc is used, because in the literature there is evidence that zinc shows inhibitory effects on lipid peroxidation (Chvapil, 1973). In general terms, zinc is interfering in some way with the attack of free radicals generated within the system, therefore the reaction sequence of lipid peroxidation is blocked or diminished. Chvapil, (1973) reported that the inhibition of the formation of MDA in liver microsomes by zinc was paralleled by a slower oxidation of NADPH and that the action is one of several relationships whereby zinc may interfere with the oxidation of unsaturated fatty acids. The protective effect of zinc may be better understood as a mechanism that stabilizes the cellular membranes making them more resistant to free radical damage. Willson, (1977) postulated that zinc within cells, may bind to critical thiol groups which normally bind iron (Scheme 1). Such a mechanism could equally apply to decompartmentalized iron and oxygen, in which case zinc inhibits the formation of the damaging superoxide radical (Scheme 2).



(193)

Scheme 1.



(194)

Scheme 2.

Assuming that the last mechanism could be occurring, then that might, in part, explain the action of $O_2^{\cdot -}$ in incubated microsomes.

There is some evidence about the effects of Ca^{2+} and Mg^{2+} on lipid peroxidation. Wills (1969a) reported that at concentrations of 10 mM (Mg^{2+} and Ca^{2+}) in the ascorbate/NADPH system the rate of lipid peroxidation was 140% and 3%, respectively. The results of (Table 9) show that Ca^{2+} at the quoted concentrations can protect the microsomal membrane from lipid peroxidation. Mg^{2+}, however, induced an activating effect. Peters and Fouts, (1970), concluded from the studies in vitro that magnesium activation of microsomal metabolism may be the result of an effect of magnesium in altering the microsomal membrane structure which, in turn, allows a more rapid interaction of the enzyme and substrate, thereby enhancing the flow of electrons from the electron-donor through several of the various chain components to the substrate. The inhibitory effects of Co^{2+} may be linked with complex formation with the aminoacids present in the surrounding medium; in fact high stability constants of cobalt-glycine; cobalt-valine; and cobalt-histidine were reported by (Martell and Calvin, 1956). The MDA values found for these metals in the microsomes incubated with dihydroxyfumaric acid are in close agreement with those reported on peroxidised microsomes (Nakano and Obo, 1979; Wills, 1969a). The catalytic effect of Ni^{2+} in promoting lipid peroxidation seems to be known; in fact, Hasan and Ali (1981), studied the effects

of Ni^{2+} at inducing lipid peroxidation in preparations of different regions of the rat brain.

The results obtained for Mn^{2+} gave strong evidence of inhibition of MDA production induced by Fe^{2+}/dihydroxy-fumaric acid on liver microsomes. A similar antioxidant effect of Mn^{2+} has been described by (Inouye, et al., 1978; 1980), when inhibition of the actions of sulphite radicals in lung lipid extracts, liver mitochondria and microsomes with additions of Mn^{2+} was achieved. Also, the inhibitory effect of Mn^{2+} on lipid peroxidation of corn oil induced by sulfite was studied by Kaplan, et al., (1975). More recently, Stacey and Klaassen, (1981), showed the interactions between Cd and other metal ions in rat hepatocytes producing lipid peroxidation inhibited by Mn^{2+} and Zn^{2+}. Radomski and Wood, (1970), studied the protective effect of Mn^{2+} and Zn^{2+} against exposure to hyperbaric O_2, and Robinson, (1965), mentioned the antioxidant action of $CoCl_2$ and $MnCl_2$ on microsomes peroxidised by ascorbate. All the cases mentioned are proof of the antioxidant effect that Mn^{2+}, Co^{2+} and Zn^{2+} can produce on systems affected by the oxidant action of ascorbate, heavy metal toxic ions, etc. Given these results, it was thought pertinent to examine in detail the inhibitory action of Mn^{2+} on microsomal lipid peroxidation stimulated by Fe^{2+}/dihydroxyfumaric acid. The results obtained in Table 10 suggest that the inhibitory action of Mn^{2+} can occur because: first, Mn^{2+} acts as an effective scavenger of $O_2^{\cdot -}$ and blocks reaction (138),

therefore the subsequent reactions must be blocked and hence the action of free radicals species generated is abolished; the second alternative is that Mn^{2+} by a displacement mechanism may be occupying the place of Fe^{2+} within the system and inhibiting the subsequent initiation and propagation reactions. Both processes may occur simultaneously, which would give support for the good inhibitory effects of Mn^{2+}. Finally, there is evidence in the literature of the inhibitory effects of Mn^{2+} on different systems stimulating lipid peroxidation in microsomes. The listed values in Table 16 show the effectiveness of Mn^{2+}:

Table 16: Antioxidant effect of Mn^{2+} on lipid peroxidised microsomes. (Taken from Slater, 1979.)

Model system studied	Mn^{2+}	
	Effect (%)	Concentration (µmol/l)
NADH/PMS/NBT	50	0.1
NADPH-lipid peroxidation	46	126
NADPH-ADP/Fe^{2+} - lipid peroxidation	44	2
CCl_4-stimulated lipid peroxidation	42	126

Considering that in the four systems used the formation of $O_2^{\cdot -}$ is involved and since Fe^{2+} participates as a component of the pro-oxidative system NADPH-ADP/Fe^{2+}, it

can be suggested that there is a common mechanism of antioxidant action of Mn^{2+} between these and Fe^{2+}/dihydroxyfumaric acid system.

Extrapolating the results obtained to an *in vivo* model, most metals do not exist solely in the free ionic state but are frequently complexed with aminoacids, proteins or other special molecules such as the porphyrins. The experiments here have established that in the range of normal physiological pH and temperature, the oxidation of unsaturated fatty acids of membranes could be catalysed by certain metals and thus, *in vivo*, non-enzymatic, metal catalysed formation of lipid peroxides may be possible. Nevertheless, it is probable that there are special sites within cells where the local concentration of metal is much greater than of the average for the whole tissue. The activity of these catalysts will vary from tissue to tissue and from one subcellular location to another and will depend on the localised concentration of catalyst, concentration of reducing agent, pH and on certain other factors.

C H A P T E R 5

C O N C L U S I O N S

Using the Brunel pulse radiolysis facilities, it has been shown that dihydroxyfumaric acid can readily enter into free radical reactions. Many of the rate constants obtained for the experiments done are new and not yet published, others are in good agreement with the results previously reported. The properties of ABTS and its radical cation, particularly its high extinction coefficient at 414 nm, appeared to make it suitable as a reference solute to determine the rate constants of the reaction of $OH^{\cdot}$, $N_3^{\cdot}$ and NO_2 with dihydroxyfumaric acid. The p-nitroacetophenone (PNAP) proved to be a convenient marker to follow the reactions of isopropanol radical with thiol compounds and iron complexes chelates. The findings on the reactions of halogenated peroxy radicals with β-carotene can be relevant for understanding the protective action of carotenoids, in vivo. The kinetics values reported here might differ substantially from those found in vivo.

The spontaneous autoxidation of dihydroxyfumaric acid in solution makes the mechanism of free radicals reactions where dihydroxyfumaric acid is involved, under circumstances of different dose rates, pH and oxygen conditions, difficult to follow. Such high G-values found are not common in radiation chemistry. This feature means that during the destruction of dihydroxyfumaric acid, free radical chain reactions may result in the decomposition of a number of molecules of the starting material from a

single free radical step. The use of absorption spectroscopy for the radical detection at room temperature is limited to the study of unstable free radicals like those from dihydroxyfumaric acid, and is not suitable by normal spectroscopy at room temperature; therefore the applicability of very fast techniques as pulse radiolysis increases the probability of improving our understanding of the reactions of free radicals with very short life.

The value of lipid peroxidation studies with incubated microsomes has allowed the rapid evaluation of the inhibitory capacity of compounds which can be antioxidants or effective free radicals scavengers. The results herein obtained show that a variety of drugs, such as promethazine, propyl gallate and HDC at the quoted concentrations had different abilities to protect the microsomal membrane against free radical attack. The binding ability of chelating agents was important to demonstrate the importance of iron ion to catalyse reactions of free radicals. Also, the inhibitory effects of Mn^{2+} added in the system Fe^{2+}/dihydroxyfumaric acid to induce lipid peroxidation on microsomes is important to understand the role and interactions of metal ions at cellular level.

From the evidences found, it is evident that dihydroxyfumaric acid is a useful source to generate a sustained flux of $O_2^{\cdot -}$. Authors such as Fischer and Hamburger, (1981), and Autor, et al., (1980) have selected dihydroxyfumaric acid to demonstrate the importance of

free radical damage to cells and tissue injury and the role of superoxide dismutase and catalase to prevent the toxic effect of the free radicals generated from dihydroxyfumaric acid.

Finally, it is important to point out that the lipid peroxidation model on incubated microsomes with agents capable to induce lipid peroxidation is a key to extrapolate results on in vivo systems when drugs with clinical use produce undesirable effects, for instance, the anesthetics chloroform, halothane (Ugazio, 1978), or aminopyrine (Ries and Sahud, 1975), among others.

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REFERENCES

Adams, G.E., Boag, J.W., Currant, J., and Michael, B.D. (1965a) in: Pulse Radiolysis. Eds.- M. Ebert, J.P. Keene, A.J. Swallow and J.H. Baxendale. Academic Press, New York and London. 117 - 129

Adams, G.E., Boag, J.W., Currant, J., and Michael, B.D. (1965b) in: Pulse Radiolysis. Eds.- M. Ebert, J.P. Keene, A.J. Swallow and J.H. Baxendale. Academic Press, New York and London. 131 - 143

Adams, G.E., McNaughton, G.S., Michael, B.D. (1967). Chem. of Ionisation and Excitation. Eds.- G.R.A. Johnson, G. Scholes, Taylor and Francis. 281 - 293

Adams, G.E., McNaughton, G.S., and Michael, B.D. (1968). Trans. Faraday Soc. 64, 902 - 910

Adams, G.E. (1972) in: Advances in Radiation Chemistry. Eds.- M. Burton and J.L. Magee. Wiley and Sons, London. 3, 125 - 208

Adams, G.E., Aldrich, J.E., Bisby, R.H., Cundall, R.B., Redpath, J.L. and Willson, R.L. (1972). Radiat.Res. 49, 278 - 289

Adams, G.E. and Willson, R.L. (1973). J. Chem. Soc., Faraday Translations I, 69, 719 - 729

Adrien, A. (1973). Metal-Binding Substances in: Selective Toxicity. The physico-chemical basis of therapy. Fifth Edition. Chapman and Hall, Ltd., London. 365

Alper, T., Hodgkins, B., (1969). Mutat. Research 8(1), 15 - 23

Allen, A.O. (1961) in: The Radiation Chemistry of Water and Aqueous Solutions. D. van Nostrand Company Inc., Princeton, New Jersey, Toronto, London, New York. 21

Anbar, M., Bambenek, M. and Ross, A.B. (1973). Selected specif rates of reactions of transient form of water in aqueous solution. I. Hydrated Electron, NSRDS-NBS, 43, Washington, D.C.

Anderson, R.F. and Patel, K.B. (1978). Photochem. Photobiol., 28, 881 - 885

Ashley, P.L. and Griffin, B.W. (1980). Mol. Pharmac. 19, 146 - 152

Autor, A.P., McLennam and Fox. A.W. (1980). oxygen free radicals generated by dihydroxyfumarate and ionizing radiation: Cytotoxic effect on isolated pulmonary macrophages: in: Molecular Basis of Environmental Toxicity, Ed.- Bhatnagar, R.J., Ann Arbor Press, Ann Arbor. 51 - 67

Babior, B.M., Kipnes, R.S. and Curnutte, J.T.(1973).J.Clin. Invest., 52, 741 - 744

Bahnemann, D. (1981). Ph.D. thesis, Technical University, Berlin.

Barber, A.A., and Bernheim, F.(1967) in: Advances in Gerontological Research, Ed.- B.L. Strehler, Academic Press, New York. 2, 355 - 403

Baxendale, J.H., Bevan, P.L.T., Scott, D.A. (1968). Trans. Faraday Soc., 64, 2389 - 2397

Bielski, G.H.J. and Allen, A.O. (1977), J. Phys. Chem., 81, 1048 - 1050

Bindoli, A. and Cavallini, L. (1980). Molec.Biology.Med. 5, 1 - 8

Burgoin, E. (1874). Compt. rend., 79, 1053

Bus, J.S. and Gibson, J.E. (1979). Rev. Biochem. Toxicol. 1, 125 - 149

Cercek, B., Ebert, M. and Swallow, A.J. (1964) Science, 145, 919 - 920

Cohen, G. and Heikkila, R.E. (1974). J.Biol.Chem. 249, 2447 - 2452

Cohen, G. and Sinet, P.M. (1981). oxygen consumption during a Fenton-type Reaction between hydrogen peroxide and an Iron-chelate Fe(II)-DTPA. In oxygen and oxy-radicals in chemistry and biology. Eds.- M.A.J. Rodgers and E.L. Powers. Academic Press. 45 - 54

Czapski, G. and Ilan, Y.A. (1978). Photochem. Photobiol. 28, 651 - 653

Chance, B., Sies, H. and Boveris, A. (1979). Physiol.Rev. 59, 527 - 605

Cheeseman, K.H. (1982). in: Free Radicals, Lipid Peroxidation and Cancer. Eds.- D.C.H. McBrien and T.F. Slater. Academic Press. 197 - 214

Chow, C.K. and Tappel, A.L. (1972). Lipids. 7, 518 - 524

Chvapil, M. (1973). Life Sciences, 13, 1041 - 1049

Daley, R.D. (1972). in: Analytical Profiles of Drug Substances. Ed.- K. Florey. Academic Press, New York and London, 1, 119

Dawe, E.A. and Land, E.J. (1975). J.C.S. Faraday I, 71, 2162 - 2169

de Kruijff, B., Cullis, P.R. and Verkleij, A.J. (1980). TIBS. 79 - 81

Del Maestro, R.F. (1979). Diss. Acta Universitatis Upsaliensis, Uppsala, Abstr. 340

Del Maestro, R.F. (1980). Acta Physiol. Scand., Suppl. 492, 153 - 168

Demopoulos, H.B. (1973). Fed.Proc., 32, 1859 - 1861

Dewhurst, H.A. (1952). Trans.Faraday. Soc., 48, 905 - 911

Dianzani, M.U. and Ugazio, G. (1978) in: Biochemical Mechanisms of Liver Injury. Ed.- T.F. Slater. Academic Press, Chapter 18, 669 - 707

Di Luzio, N.R. and Hartman, A.D. (1967). Federation Proc. 26, 1436

Di Luzio, N.R. (1973). Fed.Proc. 32, 1875 - 1881

Donovan, D.H. and Menzel, D.B. (1978). Experientia (Basel). 34, 775 - 776

Dorfman, L.M. and Adams, G.E. (1973). Reactivity of hydroxyl radicals in aqueous solutions, NSRDS-NBS, 46, Washington, D.C.

Eichenberger, K., Böhni, P., Winterhalter, K.H., Kawato, S. and Richter, Ch. (1982). Febs Letters. 142, 59 - 62

Esterbauer, H. and Slater, T.F. (1981). IRCS, Medical Science, 9, 749 - 750

Fee, J.A. and Teitelbaum, H.D. (1972). Biochem. Biophys. Res. Comm., 49, 150 - 158

Fenton, H.J.H. (1894). J. Chem. Soc., 65, 899

Fenton, H.J.H., (1895). Brit. Assoc. Advance Sci. Rep., 663

Fenton, H.J.H. (1896). J. Chem. Soc., 69, 546

Fenton, H.J.H. (1898). ibid., 73, 78

Fenton, H.J.H. and Wilkis, W.A.R. (1912), ibid., 101, 1570

Fisher, L.J. and Hamburger, S.A. (1981). Endocrinology. 108, 2331 - 2335

Flamm, E.S., Demopoulos, H.B., Seligman, M.L., Tomasula, J.J., De Crescito, V. and Ransohoff (1977). J. Neurosurg. 46, 328 - 335

Flamm, E.S., Demopoulos, H.B., Seligman, M.L., Mitamura, J.A., Ransohoff, J. (1979) in: Neural Trauma. Eds.- Popp, A.J., Bourke, R.S., Nelson, L.R.R., New York. 289 - 296

Fong, K.L., McCay, P.B., Poyer, J.L., Keele, B.B. and Misra, H. (1973). J. Biol. Chem., 248, 7792 - 7797

Forni, L.G., Monig, J., Mora-Arellano, V.O. and Willson, R.L. (1983). J. Chem. Soc., Perkin Trans. 2, in press.

Franke, W., and Brathun, G. (1931). Liebigs Ann., 487, 1

Fricke, H. and Hart, E.J. (1966) in: Radiation Dosimetry. Eds.- F.H. Attix and W.C. Roesch. Academic Press, New York

Fridovich, I. (1974). New Engl. J. Med. 290, 624 - 625

Fridovich, I. (1978). Photochem. Photobiol. 28, 733 - 741

Fujimaki, M. and Morita, M. (1968). Agric. Biol. Chem., Tokyo, 32, 574

Fukuzawa, K., Chida, H., Tokumura, A. and Tsukatani, H. (1981). Arch. Biochem. Biophys., 206, 173 - 180

Goldberg, B. and Stern, A. (1976). Biochem. Biophys. Acta. 437, 628 - 632

Goldberg, B. and Stern A. (1977a). Arch. Biochem. Biophys. 178, 218 - 225

Goldberg, B. and Stern A. (1977b). Acta. Biol. Med. Cer., 36, 731 - 734

Goldstein, B.D. and Balchum, O.J. (1967). Proc. Soc. Exptl. Biol. Med., 126, 356 - 358

Goldstein, B.D., Buckley, R.D., Cárdenas, R., Balchum, O.J., (1970). Science, 605 - 606

Gratzel, V.M., Henglein, A. and Taniguchi, S. (1970). Pulsradiolytische Beobachtungen usw. Bd. 74 Nr.3, 292 - 298

Griffin, B.W. (1977). Febs Letters. 74, 139 - 143

Gupta, M.P. (1953). J. Amer. Chem. Soc., 75, 6312 - 6313

Gutteridge, J.M.C., Richmond, R. and Halliwell, B. (1979) Biochem. J. 184, 469 - 472

Haber, F. and Weiss, J. (1934). Proc. R. Soc. A., 147, 332 - 351

Halliwell, B. and Ahluwalia, S. (1976). Biochem J., 153, 513 - 518

Halliwell, B. (1977). Biochem. J. 163, 441 - 448

Halliwell, B. and De Rycker, J. (1978). Photochem. Photobiol., 28, 757 - 763

Hamberg, M. and Samuelsson, B. (1974). Proc. Nat. Acad. Sci., 71, 3400 - 3404

Hardwick, T.J. (1952). Can. J. Chem., 30, 17

Hart, E.J. and Anbar, M. (1970). The Hydrated Electron, Wiley Interscience, N.Y.

Hartree, E.F. (1953). J. Amer. Chem. Soc., 75, 6244 - 6249

Hartroft, W.S. (1965) in: Metabolism of lipids as related to atherosclerosis. Ed.- F.A. Kummerow, Springfield: Thomas, 18

Hasan, M.,and Ali, F.S. (1981). Toxicol.Appl.Pharmacol., 57, 8 - 13

Haugaard, N. (1968). Physiol. Rev., 48, 311 - 373

Hay, R.W. and Harvie, S.J. (1965). Aust. J. Chem., 18, 1197 - 1209

Hayon, E. and Simic, M. (1970). J. Amer. Chem. Soc., 92, 7486 - 7487

Heaton, F.W. and Uri, N. (1961). J. Lipid Res., 2, 152 - 160

Hochanadel, C.J. (1952). J. Phys. Chem., 56, 587 - 594

Holman, R.T. (1954) in: Progress in the Chemistry of Fats and other Lipids. Eds.- R.T. Holman, W.D. Lundberg, and T. Malbin. Pergamon, London. 2, 51

Hunter, E.E., Scott, A., Hoffsten, P.E., Gebicki, J.M., Weinstein, J. and Scheneider, A. (1964). J. Biol. Chem., 239, 614 - 621

Inouye, B., Ikeda, M., Ishida, T., Ogata, M., Akiyama, J. and Utsumi, K. (1978). Toxicol. Appl. Pharmacol., 46, 29 - 38

Inouye, B., Morita, K., Ishida, T. and Ogata, M. (1980). Toxicol. Appl. Pharmacol., 53, 101 - 107

Johnson, G.R.A., Scholes, G. and Weiss, J. (1951). Science, 114, 412

Johnson, G.R.A., Scholes, G. and Weiss, J. (1953). J. Chem. Soc., 3091 - 3099

Kaplan, D., Mcjilton, C. and Luchtel, D. (1975). Arch. Environ. Health, 30, 507 - 509

Kaufman, R.E., Balcerzak, S.P. and Metz, E.N. (1973). Clin. Res., 21, 558

Kellogg,E.W., Fridovich, I. (1977). J. Biol. Chem., 252, 6721 - 6728

Kimball, R.E., Reddy, K., Peirce, T.H., Schwartz, L.W., Mustafa, M.G. and Cross, C.E. (1976). Am. J. Physiol., 230, 1425 - 1431

King, M.M., Lai, E.K. and McCay, P.B. (1975). J. Biol. Chem., 250, 6496 - 6502

Klein, N.A. and Toppen, D.L. (1978). J. Am. Chem. Soc., 100, 4541 - 4543

Kornbrust, D.J. and Mavis, R.D. (1980). Mol. Pharmacol., 17, 400 - 407

Koster, J.F. and Slee, R.G. (1980). Biochem. Biophys. Acta. 620, 489 - 499

Krinsky, N.I. (1974). Science, 186, 363 - 365

Kunimoto, M., Inoue, K. and Nojima, S. (1981). Biochem. Biophy. Acta., 646, 169 - 178

Laroff, G.P. and Fessenden, R.W. (1971). J. Chem. Phys., 55, 5000

Leipzing, R.M., Brewer, G.J. and Kruckeberg, W.C. (1975). Prog. Clin. Biol. Res., 1, 711

Lown, J.W. and Soo, K.S. (1977). Biochem, Biophys, research commun., 77, 1150 - 1157

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randal, R.J. (1951), J. Biol. Chem., 193, 265 - 275

Lynch, R.E. and Fridovich, I. (1978), J. Biol. Chem., 253, 1838 - 1845

Mahood, J.S., Packer, J.E., Searle, A.J.F., Willson, R.L. and Wolfenden, B.S. (1980) in: Phenothiazines and Structurally Related Drugs: Basic and Clinical Studies. Eds.- E. Usdin, H. Eckert and I.S. Forrest, Elsiever, Amsterdam, 99 - 102

Malvy, C., Paoletti, C., Searle, A.J. and Willson, R.L. (1980). Biochem. Biophys. Research Commun., 95, 734 - 737

Martell, A.E. and Calvin, M. (1956). Chemistry of the Metal Chelate Compounds, Prentice Hall, N.J., 514

Mathews, M.M. (1964), Nature, London. 203, 1092

May, H.E. and McCay, P.B. (1968). J. Biol. Chem., 243, 2296 - 2305

McCay, P.B. and Poyer, J.L. (1976) in: the Enzymes of Biological Membranes., Ed.- A. Martonosi, J. Wiley, New York, 239 - 256

McCord, J.M. (1974), Science, 185, 529 - 531

McGinness, J.E., Proctor, P.H., Demopoulos, H.B. Hokanson, J.A. and Kirkpatrick, D.S. (1978), J. Physiol. Chem. Phys., 10, 3

Mead, J.F. (1952), Science, 115, 470

Michelson, A.M. and Durosay, P. (1977), Photochem. Photobiol., 25, 55 - 63

Mills, G.C. (1957), J. Biol. Chem., 229, 189 - 197

Mora-Arellano, V.O., Wolfenden, B.S. and Willson, R.L. (1983), Repair of alcohol free radicals by hydrogen transfer from sulphydiyl compounds: Determination of absolute rate constants by pulse radiolysis (To be published).

Nakano, K. (1978). Acta. Med. Uni. Kayoshima, 20, 119 - 133

Nakano, K. and Obo, F. (1979), Biochem. Biophys, Res. Commun., 89, 1239 - 1244

Neta, P. and Fessenden, R.W. (1972), J. Phys. Chem., 76, 1957 - 1961

Nelson Research and Development Company (1979), Demande de brevet d'invention, No. 77 19483. No. de publication 2395034. Republique Francaise. Institut national de la propietie industrielle, Paris, 21

O'Brien, P.J. (1969). Can. J. Biochem, 47, 482 - 485

Orrenius, S., Dallner, G. and Ernster, L. (1964), Biochem. Biophys. Res. Commun., 14, 329

Packer, L., Deamer, D.W. and Heath, R.L. (1967). in: Advances in Gerontological Research, Ed.- B.L. Strehler, New York, Academic, 2, 77

Packer, J.E., Slater, T.F. and Willson, R.L. (1978). Life Sciences, 23, 2617 - 2620

Packer, J.E., Willson, R.L., Bahnemann, D. and Asmus, K-D, (1980). J. Chem. Soc., Perkin II, 296 - 299

Packer, J.E., Mahood, J.S., Mora-Arellano, V.O., Slater, T.F. and Willson, R.L. (1981). Biochem. Biophys. Research Commun., 98, 901 - 906

Packer, J.E., Mora-Arellano, V.O. and Willson, R.L. (1983) (To be published)

Pederson, T.C. Buege, J.A. and Aust, S.D. (1973). J. Biol. Chem., 248, 7134 - 7141

Pederson, T.C. and Aust, S.D. (1975). Biochem. Biophys. Acta., 385, 232 - 241

Pelizzetti,E., Meisel, D., Mulac, W.A. and Neta, P. (1979), J. Am. Chem. Soc., 101, 6954 - 6959

Perkins, E.G., Joh, T.H. and Kummerow, F.A. (1965). in: Metabolism of Lipids as related to atherosclerosis., Ed.- F.A. Kummerow, Springfield. Thomas, 48

Peters, M.A. and Fouts, J.R. (1970). J. Pharmacol. Exp. ther., 173, 233 - 241

Plaa, G.L. and Witschi, H. (1976). Ann. Rev. Pharmacol., 16, 125 - 141

Porter, D.J.T. and Ingraham, L.L. (1974). Biochem. Biophys, Acta., 334, 97 - 102

Pryor, W.A. (1973), Fed. Proc. Fed. Am. Soc. Exp. Biol., 32, 1862 - 1869

Pryor, W.A. (1976) in: Free Radicals in Biology, Ed.- W.A. Pryor, Academic Press, London, 1, 1 - 49

Quintiliani, M., Badiello, R., Tamba, M., Esfandi, A. and Gorin, G. (1977), Int. J. Radiat. Biol., 32, 195 - 202

Radomski, M.W. and Wood, J.D. (1970), Aerosp. Med., 41, 1382 - 1387

Recknagel, R.O. (1967), Pharmacol. Rev., 19, 145 - 208

Redpath, J.L. (1973), Rad. Res., 54, 364 - 374

Redpath, J.L. and Willson, R.L. (1973), Int. J. Radiat. Biol., 23, 51 - 65

Richter, C., Wendel, A., Wesser, U., and Azzi, A. (1975), Febs. Letters., 51, 300

Ries, C.A., Sahud, M.A. (1975), J. Am. Med. Ass., 231, 352 - 355

Robinson, J.D. (1965), Arch. Biochem. Biophys, 112, 170 - 179

Romer, K.G., Kamal, A.M., Freundt, K.J. (1981). Proceedings of the Symposium on Chemical Indices and Mechanisms of organ-directed toxicity, Barcelona, Spain, 4-7 March, 1981. U.K. Pergamon Press Ltd., Headington Hill, Hall, Oxford, England, 205 - 210

Rouser, G.G., Nelson, J., Fleisher, S. and Simmon, G. (1968) in: Biological Membranes, Ed.- D. Chapman, Academic Press, N.Y., 5

Ruzicka, F.K., Beinert, H., Schepler, K.L., Dunham, W.R. and Sands, R.H. (1975), Proc. Nat. Acad. Sci., 72, 2886 - 2890

Sachs, C. and Jonsson, G. (1975), Biochem. Pharmacol., 24, 1 - 8

Saslaw, L.D., Corrwin, L.M. and Walavdekar, V.S. (1966), Arch. Biochem. Biophys, 114, 61 - 66

Searle, A.J.F. (1981), Ph.D. thesis, Brunel University.

Searle, A.J.F. and Tomasi, A. (1982), J. Inorg. Biochem., 17, 161 - 166

Seeman, P.J., Weinstein. (1966), Biochem. Pharmac., 15, 1737

Seligman, M.L. and Demopoulos, H.B. (1973), Ann. N.Y. Acad. Sci., 222, 640 - 667

Serfass, R.E. and Ganther, H.E. (1976), Life Sci., 19, 1139 - 1144

Sinnhuber, R.O. and Yu, T.C. (1958). Fd. Res., 23, 626 - 634

Slater, T.F. and Riley, P.A. (1966), Nature, London, 209, 151 - 154

Slater, T.F. (1972). Free Radicals Mechanisms in Tissue Injury, Pion Ltd., London.

Slater, T.F. (1979) in: Oxygen Free Radicals and Tissue Damage, CIBA Foundation Symposium 65 (New Series), Excerpta Medica, Amsterdam, 143 - 176

Sogo, P.B., Pon, N.G. and Calvin, M. (1957), Proc. Natl. Acad. Sci., 43, 387

Stacey, F.W. and Harris, J.F. (1963).in: Organic Reactions, Eds.- R.A. Adams, A.H. Blatt, V. Boekeide, T.L. Cains, A.C. Cope, D.Y. Curtin and C. Niemann, J. Wiley. New York, 14, 91

Stacey, N.H., Klaassen, C.D. (1981). J. Toxicol. Environm. Health, 7, 149 - 158

Stein, G. (1971). Israel J. Chem., 9, 413 - 418

Stier, A. (1968). Anesthesiology, 29, 388

Sutherland, M.W. and Gebicki, J.M. (1982), Arch. Biochem. Biophys., 214, 1 - 11

Sutton, H.C., Adams, G.E., Boag, J.W., Michael, B.D. (1965), in: Pulse Radiolysis. Eds.- M. Ebert, J.P. Keene, A.J. Swallow, J.H. Baxendale. Academic Press, 61 - 81

Svingen, B.A., Buege, J.A., O'Neal, F.O. and Aust, S.D. (1979). J. Biol. Chem., 254, 5892 - 5899

Swern, D. (1961).in: Autoxidation and Antioxidants, Ed.- W.O. Lundberg. Wiley Inter-science, New York. 1

Takala, I., Niinikoski, J. (1974). Acta Chir. Scand. 140(3), 167 - 170

Tappel, A.L. (1961).in: Autoxidation and Antioxidants, Ed.- W.O. Lundberg. Wiley Inter-science, New York. 325

Tappel, A.L. (1973). Fed. Proc., 32, 1870 - 1874

Thomas, J.K., Rabani, J., Matheson, M.S., Hart, E.J. and Gordon, S. (1966). J. Phys. Chem., 70, 2409 - 2410

Thomas, H.V., Mueller, P.K. and Lyman, R.L. (1967). Science, 159, 532 - 534

Ugazio, G. (1978).in: Biochemical Mechanisms of Liver Injury, Ed.- T.F. Slater, Academic Press, London, New York and San Fco., Chapter 19, 709 - 743

Van Dyke, R.A. and Chenoweth, M.B. (1965). Biochem. Pharmac., 14, 603

Walling, C. (1975). Acc. Chem. Res., 8, 125 - 131

Walling, C. and Huyser, E.S. (1963).in: Organic Reactions, Eds.- R. Adams, A.H. Blatt, V. Boekeide, T.L. Cains, A.C. Cope, D.Y. Curtin and C. Niemann. J. Wiley, New York, 13, 91

Walling, C., Partch, P.E. and Weil, T. (1975). Proc. Natl. Acad. Sci., 72, 140

Wilbur, K.M., Bernheim, F., Shapiro, O.W. (1949). Arch. Biochem. Biophys., 24, 305 - 313

Williams, M.L., Shott, R.J., O'Neal, P.L., Oki, F.A. (1975). New Engl. J. Med., 292, 887 - 890

Wills, E.D. (1965). Biochem. Biophys. Acta., 98, 238 - 251

Wills, E.D. (1966). Biochem. J., 99, 667 - 676

Wills, E.D. (1969a). Biochem. J., 113, 315 - 324

Wills, E.D. (1969b). Biochem. J., 113, 325 - 332

Wills, E.D. (1969c). Biochem. J., 113, 333 - 341

Willson, R.L. (1970). Int. J. Radiat. Biol., 17, 349

Willson, R.L. (1971). Trans. Faraday Soc., 67, 3008 - 3019

Willson, R.L., Greenstock, C.L., Adams, G.E., Wageman R. and Dorfman, L.M. (1971). Int. J. Radiat. Phys. Chem., 3, 211

Willson, R.L. (1977). in: Iron Metabolism. Ciba Foundation Symposium 51 (New Series). Published by Elsevier/Excerpta Medica/North-Holland and Elsevier/North-Holland, Inc. 331 - 354

Willson, R.L. (1978). in: Biochemical Mechanisms of Liver Injury, Ed.- T.F. Slater, Academic Press, 123 - 224

Wolfenden, B.S. (1982). Ph.D. thesis, Brunel University.

Wolfenden, B.S. and Willson, R.L. (1982). J. Chem. Soc. Perkin Trans.II, 805 - 812

Yamazaki, I., Mason, H.S. and Piette, L. (1960). J. Biol. Chemi., 235, 2444 - 2449

Yamazaki, I. and Piette. L.H. (1963). Biochem. Biophys. Acta., 77, 47 - 64

Yu, W.H.A., Yu, M.C., and Young, P.A. (1974). Exp. Molec. Path., 21, 289

APPENDIX

The calculations performed for the different results in this work were obtained directly from the calculator, applying the following procedure:

Statistic	Evaluated as
Sample standard deviation	σ_{n-1}
Population standard deviation	σ_n
Arithmetic mean	$\bar{x}$
Number of data	n
Sum of value	Σx
Sum of square value	Σx^2

The sample standard deviation σ_{n-1} was derived as follows:

$$\sigma_{n-1} = \sqrt{\frac{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}{n-1}}$$

The population standard deviation (σ_n) was defined as:

$$\sigma_n = \sqrt{\frac{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}{n}}$$

and to the arithmetic mean $\bar{x}$ was obtained as:

$$\bar{x} = \frac{\Sigma x}{n}$$

All lipid peroxidation results obtained in this work are the average of no less than an individual experiment in triplicate, and each value is plotted on a graph or listed in Tables.