Oxidative Stress, Glycation and Transition Metals in Diabetic Complications

by

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To my wife Qionglin, my son Hanyu and my parents

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ABBREVIATIONS

AGE	=	Advanced Glycation Endproducts
ARIs	=	Aldose Reductase Inhibitors
ATP	=	Adenosine triphosphate
BCA	=	2,2'-bicinchoninic acid
BHT	=	Butylated hydroxytoluene
BuOOH	=	t-butyl hydroperoxide
CML	=	Carboxymethylysine
CMhL	=	Carboxymethylhydroxylysine
CuOOH	=	Cumene hydroperoxide
DMEM	=	Dulbecco's Modification of Eagles Medium
DNA	=	Deoxyribonucleic acid
DTPA	=	Diethylenetriaminepenta-acetic acid
EDDA	=	Ethylenediaminediacetic acid
EDTA	=	Ethylenediaminetetraacetic acid
FOX	=	Ferrous Oxidation -Xylenol orange
FOXRS	=	FOX-reactive substances
GC-MS	=	Gas chromatography-mass spectrometry
GSH	=	Glutathione (reduced form)
GSHPx	=	Glutathione peroxidase
GSSG	=	Glutathione (oxidized form)
HbA_{1c}	=	Glycated haemoglobin
HDL	=	High density lipoprotein
H_2O_2	=	Hydrogen peroxide
$HO^{\cdot}$	=	Hydroxyl radical
HPLC	=	High-performance liquid chromatography
LDL	=	Low density lipoprotein
$NaCNBH_3$	=	Sodium cyanoborohydride
NAD	=	Oxidized nicotinamide-adenine dinucleotide
NADPH	=	Reduced nicotinamide-adenine dinucleotide phosphate
NTA	=	Nitrilotriacetic acid
$O_2^{-\cdot}$	=	Superoxide anion
OP	=	Orthophenanthroline
PBS	=	Phosphate buffered saline
PMNs	=	Polymorphonuclear leukocytes
$R^{\cdot}$	=	Alkyl radical
$RO^{\cdot}$	=	Alkoxyl radical
$ROO^{\cdot}$	=	Peroxyl radical
ROOH	=	Lipid hydroperoxide
SOD	=	Superoxide dismutase
STZ	=	Streptozotocin
TBA	=	Thiobarbituric acid
TBARS	=	TBA-reactive substances
TMG	=	Tetramethyleneglutarate
VLDL	=	Very low density lipoprotein
WHHL	=	Watanabe heritable hyperlipidemic

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ABSTRACT

This thesis discusses the role of transition metal catalysed oxidative reactions in the development of diabetic complications and ageing through *in vitro* studies. The possible relationship between metal-dependent oxidative stress and non-enzymatic glycosylation (glycation), as well as polyol pathway metabolism, is also discussed. The experimental research reported in this thesis includes the following:

1). A simple and sensitive method for the direct measurement of peroxides is described. The method is based on the principle of the rapid peroxide-mediated oxidation of Fe^{2+} to Fe^{3+} under acidic conditions. The latter, in the presence of xylenol orange, forms a Fe^{3+}-xylenol orange complex which can be measured spectrophotometrically at 560nm. By pre-treatment with enzymes known to metabolize peroxides, it has been shown that the assay measures hydrogen peroxide (H_2O_2) and lipid hydroperoxides specifically. The Ferrous Oxidation/Xylenol orange (FOX) assay has been validated in the study of H_2O_2 generation during glucose "autoxidation", as well as lipid peroxidation of low density lipoprotein (LDL), phosphatidyl choline liposomes and red blood cell membranes.

2). The interaction between glycation and peroxidation in determining the toxicity of *in vitro* glucose-modified LDL to a fibroblast cell line is examined. Such modified LDL is cytotoxic, but this is determined primarily by the extent of metal-catalysed peroxidation of the lipoprotein, and that glycation may enhance (by an unknown mechanism) the cytotoxic effect of oxidized LDL.

3). Some experimental inhibitors (sorbinil, Al-1576 and ONO-2235) of the enzyme aldose reductase (implicated in diabetes mellitus via its ability to catalyse glucose reduction to sorbitol) are potent inhibitors of transition metal-catalysed ascorbate oxidation, and inhibit H_2O_2 formation by monosaccharide "autoxidation". This inhibition appears to be dependent upon the presence of a spirohydantoin group and/or similar structure. Furthermore, these compounds can also inhibit copper/ascorbate induced cytotoxicity in fibroblasts as judged by thymidine incorporation. It is conceivable that the copper and iron-binding capacity of these compounds may contribute to some of their observed biological effects and may provide a starting point for a new generation of experimental drugs for the treatment of diabetic complications.

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CHAPTER 1 GENERAL INTRODUCTION

1. 1. Diabetic complications and ageing

Diabetes Mellitus is a complex syndrome resulting from both genetic and environmental factors. It is characterized by chronic hyperglycaemia, absolute or relative deficiency of insulin, and disordered carbohydrate, lipid and protein metabolism. It is also associated with the development of various complications. Although it has been recognized for at least 3000 years, it is still imperfectly understood. According to current concepts, diabetes can be subdivided into two broad groups: Type I (Insulin Dependent Diabetes Mellitus, IDDM) and Type II (Non-Insulin Dependent Diabetes Mellitus, NIDDM).

Diabetic complications include 1) microangiopathy, such as retinopathy and nephropathy, 2) macroangiopathy, i.e. accelerated atherosclerosis and 3) various other complications including cataract, neuropathy and increased tendency to infection.

According to epidemiological studies in Americans, approximately 40% of the elderly have significant alterations in carbohydrate tolerance [Lipson 1986]. Although type I diabetes are predominantly "juvenile-onset", over 10% of those aged over 65 require the treatment of insulin [Kilvert 1984]. About 70% to 80% of diabetes are type II diabetes in Westen countries and the prevalence of this type diabetes clearly increases with age. In those between the ages of 30 and 50, the prevalence is about 3% to 5%. It increases to approximately 10% by the age of 60 years and about 16% to 20% of those 80 years old [Lipson 1986; Bennett 1984]. Because of its marked increase in prevalence with ageing, diabetes also represents a unique systemic chronic disease with which to study the elderly.

1. 2. Hypotheses about the development of diabetic complications

The cause of the diabetic complications is still an open question.

Since hyperglycaemia is the common symptom in diabetes, it is suggested that the elevated blood glucose is associated with the development of diabetic complications [Pirart 1978; Brownlee & Cerami 1981; Cogan et al 1984]. Although there are different views about the contribution of hyperglycaemia in diabetes [Wolff 1991], various cytotoxic roles of glucose have been proposed during the past three decades, including non-enzymatic glycosylation (glycation) [Brownlee and Cerami 1981], polyol pathway metabolism [Van Heyningen 1962; Kinoshita et al 1962] and oxidative stress [Wolff 1987; Wolff et al 1991; Baynes 1991]. These hypotheses are discussed separately in following sections.

1. 3. The role of oxidative stress in diabetes

1.3.1. General chemistry of free radical reactions

Free radicals can be termed as the species which are capable of independent existence and contain one or more unpaired electrons. Radicals can be formed by the loss of a single electron from a non-radical, or by the gain of a single electron by a non-radical. It can also happen when a covalent bond in a molecule is broken. If one electron from each of the pair shared remains with each atom, the process is called homolytic fission with the formation of two uncharged free radicals, such as hydroxyl radical ($HO^{\cdot}$), alkyl radical ($R^{\cdot}$), alkoxyl radical ($RO^{\cdot}$) and peroxyl radical ($ROO^{\cdot}$). The energy required for the breakage of a covalent bond can be provided by heat, electromagnetic radiation, chemical reactions or enzymatic reactions. It is possible to have charged species which also have an unpaired electron; those are called radical anions ($A^{-\cdot}$) and radical cations ($B^{+\cdot}$). These radicals can be generated by one-electron reduction, addition of one electron, or oxidation with the loss of one electron.

Molecular oxygen (O_2) can accept a single electron from other compounds, such as transition metals and NADPH oxidase, to form superoxide anion ($O_2^{-\cdot}$). Adding another electron and two hydrogen ions yields hydrogen peroxide (H_2O_2); fission of peroxide yields $HO^{\cdot}$. Another reactive species of oxygen is singlet oxygen ($^1O_2$). Although $^1O_2$ has same number of electrons as O_2, the two outer electrons spin in opposite directions make $^1O_2$ more reactive than O_2. Of these reactive oxygen species, $HO^{\cdot}$ is the

most reactive radical in biological systems and reacts as soon as it is formed with any nearby compound such as lipid, protein or DNA, to stimulate continuous radical reaction with the formation of different reactive radicals, e.g. R$^{\bullet}$, RO$^{\bullet}$, ROO$^{\bullet}$ and organic hydroperoxide (ROOH). The formation of those reactive oxygen species may worsen the damage in the body.

1.3.2. The role of transition metals in free radical reactions

Free radicals can be generated, *in vitro* or *in vivo*, by a non-enzymatic route which is dependent on the presence of trace amounts of transition metal ions. In the presence of the reduced form of metal ions ($Me^{(n-1)+}$), either ferrous (Fe^{2+}) or cuprous (Cu^{+}), oxygen reacts spontaneously to form $O_2^{-\bullet}$.

$$Me^{(n-1)+} + O_2 \longrightarrow Me^{n+} + O_2^{-\bullet}$$

$O_2^{-\bullet}$ is a reducing agent for metal ions accompanied by the dismutation of superoxide to H_2O_2.

$$Me^{n+} + 2\ O_2^{-\bullet} + 2\ H^{+} \longrightarrow Me^{(n-1)+} + H_2O_2 + O_2$$

In the absence of catalase or peroxidases the decomposition of H_2O_2 and the generation of extremely reactive OH$^{\bullet}$ can be catalysed by transition metal ions, through the Fenton reaction [Fenton 1894] or the metal-catalysed Haber-Weiss reaction [Haber & Weiss 1934].

Fenton reaction

$$Me^{(n-1)+} + H_2O_2 \longrightarrow Me^{n+} + HO^{\bullet} + OH^{-}$$

Metal-catalysed Haber-Weiss reaction

$$Me^{n+} + O_2^{-\bullet} \longleftrightarrow Me^{(n-1)+} + O_2$$

$$Me^{(n-1)+} + H_2O_2 \longrightarrow Me^{n+} + HO^{\bullet} + OH^{-}$$

$$\text{NET:}\quad O_2^{-\bullet} + H_2O_2 \xrightarrow[\text{catalyst}]{\text{metal}} O_2 + HO^{\bullet} + OH^{-}$$

The Fenton reaction is demonstrated *in vitro* by adding iron salts to a peroxide solution [Fenton 1894]. After a short burst of HO$^{\cdot}$ formation, however, the reaction rate slows down as the metal ion is completely converted to the oxidized form. *In vivo*, as well as *in vitro*, other reducing agents exist, e.g. ascorbate [Winterbourn 1979; Winterbourn 1981; Rowley & Halliwell 1983], reduced glutathione and other thiols [Rowley & Halliwell 1982a], reduced nicotinamide complex NADH, NADPH [Rowley & Halliwell 1982b], which may perform a function similar to superoxide to return the metal ions to the reduced state and permit the continued formation of oxygen radicals. In the Haber-Weiss reaction, the transition metals are recycled between $Me^{(n-1)+}$ and Me^{n+} states. Thus the redox metals can catalyse the formation of HO$^{\cdot}$ and $O_2^{-\cdot}$ continuously, either *in vitro* or *in vivo* through the above reactions.

1.3.3. Biological damage by free radicals

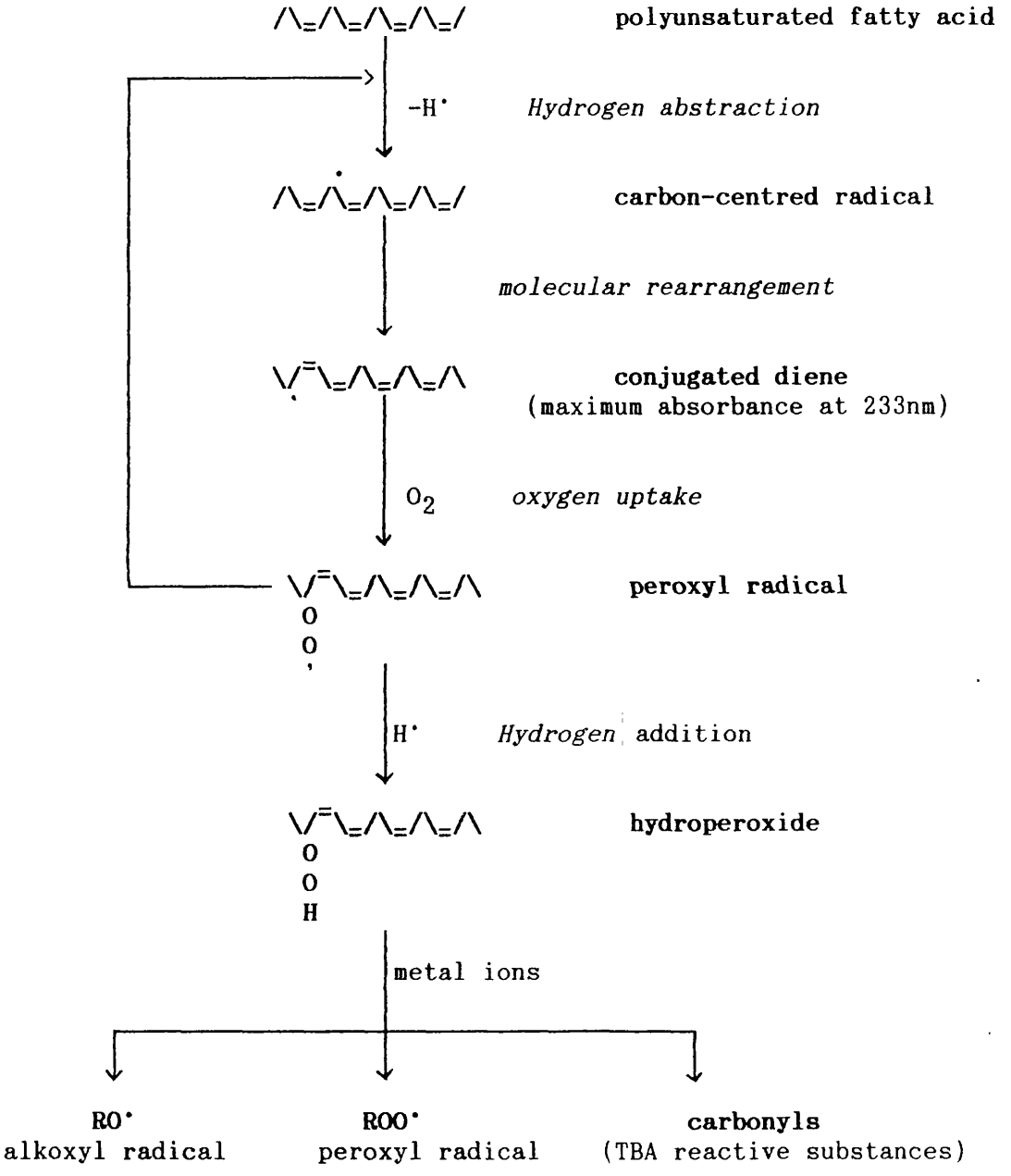
Reactive oxygen species are believed to be formed by a number of processes [Halliwell & Gutteridge 1984; Halliwell & Gutteridge 1989]. Any molecule in a biological system is a target for reaction with those reactive radicals. The hydroxyl radical will react with any species in its proximity and will initiate lipid peroxidation, damage proteins, oxidize carbohydrates and attack DNA and other intracellular components, depending on its site of production.

1.3.3.1. Lipid peroxidation

1.3.3.1.1. Chemistry of lipid peroxidation

Free radicals, such as HO$^{\cdot}$, can attack the polyunsaturated fatty acids contained in the phospholipids of biomembranes and thereby initiate lipid peroxidation by abstracting hydrogen from the carbon atoms between double bonds of unsaturated fatty acid and form alkyl radical. The double bonds in the alkyl radical rearrange, yielding a conjugated dieneyl radical which reacts with oxygen to form a peroxyl radical. The latter abstracts hydrogen further from other molecules of unsaturated fatty acid to form hydroperoxide and cause a chain reaction of lipid peroxidation as shown in Figure 1.1.

FIGURE 1.1 LIPID PEROXIDATION



In the presence of transition metal ions, lipid peroxides decompose to form reactive RO·, ROO· and non-radical lipid peroxidation products, such as carbonyls. RO· and ROO· can then initiate new rounds of lipid peroxidation and propagate further chain reactions.

$$Me^{(n-1)+} + ROOH \longrightarrow Me^{n+} + RO\cdot + OH^-$$

$$Me^{n+} + ROOH \longrightarrow Me^{(n-1)+} + ROO\cdot + H^+$$

1.3.3.1.2. Biological relevance of lipid peroxidation

The transition-metal-catalysed chain reactions of lipid peroxidation are significant in biological tissues. Lipid peroxidation destroys the biomembranes in which it occurs and also inactivates some membrane enzymes and membrane receptors [Marshansky et al 1983]. It is well documented that lipid peroxidation induces changes in the structure of plasma membranes as well as intracellular membrane and leads to changes in fluidity and permeability [Plaa & Witschi 1976] and eventually lysis of the cells [O'Malley et al 1966]. Lysis of cells can also be induced by releasing hydrolytic enzymes from peroxidized lysosomes [Hornby & Crivello 1983]. The stable products of lipid peroxidation, such as malondialdehyde and other aldehydes, can also react with different proteins and enzymes [reviewed by Tappel 1973; Esterbauer 1985; Wolff et al 1986; Kikugawa & Beppu 1987], leading to cross-linking or polymerization and loss of biological activity of these proteins. Furthermore, the products of lipid peroxidation have been found to react with DNA *in vitro* [Inouye 1984], which can lead to impaired cell proliferation.

On the other hand, lipid peroxidation *in vivo* may not only cause the destructive effects on macro-molecules, but also initiate metabolic abnormalities. For example, nanomolar levels of lipid peroxides stimulate cyclooxygenase activity [Hemler et al 1979; Hemler and Lands 1980; Cleland 1984] and experiments with synthetic hydroperoxides demonstrate that metabolic interference might be more important for cellular damage than initiation of new free radical processes [Moore et al 1983; Wolff & Jiang 1991].

1.3.3.2. Protein oxidation

The site-specific, metal-catalyzed protein oxidation was reviewed more recently [Stadtman 1990; Garland 1990; Stadtman & Oliver 1991]. Exposure of proteins or enzymes to free radical generating systems [Stadtman 1990] *in vitro* leads to oxidation of amino acids [Stadtman 1990] and inactivation of enzymes [Hodgson & Fridovich 1975], followed by polymerization (crosslinking) and/or cleavage (fragmentation) of the peptide chain [Amici et al 1989; Garland et al 1986; Wolff & Dean 1986]. Most amino acid residues in proteins, such as histidine, tyrosine, tryptophan, methionine, lysine and cysteine, are unusually sensitive to oxidation, probably because of their association with metal ion and haeme binding sites. The oxidation of histidine, cysteine, tyrosine and lysine residues may lead to the loss of enzyme activities. The oxidation of sulphur-containing amino acids leads to the formation of methionine sulphoxide and oxidation of cysteine residue leads to formation of intra- and/or inter-molecular disulphide crosslinks in proteins [Aruoma & Halliwell 1989; Maier et al 1989; Takahashi & Goro 1990]. Prolonged and extensive oxidation of protein will lead to chemical hydrolysis with the formation of carbonyl compounds [Wolff & Dean 1986; Wolff et al 1986]. Oxidized proteins are more susceptible to enzymatic hydrolysis than native protein [Wolff & Dean 1986; Wolff et al 1986].

Perhaps the most widely studied biological proteins are low density lipoprotein (LDL) and lens proteins. LDL is composed of lipids and apoproteins. It is suggested that oxidation of LDL and subsequent uptake of oxidized LDL by monocytes / macrophages to form foam cells are important events in atherogenesis (reviewed by Steinberg et al [1989] and Steinbrecher et al [1990]). Increases in methionine sulphoxide and SH oxidation in lens protein are associated with the development of human cataracts [Harding & Crabbe 1984; Spector 1985].

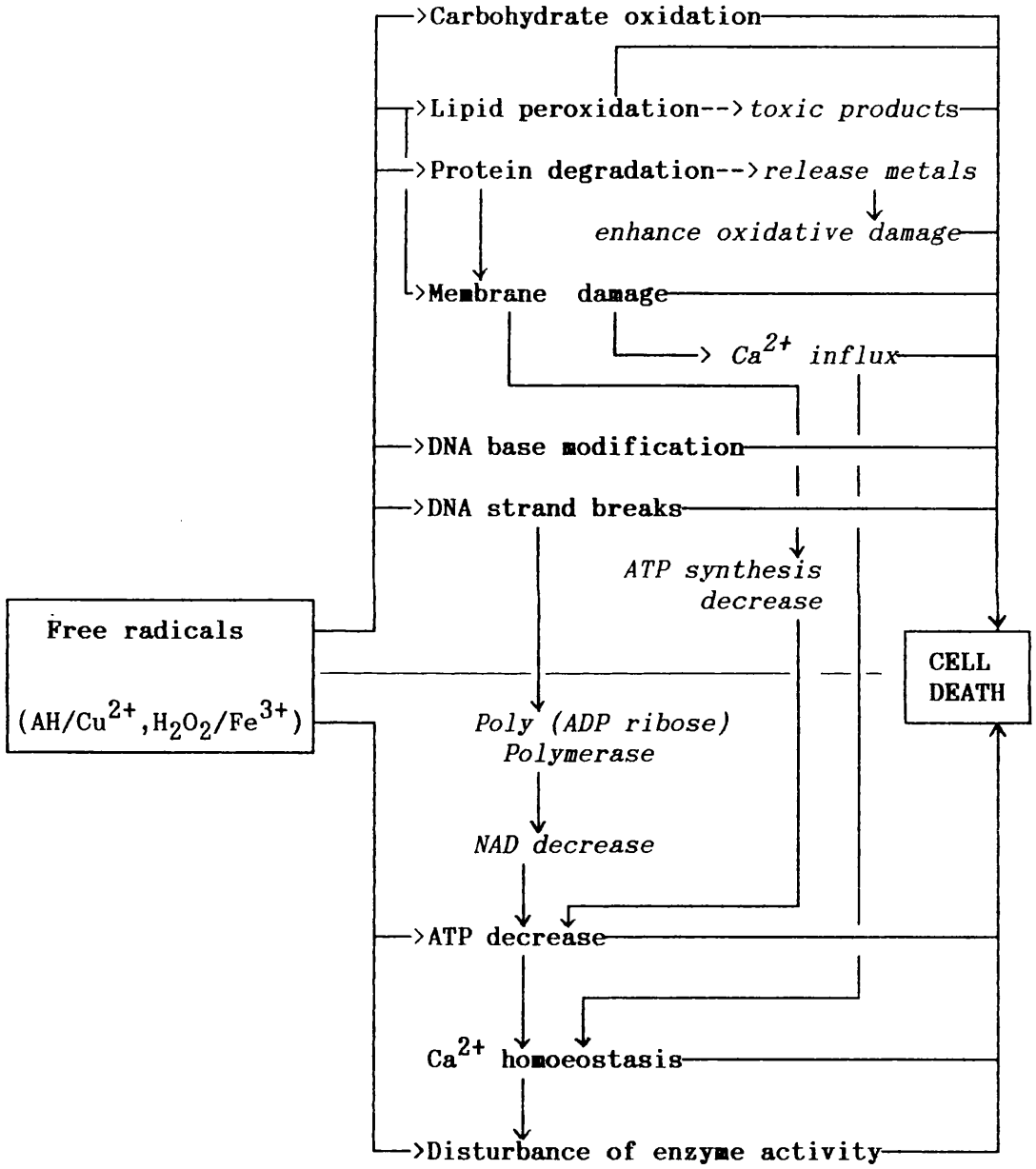
1.3.3.3. DNA damage and cytotoxicity

Although cells possess antioxidant systems to trap or inactivate reactive oxygen species, the antioxidant defence could become overwhelmed once cells are exposed to an excessive free radical generating system, such as H_2O_2/Fe^{3+}, ascorbate/Cu^{2+} or neutrophils [Jonas et al 1989; Samuni et al 1983; Van Kessel & Verhoef 1990]. Then reactive oxygen species, such as

HO•, can directly attack carbohydrates, proteins and lipids in cellular membranes, as well as DNA. The oxidation of carbohydrates, phospholipids and proteins in cellular membranes may lead to the loss of cell membrane integrity and thus cell death. Not just lipid hydroperoxides [Sandstrom 1991], but also the end products of lipid peroxidation (such as hydroxynonenals) are toxic to cells [Benedetti et al 1984]. Protein degradation may release the bound metals, which may catalyse further oxidative damage. Reactive oxygen species formed close to DNA can attack bases and deoxyribose of DNA [Aruoma et al 1991; Gutteridge 1984]. This attack may cause base hydrolysis and strand breaks, and thus cell death if this damage overwhelms the DNA repairing capacity of the cells [Mello-Filho et al 1984; Hoffmann et al 1984].

On the other hand, a serious cellular metabolic disturbance can occur after free radical damage. First, the free radical attack leads to the changes of enzyme activity. For example, nanomolar levels of lipid peroxides or micromolar levels of H_2O_2 stimulate cyclooxygenase activity and thus influence arachidonic acid metabolism, i.e. enhance enzymatic peroxidation of the unsaturated fatty acid [Hemler et al 1979; Hemler & Lands 1980; Cleland 1984]. Secondly, DNA damage (through activating poly-(ADP ribose) polymerase) and mitochondrial damage (through an effect on respiratory chain of phosphorylation) may lead to the depletion of adenosine triphosphate (ATP) [Schtaufstatter et al 1986; Spragg et al 1985]). Finally, membrane injury, destruction of intracellular thiol homoeostasis and exhaustion of the intracellular ATP pool may lead to the alteration of intracellular Ca^{2+} homoeostasis [Orrenius et al 1988; Boobis et al 1989]. The latter event may lead to activation of Ca^{2+} -dependent catabolic processes (such as activation of endonucleases, protein degradation and phospholipid hydrolysis) and thus cell death [Orrenius et al 1988; Cantoni et al 1989]. Figure 1.2 summarises the possible mechanisms of free radical induced cytotoxicity.

Figure 1.2 Mechanisms of free radical induced cytotoxicity



Note: AH = ascorbic acid

1.3.3.4. Enzymatic and chemical antioxidant systems

Oxidative damage in diseases may result either from increased oxidative stress or from a decrease in antioxidant defence mechanisms. The latter includes enzymatic and chemical antioxidant systems.

The major antioxidant enzymes are catalase, glutathione peroxidase (GSHPx) and superoxide dismutase (SOD). They can destroy H_2O_2, ROOH and $O_2^{-\cdot}$, the precursors of $HO^{\cdot}$, through the following reactions:

$$2\ H_2O_2 \xrightarrow{\text{Catalase}} 2\ H_2O + O_2$$

$$H_2O_2 / ROOH + 2\ GSH \xrightarrow{\text{GSHPx}} GSSG + 2\ H_2O / ROH$$

$$2\ O_2^{-\cdot} + 2\ H^{+} \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

Chemical antioxidants include the following: (1) free radical scavengers, such as alcohols, ascorbate, urate and glutathione (GSH); (2) lipid soluble chain breaking inhibitors of lipid peroxidation, such as vitamin E, probucol, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA); and (3) transition metal chelators, such as desferoxamine, ethylenediaminetetraacetic acid (EDTA) and diethylenetriaminepenta-acetic acid (DTPA).

1.3.4. Evidence of oxidative stress in diabetes and ageing

There is direct and indirect evidence that the concentrations of oxidising species and oxidised products are increased in the tissues in diabetes and during ageing.

1.3.4.1. Lipid peroxidation in diabetes and ageing

Several investigations have been designed to compare lipid peroxidation products in normal and age-matched diabetic plasma and tissues with the thiobarbituric acid (TBA) assay. Kaji et al [1985] reported that lipid peroxide levels in diabetic plasma were significantly higher than in healthy individuals. A possible association between diabetic retinopathy

and oxidative activity is also suggested by studies of Uzel et al [1987] which demonstrated significantly increased plasma lipid peroxide levels in Type II diabetic patients with retinopathy, as compared to patients without retinopathy. Nishigaki et al [1981] reported that the level of serum lipid peroxide generally increased less than 2-fold in diabetes patients and their results showed that large fractional increase in lipid peroxide occurred in the high density lipoprotein (HDL), and in the LDL fractions. The LDL and HDL (isolated from type II diabetes) was found more susceptible to oxidant stress *in vitro* than control lipoproteins and the increased oxidizability of lipoproteins in diabetic patients can be decreased by probucol therapy [Babiy et al 1992]. Sato et al [1979] reported that the increase in lipid peroxides was observed only in diabetic patients with angiopathy while those without complications had normal levels of plasma lipid peroxides. Another study, Simonelli et al [1989], observed lipid peroxidation in human clear lens, senile cataract lens and diabetic cataract lens and demonstrated that cataract lenses contained more malondialdehyde than did clear lenses and the level was higher in diabetes. Recently, it is reported that erythrocytes of type II diabetic subjects were found to have higher levels of malondialdehyde and lower activity of Na^+-K^+ -ATPase, as compared with the erythrocytes from healthy individuals [Rajeswari et al 1991].

Collier and colleagues [1988] noted that the levels of linolate-derived diene conjugate and the ratio of the diene conjugated to normal linolate were actually lower in diabetic patients with retinopathy. This observation seems to contradict the earlier investigations. It is, however, difficult to clarify the relationship between the measurement of conjugated diene and TBA reactive materials *in vivo*. Diene conjugate is an intermediate in the formation of lipid peroxides and may not be stable *in vivo*. On the other hand, the TBA assay may measure the stable end products of lipid peroxidation and the products of arachidonate metabolism [Hayaishi & Shimizu 1982]. Furthermore, Waitzmann [1979] showed that levels of certain prostaglandins were elevated in diabetes subjects and may be highest in diabetes with angiopathy. This may suggest that the increased level of TBA reactive substances (TBARS) in diabetes may originate from prostaglandin metabolism.

The increase in lipid peroxidation in diabetic patients is also consistent with work on diabetic animals. In streptozotocin (STZ)-diabetic rats, the level of kidney and retinal lipid peroxides increased about 2-fold [Nishimura and Kuriyama 1985]. The increase in lipid peroxides in both retina and kidney was associated with a decrease in the concentration of lipid soluble antioxidants and was inhibited by insulin treatment. The level of TBARS was also elevated in erythrocytes of STZ-diabetic rats [Jain et al 1990]. Morel and Chisolm [1989] reported that levels of lipid peroxidation products were increased in very low density lipoprotein (VLDL) and LDL isolated from STZ-induced diabetic rats and these lipoproteins were cytotoxic to cultured fibroblasts. The cytotoxicity was prevented by treating the animals with antioxidants, such as vitamin E and probucol. Of greater interest, insulin treatment also prevented the oxidative changes of VLDL and LDL and cytotoxicity in the diabetic animals. More recently, Armstrong and Al-Awadi [1991] reported that lipid peroxidation in plasma, as measured with the TBA assay, was increased in diabetic rats and the time-related increase of lipid peroxidation was correlated with the severity of retinopathy.

It was reported [Suematsu et al 1977] that there was an age- dependent increase in serum lipid peroxides in normal subjects and that the level was low in childhood and was highest for subjects aged 50 to 70. It was also reported [Hagihara et al 1984] that the age dependent increase in serum lipid peroxides was mainly due to the increased levels of lipid peroxides in serum LDL and VLDL, but not HDL, fractions in elderly subjects. It was also interesting to note that lipid soluble antioxidant a-tocopherol and high dose ascorbate reduced the levels of serum lipid peroxides in the elderly [Wartanowicz et al 1984].

1.3.4.2. Protein oxidation in diabetes and ageing

As reviewed above (section 1.3.3.2), the site-specific, metal-catalysed oxidation of proteins, including inactivation of enzymes, is an important aspect of oxidative damage in biological systems. It is well established that the inactive or less active forms of several enzymes accumulate in cells during ageing [Gershon & Gershon 1970; Stadtman 1986; Stadtman 1988]. Protein carbonyl derivatives in cultured human fibroblasts are increased in

proportion to the age of the fibroblast donor, and are much higher in cultured fibroblasts of individuals with ageing diseases, such as Werner's syndrome, than in those of age-matched controls [Oliver et al 1987; Stadtman et al 1989]. Also, the content of oxidized protein in human red blood cells increases with the age of the cell [Oliver et al 1987; Stadtman et al 1989].

Some age- and diabetes-related diseases, such as cataract and atherosclerosis, have also been investigated in terms of protein oxidation. Garland and colleagues [Garland 1990; Garland et al 1988] reported that there is a significant age-dependent increase in the protein carbonyl content of human cataract lens, and that the level of oxidized proteins is several times higher than in normal lenses. They also showed that the age- and cataract-related increase in the level of oxidized proteins can be mimicked by exposure of bovine crystallin to the ascorbate, O_2 and Cu^{2+} or Fe^{3+} system *in vitro* [Garland et al 1986]. The indication of oxidation of lipoproteins in atherosclerosis *in vivo* is the description of auto-antibodies to oxidized LDL produced in atherosclerotic human and animal sera [Palinski et al 1989]; the changes of physicochemical property of LDL isolated from animal atherosclerotic artery wall [Daugherty et al 1988]; and the studies using probucol (an antioxidant) to inhibit LDL oxidation which leads to decrease in the rate of progression of atherosclerosis *in vivo* [Parthasarathy et al 1986; Carew et al 1987; Daugherty et al 1991]. Although the antioxidant mechanisms of probucol remain unclear, it is interesting to note that probucol inhibits transition metal Cu^{2+}-induced human LDL oxidation *in vitro*, including a decrease in generation of fluorescence, prevention of the loss of the ability to bind highly reactive heparin fractions and as well as the inhibition of the modification of amino groups in the LDL surface [McLean and Hagaman 1989; Parthasarathy et al 1986].

1.3.4.3. Changes of antioxidant systems in diabetes

Some evidence has been obtained for alternations in the activity of tissue SOD in both experimental and clinical diabetes. Matkovics et al [1982] found that alloxan- or streptozotocin (STZ)-induced diabetic rats had decreased total SOD activity in liver, kidney, spleen, heart, testis,

pancreas, skeletal muscle and erythrocytes. Crouch et al [1978] found that STZ-diabetic rats had decreased Cu-ZnSOD activity in erythrocytes and retina, but not in other tissues. Other studies demonstrated that the SOD activity in STZ-diabetic rats was decreased in retina [Nishida et al 1984], renal cortex and bowel mucosa [Loven et al 1982], and kidney, erythrocytes and liver [Loven et al 1986]. It is interesting to note that both oral glutathione and intramuscular insulin elevated the activity of SOD [Loven et al 1983; Loven et al 1986]. This indicates that the severity of diabetes may affect the SOD activities and the antioxidant can restore their level.

A number of studies have also been carried out on the activities of SOD in human diabetes. It was reported that erythrocyte Cu-ZnSOD was reduced in type I diabetic children when compared to controls [Wataa et al 1986]. Subjects with type II diabetes maintained on oral hypoglycaemic agents had nearly 1 fold decrease in erythrocyte Cu-ZnSOD activity [Matkovics et al 1982]. The substrate of SOD, the superoxide ion, was significantly elevated in diabetic polymorphonuclear leukocytes (PMNs), particularly in patients with type II diabetes [Nath et al 1984]. This elevation contributed to reduction in the activities of the cytoplasmic and mitochondrial SOD. It was reported that the percentage of the glycated form of Cu-ZnSOD, which had lower enzymatic activity, was significantly increased in the erythrocytes of diabetic patients as compared to normal erythrocytes [Arai et al 1987b]. Thus, mechanisms for the inactivation of Cu-ZnSOD by glycation or reduction of SOD levels by elevated superoxide seen *in vivo* during diabetes were suggested.

There have been a number of reports about the activity changes of other antioxidant enzymes, such as catalase and glutathione peroxidase, in experimental and clinical diabetes. However, the results were frequently inconsistent and contradictory. Some authors reported that catalase activities were decreased in liver [Bitar & Weiner 1983] and muscles [Lammi-Keefe et al 1984] from diabetic rats, but others reported increased activity of the enzyme in diabetic rat tissues, such as kidney, liver and erythrocytes [Matkovics et al 1982]. In humans, there was no change in catalase activity of erythrocytes of type II diabetic subjects [Matkovics et al 1982], or in erythrocytes of type I diabetic subjects on insulin maintenance [Hagglof et al 1983]. However, Wataa et al [1986] reported an

elevation in catalase activity in erythrocytes of diabetic children. Similarly, there appear to be no consistent changes in the activities of glutathione peroxidase. In one study, glutathione peroxidase activity was increased in erythrocytes from type II diabetics [Matkovics et al 1982], but decreased in another study [Uzel et al 1987], and showed no changes in a third [Kaji et al 1985]. Glutathione peroxidase activity in erythrocytes and lymphocytes from type I diabetics maintained on insulin was not significantly changed [Hagglof et al 1983], but plasma level of glutathione peroxidase was elevated in type II diabetics [Uzel et al 1987].

The chemical antioxidants, such as glutathione, ascorbate and vitamin E, are also altered in experimental and human diabetes. It was reported that the hepatic glutathione level decreased in STZ-diabetic rats [Loven et al 1986; Hassing et al 1979] and treatment with insulin restored its concentration to normal level [Loven et al 1986]. Serum glutathione levels in type II and type I diabetic patients were also lowered [Awadallah et al 1978a & 1978b]. Total glutathione content in normal human erythrocytes decreased with age of the cells and a similar decrease occurred with cells from type II diabetics [Gandhi & Chowdhury 1977]. Both total glutathione and reduced glutathione were significantly decreased in diabetic patients as compared to normal subjects [Illing et al 1951]. Moreover, Uzel et al [1987] reported that erythrocyte level of glutathione was decreased 10-20% in type II diabetics, concomitant with a 40% increase in lipid peroxides in erythrocyte membranes; these changes were more significant in patients with retinopathy. Reduced glutathione levels in PMNs [Chari et al 1984] and in platelets [Thomas et al 1985] of diabetic patients were significantly decreased.

Ascorbate metabolism has been reported to be changed in diabetes mellitus [Taylor et al 1988]. Plasma levels of ascorbic acid were decreased in both human and animal diabetes [Chatterjee and Banerjee, 1979; Som et al 1981; Jennings et al 1987; McLennan et al 1988; Yue et al 1989]. At the same time there was an increase in the dehydroascorbate : ascorbate ratio [Chatterjee and Banerjee, 1979; Som et al 1981; Jennings et al 1987]. It was also reported that the levels of ascorbate were decreased in white blood cells from clinical diabetics [Chen et al 1983] and in some tissues from STZ-diabetic rats [Yew 1983].

Likewise, platelet vitamin E level was depressed 66% in rats with STZ-induced diabetes, an effect which was reversed by dietary supplementation with vitamin E [Karpen et al 1982]. Another study found no change in serum vitamin E in rats with STZ-induced diabetes of 1 to 2 weeks duration, but a 26% decrease in serum vitamin E and 92% decrease in hepatic vitamin E in rats with STZ-induced diabetes of 20 weeks duration [Higuichi, 1982]. In clinical studies, vitamin E content was significantly reduced in platelets from type I diabetics, when compared with platelets from control subjects [Karpen et al 1984; Karpen et al 1985], and the decreased content of vitamin E coincided with the increased level of thromboxane A_2 (a stimulator of platelet aggregation) in platelets from diabetics [Karpen et al 1984]. The vitamin E level in platelets was also significantly decreased in type II diabetics, especially in those with retinopathy, as compared with normal subjects [Watanabe et al 1984]. The decrease of vitamin E contents had positive correlation with the platelet aggregation and thromboxane B_2 contents in platelets [Watanabe et al 1984]. Thus, it is suggested that the reduced vitamin E levels in diabetic platelets can contribute to enhanced platelet thromboxane production and aggregation which relate to the development of vascular complications.

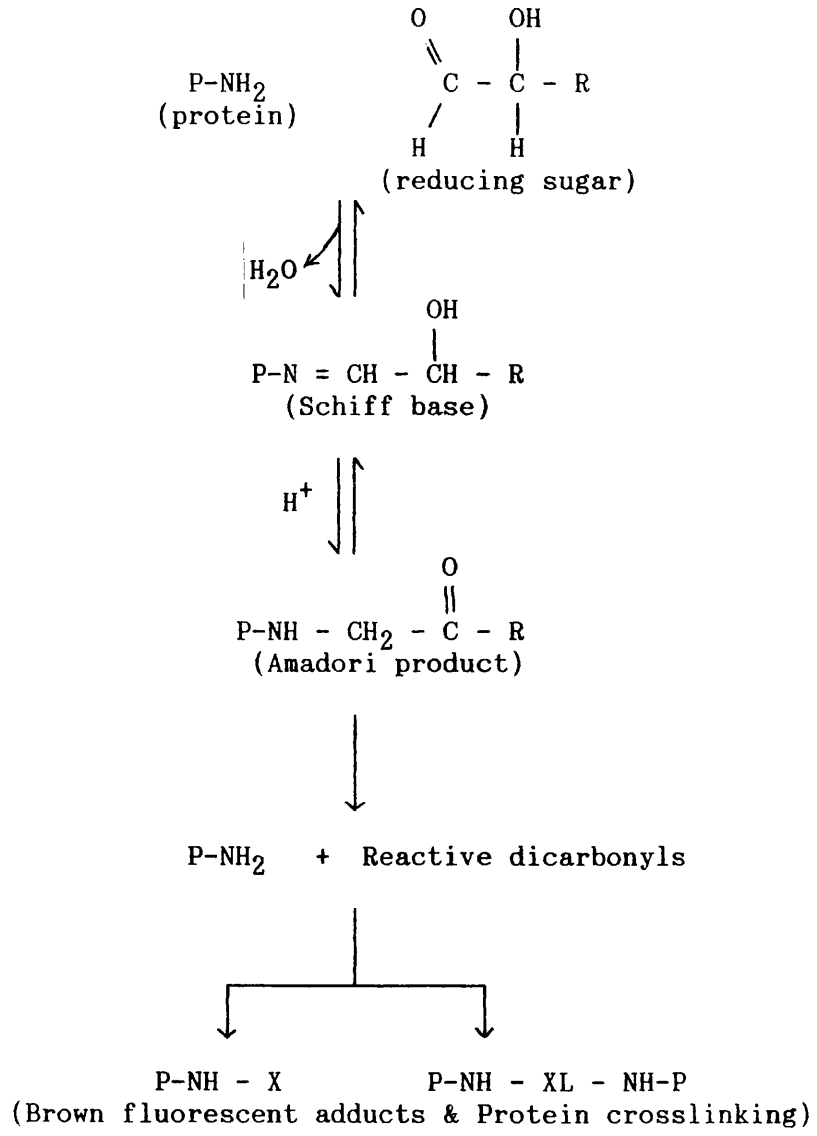
1.3.5. Glycation in ageing and diabetic complications

Glycation is a modification of proteins, occurring initially with the formation of Schiff base intermediate between glucose and amino groups on protein, followed by the formation of the ketoamine (fructoselysine) by an Amadori rearrangement. Evidence that this reaction occurs *in vivo* was obtained through the measurement of glycated haemoglobins which were elevated in diabetes [Trivelli et al 1971]. The extent of haemoglobin glycation is now used as a index of long-term glycaemia in the clinical management of diabetes [Kennedy and Baynes 1984]. Glycated proteins also undergo further reactions. The Amadori product subsequently degrades into alpha-ketoaldehyde compounds, such as 1- and 3-deoxyglucosones. These secondary compounds are more protein-reactive than the parent monosaccharide [Njorge et al 1987; McLaughlin et al 1980] and can react with proteins to form cross-links, as well as chromo/fluorophoric adducts called Advanced Glycation Endproducts (AGE) or Maillard products (Figure 1.3) which result in the protein becoming "browned", fluorescent and cross-

linked *in vitro* [Brownlee et al 1984; Monnier 1989]. One postulated cross-linking and fluorescent AGE found in hydrolysates of *in vitro* glycated protein has been identified as 2-furoyl-4[5]-[2-furanyl]-1-H-imidazole (FFI) [Pongor et al 1984]. This compound has been suggested to be relevant to protein cross-linking and fluorescence *in vivo* since a specific macrophage receptor has been identified which recognizes protein which has been exposed to glucose *in vitro* [Vlassara et al 1986]. The number of these specific receptors was found to be higher in macrophages of diabetic animals than in normal animals [Vlassara 1988]. The process of glycation and browning of proteins is thought to contribute to the development of ageing and the complications of diabetes through alteration in the crosslinking of long-lived proteins such as collagens [Monnier et al 1984; Monnier et al 1986] and lens proteins [Monnier et al 1979; Harding 1985]. Similarly, extensive glycated LDL becomes poorly recognized by fibroblasts and preferentially accumulated by macrophages [Saski and Cottam 1982; Steinbrecher & Witztum 1984; Lopes-Virella et al 1988]. This has been suggested to contribute to plasma LDL accumulation and atherosclerosis in diabetes [Witztum et al 1982]. Furthermore, SOD loses activity when exposed to glucose *in vitro* [Arai et al 1987a], higher levels of glycated erythrocyte SOD are found in aged erythrocytes and in diabetes [Arai et al 1987b].

The significant role of glycation on the aspects of "browning" or fluorescence of long-lived proteins, such as skin collagens and lens crystallins , in diabetes and ageing is established by a number of studies. For example, aspirin and its analgesics, which protect against cataract [Cotlier & Sharma 1981; Harding & Van Heyningen 1988] and prevent collagen abnormalities [Yue et al 1984], are able to inhibit glycation of collagen [Yue et al 1984] and lens proteins [Huby & Harding 1988; Ajiboye & Harding 1989]. Aminoguanidine, an inhibitor of advanced glycation [Lewis & Harding 1990], is also able to prevent collagen cross-linking and the generation of collagen fluorescence both *in vitro* and *in vivo* [Brownlee et al 1986], and retard albuminuria in diabetic rats [Soules-Liparota et al 1991].

Figure 1.3 General scheme of protein glycation (Maillard reaction) (adapted from Monnier 1989)

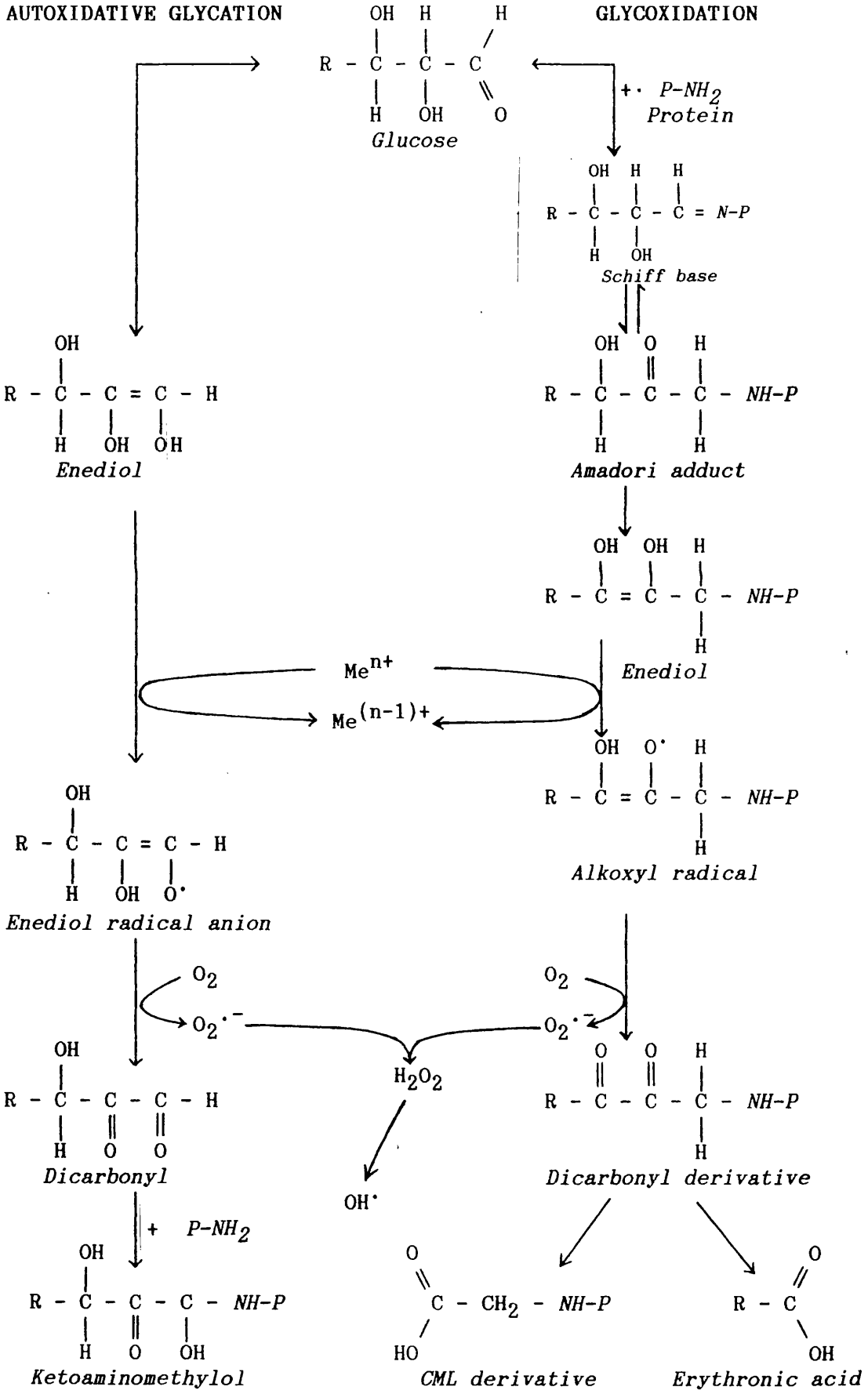


Although age-related skin collagen fluorescence is increased in diabetes, however, the correlation with arterial and joint stiffness, as well as with retinopathy, is weak (r = 0.47) and nonexistent for nephropathy [Monnier et al 1986]. On the other hand, it has been shown that there is little change in lens crystallin glycation with age of rats although lens browning is increased [Swamy and Abraham 1987; Perry et al 1987]. It is possible that, based on the knowledge referred to above, there may be some other factors involved in tissue browning and damage in ageing and diabetes.

1.3.6. Autoxidative glycation and glycoxidation

The term *Autoxidative glycation* was introduced by Wolff [1987] to describe the "autoxidation" of reducing sugars catalysed by trace amount of transition metals and its role in oxidative modification and cross-linking of proteins [Wolff et al 1991]. Glucose and other alpha-hydroxyaldehydes can be oxidized to more reactive alpha-ketoaldehydes with the formation of oxidizing intermediates, the former are then able to react with proteins to form ketoimine adducts (Figure 1.4). Evidence suggests that free radicals, such as $OH^{\cdot}$ [Hunt et al 1988], slowly produced by glucose "autoxidation" are a substantial cause of the structural damage and glycoflurophore generation in protein which is exposed to glucose *in vitro* [Hunt et al 1988; Fujimori 1989; Hunt & Wolff 1991]. Autoxidative glycation of low density lipoprotein by glucose *in vitro*, with the formation of $OH^{\cdot}$, also lead to oxidation [Sakurai et al 1991] and fragmentation of the lipoprotein, as well as peroxidation of associated lipids [Hunt et al 1990]. The generation of free radicals and the consequent protein damage by autoxidative glycation are transition metal dependent, because not only hydroxyl radical scavenger, but also (and more powerfully) metal chelating agents, such as DTPA or EDTA, inhibit the formation of $OH^{\cdot}$, as well as protein fragmentation and protein browning [Hunt et al 1988; Hunt et al 1990; Sakurai et al 1991]. In contrast, if protein glycation is induced by glucose in the presence of sodium cyanoborohydride ($NaCNBH_3$), which provides a reducing environment but also greatly increases the rate of glucose attachment by trapping the Schiff base, then practically no fluorophore development is observed [Wolff & Dean 1987]. More recently, Ceriello et al [1991] reported that the level of glycated haemoglobin

Figure 1.4. Pathways of autoxidative glycation and glycoxidation



(HbA_1c) in diabetic subjects was decreased after administration of vitamin E for 1 to 2 months. The mechanism of the inhibitory effect of vitamin E on the formation of labile glycosylation is unknown. It may be due to the antioxidant activity of the antioxidant, which may inhibit the "autoxidation" of glucose *in vivo* and the formation of ketoaldehyde. The latter is also reactive to protein according to Wolff & Dean [1987].

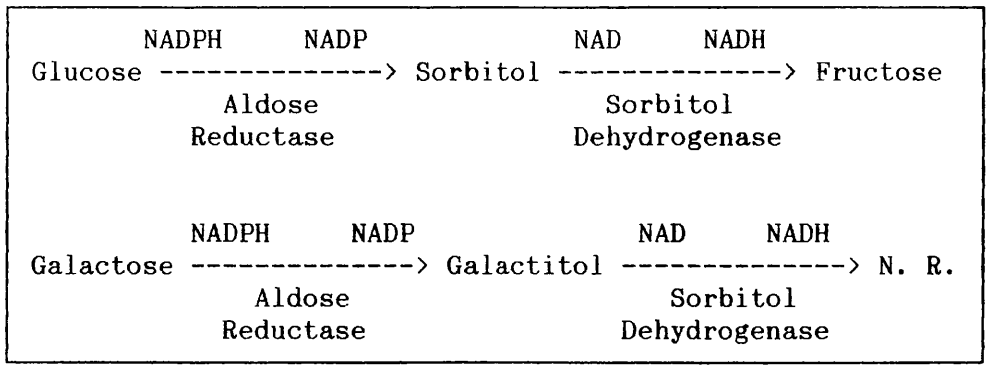
Glycoxidation, on the other hand, was introduced to describe the oxidative damage of proteins after modification by glucose [Baynes 1991]. It has become apparent that glycated proteins may also serve as sources of oxygen radicals. Hicks et al showed that not only autoxidation of glucose, but also glycated proteins, stimulate the peroxidation of lipids *in vitro* [Hicks et al 1988]. The peroxidation was partially inhibited by deferoxamine, indicating the involvement of metal ion catalysis. It has also been shown that the glycated proteins or Amadori adducts, catalysed by transition metals, cause oxidative damage of amino groups in protein [Kawakishi et al 1990], protein fragmentation [Cheng et al 1991], and DNA strand breaks [Kashimura et al 1986] *in vitro*. Furthermore, the Amadori adduct itself is able to oxidize in the presence of oxygen and transition metals, leading to the formation of $O_2^{-\cdot}$ [Sakurai & Tsuchiya 1988; Azevedo et al 1988], the release of erythronic acid and the formation of carboxymethylated lysine residues [Ahmed et al 1986; Baynes 1991], such as carboxymethylysine (CML) and carboxymethylhydroxylysine (CMhL). It is believed that CML and CMhL are the products of oxidative cleavage of the Amadori adducts [Dunn et al, 1990; 1991]. The only browning product found in collagen in diabetic patients and the elderly was pentosidine [Sell & Monnier 1990], which was formed during browning of protein by sugars *in vitro* [Sell & Monnier 1989] and its formation is also inhibited under anaerobic conditions [Baynes 1991]. These compounds, e.g. CML, CMhL and pentosidine, were termed *glycoxidation products* by Baynes and colleagues and were used as the biomarker of carbohydrate-dependent damage to protein [Dyer et al 1990; Sell & Monnier 1990]. Some investigations proved that these glycoxidative products and total fluorescence increase coordinately in collagen with age and at an accelerated rate in diabetes [Dunn et al 1991; Sell & Monnier 1990]; thus they provide evidence of increased oxidative damage, catalysed by transition metals, to collagen in diabetes and ageing.

In conclusion, high blood glucose (in the presence of trace amount of transition metals) may contribute to oxidative tissue damage through two pathways: 1) oxidative modification of protein by the products of glucose autoxidation, including reactive oxygen species and reactive alpha-ketoaldehydes; and 2) glycoxidation. The latter also generates free radicals at the site of protein modification and causes site-specific protein oxidative cleavage.

1.3.7. Polyol pathway and aldose reductase inhibition

Aldose reductase (EC 1.1.1.21) and sorbitol dehydrogenase (EC 1.1.1.14) are the enzymes that constitute the polyol pathway. In this pathway, aldose reductase, utilizing reduced nicotinamide-adenine dinucleotide phosphate (NADPH), reduces the aldehyde form of glucose or galactose to sorbitol or galactitol, respectively; and galactose is a better substrate for aldose reductase than glucose. Sorbitol, but not galactitol, is oxidized to fructose by sorbitol dehydrogenase, which requires the cofactor oxidized nicotinamide-adenine dinucleotide (NAD) (Figure 1.5). Studies have revealed that the enzymes of polyol pathway are present in many mammalian tissues, including those in which diabetes-associated pathology develops. Despite the ubiquity of this pathway, its function has remained a mystery.

Figure 1.5. Polyol pathway metabolism



Note: N.R. = no reaction

Possible mechanisms that cause cellular pathology by activating the polyol pathway were indicated from studying diabetic and galactosemic cataracts in

animals. Van Heyningen [1959; 1962] initially induced experimental cataracts in animals by raising the plasma level of aldose sugar and demonstrated that the accumulation of polyol in the lens was directly related to the rapidity with which cataract develops. Similarly, when rabbit lenses were incubated *in vitro* in the presence of high concentrations of galactose, cataract developed, which could be prevented by the addition of aldose reductase inhibitor to the medium [Kinoshita et al 1968]. Kinoshita and co-workers further proposed that the accumulation of polyols initiated the development of cataracts through an osmotic process [Kinoshita et al 1963; Kinoshita 1974]. The "Osmotic hypothesis" suggests that the activity of aldose reductase, which converts the sugar to polyol, is increased in hyperglycaemic conditions. Because the cell membranes are relatively impermeable to polyols [Wick & Drury 1951], sorbitol or galactitol accumulates to high levels, which might create a hypertonic condition that might lead to influx of water with resultant cellular oedema, metabolic dysfunction, and eventual cell death.

Since aldose reductase is found in most tissues [Ludvigson & Sorensen 1980; Akagi et al 1983; Akagi et al 1987] associated with diabetic complications, it is suggested that the polyol pathway, through an osmotic effects, may contribute to the development of other diabetic complications, such as neuropathy, retinopathy, angiopathy, and nephropathy [Cogan et al 1984; Kador et al 1985]. Sorbitol has also been found in increased concentrations in lens, erythrocytes, and nerves of diabetic animals and diabetic patients [Pirie and Van Heyningen 1964; Gabbay 1973; Ludvigson & Sorensen 1980]. This indicates a link between sorbitol formation and diabetic complications.

In order to develop new drugs for the treatment of diabetic complications, aldose reductase inhibitors (ARIs) which block the polyol pathway have been applied to animal studies and clinical trials. Some ARIs, such as sorbinil and tolrestat, can inhibit the development of sugar (experimental galactose and diabetic) cataracts in animal studies [Kador et al 1985]; inhibit retinal capillary basement membrane thickening [Robison et al 1988; Robison et al 1983] of galactosemic rats; decrease permeability of blood-retinal barrier [Lightman et al 1987; Williamson et al 1985] of galactosemic and diabetic rats; improve nerve conduction velocity in diabetic rats [Mayer &

Tomlinson 1983]. These observations seem to provide further support for the polyol pathway hypothesis.

However, after two decades of influence, the hypothesis is still questionable [reviewed recently by Harding 1991]. In diabetic rat experiments the sorbitol level became very high but the lenses stayed clear and swelling was negligible [Coulter et al 1986; Cheng et al 1989]. Although there is closer inverse relationship between the tissue myo-inositol content and its water content, the use of diets supplemented with myo-inositol does not reduce the elevated tissue levels of sorbitol and fructose; but it does delay or prevent the development of cataract and impaired conduction velocities of peripheral nerve [Mayer & Tomlinson 1983] in lens and nerve of animals with experimental diabetes. This further permitted a dissociation of polyol pathway activity from the biochemical and functional changes that occur in galactosemic or diabetic animals.

Usually, the activity of aldose reductase in tissue homogenates is estimated *in vitro* by measuring the consumption of NADPH in the presence of aldoses, such as DL-glyceraldehyde. Glyceraldehyde as well as glucose, however, is prone to autoxidation catalysed by trace amount of metals *in vitro* [Wolff et al 1984]. The formation of free radicals during autoxidation of glyceraldehyde may contribute to NADPH oxidation *in vitro* assay. The further questions, concerning the role of aldose reductase and its inhibitors in oxidation of NADPH, were raised by Wolff & Crabbe [1985].

Furthermore, much of the evidence for the role of polyol metabolism in diabetic complications comes from studies of experimental diabetes. But there are important differences between experimental and human diabetic complications. For example, although the osmotic effect of accumulated polyol, as some evidence suggests, plays a role in galactosaemic cataract, it may be of little importance in human diabetic cataracts. The human lens contains low levels of measurable aldose reductase activity and high levels of polyol dehydrogenase so that any sorbitol formed would be rapidly converted into fructose to be further metabolized [Jedziniak et al 1981]. Therefore, there is no marked difference between the low levels of sorbitol found in senile cataracts extracted from diabetics and non-diabetics [Heaf & Galton 1975; Pfaffenberger et al 1976; Lerman & Moran 1988].

1.4. The purpose of the thesis

This thesis addresses the question of the role of transition metal catalysed oxidative reactions in the development of diabetic complications and ageing through *in vitro* studies. For this purpose, a sensitive and convenient method needed to be developed for the detection of low level H_2O_2 and lipid peroxide. A new method, which is based on the principle of the rapid peroxide-mediated oxidation of Fe^{2+} to Fe^{3+} under acidic conditions, is described and applied to the studies of H_2O_2 generation by glucose "autoxidation" during albumin glycation and lipid peroxidation during LDL glycation.

The toxic effect of glucose modified LDL on fibroblasts will be used as the model of tissue damage in diabetes. The possible role of glycation and/or transition metal catalysed oxidative modification of LDL in cellular damage is examined.

In addition, the effects of some experimentally established inhibitors of aldose reductase, such as sorbinil, AL-1576 and ONO-2235, on transition metal catalysed ascorbate oxidation, the consequent cellular damage *in vitro* and possible mechanisms are also examined.

CHAPTER 2 MATERIALS AND METHODS

2. 1. Materials

Xylenol orange ((o-cresolsulfonphthalein-3'-3''-bis(methyliminodiacetic acid Sodium salt)) was obtained from Aldrich. Ammonium ferrous sulphate, H_2O_2, cumene hydroperoxide (CuOOH), t-butyl hydroperoxide (BuOOH), di-cumyl peroxide, butylated hydroxytoluene (BHT), potassium bromide (KBr), cupric sulphate, reduced glutathione (GSH), $NaCNBH_3$, thiobarbituric acid (TBA), linoleic acid, acetic acid (glacial), potassium iodide, EDTA, DTPA, cation exchange resin (chelex 50-100 dry mesh), phosphatidyl choline (Egg Yolk, sigma Type V-E), lipoxidase (EC 1.13.11.12, Type V, soybean), catalase (Type C-40), glutamine, glutathione peroxidase (Bovine Erythrocyte Type G6137), phospholipase A_2 (Porcine Pancreas Type P6534) and formic acid were obtained from Sigma (Poole, Dorset) and were of the highest purity available. Benzoyl peroxide and lauroyl peroxide were bought from Fluka (Glossop, Derbyshire). Methanol was purchased from British Drug Houses (BDH, Limited, Poole, England).

The aldose reductase inhibitor sorbinil was generously provided by Pfizer UK. AL-1576 was kindly provided by Alcon Inc. ICI 105552 was donated by ICI Pharmaceuticals (UK). ONO 2235 was the gift of ONO Pharmaceutical company. 3,3-tetra-methylene glutamic acid was obtained from Sigma. Al-1576, sorbinil and ICI 105552 solutions were prepared by rapid dissolving the substance in dilute alkali (10mM NaOH) followed by rapid neutralization with dilute HCL. ONO 2235 was dissolved in absolute ethanol.

Radiochemicals ([U-^{14}C]-glucose and [methyl-^{3}H]-thymidine) were obtained from Amersham International (UK). Liquiscint (liquid scintillation counting solution) was obtained from National Diagnostics (New Jersey, USA).

Dulbecco's Modification of Eagles Medium (DMEM), sterile foetal calf serum, gentamycin (50mg/ml), trypsin-EDTA (0.05% trypsin, 0.02% EDTA) and sterile 1 x phosphate buffered saline (PBS) were purchased from Flow Laboratories (Irvine, Scotland). Multiwell (24-well) tissue culture plates, sterile polystyrene conical tubes and flasks were obtained from Falcon, Becton Dickinson (New Jersey).

A Scanning Spectrophotometer (PU 8720 UV/VIS), the product of Philips (London, England), a Liquid Scintillation Counter (TRI-CARB 460C), the product of Packard (Berks, England), an Ultracentrifuge(Centrikon T-1065), the product of Kontron Instruments (Herts, England), and a Sigma 201-M microcentrifuge, the product of Laboratory Centrifuges GmbH (Osterode am Harz, Germany), were employed.

2. 2. Methods

Part A. General methods

2.2.1. Lipoprotein preparation

Human LDL was isolated from the plasma of normal volunteers in the density range 1.019 - 1.063 g/ml by sequential ultracentrifugation as previously described [Hatch & Lees 1968]. In brief, whole blood was obtained by venipuncture and collected in tubes with the chelating agent EDTA (1mg/ml) to prevent oxidation and coagulation. The blood was centrifuged for 10 minutes at 3500 rpm, at 4^{o}C. Pooled plasma solvent density was adjusted to 1.019 g/ml with a high density salt solution (containing NaCl, KBr and 1mg/ml EDTA). After centrifugation for 18 hours at 100,000 g, at 16^{o}C, the top layer of supernatant was removed. The density of pooled infranatant was adjusted to 1.063 g/ml and was recentrifuged as before. The LDL, which floats at a relative density of 1.063, was collected and exhaustively dialysed for 48 hours at 4^{o}C against 2 changes of 200 volumes of phosphate-buffered saline (PBS: pH 7.4). In order to prevent LDL oxidation during dialysis, the buffer contained a cation exchange resin (chelex 100 dry mesh) to chelate transition metals.

2.2.2. Liposome preparation

The liposomes were prepared as the routine method in our laboratory. 2ml of 100mg/ml phosphatidyl choline solution was dried under nitrogen and then 10ml PBS (10mM, pH 7.4) was added. The mixture was then kept in the dark in ice for 1 hour with gentle shaking for 30 seconds every 15 minutes to ensure that the dried phosphatidyl choline resuspended in PBS. The suspension was then ultrasonicated on ice for 2 minutes to make small liposomes and was ready for experiments.

2.2.3. Linoleic acid hydroperoxide preparation

Linoleic hydroperoxide was prepared by a method described previously [Gardner 1975; Hicks & Gebicki 1979]. Linoleic acid (10 mM) was oxidized by exposure to lipoxidase (30,000 units/ml) in borate buffer (10 mM, pH 9.0) at 37^{o}C. After incubation for 45 minutes, 1ml sample was dissolved in 10ml pure methanol. The concentration of hydroperoxide in methanol was quantified at 233 nm using the molar extinction coefficient (for conjugated diene) of 2.8 x 10^{4} M^{-1} cm^{-1} [Pryor & Castle 1984].

2.2.4. Preparation of red blood cells and their membranes

Whole blood was obtained from healthy volunteers by venipuncture and collected in tubes containing 3.8% sodium citrate to prevent coagulation (blood : 3.8% sodium citrate (v/v) = 9 : 1). Blood was then centrifuged at 3500 rpm for 10 minutes and the plasma and white cells were removed. The red blood cells were washed 3 times with 10mM PBS (pH 7.4). 10% (v/v) of packed red blood cells were incubated in 10mM PBS with various additions as designated. In order to lyse red blood cells, 500µl sample (at defined time intervals of incubation) was added to 10mls of 5mM potassium phosphate buffer (pH 8). After shaking vigorously the mixture was centrifuged at 15,000 rpm for 10 minutes to sediment cell membranes. The resultant ghosts (membranes) were transferred to 1.5ml microcentrifuge tubes and were washed twice with the lysis buffer. The resultant pellets were used for the measurement of lipid peroxides.

2.2.5. Measurement of protein content

Protein content was measured with bicinchoninic acid (BCA) assay [Smith et al 1985] in which Cu^{2+} is reduced by protein to Cu^{+} under basic condition and then BCA reacts with Cu^{+} with the formation of purple colour complex at 562nm. Standard BCA reagent A (carbonate/bi carbonate buffer and BCA in 0.1M NaOH) and reagent B (4% $CuSO_4$) were bought from LIC Pierce (Luton, England). 50µl of diluted protein sample was added to 2ml freshly prepared mixture of reagent A and B (50:1) and incubated at 37°C for 1 hour. Meanwhile, standard bovine serum albumin (1-20µg/ml) was used for standard curve. After incubation, absorbance was read at 562nm. Protein concentration was calculated with extinction coefficient given by standard curve.

2.2.6. Measurement of lipid peroxide with FOX-2 assay

Lipid hydroperoxides were measured in methanolic extracts of LDL or liposome samples by their oxidation of Fe^{2+} to Fe^{3+} in 25mM H_2SO_4 in the presence of xylenol orange after removal of hydrogen peroxide using catalase. Ordinarily, a 0.1 or 0.2ml methanol-extracted LDL or liposome sample was added to 0.9 or 0.8ml FOX-2 reagent (detail see chapter 3) and incubated at room temperature for 30 minutes. Then absorbance was read at 560nm. Standard H_2O_2 from Sigma was used to calibrate the assay.

2.2.7. Measurement of lipid peroxide with iodometric assay

Linoleic hydroperoxide was determined using an established iodometric method [Hicks & Gebicki 1979], based on the oxidation of I^- to I_2 by peroxides. Solution A consisted of 1:1 mixture of HPLC-grade methanol and glacial acetic acid containing 1 mg/ml EDTA (disodium salt) degassed with a stream of nitrogen for 20 minutes. Solution B consisted of HPLC-grade methanol degassed with N_2 for 20 minutes followed by addition of 20% w/v potassium iodide and degassed for a further 20 minutes. One ml of solution A was added to 1.5 ml of solution B in a 3 ml cuvette adapted to permit degassing with nitrogen as well as the addition of small volumes of fluid under anoxic conditions. The cuvette contents were treated with N_2 for 5 minutes and the cuvette was sealed. Absorbance was monitored at 290 nm for 5 minutes. The solution was discarded if absorbance increased by more than

0.005 AU. A small volume (1 - 10 µl) of a N_2-treated standard hydroperoxide solution was then injected. The cuvette contents were gently mixed by careful agitation. Absorbance at 290nm was monitored for 5 minutes after which time no further absorbance change was observed. A standard curve generated with H_2O_2 gave an extinction coefficient for tri-iodide at 290nm of 4.44 x 10^4 M^{-1} cm^{-1}, in good agreement with the value published previously [Hicks & Gebicki 1979].

2.2.8. Measurement of conjugated diene

Conjugated diene, an intermediate product of lipid peroxidation, absorbs maximally at 233nm wavelength and was detected by UV spectophotometry. Its concentration was calculated using an extinction coefficient of 2.8 x 10^4 $M^{-1}cm^{-1}$ [Pryor & Castle 1984]. A 0.2ml methanol-extracted LDL or liposome sample was added to 0.8ml pure methanol in a cuvette and the absorbance read at 233nm. To correct for the absorbance of phosphatidyl choline at 233nm, commercial phosphatidyl choline was diluted to 10mgs/ml with methanol and dried under nitrogen to remove chloroform and redissolved in 0.9ml methanol and 0.1ml PBS.

2.2.9. Measurement of stable end product of lipid peroxidation with TBA assay

Lipid peroxides decompose to beta-aldehydes, such as malondialdehyde, under acidic conditions in boiling water. The end beta-aldehyde products of lipid peroxidation, were measured using the TBA assay. 0.1ml LDL (LDL samples were dialysed as described in Chapter 4) or liposome sample was mixed with 1ml of 0.67% TBA and 0.5ml 20% trichoroacetic acid, and incubated at 100°C for 20 minutes. After cooling, the reaction mixture was centrifuged at 4,000 rpm for 5 minutes and the absorbance of the supernatant read at 532nm. The concentration of TBARS was calculated using an extinction coefficient of 1.56 x 10^5 M^{-1} cm^{-1} [Slater & Sawyer 1971].

2.2.10. Measurement of H_2O_2 with FOX-1 assay

Glucose or glyceraldehyde was incubated in potassium phosphate buffer (pH 7.4, 10mM) at 37°C under sterile condition. After incubation for 18 hours, 100µL samples were added to 900µL of FOX-1 reagent (100µM xylenol

orange, 250µM Fe^{2+} and 100mM sorbitol dissolved in 25mM H_2SO_4). Absorbance was read at 560nm after 30 minutes incubation at room temperature. Values (peroxide concentration in the sample) are the mean plus/minus standard deviation obtained from duplicate assays. The colour development was due to H_2O_2 in the samples. This was confirmed by addition of catalase (Sigma Type C-40) to the sample for 10 min (at a concentration of 100u/ml) prior to addition of reagent. Standard H_2O_2 from Sigma was used for standard curve during every experiment.

2.2.11. Analysis of LDL glycation

Protein glycation was determined with the method described previously [Wolff & Dean 1987]. LDL was incubated with 25mM glucose (containing 10µCi/ml of [U-^{14}C]-glucose) in the presence or absence of 25 mM $NaCNBH_3$ under sterile conditions. After dialysis of incubated LDL, 0.5ml aliquots were withdrawn from incubation mixtures, precipitated by addition of 200ul of 30% (w/v) trichloroacetic acid (TCA) and centrifuged at 12,000 g for 3 min. The resultant pellet was washed twice in 5% TCA, redissolved in 200 mM potassium phosphate (pH 7.4), by incubation at 37^{o}C for 15 hours, and reprecipitated with 5% TCA. The supernatant was removed. The pellet was washed once more in 5% TCA, redissolved in 0.5ml formic acid. The extent of ^{14}C-glucose incorporation was then measured by scintillation counting.

3.2.12. Oxygen consumption measurements

Oxygen uptake measurements were performed in a Clark-type electrode (Yellow Springs Instruments, Ohio, USA) in a 10ml reaction volume at room temperature. Oxygen uptake in the reaction mixture was initiated by the injection of a small volume (not more than 100µL) of stock H_2O_2 solutions. Starting O_2 concentration was taken as 190µM. The O_2 concentration in the acidic assay system was not markedly lower than that in well-aerated phosphate-buffered saline, pH 7.4.

2.2.13. Statistics

Unless otherwise indicated the data are presented as mean ± standard deviation obtained from quadruplicate assays and are representative of at least two experiments. Statistical analyses were performed by Pearson's correlation and Student's paired t-test (with Unistat III computer program).

Part B. Cell culture

2.2.14. Cells and culture conditions

A Chinese hamster fibroblast (V-79) cell line (from European Animal Study) was used. The cells were grown in a monolayer culture in Dulbecco's Modification of Eagles Medium (DMEM) containing 10% foetal calf serum, 2mM glutamine and 50µg/ml gentamycin. Cell culture was performed in an incubator in which humidity (99% relative humidity), temperature (37°C) and atmosphere (5% CO_2) were regulated.

2.2.15. Assessment of cytotoxicity

Cytotoxic effects of incubated LDL (Chapter 4) or Ascorbate/copper (Chapter 5) were assessed by monitoring DNA synthesis (incorporation of ^{3}H-thymidine into TCA-precipitable material) by V-79 fibroblasts after exposure to incubated LDL or Ascorbate/copper. Quadruplicate monolayer cultures of V-79 cells were grown overnight (12 hours) in DMEM containing 10% foetal calf serum, 2mM glutamine and 50µg/ml gentamycin. Cells were washed twice with PBS before the change of medium (DMEM for LDL experiments and chelex-treated PBS with Ca^{2+} and Mg^{2+} for ascorbate/Cu^{2+} experiments). After exposure to LDL or ascorbate/Cu^{2+} for a certain time the cells were washed twice and the medium was changed again. Then 0.1µCi/ml [methyl-^{3}H]-thymidine (in the presence of 5µM cold thymidine) was added to the medium, and left for a further 4 hours. The cells were not confluent at this stage. Cells were washed twice with PBS and harvested with 0.5ml 0.05% trypsin-0.02% EDTA (Flow Laboratories) and lysed by incubation with 0.5ml 300mM NaOH and 100µl 10% sodium dodecyl sulphate (SDS) for 30 mins. Macromolecules were precipitated by 20% TCA using 100µls of 1mg/ml DNA

as carrier. The resulting TCA insoluble pellet was washed with 5% TCA and ethanol prior to dissolution in 0.5ml formic acid. Then 5ml liquid scintillation counting solution was added and the extent of ^{3}H-thymidine incorporation was then measured by scintillation counting.

CHAPTER 3 THE DEVELOPMENT OF A NEW ASSAY FOR MEASUREMENT OF PEROXIDES

3. 1. Introduction

Increasing interest in assessment of peroxides in biological samples *in vitro* and *in vivo* studies requires a simple and reliable assay for their measurement. However, many of the currently employed assays are either nonspecific, or can be inconvenient and problematic [Hicks & Gebicki 1979; Frew et al 1983; Gutteridge & Halliwell 1990]. For example, iodometric assay relies upon strict anoxia [Hicks & Gebicki 1979]. Measurement of conjugated diene by UV absorbance determination on extracted lipids can be problematic when used to human tissue since other materials in human tissue can absorb at the same wavelength [Gutteridge & Halliwell 1990]. The most widely used assay for the measurement of lipid peroxides is probably the TBA assay. However, the TBA assay can also react with carbohydrate, amino acids and prostaglandins [Gutteridge & Halliwell 1990; Hayaishi and Shimizu 1982]. Moreover, administration of aspirin, which inhibits prostaglandin cycle-oxygenase and blocks prostaglandin synthesis, reduced by 30-60% the amount of serum TBA reactive substances in rabbits [Hayaishi and Shimizu 1982]. Thus, a substantial fraction of serum TBARS may be derived from enzymatic source, and this fraction may be important in diabetes and ageing since total lipid peroxides (judged by TBA assay) are significantly increased in diabetes [Sato et al 1979] and in the elderly [Suematsu et al 1977].

In recent years, methods based on high-performance liquid chromatography (HPLC) [Funk et al 1987; Yamamoto et al 1990; Nakamura & Maeda 1991] and gas chromatography-mass spectrometry (GC-MS) [Van Kuiji et al 1990] have been developed for detecting peroxides. HPLC technique has also been developed for the determination of aldehydic lipid peroxidation products [Esterbauer & Cheeseman 1990]. Although these methods are sensitive for the measurement of lipid peroxides, the establishment of specialized HPLC or GC-MS systems can be problematic. Enzyme associated methods, such as

glutathione peroxidase assay, peroxidase assay and cylooxygenase assay, can be sensitive for the measurement of peroxides [Frew et al 1983; Gutteridge & Halliwell 1990]. However, these methods can be expensive and may be interfered with by organic solvents which are necessary for extracting lipid peroxides in biological samples.

Gupta [1973] initially described a method for detection of H_2O_2, which involves the oxidation of Fe^{2+} by peroxides at low pH in the presence of the ferric-complexing dye xylenol orange. However, this assay cannot be applied to the measurement of lipid peroxide since an undesirable chain reaction occurs between lipid peroxide and metal ions during assaying. One clear aim of this thesis is to develop a simple system which can be applied to the study of lipid peroxidation as well as measurement of water-soluble peroxides. I have modified the Ferrous oxidation/xylenol orange method (FOX assay) which can be used to quantify peroxides at the nanomolar level in aqueous and lipid systems. The FOX assay has been applied to the study of glucose autoxidation during protein glycation, and lipid peroxidation in phosphatidyl choline liposomes, human LDL and erythrocyte membranes. The FOX assay was also compared with the currently widely used iodometric assay, TBA assay and conjugated diene measurement in the study of lipid peroxidation.

3. 2. Methods

3.2.1. Anoxic reaction conditions

To assess the effect of oxygen on the yield of colour in the FOX-1 system, 25ml volumes of 100µM xylenol orange, 250µM Fe^{2+}, 100mM sorbitol in 25mM H_2SO_4 were flushed with nitrogen for 15 mins in a vacuum flask (fitted with a rubber stopper) and then evacuated through a side arm for a further 15 mins. At this time, a small amount of concentrated (1mM) H_2O_2 was injected through the stopper. 25mls of well-aerated reaction solution were used as control. Reaction mixtures were incubated at room temperature for 30 minutes before reading absorbance at 560nm.

3.2.2. Liposome incubation

Liposomes were incubated at a final concentration of 10mgs/ml in 10mM PBS (pH 7.4) with suitable additions in a shaking water bath at 37°C. Liposomes (0.1ml) were removed at appropriate intervals, dissolved in 0.9ml methanol and centrifuged at 12,000 g for 3 minutes. 0.1ml of the supernatant was used for the measurement of lipid peroxide or conjugated diene. Another 0.1ml liposome sample was used directly for the measurement of TBA-reactive substances (for details see Chapter 2, Section 2.2.9).

3.2.3. LDL incubation

LDL (2mg/ml) was incubated alone (control) or with 10μM Cu^{2+} in 100mM potassium phosphate buffer (KP, pH 7.4) at 37°C for 7 days. Before incubation, potassium phosphate buffer was treated with chelating resin and filtered (0.2 μm pore size) to limit contamination with transition metals and microbes. Samples were taken before incubation and after incubation at appropriate intervals and were used for the measurement of TBARS, lipid peroxide and conjugated diene.

3.2.4. Pre-treatment with peroxide-metabolizing enzymes

LDL or liposome, pre-incubated with Cu^{2+}(10μM), was incubated with different enzymes, known to metabolise peroxides, in 10mM PBS (pH 7.4) at 37°C in a shaking water bath (for details see Legends to the Figures). After incubation for 30 minutes, a methanol-extracted sample was used for the measurement of lipid peroxides and conjugated dienes as described in Chapter 2. The incubation samples were directly used to measure TBARS using the methods described in Chapter 2 and in the Legends to Figures.

3.3. Results

3.3.1 The chemistry of FOX assay

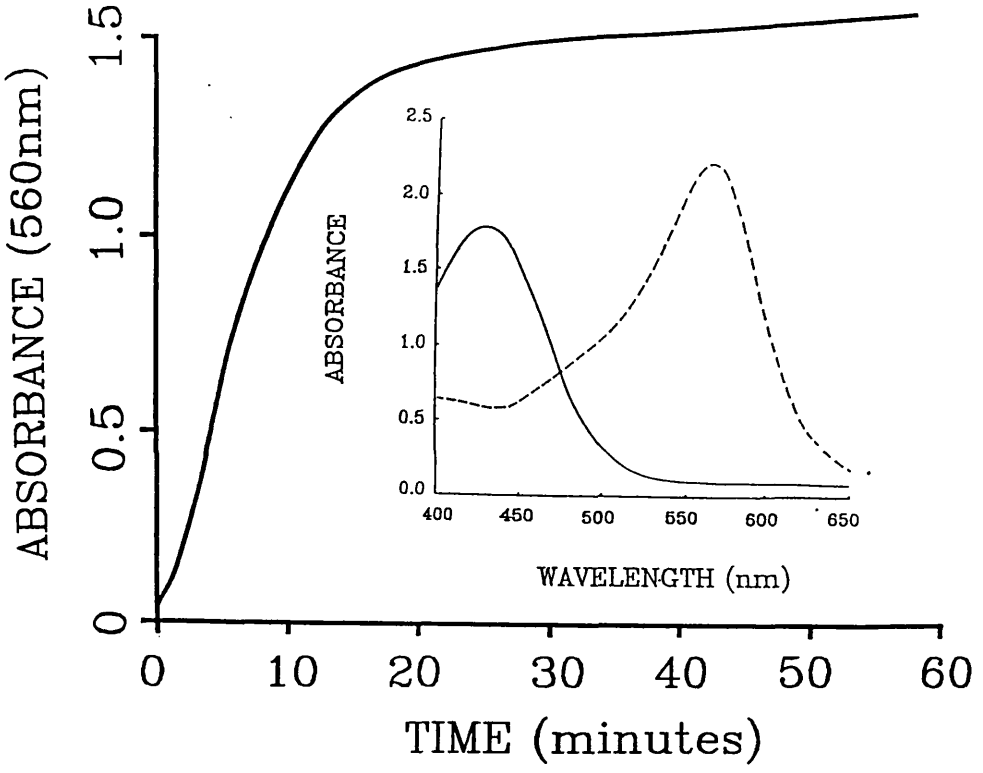
Ferrous ion (Fe^{2+}) is relatively stable to autoxidation in dilute acid, but can be oxidised by peroxides to yield Fe^{3+} and hydroxyl/alkoxyl radicals in

a well-known (but simplified) reaction which proceeds rapidly at room temperature [Koppenol & Butler 1985].

$$Fe^{2+} + ROOH \longrightarrow Fe^{3+} + RO. + OH^-$$

Detection of Fe^{3+} ions formed in a reaction mixture consisting of ferrous sulphate in dilute H_2SO_4 on addition of peroxide can be achieved using the dye xylenol orange, which binds Fe^{3+} forming a complex which absorbs strongly 560nm in dilute acid [Figure 3.1]. Figure 3.1 also shows that the reaction between H_2O_2 and the mixture of ferrous and xylenol orange in acid condition. The colour was stable overnight at room temperature. The extinction coefficient of the Fe^{3+}-Xylenol Orange complex at 560nm (the absorbance maximum) was determined using freshly-made acidic solutions of ferric chloride and determined to be 1.5×10^4 $M^{-1}cm^{-1}$, in good agreement with data reported previously [Gupta 1973]. Preliminary experiments showed that the best linearity with respect to peroxide concentration in the 1-10µM range was obtained with a reaction mixture consisting of 250µM ammonium ferrous sulphate and 100µM xylenol orange in 25mM H_2SO_4.

Figure 3.1. Spectral characteristics of ferric-xylenol orange complex and time course



Time course : *The development of colour at room temperature using 100µM xylenol orange, 250µM Fe^{2+} and 30 µM H_2O_2 in 25mM H_2SO_4.*
Trace is representative of 2 determinations.

Spectrum: *100 µM xylenol orange and 250 µM Fe^{2+} in 25 mM H_2SO_4 before (———) and after (- - -) the addition of 85 µM H_2O_2 and incubation for 45 minutes at room temperature.* Spectra are representative of 2 determinations.

3.3.2. The effects of sorbitol and methanol on colour development

Gupta [1973] refers to interference by organic compounds, such as benzene and hydroquinone, when applying the xylenol orange technique to peroxide determination. He applied sodium chloride to eliminate the effect of organic compounds on colour yield.

In the course of experiments investigating this problem in the above peroxide assay system, sodium chloride had no effect on colour development. Interestingly, it was observed that sorbitol (when added to the unmodified mixture) produced a rapidly-developing and stable signal with H_2O_2 (Table 3.1). The colour yield by the addition of sorbitol may be due to carbon-centred radicals related chain oxidation of ferrous ion.

Table 3.1 The effects of sorbitol and methanol on colour development

	ABSORBANCE (560nm) (mean ± SD)
Control	0.071 ± 0.001
Sorbitol (100mM)	0.695 ± 0.008
Sorbitol (1M)	0.584 ± 0.006
Methanol (75mM)	0.143 ± 0.009
Methanol (75mM)+ Sorbitol (100mM)	0.210 ± 0.001
Methanol (75mM)+ Sorbitol (1M)	0.404 ± 0.004

The reaction of H_2O_2 (3μM) with 100μM xylenol orange and 250μM Fe^{2+} in 25mM H_2SO_4 in the presence or absence of methanol and sorbitol at different concentrations. The reaction mixture was kept at room temperature for 40 minutes before the absorbance was read at 560nm. Data are the mean ± SD of duplicate determinations.

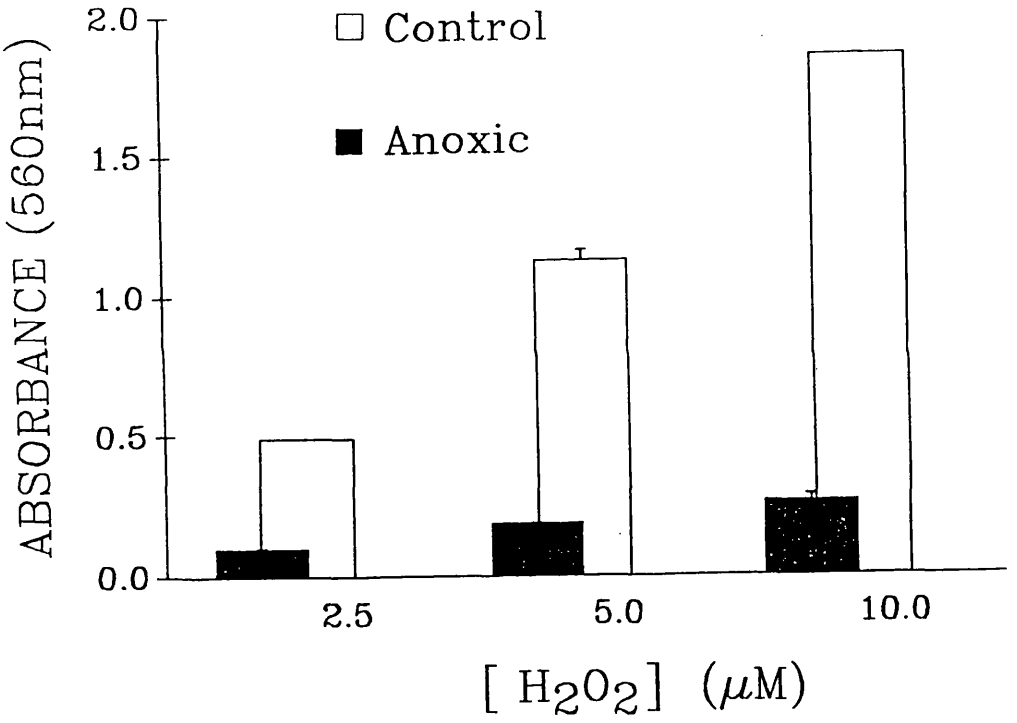
Since we also wished to measure peroxides in methanol extracts (see above) I investigated in detail the effect of methanol on the colour enhancement produced by sorbitol (Table 3.1). Methanol inhibited the colour

development produced by sorbitol but this could be partially overcome by increasing the concentration of the latter monosaccharide. Increasing the concentration of sorbitol above 100mM caused a diminution of the colour yield (Table 3.1).

3.3.3. Chain oxidation and a role for oxygen

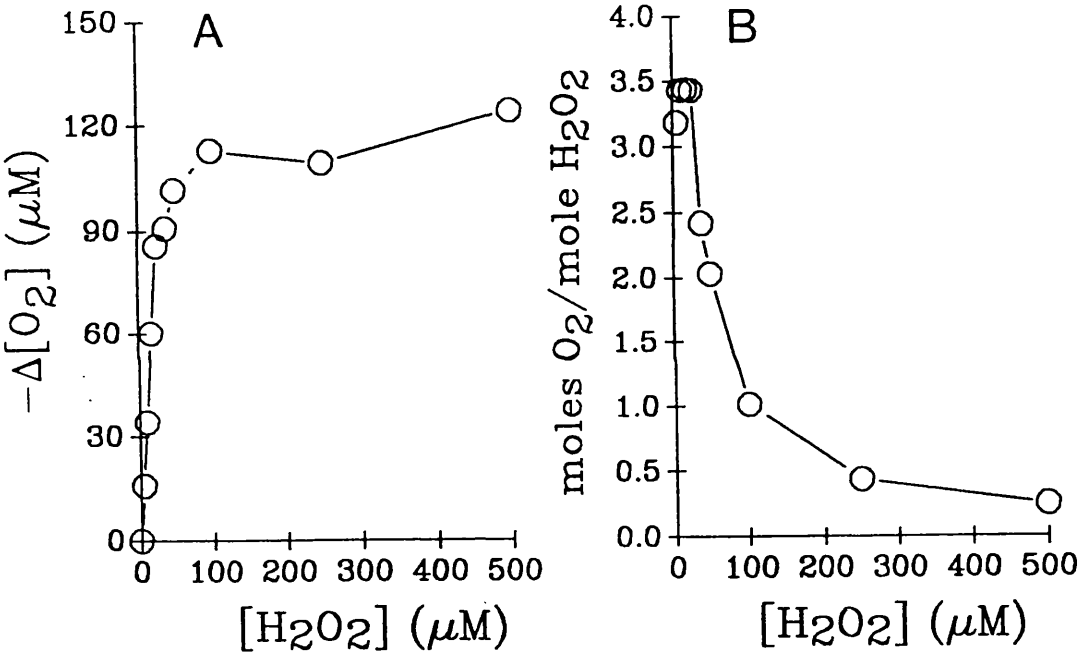
The chain oxidation of Fe^{2+} by peroxide in the presence of sorbitol suggested a role for oxygen in the process since the carbon-centred radicals formed from the attack of hydroxyl radicals on sugars are known to react rapidly with molecular oxygen yielding peroxyl radicals [Bothe et al 1978]. This was illustrated by the 5- to 7-fold lower yield of colour under anoxic conditions (Figure 3.2). Studies of oxygen consumption (using a "Clark-Type" electrode) in the reaction mixture showed that the system was not driven to anaerobic, even at a concentration of 100µM H_2O_2, but reached a plateau at which approximately 65% of the total O_2 was consumed (Figure 3.3). At higher concentrations of H_2O_2 fewer moles of O_2 were consumed per mole H_2O_2 added than at lower concentrations (Figure 3.3). Little O_2 was consumed in the absence of sorbitol.

Figure 3.2. The effect of oxygen on colour development



25mls of reagent solution (250μM Fe^{2+}, 100μM xylenol orange and 100mM sorbitol in 25mM H_2SO_4) were flushed with nitrogen for 15 mins in a vacuum flask (fitted with a rubber bung) and then evacuated through a side arm for a further 15 mins. At this time, a small bolus of concentrated (1mM) H_2O_2 was injected through the bung. The volumes added were 63μL peroxide to give a concentration of 2.5μM; 125μL to give 5.0μM: and 250μL to give 10μM. 25mls of well-aerated reagent were used as control. Reaction mixtures were incubated at room temperature for 30 minutes before reading absorbance at 560nm.

Figure 3.3. Oxygen consumption in the peroxide-detecting reagent

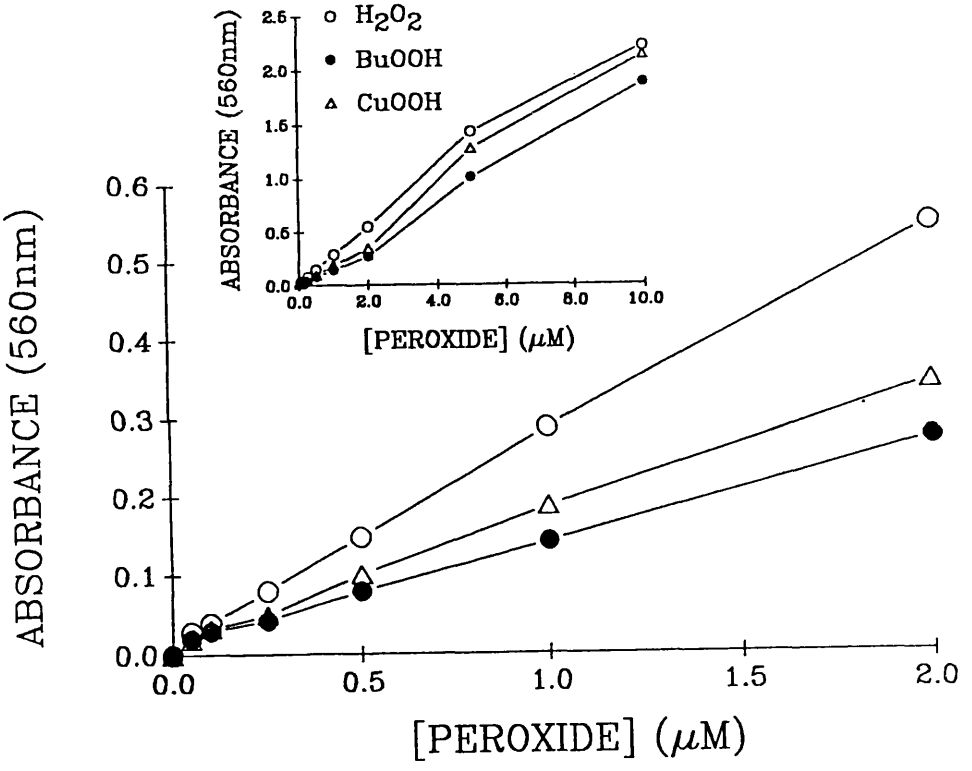


Oxygen uptake measurements were performed in a 10ml reaction volume at room temperature. Oxygen uptake in the reaction mixture was initiated by the injection of a small volume (not more than 100µL) of stock H_2O_2 solutions. Starting O_2 concentration was taken as 190µM. The O_2 concentration in the acidic assay system was not markedly lower than that in well-aerated phosphate-buffered saline, pH 7.4. The absolute value of consumed oxygen concentration (A) and of mole oxygen consumed by per mole H_2O_2 (B) are presented. Data are the average of 2 determinations. Coefficient of variation was less than 15%.

3.3.4. Chain length

Standard curves of H_2O_2, t-butyl peroxide (BuOOH) and cumene peroxide (CuOOH) in FOX-1 assay (250µM Fe^{2+}, 100µM xylenol orange, 25mM H_2SO_4 and 100mM sorbitol) are shown in Figure 3.4. It was observed that the peroxides were reactive in the order H_2O_2 > CuOOH > BuOOH and showed slight curvilinear trends but gave reproducible data (coefficient of variation typically less than 4%). The "extinction coefficients" (estimated by curve fitting to a first-degree function) were for H_2O_2 2.7 x $10^5 M^{-1}cm^{-1}$; for CuOOH 1.7 x $10^5 M^{-1}cm^{-1}$ and for BuOOH 1.3 x $10^5 M^{-1}cm^{-1}$. Given that the extinction coefficient of the Fe^{3+}-xylenol orange complex is 1.43 x 10^4 $M^{-1}cm^{-1}$, it follows that there is substantial chain oxidation of Fe^{2+} in the assay system. Approximately 19 moles of Fe^{2+} are oxidised to Fe^{3+} for each mole of H_2O_2 and somewhat fewer for the other peroxides studied.

Figure 3.4. Standard curves of H_2O_2, t-butyl peroxide and cumene peroxide measured with FOX-1 reagent.



Peroxides, in the range 0-2μM and 0-10μM in aqueous solution, were measured with 100μM xylenol orange, 250μM Fe^{2+} and 100mM sorbitol in 25mM H_2SO_4.

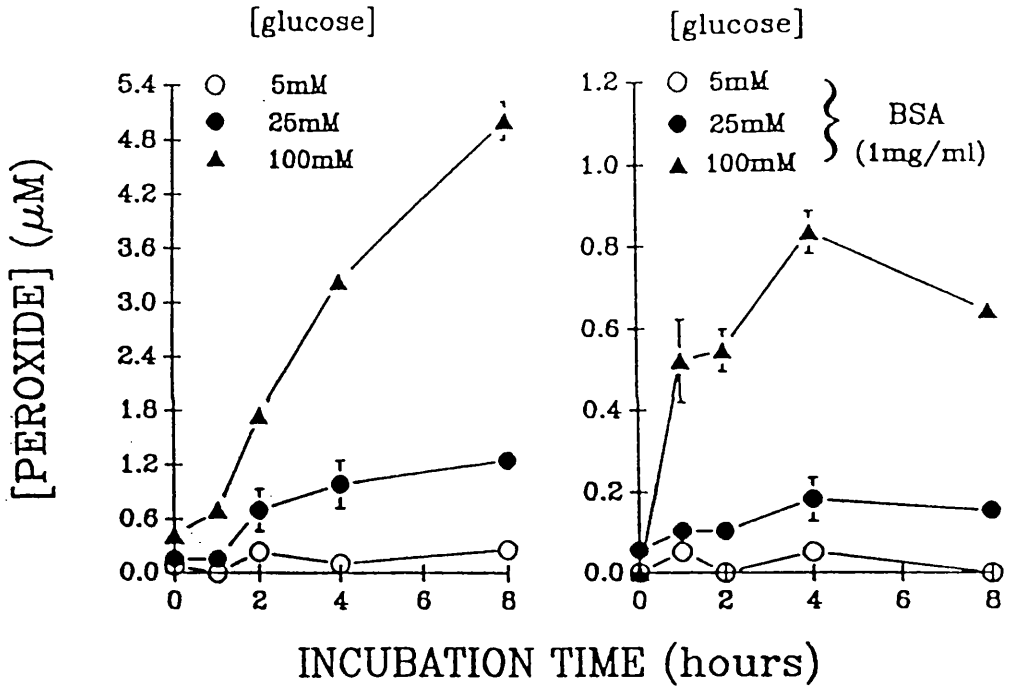
3.3.5. Validation of the FOX-1 assay in a study of glucose oxidation

The FOX-1 assay has been validated by examining the formation of H_2O_2 by "autoxidising" glucose. Transition metal-catalysed glucose oxidation has been suggested to be a factor potentially responsible for protein damage in experimental models of diabetes mellitus [Wolff & Dean 1987; Hunt et al 1988]. However, the production of H_2O_2 by glucose in the presence of protein has previously only been inferred by effects of catalase [Wolff & Dean 1987; Hunt et al 1988] since the steady-state levels are too low for reliable measurement by other methods [Wolff & Dean 1987]. In contrast, using the assay described here, the accumulation of H_2O_2 with respect to time and concentration can be monitored. Glucose (5 to 100mM) was incubated in 10mM potassium phosphate buffer for up to 20 hours at 37°C under sterile conditions (Figure 3.5) with differences in peroxide level in the range 0-200nM clearly observed. Catalase completely abolished colour development when added prior to the peroxide reagent demonstrating that H_2O_2 was being measured.

In the presence of serum albumin (1mg/ml) the steady-state levels of H_2O_2 detected were approximately 6-fold lower than in the absence of the protein (Figure 3.5). Albumin chelates copper ion [Halliwell 1988] and thus may inhibit glucose "autoxidation" via chelation of trace amounts of copper. Reaction of the open-chain form of glucose with protein amino groups will also retard the formation of H_2O_2. Although the steady-state concentrations of H_2O_2 are low in absolute terms they are in the range expected to contribute to protein damage [Hunt et al 1988].

However, the subsequent presence of albumin (final concentration of 100µgs/ml) in the peroxide-measuring reaction mixture after incubation caused no decrease in H_2O_2 recovery since BSA does not bind iron. Calculated levels of H_2O_2 agreed well with that estimated using the extinction coefficient determined above, or derived from spiking identical glucose/protein samples with small concentrations of H_2O_2.

Figure 3.5. The production of H_2O_2 by autoxidising glucose



Glucose (5mM, 25mM and 100mM) with or without the presence of 1mg/ml bovine serum albumin was incubated in potassium phosphate buffer (pH 7.4, 10mM) at 37°C. After incubation, 100μL samples were added to 900μL of reagent (100μM xylenol orange, 250μM Fe^{2+} and 100mM sorbitol in 25mM H_2SO_4). Absorbance was read at 560nm after 40 minutes incubation at room temperature following a 2 minute centrifugation at 12 000g to remove any flocculated protein. Values (peroxide concentration in the sample) are the mean ± standard deviation obtained from duplicate assays. Native (but not heat-denatured) catalase (Sigma Type C-40) added to the sample (at a concentration of 100 units/ml) prior to addition of reagent abolished the signal.

3.3.6. Undesirable chain reactions in the measurement of lipid peroxide and their control

Phosphatidyl choline liposomes were chosen as a model for biological lipid peroxidation and known strategies were adopted to stimulate their peroxidation, such as incubation with 5μM Cu^{2+}. It was observed, however, that liposomes were extremely prone to peroxidation in the presence of Fe^{2+} under acidic conditions and this led to high levels of colour development even under conditions where the extent of pre-assay peroxidation was expected to be minimal (Figure 3.6). For example, the addition of 5μM Cu^{2+} pre-incubated liposomes, solubilised in methanol, to an assay system consisting of 100μM xylenol orange and 250μM Fe^{2+} in 25mM H_2SO_4 led to the development of intense colour, with no clearly defined endpoint (Figure 3.6: Curve A). This is presumably due to the small quantities of peroxide present in the sample. Such peroxide contamination would initiate uninhibited lipid peroxidation in the assay.

(1) ROOH + Fe^{2+} ———> RO. + OH^- + Fe^{3+}

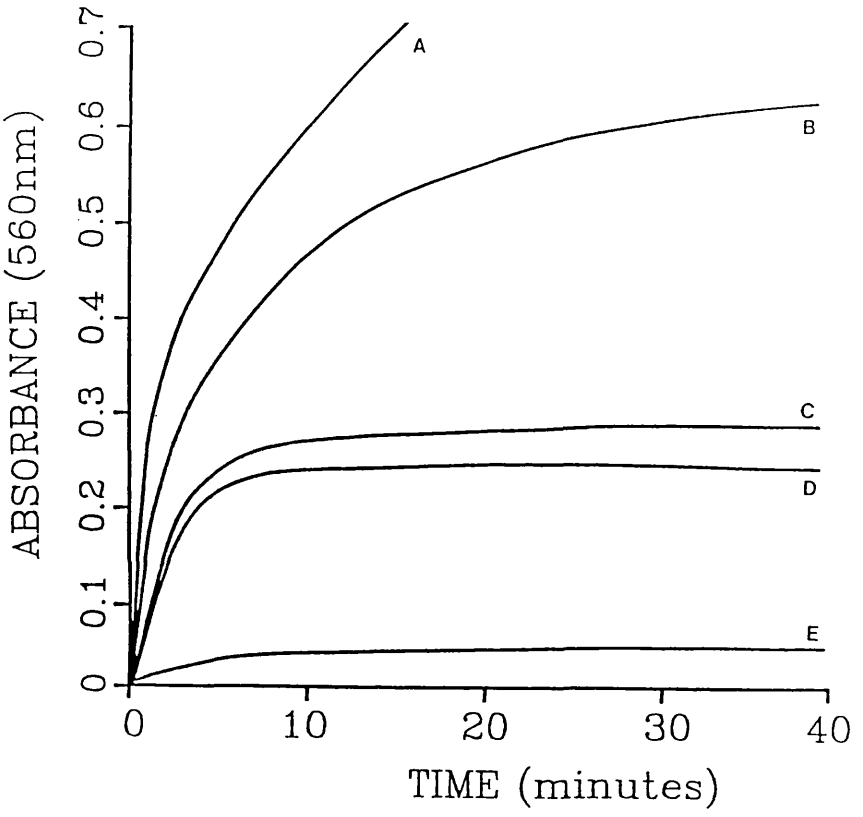
(2) RH + RO. ———> R. + ROH

(3) R. + O_2 ———> ROO.

(4) ROO. + RH ———> ROOH + R. ------> etc

The inclusion of the peroxidation inhibitor butylated hydroxytoluene (BHT) to the assay strongly inhibited this undesirable chain reaction (Figure 3.6). A high concentration of BHT (4mM) was required to achieve a definable endpoint (Figure 3.6: Curve C & D). The endpoint was greater when the liposomes were pre-peroxidised by incubation with 5μM Cu^{2+} for 2 hours (Figure 3.6: Curve C & D) than that of freshly prepared liposome (Figure 3.6: Curve E), indicating the detection of peroxide present in the liposomes prior to assay. The high concentration of chain-breaking antioxidant required is presumably a reflection of the very high rate of initiation and propagation of lipid peroxidation which can occur in the lipid-rich iron-containing assay environment. BHT, in this model system, is thus required at high concentrations but may be less critical in circumstances where the concentration of peroxidisable lipid is lower. The influence of BHT is to inhibit reaction (2) by direct reaction with RO. formed in reaction (1):

Figure 3.6. Undesirable chain oxidative reactions and their control.



Curve A: *Preincubated liposomes (final concentration of phosphatidyl choline 65μM) in 100μM xylenol orange and 250μM Fe^{2+} in 25mM H_2SO_4 at room temperature.*

Curve B: *Preincubated liposomes in 250μM Fe^{2+}, 100μM xylenol orange, 2mM BHT and 25M methanol in 25mM H_2SO_4 at room temperature.*

Curve C: *Preincubated liposomes in 250μM Fe^{2+}, 100μM xylenol orange, 100mM sorbitol, 4mM BHT and 25M methanol in 25mM H_2SO_4 at room temperature.*

Curve D: *Preincubated liposomes in 250μM Fe^{2+}, 100μM xylenol orange, 4mM BHT and 25M methanol in 25mM H_2SO_4 at room temperature.*

Curve E: *Fresh liposomes in 250μM Fe^{2+}, 100μM xylenol orange, 4mM BHT and 25M methanol in 25mM H_2SO_4 at room temperature.*
All curves are representative of two experiments.

(5) RO. + BHT ——> ROH + BHT semiquinone radical

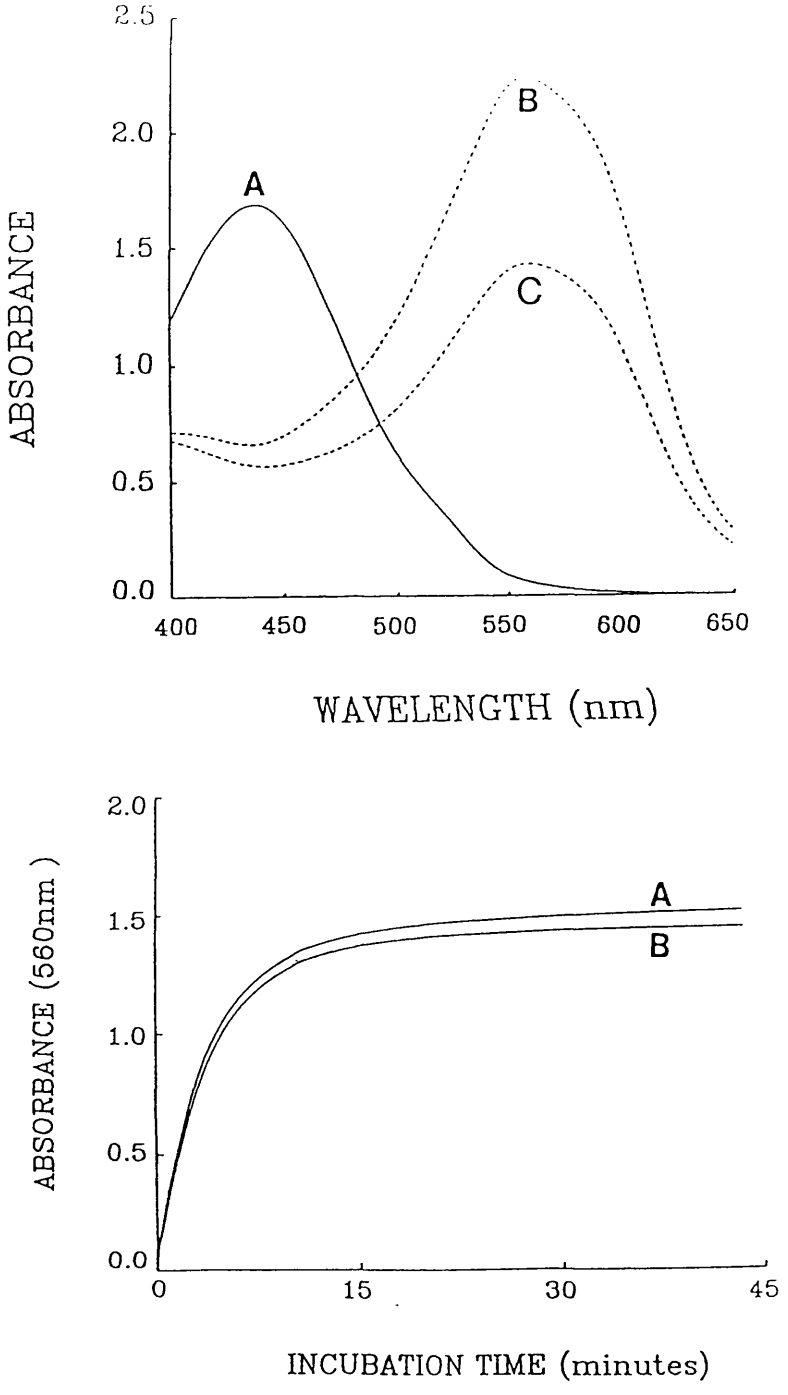
However, the addition of BHT and methanol predictably diminished the colour enhancement produced by sorbitol (Figure 3.6: Curve C & D). However, BHT has no effect on the measurement of H_2O_2 with FOX 1 assay.

3.3.7. Composition of FOX-2 assay and reactivity of peroxides

Thereafter, the FOX-2 assay developed here for the study of lipid peroxidation, reagent contains pure methanol (90%), H_2SO_4 (25mM), BHT (4mM), xylenol orange (100µM) and ammonium iron(II) sulphate hexahydrate (250µM). High concentration of BHT is employed to break the undesirable chain reaction during assaying. A high concentration of methanol (90%) is necessary in the FOX-2 reagent for dissolving BHT. Sorbitol was removed from the FOX-2 reagent since there was no big difference in colour development in the assay with or without addition of sorbitol (Figure 3.6: C & D).

Generally, 0.1 ml of methanol-extracted liposomes or LDL samples (oxidized with Cu^{2+}) were mixed with 0.9 ml FOX-2 reagent and incubated for 30 minutes at room temperature prior to absorbance measurement. Figure 3.7 shows that the reaction between H_2O_2 or linoleic hydroperoxide and FOX-2 reagent was completed in 15 minutes with the formation of Fe^{3+}-xylenol orange complex, which absorbs at 560nm. The colour was stable overnight at room temperature.

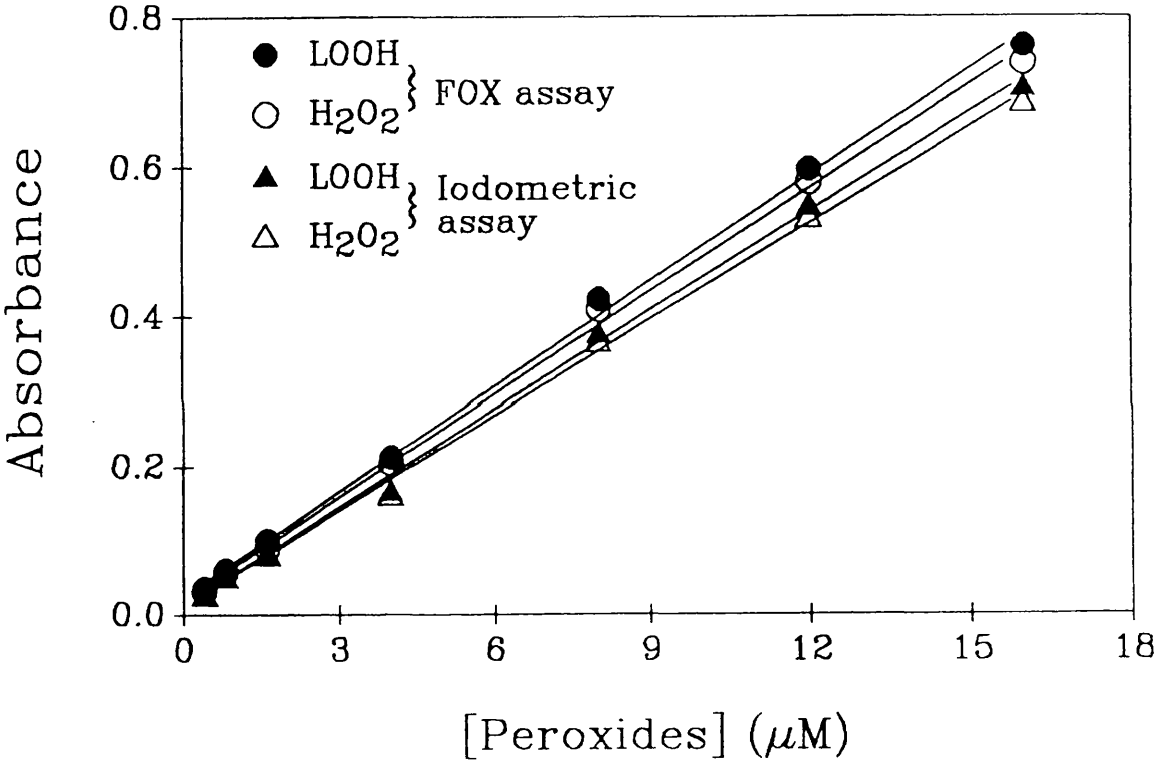
Figure 3.7. Spectral characteristics of FOX-2 and time course



Spectrum: *100 µM xylenol orange and 250 µM Fe^{2+}, 25 mM H_2SO_4, 4 mM BHT in 90% methanol before (A) and after the addition of 50 µM H_2O_2 (B) or 25 µM linoleic acid hydroperoxide (C) and incubation for 45 minutes at room temperature.*

Time course : *The development of colour at room temperature using FOX-2 reagent (100µM xylenol orange, 250µM Fe^{2+}, 4mM BHT, 25mM H_2SO_4 in 90% methanol and 10% distilled water) in the presence of 30 µM linoleic hydroperoxide (A) or 30 µM H_2O_2 (B).*

Figure 3.8. Standard curves of hydrogen peroxide (H_2O_2) and linoleic hydroperoxide (LOOH) in the range of 0-16 µM in FOX-2 assay.



Peroxides were measured with the FOX-2 assay and the iodometric assay (details see Materials and Methods). Linoleic acid (dissolved in methanol and kept at -20°C) did not generate any colour either in the FOX-2 assay or in iodometric assay at the same concentration as linoleic hydroperoxide.

H_2O_2 and linoleic hydroperoxide were used as standard peroxides for measurement of the apparent extinction coefficient in the modified FOX-2 assay and for comparison with the iodometric assay. The results (Figure 3.8) show that H_2O_2 and linoleic hydroperoxide give apparent extinction coefficients for these peroxides at 560 nm of 4.56×10^4 and 4.70×10^4 $M^{-1}cm^{-1}$, respectively. Comparison of the FOX method with the iodometric assay [Hicks & Gebicki 1979] shows that the methods have similar sensitivities towards H_2O_2 and linoleic hydroperoxide (Figure 3.8). It is clear that limited chain-oxidation of Fe^{2+} still occur, approximately 3 moles of Fe^{3+} formed per mole hydroperoxide. Nevertheless, the colour development is stable after incubation of peroxides with FOX-2 reagent for 30 minutes at room temperature, and this makes the FOX-2 assay more sensitive to peroxide measurement.

Table 3.2 Reactivity of various peroxides to FOX-2 assay

	Percentage reactivity (relative to H_2O_2)
Hydrogen peroxide	**100%**
Linoleic hydroperoxide	**103%**
t-Butyl hydroperoxide	**96%**
Cumene hydroperoxide	**98%**
Di-cumyl peroxide	**12%**
Benzoyl peroxide	**9%**
Lauroyl peroxide	**21%**

100% refers to an apparent "extinction coefficient" of 4.56×10^4 $M^{-1}cm^{-1}$ for H_2O_2 in the FOX-2 reagent. Note that the extinction coefficient of the Fe^{3+}-xylenol orange complex is 1.5×10^4 $M^{-1}cm^{-1}$ and thus there is a chain-length of 3 moles Fe^{2+} oxidised per mole H_2O_2 under these conditions.

Reactivity of other peroxides in the FOX-2 assay was tested using standard commercial t-butyl hydroperoxide (BuOOH), cumene hydroperoxide (CuOOH), di-cumyl peroxide, benzoyl peroxide and lauroyl peroxide. Table 3.2 (see above) lists the reactivity of these peroxides with the FOX-2 reagent.

Acylhydroperoxides and alkylhydroperoxides have similar reactivity with FOX-2 reagent as H_2O_2. The endoperoxides exhibit lower or no reactivity in the assay.

3.3.8. Comparison of lipid peroxides with conjugated diene and TBARS in oxidized phosphatidyl choline liposome and LDL

Lipid peroxidation is catalysed by trace amounts of transition metals, such as Fe^{2+} and Cu^{2+} [Chan et al 1982; Steinberg et al 1989]. Liposomes (10mgs/ml in 10mM PBS, pH 7.4) were incubated alone or with 10μM Cu^{2+} at 37°C in a shaking water bath. Figure 3.9 shows that the formation of lipid peroxides (measured by FOX-2 assay), conjugated diene and TBARS is time dependent. However, the concentration of lipid peroxide was slightly higher than that of conjugated diene, whereas the values estimated for TBARS given as malondialdehyde equivalents were much lower.

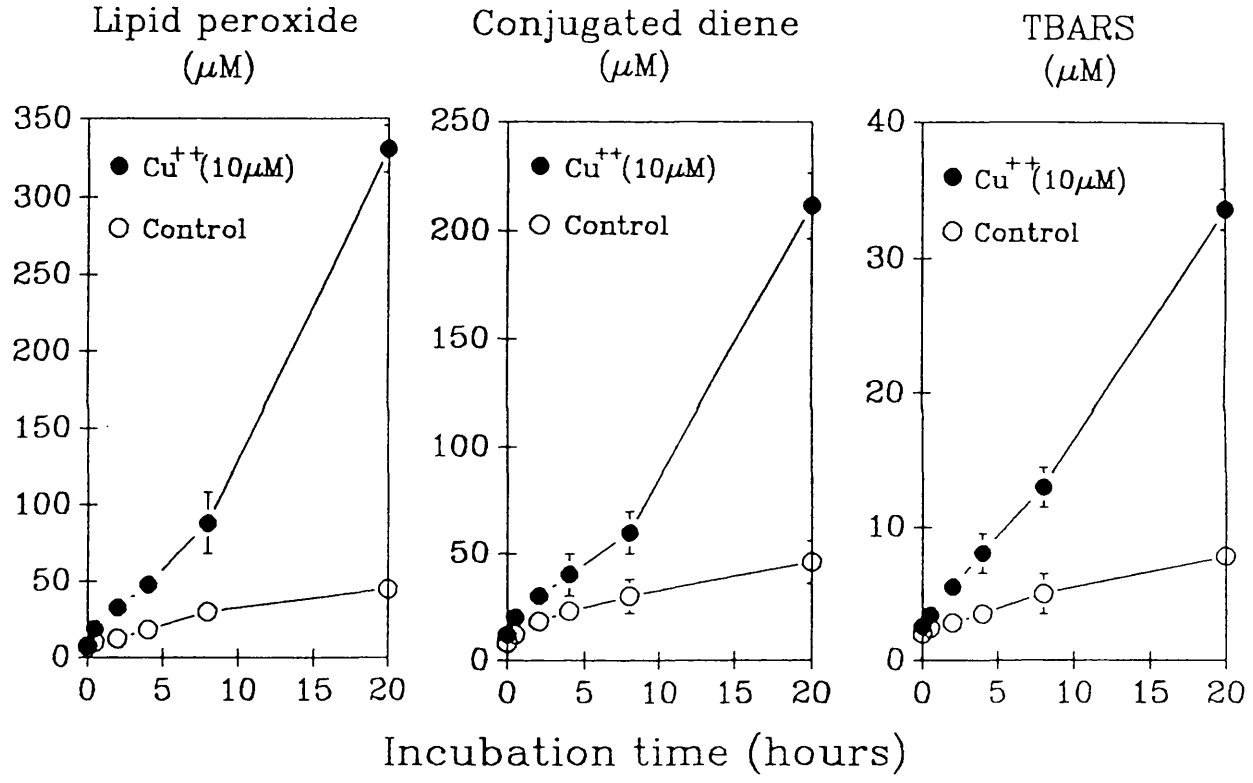
In the LDL oxidation experiment, 2 mg/ml of LDL were incubated with 10 μM Cu^{2+} in 100 mM potassium phosphate buffer (pH 7.4) at 37°C. The values of lipid peroxides (FOX-reactive substances: FOXRS) generated upon oxidation of LDL were compared with those of the conjugated diene and TBARS, measured at different time points. Figure 3.10 shows a slight increase in the concentrations of lipid peroxides, conjugated diene and TBARS in a LDL sample after incubation for 7 days in the absence of added catalyst. There was, by contrast, a large accumulation of lipid peroxides, conjugated diene and TBARS in LDL incubated in the presence of Cu^{2+} over the first 24 hours. The levels of lipid peroxides and TBARS then dropped dramatically, as reported by El-Saadani et al [1989]. However, the level of conjugated diene, as judged by absorbance at 233nm, continually increased in Cu^{2+}-incubated LDL over 7 days' oxidation. The time-related changes in the level of lipid peroxides can be explained by the activity of Cu^{2+}, which catalyses lipid peroxidation, as well as peroxide decomposition [O'Brien 1969]. After 24 hours' incubation, lipid peroxidation of LDL reaches the highest level and then decomposition of lipid peroxides becomes the major process of LDL oxidation with the formation of carbonyls, including malondialdehyde and other aldehydes [El-Saadani et al 1989; Esterbauer et al 1987; Frankel 1987; Gutteridge et al 1982]. Malondialdehyde and other aldehydes, which are major TBA reactive

substances, form polymalondialdehyde and aldehyde polymers which appear less reactive to TBA [Gutteridge 1975]. They are also able to form conjugated Schiff bases, which absorb in the ultraviolet region [Gutteridge & Kerry 1981], by covalent attachment to the amino group of protein [Kikugawa & Beppu 1987; Steinbrecher et al 1987]. The reaction of carbonyls with lipoprotein, after a long incubation at 37°C, may contribute to the absorbance increase at 233nm and the decrease in the level of TBARS [El-Saadani et al 1989]. Other oxidation products in oxidised LDL may also contribute to the absorbance increase at 233nm.

3.3.9. Confirmation of peroxide content in LDL and liposome

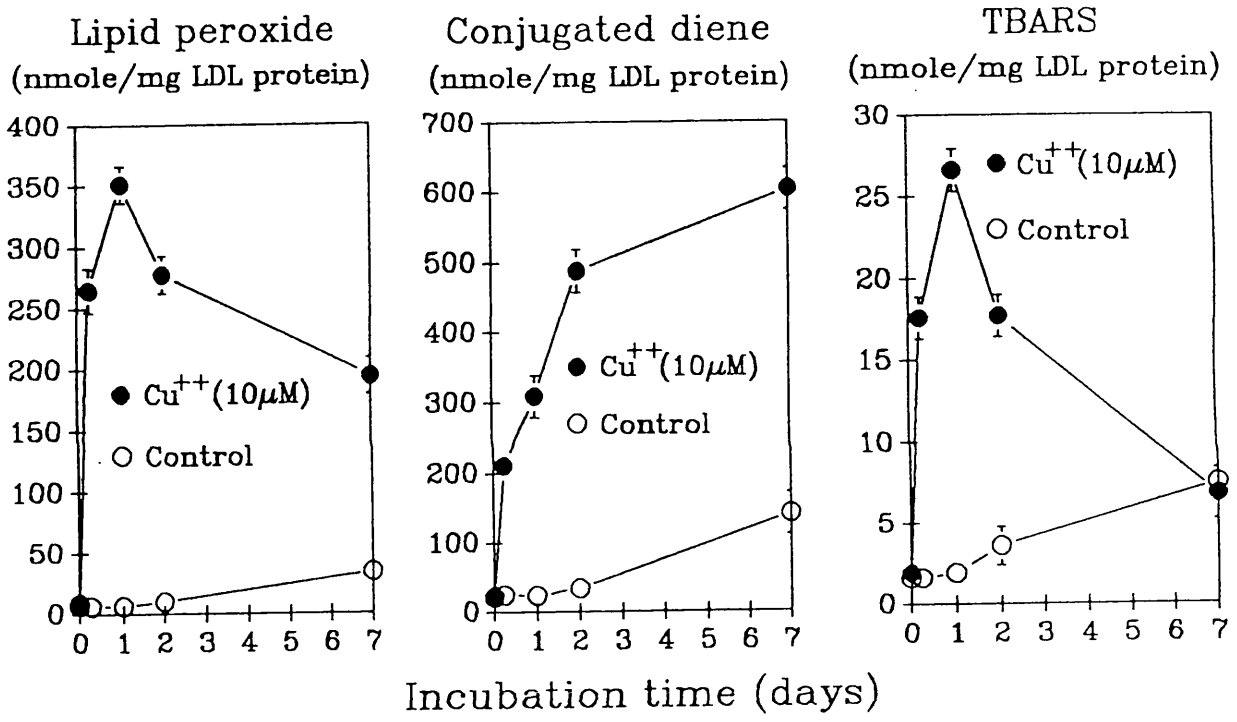
The substances reactive with FOX-2 reagent in LDL and liposome were confirmed as lipid peroxides by pre-treatment with enzymes involved in peroxide metabolism. It is well known that glutathione peroxidase can metabolize peroxides, such as phospholipid hydroperoxide, fatty acid hydroperoxide, cholesterol ester hydroperoxide, and H_2O_2, in the presence of GSH and phospholipase A_2 [Grossman & Wendel 1983; Van Kuijk et al 1989; Thomas & Girotti 1988]. The latter hydrolyses esterified peroxide [Grossman & Wendel 1983; Van Kuijk et al 1989; Thomas & Girotti 1988]. In the experiment shown in Figure 3.11, LDL and liposome were preincubated with 10μM Cu^{2+} for 6 hours and 20 hours, respectively. To the samples of oxidized LDL (Figure 3.11 A) and liposomes (Figure 3.11 B) were added (A) control buffer, (B) catalase and (C) GSH, glutathione peroxidase and phospholipase A_2. After incubation for 30 minutes at 37°C, the yields of FOX-reactive substances (FOXRS), conjugated diene and TBARS were compared. Catalase reduced the colour yield of FOX by 10-15%, which indicates the formation of H_2O_2 during oxidation of LDL and liposomes, while glutathione peroxidase, in the presence of GSH and phospholipase A_2, produced a 90-95% decrease in the colour yielded with FOX assay. By contrast, levels of conjugated diene and TBARS were not influenced by this enzyme pre-treatment.

Figure 3.9. The accumulation of lipid peroxides (FOXRS), conjugated diene and TBARS in liposomes



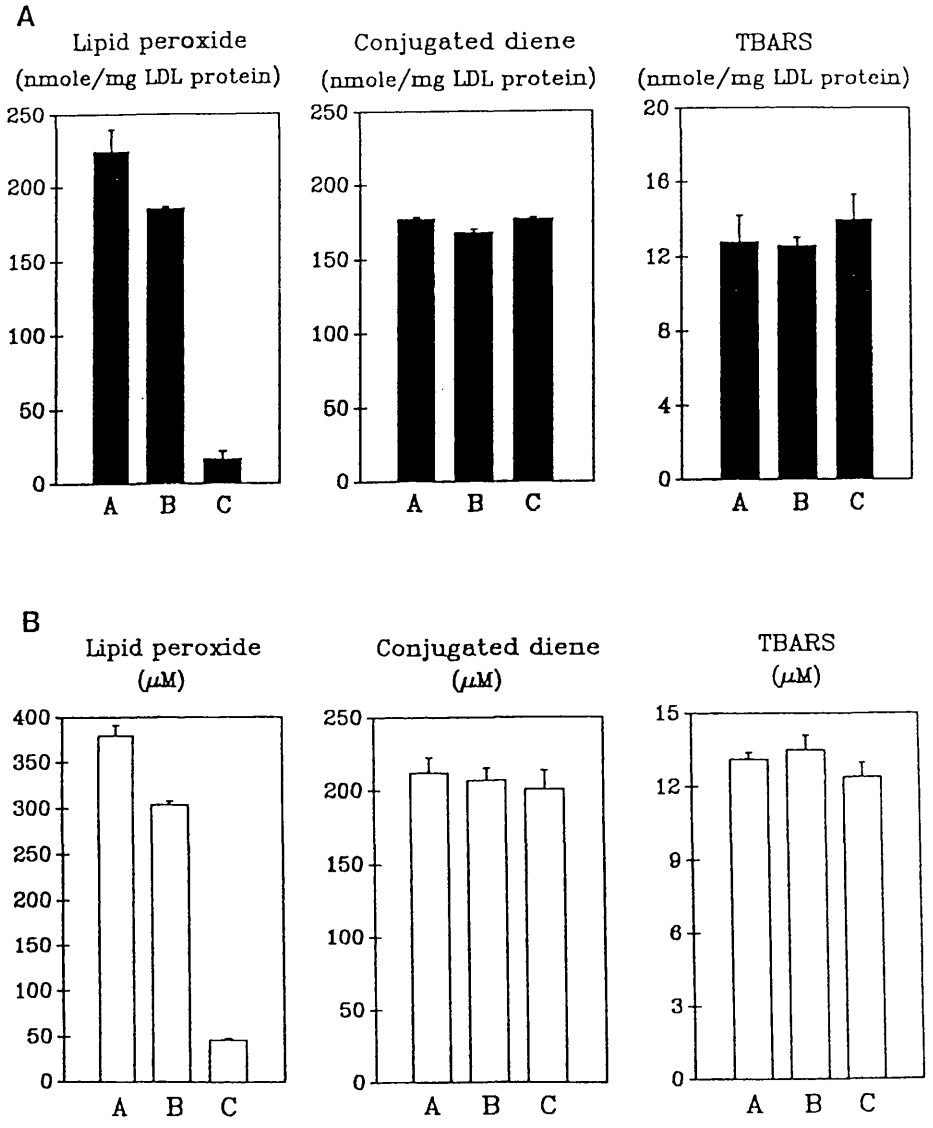
Liposomes (10mg/ml) were incubated alone (control) or with 10μM Cu^{2+} in PBS (10mM, pH 7.4) at 37°C for 20 hours. Samples were taken at zero time and at varying time intervals up to 20 hours. 0.1 ml of the above sample was directly used for the measurement of TBARS (details see Materials and Methods section). Also, 0.1 ml incubated sample was dissolved in 0.9 ml pure methanol and after centrifugation at 12,000g for 3 mins, 0.1 ml supernatant was mixed with FOX-2 reagent for the measurement of lipid peroxides and 0.2 ml supernatant was used for detection of conjugated diene.

Figure 3.10. The accumulation of lipid peroxides (FOXRS), conjugated diene and TBARS in LDL



LDL (2mg/ml) was incubated alone (control) or with 10μM Cu^{2+} in 100mM potassium phosphate buffer (KP, pH 7.4) at 37°C for 7 days. Before incubation, potassium phosphate buffer was treated with chelating resin and filtered (0.2 μm pore size) to limit contamination with transition metals and microbes). Samples were taken before incubation and after incubation for 6, 24, 48 and 168 hours. 0.1 ml of the above sample was directly used for the measurement of TBARS (for details see Chapter 2). Also, 0.1 ml incubated samples were mixed with 0.05 ml of catalase (500 units/ml) at 25°C for 15 mins. Then 0.85 ml methanol was added and mixed for 15 mins to extract lipid peroxides. After centrifugation at 12,000g for 3 mins, 0.1 ml supernatant was used for the measurement of lipid peroxides with the FOX-2 reagent. For detection of conjugated diene, 0.1 ml incubated samples were directly mixed with 0.9 ml methanol for 15 mins. After centrifugation, 0.2 ml supernatant was used for the detection of conjugated diene.

Figure 3.11. The comparison of FOXRS with conjugated diene and TBARS after pre-treatment with enzymes of lipid peroxides in LDL and liposomes.



LDL (2mg/ml, Figure 3.11 A) and liposomes (10mg/ml, Figure 3.11 B) were preincubated with 10 μM Cu^{2+} in 10mM PBS, (pH 7.4) for 6 hours and 20 hours, respectively. LDL and liposome samples (diluted 2-fold in PBS) were exposed in PBS for 30 minutes at 37°C to (B) Catalase (100 units/ml) and (C) GSH (500μM), glutathione peroxidase (10 units/ml) and phospholipase A_2 (10 units/ml). (A) represents the control. At the end of the incubation period the samples were diluted 1:9 with methanol and centrifuged at 12,000g for 3 minutes. 0.2 ml supernatant was then analyzed for peroxides and conjugated diene as described in Materials and Methods. 0.1 ml enzyme-treated sample was directly used for the detection of TBARS.

3.3.10. Validation of FOX-2 assay in the measurement of peroxidation in erythrocyte membranes

FOX-2 assay was further used to measure lipid peroxide in red blood cell membranes. Red blood cells were exposed to BuOOH *in vitro*, in the presence of Cu^{2+}, and in the presence or absence of Trolox, a water-soluble analogue of vitamin E. The contents of lipid peroxide and TBARS in red blood cell membranes are shown in table 3.3. In the case of this biological membrane it was found that TBARS was higher, both at zero time, and during the incubation, than ROOH. It is clear, however, that there are increases in TBARS and ROOH, both with respect to time in the control incubated cells, as well as a result of the specific oxidative stress associated with Cu^{2+}/BuOOH. Trolox had an inhibitory effect on both TBARS and ROOH accumulation. The reversal of the TBARS/ROOH ratio observed in this instance reflects either a profoundly different mechanism of lipid peroxidation or the possibility that the membrane-associated TBARS may be derived from other sources, such as oxidising surface sugars.

TABLE 3.3 Lipid peroxidation of red blood cell membranes

	ROOH (μM)		TBARS (μM)	
Incubation time (hours)	2	6	2	6
Control	7.4 ± 0.2	14.2 ± 0.5	11.9 ± 0.4	20.7 ± 3.4
BuOOH + Cu^{2+}	30.3 ± 2.6	44.2 ± 4.4	40.8 ± 5.4	53.2 ± 6.0
BuOOH + Cu^{2+} + Trolox	22.5 ± 2.3	30.8 ± 4.8	29.2 ± 4.0	38.8 ± 5.6

Red blood cells were prepared with the method described in chapter 2. 10% of packed Cells were exposed to various combinations of BuOOH (1mM), Cu^{2+} (20μM) and Trolox (2mM) in 10 mM PBS (pH 7.4) at 37°C. At appropriate intervals, samples (500μL) were added to 10ml of 5mM potassium phosphate buffer (pH 8) and centrifuged at 15,000 rpm for 20 minutes at 4°C. After washes as described in chapter 2, cell membranes were extracted by vortexing with 1ml of methanol (HPLC Grade, British Drug Houses, Poole, UK) for 15 minutes. Protein was precipitated by centrifugation at 12,000rpm for 2 minutes. The supernatant was retained for analysis. The formation of lipid peroxide (ROOH) and thiobarbituric acid reactive substances (TBARS) in red blood cell membrane after exposure were measured with the methods described in chapter 2. Concentrations (μM) of ROOH and TBARS in the red blood cell incubation mixture are expressed as the mean ± SD of triplicate assays. Prior to incubation the concentrations of ROOH and TBARS were 0.9 ± 0.1μM and 3.2 ± 0.3μM respectively.

3.4. Discussion

3.4.1. Sensitivity of the FOX assays

The FOX-1 assay consisting of Fe^{2+}, xylenol orange, H_2SO_4 and sorbitol, is simple and sensitive. FOX-1 assay can be used for the measurement of peroxides, such as H_2O_2, in aqueous extracts. BHT can be added and sorbitol can be removed in the FOX-2 assay (a methanol-containing version of the FOX-1 assay) to avoid potential problems of lipid peroxidation within the assay itself. The FOX-2 assay can be used for the measurement of peroxides in methanolic extracts.

Chain oxidation of Fe^{2+} in the presence of sorbitol allows the FOX-1 method to assess difference of peroxide concentration in the 20 - 100nM range, assuming the availability of a spectrophotometer which can reliably register absorbance changes of 0.005 AU. This renders it 20 times as sensitive as the horseradish peroxidase-catalysed coupling of 4-aminoantipyrine/phenol or the more complex glutathione peroxidase/reductase systems recommended by Frew and colleagues [1983]. Furthermore, it is as sensitive as, but simpler than, the methods based upon the anoxic haematin-catalysed oxidation of dichlorofluorescin to dichlorofluorescein [Cathcart et al 1983], or electroanalytical approaches [Funk et al 1987]. The FOX-2 method, for use with materials which are not water-soluble or prone to lipid peroxidation, has a sensitivity of about 500nM.

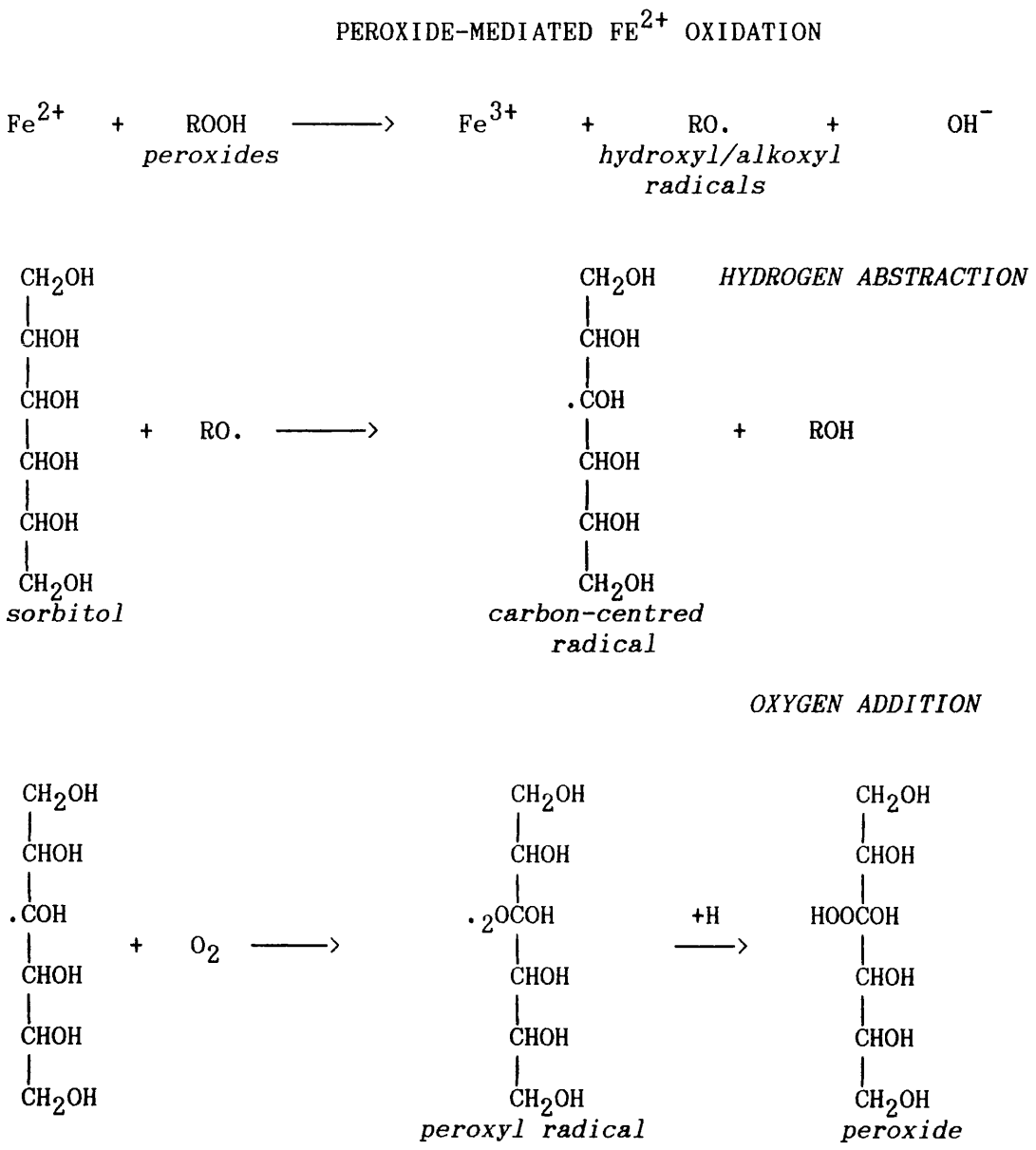
3.4.2. A suggested reaction mechanism

Understanding of the mechanism of the reactions leading to colour development is necessary in order to be able to judge those factors which might interfere. A tentative sequence of chemical events in the peroxide-detecting reaction is given in the Figure 3.12. An important component of the chain reaction is the reaction of hydroxyl/alkoxyl radicals with sorbitol. Sorbitol, like other molecules possessing aliphatic C-H groups, is oxidised by these radicals to carbon-centred radicals which react further with oxygen generating H_2O_2 via hydroperoxyl radicals [Koppenol &

Butler 1985]. Chain-propagation of Fe^{2+} oxidation is ascribed to the production of further H_2O_2 from hydroperoxyl radical ($HO_2.$), formed subsequent to the reaction of O_2 with carbon-centred sorbitol radicals (Figure 3.12). In principle this reaction should become self-prolonging. Competitive radical reactions, such as dimerization of carbon-centred radicals, must limit the reaction extent. This speculation is strengthened by the inhibitory effect of methanol upon the colour yield with sorbitol and by the decreased consumption of O_2 at higher peroxide concentrations. The greater colour yield with H_2O_2 compared with butyl peroxide and cumene peroxide in the aqueous system (Figure 3.4) may be ascribed to a greater rate of reaction of the hydroxyl radical with sorbitol, than is the case for the cumenoxyl or t-butyloxyl radicals.

The ability of some hydroxyl radical scavengers (such as sorbitol) to promote substantial Fe^{2+} oxidation, but not others (such as methanol) may be related to a relatively slower rate of dimerization of the formers' carbon-centred radicals through greater steric hindrance or lower diffusion rate. This would allow these larger radicals greater opportunity for reaction with molecular oxygen. This is consistent with the observation that methanol suppresses colour-enhancement produced by sorbitol (Table 3.1) since hydroxymethyl radicals derived from methanol would quench glucitoyl radicals by rapid dimerization. In contrast, the possibility that the colour enhancement is related to any metal-binding capacity of the polyhydroxylated compounds is not supported by the observation that colour yield diminishes as sorbitol concentration is increased (Table 3.1).

Figure 3.12. Chain-propagation of Fe^{2+} oxidation enhanced by sorbitol



3.4.3. Interference in the measurement of peroxides with FOX assays

In their application to different biological systems the FOX assays may be subject to interference by redox materials, present in biological extracts, which may oxidise generating H_2O_2. For example, in a linoleic hydroperoxide (4 µM) recovery experiment it was observed that ascorbic acid, 100 µM in the assay, slowly contributed to colour development (35% and 20% increase in colour yield in the presence of 1µM H_2O_2 in FOX-1 system and FOX-2 system, respectively). At the same concentration, uric acid increased colour yield about 6% in FOX-1 system in the presence of 1µM H_2O_2. I also observed the effects of other materials, such as glucose (25 mM), galactose (25 mM), glycerol (25 mM), cholesterol (100 µM), triglyceride (100 µM), linoleic acid (100 µM), reduced glutathione (100 µM), vitamin E (500 µM), lens crystallins (100 µg/ml) and bovine serum albumin (100 µg/ml), on colour development in the FOX-1 and FOX-2 assay. None of these contributed to the colour yield either in the presence or absence of 4 µM linoleic hydroperoxide or 4 µM H_2O_2 in the FOX systems.

For membrane or lipoprotein peroxide determination such interference need not be considered since washing and extracting or precipitation procedures would remove these low molecular weight agents. In other circumstances, however, separation of low molecular weight components by HPLC followed by post-column derivatisation with FOX could be a useful strategy to adopt.

3.4.4. Concluding remarks

Simple and sensitive methods for the determination of peroxides will assist evaluation of the "oxidative stress" hypothesis of pathophysiology. The methods described here provide a new alternative and may be useful in this respect. The FOX assays are sensitive, convenient and simple assays for the direct measurement of H_2O_2 and lipid peroxide. By comparison with conjugated diene and TBA assays, which measure products at different stages of lipid peroxidation, the FOX assay has the advantage of direct detection of lipid peroxides. The FOX-1 assay has been applied to the study of H_2O_2 generation during lens crystallin oxidation *in vitro* (Hunt, Jiang & Wolff, unpublished data). The FOX-2 assay has been employed in a study of erythrocyte membrane peroxidation *in vitro*, and lipid peroxidation in the cataractous lens *in vivo* [Woollard et al 1990a]. It is possible to

apply the FOX-2 assay to the measurement of lipid peroxide in clinical plasma samples (if a proper extraction method for lipid peroxide is available), and in clinical red blood cell membranes. I further employed the FOX assay to the *in vitro* study of LDL oxidation induced by glucose modification, and its role in cytotoxicity (Chapter 4).

CHAPTER 4 PEROXIDATION OF LDL DURING *IN VITRO* GLYCATION: IMPLICATIONS FOR CYTOTOXICITY

4.1 Introduction

There is an increased incidence of atherosclerosis in diabetes mellitus [Garcia et al 1974]. Alterations to the functional behaviour of LDL are suggested to be involved in the macrovascular complications of diabetes mellitus via altered LDL catabolism and possibly direct cytotoxicity [Steinberg et al 1989]. For example, glycation of LDL has been suggested to contribute to atherosclerosis in diabetes since LDL incubated with glucose *in vitro* becomes poorly recognised by fibroblasts and might thus accumulate in the macrophages *in vivo* [Saski & Cottam 1982; Steinbrecher & Witzum 1984].

As described in chapter 1, however, there is evidence suggesting the involvement of oxidative stress in the development of diabetic complications. Glucose may contribute directly to such oxidative stress since this sugar and the glycated protein can oxidise in the presence of transition metals [Wolff & Dean 1987; Hunt et al 1988; Baynes 1991], generating reactive free radicals and peroxides. It has been shown that glucose can induce lipid peroxidation in LDL [Hunt et al 1990]. This raises the possibility that peroxidation occurring during *in vitro* LDL glycation may contribute to observed functional alterations of the lipoprotein.

The possibility of peroxidation accompanying LDL glycation is important since oxidised LDL has been shown to be cytotoxic to cultured fibroblasts and endothelial cells [Morel & Chisolm 1989; Morel et al 1983; Henriksen et al 1979; Hessler et al 1983]. The relative importance of glycation as well as concomitant oxidative reactions thus requires evaluation with respect to LDL-cell interactions.

In this chapter, I compare the possible role of oxidative reactions and glycation in cytotoxicity of glucose-modified LDL *in vitro*. Lipid peroxidation was used as the index of oxidative modification of LDL. The availability of the sensitive and simple FOX assay, described in chapter 3, allows the accurate measurement of lipid peroxidation in LDL modified by glucose.

4.2. Methods

4.2.1. Exposure conditions of human LDL

Human LDL was prepared from normal subjects as previously described (Chapter 2). Incubations of LDL were performed under sterile conditions using filter sterilised solutions containing 0.01 % (w/v) sodium azide and under the particular conditions described in legends to figures. In general, LDL was incubated with 25mM glucose containing trace amounts of U-^{14}C-glucose in the absence of other factors or in the presence of 25mM $NaCNBH_3$ (which provides a reducing environment and traps the labile Schiff base thus increasing extent of glycation), 1mM EDTA (a transition metal chelator) or in the presence of 50µM copper sulphate to stimulate peroxidation. At appropriate time intervals (up to one week), samples were withdrawn and dialysed extensively in the presence of chelex as described in chapter 2 (section 2.2.1).

4.2.2. Analysis of LDL glycation and peroxidation

After dialysis of incubated LDL, glycation and lipid peroxidation were measured with standard methods as described in Chapter 2 (Section 2.2.4 - 2.2.10).

4.2.3. Assessment of LDL cytotoxicity

Cytotoxic effects of incubated LDL were assessed by ^{3}H-thymidine incorporation assay with V-79 cells after exposure to incubated LDL. Cells (5 X 10^4/ml) were grown overnight (12 hours) as a monolayer in DMEM containing 10% foetal calf serum, 2mM glutamine and 50µg/ml gentamycin. The medium was changed, pre-exposed/control LDL was added (250µg LDL

protein/ml cell culture medium), and the cells were allowed to grow for a further 16 hours. The medium was changed again, 0.1μCi/ml [methyl-^{3}H]-thymidine (in the presence of 5μM cold thymidine) was added, and left for a further 4 hours. Then ^{3}H-thymidine incorporation was assessed with standard assay (Chapter 2, Section 2.2.15).

4.3. Results

4.3.1. Glucose modification of human LDL: lipid peroxidation and glycation

Glucose slowly oxidises, under physiological conditions, *in vitro*. This oxidative process is dependent on the presence of catalytic transition metals, such as copper or iron, and generates hydrogen peroxide, hydroxylating and oxidizing agents as well as stable carbon products. It has previously been shown that this process leads to a time dependent increase in the accumulation of lipid peroxide in LDL incubated with glucose [Hunt et al 1990].

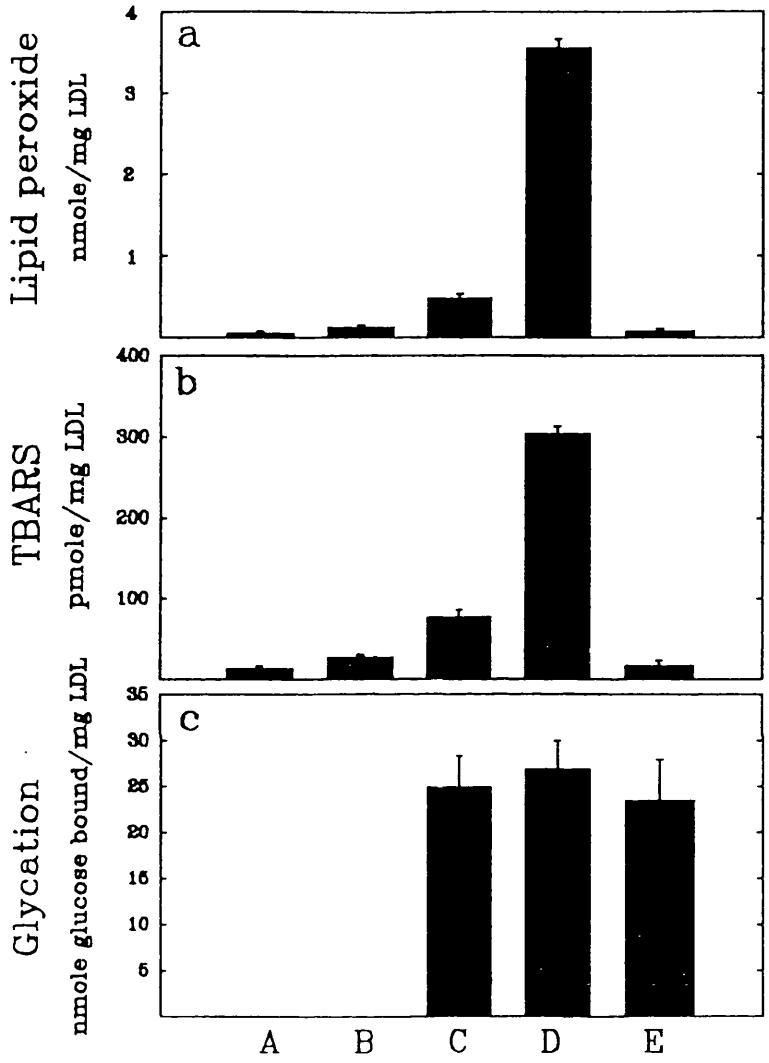
Figure 4.1a and 4.1b illustrate the accumulation of lipid peroxides (detectable by both the xylenol orange assay and the TBA assay) within LDL after a 7 day period of incubation under various conditions. The lowest level of lipid peroxide was present in the unincubated LDL (Figure 4.1a & 4.1b, Sample A), which was kept in buffer containing chelex 100 at 4^{o}C. LDL, incubated in the absence of additives for 7 days at 37^{o}C, accumulated small amounts of TBARS and peroxide (Figure 4.1a & 4.1b, Sample B) relative to unincubated LDL (Figure 4.1a & 4.1b, Sample A). As observed in Chapter 3, greater amounts of peroxide than TBARS were consistently observed. LDL incubated with 25mM glucose (Figure 4.1a & 4.1b, Sample C) contained greater amounts of TBARS and lipid peroxide than in the control incubation (Figure 4.1a & 4.1b, Sample B) confirming that high levels of glucose contribute to oxidation of LDL, at least *in vitro*. However, since glucose "autoxidation" is catalysed by transition metal ions glucose produces no increase in lipid peroxide or TBARS content relative to the control incubation in the presence of the metal chelator, ethylenediaminetetra-acetic acid (EDTA) (Figure 4.1a & 4.1b, Sample E). Lipid peroxide

accumulation could be further elevated by the presence of additional copper ions (Figure 4.1a & 4.1b, Sample C) or decreased by the presence of 1mM EDTA (Sample E). By contrast, there was no significant difference in the levels of glycation of LDL when exposed to glucose alone (Figure 4.1c, Sample C) or with the addition of copper (Figure 4.1c, Sample D) or EDTA (Figure 4.1c, Sample E). In other words, conditions of glycation can be arranged, so that there are varying degrees of peroxidation, but little change in glycation. This allows us to examine the possible interactions between glycation and peroxidation in cytotoxicity of *in vitro* glycated LDL.

4.3.2. The effects of glucose modification of human LDL upon cell growth and viability

Cytotoxicity of the pre-incubated LDL was assessed by [^{3}H]-thymidine incorporation into V-79 fibroblast DNA after exposure to LDL. Figure 4.2 illustrates ^{3}H-thymidine uptake by the fibroblasts treated by LDL, which was incubated for 7 days under various conditions. Cells treated with incubated LDL (Figure 4.2, Sample B) take up less thymidine, but in this respect are not significantly different from cells treated with unincubated LDL (Figure 4.2, Sample A). Thymidine incorporation was significantly decreased ($P<0.05$) in the cells exposed to glucose- treated LDL (Figure 4.2, Sample C) compared with the cells exposed to LDL incubated with no additives (Figure 4.2, Sample B). Indeed, LDL treated with glucose in the presence of copper (Figure 4.2, Sample D) is more toxic than, $P<0.05$, LDL pre-exposed to glucose alone (Figure 4.2, Sample D) as expected from the contents of lipid peroxide (Figure 4.1a & 4.1b). By contrast, LDL incubated with glucose in the presence of EDTA (Figure 4.1a & 4.1b, Sample E) contains less lipid peroxide than LDL incubated with glucose alone (Figure 4.1a & 4.1b, Sample C) and is thus less cytotoxic to fibroblasts ($P<0.05$, Figure 4.2, Sample E & C). Thus, the cytotoxicity of LDL resulting from pre-exposure to glucose with or without additives appears to involve transition metal catalysed lipid peroxidation.

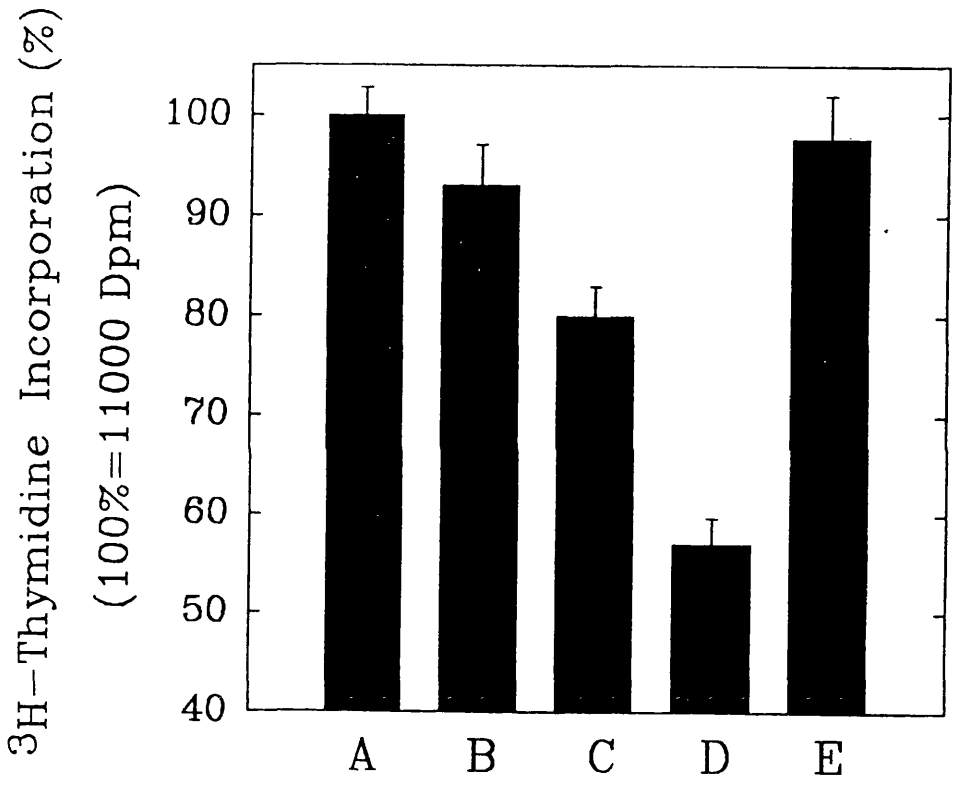
Figure 4.1. LDL lipid peroxidation and LDL glycation



LDL lipid peroxidation - the generation of lipid peroxides and thiobarbituric acid reactive substance: *LDL (5mg/ml) was incubated under various conditions in the presence of 100mM potassium phosphate (pH 7.4) at 37°C over a period of 7 days. LDL was incubated with [B] buffer alone, [C] 25mM D-glucose, [D] 25mM D-glucose and 50µM copper sulphate, and [E] 25 mM D-glucose and 1 mM EDTA. Sample [A] represents LDL incubated at 4°C in the buffer containing Chelex 100. After exposure, lipid peroxide and TBARS content of the LDL were measured as described in Methods and Materials. Values are mean ± SD of quadruplicate assays. Exposure of LDL in the presence of copper with (sample D) glucose permitted a substantial accumulation of peroxide ($P<0.0005$) when compared to LDL exposed to buffer alone (Sample B). The inclusion of glucose (Sample C) increased the level of peroxide accumulation over glucose deficient conditions (Sample B) of LDL exposure ($P<0.05$).*

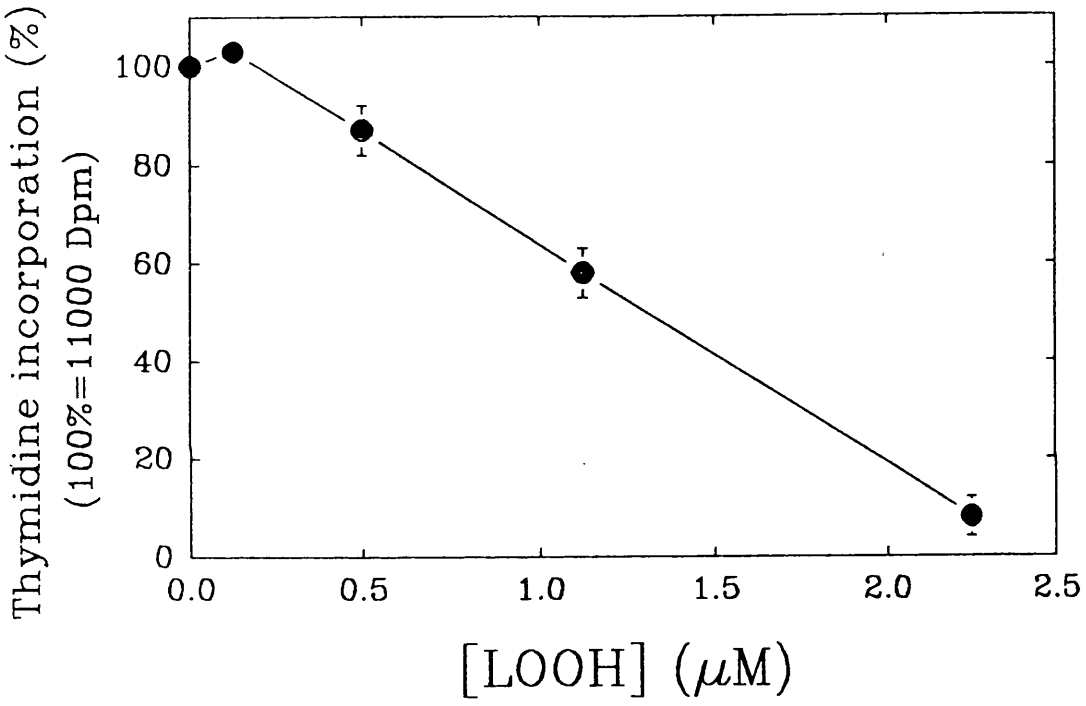
LDL glycation: *LDL (5mg/ml) was incubated under various conditions in the presence of 100mM potassium phosphate (pH 7.4) at 37°C over a period of 7 days. After exposure, LDL was assessed for radiolabelled glucose attachment as described above. Values are mean ± SD of quadruplicate assays. There are no significant difference between Samples C, D and E.*

Figure 4.2. LDL fibroblast toxicity



Previously exposed LDL was dialysed extensively and assessed for its effect upon cell viability, as monitored by ^{3}H-thymidine incorporation as described above. LDL was previously incubated with [A] buffer containing Chelex 100 at 4^{o}C, [B] buffer alone at 37^{o}C, [C] 25mM glucose at 37^{o}C, [D] 25mM D-glucose and 50µM copper sulphate at 37^{o}C, and [E] 25mM D-glucose and 1mM EDTA. Values are expressed as a percentage of thymidine incorporation in identical cell cultures during which cells were exposed to buffer alone (100% = 11000 Dpm). Values are the mean ± SD of quadruplicate assays. Sample B is significantly different from Sample C (P<0.05) and Sample D (P<0.01). Sample C is significantly different from Sample D (P<0.05). There is no significant difference between Samples A and B.

Figure 4.3. The effect of LDL-associated lipid peroxide upon ^{3}H-thymidine incorporation



LDL containing a known level of xylenol orange-detectable lipid peroxide (LOOH) was prepared by incubation with copper and glucose. After exhaustive dialysis the effect of various concentrations of LDL-associated lipid peroxide was examined for its effect upon ^{3}H-thymidine incorporation by fibroblasts. Lipid hydroperoxide concentrations indicated refer to concentrations within medium to which cells were exposed. Values are mean ± SD of quadruplicate assays. The inhibition of thymidine incorporation correlated with LDL peroxide content ($R=-0.996$, $P<0.0001$, $n = 5$).

Figure 4.3 further demonstrates that cytotoxicity of glucose modified LDL correlates well with the content of lipid peroxide in LDL (r=-0.996). A control pre-incubated sample containing no LDL but all the other components used throughout the incubation of LDL samples was exhaustively dialysed and was found to possess no cytotoxic activity. This indicated that dialysis successfully removed all LDL-free agents which might have contributed to the observed cytotoxic effects of LDL.

4.3.3. Does glycation alone contribute to cytotoxicity of glucose modified LDL?

Modification of LDL amino groups by glucose does not participate in LDL cytotoxicity directly. This was demonstrated by further studies during which oxidative reactions were limited and glycation enhanced by the use of $NaCNBH_3$. Essentially, this involved the short term exposure of 5mg/ml LDL to 25mM glucose in the presence of 25mM $NaCNBH_3$. Such an approach provides a reducing environment and permits the reduction of Schiff bases to stable glucose-amino acid adducts, whilst at the same time avoiding oxidative events. Glycation and the accumulation of lipid peroxide is shown in Table 4.1. Over a 1 day exposure of LDL to glucose plus $NaCNBH_3$, only low levels (0.028 nmole lipid peroxide/mg LDL) of lipid peroxide accumulated. The attachment of glucose in the presence of $NaCNBH_3$ (Table 4.1), on the other hand, exceeded that obtained in the absence of $NaCNBH_3$ over the 7 day exposure as described and shown in Figure 4.1c. However, Table 4.1 shows that the effect of this LDL upon thymidine incorporation was no different to that observed when LDL was incubated in the absence of other components, ie non-glycated but mildly peroxidised LDL (Table 4.1). Thus, glycation itself, if not accompanied by accumulation of lipid peroxides, does not lead to LDL cytotoxicity via the modification of LDL amino acid residues (Table 4.1 and Figure 4.1, Sample E).

TABLE 4.1 The effect of glycated LDL under reducing condition on viability of fibroblasts:

	^{3}H-thymidine Incorporation (%) 100%=12500 dpm	Glycation nmole glucose bound/mg LDL	Lipid Peroxide nmole/mg LDL
1) Control (buffer)	100 ± 5	ND	ND
2) LDL (5mg/ml)	93 ± 6	ND	0.128 ± 0.020
3) + 25mM Glucose	91 ± 7	3.5 ± 1.0	0.140 ± 0.020[a]
4) + 25mM Glucose + 25mM $NaCNBH_3$	95 ± 4	38.0 ± 1.0*	0.028 ± 0.010[b]
5) + 25mM $NaCNBH_3$	94 ± 7	ND	0.021 ± 0.008[c]

**, P < 0.001 (group 4 cf group 3); a, P > 0.05 (group 3 cf group 2); b, P < 0.005 (group 4 cf group 3); c, P < 0.005 (group 5 cf group 2) There is no significant difference between these groups for thymidine incorporation.*

LDL lipid peroxidation and glycation under reducing conditions- LDL (5mg/ml) was exposed to 25mM D-[U-^{14}C]-glucose (10μCi/ml) in the presence or absence of 25mM sodium cyanoborohydride ($NaCNBH_3$) over a period of 1 day at 37°C. Control incubations of LDL incubated with buffer alone or with 25mM $NaCNBH_3$ are shown. Levels of lipid peroxide and glycation were assessed as described in the text. Previously exposed LDL was dialysed extensively and assessed for its effect upon cell viability, as monitored by ^{3}H-thymidine incorporation as described above. Values are expressed as a percentage of thymidine incorporation in identical cell cultures during which cells were exposed to buffer alone as control group (100% = 12500 Dpm). Values are the mean ± SD of quadruplicate assays. ND: not detected.

4.3.4. Comparison of cytotoxicity induced by copper alone- and copper/glucose- modified LDL

Table 4.2 shows data suggesting that LDL modified by glucose in the presence or absence of copper ion is toxic to V-79 cells and this toxic effect appears to positively correlate with the content of lipid peroxide, ie the higher the level of lipid peroxide in LDL, the more toxic the LDL is to the cells. However, the possible contribution of glycation to cytotoxicity of LDL modified by glucose and accompanied by lipid peroxidation remains uncertain. In order to evaluate this possibility, experiments were designed to compare the contributions of lipid peroxide and glycation to cytotoxicity induced by LDL pre-exposed to 50μM Cu^{2+} alone or 25mM glucose plus 50μM Cu^{2+} for 7 days at 37°C. Table 4.2 shows the slight increases in the content of lipid peroxide and TBARS in LDL incubated in buffer at 37°C in comparison with LDL kept in buffer containing chelex 100 at 4°C. Substantial amounts of TBARS and lipid peroxide accumulate in LDL incubated with 50μM Cu^{2+}, but this is only slightly increased by the inclusion of 25mM glucose and the difference is not significant. The contribution of catalytic metal to peroxide accumulation is thus much greater than that of glucose autoxidation. A high extent of glycation was detected in LDL exposed to glucose and copper (Table 4.2). Interestingly, thymidine incorporation was significantly decreased in the cells exposed to 50μM Cu^{2+} treated LDL ($P<0.05$) and in the cells exposed to LDL pre-treated with 25mM glucose plus 50μM Cu^{2+} ($P<0.01$) compared to the cells exposed to LDL pre-incubated with no additives at 37°C (Table 4.2). Indeed, LDL treated with glucose in the presence of copper is toxic to a greater extent than would have been expected on the basis of peroxide content alone by comparison with LDL pre-exposed to copper alone ($P<0.05$, Table 4.2). Thus, the cytotoxicity of LDL resulting from pre-exposure to glucose with or without addition of copper appears to involve lipid peroxidation, and is enhanced by glycation.

TABLE 4.2 The comparison of the cytotoxicity induced by copper- and copper/glucose- modified LDL

Groups	thymidine Incor.(%)	Glycation nmole glucose bound/mg LDL	Lipid Peroxide nmole/mgLDL	TBARS pmole/mgLDL
1). LDL Chelex/4^{o}C	100 ± 4	ND	0.05 ± 0.02	14 ± 3
2). LDL 37^{o}C	93 ± 5 ^{d}	ND	0.13 ± 0.03	28 ± 2
3) LDL (37^{o}C) + 50μM Cu^{2+}	72 ± 5 ^{a}	ND	3.40 ± 0.10*	285 ± 9*
4) LDL (37^{o}C) + 50μM Cu^{2+} + 25mM Glucose	57 ± 3 ^{b,c}	27 ± 3	3.56 ± 0.08$^{*,@}$	305 ± 8$^{*,@}$

a, P<0.05 (group 3 cf group 2); b, P<0.01 (group 4 cf group 2);
c, P<0.05 (group 4 cf group 3); d, P>0.05 (group 2 cf group 1).
**, P<0.001 (group 3 or 4 cf group 2); @, P<0.05 (group 4 cf group 3);*

LDL (5mg/ml) was exposed to 25mM D-[U-^{14}C]-glucose (10μCi/ml) in the presence or absence of 50μM copper over a period of 7 days at 37^{o}C. Control incubations of LDL incubated with chelex 100 treated buffer at 37^{o}C or with buffer plus chelex 100 at 4^{o}C are shown. Levels of lipid peroxide, TBARS (thiobarbituric acid reactive substances) and glycation were assessed as described in the text. Previously exposed LDL was dialysed extensively and assessed for its effect upon cell viability, as monitored by ^{3}H-thymidine incorporation as described above. Values are expressed as a percentage of thymidine incorporation in identical cell cultures during which cells were exposed to buffer alone as control group (100% = 11000 dpm). Values are the mean ± SD of quadruplicate assays. Copper alone and copper/glucose modified LDL are toxic to cells and are significantly different from LDL incubated in buffer at 37^{o}C upon thymidine incorporation (P<0.05 for copper treated LDL; P<0.01 for copper/glucose treated LDL). There is significant difference between copper alone treated LDL and copper/glucose treated LDL upon thymidine incorporation by V-79 cells (P<0.05). ND: not detected.

4.4. Discussion

The contribution of high blood glucose to diabetic complications is often discussed in terms of glycation, by the exposure of proteins to high levels of glucose over long periods *in vitro* [Brownlee et al 1984]. However, many of the observed changes resulting from such exposure may be due to oxidative damage since reactive oxygen species are generated during glycation [Sakurai & Tsuchiya 1988; Azevedo et al 1988; Jiang et al 1990; Mullarkey et al 1990].

The purpose of the designed experiments described in this chapter is to investigate the possible role of glycation and peroxidation in cytotoxicity induced by glucose modified LDL. LDL was exposed to glucose with or without the addition of transition metal copper, EDTA or $NaCNBH_3$ *in vitro*. After intensive dialysis, the pre-incubated LDL was added to the medium of V-79 fibroblasts. Since the thymidine incorporation assay is a sensitive method for measuring cellular damage, the effect of the LDL upon the cells was tested by measuring thymidine incorporation into DNA.

The results of this study show that the exposure of LDL to glucose induces the peroxidation of lipid in LDL (Figure 4.1). Interestingly, this oxidative modification of LDL by glucose renders LDL cytotoxic (Figure 4.2). Although lipid peroxidation induced by glucose and/or glycated protein in liposome and LDL has been shown before [Hicks et al 1988; Hunt et al 1990], the results reported here are the first observations of a direct cytotoxic effect of glucose modified LDL *in vitro*. It is by no means yet certain that the toxicity of glucose modified LDL is due to lipid peroxide or its aldehyde products carried by LDL or oxidised apoprotein moieties. Whatever the mechanism it was, however, increased content of lipid peroxides (estimated either by TBA assay or FOX assay) in LDL which were associated with the oxidised LDL cytotoxicity (Figure 4.3).

Furthermore, the exposure of LDL to glucose with Cu^{2+} increased the content of lipid peroxides and thus increased the toxic effect of the pre-incubated LDL upon cells (Figure 4.1 & 4.2). LDL incubated with a metal chelator, e.g. EDTA, contained a very low level of lipid peroxides and was

not toxic to cells (Figure 4.1 & 4.2). In the case of exposure of LDL to glucose alone (Figure 4.1 & 4.2), transition metal catalysed oxidation was also the cause of lipid peroxidation and cytotoxicity since transition metals are available in most experimental buffers (such as about 1 µM Copper and 5 µM iron in 100mM potassium phosphate buffer). This further suggests that transition metal seems to be an essential factor in the oxidation of glucose incubated LDL rendering the cytotoxic. Of course, the cellular effect of glucose incubated LDL observed *in vitro* may be very different to that which occurs *in vivo*. Again, the determining factor for initiating LDL oxidation and the consequent cellular damage may be the availability of decompartmentalized transition metals *in vivo*.

LDL glycation alone does not contribute to cytotoxicity of LDL pre-incubated with glucose. This was shown by the lack of cytotoxic effects of LDL (Figure 4.2 & Table 1) after exposure to glucose together with EDTA (to prevent LDL oxidation) or with $NaCNBH_3$ (to enhance glycation and inhibit oxidation of LDL). However, LDL pre-incubated with glucose and Cu^{2+} contains a slightly higher level of lipid peroxides but is much more toxic to the cells than the LDL exposed to Cu^{2+} alone (Table 4.2). This indicates that glycation of LDL may enhance LDL cytotoxicity if LDL oxidation occurs simultaneously. The cytotoxic effect of glycated LDL may be due to its consequent oxidative modification of lipid and/or lipoprotein moieties in LDL. However, this experiment cannot identify the exact cytotoxic substances and further work is required.

There are some limitations to the experimental system described in this chapter. For example, Cu^{2+} was used to catalyse oxidation during LDL incubation and 50µM of free Cu^{2+} is unlikely to be achieved in human tissue *in vivo*. Such a high concentration of Cu^{2+}, however, can be applied to LDL incubation *in vitro* to ensure the oxidation of LDL, and can be used as a model to distinguish oxidative modification from glycation.

CHAPTER 5 SPIROHYDANTOIN INHIBITORS OF ALDOSE REDUCTASE INHIBIT TRANSITION METAL CATALYSED OXIDATIONS

5.1. Introduction

High blood glucose, as described in chapter 1, may contribute to the development of diabetic complications through polyol pathway metabolism. Many inhibitors of aldose reductase (the key enzyme for polyol pathway) have been synthesized and have been applied to experimental studies and clinical trials for diabetic complications [Dvornik & Porte, 1987]. There is evidence that some ARIs can prevent or inhibit the development of sugar cataract or experimental diabetic cataract [Dvornik & Porte, 1987], and prove beneficial in the treatment of diabetic neuropathy [Jaspan et al 1985; Pfeifer 1986]. However, the precise mechanism of ARIs' inhibitory effects on the development of diabetic complications remains unknown. It may be a result of activity distinct from the inhibition of polyol accumulation. For example, tolrestat inhibits plasma ascorbic acid depletion [McLennan et al 1988], and sorbinil decreases the increased level of lipid peroxidation [Yeh & Ashton 1990] associated with experimental diabetes, but by unknown mechanisms. Moreover, sorbinil inhibits free radical NADPH oxidation stimulated by transition-metal catalysed monosaccharide enediol oxidation *in vitro* [Wolff & Crabbe 1985]. These findings imply that the pharmacological effects of these ARIs on the development of diabetic complications may be due to their antioxidant activities *in vivo*.

In order to test the possible antioxidant activity of ARIs, transition metal catalysed ascorbate oxidation was used as a simple oxidation system *in vitro*. The effects of ARIs on H_2O_2 formation by monosaccharide autoxidation were examined with the FOX-1 assay described in Chapters 2 & 3. Furthermore, the effects of ARIs on biological tissue damage induced by copper/ascorbate system were also evaluated *in vitro*. The following results show that some ARIs are potent chelators of transition metals, such as copper and iron, and thus possess the potential for antioxidant effects.

These observations may explain some of the biological effects of these inhibitors and suggest a starting point for the design of a new generation of experimental and therapeutic compounds which may diminish diabetic complications.

5.2. Methods

5.2.1. Ascorbate oxidation

All solutions were prepared in Chelex-treated (50-100 Dry Mesh; Sigma) double distilled water. Solutions were stored over chelex and made fresh each day. Ascorbate oxidation was monitored over an initial 3 minute period at 265nm in a Pye Unicam 8720 UV/VIS spectrophotometer at 37°C. Ascorbate oxidation was catalysed by Cu^{2+} (copper sulphate), Cu^{2+}-1,10 orthophenanthroline (OP) complex, Fe^{3+} (ferric chloride) or Fe^{3+}-ethylenediaminetetra-acetic acid (EDTA) and H_2O_2 (see Legends to Figures). The reaction was initiated by addition of ascorbate (final concentration, 100µM). The oxidation rate of ascorbate was calculated from extinction coefficient, 12.5 $nM^{-1}cm^{-1}$, which was extablished by measuring absorbance at 265nm with different concentrations of standard ascorbic acid. Low baseline rates of ascorbate oxidation were ensured by prior chelex treatment of stock solutions and water used to dissolve components. This yielded very reproducible rates of oxidation (coefficient of variation <2%).

5.2.2. Incubation of monosaccharides and H_2O_2 measurement

Glucose or glyceraldehyde (with or without the additives) was incubated in potassium phosphate buffer (pH 7.4, 10mM) at 37°C under sterile conditions. After incubation for appropriate times, 100µl samples were added to 900µl of reagent (100µM xylenol orange, 250µM Fe^{2+} and 100mM sorbitol in 25mM H_2SO_4). Absorbance was read at 560nm after 30 minutes incubation at room temperature. Values (peroxide concentration in the sample) are the mean plus/minus standard deviation obtained from duplicate assays.

5.2.3. Cell culture and viability test

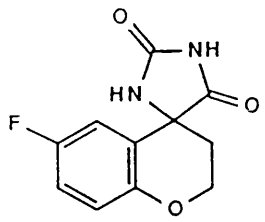
The cytotoxic effects of copper/ascorbate upon V-79 fibroblasts was assessed by radiolabelled thymidine incorporation. Cells (10^5 /ml) were grown overnight as a monolayer in DMEM containing 10% foetal calf serum, 2mM glutamine and 50µg/ml gentamycin. Cell culture was performed in an incubator with standard conditions described in Chapter 2. The medium was then aspirated and cells were washed twice with Chelex treated PBS. The washed cells were incubated in Chelex treated PBS, containing 0.2 mg/ml of Ca^{2+} and 0.2 mg/ml of Mg^{2+}, for 1 hour at 37°C. Copper and ascorbate (with or without ARIs) were added to the PBS and cells were allowed to grow for further 30 minutes in the same conditions. PBS was then aspirated and cells were washed twice with DMEM. Cells were allowed to grow for a further 4 hours in DMEM with 0.1µCi/ml [Methyl-^{3}H]-Thymidine (5µM cold thymidine). ^{3}H-thymidine incorporation into the cells was then assessed by the standard method described in Chapter 2.

5.3. Results

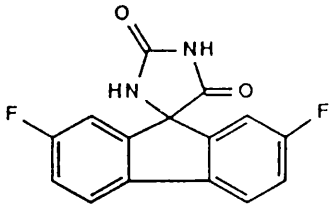
5.3.1. Spirohydantoin ARIs inhibit copper catalysed ascorbate oxidation

The structures of the aldose reductase inhibitors tested are shown in Figure 5.1. The spirohydantoin compounds sorbinil and AL-1576 inhibit copper-catalysed ascorbate oxidation whereas the carboxylate inhibitors ICI 105552 and tetramethyleneglutarate (TMG) had no inhibitory effect over the time scale (3 minutes) of ascorbate oxidation studied (Table 5.1). ONO-2235 had only a little effect on ascorbate oxidation within 3 minutes incubation. Consistent with the observation that hydantoin inhibitors block copper-catalysed ascorbate oxidation, it was found that hydantoin itself had a moderate inhibitory effect. Table 5.1 also shows the inhibitory behaviour of known metal-chelating reagents for comparison. The inhibitory effect of sorbinil and AL-1576 on Cu^{2+}-catalysed ascorbate oxidation was less than the inhibition achieved by ethylenediaminedia-acetic acid (EDDA), nitrilotriacetic acid (NTA) and 2,2'-bicinchoninic acid (BCA).

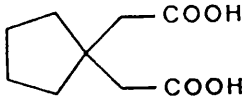
Figure 5.1. The structure of aldose reductase inhibitors.



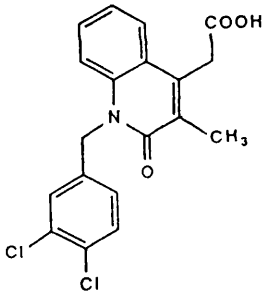
CP-45,634
Sorbinil



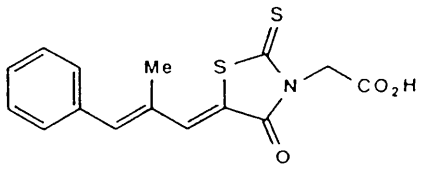
AL-1576



AY-20,037 (TMG)



ICI-105,552



ONO-2235
Epalrestat

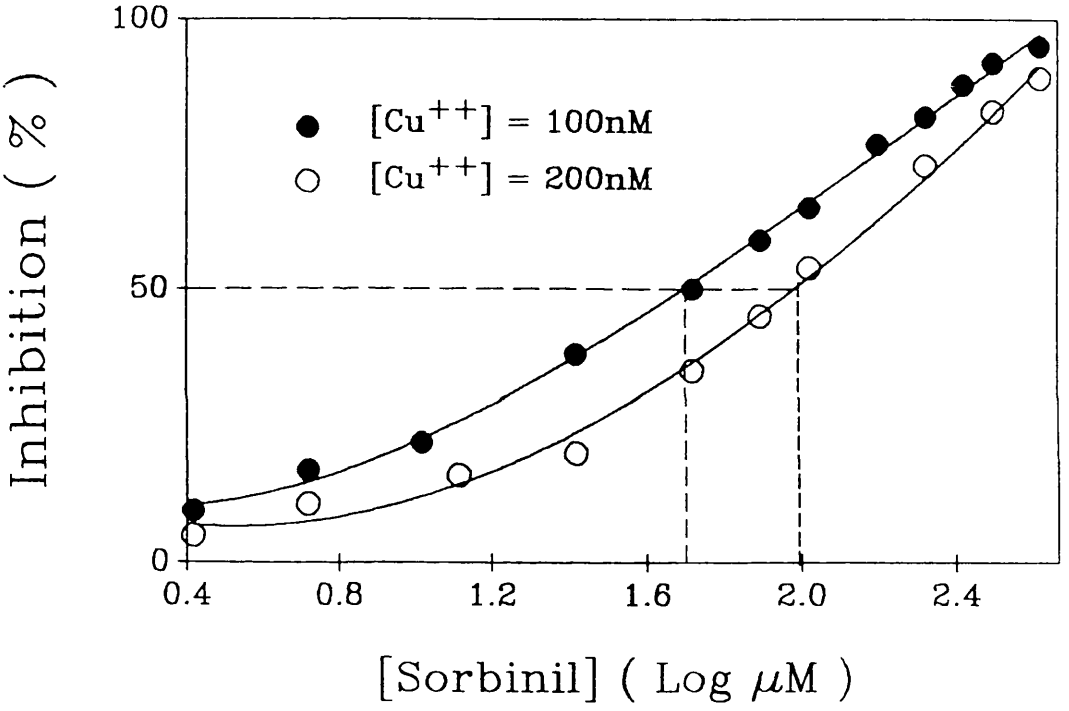
Table 5.1. The effects of some aldose reductase inhibitors on Cu^{2+}-induced ascorbate oxidation

	Concentration (µM)	Ascorbate oxidation rate (nmoles/min)	Percentage activity
Control (Cu^{2+})		7.84 ± 0.08	100
Sorbinil	100	3.60 ± 0.08	45.9
AL-1576	100	3.04 ± 0.04	38.8
ONO 2235	100	7.68 ± 0.16	98
ICI 105552	100	8.08 ± 0.16	103
TMG	100	7.52 ± 0.08	95.9
Hydantoin	100	6.24 ± 0.16	79.6
EDDA	100	0.24 ± 0.04	3
NTA	100	0.24 ± 0.04	3
BCA	10	0.24 ± 0.04	3

Copper sulphate was dissolved in chelex treated distilled water. Other reagents were prepared and treated as described in Methods. Final concentrations of ascorbate, potassium phosphate buffer (pH 7.4) and Cu^{2+} were 100µM, 20mM and 200nM, respectively. Ascorbate oxidation rate ($-OD_{265nm/min}$) was monitored at 37°C for 3 minutes. Results are expressed as means+/-SD of 4 measurements.

Figure 5.2 gives log dose-inhibition curves for sorbinil at 2 concentrations of added catalytic copper (100 nM and 200nM) showing that at lower concentrations of copper the "IC_{50}" for inhibition by sorbinil is decreased. The IC_{50} for sorbinil, in the presence of 200nM Cu^{2+}, is 95µM and decreases to 50µM when Cu^{2+} concentration reduces to 100nM (Figure 5.2).

Figure 5.2. The inhibitory effect of sorbinil on Cu^{2+}-induced ascorbate oxidation



Cu^{2+} was dissolved in chelex treated distilled water. Sorbinil and ascorbate stock solutions and potassium phosphate buffer (pH 7.4) were made using chelex-treated double distilled water. 20mM potassium phosphate buffer, 100μM ascorbate, 100nM or 200nm Cu^{2+} and 2.5 - 600μM sorbinil were present in 1 ml reaction volume. Ascorbate was added last. Ascorbate oxidation rate($-OD_{265}$nm/min) was monitored at 37°C for 3 minutes. The figure shows the percentage inhibition of ascorbate oxidation against concentration (Log μM) of sorbinil.

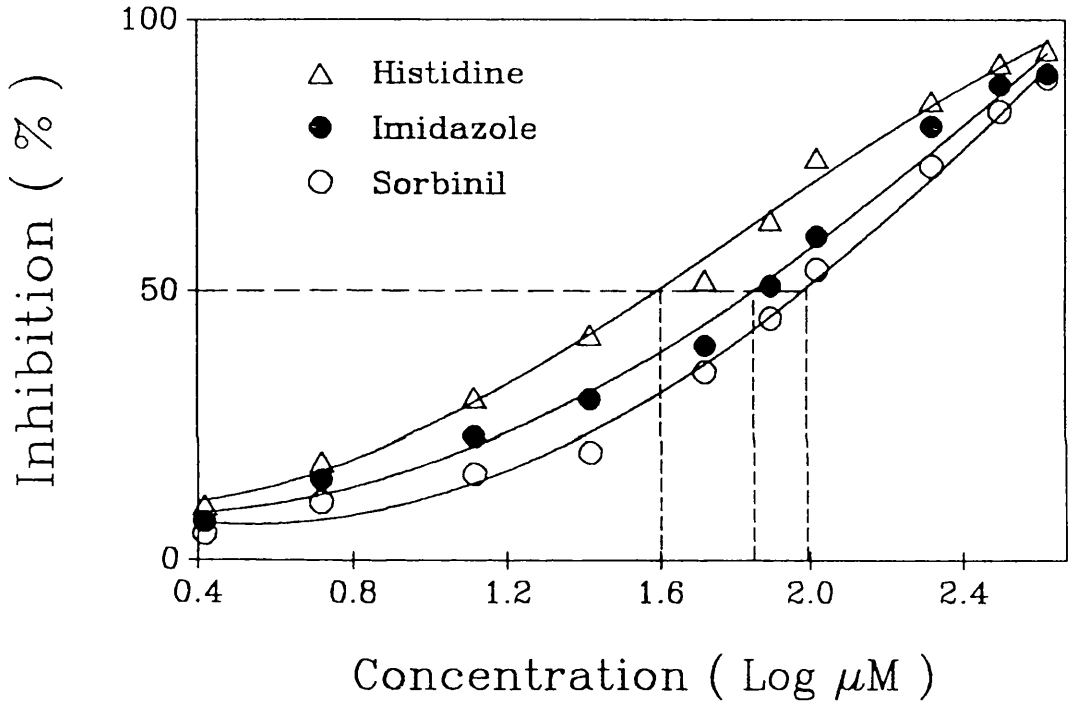
5.3.2. The possible mechanism of the effects of spirohydantoin ARIs on copper catalysed ascorbate oxidation

The inhibitory effect of a compound on Cu^{2+}-catalysed ascorbate oxidation may, in principle, be due to its ability to chelate the metal and/or scavenge free radicals. In order to investigate the possible mechanisms of spirohydantoin ARIs' effects on ascorbate oxidation, sorbinil was compared to the selective copper-complexing agents histidine and imidazole. Figure 5.3 shows log dose-inhibition curves for sorbinil, histidine and imidazole in the presence of 200nM copper ion. The IC_{50}'s for the 3 compounds (for histidine 38μM, for imidazole 75μM and for sorbinil 95μM) are within the same range.

Similarly, when ascorbate oxidation was catalysed by Cu^{2+} in the presence of orthophenanthroline (OP) (Figure 5.4) there was little evidence of inhibition by sorbinil until very high (> 500μM) concentrations were achieved (Figure 5.4). Imidazole, at all concentrations, was found to be inert with respect to inhibition of ascorbate oxidation catalysed by copper-orthophenanthroline (Figure 5.4). Taken together, these data would seem to suggest the inhibitory effect of sorbinil on copper catalysed ascorbate oxidation, like that of imidazole, may be mainly due to its Cu^{2+} chelating ability.

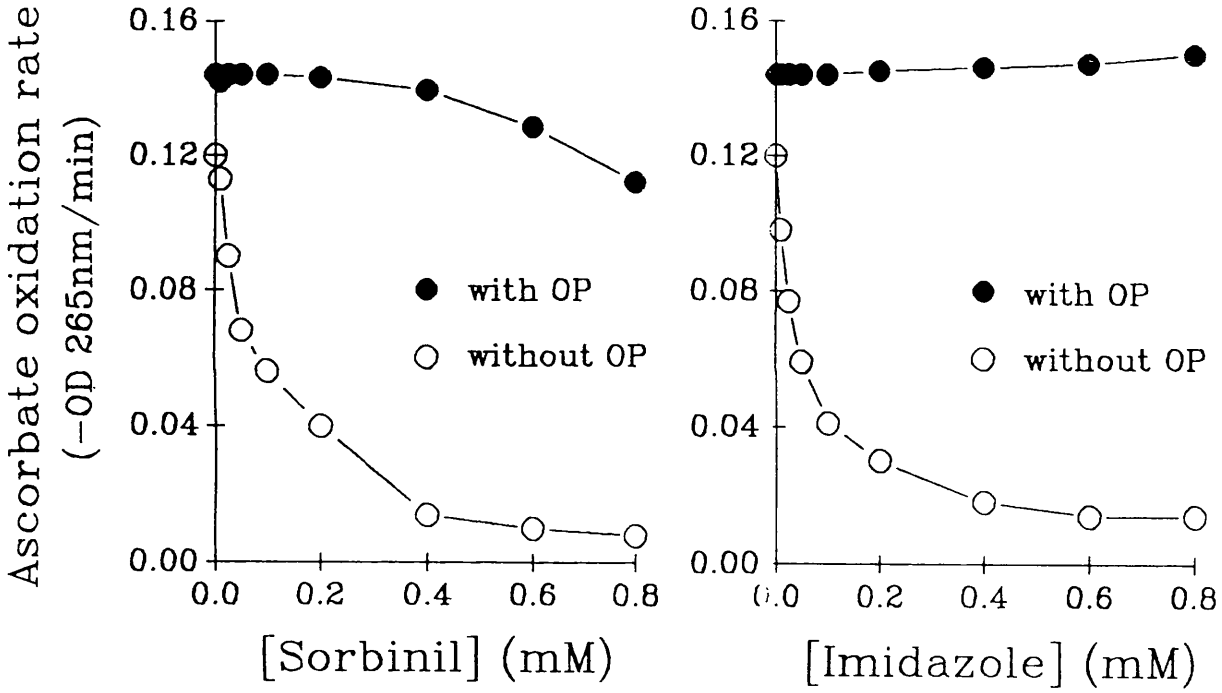
In addition, it can be seen that the inhibitory effect of sorbinil and/or AL-1576 decreases as Cu^{2+} concentration is increased for a fixed concentration of the ARIs (Figure 5.5). By contrast, DTPA was a potent inhibitor over the entire Cu^{2+} concentration range. This further indicated that spirohydantoin ARIs are weak copper chelators. However, ethanol (at the concentration of 1mM) had no effect on ascorbate oxidation thereby further excluding a role for hydroxyl radical scavenging in the inhibitory effect of the ARIs.

Figure 5.3. Comparison of the effects of sorbinil, histidine and imidazole on Cu^{2+}-induced ascorbate oxidation



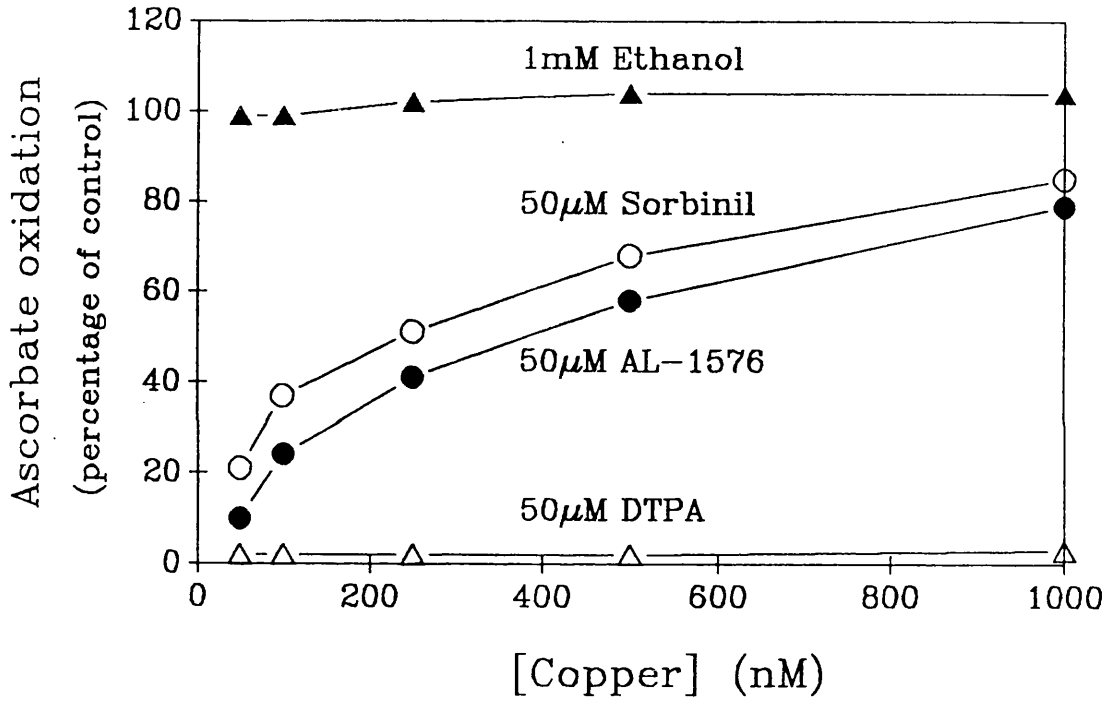
The inhibitory effects of different concentrations (1-600μM) of sorbinil, histidine and imidazole were observed under the conditions described above. Ascorbate oxidation was initiated by the addition of 150nM Cu^{2+}.

Figure 5.4. The effects of sorbinil and imidazole on Cu^{2+} or Cu^{2+}-orthophenanthroline induced ascorbate oxidation



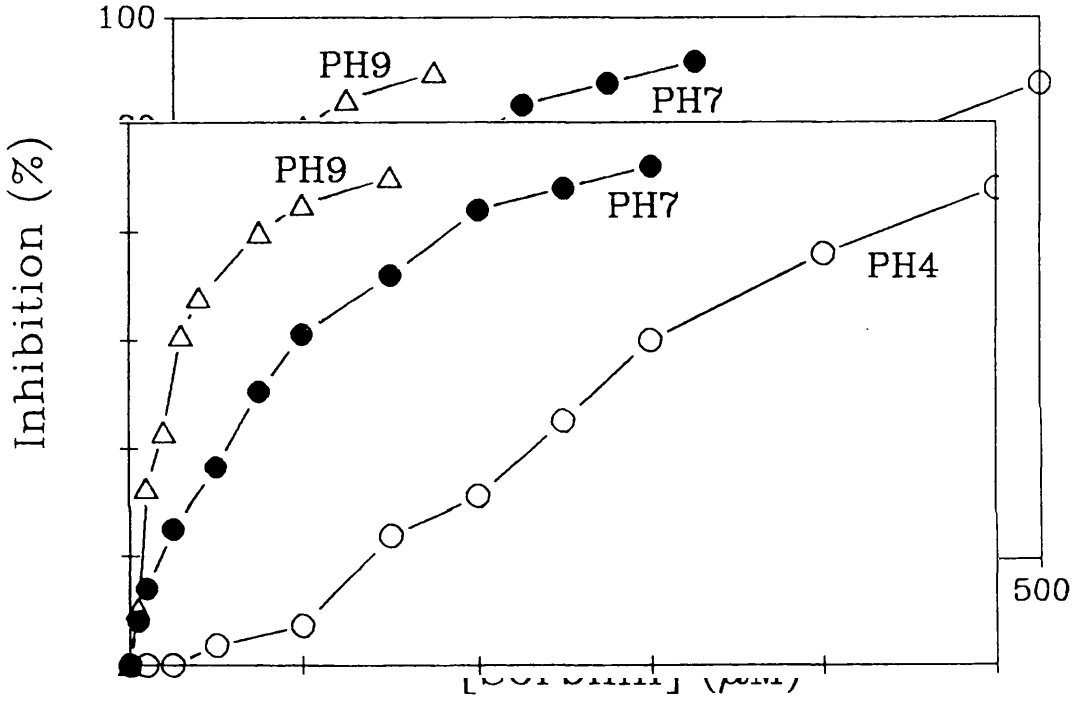
Ascorbate oxidation was induced by Cu^{2+} or Cu^{2+}-OP complex in potassium phosphate buffer (pH 7.4). Cu^{2+} and OP were added to the cuvette and mixed in buffer prior to the addition of 5 to 800µM sorbinil or imidazole. The final concentrations of Cu^{2+}, OP, ascorbate and potassium phosphate in the cuvette were 500nM, 30µM, 100µM and 20mM, respectively.

Figure 5.5. The effects of sorbinil, AL-1576, DTPA and ethanol on ascorbate oxidation induced by different concentration of Cu^{2+}



Ascorbate (100μM) oxidation was initiated by the addition of different concentrations of Cu^{2+} (in the range 50nM to 1000nM) in 20mM potassium phosphate buffer (pH 7.4) at 37°C and monitored for 3 minutes. The effects of sorbinil (50μM), AL-1576 (50μM), DTPA (50μM) and ethanol (1mM) were examined.

Figure 5.6. The effect of pH on the inhibitory effect of sorbinil on Cu^{2+}-induced ascorbate oxidation



20mM potassium phosphate buffer of varying pH values (4.0, 7.0 or 9.0) was used to see the effects of pH on the inhibitory effect of sorbinil on ascorbate oxidation. In 1ml reaction volume, 150nM Cu^{2+}, 1-500µM sorbinil and 100µM ascorbate were used. The treatment of solutions and the condition for the measurement of ascorbate oxidation rate were the same as described above. The figure shows the percentage inhibition of ascorbate oxidation against concentration (µM) of sorbinil.

The inhibitory effect of sorbinil is strongly pH dependent (Figure 5.6) which might be explained by enolization of the spirohydantoin group at higher pH values and thus providing a greater availability of nitrogen lone pair electrons. These play an important role in the chelating ability of other nitrogen-containing copper-complexing agents. Neither magnesium, zinc nor calcium decreased the inhibition of Cu^{2+}-catalysed ascorbate oxidation by sorbinil and AL-1576 (Table 5.2) indicating that neither of the ARIs has any effects on magnesium, zinc or calcium. Table 5.2 also shows that zinc itself can inhibit copper induced ascorbate oxidation. This observation is in agreement with the view that zinc is a non-transition element and thus can inhibit transition metal catalysed oxidation by displacing transition metal from its binding site [Willson 1978].

Table 5.2. The influence of magnesium, zinc and calcium on the inhibitory effects of sorbinil and AL-1576 on Cu^{2+}-catalysed ascorbate oxidation.

	Ascorbate oxidation rate $-OD_{265}$/min	nmole/min
Control (KP alone)	0.002 ± 0.001	0.16 ± 0.08
Control (200nM Cu^{2+})	0.131 ± 0.004	10.48 ± 0.32
+ Ca (50µM)	0.133 ± 0.002	10.64 ± 0.16
+ Mg (50µM)	0.129 ± 0.003	10.32 ± 0.24
+ Zn (50µM)	0.110 ± 0.003	8.80 ± 0.24
Cu^{2+} + sorbinil (100µM)	0.067 ± 0.002	5.36 ± 0.16
+ Ca (50µM)	0.069 ± 0.003	5.52 ± 0.24
+ Mg (50µM)	0.065 ± 0.002	5.20 ± 0.16
+ Zn (50µM)	0.051 ± 0.004	4.08 ± 0.32
Cu^{2+} + AL-1576 (100µM)	0.056 ± 0.002	4.48 ± 0.16
+ Ca (50µM)	0.059 ± 0.004	4.72 ± 0.32
+ Mg (50µM)	0.057 ± 0.003	4.56 ± 0.24
+ Zn (50µM)	0.043 ± 0.002	3.44 ± 0.16

Copper sulphate, magnesium, zinc and calcium was dissolved in chelex treated distilled water. Other reagents were prepared and treated as described in Methods. Final concentrations of ascorbate, potassium phosphate buffer (pH 7.4), Cu^{2+}, sorbinil and AL-1576 were 100µM, 20mM, 200nM, 100µM and 100µM, respectively. Ascorbate oxidation rate ($-OD_{265nm/min}$) was monitored at 37°C for 3 minutes. Results are expressed as means+/-SD of 4 measurements.

5.3.3. Effects and the possible mechanism of ARIs on iron catalysed ascorbate oxidation

Similarly, sorbinil and AL-1576 also had some influence upon ascorbate oxidation catalysed by Fe^{3+} alone, or Fe^{3+} in the presence of EDTA and hydrogen peroxide (H_2O_2) (Table 5.3). In the presence of Fe^{3+} (20µM) alone the rate of ascorbate oxidation was low relative to that seen with Cu^{2+} (Table 5.3), as reported previously [Wolff, Wang & Spector 1987], but was accelerated over 10-fold by the simultaneous addition of EDTA (100µM) and H_2O_2 (2mM). Sorbinil had negligible inhibitory effect upon Fe^{3+}-EDTA-H_2O_2-catalysed ascorbate oxidation (Table 5.3) but inhibited ascorbate oxidation catalysed by free Fe^{3+} by 14%. AL-1576 was a more effective inhibitor than sorbinil in both cases (Table 5.3) and inhibited Fe^{3+}-EDTA-H_2O_2-catalysed oxidation of ascorbate by 21% and that catalysed by free Fe^{3+} by 50%. Neither TMG, ICI 105552 nor ONO 2235 had any effect upon Fe^{3+}- or Fe^{3+}-EDTA-H_2O_2-catalysed ascorbate oxidation (Table 5.3) as observed also in the case of Cu^{2+}-catalysed ascorbate oxidation.

Table 5.3 The effects of some aldose reductase inhibitors on Fe^{3+}- or Fe^{3+}-EDTA-H_2O_2-catalysed ascorbate oxidation

	Ascorbate oxidation rate $-OD_{265nm/min}$	nmoles/min	Percentage activity
Fe^{3+}	0.028 ± 0.002	2.24 ± 0.16	100
+ sorbinil (100µM)	0.024 ± 0.002	1.93 ± 0.16	86
+ AL-1576 (100µM)	0.014 ± 0.004	1.12 ± 0.32	50
+ ONO-2235 (100µM)	0.026 ± 0.003	2.08 ± 0.24	91
+ TMG (100µM)	0.030 ± 0.003	2.40 ± 0.24	107
+ ICI 105552 (100µM)	0.028 ± 0.001	2.24 ± 0.13	100
Fe^{3+}-EDTA-H_2O_2	0.476 ± 0.007	38.08 ± 0.56	100
+ sorbinil (100µM)	0.486 ± 0.008	38.88 ± 0.64	102
+ AL-1576 (100µM)	0.378 ± 0.005	30.24 ± 0.40	79
+ ONO-2235 (100µM)	0.473 ± 0.003	37.84 ± 0.24	99
+ TMG (100µM)	0.480 ± 0.005	38.40 ± 0.40	101
+ ICI 105552 (100µM)	0.478 ± 0.003	38.24 ± 0.24	100

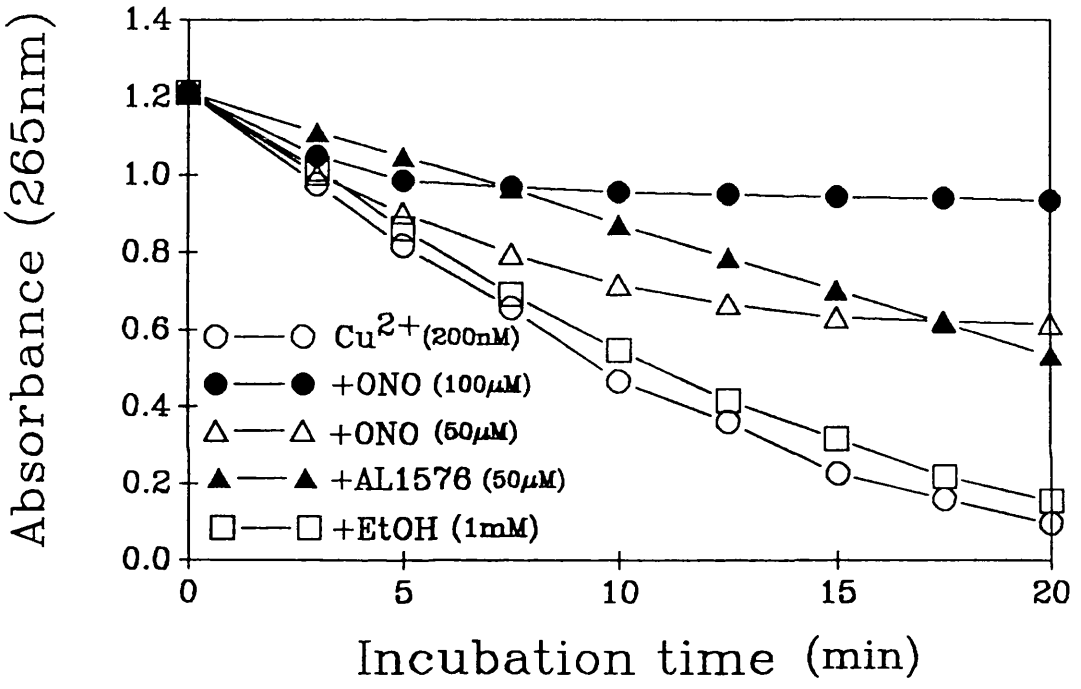
Ascorbate oxidation was induced by 20µM $FeCl_3$ or by 20µM $FeCl_3$, 100µM EDTA and 2mM H_2O_2 in 20mM sodium carbonate buffer (pH 7.4) at 37°C. The effects of ARIs on Fe^{3+} or Fe^{3+}-EDTA/H_2O_2 - induced ascorbate oxidation ($-OD_{265nm/min}$) were monitored for 3 minutes. Ascorbate oxidation rate is given as nmoles of ascorbate oxidised per minute. Results are expressed as means+/-SD of 4 measurements.

5.3.4. Unexpected inhibitory effect of ONO-2235 on Cu^{2+}-catalysed ascorbate oxidation

In previous experiment (Table 5.1), ONO-2235 had little effect on Cu^{2+}-catalysed ascorbate oxidation which was monitored for first 3 minutes. However, when ONO-2235 was incubated with Cu^{2+} and ascorbate in 20mM potassium phosphate buffer (pH 7.4) for up to 20 minutes at 37°C, ascorbate oxidation was inhibited (Figure 5.7). This inhibitory effect was ONO-2235 concentration and incubation time dependent. Interestingly, the kinetics of ascorbate oxidation inhibited by ONO-2235 were different from those affected by AL-1576 (Figure 5.7). As we can see in figure 5.7, the concentration of ascorbate remained higher in the reaction mixture in which ONO-2235 was included than in the mixture containing AL-1576 after incubation for 20 minutes. It is certain that the inhibitory effect of ONO-2235 on ascorbate oxidation was not due to the effect of ethanol (the solvent of ONO-2235 presented in reaction mixture) since the same concentration of ethanol had no effect on ascorbate oxidation (Figure 5.7).

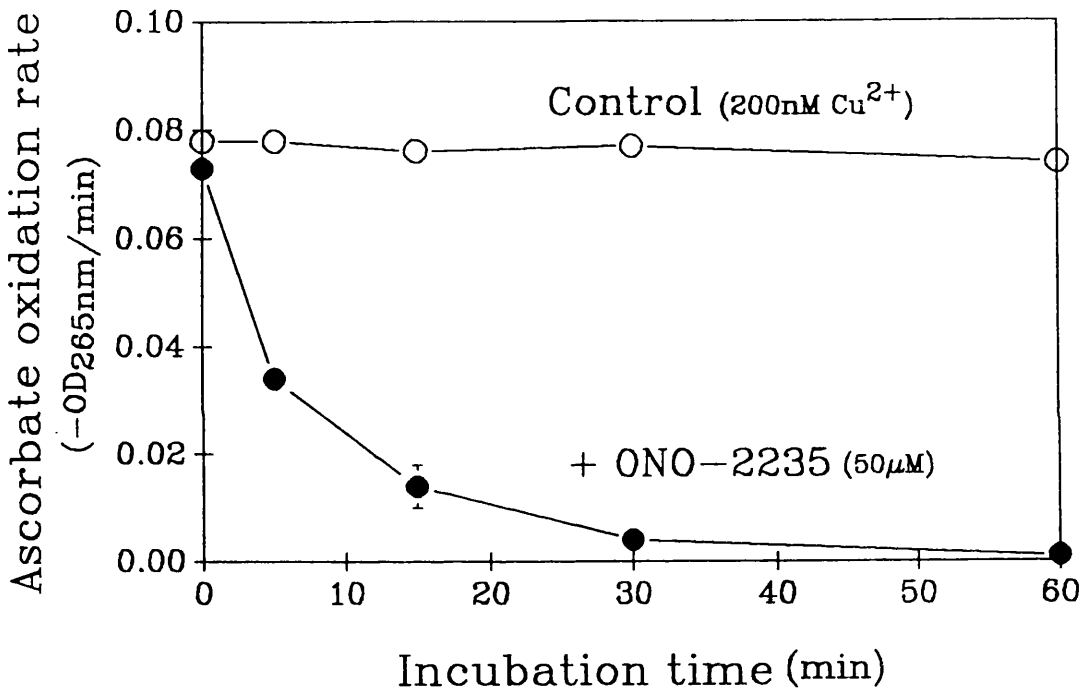
Figure 5.8 shows the results of ascorbate oxidation induced by Cu^{2+} alone or Cu^{2+} and ONO-2235 mixture, which were incubated at 37°C for 5 to 60 minutes before the addition of ascorbate to initiate oxidation-reduction reactions. Interestingly, the longer Cu^{2+} was incubated with ONO-2235 in 20mM potassium phosphate buffer (pH 7.4), the less ascorbate was oxidised. However, ascorbate oxidation was not affected by incubation of Cu^{2+} in the absence of ONO-2235 in the same buffer. This may indicate the interaction between Cu^{2+} and ONO-2235, which lead to the inhibitory effect of ONO-2235 on ascorbate oxidation.

Figure 5.7. Unexpected inhibitory effect of ONO-2235 on ascorbate oxidation



Ascorbate oxidation was induced by 200nM Cu^{2+} in 20mM potassium phosphate buffer (pH 7.4) with or without the addition of 50μM AL-1576, 50μM ONO-2235, 100μM ONO-2235 or 100μM ethanol. Ascorbate oxidation ($-OD_{265nm}$) was monitored at 37°C for up to 20 minutes. Cu^{2+} was dissolved in chelex treated distilled water. AL-1576 and ascorbate stock solutions and potassium phosphate buffer (pH 7.4) were made using chelex-treated double distilled water. ONO-2235 stock solution was made with pure ethanol.

Figure 5.8. Pre-incubation Cu^{2+} with ONO-2235 leads inhibition of ascorbate oxidation

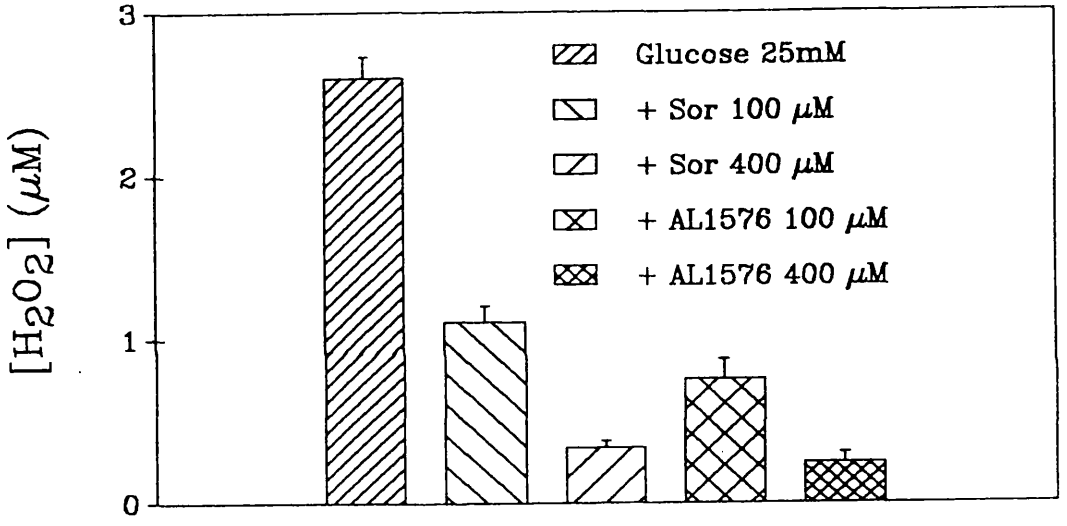


Cu^{2+} (200nM) in the presence or the absence of ONO-2235 (50μM) was incubated in 20mM potassium phosphate buffer (pH 7.4) at 37°C for 5 to 60 minutes. Then ascorbate oxidation was initiated by addition of the incubated mixture at the appropriate intervals. Ascorbate oxidation rate ($-OD_{265}$nm/min) was monitored for 3 minutes at 37°C.

5.3.5. Effects of sorbinil and AL-1576 on the generation of hydrogen peroxide by monosaccharide autoxidation

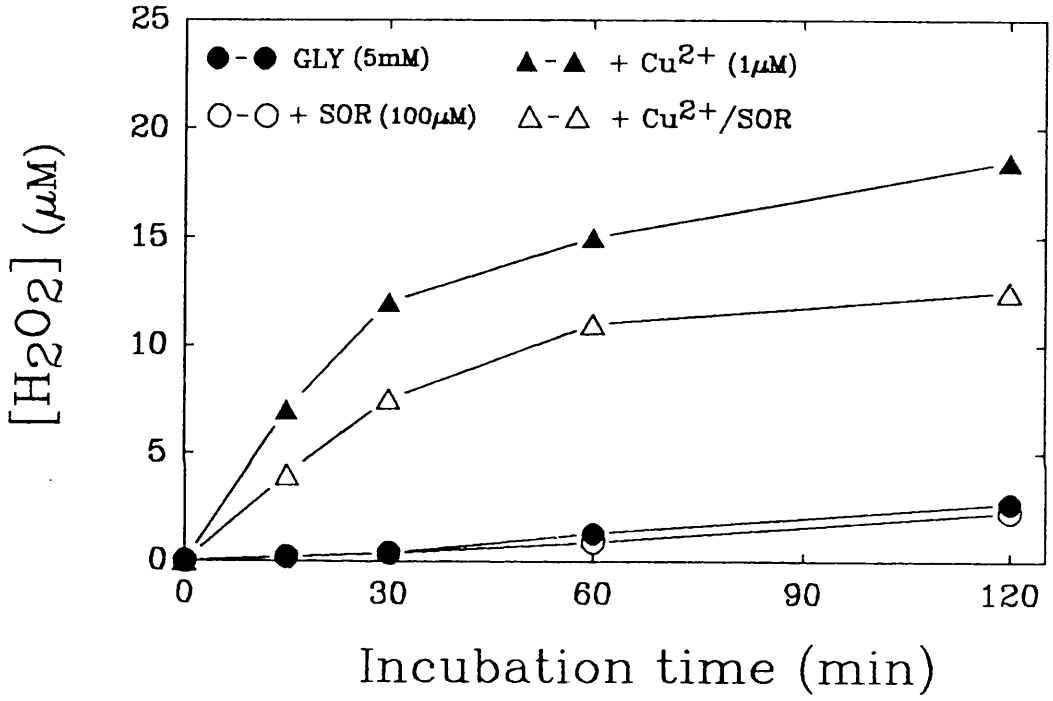
Monosaccharides, such as glucose and glyceraldehyde, are prone to transition metal catalysed autoxidation with the formation of $O_2^{-\cdot}$, H_2O_2 and $^{\cdot}OH$ [Thornalley et al 1984; Wolff et al 1984]. Using the FOX-1 assay described in chapter 3, the accumulation of H_2O_2 with respect to incubation time and concentration of glucose can be monitored as described in chapter 3 (Figure 3.5). Comparably, a low level of H_2O_2 was generated when glucose (25mM) was incubated in 10mM potassium phosphate buffer for 18 hours at $37^{o}C$ under sterile conditions (Figure 5.9). When glucose was incubated with the addition of sorbinil or AL-1576, H_2O_2 formation was significantly decreased (Figure 5.9). The higher the concentration of ARIs added to the incubation, the less H_2O_2 was formed. Similarly, Figure 5.10 shows time-related accumulation of H_2O_2 during glyceraldehyde oxidation induced by copper. Interestingly, sorbinil demonstrated the time-related inhibitory effect on H_2O_2 formation by copper induced glyceraldehyde oxidation.

Figure 5.9. The effects of sorbinil and AL-1576 on H_2O_2 formation by glucose autoxidation



Glucose (25mM) with or without the additions of sorbinil or AL-1576 (100μM or 400μM) was incubated in potassium phosphate buffer (pH 7.4, 10mM) at 37°C under sterile conditions. After incubation for 18 hours, 100μL samples were added to 900μL of reagent (100μM xylenol orange, 250μM Fe^{2+} and 100mM sorbitol in 25mM H_2SO_4). Absorbance was read at 560nm after 30 minutes incubation at room temperature. Values (peroxide concentration in the sample) are the mean plus/minus standard deviation obtained from duplicate assays. Catalase (Sigma Type C-40) added to the sample (at a concentration of 100u/ml) prior to addition of reagent abolished the colour development.

Figure 5.10. The effect of sorbinil on H_2O_2 formation by glyceraldehyde autoxidation



Glyceraldehyde (5mM) with or without the additions of Cu^{2+} (1μM) and/or sorbinil (100μM) was incubated in potassium phosphate buffer (pH 7.4, 10mM) at 37°C under sterile conditions. After incubation for 2 hours, 100μL samples at different time intervals were added to 900μL of reagent (100μM xylenol orange, 250μM Fe^{2+} and 100mM sorbitol in 25mM H_2SO_4). Absorbance was read at 560nm after 30 minutes incubation at room temperature. Values (peroxide concentration in the sample) are the mean plus/minus standard deviation obtained from duplicate assays. Catalase (Sigma Type C-40) added to the sample (at a concentration of 100u/ml) prior to addition of reagent abolished the colour development.

5.3.6. Effects of sorbinil and AL-1576 on copper/ascorbate induced cytotoxicity

Cu^{2+} and ascorbate were used to induce cytotoxicity on the V-79 cell line. The effects of sorbinil and AL-1576 on oxidative cellular damage upon the fibroblasts was assessed by ^{3}H-thymidine incorporation with this model *in vitro*.

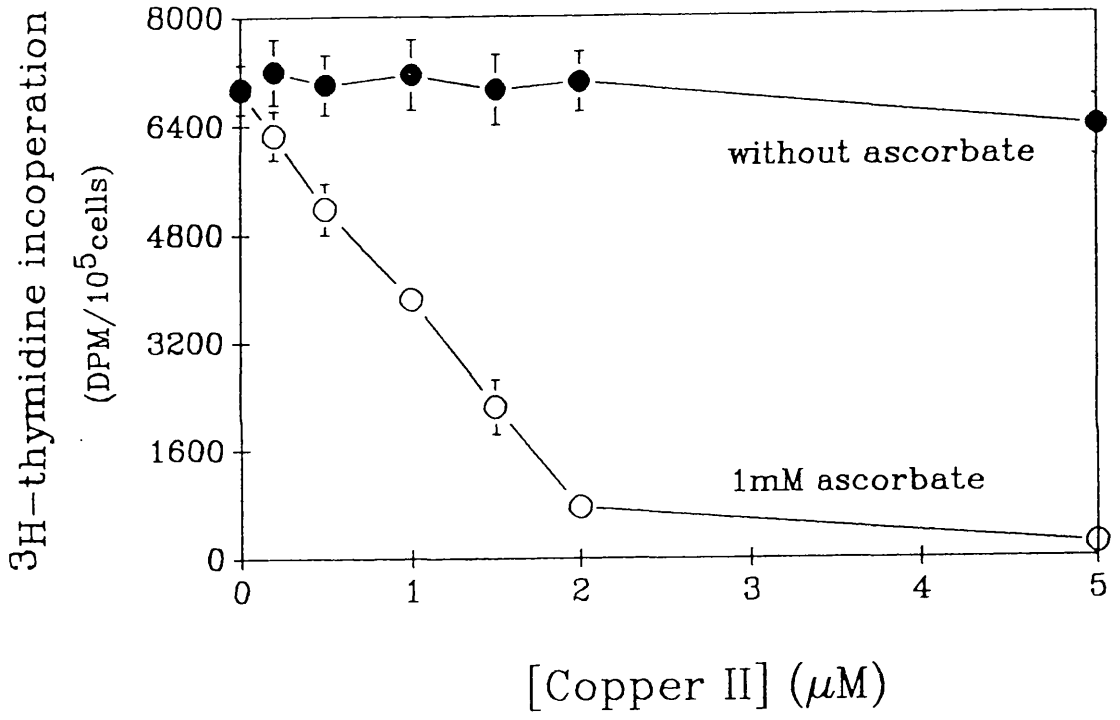
5.3.6.1. Model of oxidative cytotoxicity

It was reported previously that transition metal copper catalysed ascorbate oxidation can induce oxidative cellular damage *in vitro* [Samuni et al 1983; Bram et al 1980; Wolff et al 1987]. In this experiment V-79 cells were exposed to different concentrations of Cu^{2+} with or without the addition of 1mM ascorbate in chelex treated PBS for 30 minutes at 37^{o}C (Figure 5.11). Copper (II) at low concentration (less than 5μM in medium) showed no toxic effect on the cells in the absence of ascorbate (Figure 5.11). In the presence of 1mM ascorbate, however, copper (II) demonstrated the dose-dependent toxic effect upon V-79 cells detected by ^{3}H-thymidine incorporation (Figure 5.11).

5.3.6.2. Inhibitory effects of sorbinil and AL-1576 on cytotoxicity

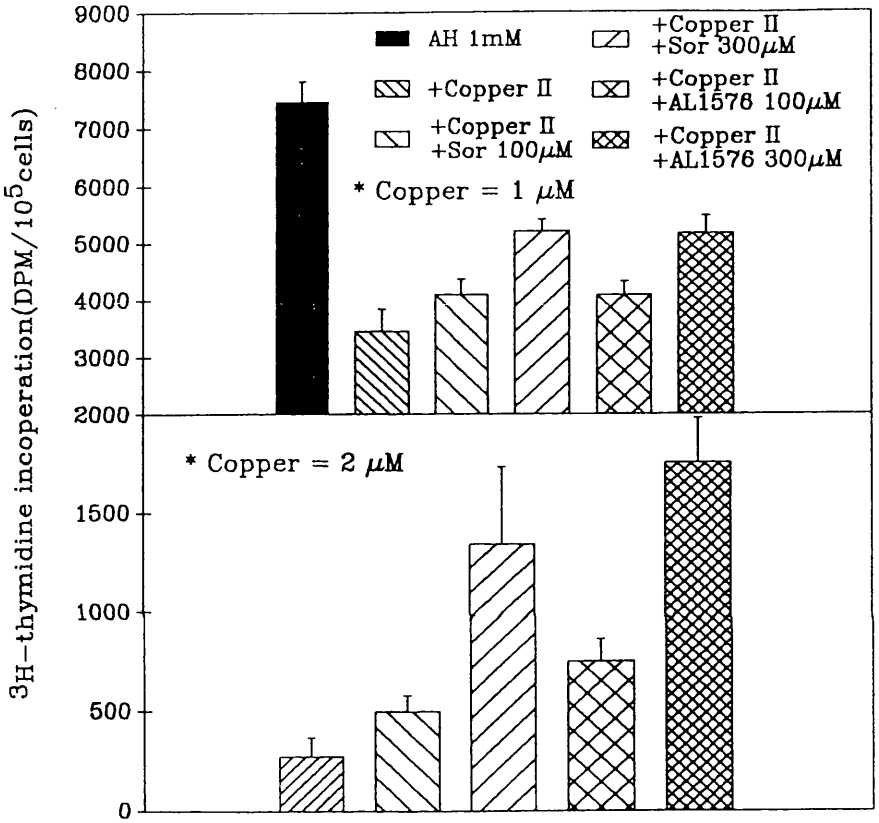
Figure 5.12 shows the inhibitory effects of sorbinil and AL-1576 on Cu^{2+}/ascorbate induced cytotoxicity. As in the previous experiment, copper concentration dependent decrease in thymidine incorporation was induced in the cells exposed to 1mM ascorbate plus 1μM or 2μM Cu^{2+} in PBS (control groups in Figure 5.12). Interestingly, when copper and ascorbate were added to the cells in PBS together with different concentration of sorbinil or AL-1576, their cytotoxic effects were significantly reduced (Figure 5.12). Again, the recovery of thymidine incorporation was dose-dependent on the concentration of sorbinil or AL-1576.

Figure 5.11. Copper (II) and ascorbate induced cytotoxicity



The effects of different concentrations of Cu^{2+}, in the presence of or the absence of 1mM ascorbate, on viability of V-79 cells were examined by ^{3}H-thymidine incorporation assay. Cells (10^5/ml) were cultured in chelex treated PBS for 1 hour before exposure to Cu^{2+} and ascorbate for another 30 minutes. ^{3}H-thymidine incorporation was then assessed by the method described in Materials and Methods. Results are expressed as a mean ± standard deviation obtained from quadruplicate assays.

Figure 5.12. The effects of sorbinil and AL-1576 on Cu^{2+}/ascorbate induced cytotoxicity



V-79 cells (10^5/ml) were exposed to 1mM ascorbate with or without the additions (as labelled in the figure) in chelex treated PBS for 30 minutes after pre-incubation in PBS for 1 hour. ^{3}H-thymidine incorporation was then assessed by the method described in Materials and Methods. Results are expressed as a mean ± standard deviation obtained from quadruplicate assays. Note: AH=ascorbate, Sor=sorbinil.

Paired t-test:

AH cf *AH+1μM* Cu^{2+}, P<0.01; *AH* cf *AH+2μM* Cu^{2+}, P<0.0005;

AH+1μM Cu^{2+} cf *AH+1μM* Cu^{2+}*+100μM sor*, P=0.24; *AH+1μM* Cu^{2+} cf *AH+1μM* Cu^{2+}*+300μM sor*, P<0.04; *AH+1μM* Cu^{2+} cf *AH+1μM* Cu^{2+}*+100μM AL1576*, P=0.23; *AH+1μM* Cu^{2+} cf *AH+1μM* Cu^{2+}*+300μM AL1576*, P<0.03; *AH+2μM* Cu^{2+} cf *AH+2μM* Cu^{2+}*+100μM sor*, P<0.03; *AH+2μM* Cu^{2+} cf *AH+2μM* Cu^{2+}*+300μM sor*, P<0.01; *AH+2μM* Cu^{2+} cf *AH+2μM* Cu^{2+}*+100μM AL1576*, P<0.01; *AH+2μM* Cu^{2+} cf *AH+2μM* Cu^{2+}*+300μM AL1576*, P<0.002;

AH+1μM Cu^{2+}*+100μM sor* cf *AH+1μM* Cu^{2+}*+300μM sor*, P<0.005; *AH+2μM* Cu^{2+}*+100μM sor* cf *AH+2μM* Cu^{2+}*+300μM sor*, P<0.02; *AH+1μM* Cu^{2+}*+100μM AL1576* cf *AH+1μM* Cu^{2+}*+300μM AL1576*, P<0.04; *AH+2μM* Cu^{2+}*+100μM AL1576* cf *AH+2μM* Cu^{2+}*+300μM AL1576*, P<0.01;

5.4. Discussion

It has been suggested that some complications of diabetes mellitus may derive from oxidative tissue damage catalysed by "decompartmentalised" transition metals [Wolff et al 1991]. Thus, developing low toxicity antioxidants, such as weak metal-chelating agents, may be one possible way of blocking the development of diabetic complications.

The data described above suggest that sorbinil and AL-1576 have the ability to chelate redox active transition metal ions, and thus inhibit transition-metal catalysed ascorbate oxidation via this mechanism. This ability seems dependent upon the presence of a spirohydantoin group within the compounds. This is suggested by (1) structural analogy of the hydantoin group with imidazole and Histidine; (2) by comparison of those structures which inhibit oxidation with those which do not; (3) by the ability of hydantoin itself to inhibit copper-catalysed ascorbate oxidation partially; (4) by implication of a role for nitrogen lone pair electrons on the basis of greater inhibition of ascorbate oxidation at higher pH. The inhibitory effect of sorbinil or Al-1576 on copper catalysed ascorbate oxidation seems not be due to the free radical scavenging activity, since ethanol (a hydroxyl radical scavenger) has no effect on ascorbate oxidation (Figure 5.5). In addition, sorbinil (only at an high concentration) slightly inhibits ascorbate oxidation catalysed by phenanthroline-bound copper (Figure 5.4).

No specific mechanism can be advanced to account for the proposed binding of copper and iron to the compounds except to note that the hydantoins bear some chemical similarity to the 1-alkyl-2-methyl-3-hydroxypyrid-4-ones which are potent iron chelating agents [Jeremy et al 1988]. However, the affinity constants, at least for Cu^{2+}, would have to be lower than the respective affinity constants of the polynitrogenated and polycarboxylated compounds EDTA, NTA and BCA for Cu^{2+} (Table 5.1). The mechanism of Cu^{2+} chelation by sorbinil is different from that of imidazole since sorbinil, but not imidazole, is able to inhibit Cu^{2+}-orthophenanthroline-catalyzed ascorbate oxidation. This presumably relates to the ability of sorbinil to approach sites on the metal which are required for ascorbate oxidation.

Similarly, it is difficult to deduce, from the above results, the exact mechanism of the effect of ONO-2235 on binding copper and thus inhibiting ascorbate oxidation. Although it is certain that ONO-2235 has a different pattern from AL-1576 in inhibiting copper catalysed ascorbate oxidation, the structure of the hydantoin group in sorbinil and AL-1576 is somewhat similar to the structure of nitrogen related five ring and carboxyl group in ONO-2235. This structure of ONO-2235 may relate to the interaction between copper and ONO-2235. The effect of ONO-2235 on ascorbate oxidation may be due to its ability to precipitate copper ions.

Whatever the precise chelating mechanism is, the ability of sorbinil and AL-1576 to bind copper and thus to inhibit the cytotoxic effect induced by copper catalysed ascorbate oxidation, further suggests their potential role in preventing or limiting oxidative tissue damage (Figure 5.12). Additionally, the ability of sorbinil or AL-1576 to inhibit H_2O_2 formation by transition metal catalysed sugar "autoxidation" (Figure 5.9 & 5.10), is consistent with previous observations [Wolff & Crabbe 1985], in which sorbinil blocks free radical NADPH oxidation stimulated by transition-metal catalysed monosaccharide enediol oxidation *in vitro*. This indicates that the metal-chelating capability of sorbinil, AL-1576 or ONO-2235 may be important in their effects of inhibiting hyperglycaemic tissue damage in diabetes.

CHAPTER 6 GENERAL DISCUSSION

6.1. Transition metal, lipid peroxidation and cytotoxicity of oxidised LDL

As discussed earlier (chapter 1, Figure 1.1), polyunsaturated fatty acids are prone to transition metal-catalysed oxidation [Halliwell & Gutteridge 1989; Braughler et al 1986; Chan et al 1982]. Lipid peroxidation does not only act as a mediator which translates the reactivity of transition metals and primary free radicals into lipid degradation products, but also can act as an amplifier for the potential reactivity of transition metals and primary free radicals, since the chain reaction or propagation in lipid peroxidation lead to the formation of a great number of lipid peroxides, alkyl radicals and alkoxyl radicals. It seems reasonable to assume that these reactive oxygen species, in the presence of oxygen-activating transition metals, can overwhelm the antioxidant systems and destroy the biological substances in which they were generated. For instance, extensive peroxidation *in vitro* leads to loss of cell membrane integrity as well as inactivating enzymes.

The observations reported in chapter 4 also confirmed that oxidised LDL (induced by copper) is toxic to fibroblasts (Figure 4.2) and this cytotoxic effect correlated well with the content of confirmed lipid peroxides in LDL (Figure 4.3). Metal-chelating agents can prevent such lipid peroxidation and cytotoxicity *in vitro*, suggesting the involvement of transition metals (Figure 4.1 & 4.2). This observation is consistent with the previous reports [Morel et al 1983; Hessler et al 1983]. Interestingly, Morel and Chisolm [1989] also reported that lipoproteins (VLDL and LDL) from diabetic rats did indeed undergo an accelerated accumulation of peroxide and were toxic to human fibroblasts *in vitro*. The increased lipid peroxidation in lipoproteins isolated from diabetic patients [Nishigaki et al 1981] and the elderly [Hagihara et al 1984] may reflect the increased stress of oxidative tissue damage *in vivo*.

Perhaps the oxidation of apoproteins in LDL is also important to the tissue damage in diabetes, but there is no evidence yet to prove that this occurs in diabetes.

6.2. Glycation and oxidation in cytotoxicity of glucose modified LDL

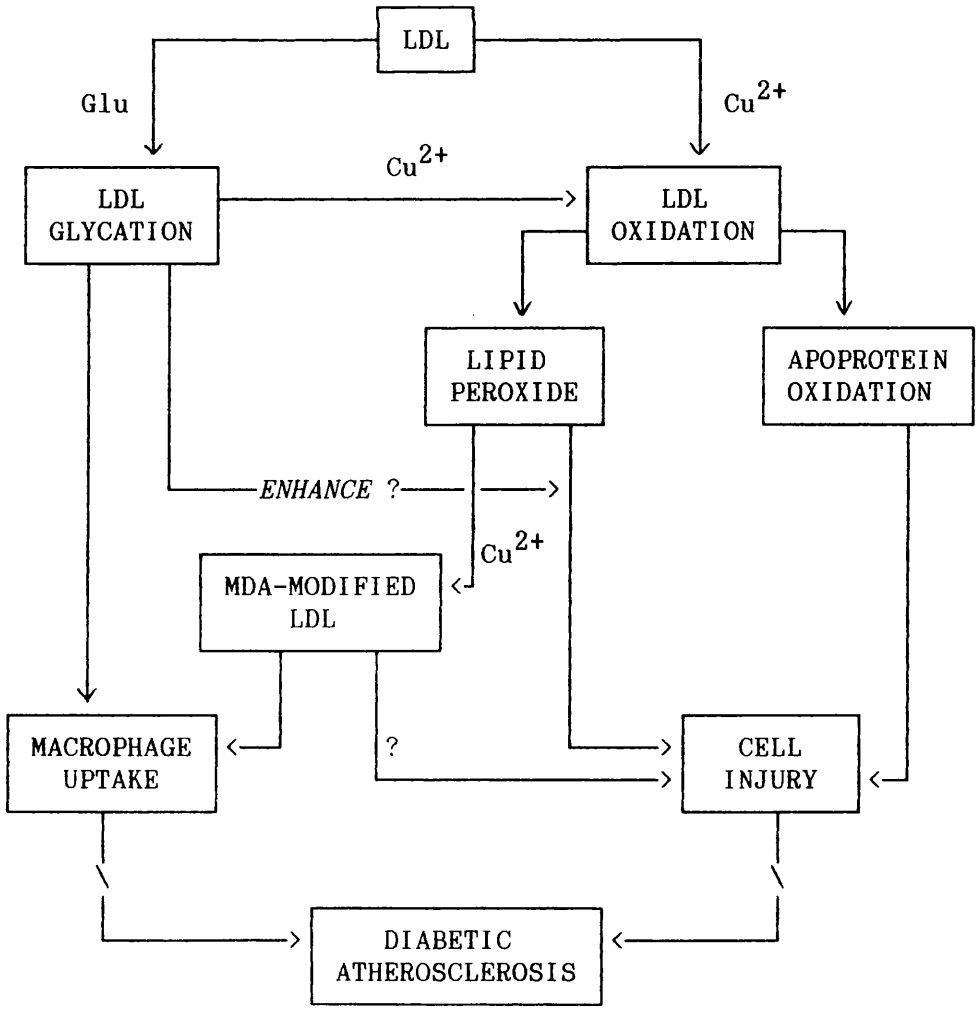
It is widely acknowledged that glycation of LDL may be important in the development of diabetic atherosclerosis, since glycated LDL is poorly recognized by fibroblast receptors and accumulates in macrophage [Saski & Cottam 1982]. The latter event leads to the formation of foam cells in arterial walls as proposed by Goldstein and Brown [1977]. Atherosclerosis in diabetes, however (as originally observed by Pirart [1978]), is only indirectly related to poor glycaemic control so that other aetiological factors need to be considered.

On the other hand, investigations have shown that oxidation of LDL also leads to numerous changes in its biological properties that could be of pathogenetic importance, including increased cytotoxicity, uptake in macrophages, and chemotactic activity for monocytes [reviewed by Witztum & Steinberg 1991]. It has also been shown that LDL exposed to glucose exhibits a time dependent and transition metal dependent accumulation of lipid peroxides [Hunt et al 1990]. Transition metals are available in most experimental buffers and total transition metal (such as copper) may be increased in diabetic patients [Locke et al 1932; Mateo et al 1978; Noto et al 1983] and in the elderly [Harman 1965]. It is conceivable that glycation either occurring *in vitro* or *in vivo* may be accompanied by oxidative reactions. Thus, oxidative events associated with the exposure of LDL to glucose may contribute to cytopathological effects of glycated LDL.

The investigation reported in chapter 4 first demonstrated that exposure of LDL to glucose *in vitro* was associated with peroxidation, and that glucose-incubated LDL was toxic to fibroblasts. It was also suggested that although LDL modified by glucose in the absence of peroxidation, i.e. glycation alone, had no effect upon cell proliferation, glycation of LDL might enhance cytotoxicity of oxidised LDL by unknown

mechanisms. Therefore, there is no evidence for suggesting that either glucose-induced peroxidation, or the attachment of glucose to LDL amino groups, are independent cytotoxic factors, at least *in vitro*. The data are, however, consistent with a hypothesis that there is a synergistic cytotoxic reaction between peroxidation and glycation of LDL induced by glucose. One possibility is that peroxidised material of LDL causes cellular damage and glycation of the LDL enhances uptake of peroxidised material into critical cellular sites. It may also be due to enhancement of oxidation of protein moieties in LDL by glycation. Figure 6.1 summarizes the possible interaction of LDL glycation and oxidation in the development of diabetic atherosclerosis. However, the precise mechanisms of the cytotoxic effect of glucose modified LDL need further investigation.

Figure 6.1. The possible interaction of LDL glycation and oxidation in the development of diabetic atherosclerosis.(Note: Glu = glucose)



The possible interaction between LDL oxidation and glycation upon cells gives support to the results reported by Morel & Chisolm [1989]. They reported that the treatment of diabetic rats with antioxidants, such as vitamin E or probucol, inhibited lipid peroxidation of lipoproteins and thus prevented cytotoxicity of those lipoproteins *in vitro*. The mechanism of lower cytotoxicity of the lipoproteins isolated from diabetic rats treated with insulin, however, was unknown [Morel & Chisolm 1989]. The results reported in chapter 4 shows that glycation of LDL was associated with lipid peroxidation (Figure 4.1) and glycation might enhance oxidative cellular damage induced by glucose and metal ion modified LDL (Figure 4.2 & Table 4.2). These may explain why the lipoprotein (isolated from insulin treated diabetic rats) was less toxic to cells *in vitro*, since blood glucose was lowered and thus LDL glycation was decreased in diabetic rats treated with insulin *in vivo* [Morel & Chisolm 1989].

6.3. Monosaccharides autoxidation as a source of reactive oxygen species in diabetes

In addition to the glycation reactions, monosaccharides (such as glucose) are prone to transition metal-catalysed oxidation. Monosaccharides can enolize and therefore reduce transition metal and molecular oxygen sequentially, yielding $O_2^{-\cdot}$, $OH^{\cdot}$ and dicarbonyls (*a*-ketoaldehyde) [Wolff et al 1984; Wolff & Dean 1987; Thornalley et al 1984; Thornalley & Stern 1984]. Experiments *in vitro* show that glucose incubated with protein undergoes a similar process with the formation of $O_2^{-\cdot}$ and $OH^{\cdot}$ [Wolff & Dean 1987; Hunt et al 1988]. The latter can attack glycated protein and induce site-specific damage, including the oxidation of amino acids in protein, the generation of fluorophore (protein browning) and the fragmentation of protein [Wolff et al 1987; Hunt et al 1988; Hunt & Wolff 1991]. It has been suggested that the generation of oxidative intermediates and protein damage are transition metal dependent [Wolff et al 1991]. Metal-catalysed monosaccharide oxidation may be one process contributing to oxidative damage within the monosaccharide-stressed tissue.

There is little doubt that free radicals and transition metals are involved in protein alterations after exposure to glucose *in vitro*. The production of H_2O_2 by glucose in the presence of protein, however, has only been indicated by the inhibitory effect of catalase on protein fragmentation, since the steady-state concentrations of H_2O_2 are too low for measurement by most methods [Hunt et al 1988]. The accumulation of H_2O_2 with respect to time and concentration can be monitored using a sensitive assay based upon the amplified oxidation of Fe^{2+} to Fe^{3+} in the presence of xylenol orange (Figure 3.5). This proves that H_2O_2 is formed during protein glycation and glucose autoxidation. Spirohydantoin ARIs, however, inhibit H_2O_2 formation during monosaccharide autoxidation (Figure 5.9 & Figure 5.10). The latter event may also account for the antioxidant activity of sorbinil *in vivo* in diabetic rats [Yeh & Ashton 1990].

6.4. Ascorbate oxidation as the source of reactive oxygen species in diabetes and ageing

In addition to monosaccharide, other redox materials in diabetic subjects, such as ascorbate, may be important in the generation of oxidative intermediates and the consequent tissue damage because of their capability to reduce transition metals. Moreover, diabetes mellitus is also accompanied by disturbance of ascorbic acid metabolism [Chatterjee & Banerjee 1979; Jennings et al 1987; Som et al 1981; Taylor et al 1988]. This metabolic abnormality is associated with diabetic complications [Taylor et al 1988]. It is well known that ascorbic acid is sensitive to oxidation and its autoxidation is transition metal dependent [Buettner 1988; Halliwell et al 1976]. More recently, Hunt and Wolff compared protein (lens crystallin and serum albumin) modification induced by autoxidation of glucose, ascorbic acid and arachidonic acid, and demonstrated that ascorbate and arachidonate, in the presence of trace amounts of Cu^{2+}, were more powerful for fragmenting and browning proteins than glucose [Hunt & Wolff 1990].

Ascorbate (AH), in the presence of oxygen, can reduce free or loosely bound transition metals (Me^{n+}(~~biol)) to the reduced form of the metal ($Me^{(n-1)+}$(~~biol)) with the formation of dehydroascorbate (DHA) and superoxide ion ($O_2^{-\cdot}$) at the metal binding site. The latter will dismutate to form H_2O_2 which, catalysed by transition metals, decomposes into $HO^{\cdot}$.

$$AH + O_2 + Me^{n+}(\sim\sim biol) \longrightarrow DHA + O_2^{-\cdot} + H^+ + Me^{(n-1)+}(\sim\sim biol)$$

$$O_2^{-\cdot} + O_2^{-\cdot} + 2H^+ \longrightarrow H_2O_2 + O_2$$

$$H_2O_2 + Me^{(n-1)+}(\sim\sim biol) \longrightarrow HO^{\cdot} + OH^- + Me^{n+}(\sim\sim biol)$$

The reactive oxygen radicals can cause DNA damage [Chiou 1983; Kazakov et al 1988; Wang et al 1989], cytotoxicity [Bram et al 1980; Samuni et al 1983; Wolff et al 1987], enzymatic inactivation [Orr 1967a; Orr 1967b; Shinar et al 1983; Nakanishi et al 1985; Alonso and Rubio 1987], protein structure changes [Marx et al 1985; Bensch et al 1985; Garland et al 1986] and monosaccharide oxidation [Aruoma et al 1987]. Ascorbate is toxic to cells in the presence of trace amounts of Cu^{2+} (concentration down to 0.2 µM in the medium), but is not toxic to cells in the absence of Cu^{2+} (Figure 5.11). This indicates that free or loosely bound transition metals are essential for the cytotoxic effect of the pro-oxidant, i.e. ascorbate.

The decreased level of ascorbate and the increased ratio of dehydroascorbate / ascorbate in plasma and tissue of diabetic subjects [Jennings et al 1987; Som et al 1981] is in agreement with the view that metal catalysed oxidation is increased *in vivo*. Recently, Dunn et al [1990] reported that CML was also formed on reaction of ascorbate with proteins under metal-catalysed autoxidation condition. Since CML accumulates with age and at an accelerated rate in diabetes in both lens and collagen [Dunn et al 1989 & 1991; Baynes 1991], the detectable CML in diabetes and ageing may be the result of protein damage induced by transition metal catalysed oxidation, such as glucose oxidation and possible ascorbate oxidation *in vivo*.

Again, the inhibitory effects of spirohydantoin ARIs on copper induced ascorbate oxidation and associated cytotoxicity (Figure 5.12) further indicate their antioxidant activity. If there is increased oxidative tissue damage catalysed by transition metals in diabetes, then the antioxidant capability of those ARIs might account for their inhibitory effect on the development of diabetic complications [Pfeifer 1986].

6.5. Oxidative stress may be a common pathway in the development of diabetic complications

Oxidative stress may be defined as the increased steady-state of reactive oxygen species in biological systems and its consequent tissue damage. Transition metal catalysed oxidation of lipids, glucose and ascorbate may be the source of reactive oxygen species in diabetes. Furthermore, peroxides may influence arachidonic acid metabolism. For example, nanomolar levels of lipid peroxides or micromolar levels of H_2O_2 stimulate cyclo-oxygenase activity. In fact, the continued activity of the enzyme is dependent upon the continuous availability of peroxide stimulators of the enzyme, produced either as a result of leucocyte activity, or transition metal- or lipoxygenase-catalyzed oxidation of unsaturated fatty acids [Hemler et al 1979; Hemler & Lands 1980; Cleland 1984]. Small alterations in "peroxide tone" (the environmental peroxide level) may contribute to abnormalities in arachidonic acid metabolism.

Since cyclo-oxygenase activity appears to be increased in diabetes [Karpen et al 1982; Butkus et al 1980], arachidonic acid metabolism, which forms the endo- and hydro-peroxide intermediates (prostaglandins, thromboxanes and leukotrienes), may be another source of reactive oxygen species and contribute to tissue damage in diabetes mellitus [Metz, 1983]. It is proposed that the increased prostaglandin intermediates in diabetes may contribute to diabetic microangiopathy through their effects on platelet aggregation [Waitzman 1979; Butkus et al 1980]. Therefore, enzymatic oxidation and "peroxide tone" may be the other source of reactive oxygen species and cause accumulation of peroxides implicated in angiopathy in diabetes [Waitzman 1979; Butkus et al 1980].

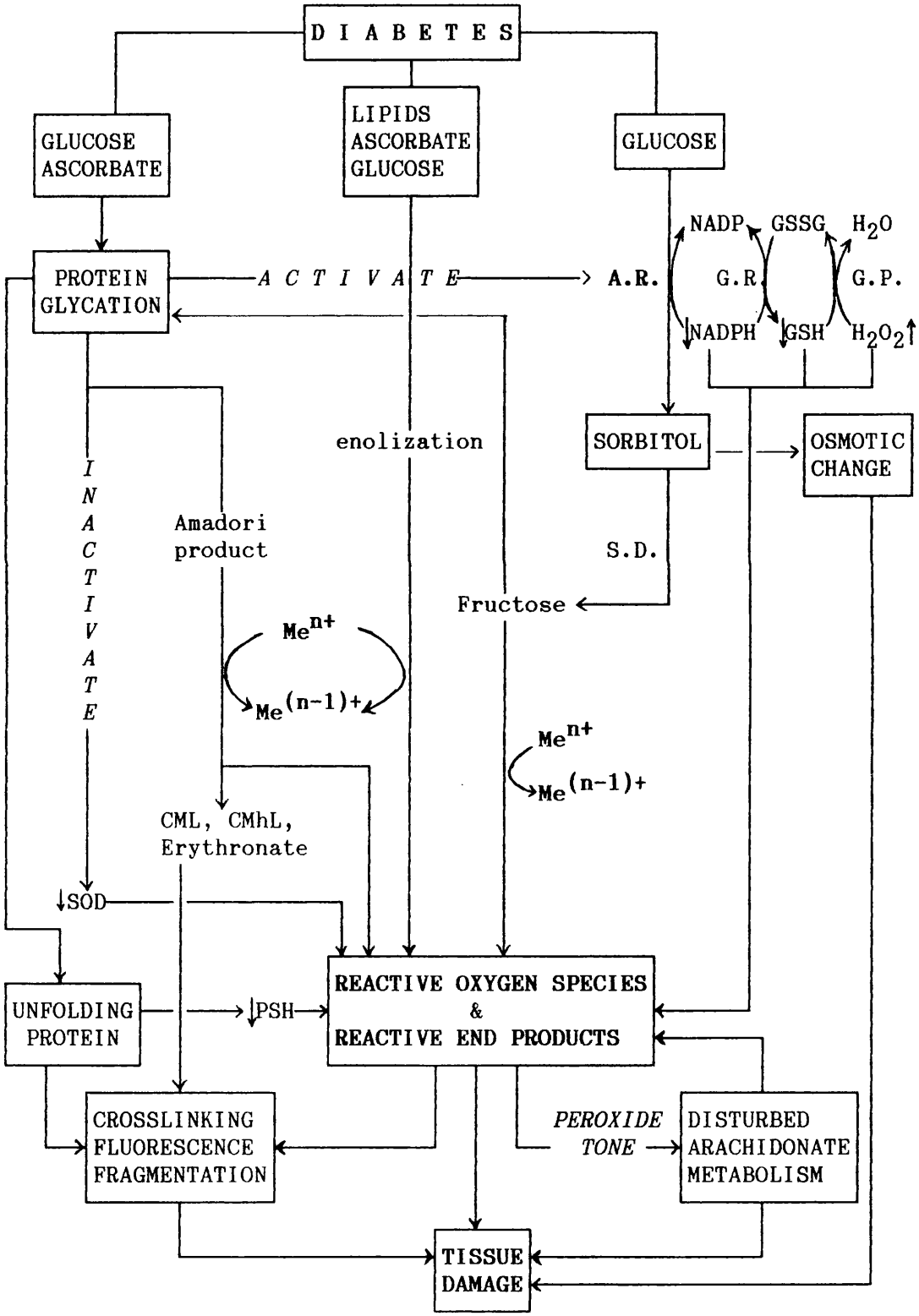
Interestingly, protein glycation may also contribute to oxidative stress through oxidative glycation and/or glycoxidation pathways with the formation of reactive oxygen species and reactive end products (as described in chapter 1). It is suggested that small amounts of transition metals are required for protein fluorescence produced by glucose since free radical oxidative reactions of the reducing sugar are involved [Wolff &

Dean 1987; Hunt et al 1988]. Other physiological reducing agents, such as ascorbic acid and unsaturated fatty acids are more efficient protein browning or/and cross-linking agents than glucose *in vitro*, if their oxidation is permitted by the presence of transition metals [Gutteridge 1985; Ortwerth et al 1988; Koller et al 1986; Hunt & Wolff 1990]. In addition, glycation of SOD causes inactivation of the antioxidant enzyme and thus may cause the accumulation of reactive oxygen species. Furthermore, it is suggested that aldose reductase can be activated by glycation [Srivastava et al 1986; Srivastava et al 1989], and monoaminoguanidine (the inhibitor of advanced glycated end product formation) also inhibits the activity of aldose reductase both *in vitro* and *in vivo* [Kumari et al 1991]. The activated aldose reductase may initiate further oxidative reactions; this is discussed later.

It has been hypothesized that the polyol pathway metabolism also contributes to oxidative tissue damage. First, it is suggested that aldose reductase may render the lens more susceptible to oxidative injury by lowering its redox status [Barnett et al 1986; Srivastava et al 1989; Wolff 1987] since the activation of aldose reductase by glycation (induced by galactose or glucose) may divert substantial NADPH from the enzyme glutathione reductase, which also requires NADPH as cofactor for its contribution to lens antioxidant defence. This may be significant to galactose-fed animals (which have GSH depletion [Lou et al 1988]), but of little importance to humans because of the low activity of aldose reductase in human tissue [Lerman & Moran 1988; Jedziniak et al 1981]. Secondly, the final product of the polyol pathway, i.e. fructose, is prone to transition metal-catalysed oxidation with the formation of reactive oxygen species [Jiang et al 1990]. Fructose also causes modification of protein with the formation of browning product [McPherson et al 1988]. Again, fructose induced tissue damage may be less important than glucose in diabetics because of the low activity of aldose reductase in human tissue [Lerman & Moran 1988; Jedziniak et al 1981].

It could be suggested that oxidative stress may be a common pathway involved in tissue damage in ageing and diabetes (summarized in Figure 6.2). There is no direct evidence which has shown an increase in the steady-state levels of reactive oxygen species or an increase in the rate of oxidant production in tissue from diabetic patients or the elderly.

Figure 6.2. The possible relationship between protein glycation, oxidative stress and polyol pathway metabolism in the development of diabetic complications.



Note: SOD = superoxide dismutase; PSH = protein thiol; CML= Carboxymethylysine; CMhL = Carboxymethylhydroxylysine; A.R. = aldose reductase; S.D.= sorbitol dehydrogenase; G.R.= glutathione reductase; G.P.= glutathione peroxidase.

However, the increase of the levels of end products of lipid peroxidation and protein oxidation, as well as the decline of antioxidants (such as vitamin E and ascorbate) in tissues (Chapter 1), may indicate the increase of oxidative stress in diabetes and the elderly.

6.6. The availability of transition metals in diabetes and ageing

Transition metals, as described in Chapter 1, can catalyse the oxidation of reducing agents (including monosaccharides, ascorbate and the unsaturated fatty acids) with the formation of reactive oxygen species, and catalyse oxidative glycation and glycoxidation; transition metals are also associated with site-specific protein damage and initiate cellular and DNA damage through Fenton and Haber-Weiss reaction. The major question which then arises is whether iron or copper catalysts are available for oxidative reactions *in vivo*. These metals are normally bound by specific binding proteins, such as ceruloplasmin, transferrin, ferritin, hemoglobin, myoglobin, albumin and various enzymes. Thus, the normal concentration of free metal ions *in vivo* may be in the nanomolar range. However, there may be some loosely bound metals, such as those bound to citrate, amino acids, phosphate and ADP, available for catalysing free radical reactions described earlier. Metal ions may be released from cellular compounds during cell damage [Minotti et al 1991] or from the binding proteins during their degradation [Gutteridge 1986; O'Connell et al 1986; Winterbourn et al 1991], that may enhance further free radical reactions and protein degradation [Stadtman 1990]. Moreover, albumin bound copper is still capable of catalysing ascorbate oxidation, although the oxidation rate is much lower than that catalysed by the same concentration of free copper. But in the case of chronic disease, such as diabetic complications, such slow oxidation may be significant to the development of those diseases.

The total copper levels are higher in diabetic individuals than in normals [Locke et al 1932], and are highest in diabetics with angiopathy and/or alterations in lipid metabolism [Mateo et al 1978; Noto et al 1983]. It is not clear whether this copper increase is caused by an increase in caeruloplasmin or represents an increase in the pool of copper associated with albumin or low molecular weight chelates. However, a small proportion of plasma copper is attached to amino acids and serum albumin [Lau & Sarkar

1981; McGahan & Bito 1983; Gutteridge et al 1985] and copper may participate in oxidative reactions in this form. A role for copper in the pathogenesis of diabetic complications is also suggested by the observation that levels of copper are higher in cataractous than clear lenses [Nath et al 1969]. Furthermore, the increased carbonyl products of site-specific, metal-catalysed oxidation of aged lens proteins [Garland 1990; Stadtman & Oliver, 1991], as well as the increased oxidised products of glycated proteins (catalysed by transition metals) in aged and diabetic lens and collagen [Ahmed et al 1986; Baynes 1991], suggest that transition metal is available in the form permitting such potentially pathological oxidations *in vivo*.

Iron may also contribute to oxidative stress since diabetes is found commonly in transfusion siderosis, dietary iron overload and idiopathic haemochromatosis [McLaren et al 1983; Phelps et al 1989]. Iron also appear to be elevated in the lens with cataract [reviewed by Garland 1990]. Patients with classical iron overload often possess low levels of serum and white blood cell ascorbic acid [Cohen et al 1981; Nienhuis 1981]. In diabetes, levels of plasma and white blood cell ascorbic acid are lower (despite similar levels of intake and excretion), and oxidation of this antioxidant to dehydroascorbate is higher than in normal individuals [Jennings et al 1987; Som et al 1981]. This suggests that oxidative stress, perhaps initiated by transition metals, may contribute to the pathogenesis of diabetes and its complications [Oberley 1988; Wolff 1987].

Ageing has also been proposed to possess a free radical component [Harman 1965; Harman 1981]. Both plasma copper [Harman 1965] and peroxide [Suematsu et al 1977] levels increase with age in man. The levels of lens Cu^{2+} increase with age but are 4-fold higher in cataract than in age-matched clear lenses [Nath et al 1969]. This suggests that transition metal initiate oxidative stress may also contribute the ageing process.

6.7. The metal-chelating ability of ARIs provides a clue for developing new drugs

If ARIs prove beneficial in the treatment of diabetic complications, such as neuropathy [Pfeifer 1986; Mori et al 1988] and early retinopathy [Hotta

et al 1988], it may be as a result of activity distinct from the inhibition of polyol accumulation. It is conceivable that the secondary activity (the metal-chelating ability) of some ARIs may contribute to some of the observed biological effects which are not easily explained by simple inhibition of aldose reductase activity. For example, the inhibitory effect of sorbinil on increased kidney glomerular filtration rate observed in experimental diabetes appears not to be related to inhibition of glomerular polyol production but rather suppression of the formation of vasodilatory prostaglandins [Craven & DeRubertis 1988]. Sorbinil can also block lipid peroxidation in the diabetic rat lens [Yeh & Ashton 1990], and prevent the formation of non-tryptophan fluorescence and high molecular weight protein in lens crystallins of galactosaemic rats [Nagaraj & Monnier 1990]. In addition, AL-1576 is able to retard naphthalene induced cataract [Lou et al 1990]. Aldose reductase is difficult to implicate in this form of cataract, but it may involve oxidation of lens components, such as glutathione and ascorbate. Similarly, another aldose reductase inhibitor, e.g. tolrestat, inhibits the plasma ascorbic acid depletion associated with experimental diabetes, but by unknown mechanisms [McLennan et al 1988]. Finally, the aldose reductase inhibitor statil improves erythrocyte deformability in patients, and this effect is not related to lowering of erythrocyte polyol levels but appears to be a separate membrane effect [Rillaerts et al 1988].

The therapeutic efficacy of ARIs in human diabetes mellitus is not well established. No judgement can be made concerning the extent to which the metal complexation by spirohydantoin drugs and ONO compound may relate to the efficacy of the compounds in treatment of the diabetic complications. Most clinical trials of these drugs have been performed to assess efficacy against diabetic neuropathy and retinopathy. Although some trials have indicated that aldose reductase inhibitors produce objective benefit at least in terms of an increase in nerve conduction velocity [Jaspan et al 1985; Sima et al 1988; Hotta et al 1988], other studies have shown no such effect [Jennings et al 1990; O'Hare et al 1988; Martyn et al 1987].

Although aldose reductase was originally implicated as a causative factor of cataract in diabetes, the only drugs which appear to have a protective effect against human cataract are aspirin, paracetamol and other "aspirin-like analgesis" [Harding & Van Heyningen 1988]. The latter drugs, however,

are poor inhibitors of aldose reductase [Sharma & Cotlier 1982]. It is hypothesized that the therapeutic effect of these drugs on cataract is due to their modification on lens proteins [Ajiboye & Harding 1989], but these drugs do also show various forms of antioxidant activity, including metal chelation [Woollard et al 1990b; Kennedy et al 1990; Aruoma & Halliwell 1988]. This indicates that the inhibitory effects of these ARIs on the development of diabetic complications may be also associated with their antioxidant activities *in vivo*.

Of note here is that the IC_{50} for inhibition by sorbinil or AL-1576 is decreased when the concentration of copper used to catalyse ascorbate oxidation is decreased (Figure 5.5). This may be of relevance to the question of the level of decompartmentalized transition metal *in vivo* and whether transition metal-binding capacity contributes to biological effects of the drugs. Plasma steady-state levels of sorbinil during its administration in clinical trials are in the range 10-60μM [Christensen et al 1985] which would be the concentrations necessary for chelation of low levels of free transition metal ion. In conclusion, the design of selective metal-complexing agents might be a useful experimental approach to the treatment of the complications of diabetes mellitus.

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APPENDIX:

Publications

1. Jiang,Z.Y., Woollard,A.C.S. and Wolff,S.P. (1990) Hydrogen peroxide production during experimental protein glycation. **FEBS Letters 268: 67-71.**

2. Jiang,Z.Y., Woollard,A.C.S. and Wolff,S.P. (1991) Lipid peroxide measurement by oxidation of Fe^{2+} in the presence of xylenol orange: comparison with the TBA assay and iodometric method. **Lipids 26: 853-856.**

3. Jiang,Z.Y., Zhou,Q.L., Eaton,J.W., Koppenol,W.H., Hunt,J.V. and Wolff,S.P. (1991) Spirohydantoin inhibitors of aldose reductase inhibit copper-catalysed ascorbate oxidation *in vitro*. **Biochemical Pharmacology 42: 1273-1279.**

4. Wolff,S.P., Jiang,Z.Y. and Hunt,J.V. (1991) Protein glycation and oxidative stress in diabetes mellitus and ageing. (A review) **Free Radical Biology & Medicine 10: 339-352.**

5. Wolff,S.P. and Jiang,Z.Y. (1991) Free radicals and cataract: diract chemical damage or exaggerated physicological responc? In **"Eye lens membranes and aging: Topics in aging research in Europe, Vol. 15" (Eds. Vrensen,G.F.J.M. and Clauwaert,J.) pp 221-231.** Eurage, The Netherlands.

6. Jiang,Z.Y., Hunt,J.V. and Wolff,S.P. (1992) Ferrous ion oxidation in the presence of xylenol orange for detection of lipid hydroperoxide in low density lipoprotein. **Analytical Biochemistry 202: 384-389.**

7. Hunt,J.V., Jiang,Z.Y. and Wolff,S.P. (1992) Formation of hydrogen peroxide by lens proteins: Protein-derived hydrogen peroxide as a potential mechanism of oxidative insult to the lens. **Free radical Biology & Medicine (In press)**

8. Bascal,Z.A., Hunt,J.V., Jiang,Z.Y., Wolff,S.P. and Woollard,A.C.S. Oxidative stress, hyperglycaemia and protein glycation. In **"Proceedings of the international symposium on proteins in life and in environment-1991" (Ed. Srinnivasan,A.)**
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9. Jiang,Z.Y., Hunt,J.V. and Wolff,S.P. Peroxidation of LDL during *in vitro* glycation: Implications for cytotoxicity. (Submitted)

10. Jiang,Z.Y., Lightman,S. and Wolff,S.P. Oxidative stress and transition metal in diabetic complication. a review (Submitted)