METHODOLOGY FOR THE ASSESSMENT OF LIPID PEROXIDATION

IN VIVO AND IN VITRO

by

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SUMMARY

Radical attack on polyunsaturated fatty acids in biological systems has been implicated in the pathogenesis of a number of important diseases, including atherosclerosis and cancer. The analytical methods available to study this phenomenon are extremely varied, and most methods provide data which is difficult to interpret, either because of interference or because of lack of specificity. Those methods which are specific often measure late products of lipid peroxidation, such as aldehydes.

Phospholipid hydroperoxides are suitable indicators of lipid peroxidation, however current methodology for these compounds is limited, particularly for analysis of tissue samples. Gas chromatography (GC) and high performance liquid chromatography (HPLC) were investigated as techniques for the analysis of phospholipid hydroperoxides. GC and reversed phase HPLC both proved unsuitable for the analysis of hydroperoxides as resolution of the required analytes could not be achieved. The application of normal phase HPLC separations was investigated. Partial resolution of oxidised phospholipids from parent phospholipids was observed using this mode of HPLC, however the use of aminopropyl bonded silica columns with methanol/pentane/water eluents enabled coelution of all species within a class, while maintaining resolution of classes.

Detection systems for sensitive and selective post column measurement of phospholipid
hydroperoxides were investigated. Reductive mode electrochemical detection was found to be unsuitable for use with normal phase separations of phospholipids due to interference from non-hydroperoxide phospholipid species. Post column chemiluminescence detection was optimised for use with normal phase chromatography. It proved to be sensitive, having a limit of detection on 30pmol hydroperoxide on column, and did not suffer from interference from co-eluting species.

Problems were encountered when trying to apply HPLC analysis with chemiluminescence detection to the analysis of phospholipid hydroperoxides in animal tissues such as rat liver. The hydroperoxides were extremely unstable during lipid extraction.

Standard methods for stabilising hydroperoxide groups, for example by either antioxidation or chelation of transition elements, were ineffective. The consequences of this finding on accepted practice of lipid analysis are discussed. A suitable alternative stabilisation method was developed, and used as the basis of a method for rapid extraction of phospholipids and phospholipid hydroperoxides from animal tissues.

Extraction by low temperature homogenisation with rapid filtration, followed by normal phase HPLC with chemiluminescence detection was found to be suitable for the measurement of phospholipid hydroperoxides in tissues.
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<tr>
<td>BHT</td>
<td>Butylated hydroxytolene</td>
</tr>
<tr>
<td>CZE</td>
<td>Capillary zone electrophoresis</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ESR</td>
<td>Electron spin resonance</td>
</tr>
<tr>
<td>FAME</td>
<td>Fatty acid methyl ester</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>GSH</td>
<td>Reduced glutathione</td>
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<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>k'</td>
<td>Capacity factor</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>N</td>
<td>Theoretical plates</td>
</tr>
<tr>
<td>PTFE</td>
<td>Polytetrafluoroethylene</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>Rs</td>
<td>Resolution</td>
</tr>
<tr>
<td>SPE</td>
<td>Sold phase extraction</td>
</tr>
<tr>
<td>TBA</td>
<td>Thiobarbituric acid</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
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1.1 General Introduction

Deterioration of fats in foods has been recognised since ancient times, principally in relation to rancidity of butter and other animal fat products. The involvement of oxygen in processes causing rancidity was discovered as early as 1820 by De Saussure, and the principal mechanisms involved in the oxidation of unsaturated fats was illustrated in the 1940's as a result of the work of Criegee (Criegee et al, 1939), Bolland and Bateman (Bolland, 1949; Bateman, 1954). This work clearly established that addition of oxygen to unsaturated lipids required a prior reaction of the lipid molecule with a free radical.

A free radical is defined as any species that contains one or more unpaired electrons (Halliwell, 1993), in consequence radicals are often very highly reactive. The importance of radical reactions in living systems was first noticed in the 1950's when observations on the effect of ionising radiation on tissues led to the realisation that the damage occurring during irradiation was caused by free radicals produced in water (Horgan and Philpot, 1954). Irradiation of animal tissues produced chemical changes similar to those encountered in ageing tissues which had not been irradiated, and free radicals were thus implicated in the ageing process. In addition research on the mechanisms of action of a variety of toxic compounds, particularly carbon tetrachloride, in the 1960's demonstrated that these compounds exerted their effects as a result of the production of free radicals during metabolism (Horton and Fairhurst, 1987). Such data has prompted a great deal of interest in the role of free radicals in toxicity and disease processes, with the result that
free radical mechanisms are now implicated in the aetiology and/or pathogenesis of a great many diseases, including heart disease and cancer (Halliwell and Gutteridge, 1989; Begin, 1987; Dargel, 1992; Dianzani, 1989).

One important manifestation of free radical processes in biological systems is their effect on lipids. Oxidative deterioration of polyunsaturated fatty acid groups in animal and plant cells has an extremely deleterious effect on cell function. This process of oxidative deterioration of unsaturated lipids, with its wide variety of possible end products and potential affects on cellular function, is referred to as lipid peroxidation. The diversity of free radical reactions with lipids is multiplied in biological systems, due to the variety of other molecules that are available for interaction with lipid peroxides. The radical species responsible for initiation of lipid peroxidation \textit{in vivo} is still the focus of a great deal of scientific debate. Table 1.1 shows some of the oxygen radicals and reactive oxygen species thought to be involved in lipid peroxidation in biological systems with their mechanisms of production.

Lipids are not the only biomolecules susceptible to a free radical attack. Among the classes of compounds that have been shown to react with oxygen centered free radicals are carbohydrates (Moorhouse \textit{et al}, 1985), proteins (Schnessler and Schilling, 1984; Kim \textit{et al}, 1985; Stadtman and Oliver, 1991) and Deoxyribonucleic acid (DNA) (Halliwell and Aruoma, 1991). While these reactions can cause damage to the cell, the products of these reactions are relatively inert.

In contrast, the reaction of free radicals with unsaturated lipid molecules leads to
formation of a variety of products, many of which are extremely toxic and capable of causing extensive damage to cells. Lipid peroxidation has been observed in a wide variety of diseases and toxic processes (Halliwell and Gutteridge, 1989; Comporti, 1993; Halliwell, 1993; Bus and Gibson, 1979; Dargel, 1992; Dix and Aibens, 1993; Horton and Fairhurst, 1987). In many disease states which exhibit increased lipid peroxidation its role

Table 1.1 Free Radical Species Hypothesised as being active in Biological Systems

<table>
<thead>
<tr>
<th>Radical Species</th>
<th>Source</th>
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<tbody>
<tr>
<td>Hydroxyl radical (OH·)</td>
<td>Fenton reactions. Ionising radiation of water</td>
</tr>
<tr>
<td>Superoxide radical (O₂⁻)</td>
<td>Electron leakage from electron transport systems. Respiratory burst of phagocytic cells</td>
</tr>
<tr>
<td>Alkyl hydroperoxy radical (ROO·)</td>
<td>Hydrogen abstraction by radical attack followed by molecular oxygen addition. Direct attack of singlet oxygen.</td>
</tr>
<tr>
<td>Alkoxy radical (RO·)</td>
<td>Metal catalysed scission of alkyl hydroperoxides.</td>
</tr>
<tr>
<td>Perhydroxyl radical (HO₂⁻)</td>
<td>Leakage from dismutation reactions of superoxide radical.</td>
</tr>
</tbody>
</table>
in the aetiology of the disease is unclear. This is often due to the difficulty in deciding whether lipid peroxidation is a cause or an effect of the disease. Cell death and necrosis of tissues caused by the disease process can result in a considerable increase in peroxidation. In consequence, evidence of gross lipid peroxidation does not prove a primary involvement of free radicals in the mechanism of disease. Such involvement must either be demonstrated as taking place prior to necrosis or shown to be unrelated. While the role of lipid peroxidation is not clear in many of these diseases, in some of them peroxidation has been postulated as important to the pathogenesis of the disease. This is certainly the case with atherosclerosis. This disease is characterised by localised thickening of the arteries by fibrous plaques and peroxidation is strongly implicated in plaque formation (Chakravarti et al., 1991; Halliwell and Gutteridge, 1989 and 1990; Dargel, 1992; Carew et al., 1987). Some of the products of lipid peroxidation, in particular the aldehydes, have also been shown to directly attack DNA, leading to speculation that lipid peroxidation may be involved in some types of carcinogenesis (Dianzani, 1989). A simplified diagram illustrating the main products and relationships in lipid peroxidation is given in figure 1.1

The study of lipid peroxidation has long been hampered by the unsuitability of analytical techniques available for the measurement of the products of peroxidation in vivo (Slater, 1984; Gutteridge and Halliwell, 1990). Lack of sensitivity or specificity have often caused problems in the interpretation of data from studies of peroxidation. Consequently a great deal of research has been conducted either to critically evaluate the available methods, or to develop techniques with the requisite sensitivity and specificity (Halliwell and Chirico, 1993). In particular chromatographic methods have been used to great effect to improve
Figure 1.1 General Reactions of Lipid Peroxidation
the specificity of some important assays. This is certainly true of the often used thiobarbituric acid (TBA) test (see section 2.1.2a), where high performance liquid chromatography (HPLC) separation allows measurement of specific TBA adducts. However, significant problems still exist in the measurement of initial products of lipid peroxidation such as hydroperoxides. These relatively unstable compounds have not been satisfactorily measured in animal tissues such as liver. In consequence lipid peroxidation in these tissues can only be studied by interpretation of data from indirect analytical procedures.

1.2 Free Radical Reactions of Lipids

Free radical reactions can be classified into three groups, namely initiation, propagation and termination (Horton and Fairhurst, 1987). Initiation may be defined as the process by which radicals are generated, propagation as the process of radical transfer whereby radicals are conserved as peroxidation reactions proceed, and termination reactions are those processes whereby a radical is destroyed or altered to a non-radical state. Examples of these reactions are shown in figure 1.2. The initiation, propagation and termination reactions all occur within peroxidation reactions of polyunsaturated fatty acid groups. Fatty acids are present in biological systems either free, or esterified within larger molecules such as phospholipids or acylglycerols. The structure of a typical unsaturated fatty acid, and that of triacylglycerol and some of the more common phospholipids are shown in figure 1.3.
1) Initiation

\[ R - R \rightarrow R\cdot + R\cdot \]

eg. Fenton Reaction

\[ \text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^+ + \text{OH}^{-} + \cdot \text{OH} \]

2) Propagation

\[ R\cdot + \text{RH} \rightarrow R\cdot + \text{RH} \]

eg. Hydrogen abstraction

\[ \cdot \text{OH} + \text{RH} \rightarrow \text{H}_2\text{O} + \text{R} + \cdot \text{H} \]

3) Termination

\[ R\cdot + R\cdot \rightarrow R - R \]

eg. Dimerisation of Hydroperoxide Radicals

\[ \text{ROO} \cdot + \text{ROO} \cdot \rightarrow \text{ROOR} + \cdot \text{O}_2 \]

Figure 1.2 Free Radical Reactions
Figure 1.3  Lipid Structures
1.2.1 Initiation of lipid peroxidation

The mechanism of initiation of lipid peroxidation in unsaturated fatty acids has been well established for many years, based on the work of Criegee (Criegee et al, 1939) and Bolland (Bolland, 1949) described above. The normal structure of unsaturated fatty acid chains in biological systems has double bonds separated by an alkyl group. This configuration causes the bis-allelic hydrogen atoms to be more weakly held and hydrogen atoms in this position are therefore prone to abstraction by radical groups, thus forming an alkyl radical. This reaction is illustrated below.

\[
\text{OH}^\cdot + \text{RCH} = \text{CH} - \text{CH}_2 - \text{CH} = \text{CHR} \rightarrow \\
\text{H}_2\text{O} + \text{RCH} - \text{CH} - \text{CH} - \text{CHR} + \cdot
\]

Following hydrogen abstraction, molecular oxygen can then react with the alkyl radical to produce a peroxy radical. This mechanism involves rearrangement of the molecule to produce a conjugated diene structure, as illustrated below.

\[
\text{RCH} = \text{CH} - \text{CH} - \text{CH} = \text{CHR} + \text{O}_2 \rightarrow \text{RCH} = \text{CH} - \text{CH} = \text{CHR} + \cdot
\]

Initiation of lipid peroxidation in cells or in subcellular fractions has been the subject of keen discussion over the last several years. A number of free radical initiators have been put forward but it has been difficult to clearly identify any one of them as the primary initiator in biological systems. This is due to the complexity of the systems being studied.
The hydroxyl radical, and the perhydroxyl radical are both capable of hydrogen abstraction (Bielski et al, 1983; Patterson, 1981) while dioxygen and superoxide radicals are not sufficiently reactive to initiate peroxidation (Bielski and Cabelli, 1991) (see table 1.1). However, superoxide is capable of being reduced to the perhydroxy radical at reduced pH's (Bielski and Cabelli, 1991) and this in turn is capable of hydrogen abstraction.

The hydroxyl radical is the most reactive of the possible candidates for *in vivo* initiation of lipid peroxidation. It can be formed by the reaction of ferrous iron with hydrogen peroxide, (the Fenton reaction):

\[ \text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^- \]  

(3)

An early observation was that ferrous iron, or ferric iron in the presence of reducing agents, stimulated lipid peroxidation in tissue homogenates, or microsomal fractions (Warburg, 1914; Kohn and Liversedge, 1994; Elliot and Libet, 1944). It appeared likely that the Fenton reaction was taking place under these conditions.

Experiments were conducted in a variety of *in vitro* peroxidation systems such as microsomal suspensions containing iron, adenosine triphosphate (ATP) and reduced nicotinamide adenosine diphosphate (NADPH) (Fong et al, 1973), microsomal suspensions containing ethylenediamine tetracetic acid (EDTA), ferrous iron and NADPH (Lai and Piette, 1977; Lai and Piette 1978; Gutteridge, 1984), xanthine oxidase, and ferric chloride (Girotti and Thomas, 1984; 1984a), and microsomal liposomes with ferrous chloride and hydrogen peroxide (Minotti and Aust, 1987) amongst many others. The
effects of superoxide dismutase, catalase and hydroxyl radical inhibitors vary considerably between these systems. In an attempt to rationalise the often conflicting results obtained from these experiments Minotti and Aust (Minotti and Aust, 1987) proposed that an Fe$^{2+}$-Fe$^{3+}$ complex, or perhaps an Fe$^{2+}$ dioxygen complex might be the initiator of peroxidation. Such a complex has never been convincingly demonstrated by experiment. Indeed the length of lag phase before peroxidation found in ferrous iron seeded systems can be reduced by lead and aluminium as well as by ferric iron (Aruoma et al, 1989) indicating that a specific complex is not required.

Another approach to the problem has been to use electron spin resonance (ESR) to look directly for the hydroxyl radical. The technique relies on the paramagnetic properties of unpaired electrons. When electromagnetic radiation is applied to a species with unpaired electrons, the electrons will absorb energy at specific wavelengths. The absorption spectrum of a particular radical is unique and can be used to identify known species, or to provide structural information about unknowns. Unfortunately hydroxyl radicals are so reactive that they do not exist for long enough to be measured directly by ESR. This difficulty is overcome by reacting the radical with compounds such as 5,5-dimethyl-1-pyrroline-N-oxide to produce more easily detected long lived radical species, a technique known as spin trapping. ESR spin trapping techniques have been used in an effort to directly detect hydroxyl radicals in biological systems. Some workers have reported success in this area (Finkelstein et al, 1979; Burkitt and Mason, 1991) however a number of problems have been identified when applying this technique to biological materials. The principal is that most spin trapping reagents are disruptive to biological systems at the concentrations required to perform the analysis (Babbs and Steiner, 1990). The other
major difficulty is the reactivity of spin trapping agents with other agents in biological systems. Direct spin traps with 5,5-dimethyl-l-pyrroline N-oxide (DMPO), N-tert-butyl-nitrone (PBN) and α-(4-pyridyl 1-oxide) N-tert butyl nitrone (4POBN) and the hydroxyl radical are all known to be mimiced by reactions with other species (Rosen et al, 1994). Other reagents, such as dimethyl sulfoxide (DMSO), have been used to produce secondary radicals with the hydroxyl radical, which can react with the spin trapping reagent. This technique can increase the specificity of the method, but when applied to the analysis of endothelial cells undergoing chemically induced lipid peroxidation there was no evidence of hydroxyl radical formation (Rosen and Freeman, 1984). The use of the same technique with human neutrophil preparations did detect extracellular hydroxyl radical production (Rosen et al, 1988). At present these technical difficulties mean that ESR, while being the primary technique for the measurement and characterisation of radicals in vitro, has shed little light on the nature of the initiating species of lipid peroxidation.

An alternative approach to determining whether the hydroxyl radical is the initiator of peroxidation is to see if products of peroxidation are typical of hydroxyl radical attack. The hypothesis is that the fingerprint of reaction products will vary between initiation mechanisms, and therefore that products from hydroxyl radical attack will show a characteristic pattern (Halliwell, 1993). Such products of radical attack on DNA have been studied by both high performance liquid chromatography with electrochemical detection (HPLC-EC) (Floyd et al, 1986) and by gas chromatography with mass spectrometry (GC-MS) (Dizdaroglu et al, 1991). The HPLC-EC method measures 8-oxo-guanine and has been used to show that oxidative stress in whole cells and tissues
can directly lead to chemical alteration of DNA (Ames, 1989; Fraga et al, 1990; Floyd, 1990). The measurement of 8-hydroxyguanine alone does not provide conclusive evidence of the role of hydroxyl radicals in this process. The use of GC-MS allows a larger group of products of free radical attack on DNA bases to be detected. Products characteristic of hydroxyl radical attack in DNA have been observed in murine hybridoma cells treated with hydrogen peroxide (Dizdaroglu et al, 1991).

At present the exact nature of the initiator of lipid peroxidation is still uncertain. The main contender remains the hydroxyl radical, but it is likely that a number of complementary mechanisms are working to produce the observed results.

1.2.2 Propagation of lipid peroxidation

Propagation reactions of lipid peroxidation yield a wide variety of products, many of which have potentially deleterious effects on biological systems. These reactions have been extensively studied in vitro with respect to fatty acid hydroperoxides (Porter, 1984; Frankel, 1987), and can be classified into three major types:

- hydrogen abstraction to form a new radical
- decomposition of hydroperoxides
- cyclization

In the first of these the peroxy radical itself reacts with a bis allelic methylene group to abstract hydrogen, thus forming a hydroperoxide and an alkyl radical (Demopoulos,
1973). Decomposition of hydroperoxides either thermally or by metal catalysis, leads to formation of an alkoxy radical which can subsequently form either epoxides (Hamberg, 1975) or alkanes and aldehydes (Esterbauer, 1982). These reactions are shown in figure 1.4.

Figure 1.4 Breakdown Reactions of Lipid Hydroperoxides

Fatty acids containing more than three double bonds are capable of forming hydroperoxide epidioxides and hydroperoxide bicycloendoperoxides from dihydroperoxide precursors.
(Neff et al., 1981; Toyoda et al., 1984; Porter et al., 1976) and these compounds are thought to be the precursors for malondialdehyde (Pryor et al., 1976), a compound commonly assayed as an indicator of lipid peroxidation. Examples of some of the general structures of these compounds are given in figure 1.5.

\[ \begin{align*}
\text{R'} & \quad \text{R} \\
\text{O} & \quad \text{O} \\
\text{OOH} & \\
\end{align*} \]

Hydroperoxyepidioxide

\[ \begin{align*}
\text{R} & \\
\text{O} & \quad \text{O} \\
\text{O} & \quad \text{O} \\
\text{OOH} & \\
\end{align*} \]

Hydroperoxybiepidioxide

\[ \begin{align*}
\text{R} & \\
\text{O} & \quad \text{O} \\
\text{O} & \\
\end{align*} \]

Bicyclicendoperoxide

Figure 1.5  Cyclic Compounds Formed During Lipid Peroxidation

All of the propagation products seen in vitro can also be identified in vivo (Frankel, 1987). In addition breakdown of peroxy and alkoxy radicals in vivo can produce a number of aldehydes and alkanes (Schauenstern et al., 1977) and these have been detected in microsomal systems and rat liver hepatocytes (Esterbauer et al., 1982; Poli et al., 1985). The aldehydes are of particular interest as these reactive molecules are often extremely toxic and are capable of causing damage far from their site of origin (Poli et al., 1987) (see also section 1.2.4). Aldehydes produced by peroxidation reactions have been shown to react with proteins (Esterbauer, 1993; Chio and Tappel, 1969) and DNA (Emerit et al.,
1991) thereby disrupting normal cell function. The bicycloendoperoxides may also be of interest as they have structural similarities to prostaglandins (Frankel, 1987). Prostaglandins have very potent effects on a number of biological processes and inappropriately formed compounds mimicking these effects might have serious consequences in vivo. Indeed fatty acid hydroperoxides have been shown to potentiate mesentary artery vasoconstriction responses in vitro, in a similar fashion to prostaglandins (Hubel et al, 1993). Little is known at present about the biochemical activities of the cyclic peroxide groups produced during lipid peroxidation.

1.2.3 Termination Reactions of Lipid Peroxidation

Termination reactions are the result of two radicals combining to form non-radical products. Given the excess concentrations of non-radical molecules in any given reaction mixture propagation reactions are more likely to occur than termination reactions (Halliwell, 1993).

The obvious termination reaction is that of combination of lipid radical with lipid radical to form a lipid cross linkage. Linoleic acid hydroperoxides have been shown to form dimers by forming either peroxy or ether linkages. These dimers can decompose to form hydroxy aldehydes, nonenal, aldehyde esters and oxygenated aldehyde esters (Miyashita et al, 1985). Peroxidation has been demonstrated to reduce the fluidity of cell membranes (Richter, 1987) and such linkages between lipid group would effect the mobility of lipids within the membrane. Proteins can form free radicals as a result of attack by hydroxyl and lipid radicals (Gardner, 1979; Stadtman, 1986) and such radicals can react with lipid
radicals or other protein radicals causing lipid-protein and protein-protein cross linkages. Proteins affected in this way often lose their catalytic or binding characteristics thus rendering them biochemically inactive (Hogberg et al, 1973; Jones et al, 1983). They may also link covalently with DNA and carbohydrates (Stadtman and Oliver, 1991). All these types of termination reactions can cause damage to cell membrane structure and function (Eichenburger et al, 1982; Shimasaki et al, 1984; Jain, 1984). The effects of such damage are discussed in Section 1.4 below.

1.3 Non Radical Reactions of Lipid Peroxidation

From the foregoing discussion it is apparent that the fatty acid peroxy radical is the core species involved in lipid peroxidation. Its decomposition produces the range of products associated with the effects of lipid peroxidation, and it is readily formed by free radical reactions with polyunsaturated fatty acids (PUFA's).

Free radical processes may not always be important in all pathological conditions. There are a number of mechanisms \textit{in vivo} which probably produce endogenous fatty acid hydroperoxide species and cells are unlikely to be completely free of such molecules under normal circumstances. For example lipoxygenase and cyclooxygenase enzymes produce hydroperoxides as part of the synthesis of leukotrienes and prostaglandins (Samuelsson, 1980). Hydroperoxides can also be produced by the direct addition of singlet oxygen to unsaturated aliphatic groups (Kanofsky and Axelrod, 1986; Khan, 1984; Cadenas, 1989). Lipid hydroperoxides may be formed from singlet oxygen which is produced \textit{in vivo} and the low level chemiluminescence seen in cells and tissues under oxidative stress has been
attributed to singlet oxygen decay (Wefers, 1987; Sies, 1987; Cadenas and Sies, 1984).

Dismutation of superoxide that is formed from leakage of electrons onto oxygen from various components of electron transport chains is one potential source of singlet oxygen. Although the extent of singlet oxygen yield by this mechanism is estimated at only 0.008% of the molecular oxygen produced by this reaction (Foote et al, 1980) such production may lead to small but significant formation of hydroperoxides in vivo. Other potential sources of singlet oxygen are peroxidase reactions, prostaglandin H₂ synthetase and cytochrome P₄₅₀ (Cadenas, 1989a) however not all of these sources exhibit infra red emission at 1268nm, which is unique for singlet oxygen. Phospholipid hydroperoxides are therefore produced endogenously at low levels during normal metabolic activity and will be available for further radical formation in the presence of free iron. It may be that the majority of peroxidation products are due to lipid radicals formed from these few precursors.

Radicals are not the only direct products of lipid peroxidation which may have serious effects on biological systems. Some of the most reactive products are non radical in nature. A good example of this are the aldehydes produced during the breakdown of hydroperoxides and cyclic oxygen products. As noted above, aldehydes are extremely toxic compounds (see also section 1.2.2), it is not surprising, therefore, that they have significant effects on cell function when produced during lipid peroxidation.

Aldehydes produce most of their effects by reaction with the amino groups of proteins to form Schiff's bases. Schiff's bases are fluorescent compounds. They are found in all tissues and have higher concentrations in systems where lipid peroxidation has been stimulated
(see section 2.2.3a) (Chio et al, 1969; Dillard and Tappel, 1971). Aldehydes such as malondialdehyde, which contains two carbonyl groups, are capable of reacting with two protein molecules and thus cross linking them. As with cross linking termination reactions, such reactions to proteins are likely to destroy their physiological function.

1.4 Protective Mechanisms against Lipid Peroxidation

The basic components of a system designed to stimulate lipid peroxidation, namely unsaturated fatty acids, iron and oxygen, are fundamental physiological components of most organisms. It is therefore necessary to control the storage, transport and utilisation of these components, and to remove inappropriate products of lipid peroxidation, if the organism is to function properly.

A number of mechanisms exist to protect the organism from the effects of lipid peroxidation. This topic has been reviewed a number of times (Horton and Fairhurst, 1987; Halliwell and Gutteridge, 1989; Diplock, 1994) and a number of attempts at classifying protective mechanisms have been made. In this review the mechanisms have been classified into three major classes based on the primary strategy of protection.

- Removal or transformation of reactive oxygen species
- Maintenance of membrane integrity
- Transition metal sequestration

These three mechanisms protect at molecular, cellular and systemic levels respectively.
The first mechanism renders potentially destructive products of peroxidation less harmful by transforming them into less reactive molecules. The structure of cell membranes reduces the possibilities for oxidative damage and its repair and maintenance ensures that the cell and its organelles can continue to function effectively. The final mechanism ensures that agents that are efficient catalysts for peroxidation (principally iron and other transition elements) are bound in a form that ensures that their transport and physiological function causes minimal damage to the organism as a whole.

1.4.1 Transformation of Reactive Oxygen Species

Mechanisms for transformation of reactive oxygen species (ROS) to less reactive species is perhaps the most important of the three protective strategies, in that it provides the largest number of different mechanisms. ROS transformation strategies can be sub-divided into two subgroups,

a) antioxidants, systems designed to scavage free radical species,

b) enzyme systems which catalyse the transformation of specific reactive oxygen molecules.

a) Antioxidant Control of Reactive Oxygen Species

Antioxidants work as radical sinks, by allowing active free radicals to abstract a hydrogen atom from them in a mechanism similar to that for forming a lipid radical. The radical groups so formed are relatively non-reactive and thus unable to propagate radical cascade
(see figure 1.1 and section 1.2.2). In consequence the radical chain reaction is effectively terminated.

The principle antioxidant found in biological systems is \( \alpha \)-tocopherol (also commonly referred to as vitamin E). This lipophilic compound is present in the hydrophobic centre of the lipid membrane (Buttriss and Diplock, 1984) and is able to reduce phospholipid peroxy radicals to hydroperoxides (Bogurth and Niemann, 1971). The \( \alpha \)-tocopherol radical so formed is relatively unreactive and is regenerated in turn by ascorbate (Scarpa et al, 1985). Its importance to biological systems is exemplified by the fact that \( \alpha \)-tocopherol deficiency is known to have adverse effects in a variety of mammalian species. For example, \( \alpha \)-tocopherol deficient rats and chicks are more sensitive to the toxic effects of paraquat (a free radical generating toxin) than control animals (Block, 1979; Cambs and Peterson, 1983) and deficient rats have been shown to exhale significantly higher concentrations of alkanes associated with peroxidation, and rapidly accumulate the fluorescent pigments associated with reactions of aldehyde with amino acids (Halliwell and Gutteridge, 1990) (see also section 2.2.3a). Additionally in vitro comparisons of microsomes and mitochondria have shown that organelles from vitamin E deficient rats are more susceptible to peroxidation (Tappel and Zalkin, 1959; Dillard and Tappel, 1971).

Carotenoid, ubiquinone and ubiqinol have also been cited as having antioxidant properties (Sies et al, 1992; Berger et al, 1987; Das and Ramanathan, 1992). \( \alpha \)-Tocopherol, ubiquinone, ubiqinol and the carotenoids are lipophilic molecules and as such are found predominantly associated with cell membranes. Antioxidant protection in the cytosol is provided by ascorbic acid (Vitamin C) and uric acid (Niki, 1987). Vitamin C has been
shown to regenerate α-tocopherol *in vitro* in homogenous solutions and in liposome suspensions undergoing oxidation (Packer *et al.*, 1979; Doba *et al.*, 1985). It is possible that a synergistic relationship exists between these two oxidants (and possibly others) *in vivo*.

The structures of some of the more common antioxidants are given in Figure 1.6.

![Figure 1.6 Common Intracellular Antioxidants](image)

**b) Enzymic Removal of Reactive Oxygen Species**

Enzymic removal of reactive oxygen species is widespread within the cell. Glutathione peroxidase enzymes reduce hydrogen peroxide and fatty acid hydroperoxides to hydroxides and some enzymes with this activity can directly reduce phospholipid
hydroperoxides which are free in solution or situated in the lipid bilayer (Ursini and Bindolini, 1987). In general, glutathione peroxidases require selenium for their activity and a number of diseases related to selenium deficiency have symptoms remarkably similar to those of \( \alpha \)-tocopherol deficiency. Glutathione peroxidase acts, as its name suggests, by reducing peroxides at the expense of reduced glutathione (GSH) in the following reaction.

\[
2\text{ROOH} + 2\text{GSH} \xrightarrow{\text{Glutathione Peroxidase}} 2\text{ROH} + 2\text{H}_2\text{O} + \text{GSSG} \tag{4}
\]

It is often assumed that a simple inverse correlation exists between intracellular GSH and levels of detectable peroxidation. While lowered concentrations of GSH have been shown to lead to an increased susceptibility to lipid peroxidation (Maellaro et al, 1990) and to loss of cell membrane integrity (Dargel, 1992) under certain experimental conditions, lowered GSH concentrations are not always deleterious (Smith, 1992). It appears that other protective mechanisms operate in these circumstances, implying that intracellular control of peroxidation is subtle, and not simply a matter of counteracting the reactive oxygen species with protective compounds.

Catalase also acts on hydrogen peroxide, by converting it to water and molecular oxygen. The enzyme is thought to be associated exclusively with specialised metabolic organelles called peroxisomes (Halliwell and Gutteridge, 1989). These organelles contain a variety of enzymes that can produce hydrogen peroxide, but are by no means the only intracellular source of hydrogen peroxide. However the presence of catalase in peroxisomes (cell organelles which have a high rate of peroxide production), may be of great importance in protecting the cell from the effects of reactive oxygen species. Evidence for this comes
from the action of peroxisome proliferating agents. These agents, which include compounds such as clofibrate, in addition to increasing the numbers of peroxisomes in the liver of rats and mice treated with them, also increase the activity of enzyme systems such as acyl-CoA oxidase which produce H$_2$O$_2$, without a concomitant increase in catalase activity. Livers from animals so treated show a marked increase in lipofuscin, compared with untreated animals of the same age and show an increased evidence of liver tumour formation (Reddy and Lalwai, 1989). Lipofuscin is deposited as granules in tissues and is composed of an aggregation of proteins and lipids. Increased deposition of lipofuscin is associated with ageing and lipid peroxidation (Donato, 1981) (see also section 2.2.3a).

Superoxide radicals can be formed in cells at a number of sites by leakage of electrons onto molecular oxygen. This is thought to happen in the endoplasmic reticulum by leakage from the electron transport chain (Freeman and Crapo, 1981). Although not able to initiate hydrogen abstraction, its ability to reduce iron and release it from ferritin and other iron binding compounds (Halliwell and Gutteridge, 1990), and its ability to protonate superoxide to produce the perhydroxyl radical at reduced pH's (Bielski et al, 1983), may allow the species to damage the cell.

Superoxide radical is reduced to hydrogen peroxide by the action of the enzyme superoxide dismutase (Fridovich, 1986). A number of superoxide dismutases have been isolated, varying primarily in the transition elements attached to the enzyme. Copper/zinc, manganese and iron forms have all been isolated from bacteria but only the copper/zinc and manganese forms have been detected in mammals (Fridovich, 1986).
Since the discovery of superoxide dismutase, the superoxide radical has been postulated as the primary starting point for oxygen damage in organisms (Fridovich, 1986). While it is undoubtedly true that inappropriate superoxide production can cause significant problems within cells it has recently been suggested that the superoxide/superoxide dismutase system is an intracellular protective mechanism against inappropriate oxygen attack (Winterbourne, 1993). The basis of this theory is that free radical species donate electrons to oxygen to form superoxide which is then converted to hydrogen peroxide by the action of superoxide dismutase. Superoxide therefore acts as the focus of a number of radical groups and allows the cell to respond to free radicals by a generalised response. The theory raises superoxide from the status of an unwanted toxic byproduct, to an integral part of the cells radical protection system.

1.4.2 Maintenance of Membrane Integrity

The majority of the current literature stresses the adverse effects of lipid peroxidation on the structure and function of cell membranes (Richter, 1987; Dinis et al, 1993). However, this ignores the structural protection provided by the lipid bilayer. The unsaturated fatty acid groups are held within the body of the membrane and thus physically shielded from radical attack. The reaction of hydroxyl radicals with lipid micelles has been shown to be an order of magnitude less than with monomeric PUFA's (Patterson, 1981). Any lipid radicals formed within the bilayer are scavenged by the α-tocopherol molecules located in the membrane to produce non-radical hydroperoxides (Diplock, 1994). These hydroxides and hydroperoxides can then be cleaved by the enzyme phospholipase A2 and rendered more readily available for peroxidase reduction in the cytosol. Phospholipase
A$_2$ has also been shown to increase its activity in proportion to the extent of lipid peroxidation (Sevanian et al, 1983).

The capacity of the cell to repair the cell membrane and maintain its integrity is essential to cell survival. When carbon tetrachloride is used to induce damage to hepatocytes, these cells only reach a point where death is inevitable when membrane functions, such as maintenance of intracellular calcium levels, start to fail (Recknagal and Glenda, 1973; Shanee et al, 1979; Ungemach, 1987). At this point the intracellular concentrations of GSH are not significantly depleted (Krebs et al, 1978) indicating that depletion of at least one biochemical protection system in the liver is not related to membrane failure.

The role of intracellular membranes in compartmentalising cells may have implications for the control of free radical processes in general and lipid peroxidation in particular. Not only does compartmentalisation isolate areas of the cell that have high peroxidation capabilities such as mitochondria, peroxisomes and the endoplasmic reticulum, it may also allow the fine tuning of mechanisms of protection against free radical damage as witnessed by the compartmentalisation of catalase within peroxisomes, and the different pools of GSH found within the cell (Meredith and Reed, 1982).

1.4.3 Transition Element Sequestration

The active catalytic role of iron and other transition elements in initiation and propagation of lipid peroxidation means that there is a need for tissues to avoid inappropriate release of these elements in order to avoid free radical damage (Halliwell and Gutteridge, 1986).
A number of transport and storage molecules have been identified for iron and other transition elements. Transferrin and ferritin are proteins used for the transfer and storage, respectively, of iron. Iron complexed with these proteins has been shown to be unable to catalyse peroxidation (Halliwell and Gutteridge, 1986). Similarly, ceruloplasmin sequesters 95% of human plasma copper, the rest being bound to histidine, albumin and small peptides (Halliwell and Gutteridge, 1990).

The efficiency of these systems ensures that little unbound iron or copper is available for generation of oxygen free radicals in vivo. The bleomycin assay has been used to measure bound iron in vivo. This assay is based on the iron catalysed breakdown of DNA by the glycopeptide antibiotic bleomycin. The method has been used to show that free iron in normal human plasma is present at concentrations of less than 0.5μM (Gutteridge et al, 1982). The importance of these sequestration proteins in the body is highlighted by the deleterious effects observed in tissues where they are depleted. In tissues naturally deficient in iron binding capacity, such as the central nervous system, mechanical damage leading to release of metal ions into these tissues has been shown to stimulate lipid peroxidation which may result in substantial tissue damage (Stocks et al, 1974; Halliwell and Gutteridge, 1985).

1.5 Stimulation of Lipid Peroxidation by Toxic Chemicals

1.5.1 Role of Lipid Peroxidation in Toxic Mechanisms

A large number of toxic compounds are known to exert their toxic effects by stimulating
lipid peroxidation. This topic has been extensively reviewed in the recent past (Horton and Fairhurst, 1987; Mason and Chigwell, 1994) and therefore this discussion will only highlight the salient features of the topic. The reader is referred to the cited reviews for a more extensive discussion of this subject.

Xenobiotic stimulation of lipid peroxidation can occur via two major mechanisms. Toxic compounds can either be metabolised to a free radical group or they can deplete or inactivate the protective mechanisms of the cell.

a) Metabolism to Free Radicals

A number of compounds can be directly metabolised to free radicals capable of initiating lipid peroxidation. The best known example of this are the halogenated alkanes, in particular, carbon tetrachloride. Carbon tetrachloride is reduced by NADPH-cytochrome P_{55} reductase in the liver to produce the trichloromethyl radical \( \text{CCI}_{3} \) (Slater, 1966). This radical has been shown to initiate peroxidation \textit{in vitro} (Glende and Recknagel, 1969). Organotin compounds are also metabolised in a similar fashion (Wiebkin \textit{et al}, 1982).

Many of the compounds which are metabolised to radicals are thought to produce superoxide by redox cycling. Compounds which act in this way appear to produce organ specific peroxidation. For example paraquat promotes peroxidation in the lung (Bus \textit{et al}, 1976), adriamycin in the heart (Olson \textit{et al}, 1981) and nitrofurans in the brain, testes and kidney (Decampo \textit{et al}, 1981).
Few of these mechanisms are well understood in detail, particularly with respect to the role of lipid peroxidation in the process, and there is often controversy over mechanisms of action. For example there is now some doubt regarding the role of the trichloromethyl radical in membrane damage (Le Page et al, 1988) and the induction of lipid peroxidation in the lung as a result of paraquat intoxication has been questioned (Sato et al, 1992; Shu et al, 1979). When reviewing some of this data Horton and Fairhurst (1987) concluded that differences in interpretation were exacerbated by problems in analytical technique.

b) Depletion of Protective Mechanisms

A number of chemical entities are known to affect various aspects of the glutathione peroxidase system. Silver, cadmium, mercury and lead all inhibit enzymes of the system. Other chemicals are known to deplete cytosolic glutathione. Reduced glutathione can be oxidised by diazenes such as diamide (Kosower and Kosower, 1978). The reaction of this compound with reduced glutathione is given below.

\[
\begin{align*}
\text{(CH}_3\text{)}_2\text{N} &- \text{C} - \text{N} = \text{N} - \text{C} - \text{N} \left(\text{CH}_3\right)_2 + 2\text{GSH} \rightarrow \text{GSSG}^+ \\
\text{Diamide} & \\
\text{(CH}_3\text{)}_2\text{N} - \text{C} - \text{N} - \text{N} - \text{C} - \text{N} \left(\text{CH}_3\right)_2 \\
\text{O} & \quad \text{H} \quad \text{H} \quad \text{O}
\end{align*}
\]

Administration of these agents caused lipid peroxidation and severe cell damage, however recovery of levels of GSH were rapid unless the compound was also metabolised to a free radical.
More commonly, xenobiotic compounds are conjugated to glutathione by the action of glutathione 5-transferases, causing depletion of glutathione that cannot be readily regenerated and leading to cell damage and increased malondialdehyde concentrations (as determined by the Thiobarbituric acid test, see Chapter 2). A variety of compounds demonstrate this effect, including chloroform, (Eckstrom and Hogberg, 1980) acrolein (Zitting and Heinonen, 1980), paracetamol (Beales et al, 1985), diethylmaleate (Hogberg et al, 1975) and bromobenzene (Casini et al, 1982). The conclusion that glutathione reduction leads to oxidative stress may be over simplifying the situation. The toxicity of compounds associated with glutathione depletion may also involve loss of energy production, loss of ionic homeostasis and complex events involving protein thiols. Loss of glutathione may be only one part of a complex picture when considering lipid peroxidation. (Reed, 1990).

1.5.2 Environmental Pollutants and Lipid Peroxidation

The foregoing section dealt with the general involvement of lipid peroxidation in severe intoxication. Lipid peroxidation has also been implicated in the harmful effects of some compounds at low dose over long periods of time. Low level, long term exposure is a feature of environmental and occupational exposure to potentially harmful agents.

Lipid peroxidation in vivo has been demonstrated for both ozone (Goldstein et al, 1969; Sevanian et al, 1979; Sagai et al, 1987) and nitrogen dioxide (Sagai et al, 1984; Sevanian et al, 1982) at high concentrations. These compounds are atmospheric pollutants capable of causing emhasema, bronchitis and asthma but the mechanisms of long term health
effects are yet to be determined (Mustafa, 1994). A number of studies have demonstrated increased lung tumour formation in mice exposed to high ozone (Hassett et al, 1985; Last et al, 1986) and nitrogen dioxide (Adkins et al, 1985) concentrations. More importantly ambient concentrations of both pollutants have been shown to increase the incidence of lung metastasis (Richters and Kuraitis, 1981; Kobayashi et al, 1987). The involvement of free radical reactions, and lipid peroxidation in these processes appears likely, but has yet to be clearly established.

Non-malignant alveolar fibrosis (asbestosis) and malignant mesothelioma have for some time been linked with long term occupational exposure to asbestos (Shull et al, 1992). Asbestos is known to form reactive oxygen species such as superoxide (Hansen and Mossman, 1987) and hydroxyl radical (Zalma et al, 1987). Lipid peroxidation is promoted by asbestos fibres in vitro (Weizman and Weitberg, 1985; Gulumian et al, 1983). The mechanism of toxicity is related to free radicals generated by iron on the surface of asbestos fibres. (Gulumian and Kirloe-Smith, 1987). The role of lipid peroxidation in subsequent development of lung disease remains unclear, as benzo(a)pyrene adsorbed onto the asbestos fibre may also act as a carcinogen (Thomas et al, 1978).

Other environmental pollutants may also become linked with lipid peroxidation as research develops. Already some chlorinated pesticides have come under scrutiny (Vidila et al, 1990). The difficulty with this area of research is gaining unequivocal evidence that long term, and therefore slow, processes have a clear peroxidative mechanism. Present analytical techniques are unlikely to be sensitive enough to provide the required data.
1.6 *The Effects of Lipid Peroxidation in vivo*

While an understanding of the chemical mechanisms of free radical processes, in particular lipid peroxidation, in biological systems is essential, it is the effects that these mechanisms can have on living organisms which is of principal importance. The wide variety of reactions already described can have major repercussions on cellular function by altering the structure and function of cellular components such as enzymes, receptors, and cellular signalling mechanisms.

1.6.1 *Effects of Lipid Peroxidation at the Cellular Level*

Given that free radical attack will damage polyunsaturated fatty acid groups within the cell membrane, the effects on membrane structure and function will be considered first. The classic model of the lipid membrane is of a phospholipid bilayer, with proteins embedded in it either at the polar surface area (extrinsic) or more deeply embedded, possibly spanning the whole lipid bilayer (intrinsic). This basic structure may be further extended to include the positioning of $\alpha$-tocopherol and cholesterol in the hydrophobic interior of the membrane. This model of membrane structure was first proposed as the fluid mosaic model and has been elaborated and refined since being first postulated in the early 1970's (Singer and Nicholson, 1972). The fluidity of the lipid layer is thought to be important, as it allows diffusion and migration of some protein molecules within the system. However this picture of a tidy sheet of well ordered phospholipids, in which float a mixture of enzymes, receptors and transport proteins is probably naïve.
It is becoming increasingly apparent that the cell membrane is considerably more complex than the relatively simple Fluid Mosaic model. The lipid bilayer can no longer be thought of as a relatively inert matrix for active proteins. There is a considerable difference in the composition of phospholipids found on either side of most cell membranes (Richter, 1987). Certain membrane lipids, such as phosphatidyl inositol, which is metabolised as part of the inositol triphosphate signal transduction system (Rhee et al., 1989), have important roles to play in cellular control mechanisms. The traditional view of the bilayer organisation of lipids has also come into question. Although bilayer organisation is still the predominant type of phospholipid organisation in cell membranes, inverted hexagonal structures with monolayer organisation have been observed in cells and such structures have characteristics which differ from classic lipid bilayers, such as reduced fluidity and increased density of membrane proteins. Differences of this kind point to localised variations in membrane function. Inverted hexagonal structures are associated with membrane fusion, protein insertion, and increased membrane activity and are rich in phosphatidyl ethanolamine (Ellens et al., 1989; Cheng et al., 1986; Epand and Bottega, 1988). In vitro preparations of inverted hexagonal lipid structures have been shown to be more susceptible to lipid peroxidation than bilayer structures (Wang et al., 1992), and if this holds true in vivo, lipid peroxidation may predominate in protein rich areas of the membrane, thus exacerbating the effects on active membrane functions.

The fluidity of the cell membrane can be studied by the use of fluorescent probes and ESR probes. Fluorescent probes such as anilinonaphthalene sulphonate and pyrene can be excited by polarised light, and the polarity and spectra of the emissions can be used to provide information on the pH, membrane charge and viscosity of the membrane in the...
region of the probe. Nitroxide probes can be used with ESR and changes in the ESR spectra can be used to assess probe mobility, orientation, lateral diffusion and flip-flop of the probe between layers in the membrane (Richter, 1987). Most studies using fluorescent probes and ESR have shown that peroxidation in membranes causes an increase in order of the membrane indicating a loss of fluidity (Richter, 1987). Such changes have an adverse effect on membrane function in living cells and have been shown to restrict the mobility of enzyme bound proteins, such as members of the Cytochrome P450 group (Gut et al, 1985).

Peroxidation is associated with disturbances in calcium homeostasis leading to increases in cytosolic calcium concentrations, and these changes are most probably related to membrane damage (Recknagel, 1983; Orrenius et al, 1986; Albano et al, 1991; Elliott et al, 1992). Loss of calcium homeostasis has diverse effects on the cell, and leads to collapse of membrane potentials and activation of lytic enzymes (Nictera et al, 1992). Studies of this phenomenon in systems treated with carbon tetrachloride, a compound which produces free radical metabolites which stimulate lipid peroxidation (see section 1.5.1), have shown that this loss of control of calcium homeostasis corresponds with the "point of no return" where the cell cannot recover from the toxic insult and cell death is inevitable (Recknagel and Glenda, 1973; Shanne et al, 1979; Ungemach, 1987).

In recent years it has become increasingly apparent that some products of lipid peroxidation can have direct effects on cell regulation. In particular 4-hydroxynonenal, an aldehyde associated with lipid peroxidation, has been shown to inhibit cell proliferation in cell culture (Poot et al, 1988; Kaneko et al, 1988), and to have chemotactic activity
(Curzio et al, 1990). It has also been shown to stimulate adenylate cyclase (Paradisi et al, 1985) and phospholipase C activity (Rossi et al, 1991). Both of these enzymes are involved with regulation of cell receptors and secondary messenger mechanisms. Lipid hydroperoxides and hydroxy fatty acids have also been shown to have effects on cell proliferation and other cellular responses, including reduction of protein kinase activity, (Begin, 1987; Bull et al, 1988; Craig et al, 1990) and the possibility of using hydroxy fatty acids as anti tumour agents has been suggested (Masotti et al, 1993). The exact mechanisms by which these compounds achieve these effects is not known and the picture is confused by the fact that α-tocopherol, the most important intracellular antioxidant, has also been shown to inhibit protein kinase activity in certain cell lines and inhibit cell proliferation (Boscoboinik et al, 1991). Clearly some very subtle interactions may be taking place, but further studies are necessary to clarify this data.

While the effects of lipid peroxidation are understood in terms of the general cell pathology occurring in abnormal cells or cells exposed to toxic insult, little is known about the normal physiological role of lipid peroxidation and its end products. A better understanding of these physiological roles may help to elucidate some of the problems related to lipid peroxidation in toxic processes and disease states.

1.6.2 Lipid Peroxidation and Disease

Of the 600,000 or so deaths which occur in the UK each year, in 1992 over 70% were from circulatory disease or cancer (Church, 1994). Both of these conditions are believed to be associated with lipid peroxidation (Dargel, 1992; Halliwell and Gutteridge, 1989 and
1990), and therefore from an economic if not a humanitarian point of view, research into the role of lipid peroxidation in these diseases is important. However the list of other diseases which have been linked to lipid peroxidation is extensive (table 1.2), and a considerable amount of research is being conducted into the role of lipid peroxidation in disease. There are many reviews of the role of free radicals and lipid peroxidation in disease (Begin, 1987; Dargel, 1992; Halliwell and Gutteridge, 1990). This review will therefore cover the general state of knowledge in the major conditions associated with lipid peroxidation, but with emphasis on recent developments.

Table 1.2 Diseases Associated with Free Radicals and Lipid Peroxidation

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<tr>
<td>AIDS</td>
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<td>Alzheimer's Disease</td>
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<td>Angina</td>
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<td>Arthritis (rheumatoid)</td>
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<td>Ischaemia/reperfusion injury</td>
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<td>Keshan's syndrome (selenium deficiency)</td>
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<td>Thyroid Disease</td>
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<td>Vitamin E deficiency</td>
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36
a) Atherosclerosis

Heart disease is the single largest cause of death in the developed world, and ischaemic heart disease as a result of atherosclerosis accounts for the vast majority of heart conditions (Dargel, 1992). Atherosclerosis is characterised by a thickening of the walls of blood vessels leading to occlusion of blood flow. Restricted blood flow results in ischaemia which causes potentially severe localised tissue damage due to oxygen starvation. In its most extreme manifestations the disease can cause stroke or myocardial infarction, which may result in death or disablement for the sufferer (Chakravarti et al, 1991). A number of factors are known to increase the risk of cardiovascular disease, and these include smoking, hypertension, elevated blood cholesterol levels and hyperlipidaemia (Gotto and Farmer, 1988). Increased blood concentrations of high density lipoproteins (HDL) relative to low density (LDL) and very low density lipoproteins (VLDL) has been shown to reduce the risk of cardiovascular disease.

The role of lipid peroxidation in the formation of atherosclerotic plagues is now widely accepted (Halliwell and Gutteridge, 1990; Chakravarti et al, 1991; Dargel, 1992). Physical damage to vascular endothelial cells may result in peroxidative damage that sequentially leads to plaque formation (Hennig and Chow, 1988), and lipid peroxides present in LDL may cause physiological changes in cell walls, including stimulation of endothelial cells to produced macrophage chemoattractants and increasing uptake of cholesterol into macrophages (Esterbauer et al, 1990; Esterbauer et al, 1990a).

Most of the evidence for lipid peroxide involvement in atherosclerosis is indirect.
Injections of high concentrations of linoleic acid hydroperoxides into rabbits caused development of lesions on the aorta (Cutler and Schneider, 1974; Yagi et al, 1981). Peroxidised LDL has been shown to have toxic effects on endothelial cells and to stimulate the release of a chemotactic factor from these cells in non-toxic concentrations in vitro (Berliner et al, 1990). It has also been demonstrated that probucol, an anticholesterolaemic drug with powerful antioxidant properties, is antiatherogenic in rabbits. This effect appears to be related to the drugs antioxidant properties rather than its anticholesterolaemic action (Carew et al, 1987; Kita et al, 1987; Smith et al, 1992). Probucol has also been shown to have a protective effect on cultured endothelial cells exposed to both cumene hydroperoxide and oxidised LDL (Kazuya, 1991), and to reduce concentrations of plasma lipid peroxides in man (Paterson et al, 1992).

Recent work has shown that the contents of atherosclerotic lesions stimulate peroxidation in rat liver microsomal preparations (Smith et al, 1992), however in this study no direct measurements of lipid peroxides were undertaken, and no comparison with normal tissue was conducted. More interesting is the observation that in healthy volunteers approximately 85% of the oxidised lipids were found in HDL. The beneficial role of HDL may be explained by this type of lipoprotein accumulating oxidised lipids and making them unavailable to LDL (Bowry et al, 1992).

b) Cancer

Chemical carcinogenesis is a topic that has been frequently reviewed in the literature, and the principles of initiation, promotion and progression are generally established. The
detailed mechanisms of carcinogenesis are not clearly understood (Williams and Weisburger, 1986).

It has been postulated for many years that oxygen free radicals and lipid peroxidation products are involved in some types of carcinogenesis. As we have seen it is certainly the case that the hydroxyl ion is capable of reaction with DNA, and that changes to DNA occur under conditions of oxidative stress (Ames, 1989; Fraga et al., 1990; Floyd, 1990). A number of other observations link lipid peroxidation with cancer, although there is dispute over much of the data. An early observation was that transformed cells, that is cells which have lost their normal proliferative controls and have formed tumours, are often found to have different susceptibilities to peroxidation, and to have different activities of antioxidant enzyme systems to those of the parent tissue type. This is often reported as a decrease in susceptibility to peroxidation and antioxidant systems (Dargel, 1992; Bartoli et al., 1988; Dianzani, 1989). While this is true of certain commonly studied cell types, such as hepatomas (Dianzani et al., 1984) other tumour cells have the reverse pattern, such as breast tumour cells (Hietanen et al., 1986; Kumar et al., 1991) and endometrial cancer (Punnonen et al., 1993). However the alteration in peroxidative and antioxidant status appears to be common to all tumours, regardless of the nature of alteration.

Another link is that many peroxidative compounds are involved with tumour promotion. Certain organic peroxides act as tumour promoters and certain reactive oxygen generating systems can mimic the actions of tumour promoters (Taffe and Kensler, 1989). Various mechanisms have been postulated for tumour promotion by reactive oxygen species.
These include epigenetic modulation by oxidation of thiols, amino acids or metal ions, and genetic modulation by DNA base modification or DNA strand breakage (Taffe and Kensler, 1989).

In recent years a membrane cluster hypothesis of carcinogenesis has been put forward (Beech, 1993). This hypothesis is based on the observation that intracellular clusters of membrane proteins may form in response to certain types of bioelectric field (Beech, 1993). Such clusters may lead to depolarisation of the membrane and subsequent mitosis. Under prolonged stimulation such clusters hypothetically become permanent and may be conserved and passed to daughter cells during mitosis, leading to sustained repetitive cell division to produce a tumour. As yet no experimental link between oxidative attack of membranes and cluster formation has been made.

The antioestrogenic agent, tamoxifen, is widely used in the treatment and prophylaxis of breast cancer (Jordan, 1992). Although this compound is thought to exhibit its beneficial effects through its antioestrogenic properties, it also has antioxidant properties which may be relevant to its therapeutic effects (Wiseman et al, 1993). It has been recently shown that an active metabolite of tamoxifen, 4-hydroxytamoxifen, can be incorporated into the lipid bilayer and induce structural changes in lipid membranes (Custodio et al, 1993). How these two properties of the drug may be related to its anticancer effects is as yet unknown.

c) Acquired Immune Deficiency Syndrome (AIDS)

One of the more interesting developments in the last few years has been a link between
reactive oxygen species, and expression and control of the human immune deficiency virus (HIV) which is the causal agent in Acquired Immune Deficiency Syndrome (AIDS). A number of studies have shown that the oxidative burst of neutrophils and mononuclear phagocytes is reduced in HIV infection (Spear et al, 1990; Muller et al, 1990; Bruro- Ceullar et al, 1992). Certain reactive oxygen species such as hydrogen peroxide and hydroxyl radical have been shown to induce the production of the transcription factor nuclear factor \(\kappa\)B (NF-\(\kappa\)B) which is known to activate expression of the viral gene (Schreck et al, 1991; Schreck et al, 1992). A variety of antioxidants have been shown to reduce HIV replication in infected cells (Packer and Suzuki, 1993) and this effect may be due to their antioxidant actions. This observation has promoted the suggestion that antioxidant therapy may be of benefit to HIV positive patients in delaying onset of the overt disease (Packer and Suzuki, 1993; Muller, 1992), however the exact role of reactive oxygen species remains unclear.

d) Inflammatory Diseases and Autoimmune Diseases

The normal inflammatory response involves the migration of macrophages and neutrophils to the site of infection. These cells secrete a large number of mediators of the inflammatory response, and amongst these are a number of reactive oxygen species. These species cause a significant amount of tissue damage. Increases in plasma levels of conjugated dienes, lipid hydroperoxides and malondialdehyde (MDA) have been reported in plasma during acute inflammatory reactions (Ward et al, 1986; Dormandy, 1989). This process, while damaging tissue, has biological advantage in that these agents act as chemoattractants for a range of immunological cells, and may also directly attack bacterial
infections.

It has been suggested that where inflammation occurs, associated lipid peroxidation may cause alteration of macromolecules to an extent where antibodies are produced against these same macromolecules (Lunec et al, 1985). These types of antibodies are a feature of autoimmune diseases such as rheumatoid arthritis and systemic lupus erythmatosis (SLE) (Lunec et al, 1985; Blount et al, 1991). In certain inflammatory conditions such as rheumatoid arthritis the symptoms of the condition are accelerated by bleeding into the synovial fluid caused by mechanical damage to the vascularisation. It has been postulated that some of the inflammation and tissue damage may be caused by the freeing of iron from haemoglobin leading to free radical formation and subsequent peroxidation (Winyard et al, 1987). However, the low levels of free iron measured in synovial fluids from rheumatoid arthritis sufferers (Gutteridge, 1987) does not support this hypothesis.

e) Thyroid Disease

The thyroid gland produces two hormones, thyroxine and triiodothyronine, which can control the overall systemic metabolic rate. The clinical consequences of over production of these hormones (hyperthyroidism) are usually those of increased heart rate, palpitations, rapid bowel rate and loss of weight; while underactivity (hypothyroidism) leads to slowed heart rate, drying and thickening of the skin and intolerance to cold. Hyperthyroidism has long been known to stimulate oxidative metabolism (Kubista et al, 1971; Winder et al, 1975). Such stimulation leads to thyrotoxic myopathy (Kazakov et al, 1986). Experimentally induced hyperthyroidism in rats causes a significant drop in the
activity of glutathione peroxidase in heart, liver and muscle (Asayama et al, 1987). These same animals showed significant increases in mitochondrial superoxide dismutase and increased concentrations of thiobarbituric acid reactive substances in heart and soleus muscles (Asayama et al, 1987). Given this trend towards increased protection against peroxidation it may be that some of the adverse effects of thyrotoxicosis are linked to lipid peroxidation.

1.7 Summary

Our view of free radical processes and the role of lipid peroxidation in biological systems is changing. The emphasis is moving away from a perception of peroxidation as a damaging phenomenon alone, to one where peroxidation has a role to play in cellular and perhaps systemic control. The nature of this role is not well understood but appears to be intimately linked with intracellular, and possibly intercellular, signalling and therefore may be another on the growing list of functions for the cell membrane.

This changing view of peroxidation requires detailed experimental work if the full role of lipid peroxidation in normal and abnormal cell function is to be understood. The keys to this understanding are the analytical techniques required to identify and quantitate the changes in lipids which occur as a function of these processes.
CHAPTER 2: ANALYTICAL METHODS FOR THE ASSESSMENT AND MEASUREMENT OF LIPID PEROXIDATION

2.1 General Introduction

One of the major handicaps in lipid peroxidation research is the absence of sensitive and accurate methods for measurement of lipid peroxidation products. A very large number of analytical approaches have been applied to the study of lipid peroxidation. This search for adequate methodology has been driven by the limitations of the existing techniques, and the need often to apply more than one analytical technique in order to be able to adequately interpret experimental data.

In describing such a vast array of methods a major difficulty is classifying them. Methods can be divided into two major classes, (a) general non chromatographic methods and, (b) specific and chromatographic methods, but even within these classes are many subclasses of method. Historically most methods have been of the general, non chromatographic category, and the vast majority of lipid peroxidation estimations continue to be of this type. However improvements in chromatographic techniques coupled with advances in detection systems have led to an increased application of chromatography to the study of lipid peroxidation.

The classification of methods in this way mirrors the two types of strategy employed for quantitative assessment of peroxidation. The most common approach is to view lipid peroxidation as a coherent single process and try to measure the extent of this process by
quantitation of a general marker of peroxidation. Such an approach is flawed in that lipid peroxidation is essentially a heterogenous process. Results obtained by these methods may not accurately reflect the nature of the system being studied, particularly when the mechanisms of peroxidation are not well understood. The other approach is to accurately quantify a specific reaction product, or class of products of peroxidation. This way of addressing the problem provides highly specific data for the particular analyte being studied but may not provide information on the peroxidative process as a whole. This latter approach often requires extensive analytical expertise.

Consequently the choice of analytical method depends greatly on the objective of the experiment, and the nature of the peroxidative system under study. It is often necessary to employ more than one method in order to be able to interpret experimental data correctly.

2.2 Non-chromatographic methods of lipid peroxidation measurement

By far the most commonly used methods of experimental assessment of lipid peroxidation are non-chromatographic and include spectrophotometric, fluorescent, chemiluminescent and enzymic techniques. All these procedures have certain traits in common. Methods in this category do not directly measure a specific reaction product and thus may give rise to misleading results if experimental conditions are not well understood. Also many of the methods measure propagation products rather than the earlier products of initiation. Consequently data from these assays can be difficult to interpret unless great care is taken with design experiments as artifactual increases in peroxidation often arise during
experimental procedures.

2.2.1 Spectrophotometric determination

Spectrophotometric methods probably account for the vast majority of lipid peroxidation determinations. They include the Thiobarbituric acid (TBA) test, which is used in almost all biologically related studies on lipid peroxidation, as well as the conjugated diene test and direct assay techniques for peroxide groups.

a) Thiobarbituric Acid Test

The TBA test is based on the reaction of thiobarbituric acid with malondialdehyde (MDA), a product of endoperoxide breakdown to form a reaction product with absorption at 532-535nm (Slater, 1984). The method was originally developed as a test for milk rancidity in the 1930's but was later shown to be applicable to tissue samples (Wilbur et al, 1949).

Despite the very wide application of the method in peroxidation studies, the assay has been shown to be very non-specific. Many compounds are known to form interfering chromogens with TBA including endogenous aldehydes and carbohydrates (Moorhouse et al, 1985). The TBA method also generates MDA from lipid hydroperoxides and cyclic endoperoxides under the assay conditions when free transition elements are available (Gutteridge and Quinilan, 1983). For this reason very few papers now refer to the measurement of MDA when discussing TBA test results, but use the term thiobarbituric
acid reacting substances (TBARS) to more accurately reflect the nature of the assay. The method continues to be applied, primarily because of its ease of use, and because, in spite of the difficulties in interpretation, it has been shown to correlate well with other methods of lipid peroxidation measurement (Slater, 1984).

b) Measurement of Conjugated Dienes

The formation of conjugated diene groups in polyunsaturated fatty acids which have undergone radical attack has been widely used as a method of assessment for lipid peroxidation, based on the ultra violet (UV) response at 230-235nm of conjugated diene groups in polyunsaturated fatty acids (Halliwell and Gutteridge, 1989; Slater, 1984). The major problem with direct measurement of conjugated dienes in biological samples is the potential for interference from endogenous materials at this wavelength. Analysis of conjugated dienes from lipid extracts at 235nm involves the measurement of the required absorbance maxima as a small shoulder on a large background absorbance (Slater, 1984). The use of second derivative spectroscopy, allowing clearer discrimination of 235nm response against background, has assisted in improving the data from such assays (Corongui and Milia, 1983). However another difficulty with the technique is that some endogenous lipid conjugated dienes are demonstrably not formed by free radical attack, for example, octadeca-9,11-dienoic acid, a fatty acid found in human serum (Dormandy and Wickens, 1987). The interference, inherent in this approach, can be avoided by combining the technique with separation methods such as HPLC (Dormandy and Wickens, 1987), thus allowing the lipid dienes to be measured in isolation.
c) Iodometric Peroxide Measurement

The measurement of the highly reactive peroxide group is an obvious strategy for assessment of lipid peroxidation and has the benefit of measuring a functional group which is central to the process. Techniques using reactions specific to the peroxide functional group have been available for many years, particularly for assessment of levels of peroxidation in foodstuffs. The principle method is the iodometric assay based on the production of iodine from potassium iodide (Lea, 1931) in the following reaction.

\[
\text{ROOH} + 2\text{I}^- + 2\text{H}^+ \longrightarrow \text{I}_2 + \text{ROH} + \text{H}_2\text{O} \quad (6)
\]

The iodine evolved is measured either titrimetrically (Lea, 1931;) or by monitoring absorption at 360nm (Hicks and Gerbicki, 1979). The major disadvantage of the method is that a significant positive bias may occur from oxidation of the potassium iodide by atmospheric oxygen and strict precautions must be taken to exclude oxygen from all reagents (Lea, 1931; Buege and Aust, 1978; Hicks and Gerbici, 1979). In practice this is extremely difficult and the method has not been widely adopted for assessment of lipid peroxidation, but is primarily used to assess peroxide levels in lipid hydroperoxide preparations used as calibrants in other, more robust, assays. Recently a stabilised reagent for iodometric assay of lipid peroxides has been applied to the analysis of hydroperoxides in serum lipoproteins (El-Saadarin et al, 1989) and this approach may allow this method to be more extensively used in biological matrices. However, the assay also detects hydrogen peroxide and therefore results from such assays could only give a total peroxide value for the tissue unless the method was combined with some form of sample
preparation.

d) Summary

All of the spectrophotometric techniques described have been relatively widely applied in the study of lipid peroxidation and are used extensively for tissue analysis of lipid peroxidation. They lack specificity as they do not measure clearly identified products of lipid peroxidation, and are prone to interference from other compounds present in experimental samples. It is difficult to find reaction mechanisms cited for many of these methods, including TBA, which implies that an understanding of how these methods actually work is often lacking. Such a situation makes interpretation of data even more difficult. However they are extremely easy to use and have therefore been widely applied in experimental situations where a general indication of level of peroxidation is required.

2.2.2 Enzymic methods

A number of enzymic methods for assessment lipid peroxidation have been developed, including glutathione peroxidase-reductase systems (O'Gara et al, 1989), cyclooxygenase activation (Pendleton and Lands, 1987) and the peroxidase activity of haemoglobin (Matsushita et al, 1987). A number of problems exist with this type of methodology, not least the fact that their application to biological systems is prone to interference by endogenous enzymes and substrates. The methods do, however, have the advantage of greater sensitivity than the iodometric systems.
a) Glutathione Peroxidase Assay

The glutathione peroxidase-reductase system is a coupled enzyme reaction. The reduction of peroxides by glutathione peroxidase depletes reduced glutathione which is subsequently replenished by glutathione reductase.

\[
2\text{ROOH} + 2\text{GSH} \xrightarrow{\text{Glutathione Peroxidase}} 2\text{ROH} + 2\text{H}_2\text{O} + \text{GSSG}
\]  \hspace{1cm} (7)

\[
\text{GSSG} + 2\text{NADPH} \xrightarrow{\text{Glutathione Reductase}} 2\text{GSH} + 2\text{NADP} \hspace{1cm} (8)
\]

The assay is monitored by the reduction in NADPH. The method is limited because the activity of glutathione peroxidase varies depending on hydroperoxide species and requires catalase to be present in the assay system to avoid interference from hydrogen peroxide (O'Gara et al, 1989). Also endogenous glutathione reductase and peroxidase will significantly affect results from tissue analysis. Consequently the method is only suitable for \textit{in vitro} investigations.

b) Cyclooxygenase Activation Assay

Lipid hydroperoxides are known to stimulate prostaglandin H synthase (PHS) activity (Hember and Lands, 1980) and this response has been used as the basis of an assay of lipid hydroperoxides in biological matrices (Pendleton and Lands, 1987). Unfortunately PHS exhibits some unusual properties which adversely affect the application of the method. The enzyme has dual activity. In addition to converting arachidonic acid to prostaglandin G\textsubscript{2},
it also has a peroxidase function which converts prostaglandin G₂ to prostaglandin H₂. The enzyme can only catalyse a limited number of conversions to prostaglandin H₂ before being totally inactivated (Eling et al., 1990). In consequence the assay monitors the reduction in lag time of the enzyme occasioned by the peroxide stimulation rather than the simpler procedure of monitoring the reaction rate (Pendleton and Lands, 1987). Quantitation thus relies on identifying the start of the reaction, in this case by measuring uptake of oxygen. Instead of measuring the reaction over a relatively long period of time, as would be the case with a rate measurement, the analyst has to ascertain when the oxygen consumption starts. This type of measurement is prone to large errors. In addition, cyanide, phenol, ethane and glutathione peroxidase are known to affect the lag time by either competing for the haem activation binding site of the enzyme or scavenging free radical intermediates (Pendleton and Lands, 1987) further limiting the application of the method. It has also been suggested that differing hydroperoxides are likely to have varying levels of stimulation (Halliwell and Gutteridge, 1989). All of these factors mitigate against the assay system being adopted as a general method for biological samples.

c) Haemoglobin Assays

A number of workers have used the peroxidase activity of haemoglobin, linked with suitable dyes as hydrogen donors, to measure peroxide levels. The dyes used have included sesamol dimers (Kikugawa et al., 1985) and a methylene blue derivative (Oshishi et al., 1985). These methods have been suggested as suitable for analysis of peroxides in foodstuffs and biological materials, however they show variable responses to different peroxidised lipids and, in particular, show a reduced response to phospholipid and
triglyceride hydroperoxides. It has therefore been recommended that these methods only be used in the analysis of pure lipid systems or after fractionation of the lipids from biological samples (Matsushita et al, 1987). It may be that these techniques will find application as post column reaction methods for chromatographic procedures although they lack the sensitivity of other post column reaction methods which are available.

d) Summary

The warning against using haemoglobin assays unless the sample had been purified (Matsushita et al, 1987) might easily also be applied to the other enzyme assays described. While these systems have the advantage of being sensitive, they are extremely prone to problems of interference and are therefore unsuitable for use with tissue samples.

2.2.3 Luminescence Techniques

The luminescent properties of molecules offer a sensitive and selective method of measurement of these molecules. Luminescent molecules are capable of absorbing energy and then emitting energy of a specific wavelength by dropping back to the ground state. The specific nature of this emission, or fluorescence, ensures the specificity of the method (Baeyens et al, 1989). Two techniques exist for applying this energy to the system. It can be applied as light of a specific wavelength (photoluminescence) or using the energy derived from a specific chemical reaction (chemiluminescence).

Photoluminescent and chemiluminescent techniques can be used in two ways for
assessment of peroxidation. Direct measurement can be either to measure photoluminescent products of lipid peroxidation, or to detect the low level luminescence in tissues associated with free radical reaction processes. Alternatively, indirect techniques can be employed by either forming photoluminescent derivatives with lipid peroxidation products, or using chemiluminescent reactions involving lipid peroxides as the basis of assay methods.

a) Lipofuscin Measurement

Photoluminescence can be used for the measurement of lipofuscin in tissues. Lipofuscin is a yellow brown, lipid rich material, and is deposited as cytoplasmic granules which accumulate in a variety of tissues. The presence of these granules in tissues is strongly linked with peroxidation and ageing (Donato, 1981). Lipofuscin has a characteristic fluorescent spectrum and a number of procedures based on extraction and fluorescent measurement of lipofuscin have been described as methods to measure lipid peroxidation in tissues (Dillard and Tappel, 1984; Tsuchida et al., 1987). There is much controversy over the exact chemical nature and source of lipofuscin. It is thought to be composed of lipids and amino acid components and therefore most probably consists of Schiff's bases formed by reactions between aldehydes and proteins (Donato, 1981). There is general agreement that its formation is related to free radical processes, the primary evidence being the good correlation between peroxidative processes, such as γ irradiation, and lipofuscin production (De et al., 1983), and an inverse correlation between concentrations of antioxidants such as α-tocopherol and lipofuscin production (Desai et al., 1975).
Several problems have been encountered with lipofuscin measurement. Differences between the spectral properties of lipofuscin in situ and the extracted fluorescent materials have been described (Stark et al, 1985). As lipofuscin is a heterogeneous group of similar compounds there is no single extraction procedure that will remove all lipofuscin-like substances from tissues, furthermore substantial variation in recovery of lipofuscin between tissues has been reported (Tsuchida et al, 1987). This may be due to production of differing types of lipofuscin in different tissues.

b) Chemiluminescence Measurements

Low level chemiluminescence at the whole organ, cellular and subcellular level is attributed to peroxidation processes in these systems and has been used as a direct measure of peroxidation (Bovris et al, 1980; Cadenas, 1985; Ursini et al, 1989).

This technique is the only way to directly measure peroxidation in vivo. The chemiluminescent response is stimulated by depletion of reduced glutathione and vitamin E (Ursini et al, 1989) and by dosing with organic peroxides (Barsacchi et al, 1983). However as the chemiluminescence also seems to be related to activity of cyclooxygenase (Cadenas et al, 1983) and lipoxygenase (Schulte-Herbruggen and Cadenas, 1985) the specificity of the method to free radical mechanisms is in doubt.

Chemiluminescence utilising the reaction of peroxides with luminol/heme reagents has been used by a number of workers (Iwaoka and Tabate, 1984; Yamamoto and Ames, 1987). The method has the benefit of being specific for peroxide groups (Miyazawa,
but specific species produce differing reaction rates with the reagents. Consequently results from biological matrices may be difficult to interpret and the technique has been more intensively used with HPLC to increase specificity (Yamamoto and Ames, 1987; Miyazawa, 1989). This application is more extensively discussed below (see section 2.5.4).

c) Indirect Photoluminescence

Photoluminescent derivitisation of carbonyl products of lipid peroxidation have been developed as assay systems. TBA (Yagi, 1984), l-aminopentane (lio and Yoden, 1988) and other compounds containing amino groups have been used to produce photoluminescent derivatives. However in biological systems (or indeed pure lipid assay systems containing complex mixtures of phospholipids) there is likely to be some overlap in spectra between the fluorescent adducts of interest and opportunistic reactions with endogenous carbonyl groups. This may be minimised by careful choice of excitation and emissions wavelengths, but probably not eliminated altogether.

The use of parinaric acid fluorescence as a marker of lipid peroxidation has been recently developed (Kuypers et al, 1987). Cis-parinaric acid is strongly fluorescent when partitioned into a lipophilic matrix such as a lipid bilayer or cell membrane, but loses its fluorescent properties if attacked by free radicals. In consequence the loss of fluorescence due to peroxidation can be used as a method of measuring lipid peroxidation in erythrocyte membranes (Van den Berg et al, 1988). It has also been used to assess the protective properties of antioxidants in reducing radical damage in cell membranes.
(McKenna et al, 1991) and for studying lipid peroxidation in living cells (Hedley and Chow, 1992). Although the method does not measure any specific reaction product the possibilities of the technique as a marker of lipid peroxidation at the subcellular level are exciting. In particular the use of membrane specific fluorescent probes which can be excited by cis-parinaric acid emissions has been discussed by Hedley and Chow (Hedley and Chow, 1992) as a possible way of differentiating peroxidation at the subcellular level.

d) Summary

The use of luminescent and fluorescent techniques has the distinct advantage of higher sensitivity than spectrophotometric methods and certain techniques open up analytical possibilities, such as in vivo assessment of peroxidation or subcellular imaging of lipid peroxidation, which are not practical by other approaches. Specificity for these types of assays remains a problem. While the use of fluorescent markers are potentially highly specific and sensitive, in practice they require extensive sample preparation which may result in artifact formation.

2.2.4 Summary of General Methodology

It is apparent that a wide variety of general methods for lipid peroxidation are available. However in every case there is potential for interference with the method which would lead to significant bias of the results under certain conditions, particularly in the assay of tissue samples. In order to interpret results from such assays it is extremely important to understand how the experimental conditions and nature of the assay sample will affect the
assay result. While this is true of every analytical procedure, in the case of general methods of assessment of lipid peroxidation the complexity of the system being studied combines with the lack of selectivity of the available methods to exacerbate the problem.

2.3 General History of Chromatography

Specific measurement of products of lipid peroxidation has been achieved by the use of chromatography. Chromatography can be defined as the separation of substances by differential migration against a stationary sorptive medium. (IUPAC, 1974). In practice this means the separation of mixtures of compounds by passing them, dissolved either in a gaseous or liquid mobile phase, down sorptive columns. The main breakthrough in this approach to biochemical analysis came at the turn of the century with the work of Tswett, who used large columns of various sorptive materials to separate plant pigments. This early work even included reference to purification of lecithin (phosphatidylcholine) from egg yolks (Tswett, 1903); possibly the earliest lipid chromatography described. Other workers at the turn of the century were also using sorptive columns to achieve separations of inorganic and organic salts and petroleum fractions (Reed, 1893; Day, 1903). The technique remained dormant until the 1930's when Lederer demonstrated separations of carotenes in carrot extracts and xanthophils in egg yolk (Lederer, 1972). The technique of adsorption chromatography was developed by the use of paper chromatography and thin layer plates in the 1940's and 1950's (Pelick et al, 1966).

The early 1940's saw Martin and Synge develop the theory and practice of liquid-liquid partition chromatography. By using water held in a silica gel stationary phase they were
able to separate acetylated amino acids using chloroform as the mobile phase or carrier (Martin and Synge, 1941 a and b). These systems gave poor separation of certain amino acids, and Martin and Synge predicted at this time that the use of gas liquid systems for analogous partition chromatography would give greater resolution. However it was not until 1952 that Martin's group devised the first gas liquid chromatograph (James and Martin, 1952). Gas solid chromatographs (achieving separation by adsorption rather than partition) had also been developed by Cremers group in Austria (Cremer, 1950). Gas chromatography opened up many new possibilities and the 1950's and 60's saw developments in chromatograph design, column materials and detection systems. Growth in the number of applications for gas chromatography has been very large since that time, however the technique was and is limited to analysis of volatile materials. Consequently, if not already volatile, analytes of interest have to be capable of forming volatile derivatives to be amenable to gas chromatographic analysis. Compounds not amenable to gas chromatography had either to be analysed by traditional techniques or to be separated by paper or thin layer chromatography, both of which at this time had poor resolution compared to gas chromatography.

The possibilities for high resolution liquid chromatography had already been theoretically identified in the early 1940's by Martin and Synge (Martin and Synge, 1941b). They recognised that smaller particle size of packing materials and high pressure flow of the liquid carrier should reduce the theoretical plate height of the column, thereby increasing chromatographic efficiency. This potential was not realised for many years primarily due to the absence of appropriate detection systems that would allow sensitive measurement of analytes in small volumes of liquids. This problem was finally overcome by Kirkland,
who adapted a suitable detector to the task and the technique of high pressure liquid
cromatography was made available to analytical scientists (Kirkland, 1969).

2.4 Gas Chromatography

2.4.1 Gas Chromatography of Lipids

The commonest application of gas chromatography to lipid analysis has been in the area
of fatty acid profiling. In these types of analysis total lipid extracts are separated into lipid
classes. Those classes containing fatty acid groups could then be transesterified to
produce volatile alkyl ester derivatives amenable to gas chromatography (Kuksis and
Myher, 1980; Eveshed, 1992). This reaction breaks the ester linkage of fatty acids, the
glycerol group of triglycerides and phospholipids creating instead a methyl group. This
reaction frees the fatty acid for analysis while at the same time increasing volatility of the
molecule. Such techniques have proved useful for monitoring fatty acid changes in
complex lipid classes but are time consuming and relatively complex to perform.
However, with the advent of high efficiency, capillary columns with temperature stable
stationary phases it has become possible to analyse intact lipid species such as
triacylglycerols, diacylglycerols, steryl esters and wax esters (Eveshed, 1992).

Phospholipids and sphingolipids are not suitable candidates for this type of analysis as they
are thermally labile. The analysis of these compounds has been achieved in part by
converting them to diacylglycerols by the removal of the phosphate groups using
phospholipase C (Myher and Kuksis, 1984). Phospholipid species within a class can then
be determined by high temperature gas chromatography.

Detection systems for lipids on gas chromatographic analysis are generally limited to flame ionisation detection (FID) or on line mass spectrometry (MS). Flame ionisation is a sensitive and very general detection system which detects all organic compounds. The principle of the detector is that the organic analytes are combusted in a hydrogen/air flame. This combustion yields ions which form a potential between the flame and a collector electrode which can be measured as a current. This current is linear with concentration over a wide range, and the detector has a fast response making it ideal for use with high resolution chromatography. Mass spectrometry relies on the ionisation of the analyte either by electrons or by chemical ions (usually formed by electron bombardment of methane) and subsequent measurement of the ion or ions provided. This detector can be used to provide structural information about the analyte, or if used in selective ion mode, to be a high sensitive and selective detector.

Biological samples such as animal body fluids and tissues are not suitable for direct injection into a gas chromatograph due to high proportion of non-volatile components which would block the column after very few injections and due to potential interferences from other volatile components. In consequence such samples require considerable treatment prior to analysis. This follows a number of steps

- total extraction of lipids into solvents
- separation of particular lipid class to be analysed
- where necessary, derivatisation of lipid class to volatile analyte.
Total extraction of animal tissues is most commonly performed by solvent extraction using chloroform/methanol mixtures (Folch et al, 1957; Bligh and Dyer, 1959). These techniques will extract the majority of lipophilic compounds and extracts therefore contain a great many non-lipid compounds which are not required for analysis. It is therefore common to further fractionate the lipids into general classes. This is generally achieved either by thin layer chromatography (Henderson and Tocher, 1992), adsorptive column chromatography (Hamilton et al, 1992), or by solid phase extraction (Kaluzny et al, 1985).

2.4.2 Gas Chromatography of Lipid Peroxides

A number of methods for the determination of hydroxy fatty acid derivatives of hydroperoxides by gas chromatography coupled with mass spectrometry have been described (van Kuijk et al, 1985; Hughes et al, 1986; Guido et al, 1993; Thomas et al, 1992). All these methods have similar protocols, namely extraction of the hydroperoxides, reduction of the thermally unstable hydroperoxide group to its hydroxide, and transmethylation using alkylating reagents, such as methanolic boron triflouride or diazomethane, to form hydroxy fatty acid alkyl esters suitable for gas chromatography. While in theory mass spectrometry provides a specific method of detection, these methods have been criticised because significant breakdown of lipid hydroperoxides occurs during sample extraction (Thomas et al, 1992). This problem has been addressed by reducing the hydroperoxide group within the sample prior to extraction and subsequent transmethylation to release fatty acid alkyl esters. Even with this improvement it is impossible to know what proportion of the final hydroxy fatty acid content is from
hydroperoxides present at extraction, and what proportion is from natural reductive processes which occurred pre-extraction. In addition, a recent study of transmethylation techniques has shown that these techniques produce significant structural alteration of the hydroxy fatty acids (Ulbert and Kamptner, 1992).

2.4.3 Gas chromatographic Determination of Alkanes produced during Lipid Peroxidation

The only non-invasive method of in vivo assessment of lipid peroxidation currently available is the measurement of exhaled alkanes (Riely et al., 1974). Low molecular weight alkanes are produced by breakdown of hydroperoxides. These volatile compounds are exhaled, and the exhaled breath can be analysed by gas chromatography. Direct measurement of alkanes from samples of exhaled air limits the sensitivity of the method, but allowing the accumulation of the alkanes in a controlled closed system (Lawrence and Cohen, 1984), or concentrating the alkanes by drawing the exhaled air over an adsorbent material prior to analysis can substantially lower limits of detection. Measurement of ethane and pentane (formed by the peroxidation of $\omega-3$ and $\omega-6$ polyunsaturated fatty acids respectively) have been used. Many problems have been identified with these assays. Endogenous production of alkanes by gut flora may contribute to the exhaled totals (Slater, 1984), and ethane and pentane are rapidly metabolised (Dougherty et al., 1988). Normal levels of ethane and pentane in exhaled air are relatively high (particularly pentane) and show considerable variation between individuals (Wendel, 1987). However, in spite of these problems, a considerable number of papers have been published presenting exhaled alkane data. The use of pentane as a marker of in vivo peroxidation
has recently been questioned by Kohlmuller and Kochen, who maintain that the majority of published methods for pentane are also measuring isopentane, a compound not produced by peroxidation (Kohlmuller and Kochen, 1993). They have suggested that this calls into question the majority of exhaled pentane data in the literature as results attributed to peroxidation may be caused by other metabolic changes. However, alkane determinations do provide some useful data on peroxidative status in the living organism, and in most studies this data is backed up by other determinations of lipid peroxidation.

2.5 High Performance Liquid Chromatography (HPLC)

2.5.1 HPLC of Lipids

From the earliest development of HPLC in the 1960's, the technique has found application in the analysis of lipids. HPLC has advantages in the analysis of lipids in that it is amenable to the analysis of non-volatile and thermally unstable materials. It therefore has particular significance in the determination of phospholipids and related compounds which are unsuitable for direct analysis by GC. This subject has been extensively reviewed (Christie, 1987) and little has altered in the general strategies which are applied to the analysis of lipids. This section therefore summarises those HPLC methods for analysis of lipids.

Early HPLC separations were performed on columns using polar packing materials such as silica or (less commonly) alumina. As these materials had already been used for separating lipid classes by column chromatography it was a simple and obvious progression to use HPLC to increase the resolution and sensitivity of the analytical
procedure. HPLC was therefore widely used to separate and measure lipid classes.

As HPLC became more extensively used as an analytical technique the need to chromatograph non-polar, non-volatile analytes grew. This was achieved by the covalent bonding of alkyl chains to the surface of silica particles, thereby altering the surface of the silica. There are now a considerable number of these non-polar materials available and they have been used in lipid research to separate fatty acyl lipids, such as the acylglycerols and phospholipids, into their separate species. For historic reasons these non-polar bonded phase materials were termed reversed-phase packing materials as the polarity of the silica surface had been "reversed". Reversed phase HPLC is now used for the vast majority of separations which are performed. The ability to bond to silicas has led to development of a number of polar bonded phases. Silica's bonded with diol groups and aminopropyl groups are now commercially available and have been used to achieve separation of lipid classes.

Normal and reversed phase HPLC methods have very different selectivities for lipids and there are particular problems associated with each. Normal phase chromatography of phospholipids enables the lipid classes to be clearly separated which in principle allows the profiling and quantitation of classes in biological samples to be undertaken. The problem for quantitation is that the classes are heterogenous; in other words they contain many species. The response of species to the different methods of HPLC detection often vary, making quantitation of each chromatographic peak very difficult. Elegant normal phase separations of phospholipid classes have been described with silica (Kiatarita and Bessman, 1981; Patton et al, 1982; Christie, 1985), and with 1,2-dihydroxypropyl (Briand

Reversed phase allows these species to be individually separated, but mixtures of classes lead to very complex chromatograms with overlapping peaks. Prior separation of the classes is therefore necessary. Reversed phase chromatography was first applied to separation of phosphatidylcholine species using a hydroxyalkoxypropyl sephadex column in 1975 (Arvidson, 1975). Resolution was relatively poor and only four fractions were collected. Since that time reversed phase materials have been effectively applied to the separation of lipid species. High resolution species separations using octodecyl silane bonded phase silicas have been described for phosphatidylcholine (Patton et al, 1982; Porter et al, 1979; Sotiros et al, 1985), phosphatidylethanolamine (Gross, 1985; Justin et al, 1985) phosphatidylglycerol (Smith et al, 1985) and sphingolipids (Hirabayashi et al, 1986; Jungalwala et al, 1979).

Detection has been a considerable problem in the HPLC analysis of lipids. The only universal detector currently available in HPLC is the refractive index detector, however its lack of sensitivity hampers its use in biological applications. Light scattering detection has been used for the study of lipids but has commonly been reported as being insensitive or as having a non-linear response thereby making quantitation difficult. More commonly used techniques such as ultraviolet detection have proved problematic because the low maximum absorbence of many lipids (200-210nm) restrict the types of mobile phase constituents that may be used to achieve separations, and the response between individual molecules within a class can vary widely making interpretation of data from heterogenous
sample sources difficult to interpret. The use of ultra violet transparent solvent systems such as buffered acetonitrile/water eluents or hexane/isopropanol/water eluents allowed UV detection at 205nm to be utilised (Christie, 1987). Detection at 220nm, measuring the absorbance of ester bonds, has been shown to reduce difference in response to 5% or less (Shukla et al, 1983). However the benefits are offset, as sensitivities at this wavelength are little better than those achievable with refractive index detectors (Hersloff, 1981).

Flame ionisation detectors for liquid chromatography are available but have proved unreliable primarily due to the problems of reproducibly volatising organic solvents. The difficulties in using mobile phase additives such as buffers, which are not amenable to volatisation, have made the use of this type of detection less viable for liquid chromatography. Similar problems have hampered the use of mass spectrometry as an HPLC detector, however recent developments in atmospheric pressure ionisation mass spectrometers appear likely to improve the situation.

2.5.2 HPLC of Non-Lipid Products of Lipid Peroxidation

Following the discovery that lipid peroxidation yielded a complex mixture of toxic aldehydes (Schauenstern et al, 1977; Conway et al, 1975) methods were developed to measure the carbonyl products of lipid peroxidation in tissues (Esterbauer and Cheeseman, 1990). The basis of the methodology was derivatisation in situ of all aldehydes to their dinitrophenyl hydrozones using dinitrophenyl hydrazine (DNPH). These stable derivatives could then be isolated by solvent extraction. The extracts are pre-separated by silica gel
thin layer chromatography (TLC). This facilitates removal of excess DNPH and contaminating hydrazones formed from acetone, formaldehyde and acetaldehyde, as well as allowing the aldehydes to be divided into polar and non-polar classes. These classes are then analysed by reversed phase HPLC.

These techniques have been used to study aldehyde production during lipid peroxidation in rat microsomes (Esterbauer et al, 1982; Poli et al, 1985) and rat hepatocytes (Poli et al, 1985). The technique has also been applied to whole tissue analysis to measure 4-hydroxynonenal or hexanal (Esterbauer, 1993), and these compounds have been suggested as suitable markers of lipid peroxidation.

Although these compounds are highly cytotoxic, have been shown to react with proteins and have considerable effects on the biochemical functions of cells (Esterbauer, 1993; Dianzani, 1989) they are produced from the breakdown of lipid hydroperoxides and therefore may be prone to similar artifacts during sample work up to those seen with the TBA test. In addition these compounds are freed into the cytosol and hence offer no opportunity to study lipid peroxidation in situ on the cell membrane. However these methods have provided important information about one aspect of lipid peroxidation, and the possibility exists to use the derivatisation techniques to measure aldehyde groups on phospholipids.

2.5.3 HPLC of Fatty Acid Hydroperoxides

A number of separations have been reported for fatty acid peroxides. These have been
performed on ester derivatives of fatty acid hydroperoxides (Chan and Prescott, 1975; Teng and Smith, 1985) and free fatty acid hydroperoxides (Teng and Smith, 1985; Wurzenburger and Grosch, 1984; Verhagen et al, 1978) on silica columns, although reversed phase separation of free fatty acid hydroperoxides has also been described (Kanner and Kinsella, 1983). Others have reduced the unstable hydroperoxy group to the more stable hydroxy form, using reducing agents such as sodium borohydride followed by normal phase HPLC (Chan and Prescott, 1975; Porter et al, 1980) or reversed phase HPLC (Van Rollins and Murphy, 1984; Williamson and Zurier, 1984).

The resolution of fatty acids achievable by HPLC is much less than that achieved by gas chromatography. Consequently this has meant these methods have been principally used for the study of mechanisms of fatty acid peroxidation in vitro, rather than analysis of peroxidation products formed in vivo.

2.5.4 HPLC of Phospholipid Hydroperoxides

Initial separations of peroxidised phospholipids used reversed phase HPLC techniques to study the reaction products of oxidation of phospholipids, either as individual species (Porter et al, 1980) or separation of molecular species from within a single class (Crawford et al, 1980). These workers used the characteristic absorbance of conjugated dienes at 235nm as the method of distinguishing peroxidised phospholipids from non-oxidised parent phospholipids. The resolution of peroxidised phospholipids from parent phospholipids using reversed phase chromatography is so great that Crawford et al (1980) were able to completely elute all oxidised phosphatidylcholine species prior to the first
parent phospholipid species.

Normal phase columns have been used with peroxidised lipids (Terao et al., 1985; Terao and Matsushita, 1987) however separation of parent phospholipids from peroxidised phospholipids has not been reported using these phases. Comparison of absorption at 235nm to 205nm has been suggested as a useful indication of peroxidation of phospholipid classes (Terao and Matsushita, 1987) however, given the co-elution of parent phospholipids with peroxidised species, specific detection systems are required if the peroxidised lipids are to be quantitatively measured.

2.5.5 Specific HPLC Detection Methods for Hydroperoxides

With any chromatographic procedure the use of a highly selective detection system is beneficial whenever possible as it reduces the possibility of interference. Two such systems are at present possible for the direct detection of hydroperoxides. They are electrochemical detection and post column chemiluminescence. Both techniques have been used for other analytes and have proved to be very sensitive.

Electrochemical detection exploits the ability of the analyte to be easily reduced or oxidised, which generates a current in the cell which can be amplified and recorded. Most electrochemical detectors used in HPLC are amperometric, that is they apply a fixed potential at cell surface and detect currents produced by any redox reactions that take place in the eluent at that potential. Careful control of the potential and, where necessary, prior reduction or oxidation of potential contaminants, increases the specificity of the
technique (Krstulovic et al, 1984). The use of reductive mode electrochemical detection for organic peroxides was reported by Funks group in 1985 (Funk and Baker, 1985) and subsequently applied to fatty acid hydroperoxides (Funk et al, 1987; Funk, 1987). This approach has been applied to phospholipids (Yamada et al, 1987) but has not been widely adopted, possibly because of the problems associated with reductive mode electrochemical detection.

More promising has been the application of chemiluminescence to the direct measurement of lipid hydroperoxides using post column reagent systems. The two post column reagents that have been put forward for phospholipid hydroperoxides are isoluminol-microperoxidase reagents (Yamamoto et al, 1987) and luminol-cytochrome C reagents (Miyazawa et al, 1987). The exact nature of the reaction is unknown but it is assumed that the haem catalyst causes the hydroperoxide to form the alkoxy radical. This in turn attacks the luminol group to form a radical capable of reacting with molecular oxygen to form an endoperoxide. The decay of the luminol radical generates energy as light at a wavelength of approximately 430nm (Yamamoto and Ames, 1987). In order to use such reactions as an HPLC detection system the components of the reaction must be mixed with the eluent flow prior to a chemiluminescence detector. These methods have been applied to analysis of peroxides in human plasma (Miyazawa, 1989; Frei et al, 1988) with some success but have met with limited success when applied to tissues such as liver and brain (Miyazawa et al, 1992).
2.6 Summary

The foregoing discussion has examined the mechanisms of lipid peroxidation and tried to put into context its importance in biology and medicine. It has also reviewed the wide range of analytical techniques that have been applied to the problem of lipid peroxidation in biological systems.

Two major themes become evident from a review of analytical chemistry in the study of lipid peroxidation. Firstly, while there is a considerable body of literature that implicates lipid peroxidation in a variety of diseases, results and conclusions from different experiments often conflict. In consequence the full role of lipid peroxidation remains obscure. Secondly the analytical techniques used to study peroxidation are often insensitive, or unspecific, or both. By far the most commonly cited analytical procedure is the TBA method (section 2.1.2a). This method is perhaps popular because of its ease of use, but its lack of specificity means that only the most general conclusions can be made from TBA assay data. This, to a greater or lesser extent, is true of every method described in this chapter. In many cases these methodological inconsistencies compound the problem of interpretation. Seemingly minor differences in experimental conditions may have major effects on analytical data, and these relationships are often poorly understood, particularly in tissue samples, such as blood plasma, liver, kidney and brain.

Recent developments in analytical techniques have resulted in more sensitive and selective techniques becoming available. These have been predominantly chromatographic in nature, combining the selectivity possible in the separation step of the technique with
sensitive post column detection systems. Unfortunately, in many cases the analyte measured is a late reaction product of lipid peroxidation such as an aldehyde or endoperoxide. The range of chromatographic analysis is limited. Gas chromatography often requires extensive sample preparation leading to artifact production and potentially misleading results, and HPLC divides between normal phase or reversed phase mechanisms, neither of which are entirely satisfactory for the measurement of lipid peroxides.

2.7 Aims of the study

From the discussion in the previous section it is clear that there is a need for sensitive and selective analytical methods for the assessment of lipid peroxidation. While the majority of existing chromatographic methods have problems usually with regard to sample preparation, the potential selectivity of chromatography still offers the best approach to the problem.

It was hoped to use the potential selectivity of chromatography to separate and measure phospholipid hydroperoxides by exploiting new detection possibilities. Phospholipid hydroperoxides have significant advantages over other products of lipid peroxidation in terms of the information they can provide. They are an early product of lipid peroxidation. Phospholipids are also found primarily in the cell membrane and their hydroperoxides are therefore potential candidates for subcellular studies of lipid peroxidation. Perhaps most importantly the hydroperoxide group is also highly reactive and therefore has potential for a number of sensitive detection systems. The major
difficulty inherent in the analysis of lipid hydroperoxides is their instability in biological systems.

The aim of this study was to examine methods for the extraction, chromatographic separation and detection, with the objective of finding a reliable, sensitive and selective method for the measurement of phospholipid hydroperoxides in biological materials, particularly animal tissues such as liver.
CHAPTER 3: MATERIALS AND METHODS

3.1 General Reagents and Standards

3.1.1 Gas Chromatography Gases

Helium, oxygen free nitrogen and hydrogen were supplied by BOC, Guildford, UK. Compressed air was produced by using a Jun-Air compressor (Jun-Air, Paisley, UK).

3.1.2 HPLC Solvents

HPLC grade methanol, acetonitrile, propan-2-ol and hexane were supplied by Fisons Scientific Apparatus, Loughborough, UK. Hipersolve Pentane, tetrahydorfurane and Analar Chloroform were supplied by BDH, Poole, UK. Water was prepared using an Elgastat Spectrum reverse osmosis unit.

3.1.3 Lipid Standards

Bovine liver phosphatidylcholine, Bovine liver phosphatidylethanolamine, sphingomyelin, cholesterol, distearin, tristearin, dioleine, trioleine, bovine liver phosphatidylserine and phosphatidylinositol were supplied by Sigma Chemical Company Ltd, Poole, Dorset. Fatty acid mixtures and fatty acid methyl ester mixtures were supplied by Alltech UK, Carnforth, Lancs. Rose Bengal dye for preparation of lipid peroxides was supplied by Aldrich Chemical Company, Gillingham, UK.
3.1.4 Solid Phase Extraction Cartridges

100mg C18, C8, C2 and NH\textsubscript{2} Bond Elute cartridges were supplied by Varian, Harbor City, USA, as were 300mg NH\textsubscript{2} Bond Eluts cartridges used for separation of lipid classes from lipid mixtures.

3.1.5 General Reagents

Glacial acetic acid, concentrated sulphuric acid and sodium hydroxide was supplied by BDH, Poole, UK. Buffer solutions at pH4, pH7 and pH9 for calibration of the pH meter, Tetrabutyl ammonium iodide, sodium tetraborate and ammonium acetate were supplied by BDH, Poole, UK. Methanolic boron trifluoride in methanol was supplied by Alltech UK, Carnforth, Lancs. Ammonium molybdate and Fiske-Subbarrow reagent, for lipid phosphorous determinations, microperoxidase (MP11) and 6-amino-2,3-dihydro-1,4-phththalazinedione (isoluminol) for chemiluminescence detection, potassium chloride for electrochemical detection and cumene hydroperoxide and hydrogen peroxide were supplied by Sigma Chemical Company Ltd, Poole, Dorset.

3.2 Equipment

3.2.1 HPLC Apparatus

The main HPLC apparatus used during this study comprised the following equipment in series, Beckman 110B single piston HPLC pump (Beckman RIIC Ltd., High Wycombe,
Bucks), Waters Wisp 710 HPLC autosampler, LDC Spectromonitor III UV detector (Laboratory Data Control, Stone, Staffs) and a JJ Lloyd CR 652 Chart Recorder (JJ Lloyd Instruments Ltd., Southampton). For particular experiments additional detectors were connected in series with the Spectrophotometer III. These included a Spectromonitor 3200 UV detector (Laboratory Data Control, Stone, Staffs), Coulochem 100A electrochemical detector (ESA Inc., Bedford, USA) with porous graphite working electrodes, and a Kratos FS970 fluorimeter (Kratos UK Ltd) modified for chemiluminescence detection. For certain experiments a Rheodyne 7125 manual HPLC injector (Rheodyne Inc., Berkeley, California) was used in place of the Wisp autosampler (Waters Associates, Milford, USA).

3.2.2 Gas Chromatographic Apparatus

Packed column GC was performed using a Perkin Elmer Sigma 4 Gas Chromatograph, (Perkin Elmer, Beaconsfield, UK) with a 6 meter x 4mm column packed with 10% Silar 10C on 100/120 mesh Gas Chrom QII. The column was used at 180°C isothermally, with nitrogen as the carrier gas at a flow rate of 35ml/min. Capillary GC was attempted using a 25 meter x 0.25mm Silar 10C fused silica column under a variety of temperature programmes and carrier gas flows.

*The detector was used without the lamp, and a PTFE cell assembly used(see section 4.8, page 153)
3.2.3 Chromatographic Columns and Plates

The following HPLC columns:

- 250 x 4.6mm Spherisorb ODS 1, 5μ C18 Bonded Silica
- 250 x 4.6mm Spherisorb C8, 5μ C8 Bonded Silica
- 250 x 4.6mm Spherisorb S5W, 5μ silica
- 250 x 4.6mm Spherisorb Diol, 5μ diol bonded silica
- 250 x 4.6mm Apex Amino, 5μ aminopropyl bonded silica

were supplied by Fisons Scientific Apparatus, Loughborough, UK.

PT 10% Silar 10C on 100/120 Gas Chrom QII, used as a GC packed column stationary phase, and the 25meter by 0.25mm fused silica capillary column with a 0.2μm Silar 10C film, were supplied by Alltech UK, Carnforth, Lancs.

TLC was performed on 20 x 20cm Silica Gel 60 Aluminium TLC plates, with 0.2mm layer thickness, supplied by E.Merck, Dormstadt, Germany.

3.2.4 General Laboratory Apparatus

All spectrophotometric determinations were performed on a Perkin Elmer Lambda 5 Scanning Spectrophotometer (Perkin Elmer, Beaconsfield, UK).
Acid digestions and heated reactions for lipid phosphorus determinations were conducted on a Grant BT3 (Grants Instruments Ltd, UK) variable temperature heating block.

Adjustment of pH was performed using an orion glass electrode (Orion Research Boston) connected to a PTI-15 digital pH meter (EDT Instruments, Dover, Kent).

Control of temperature to -30°C was conducted in a refrigerated bath using acetone as the cooling solvent.

Weighing of reagents was carried out on a Sartorius 2024MP five place balance (Sartorius Ltd, Belmont, Surrey) (for milligram quantities or less), or on an Ohaus Brainweigh B1500D top pan balance (Ohaus Europe Ltd, Cambridge).

Quantitative liquid transfers were performed with Gilson Pipetteman P5000, P1000 and P200 pipettes (Gilson Medical Electronics, Villiers-le-Bel, France).

Evaporation to dryness of samples was conducted on a solvent evaporator built within the laboratory. Glass tubes were individually supported and oxygen free nitrogen channelled into them via glass pasteur pipettes.

Disposable glass culture tubes with teflon lined screw caps were obtained from Corning, New York.
Disposable 3ml LP3 polypropylene tubes with polypropylene stoppers were obtained from Sarstedt, Nurbrecht, Germany.

Elution of Bond Elut cartridges was performed on a Vac-Elute SPS 24 place Vacuum Elution Manifold (Varian, Harbor City, USA).

Ultra Violet (UV) irradiation was performed with an Original Hanau Lamp containing an N15 W G1ST8 Germicidal UV source.

3.3 Chromatographic Procedures

3.3.1 Use of HPLC Apparatus

All HPLC apparatus was connected using 0.1mm internal diameter stainless steel or teflon tubing, and care was taken to ensure that all tubing lengths were kept to a minimum. Tubing was connected using swagelock 1/16" zero dead volume connectors with teflon ferrules to facilitate replumbing and to ensure correct seating of tubing after replacement of columns. Columns were connected to the system using PEEK fingertight connectors with PEEK ferrules.

All samples were taken to dryness under oxygen free nitrogen and reconstituted either in eluent (for acetonitrile/water and isopropanol/hexane/water mobile phases) or in methanol (for methanol/pentane/water mobile phases) before injection onto the chromatograph, unless otherwise stated.
Prior to use all columns were checked for efficiency by comparison with manufacturers specification. Eluents and test compounds as specified on the column test certificates were followed and no column was used that showed more than a 20% deviation in efficiency from the manufacturers test result. Once in use columns were monitored by the efficiency and resolution of phosphatidyl choline and phosphatidyl ethanolamine separation.

3.3.2 Use of HPLC Detectors

a) UV detectors were used either singly or in series to monitor column eluents at 205nm (for total phospholipid) or 235nm (for detection of conjugated dienes). When used in series low volume connections were used between the detectors. No peak broadening between detectors was apparent.

b) Electrochemical detection was performed using a single porous carbon working electrode. Potentials were varied between -0.3 to -1.5 v verses a silver/silver chloride reference electrode. 0.15M potassium chloride was used as the supporting electrolyte. Due to the high pressure drop across the electrochemical cell, this was always placed between the column and any other detector used in order to avoid pressurising delicate spectrophotometric flow cells.

c) Chemiluminescence detection was based on the method of Yamamoto et al (1987) with some modification, as described in Chapter 4, section 4.8. The post-column reagent was prepared from 70:30 Methanol/50mM sodium tetraborate. To this
was added up to 35mg/l microperoxidase and up to 400mg/l isoluminol. Solutions were sonicated to assist solution, and then pH adjusted using either concentrated sodium hydroxide solution or 10% hydrochloric acid.

Reagent was delivered using a Beckman 110B HPLC at 1.0ml/min, and mixed with the eluent flow post column using a low dead volume T connector. A polytetrafluoroethylene (PTFE) tubing delay coil was placed between the mixing connector and the modified Kratos FS970 detector.

3.3.3 Thin Layer Chromatography

All TLC was performed using pre-poured plates. Samples were manually spotted onto plates using precalibrated microcapillaries. Spots of up to 2mm in diameter were applied and allowed to dry before each re-application. Up to 250µg of each sample was applied in this manner. Samples were dissolved in chloroform methanol prior to application.

All development of TLC plates were conducted in standard TLC tanks. Eluent systems are as described in Chapter 4, section 4.4.1. Eluents were placed between 0.5 and 1.0 cm deep in the development tanks and the atmosphere within the tank was allowed to equilibrate for at least 30 minutes prior to addition of the plate. Where double development was conducted the plate was removed from the first solvent and allowed to dry horizontally prior to any subsequent development.

All plates were visualised by placing the developed plate in a TLC tank filled with iodine

*Post column eluent and reagent were mixed using a zero dead volume T connector.*
3.4 Formation of Fatty Acid Methyl Ester Derivatives

Methylation of fatty acids and transmethylation of phospholipids and acylglycerides was performed using 4% boron trifluoride in methanol. Aliquots of lipids dissolved in chloroform containing up to 1mg of each lipid were dried under oxygen free nitrogen. Residues were reconstituted in 0.5ml of tetrahydrofuran, and 1ml of 14% boron trifluoride in methanol added. Tubes were incubated at 50°C overnight. 2.5mls of water was then added to the reaction mixture. Fatty acid methyl esters were extracted into hexane by adding 2.5mls of hexane to the reaction mixture and shaking for 20 minutes. The hexane layer was removed with a pasteur pipette, and the reaction mixture was re-extracted with 2.5mls of hexane. Hexane extracts were pooled, dried under oxygen free nitrogen and reconstituted in 1ml hexane prior to gas chromatographic analysis.

3.5 Sample Preparation Techniques

All solid phase extraction work was conducted with Bond Elute luer cartridges, in conjunction with a Vac Elut 24 place Vacuum Box.

3.5.1 Normal Phase Solid Phase Extraction

Separation of phospholipids from total lipid solutions and extracts was performed by the method of Kaluzny et al (1985). Initially extractions were performed with 300mg amino
propyl Bond Elute cartridges, conditioned with 4ml of hexane. Samples containing up to 2.5mg of total lipid were applied dissolved in 200μl chloroform, and the columns washed with 4ml hexane. Cartridges were then eluted with 4ml of 2:1 chloroform/propan-2-ol, followed by 4ml of 2% glacial acetic acid in diethyl ether to remove neutral lipids (diglycerides, triglycerides and cholesterol). Phospholipids were then eluted with 4ml of methanol.

The method was also applied to 100mg columns by reducing the volumes of all eluents to 1ml. This resulted in no significant contamination of the phospholipid extract with neutral lipids, however recovery of oxidised lipid species was reduced under these conditions. Application of a further 1ml of methanol resulted in full elution of oxidised species.

3.5.2 Reversed Phase Solid Phase Extraction

Bond Elute cartridges containing 100mg of either C2, C8 or C18 were used in experiments to develop SPE methods to separate oxidised phospholipid species from parent compounds.

All reversed phase cartridges were conditioned with 2ml of methanol followed by 2ml of water. Where additional conditioning with tetrabutyl ammonium iodide or ammonium acetate was undertaken 2ml of the appropriate conditioning solution was passed through the column just prior to the water conditioning step. Up to 1mg of lipid was then applied to the column in 200μl of 10% methanol in water.
Two types of elution were conducted either repeat or sequential. For repeat elution aliquots of eluent of the same composition were repeatedly applied to the column, with an end methanol wash. In sequential elution aliquots of eluents were linearly increased in solvent strength, followed by a final methanol wash.

All eluents were examined by normal phase HPLC with both UV and chemiluminescence detection, as described above.

3.6 Lipid Phosphorus Analysis

This was conducted using a modification of the Bartlett assay (Bartlett, 1959). Samples of phospholipid of up to 500μg were dried under oxygen free nitrogen in 10ml culture tubes. These samples were then digested with 200μl 6N sulphuric acid for 4 hours at 150°C. 100μl of 30% hydrogen peroxide were added to the digest, which was then heated at 150°C for 1 hour. The digest was allowed to cool. 300μl 6N sulphuric acid, 0.9ml water, 850μl of 1.2% ammonium molybdate and 50μl of Fiske-Subbarow reagent were added. The tube contents were mixed and heated at 105°C for 15 mins. After cooling the absorbance of the solutions at 800nm was recorded.

Results were calculated by reference to a standard curve produced by analysis of known concentrations of bovine liver phosphatidylcholine.

3.7 Iodometric Determination of Lipid Peroxide Values
The method used for Iodometric determination of lipid peroxide value is that described by El-Saadarin et al (1989). The method is based on oxidative conversion of iodide to iodine with subsequent spectrophotometric measurement of iodine at 365nm.

50µl of sample containing either unknown amounts of lipid peroxide or known amounts of either cumene hydroperoxide or hydrogen peroxide were added to 2ml of iodometric reagent containing 0.2M potassium phosphate buffer (pH6), 0.12M potassium iodide, 0.15mM sodium azide, 2g/l polyethylene glycol mono(p-(1,1',3,3'-tetra methyl-butyl)-phenyl)ether, 10µM ammonium molybdate and 0.1g/l alkylbenzyl-dimethylammonium chloride. The samples were mixed for 5 seconds and then capped and left for 1 hour at room temperature in darkness. The absorbance of the samples was then measured at 365nm against a reagent blank.

Concentration of the hydroperoxides was calculated by comparison of absorbance of unknowns, with a calibration curve of known amounts of cumene hydroperoxide and hydrogen peroxide.

### 3.8 Assessment of Chromatographic Performance and Extraction Recovery

Chromatographic performance was assessed in terms of response, efficiency, capacity factor and resolution. Performance of extraction methods were assessed by calculation of recovery. The following definitions and methods of calculation have been used throughout the study.
3.8.1 Response

The detector response was calculated as attenuated signal from a given detector as recorded on chart recorder. Peak height was used as the measurement parameter.

3.8.2 Recovery

Recovery in this study was always the extraction recovery and was calculated as:

\[
\text{Recovery} \% = \frac{\text{Found Response}}{\text{Expected Response}} \times 100
\]

where found response is that measured experimentally and expected response was the response calculated from 100% recovery after correcting for known experimental losses or dilution factors.

3.8.3 Efficiency

This was calculated as number of theoretical plates (N) by the following equation:

\[
N = 5.54 \left( \frac{t_r}{W_{1/2}} \right)^2
\]

Where \( t_r \) is retention time and \( W_{1/2} \) is the peak width (measured in the same units as retention) at half height (Bidlingmeyer and Warren, 1984). Values were not converted for assymetry of peak shape. Theoretical plates are expressed as plates per column throughout this thesis.
3.8.4 Analyte Retention

This is calculated as capacity factor (k') using the following equation:

\[ k' = \frac{(t_r - t_o)}{t_o} \]

Where \( t_r \) is the retention time of analyte, and \( t_o \) is the retention time of a non retained compound.

3.8.5 Resolution of Analytes

The resolution (Rs) of two analytes is calculated by the following equation:

\[ R_s = \frac{(t_{rA} - t_{rB})}{1/2(WA + WB)} \]

Where the numerator is the difference in retention times, and the denominator is the mean peak width (measured in the same units as retention). An Rs value of 1 or more indicates baseline resolution. An Rs value of 0 indicates co-elution of analytes.
CHAPTER 4: RESULTS AND DISCUSSION

4.1 Research Strategy

The objective of this study was to develop sensitive and selective methods to measure phospholipid hydroperoxides in animal tissues. The variety of assay methods and analytical techniques available is exceptionally wide and this initially produced some problems in identifying a starting point for development. However after detailed review only a few possibilities were considered suitable for investigation.

Non-chromatographic techniques, while easy to use and requiring only a minimum of equipment, were prone to interference. In order to improve the selectivities of such methods, sample clean up or separation prior to analysis would be required, detracting from their main advantage. In addition the analytes measured in these assays are often formed late in the peroxidation pathway, and are therefore more susceptible to assay condition artifacts. These characteristics did not correspond with the objectives of the study and therefore further investigation of general methods for analysis of lipid peroxidation was not conducted.

The chromatographic techniques, on the other hand, all had some potential benefits. Gas chromatography for the measurement of fatty acid groups was a strong candidate. While the use of a mass spectrometer as a detector is out of the question for many laboratories due to the relatively high capital costs of the equipment, the possibility of separating oxidised fatty acids using the high resolution possible with high polarity capillary columns,
would allow the use of flame ionisation detection. The use of thin layer chromatography was also a possibility. While there have been marked improvements in resolution achieved with this technique, particularly with layers of smaller particulate size, there remain significant problems with quantitation. The most suitable chromatographic technique for analysis of phospholipids is high performance liquid chromatography. Phospholipid molecules can be analysed intact and without prior derivitisation. The main drawback is that current methods either separate species or classes of phospholipid. In the former case prior separation of the classes is usually required prior to analysis of the species, thus requiring extensive sample preparation. Class separations, on the other hand, provide no separation of oxidised from parent phospholipids and thus require very selective detection methods.

In order to perform a chromatographic method for phospholipids in tissues a number of analytical steps must be followed. These are:

- Extraction of lipids from tissues
- Pre-separation of phospholipids from lipophilic contaminants
- Chromatographic separation of phospholipids
- Detection and quantitation

Sample preparation and chromatographic separation are individual but complementary aspects of the assay. When considering the analysis of unstable species such as hydroperoxides in biological tissues, care in selection of sample preparation techniques is of greater relative importance than in the analysis of relatively stable analytes, where
greater flexibility in sample preparation is possible.

The initial experiments therefore concentrated on investigations of chromatography with the objective of selecting a robust and selective chromatographic separation. This would be followed by investigation of suitably sensitive detection systems for these compounds. Finally, when the basic components of the method were in place the problems associated with extraction and purification of lipid hydroperoxides from tissue samples would be addressed.

A potential problem with such a strategy is incompatibility of techniques between stages. For example, a developed separation may not be compatible with required detection techniques. To avoid this problem it is necessary to have a clear vision of the constraints that apply to the other areas of the work. For this study, end step detection was most likely to be electrochemical. The chromatography would therefore have to be isocratic, and have an aqueous component in the mobile phase so that supporting electrolytes could be added. Another constraint was that of instability of lipid hydroperoxides. Consequently the chromatography should be compatible with a fairly stable and rapid extraction procedure to minimise losses of hydroperoxide.

4.2 Gas Chromatography

The use of gas chromatography in the analysis of fatty acids from complex lipids has already been used in tandem with mass spectrometry to measure lipid hydroperoxides as their hydroxy derivatives (van Kuijk et al, 1985; Hughes et al, 1986; Guido et al, 1993,
Thomas et al, 1992). Mass spectroscopy is a most sophisticated method of detection, but purchase and manning of a mass spectrometer is also extremely costly. The possibility existed of using high resolution capillary columns to resolve all fatty acid species present and thereby separate the oxidised species so that they could be individually quantitated using flame ionisation detection. High resolution chromatography would remove the necessity of mass spectrometric detection, and make this form of analysis available to more laboratories. In addition mass spectrometric detection is most sensitive in selected ion mode, which would not allow simultaneous determinations of a range of hydroperoxide and hydroxide products. Use of mass spectrometry in the scanning mode limits the sensitivity. Consequently development of a high resolution gas chromatography method using flame ionisation detection might allow simultaneous profiling of the peroxidation products.

4.2.1 Packed Column Gas Chromatography

Before moving to capillary systems, chromatography of fatty acid methyl esters was conducted on a packed column containing a highly polar stationary phase, Silar 10C on 100/120 mesh Gas Chrom QII. Typical chromatograms of commercially available fatty acid methyl esters are shown in figure 4.1. Resolution was relatively good but the column was unable to resolve mixtures of cis and trans isomers of fatty acid methyl esters and would therefore be unlikely to separate oxidised forms of fatty acids. However the technique allowed investigation of the transmethylation derivatisations required to produce fatty acid methyl esters from complex lipids.
Figure 4.1 Separation of Fatty Acid Methyl Esters.

6M x 4mm Silar 10C stationary phase of 100/120 mesh Gas Chrom QII run at 180 °C isothermally, carrier was nitrogen at 35ml/min, detection by FID. 1μg injection of 1mg/ml total FAME.
A: Chromatography of fatty acid methyl esters.
B: Chromatography of cis and trans isomers of fatty acid methyl esters
Key numbers indicate carbon number of alkyl chain, followed by number of double bonds

4.2.2 Transmethylation of Complex Lipids

The use of boron trifluoride in methanol as a transmethylation reagent was investigated.
Using the conditions described in Chapter 3, section 3.4, a number of complex lipids and fatty acids were methylated. The results are given in table 4.1

Considerable variation in recoveries between classes and species was evident and in consequence, variation in rates of transmethylation between species within class was
likely. If this technique was to be used in the quantitative analysis of oxidised fatty acids considerable development would be required. This work was deferred while capillary chromatography was investigated.

Table 4.1 Recovery of Fatty Acid Methyl Esters (FAME) from lipids esterified with Methanolic Boron Trifluoride

<table>
<thead>
<tr>
<th>Lipid</th>
<th>% Recovery of FAME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylcholine</td>
<td>69</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>95</td>
</tr>
<tr>
<td>Diolein</td>
<td>98</td>
</tr>
<tr>
<td>Triolein</td>
<td>66</td>
</tr>
<tr>
<td>Fatty Acid 16:0</td>
<td>60</td>
</tr>
<tr>
<td>Fatty Acid 18:0</td>
<td>87</td>
</tr>
<tr>
<td>Fatty Acid 18:1</td>
<td>80</td>
</tr>
<tr>
<td>Fatty Acid 18:2</td>
<td>49</td>
</tr>
</tbody>
</table>

4.2.3 Capillary Chromatography

A 50 meter Silar 10C fused silica capillary column was investigated for the separation of fatty acid methyl esters. The results were disappointing. Little or no resolution of fatty acids could be achieved, and the chromatography that was achieved was not reproducible. A variety of carrier gas linear velocities and temperature programs were tried, but none provided a useable result. Instrumental problems were discounted as existing capillary separations could be achieved on the apparatus with no loss of resolution or efficiency. The suppliers confirmed that difficulties had been encountered with the use of Silar 10C in capillaries as irregular coating of the columns was occurring. The consequent variation in phase thickness was responsible for the irregularity of chromatography. Other, less
polar, capillary coatings would have been unlikely to have given the required resolution. Taking this and the problems with transmethylation into account investigation of gas chromatography as a technique for measurement of peroxidised lipids was not considered viable. The use of high performance liquid chromatography as a suitable technique was investigated.

4.3 Liquid Chromatography of Phospholipids and Phospholipid Hydroperoxides

4.3.1 Introduction

HPLC methods for oxidised phospholipids using either normal or reversed phase systems have been described by a number of workers (Crawford et al, 1980; Terao et al, 1984; Porter et al, 1980; Yamamoto et al, 1987; Miyazawa, 1987). Normal phase and reversed phase packings give markedly different selectivities for phospholipids, neither of which is entirely satisfactory when choosing a packing for analysis of oxidised phospholipids. Normal phase columns will not discriminate between oxidised and parent phospholipid while reversed phase columns produce extremely complex chromatograms with many overlapping peaks if multiple class separations are attempted. The possibility of resolving oxidised from parent phospholipid made reversed phase chromatography the favoured technique for initial investigation.

4.3.2 Reversed Phase Chromatography of Phospholipids

The elegant work of Crawford et al (1980) in separating phosphatidylcholine oxidation
products from parent compound using reversed phase HPLC provided the starting point for this investigation. This method described high resolution separation of parent and oxidised compounds, using octadecylsilane bonded silica (often also referred to as ODS or C18) with methanol/water eluents. Unfortunately, the method also partially resolved the oxidised species (measured as conjugated dienes at 235nm) from one another. Under most circumstances such chromatography would be highly desirable as it would increase the selectivity of the method. However in this case resolving the individual peroxide species from one another might lead to a future requirement for very high sensitivity of detection if each individual species was to be quantified. This might be difficult to achieve. The essence of the problem was how to exploit the considerable power of reversed phase chromatography to separate oxidised from parent compound, while minimising the loss of sensitivity occasioned by species separation.

The problem was tackled by attempting to develop chromatographic conditions where co-elution of the oxidised groups could be achieved, while maintaining the resolution of oxidised species from parent species. It would be necessary to study the effect of de-optimisation on the chromatography of phosphatidylcholine and phosphatidylethanolamine as these are the predominant phospholipid species present in rat liver (Christie, 1985). At the very least, resolution of these compounds from each other and from their oxidised species was required. If this was not possible then prior separation of classes would be necessary to develop a working method, thus increasing the complexity of the analytical procedure, and thereby increasing the likely loss of hydroperoxides.

Initial experiments were performed to establish the chromatographic properties of
phosphatidylcholine and phosphatidylethanolamine on reversed phase HPLC columns. Samples containing oxidised species of each phospholipid were prepared by diluting commercially available phosphatidylcholine and phosphatidylethanolamine in methanol and allowing these to autoxidise at room temperature in daylight for 24 hours. These autoxidation cocktails were then subjected to reversed phase HPLC using a range of mobile phases from 10% water in methanol up to 100% methanol.

In contrast to the results of Crawford et al (1980) elution of parent phospholipids from a 25cm C18 column proved difficult, with these compounds being retained for one to two hours. Peak spreading under these conditions meant that at lower concentrations parent phospholipids were undetectable.

Acceptable retention times were achieved with a C8 column, however, partial separation of oxidised species and parent compounds remained a feature of all chromatograms, and considerable overlap of oxidised species and parent species between classes was evident. Figures 4.2 and 4.3 show the effects of mobile phase composition on retention time and peak width of phosphatidylcholine and phosphatidylethanolamine respectively, on a 25cm C8 column. Chromatographic peaks were categorised as oxidised if they exhibited absorbance at 235nm, and as parent if they produced a 205nm trace which did not have a similar retention time to a compound identified as oxidised. Sample chromatograms of the greatest resolution between all four groups on C8 columns are shown in figure 4.4 The data supports the results of Crawford et al in so far as separation of oxidised from parent phospholipids with reversed phase chromatography is easily achievable. However resolution of all four classes was not possible under these conditions. Use of higher
percentages of water in the eluent markedly increased retention times without improving efficiency or resolution.

Figure 4.2 Effect of Mobile Phase Composition on Retention of Oxidised and Parent Phosphatidylcholine on Reversed Phase HPLC.

Sample: 1mg/ml bovine liver phosphatidyl choline in methanol, autoxidised for 24 hours in daylight at room temperature. HPLC Conditions: Column 25cm x 4.6mm Spherisorb 5μ C8 Eluents as indicated. Flow rate 1ml/min. Total Lipids detected at 205nm, 0.1 AUFS. Oxidised lipids detected at 235nm, 0.1 AUFS. Range Bars show peak width at base line. Points show retention time measured to peak maximum.

Reversed phase columns based on bonded silicas have residual unlinked silanol groups on the silica surface, and therefore retain some residual normal phase characteristics (Law et
These characteristics have been exploited with some success to improve chromatographic separations, particularly for basic drugs. An attempt was made to improve resolution of the four phospholipid classes by enhancing the residual normal phase qualities of the column, thereby increasing species discrimination in the separating species.

Figure 4.3 Effect of Mobile Phase Composition on Retention of Oxidised and Parent Phosphatidylethanolamine on Reversed Phase HPLC.

Sample: 1mg/ml bovine liver phosphatidyl ethanolamine in methanol, autoxidised for 24 hours in daylight at room temperature HPLC Conditions: Column 25cm x 4.6mm Spherisorb 5μ C8 Eluents as indicated, Flow rate 1ml/min Total Lipids detected at 205nm, 0.1 AUFS. Oxidised lipids detected at 235nm, 0.1 AUFS. Range Bars show peak width at base line. Points show retention time measured to peak maximum.
Figure 4.4. HPLC of Oxidised and Parent Phospholipids on a C8 Column.

Sample: 1mg/ml bovine liver phosphatidylyethanolamine or phosphatidylcholine in methanol, autooxidised for 24 hours in daylight at room temperature. HPLC Conditions: Column 25cm x 4.6mm Spherisorb 5μ C8. Eluent 4% water in methanol. Flow rate 1ml/min. Total Lipids detected at 205nm, 0.1 AUFS. Oxidised lipids detected at 235nm, 0.1 AUFS.

O = oxidised phospholipid
P = parent phospholipid
system. The pH of the mobile phase was varied between pH 4 and pH 10 in an attempt to ionise the compounds, and increase interaction with the silanol groups of the column. Given that both molecules contain anionic (eg; phosphate) and cationic (eg; amine) groups, it was difficult to predict exactly which pH might give the best response and consequently the range of pH's tried was only limited by the requirements of the column. The results are summarised in figure 4.5. The chromatography exhibited almost classic normal phase

![Figure 4.5](image_url)

**Figure 4.5 Effect of pH on Retention of Oxidised and Parent Phospholipids during Reversed Phase HPLC.**

Sample: 1mg/ml bovine liver phosphatidy1 ethanolamine or phosphatidylcholine in methanol, autoxidised for 24 hours in daylight at room temperature. HPLC Conditions: Column 25cm x 4.6mm Spherisorb 5μ C8. Eluents: 4% water in methanol, pH as indicated, Flow rate 1ml/min Total Lipids detected at 205nm, 0.1 AUFS. Oxidised lipids detected at 235nm, 0.1 AUFS. Data points indicate mean retention of all peaks of the designated type. pH was adjusted using hydrochloric acid and sodium hydroxide solutions, and estimated using a pH meter.
behaviour at pH 4 with phosphatidylcholine and phosphatidylethanolamine each eluting as single peaks having different retentions, albeit with very poor chromatographic efficiency. At all higher pH's clear reversed phase mechanisms were apparent as separation of all the classes into species occurred up to pH 10 (figure 4.5), while showing some convergence of the classes at pH 10, do not imply movement towards normal phase mechanisms at this pH and higher. Figure 4.6 shows the chromatograms of phosphatidylcholine at pH's 8.5 and 10, clearly showing high species resolution at these pH's, indicative of reversed phase mechanisms.

While each phospholipid class could be focused into a single peak, these peaks were wide and baseline resolution of the four groups could not be obtained. It may be that the use of reversed phase columns bound with alkyl groups of shorter chain length made expression of the normal phase characteristics easier to obtain due to the increased availability of the silanol groups. There was no indication from the work with C8 columns that this approach would lead to the resolution that was required as a move to further increase the normal phase character of the separation would have lowered the resolution of oxidised and parent phospholipids (Terao et al, 1984). Any loss of resolution would result in overlap of peaks, and thereby mutual interference of peaks.

Coelution of similar compounds to achieve a working assay has been recently applied to the screening analysis of phenols in water (Webster et al, 1993). This group reported some success when focusing phenols on very short analytical HPLC columns, but even then partial resolution was apparent even though only a semiquantifiable amalgamated

*Separation of individual phospholipid molecular species, normally associated with reversed phase separations.
Figure 4.6  Chromatograms of Reversed Phase Separations of phosphatidylcholine at pH 8.5 and 10.0

10μg of oxidised phosphatidylcholine on column. Eluents: 4% water in methanol at indicated pH. Flow rate 1.0ml/min. Detection at 235nm. Parent peaks identified by comparison of chromatogram at 205nm.
peak was produced. The approach differed slightly from the one discussed in this study, in that fusion of peaks for phenols was achieved primarily by reducing column length, rather than attempting to alter separation. The concept of peak fusion or de-optimisation may therefore be applicable in certain circumstances to one or perhaps two heterogenous group of compounds, particularly when screening assays rather than quantitative determinations are required. To expect complete success with four heterogenous groups was perhaps a little ambitious.

While not wholly successful for the purpose of this study, namely the measurement of tissue phospholipid peroxides, the separations with C8 reversed phase columns illustrated in figure 4.4 have not been reported elsewhere. The possibility exists that non-standard reversed phase packings using perhaps C10 or C12 bonded silica's might improve resolution. However such phases are not commonly available and the decision was taken to investigate other modes of separation.

4.3.3 Normal Phase Chromatography of Phospholipids

The difficulties with reversed phased separation meant that an alternative approach was required. The possibility of coelution of oxidised and unoxidised phospholipid species within a class coupled with detection systems selective for particular oxidised species, was a viable alternative. Separation of lipid classes is traditionally achieved using normal phase liquid chromatography. The use of silica thin layer chromatography has been extensively used for this purpose (Henderson and Tocher, 1992) and more recently normal phase HPLC has been used with considerable success (Christie, 1987). This approach has a

*Not available commercially.*
fundamental problem when measurement of oxidised phospholipids is considered. These compounds coelute with parent compounds on normal phase chromatographic systems (Terao et al, 1984; Matsushita, 1987), and thus may cause possible interference with the detection and quantitation of oxidised species.

Two sensitive detection techniques seemed applicable to the measurement of hydroperoxides, namely reductive mode electrochemical detection, and post column chemiluminescence. Both placed constraints on the types of mobile phase that could be used. Electrochemical detection systems require a mobile phase that can support an electrolyte and consequently a significant aqueous component would be required. Chemiluminescence detection requires an eluent miscible with the post column reagent and which would not precipitate the active components of the reagent. Neither detection system is tolerant of gradient elution. Although initial detection was conducted by UV absorbance, care was taken to use eluent systems that would be compatible with these forms of detection. This obviously placed some constraints on the eluents that could be employed.

a) Acetonitrile/Methanol/Water Eluents

The initial experiments with normal phase separation of phospholipids was based on the work of Jungalwala et al, (1979). This group achieved separations of phospholipid using gradient elution, which is incompatible with the proposed future detection strategies, but it was felt that separations of phosphatidylcholine and phosphatidylethanolamine from each other and other lipid species might be achievable with isocratic elution while
maintaining a suitable eluent composition for selective detection. In particular the mobile phases used by Jungalwala et al had a significantly larger aqueous component than other normal phase separations of phospholipid classes, an important consideration for future detection work.

A 15cm silica column and a mobile phase of 66% acetonitrile, 19.6% methanol, 13.1% water and 0.3% ammonium hydroxide at 1.0ml/minute was used initially. This gave adequate retention of phosphatidylcholine but phosphatidylethanolamine had a capacity factor of close to zero. An example of the chromatography of phosphatidylcholine is shown in figure 4.7. In addition, preliminary experiments with detection of phosphatidylcholine conjugated dienes formed by autoxidation in air at room temperature for 48 hours gave significant peaks at 235nm both near to the void volume and at the retention time of parent phospholipid. The concern was that the response near to the void volume was being produced by certain species of oxidised phosphatidylcholine. If this was the case then separation of types of oxidised groups might have been occurring.

In order to check whether oxidised phospholipid species were rapidly eluting on this system, fractions were taken from the chromatographic runs performed using conditions given in figure 4.7 and subjected to lipid phosphorus analysis by the Bartlett assay (Bartlett, 1959). The results are presented in table 4.2. No lipid phosphorus was detected at or near the void volume in these determinations. The compound absorbing at 235nm in this area of the chromatogram is therefore not a phospholipid, but most probably a breakdown product of phospholipid hydroperoxides, presumably 4-hydroxyalkenals and/or alkanes.
Figure 4.7 Normal Phase Chromatography of Phosphatidylcholine.

Column 15cm spherisorb 5 silica, mobile phase 66% acetonitrile, 19.6% methanol, 13.1% water, 0.3% ammonium hydroxide at 1.2ml/min. Detection 205nm. 2.5μg phosphatidylcholine on column. Sample was allowed to antioxidise for 24 hours prior to chromatography. C indicates peaks also giving a response at 235nm. (See Text.) Negative peak at 4mins may be due to pentane added to assist solution of phospholipid residues.

Chromatographic efficiencies for phosphatidylcholine were low compared with the theoretical plate numbers normally associated with HPLC (table 4.3) but this alone would not necessarily have excluded the use of this HPLC system. Other problems, however, were more serious. Peak tailing proved to be a problem at all eluent compositions. Only slight retention of phosphatidylethanolamine could be achieved with a maximum capacity.
Table 4.2  Recovery of Lipid Phosphorous from fractions collected for normal phase HPLC of Oxidised Phospholipids.

<table>
<thead>
<tr>
<th>Elution Time (mins)</th>
<th>% Recovery of Lipid Phosphorous</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PTE</td>
</tr>
<tr>
<td>0-2</td>
<td>0</td>
</tr>
<tr>
<td>2-4</td>
<td>80</td>
</tr>
<tr>
<td>4-6</td>
<td>22</td>
</tr>
<tr>
<td>6-8</td>
<td>0</td>
</tr>
<tr>
<td>8-10</td>
<td>0</td>
</tr>
</tbody>
</table>

factor of 0.7 achieved with 30% acetonitrile. Residues of phosphatidylcholine and phosphatidylethanolamine were also found to dissolve poorly in the eluents, causing potential problems with recoveries. Attempts to improve this by using methanol, or acetonitrile with increasing percentages of pentane were successful in dissolving phospholipid residues, but caused disruption of the chromatography by generating negative peaks and increasing baseline noise in the early part of the chromatogram. Hence it was decided to investigate other mobile phases.

An isocratic separation of phospholipid classes has been reported with an acetonitrile based eluent on a silica column (Kaitaranta and Bessman, 1981), however this method required over 45 minutes to elute phosphatidylcholine, with no greater efficiency than the separation described above.
Table 4.3 Capacity Factor and Efficiency of Phosphatidylcholine on Spherisorb Silica (15cm)

<table>
<thead>
<tr>
<th>%CH:CN*</th>
<th>Efficiency (N)*</th>
<th>k'</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>--</td>
<td>&gt;50</td>
</tr>
<tr>
<td>66</td>
<td>510</td>
<td>5.0</td>
</tr>
<tr>
<td>50</td>
<td>171</td>
<td>4.1</td>
</tr>
<tr>
<td>30</td>
<td>97</td>
<td>7.4</td>
</tr>
</tbody>
</table>

* Polar modifier used was 21:14:0.35, Methanol: Water: Conc. Ammonium Hydroxide.

b) Phospholipid Class Separations with Propanol/Water/Hexane Eluents

Propan-2-ol/water/hexane eluents have been used with a variety of normal phase columns for separation of phospholipid classes (McLuer et al., 1986; Christie, 1987). The behaviour of phosphatidylcholine and phosphatidylethanolamine with a variety of eluents composed of propan-2-ol, water and hexane was investigated on three normal phase columns, namely spherisorb silica, diol substituted silica, and aminopropyl substituted silica, all at 250nm x 4.6mm dimensions, with 5μm diameter packing materials. In these experiments 50μl injections containing 25μg of either phosphatidylcholine or phosphatidylethanolamine in eluent were injected on column. These columns were eluted with propan-2-ol/hexane/water eluents of varying composition at a flow rate of 1.0ml/minute. The eluent was monitored at 205nm and the retention time and peak width at half peak height recorded. The results from these studies are summarised in tables 4.4, 4.5 and 4.6.

* Plates per column
Table 4.4 Capacity factor, efficiency and resolution of phosphatidylcholine and phosphatidylethanolamine on a 250 x 4.6 mm, 5μm Spherisorb silica column using propan-2-ol, water, hexane eluents.

<table>
<thead>
<tr>
<th>Composition of Mobile Phase</th>
<th>Phosphatidylcholine</th>
<th>Phosphatidylethanolamine</th>
<th>R_s</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Hexane</td>
<td>% of Water in Polar Phase</td>
<td>k'</td>
<td>N</td>
</tr>
<tr>
<td>10</td>
<td>20</td>
<td>9.5</td>
<td>440</td>
</tr>
<tr>
<td>15</td>
<td>23.5</td>
<td>425</td>
<td>2.75</td>
</tr>
<tr>
<td>10</td>
<td>&gt;50</td>
<td>-</td>
<td>4.6</td>
</tr>
<tr>
<td>30</td>
<td>20</td>
<td>3</td>
<td>207</td>
</tr>
<tr>
<td>15</td>
<td>5.75</td>
<td>238</td>
<td>1.75</td>
</tr>
<tr>
<td>10</td>
<td>16.8</td>
<td>436</td>
<td>2.5</td>
</tr>
<tr>
<td>50</td>
<td>15</td>
<td>2.25</td>
<td>220</td>
</tr>
<tr>
<td>10</td>
<td>4.5</td>
<td>158</td>
<td>1.5</td>
</tr>
<tr>
<td>5</td>
<td>&gt;50</td>
<td>-</td>
<td>6.75</td>
</tr>
<tr>
<td>70</td>
<td>10</td>
<td>15</td>
<td>*</td>
</tr>
<tr>
<td>5</td>
<td>&gt;50</td>
<td>-</td>
<td>17.5</td>
</tr>
</tbody>
</table>

* = Peaks split.

Separation of phosphatidylcholine on silica showed a standard normal phase behaviour with most eluent compositions as retention of both lipids was reduced with increasing percentages of water in the eluent. Increasing the proportion of hexane increased the eluting power of the mobile phase. This is particularly apparent when eluents containing the same ratio of water to propanol are compared. For example, at a ratio of water to propan-2-ol of 1:9 capacity factor values for phosphatidylcholine are >50, 16.8 and 4.5 for 10%, 30% and 50% of hexane respectively, and a similar drop is seen at water to propan-2-ol ratios of 1:5.7. At higher proportions of hexane the column exhibited an increase of capacity factor for both phosphatidyl species, the effect being more marked.
Table 4.5  Capacity factor, efficiency and resolution of phosphatidylcholine and phosphatidylethanolamine on a 250 x 4.6 mm, 5µm Diol column using propano-2-ol/water/hexane eluents.

<table>
<thead>
<tr>
<th>Composition of Mobile Phase</th>
<th>Phosphatidylcholine</th>
<th>Phosphatidylethanolamine</th>
<th>Rs</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Hexane</td>
<td>% of Water in Polar Phase</td>
<td>k'</td>
<td>N</td>
</tr>
<tr>
<td>10</td>
<td>20</td>
<td>1.8</td>
<td>576</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>2.25</td>
<td>511</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3.5</td>
<td>506</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>11.25</td>
<td>357</td>
</tr>
<tr>
<td>30</td>
<td>15</td>
<td>2.13</td>
<td>369</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>4.18</td>
<td>625</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>14.25</td>
<td>468</td>
</tr>
<tr>
<td>50</td>
<td>10</td>
<td>2.75</td>
<td>784</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>15.5</td>
<td>492</td>
</tr>
<tr>
<td>70</td>
<td>10</td>
<td>7.5</td>
<td>666</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>15.25</td>
<td>*</td>
</tr>
</tbody>
</table>

* = Peaks split.

Table 4.6  Capacity factor, efficiency and resolution of phosphatidylcholine and phosphatidylethanolamine on a 250 x 4.6 mm, 5µm Apex Amino column using propano-2-ol, water, hexane eluents.

<table>
<thead>
<tr>
<th>Composition of Mobile Phase</th>
<th>Phosphatidylcholine</th>
<th>Phosphatidylethanolamine</th>
<th>Rs</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Hexane</td>
<td>% of Water in Polar Phase</td>
<td>k'</td>
<td>N</td>
</tr>
<tr>
<td>10</td>
<td>20</td>
<td>2.5</td>
<td>1870</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>8.5</td>
<td>1388</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>&gt;50</td>
<td>-</td>
</tr>
<tr>
<td>30</td>
<td>20</td>
<td>2.125</td>
<td>199</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>3.75</td>
<td>429</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>12.25</td>
<td>972</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>&gt;50</td>
<td>-</td>
</tr>
<tr>
<td>50</td>
<td>10</td>
<td>10.0</td>
<td>743</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>&gt;50</td>
<td>-</td>
</tr>
</tbody>
</table>
as the proportion of water in the mobile phase decreased. Peak splitting was also apparent at the highest hexane concentrations. This is indicative of species separation rather than column deterioration because no peak splitting was apparent when chromatography was repeated with eluents with lower hexane concentrations. Species separation is more typical of reversed phase mechanisms, and therefore at higher concentrations of hexane, silica columns are exhibiting reversed phase characteristics with respect to phospholipids.

Baseline resolution of the two phospholipids on silica was achieved with most eluent compositions but this also showed a marked increase with high water content or high hexane content. Resolutions in excess of 3 were achieved at these two extremes, but with the mid range eluents Rs values dropped to 0.8.

The data from the diol phase was more difficult to interpret than those of the silica phase. A decrease in the polarity of the eluent by reducing the water content increased the retention of both phospholipids and increased their resolution. However while this was true in general terms no clear pattern was apparent. For example with a ratio of water to propan-2-ol of 1:9 capacity factors were 3.5, 4.2, 2.8 and 7.5 for percentages of hexane in the eluent of 10, 30, 50 and 70 respectively, while at lower ratios of water increase in hexane concentration resulted in increases in retention for both compounds. Peak splitting was observed with the least polar eluent, but only for phosphatidylcholine, indicating that aspects of reversed phase chromatography could be achieved on diol columns, as with the silica columns, at extreme eluent compositions.
The aminopropyl column required eluents of higher polarity than required for the silica or diol columns in order to elute phosphatidylcholine and phosphatidylethanolamine. Perhaps because of this no peak splitting associated with species separation was apparent when monitoring at 205nm.

HPLC separations are routinely developed for non-lipid analytes on 25cm columns with plate numbers in the order of 10000 plates. None of the normal phase columns investigated achieved efficiencies of this magnitude, however, the results are similar to other reported isocratic separations of phospholipid classes (Kaitaranta and Bessman, 1981; Patton et al, 1982; Christie et al, 1985).

In spite of this good resolution of phosphatidylcholine and phosphatidylethanolamine was achievable from all columns with nearly all elutes examined. Both the diol and silica columns demonstrated short retention times for phosphatidylethanolamine (figures 4.8 and 4.9). Short retention times for an analyte often lead to problems with interference from non-retained impurities in biological samples such as tissues even after sample preparation.

In order to minimise this possibility capacity factor in excess of 2.5 was considered a minimum requirement. Achievement of capacity factors in excess of 2.5 for phosphatidylethanolamine with both the silica and diol columns resulted in extended retention of phosphatidylcholine. The aminopropyl phase gave higher efficiencies than the other two normal phase columns and gave acceptable resolution without extended retention for both phospholipids. In addition this was achieved with higher percentages of water and lower percentages of hexane than the other normal phase systems. Eluents
Figure 4.8
Chromatography of Phosphatidylcholine and Phosphatidylethanolamine on a 25cm Spherisorb Silica 5 Column

Eluent = 59.5% propan-2-ol, 10.5% water and 30% hexane at 1.0ml/min Detection at 205nm. 10μg phosphatidylcholine (PTC), 5μg phosphatidylethanolamine (PTE) on column
Figure 4.9 Separation of Phosphatidylcholine and Phosphatidylethanolamine on a 25cm Diol Column

Eluent = 66.5% propan-2-ol, 3.5% water and 30% hexane at 1.0ml/min Detection at 205nm. 10μg phosphatidylcholine (PTC), 5μg phosphatidylethanolamine (PTE) on column.
of this composition are more likely to be compatible with electrochemical and chemiluminescence detection.

c) Normal Phase Chromatography of Peroxidised Phospholipids

When using eluents of decreased polarity in the above experiment some columns exhibited partial retention of phospholipid species as evidenced by production of more than one peak for each phospholipid class injected on column. This was not due to any deterioration of the column, but appeared to be related to a reversed phase mechanism of separation. If this was the case then discrimination between parent compound and oxidation products was possible.

In order to determine whether such separation was occurring, and to what extent, parent and oxidised phospholipids were chromatographed under a range of eluent conditions. Phosphatidylcholine and phosphatidylethanolamine were subjected to singlet oxygen attack with Rose Bengal dye as described in Chapter 3. Irradiation was performed overnight and phospholipids were extracted from 1ml Rose Bengal solutions containing 1mg of each phospholipid, with dichloromethane. The dichloromethane layer was taken to dryness and reconstituted with 2ml of mobile phase. 20μl of this was injected on column. Total phospholipid was detected by absorbance at 205nm, and conjugated dienes were detected at 235nm. The results obtained for phosphatidylcholine and phosphatidylethanolamine are summarised in tables 4.7 and 4.8 respectively.
Table 4.7 Chromatography of phosphatidylcholine and its oxidation products on normal phase columns.

<table>
<thead>
<tr>
<th>IPA: Water: Hexane %</th>
<th>Column</th>
<th>Phosphatidylcholine</th>
<th>Oxidised Phosphatidylcholine</th>
<th>Rs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>k'</td>
<td>N</td>
<td>k'</td>
</tr>
<tr>
<td>45 5 50</td>
<td>Diol</td>
<td>3.13</td>
<td>589</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>Silica</td>
<td>31.0</td>
<td>3489</td>
<td>&gt;50</td>
</tr>
<tr>
<td></td>
<td>NH₂</td>
<td>10.0</td>
<td>743</td>
<td>12.9*</td>
</tr>
<tr>
<td>59.5 10.5 30</td>
<td>Diol</td>
<td>2.13</td>
<td>1068</td>
<td>2.13</td>
</tr>
<tr>
<td></td>
<td>Silica</td>
<td>10.0</td>
<td>670</td>
<td>12.25*</td>
</tr>
<tr>
<td></td>
<td>NH₂</td>
<td>3.75</td>
<td>781</td>
<td>4.5</td>
</tr>
<tr>
<td>72 18 10</td>
<td>Diol</td>
<td>1.75</td>
<td>1368</td>
<td>1.75</td>
</tr>
<tr>
<td></td>
<td>Silica</td>
<td>3.75</td>
<td>1183</td>
<td>3.75</td>
</tr>
<tr>
<td></td>
<td>NH₂</td>
<td>2.25</td>
<td>1910</td>
<td>2.25</td>
</tr>
</tbody>
</table>

* = Peaks splitting apparent.

Partial resolution of oxidised phospholipids from parent phospholipids was seen with all but the highest percentages of water in the eluent. Indeed, under the lowest concentrations of water, oxidised phosphatidylethanolamine was completely resolved (Rs ≥ 1) by all three columns. This resolution is observed even with eluents which had not previously shown reversed phase behaviour by partially resolving parent phospholipid species. The columns were also able to split the oxidised species into two or more peaks. While these species all contain conjugated diene groups, and are likely to be phospholipid, it is not known whether this represents partial resolution of hydroperoxides from other later products.
Table 4.8  Chromatography of phosphatidylethanolamine and its oxidation products on normal phase columns.

<table>
<thead>
<tr>
<th>IPA: Water: Hexane %</th>
<th>Column</th>
<th>Phosphatidylethanolamine</th>
<th>Oxidised Phosphatidylethanolamine</th>
<th>R_s</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>k'</td>
<td>N</td>
<td>k'</td>
</tr>
<tr>
<td>45 5 50</td>
<td>Diol</td>
<td>2.25</td>
<td>936</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>Silica</td>
<td>6.5</td>
<td>3463</td>
<td>7.13*</td>
</tr>
<tr>
<td></td>
<td>NH_3</td>
<td>12.75</td>
<td>827</td>
<td>18.0*</td>
</tr>
<tr>
<td>59.5 10.5 30</td>
<td>Diol</td>
<td>1.63</td>
<td>425</td>
<td>1.75</td>
</tr>
<tr>
<td></td>
<td>Silica</td>
<td>2.5</td>
<td>1697</td>
<td>2.75</td>
</tr>
<tr>
<td></td>
<td>NH_3</td>
<td>5.25</td>
<td>1353</td>
<td>6.0*</td>
</tr>
<tr>
<td>72 18 10</td>
<td>Diol</td>
<td>1.38</td>
<td>1389</td>
<td>1.38</td>
</tr>
<tr>
<td></td>
<td>Silica</td>
<td>1.5</td>
<td>683</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>NH_3</td>
<td>3</td>
<td>984</td>
<td>3</td>
</tr>
</tbody>
</table>

* = Peaks splitting apparent.

such as epoxides and cyclic endoperoxides or phospholipid species separation. Figures 4.10 to 4.12 illustrate the extent of the resolutions achieved with certain combinations of column and eluent composition.

Co-elution of the oxidised species with parent phospholipid while maintaining acceptable retention and resolution of classes could be most readily achieved using the aminopropyl column. This is demonstrated in figure 4.13.

It was apparent that the retention of phosphatidylcholine and phosphatidylethanolamine on both the diol and silica columns had changed between this experiment and the last. For both columns capacity factors and efficiencies
Figure 4.10 Partial Resolution of Oxidised Phospholipids from Parent Phospholipids by HPLC on a 25cm Silica Column.

Samples prepared by incubating 1 mg of each phospholipid class in 1 ml of 1 mg/ml Rose Bengal in methanol. Eluent: 10.5% water, 59.5% propan-2-ol, 30% Hexane at 1 ml/min

Column: 25 cm x 4.6 mm Spherisorb S5W 5 μ Silica

PTC = bovine liver phosphatidylecholine

PTE = bovine liver phosphatidylethanolamine

CD = Peaks showing conjugated diene response at 235 nm
Figure 4.11 Partial Resolution of Oxidised Phospholipids from Parent Phospholipids by HPLC on a 25cm Aminopropyl Column.

Samples prepared by incubating 1mg of each phospholipid class in 1ml of 1mg/ml Rose Bengal in methanol.
Eluent: 5%water, 45%propan-2-ol, 50%Hexane at 1ml/min
Column: 25cm x 4.6mm 5μm Apex Aminopropyl bonded silica.
PTC= bovine liver phosphatidylcholine
PTE = bovine liver phosphatidylethanolamine
CD= Peaks showing conjugated diene response at 235nm
Figure 4.12 Partial Resolution of Oxidised Phospholipids from Parent Phospholipids by HPLC on a 25cm Diol Column.

Samples prepared by incubating 1mg of each phospholipid class in 1ml of 1mg/ml Rose Bengal in methanol. Eluent: 5% water, 45% propan-2-ol, 50% Hexane at 1ml/min. Column: 25cm x 4.6mm Spherisorb 5μ Diol bonded silica.

PTC = bovine liver phosphatidylcholine
PTE = bovine liver phosphatidylethanolamine
CD = Peaks showing conjugated diene response at 235nm
Figure 4.13 Coelution of Oxidised with Parent Phospholipid using Normal Phase HPLC.

Samples prepared by incubating 1mg of each phospholipid class in 1ml of 1mg/ml Rose Bengal in methanol.  
Eluent: 13.5% water, 76.5% propan-2-ol, 10% Hexane at 1ml/min  
Column: 25cm x 4.6mm 5µm Apex Aminopropyl bonded silica.  
PTC= bovine liver phosphatidylcholine  
PTE = bovine liver phosphatidylethanolamine
for both phospholipids studied had altered. The use of aqueous eluents is known to be problematic for silica columns leading to changes in activity and therefore selectivity and it would appear that this also holds true for diol columns. Also, as seen with class separation experiments above, slight changes in eluent composition, particularly the percentage of water used, can markedly alter retention. Deterioration of aminopropyl columns was observed but significant changes in retention and efficiency did not occur until after repeated injection of fairly crude tissue extracts (see section 4.9). Presumably, this is caused by irreversible binding of acidic components onto the phase. However, aminopropyl phases are more robust in routine use with these eluents than either diol bonded phases or silica.

Resolution, or partial resolution, of oxidised species from parent species of phospholipid on normal phase stationary phases contradicts the previously reported behaviour of these phases. A number of workers have stated that normal phase HPLC results in coelution of the oxidised and parent species. Under most conditions resolution is so poor as to be almost non-existent and it is likely that the partial resolution has gone unnoticed, or considered insignificant. However, partial resolution may have an effect on certain methods of detection. Measurement of small concentrations of hydroperoxides, for example, as a shoulder on a much larger phospholipid peak might be less reproducible than measurement under a completely parallel elution. This may be particularly true if the parent phospholipid peak is itself a summation of a very slightly resolved species. Such effects would subtly change as retention altered, either from column deterioration or slight inconsistencies in mobile phase composition, making detection and quantitation less robust.
The ability to separate the oxidised species from other components has further significance in that the possibility exists to measure the oxidised groups within classes without interference. However, the loss of efficiency that resulted was unacceptable and this avenue was not explored further.

The aminopropyl column was chosen for future work. It was less prone to unpredictable changes in chromatographic properties, and is selective for the compounds of interest. Partial separations of oxidised species from parent molecules could conceivably give problems with detection, therefore the use of low percentages of hexane (i.e. <15%) and higher percentages of water (i.e. >10%) offered the best compromise of efficiency, resolution and potential compatibility with sensitive detection systems. The retention of other lipids likely to be present in tissue extracts was examined and the retentions of these compounds are given in table 4.9. None of these compounds coeluted with either phosphatidylcholine or phosphatidylethanolamine.

The data in table 4.9 was generated some time after the retention data in sections b) and c) above. The increase in capacity factor for phosphatidylcholine and phosphatidylethanolamine in this table compared with previous data was part of a gradual change in the column. Phosphatidylinositol and phosphatidylserine appear to bind irreversibly to the aminopropyl column, and this is likely to be the case for other compounds. The changes in the column leading to increase capacity factors did not result in resolution of oxidised and parent forms of phospholipids under eluent conditions which had previously been shown to produce coelution of these compounds.
Table 4.9 Retention of Other Lipids on Aminopropyl HPLC Columns

<table>
<thead>
<tr>
<th>Lipid Class</th>
<th>k'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylinositol</td>
<td>&gt;50</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>&gt;50</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>5</td>
</tr>
<tr>
<td>Triacylglyceride</td>
<td>0.5</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.8</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
<td>2.75</td>
</tr>
<tr>
<td>Phosphatidylethanolamine</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Column= 25cm x 4.6mm Apex aminopropyl, Eluent 72% IPA, 18% water, 10% Hexane at 1.0ml/min

(d) Methanol/Water/Pentane Eluents

Isopropanol proved to be incompatible with chemiluminescent detection systems (section 4.8, below) because of the high background luminescence. During previous work with acetonitrile based eluents, methanol and pentane were investigated as possible reconstitution eluents for phospholipid residues, and had been found to be miscible.

Both methanol and pentane proved to have no adverse effects on chemiluminescent response and it therefore seemed possible that methanol:water:pentane mobile phases might provide a suitable alternative to the isopropanol, hexane, water eluents used up to now, and behave in similar ways. An experiment was performed to establish whether this was the case. The results from this experiment are presented in table 4.10.
Table 4.10 Separation of phosphatidylcholine and phosphatidylethanolamine on aminopropyl columns using methanol:pentane:water eluents.

| Eluent Phosphatidyl- | Phosphatidyl- Phosphatidylethanolamine R<sub>s</sub> |
|---------------------|---------------------------------|---------------------------------|-------------------|
| Methanol : Pentane : Water | choline | | |  |
| 95 : 0 : 5 | 3 | 3938 | 12.2 | 2489 | 11.94 |
| 90 : 10 : 0 | 3.5 | 1794 | 12.5 | 1578 | 8.6 |
| 90 : 5 : 5 | 2.2 | 936 | 7.255 | 798 | 5.33 |
| 90 : 0 : 10 | 2.63 | 2373 | 9.08 | 2216 | 9.4 |
| 85 : 15 : 0 | 4.38 | 1138 | 26.5 | 1047 | 9.32 |
| 85 : 10 : 5 | 2.75 | 2540 | 7.75 | 2094 | 8.00 |
| 85 : 5 : 10 | 2.25 | 951 | 5.75 | 646 | 4.90 |
| 85 : 0 : 15 | 2.525 | 643 | 7.25 | 717 | 4.52 |
| 80 : 15 : 5 | 2.85 | 2716 | 10.1 | 1862 | 9.19 |
| 80 : 10 : 10 | 2.5 | 1085 | 8.13 | 2554 | 12.5 |
| 80 : 5 : 15 | 2.37 | 1247 | 8.00 | 1983 | 8.04 |

Column = 250 x 4.6nm Apex Aminopropyl.

Under these conditions the amino propyl column gave very high resolution and higher efficiency than achieved with propan-2-ol/hexane/water eluents. There was no separation of oxidised from parent group with any methanol, pentane, water eluent used, possibly due to the higher polarity of methanol compared with propan-2-ol. An example of phospholipid chromatography using methanol/water/pentane eluents is shown in figure 4.14.

4.3.4 Summary

Reversed phase and normal phase chromatography was evaluated for the separation of phospholipids (particularly phosphatidylcholine and phosphatidylethanolamine) and oxidised phospholipids. Reversed phase HPLC proved unsuitable. While some resolution of phosphatidylcholine, oxidised phosphatidylcholine, phosphatidylethanolamine and
Figure 4.14 Normal Phase HPLC of Phospholipids with Methanol/Pentane/Water Eluent.

PTC = 1mg/ml phosphatidylcholine after 4 hours Rose Bengal irradiation
PTE = 1mg/ml phosphatidylethanolamine after 4 hours Rose Bengal irradiation.
Eluent: 85% methanol, 10% pentane, 5% water at 1.0 ml/min. Column: 25cm x 4.6 mm Apex Aminopropyl Bonded Silica. 10μl of sample on column
oxidised phosphatidylethanolamine was achieved, efficiency was poor and co-elution of some groups too great to allow a viable method to be developed. Attempts to improve resolution resulted in species separation, generating complex chromatograms, difficult to interpret. A variety of normal phase columns were evaluated, with propan-2-ol/water/hexane eluents, and the aminopropyl column was found to have the most suitable retention and resolution of phosphatidylcholine and phosphatidylethanolamine, and gave more suitable results in routine use. Surprisingly, all normal phase columns were found to partially or completely resolve oxidised species from parent species under certain conditions, a result that contradicts conclusions of previous work (Terao et al, 1988).

Detection problems with propan-2-ol prompted the investigation of a new mobile phase. Methanol/water/pentane eluents were found to have similar properties to propanol/water/hexane eluents with aminopropyl columns, Methanol/water/pentane eluents also had higher efficiencies (see table 4.10) and a wider range of eluent conditions providing co-elution of oxidised and parent phospholipids.

The use of aminopropyl bonded silicas with methanol/water/pentane eluents, under eluent conditions where oxidised and parent phospholipid co-elute, was the most suitable chromatographic method for analysis of phospholipid hydroperoxides.

4.4 Phospholipid Purification

As a prerequisite to any analysis of phospholipids from a biological matrix, removal of the phospholipid from any interfering substances such as proteins, DNA and other lipid classes
is essential if clean chromatography is to be achieved and column life extended. The objective of this phase of the study was to identify a method that could separate phospholipids as a class from other lipid classes and impurities which are present in total lipid extracts from tissues.

Lipid hydroperoxides are unstable groups which are readily reduced to form hydroxylipids or broken down to form alkoxy radicals and aldehydes. For this reason extended extraction systems are not available. Two rapid separation techniques were available, namely thin layer chromatography and solid phase extraction.

Thin layer chromatography has been extensively used for lipid separations (Henderson and Tocher, 1992) and has the advantage that it is fairly rapid and easy to use. However in recent years the use of solid phase extraction has been employed increasingly for a wide variety of sample preparation applications. The technique employs bonded silicas to adsorb analytes of interest while impurities are flushed through. Suitable elution solvents can then be employed to elute the analyte prior to a suitable quantitation procedure.

Any selected technique would have to ensure that a clean fraction could be obtained rapidly with a high recovery of oxidised lipids. TLC and solid phase methods were assessed with respect to these criteria.
4.4.1 Thin Layer Chromatography

Initial experiments repeated the work of Yao and Rastetter (1985). This method used an initial development with 25:25:10:9 ethyl acetate/propan-1-ol/chloroform/0.25% potassium chloride, followed by a second development in 60:40:1:0.05 benzene/diethylether/ethanol/acetic acid. An example of separations achieved is given in figure 4.15. While resolution of phospholipids from other classes was high, separation of the phospholipids was surprisingly complex, without providing clear resolution of all groups. The development, including drying between stages took approximately 1.5 hours and plate scrapping and extraction would also have been required before subsequent quantitative analysis could have been performed. In consequence work was suspended on TLC at this stage to concentrate on solid phase extraction.

4.4.2 Solid Phase Separations of Lipid Groups

Kaluzny et al (1985) have demonstrated the rapid separation of major groups of lipids using aminopropyl solid phase extraction cartridges. Using these conditions, (as described in chapter 3, section 3.5.1) phosphatidylcholine and phosphatidylethanolamine could be completely recovered, and separated from the neutral lipids (table 4.11). However, approximately 15% of triglycerides, and all the free fatty acids are lost during this procedure. The fatty acids are potentially covalently bound to the amine group. This may cause some problems with the technique when applied to tissue extracts as free fatty
Figure 4.15 TLC of Lipid Classes

a) Initial Development with 25:25:10:9 ethyl acetate/propan-1-ol/chloroform/0.25% KCl
b) Second Development with 60:40:1:0:05 benzene/diethyl ether/ethanol/acetic acid

10μl of 2:1 chloroform/methanol solution of each lipid at concentrations of between 5 and 20 mg/l were applied at the origin.

Abbreviations: CHOL, cholesterol; FA, fatty acid; PTC, phosphatidylcholine; PTE, phosphatidylethanolamine; PTS, phosphatidylserine; TG, triglyceride (triolein); SPM, sphingomyelin.
Table 4.11 Recovery of lipid Classes using Amino Propyl Solid Phase Extraction Cartridges

<table>
<thead>
<tr>
<th>Eluents</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PTC</td>
</tr>
<tr>
<td>1. Sample loading</td>
<td>0</td>
</tr>
<tr>
<td>2. 2:1 Chloroform/1Propan-2-ol</td>
<td>0</td>
</tr>
<tr>
<td>3. 2% Acetic acid in diethyl ether</td>
<td>0</td>
</tr>
<tr>
<td>4. Methanol</td>
<td>101</td>
</tr>
</tbody>
</table>

2.5mg of each sample dissolved in 250ml chloroform placed on 300mg NH2 bond elute. Elution was performed stepwise from eluent 1-4 using 4ml of each eluent. Results are the mean of 3 experiments. PTC = bovine phosphatidylcholine, PTE = bovine phosphatidylethanolamine, FA = linolenic acid, TG = triolein, DG = Diolein.

Acids may deactivate the column. The reasons for losses of triglycerides are less easy to explain. For the purposes of this study such losses were of minor importance as only the phospholipid fraction was required for future analysis. Phospholipids were reproducibly extracted with high recovery, and with no interference from other lipid classes tested.

Similar results were obtained when the method was scaled down to 100mg cartridges, using 1ml elution volumes. Complete recovery of phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin was demonstrated. Recovery of oxidised phosphatidylcholine and phosphatidylethanolamine, as measured by 235nm absorbance after HPLC, was found to be only 80% with 1ml methanol elution, but use of a 2ml elution volume for the final methanol elution resulted in complete recovery of all conjugated dienes.
4.4.3 Summary

Solid phase extraction of phospholipids is both faster and less technically demanding than TLC, and was therefore the method of choice for phospholipid extraction. Some retention of oxidised species was apparent, but this problem was easily resolved by increasing elution volume.

4.5 Preparation of Phospholipid Peroxides

A key part of any quantitative analytical method is its calibration. Unfortunately, there are no commercially available phospholipid hydroperoxides and in consequence it is necessary to prepare these from phospholipid precursors in order to calibrate the method. This requires three steps:

1) Synthesis of the hydroperoxides
2) Purification of the hydroperoxides
3) Measurement of purity

The objective of this part of the study was to synthesise, purify and assess the yield of hydroperoxides. These materials would then be used as the calibrants for future chromatographic assays, and as spike standards to assess recovery of phospholipid hydroperoxides during tissue extraction experiments.

Preparation of peroxidised phospholipids for initial chromatographic experiments on behaviour relied on autoxidation of the phospholipid in methanol. In order to get a
measurable yield of conjugated dienes, samples had to be left for 24 hours or more in air at room temperature.

A method with a higher and more reliable yield was required. A number of methods for preparation of lipid peroxides have been discussed in the literature. They include biological preparations of tissue homogenates or microsomes supplemented with iron salts and reducing agents (Gutteridge, 1984; Minotti and Aust, 1987), enzymic systems such as xanthine oxidase (Hassan, 1984) or lipoxygenase (Kaplan and Ansari, 1984; Hamberg and Samuelsson, 1967), or chemical methods using singlet oxygen formation (Van Kuijk and Dratz, 1987).

Biological systems have been extensively used to study peroxidation systems, however in this case would have required complex sample clean up. Similar constraints apply to xanthine oxidase, as this method is performed in a biological matrix. Lipoxygenase production of hydroperoxides has been reported as not applicable to the production of phospholipid hydroperoxides (Gibian and Galaway, 1977). Singlet oxygen methods provide a simple alternative with higher yields. However the range of hydroperoxide species formed from polyunsaturated groups by singlet oxygen attack is larger than those formed from free radical initiated peroxidation (Porter, 1984; Frankel, 1987).

Chemical methods have potential advantages over biological systems for the production of hydroperoxides. The matrix in which the hydroperoxides are produced is less complex making extraction simpler and reducing the possibility of secondary reactions which might reduce the yield of hydroperoxide (for example, by iron-catalysed hydroperoxide
cleavage). The chemical methods are also simpler to perform and therefore hopefully easier to control. This is particularly true of singlet oxygen methods. For these reasons hydroperoxide preparation by singlet oxygen attack was chosen. The fact that this system produces a wider variety of hydroperoxide isomers than free radical mechanisms was not a particular problem at this stage as all isomers would coelute when chromatographed.

Initially the method was used as stated in the literature, with phospholipid exposed to high energy UV radiation in solution with Rose Bengal followed by dichloromethane extraction. Solvent was then removed by drying under oxygen free nitrogen. A minor problem was encountered in the extraction step as traces of Rose Bengal carried over into the dichloromethane. This did not interfere with the HPLC detection. During solid phase extractions of lipids using aminopropyl cartridges it was noted that Rose Bengal dye was held at the top of the cartridge. Subsequently all Rose Bengal was removed from singlet oxygen preparations by passing up to 2ml of the preparation down a 100mg NH₂ solid phase extraction column. Recoveries of conjugated dienes from Rose Bengal preparations were in excess of 95% using this method.

The spectrophotometric scans of phospholipids before and after irradiation showed a distinct absorbance peak formed at 235nm (figure 4.16). The progress of the reaction was monitored by HPLC at 235nm. Maximum diene production occurred after 5 hours of irradiation. Phospholipid hydroperoxide production also peaked at approximately 5 hours as measured by HPLC chemiluminescence later in the study. The time course data are presented in figure 4.17.
Figure 4.16. UV Scans of Bovine Liver Phosphatidylcholine Before and After 18 Hours of UV Irradiation with Rose Bengal Dye.

A: UV scan of 160mg/ml bovine liver phosphatidylcholine in methanol.
B: UV scan of 160mg/ml phosphatidylcholine after 18 hours irradiation with Rose Bengal dye. Irradiation and extraction by dichloromethane as described in text.
Figure 4.17. Time Course of Singlet Oxygen Attack on Phosphatidylcholine.

1mg phosphatidylcholine subjected to uv irradiation in 1ml of 1mg/ml Rose Bengal in methanol. Products separated from dye with aminopropyl bond elute columns prior to HPLC. Total phospholipid(205nm), conjugated diene phospholipids(235nm) and phospholipid hydroperoxide(chemiluminesce) were monitored. Results are mean and SD of three results.

Singlet oxygen reaction using Rose Bengal dye, coupled with aminopropyl solid phase extraction provides a rapid method of preparing phospholipid peroxides for subsequent experiments. The method has the additional advantage of being amenable to extraction. Passing reaction solutions through aminopropyl cartridges efficiently removes Rose
Bengal from the preparation, leaving a methanol solution of phospholipids and their oxidation products.

**Section 4.6 Electrochemical Detection of Phospholipid Hydroperoxides**

Having established chromatographic conditions for phospholipids the next stage was to develop suitable detection systems for phospholipid hydroperoxides. Ultra Violet detection at 235nm had proved useful up to this point as a method of identifying oxidised forms of phospholipids. However, for quantitative detection problems would have to be overcome. The use of 235nm absorbance as a method of detecting oxidised species is non-specific (Slater, 1984) as it measures the conjugated diene formed during oxygen addition after radical attack. This structure is conserved in the lipid oxidation products as they are transformed to hydroxides, epoxides, epidioxides and cyclicendoperoxides (see chapter 1) and in consequence is non-specific and will detect products of decomposition of hydroperoxides as well as hydroperoxides themselves. As coelution of all oxidised phospholipid species with parent phospholipid had proved to be the only rapid chromatographic method not requiring complex preseparations of the total lipid extractions, a detection method specific to phospholipid hydroperoxides was required.

Electrochemical detection exploits the current generated by oxidation or reduction of a substitute at a fixed potential. This form of electrochemical detection, termed amperometric because of the use of current as the measurement parameter, is particularly applicable to liquid chromatographic systems under isocratic conditions. Electrodes can
be placed parallel to the eluent flow and a potential can be set up across the eluent. Compounds passing this face react at the surface of the working electrode as they pass and the current produced is proportional to the concentration of compound at the cell surface at that point in time. Careful selection of potentials, and the use of preoxidation or prereduction prior to the detection cell can be used to increase the selectivity of the method.

In order to be able to measure lipid hydroperoxides it is necessary to use the detector in reductive mode. As molecular oxygen will be readily reduced in such systems care must be taken to exclude oxygen from the system if background currents are to be kept low. Consequently the mobile phase was fitted with a helium sparge, and all PTFE fittings between the reservoir and the detector were changed to PEEK fittings which are impermeable to gases.

Other groups have reported some success with reductive mode electro-chemical detection for fatty acid (Terao et al, 1988) and phospholipid hydroperoxides (Yamada et al, 1987). The phospholipid chromatography was performed using a reversed phase system, so that hydroperoxides were separated from parent compounds. Successful detection was demonstrated by Yamada et al at potentials of -0.3V using carbon electrodes. Chromatography of phospholipids was relatively inefficient using this system, with the phosphatidylcholine showing species separation, as would be expected under these conditions.

Using isopropanol/hexane/water systems discussed earlier the water phase was replaced
with 0.15% potassium chloride to support any electrochemical reaction. Injections of 100μg of oxidised phosphatidyl choline were injected onto the system at increasing potentials. The results obtained were somewhat disappointing. Chromatography of the phospholipid was not affected by the addition of salts to the eluent, and retention times and efficiencies were comparable to previous results. However little response was seen from the electrochemical detector even at potentials down to -1.5V vs Ag/AgCl reference electrode.

Changes in mobile phase constitution, including increasing the proportion of aqueous modifier and changing the electrolyte to NaOCl had no effect on response. The electrochemical detector was also checked to ensure no fault was occurring by chromatographing quinone at -0.8V.

It was noticed that the same unusual pattern was seen at all voltages, namely a slight peak within a general drop in baseline. This peak, was slightly displaced towards the tailing edge of the negative peak. It appeared that a rising peak was superimposed on a negative peak. Chromatography of phospholipids which had not been oxidised produced a substantial negative peak. The parent phospholipid was causing a drop in the background current, on which the reductive response of the hydroperoxides was superimposed, as demonstrated by the negative response of parent phosphatidylcholine, shown in figure 4.18. It would therefore be necessary to separate oxidised from parent phospholipids if this detection system was to be used as part of a quantitative assay.
Figure 4.18 Chromatography of Oxidised Phosphatidylcholine Comparing UV Detection with Reductive Mode Electrochemical Detection.

UV detection was at 235nm, 0.1 AUFS. Electrochemical detection was at 1.5v using a Coulochem 5100 porous carbon electrode assembly. Eluent: 76% propan-2-ol, 14% of 1.5g/l sodium chloride in water, 10% hexane, 1.5g/l potassium chloride. Flow rate 0.8ml/min Column 25cm x 4.6mm aminopropyl bonded silica.

A: Oxidised phosphatidylcholine, 235nm UV
B: Oxidised phosphatidylcholine, -1.5V, EC
C: Parent phosphatidylcholine, -1.5V, EC

4.7 Purification of Phospholipid Peroxides by Solid Phase Extraction

If electrochemical detection was to be a viable method for HPLC detection of phospholipid hydroperoxides, then a rapid method for removal of parent phospholipid was required.
High resolution reversed phase HPLC separations of oxidised phospholipid from their parent compounds within classes have already been demonstrated (see section 4.3.2). Separations of the oxidised and parent forms of individual phospholipid classes using reversed phase solid phase extraction columns was therefore worthy of investigation. The advantages of this technique as a preparation procedure would be simplicity and speed of use compared to HPLC. In addition, it might be possible to use solid phase extraction in simple preparation of tissue extracts, in order to prepare samples free of interference from parent phospholipids for subsequent normal phase chromatography or, if sufficiently pure, direct analysis without the need for chromatography.

4.7.1 Comparison of Reversed Phase Bond Elut Cartridges

Initial experiments used C18 Bond Elute columns with isocratic elution and measurement by HPLC analysis with detection at dual wavelength. 1mg of phosphatidylcholine was irradiated with 1ml of a 1mg/ml Rose Bengal solution and extracted using an aminopropyl cartridge. This preparation was dried under oxygen free nitrogen and reconstituted with 2ml of 10% methanol in water. 200μl of this was placed onto a 100mg C18 Bond elut cartridge which had been preconditioned with 2mls of methanol followed by 2mls of water. These cartridges were then eluted with repeated 1ml aliquots of methanol/water eluents. Eluted fractions were then analysed by HPLC using detection at 205 and 235nm to monitor for parent phospholipids and oxidised phospholipids respectively. No resolution of oxidised species from parent compounds was evident at any eluent composition.
It was realised that discrimination akin to that achieved in properly equilibrated HPLC columns was unlikely to be achieved using isocratic elution on short, non-equilibrated columns. However, using sequential elution with eluents of increasing strength, rather than isocratic elution, seemed a more promising approach. The same protocol of conditioning and loading the columns to that described for isocratic elution was followed, but elution was performed with methanol/water eluents of increasing methanol concentration. Three types of reversed phase solid phase extraction (SPE) cartridge were investigated, namely C2, C8 and C18 substituted silica cartridges. The data from this experiment is given in figure 4.19.

Based on the absorbance at 235mm and 205mm for oxidised and non-oxidised phosphatidylcholine respectively, C2 cartridges showed clear discrimination between the two types of lipid over a range of eluents from 80% methanol to 100% methanol. C8 cartridges showed discrimination at 90% methanol to 100% methanol and C18 columns showed no discrimination whatsoever.

As oxidised phosphatidylcholine would also absorb at 205nm oxidised lipids would contribute to the parent phosphatidylcholine response. In other words discrimination would be slightly greater than indicated by these results. It was decided to further investigate purification of oxidised lipids using the C2 cartridges, as this packing gave the greatest discrimination between oxidised and parent phospholipid.
Figure 4.19. Resolution of Oxidised from Parent Phosphatidylcholine using Solid Phase Extraction.

100μg of phosphatidylcholine after 4 hours Rose Bengal irradiation applied to 100mg cartridge in 200 ml 10%MeOH in water. Eluted sequentially with 1ml of each eluent. Eluted fractions analysed by HPLC at 205nm (parent phosphatidylcholine) or 235nm (oxidised phosphatidylcholine). Data points are mean and SD of 3 experiments.

4.7.2 Bond Elut Cartridge Modification

The partial resolution seen in the above experiments with Bond Elut cartridges was encouraging. The possibility of improving these resolutions by altering or enhancing the characteristics of the cartridges was therefore investigated.

Two approaches were tested to try to improve the separation of phosphatidylcholine from its oxidation products, namely addition of ammonium acetate to the eluent as a cation
exchange reagent, and conditioning the SPE column with tetrabutyl ammonium iodide in order to block residual silanol groups on the column and increase the non-polar nature of the C2 solid phase extraction cartridge. Cartridge modification to affect elution has been successfully applied to separations of basic drugs (Law et al, 1992; Law and Weir, 1992).

Similar experiments were conducted to those described above, within 200μl oxidised lipid placed onto a preconditioned C2 cartridge. One set of cartridges was given an additional conditioning step of addition of 2ml of 0.1M tetrabutylammonium iodide in 10%water in methanol. Another set was eluted with either 1mM, 10mM or 100mM ammonium acetate in the eluents. The data from these experiments are presented in figure 4.20. The addition of ammonium acetate to the eluent appears to increase the normal phase characteristics of the separation whilst in no way increasing the resolution of the oxidised species from the parent forms. The data presented is for 1mM ammonium acetate. 10mM and 100mM additions were also tested with no difference to the elution. The use of tetrabutylammonium to condition the column on the other hand does increase the discrimination the column can achieve, increasing the retention of the less polar PTC while leaving the elution of oxidised compounds relatively unaffected.

Having elucidated optimum column conditions for separation of peroxidation products from parent compounds it was then necessary to optimise the separation on C2 cartridges by a more detailed investigation of the properties of eluents at concentrations between 80% and 90% methanol, this being the range of composition of eluent where greatest resolution was achieved under optimal column conditions.
Figure 4.20 Effect of Cartridge Pretreatment on Resolution of Oxidised from parent Phosphatidylcholine.

100μg of phosphatidylcholine after 4 hours Rose Bengal irradiation applied to 100mg cartridge in 200 ml 10%MeOH in water. Eluted sequentially with 1ml of each eluent. Eluted fractions analysed by HPLC at 205nm (parent phosphatidylcholine) or 235nm (oxidised phosphatidylcholine). NA: No Additive; AA: 1mM ammonium acetate in eluents; TBA: conditioned with 0.1 tetrabutylammonium iodide. Data points are mean and SD of 3 experiments.

The set up of this experiment was somewhat different to the others in that elution was conducted on separate SPE cartridges rather than sequential elution of single cartridges as had been previously performed. Cartridges were conditioned and loaded as before, but eluted with repeated aliquots of eluents of the same composition, and finally washed with 1ml of methanol. Figure 4.21 shows the percentage elution of both oxidised and parent phospholipid with the first 1ml of the appropriate eluent. Sixty percent of the oxidised species can be eluted without elution of any component of non-oxidised phosphatidyl
Figure 4.21. Optimisation of SPE of Oxidised Phosphatidylcholine with C2 Cartridges.

100μg of phosphatidylcholine after 4 hours Rose Bengal irradiation applied to 100mg cartridge in 200 ml 10%MeOH in water. Eluted sequentially with 1ml of each eluent. Eluted fractions analysed by HPLC at 205nm (parent phosphatidylcholine) or 235nm (oxidised phosphatidylcholine). Cartridges are treated with tetra butyl ammonium iodide during conditioning. Data points are recovery from first 1ml elution of cartridges, and are mean and SD of 3 experiments

Similar experiments were undertaken with C8 Bond Elute columns, to establish whether any improvement in resolution might be achieved with these types of columns. No noticeable improvement in resolution was achieved with either tetrabutylammonium iodide or ammonium acetate conditioning. Isocratic elution at eluent compositions between 90% and 100% methanol (as illustrated in figure 4.22) showed much poorer resolution of the oxidised group than the similar experiment with C2 columns.
Figure 4.22 Optimisation of SPE of Oxidised Phosphatidylcholine with C8 Cartridges.

100µg of phosphatidylcholine after 4 hours Rose Bengal irradiation applied to 100mg cartridge in 200 ml 10%MeOH in water. Eluted sequentially with 1ml of each eluent. Eluted fractions analysed by HPLC at 205nm (parent phosphatidylcholine) or 235nm (oxidised phosphatidylcholine). Data points are recovery from first 1ml elution of cartridges and are mean and SD of 3 experiments.

4.7.3 Partial Resolution of Oxidised Species using Solid Phase Extraction

In the experiment to test isocratic elution of phospholipid from tetrabutylammonium iodide modified C2 cartridges ratios of 235nm response to 205nm response from all initial 1ml elutions remained constant at eluent compositions at or less than 85% methanol. This indicated that a pure product was being eluted. If this ratio of response is taken to be that of 'pure' oxidised phospholipid and the response of all phosphatidyl choline species at
205nm is assumed to be equal then the proportions of oxidised material in each sample can be estimated by comparing the 235/205 ratios found with that expected for oxidised compounds. These assumptions are unlikely to be completely valid in that 'pure' oxidised phospholipid is likely to be a heterogeneous mixture of dienes, and the 205nm response will almost certainly vary between species. However they are likely to be a close approximation. Using this method the percentage of oxidation products in the singlet oxygen preparation was calculated as 58% of the total, while those present in the parent phospholipid prior to singlet oxygen oxidation were calculated as 4% of the total.

Repeated elution of the tetrabutylammonium treated C2 columns at varying percentages of methanol was conducted. There was considerable difference in the elution profile of the conjugated dienes found in parent phospholipids and those found in the singlet oxygen preparations. At all elution compositions conjugated diene compounds from singlet oxygen attack eluted prior to those found endogenously in the commercial phosphatidylcholine preparation. The oxidised species in the parent PTC, being older, are probably later products of peroxidation, whereas the singlet oxygen products probably represent predominance of earlier products such as hydroperoxides. This data is presented in table 4.12.
Table 4.12 Elution of Singlet Oxygen Reaction products from C2 Solid Phase Extraction Cartridges

<table>
<thead>
<tr>
<th>Sample</th>
<th>Elution Solvent (% MEOH)</th>
<th>% of Total Oxidation Products 235nm HPLC Response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fraction 1</td>
</tr>
<tr>
<td>100µg Oxidised Phosphatidylcholine</td>
<td>82</td>
<td>30 ±2.8</td>
</tr>
<tr>
<td></td>
<td>85</td>
<td>60 ±4.5</td>
</tr>
<tr>
<td></td>
<td>87</td>
<td>84 ±5.4</td>
</tr>
<tr>
<td>100µg Phosphatidylcholine</td>
<td>82</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>85</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>87</td>
<td>71 ±2.4</td>
</tr>
</tbody>
</table>

Data are mean and SD of 3 experiments

Later in the study these experiments were repeated using the chemiluminescence method.

This detection system is specific for hydroperoxides. The data, presented in table 4.13, bears out the conclusions above. Parent phospholipid did not exhibit any chemiluminescent response, while the chemiluminescent response of singlet oxygen products clearly parallel the diene profile.

4.7.4 Summary

Solid Phase extraction did not yield complete resolution of the oxidised from unoxidised phospholipids. However it did provide useful information about the composition of the singlet oxygen preparation and can be used to rapidly purify oxidation products for subsequent calibration of future analyses albeit with a lower yield than would have been hoped for.
Table 4.13  Comparison of chemiluminescence detection and UV detection of fractions of singlet oxygen reaction products of phosphatidylcholine from C2 solid phase extraction cartridges.

<table>
<thead>
<tr>
<th>Sample elution solvent</th>
<th>% of total chemiluminescence response</th>
<th>% of total u.v response at 235nm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rose Bengal</td>
<td>Parent</td>
</tr>
<tr>
<td>82% MeOH Fraction 1</td>
<td>32 ±2.9</td>
<td>-</td>
</tr>
<tr>
<td>82% MeOH Fraction 2</td>
<td>46 ±3.2</td>
<td>-</td>
</tr>
<tr>
<td>82% MeOH Fraction 3</td>
<td>13 ±4.5</td>
<td>-</td>
</tr>
<tr>
<td>82% MeOH Methanol Wash</td>
<td>9 ±1.5</td>
<td>-</td>
</tr>
<tr>
<td>85% MeOH Fraction 1</td>
<td>71 ±5.2</td>
<td>-</td>
</tr>
<tr>
<td>85% MeOH Fraction 2</td>
<td>25 ±2.4</td>
<td>-</td>
</tr>
<tr>
<td>85% MeOH Fraction 3</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>85% MeOH Methanol Wash</td>
<td>4 ±0.6</td>
<td>-</td>
</tr>
</tbody>
</table>

Data are mean and SD of 3 experiments, sample = 100μg oxidised phosphatidylcholine. No chemiluminescent response was seen from parent phospholipid.

With regard to electrochemical detection, however, rapid removal of parent phospholipid had not been possible. This result necessitated finding a suitable alternative detection system.

4.8 Post Column Chemiluminescence Detection of Phospholipid Hydroperoxides

The lack of success in detecting phospholipid hydroperoxides by HPLC with electrochemical detection, and the problems in developing a rapid extraction technique for pure phospholipid hydroperoxides which would effectively have removed the problems
with the quantitation step, meant that an alternative approach had to be developed. Again this had to be sensitive and selective for phospholipid hydroperoxides. Post column chemiluminescence detection appeared to meet these requirements.

Chemiluminescence detection systems rely on a specific reaction of the substrate with reagents capable of luminescent response. The reagents are elevated to an exited state by the reaction and the subsequent decay of the molecule to ground state results in emission of light which can be detected. The technique is selective by virtue of the specificity of the reaction and often more sensitive than other methods because it is possible to clearly detect even a small increase in light emission against a very low background. However the coupling of this type of detection to HPLC requires the use of a post column reaction system which demands careful set up and optimisation.

HPLC methods using this form of detection for phospholipid hydroperoxides have been described by two groups (Yamamoto et al, 1987; Miyazawa et al, 1987). These methods appear to have the sensitivity and specificity required. This gave a choice between the use of isoluminol/microperoxidase (Yamamoto et al, 1987) or luminol/cytochrome C (Miyazawa et al, 1987) as the chemiluminescent reagents. In the absence of any discernable difference in performance or sensitivity between the two techniques from the published literature, the isoluminol/microperoxidase system was chosen on the basis of cost. The cost of the microperoxidase was 20% of that of cytochrome C. A diagram of the HPLC sytem used for the following experiments is shown in figure 4.23.
Initial experiments repeating the conditions described by Yamamoto et al (1987) were encouraging. These were performed away from the chromatographic system by addition of freshly prepared 4 hour Rose Bengal irradiation preparations of phosphatidylcholine, to a 50:50 mixture of chemiluminescent reagent and HPLC eluent. The chemiluminescence reagent used was that identified as the optimum by Yamamoto (1987) namely, 70:30 methanol/80mM Borax containing 25mg/l microperoxidase and 180mg isoluminol, all at pH 10. The HPLC eluent used was the optimum eluent for the aminopropyl HPLC columns, 72:18:10 propan-2-ol/water/hexane. On addition of the hydroperoxide the reagents were immediately mixed and drawn through a Kratos 970 flow cell using a luer syringe. Response was detected using the Kratos 970 photomultiplier connected to a chart recorder. Chemiluminescent responses measured at specific times post addition were found to correlate well with arbitrary concentrations of hydroperoxides produced by serial dilution.
Attempts to repeat this experiment were not successful and this failure was eventually identified as caused by the propan-2-ol used in the mobile phase by monitoring the chemiluminescence response of components of the HPLC mobile phase. Initial batches of propan-2-ol produced no interference in the chemiluminescence response but later batches were found to produce considerable chemiluminescence. This was somewhat surprising because Miyazawa et al. used this solvent in their chemiluminescence HPLC system and did not report any problems. This interference prompted the search for alternatives solvent systems which resulted in the investigation of methanol/pentane/water systems described in section 4.3.3(d) above.

Changes in the solvents used on the system lead to further difficulties. The use of organic solvents in high concentrations in the HPLC eluents had caused intermittent problems with blockage in the Kratos flow cell from the start, either by precipitation of buffer or proteins. While disruptive, the problem was intermittent and the system could still be used, however on changing to methanol/pentane/water systems these problems became worse. Reduction of the concentration of the buffer from 100mM to 50mM helped this situation somewhat without affecting the chemiluminescent response of the reagent, but the frequency of blockage would have made attempts at automation impossible. Finally the purpose built Kratos flow cell was bypassed with a home-made cell made from coiled PTFE. This alleviated all the blockage problems as the internal diameter of this tubing was slightly larger than the inlet tubing on the Kratos flow cell. The PTFE flow cell was coiled in a planar manner, from inlet in the centre to an outlet at the circumference. This cell was held in place by custom-made metal clips and then placed centrally in the flow cell chamber with the plane of the cell at 90 degrees to the normal light path, where it was
anchored to the existing inlet and outlet tubes of the flow cell. This was left in place partly to provide the anchor points, but also because it was hoped that the parabolic mirror designed to reflect back the fluorometric response to the photomultiplier would help to capture a greater proportion of the chemiluminescent signal.

Perhaps more importantly, the change in eluents produced a loss in response from the chemiluminescent reagent. This prompted a careful evaluation of the assay. In the first instance the effect of pH on the system was investigated. As before the reactions were monitored in the flow cell of the detector after addition of aliquots of Rose Bengal irradiants of phosphatidylcholine to 50:50 solutions of chemiluminescence reagent with HPLC eluent. The results are displayed in figure 4.24. This increase in chemiluminescence response with increase in pH is in contradiction to the optimum conditions reported by both Yamamoto (1987) and Miyazawa (1987). Both groups reported optimum response at pH 10 or thereabouts, either as an absolute maximum response or because of the influence of baseline noise reducing the signal to noise ratios at higher pH values. No such problems with noise were apparent during this study. It may be that there is some change in the structure of the haem catalyst for this reaction at higher pH. A distinct change in the orange colour of the reagent occurs at approximately pH 12.5, which may indicate a change in the structure of the haem group possibly leading to greater catalytic activity.
Figure 4.24. Affect of pH on Chemiluminescent Response.

Chemiluminescence reagent contained 70% methanol, 30% 50Mm borax, 25mg/l microperoxidase and 200mg/l isoluminol, with pH adjusted with either 0.1M HCl or Conc NaOH. Response measured on HPLC with eluent(5% water in methanol) and reagent both set at 1.0ml/min. Data points are mean and SD of 3 experiments.

The effect of concentrations of isoluminol and microperoxidase on response was also investigated. This was found to coincide with the conditions reported by Yamamoto et al (table 4.14) with optimum response occurring at 200mg/l isoluminol and 25mg/l microperioxidase. Reagent concentrations higher than this produced little increase in response, but conversely no decrease was seen. The response surface flattened at concentrations of microperoxidase greater than 25mg/l and concentrations of luminol greater than 200mg/l. This was advantageous as small variations in post column mixing are unlikely to have a marked effect on response. In consequence the detection system, and hence the assay should be more robust.
Table 4.14  Optimisation of Chemiluminescence Reagent Composition

<table>
<thead>
<tr>
<th>Microperoxidase Concentration (mg/l)</th>
<th>Isoluminol Concentration (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100</td>
</tr>
<tr>
<td>15</td>
<td>67</td>
</tr>
<tr>
<td>20</td>
<td>92</td>
</tr>
<tr>
<td>25</td>
<td>92</td>
</tr>
<tr>
<td>30</td>
<td>108</td>
</tr>
<tr>
<td>35</td>
<td>-</td>
</tr>
</tbody>
</table>

Results expressed as a percentage of the 25mg/l microperoxidase/200mg/l isoluminol reagent response of oxidised phosphatidylcholine on the post column HPLC. Reagent pH was maintained at 13. Results are mean of three determinations.

Chemiluminescence response is dynamic with time and is therefore often more complex to optimise than other types of post column reaction where the only consideration is measurement of end point of the reaction. The time at which the eluent flow enters the detector cell will affect the sensitivity of the detection system and mixing of reagents and residence time in the flow cell relative to the kinetics of the reaction may have effects both on sensitivity and the recorded chromatographic peak shape. The affect of time delay and cell volume on detector response are illustrated in figure 4.25. The figure takes a hypothetical (and simplified) HPLC peak and compares how varying post column delay and size of flow cell will affect chemiluminescent response based on an approximation to the chemiluminescent reaction time curve (see figure 4.26). As can be seen with a residence time of five seconds within the flow cell the peak shape and peak width are significantly affected. Reduction of the size of the flow cell, while slightly improving the peak shape reduces the sensitivity of detection.
Figure 4.25 Calculated Peak Effects of Chemiluminescent Detection Windows.

Data for curves are based on a symmetrical HPLC peak and on a time course based on figure 4.26.
A Simplified chromatographic peak
B Effect of 5 second flow cell centred on maximum response of reaction.
C Effect of 5 second flow cell centred at 20 second region of reaction response
D Effect of 3 second flow cell centred on maximum response of reaction

In order to detect the maximum chemiluminescent response it was necessary to calculate the time course of the assay. Attempts were made to record this in situ by adding an aliquot of phospholipid hydroperoxide to a mixture of the chemiluminescence reagent and HPLC eluent, aspirating this into the flow cell and then monitoring the reaction as it happened using the Kratos detector. Unfortunately, the time delay in aspiration, compounded by problems of formation of bubbles in the flow cell, meant that only the
decay part of the reaction could be monitored, while the maximum response was obscured. It was therefore necessary to vary the length of the delay coil from the mixing T-piece to the Kratos flow cell and compare the response of chromatographed phospholipid hydroperoxides with different delay coils. The results are presented in figure 4.26 and show a maximum response at approximately nine seconds.

In order to optimise the chemiluminescent response without distorting the chromatographic peak more than was necessary a time delay of eight seconds was established for routine detection, with a three second flow cell. This allowed the majority of the most efficient part of the chemiluminescent response to be recorded.

The precision and linearity of the detection system were investigated by repeated analysis of known concentrations of phosphatidylcholine hydroperoxide using the chemiluminescence HPLC system. Phospholipid hydroperoxides prepared by Rose Bengal irradiation were isolated by reversed phase solid phase extraction as described in section 4.7.2. Concentrations of hydroperoxide were determined by stabilised iodometric analysis (El Saadarin et al, 1989) using hydrogen peroxide and cumene hydroperoxide as calibrants. Assay responses from these two peroxides differed slightly, but by less than 10% and a mean of their responses was used to estimate phospholipid hydroperoxide content in the oxidised phospholipid preparation. The chemiluminescent detection system proved to be linear up to 304nmol of hydroperoxide (figure 4.27) and to have extremely good precision over the range tested (table 4.15). However the estimated limit of detection, of 30pmol, as estimated at 3 times the baseline noise, was somewhat higher than claimed by both Yamamoto (1987) and Miyazawa (1987). These groups have
Figure 4.26 Time Course of the Chemiluminescent Reaction.

Table 4.15 Precision of Chemiluminescent Detection

<table>
<thead>
<tr>
<th>pmol LOOH on column</th>
<th>%CV(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3400</td>
<td>1.93</td>
</tr>
<tr>
<td>1700</td>
<td>3.61</td>
</tr>
<tr>
<td>800</td>
<td>2.35</td>
</tr>
<tr>
<td>500</td>
<td>5.50</td>
</tr>
</tbody>
</table>

Phosphatidylcholine hydroperoxide concentration was measured by stabilised iodometric method. %CV is defined as \((SD/\text{mean}) \times 100\) (n=6). HPLC was 25 cm aminopropyl column, mobile phase 5% water in methanol at 1.0 ml/min. 10\(\mu\)l of sample injected.
Figure 4.27 Standard Curve of Phospholipid Measured By HPLC with Post-Column Chemiluminescence.

Conditions as table 4.15

reported limits of detection of 5pmol and 10pmol of hydroperoxide on column, using commercially available HPLC chemiluminescent detectors. It is likely that limits of detection could be improved in the system reported in this study. A properly engineered flow cell, with an optimised mirror system for directing emissions to the photomultiplier would help. In the short term increasing the flow cell time window would improve response. It is impossible to predict, however, what effects these modifications will have on background noise.

Given the problems of interference of parent phospholipids on electrochemical response it was necessary to determine if similar effects would be seen with chemiluminescence
detection. Aliquots of phospholipid hydroperoxides were fortified with known amounts of parent phospholipid and their chemiluminescent responses were compared. No loss of response up to concentrations of 2mg/ml of parent phospholipid could be detected (table 4.16).

Table 4.16 Effect of Total Concentration of Phosphatidylcholine on Chemiluminescence Response of Phosphatidylcholine Hydroperoxide

<table>
<thead>
<tr>
<th>Concentration of Parent Phospholipid (mg/ml)</th>
<th>Chemiluminescent Response (% of hydroperoxide response)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>111</td>
</tr>
<tr>
<td>1</td>
<td>112</td>
</tr>
<tr>
<td>0.5</td>
<td>110</td>
</tr>
<tr>
<td>0.25</td>
<td>113</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

10μl injection of 500μg/ml phosphatidylcholine subjected to four hour Rose Bengal irradiation. HPLC eluent, 95% methanol, 5% water, at 1.0ml/min. Chemiluminescence reagent 70% methanol, 30% 50mM borax, 25mg/l microperoxidase and 200mg/l isoluminol, all at pH 13, at 1.0ml/min.

Summary

The use of chemiluminescence detection has been shown to be a robust, precise and sensitive technique for the measurement of phospholipid hydroperoxides. Chemiluminescent response is not quenched by co-eluting parent phospholipids, therefore the technique is suitable for use with the normal phase HPLC methods previously developed.
4.9 Extraction of Lipid Hydroperoxides from Tissues

Analysis of lipids from tissue samples often requires extraction of the lipids from the sample matrix as a prerequisite to the quantitative analysis. Initial extraction of total lipids is most commonly performed by solvent extraction procedures such as those described by Folch et al (1957) and Bligh and Dyer (1959) using chloroform and methanol as the extraction solvents. Such methods extract the majority of lipid compounds from tissue samples and consequently the extractions contain a mixture of lipid classes, along with a range of other lipophilic impurities.

Lipid extracts can be readily purified into general lipid groups by TLC and SPE (see section 4.4 above). However little is known about the behaviour of oxidised lipid species during liquid extraction from tissues. In order to develop a method for the measurement of tissue lipid hydroperoxides a suitable extraction method was required. Major problems were anticipated with the stability of hydroperoxides during extraction, and consequently, with recovery of phospholipids from tissues.

4.9.1 Modified Folch Extraction of Rat Liver Tris Buffer Homogenates

Many previous animal studies have been conducted within the Robens Institute on compounds that might potentially produce their toxic effects, either wholly or in part, by the stimulation of lipid peroxidation in vivo. A great many tris buffer homogenates of tissues from these studies have been stored and were still available for further investigation. Initial experiments were therefore targeted at extractions from tris buffer
homogenates used for tissue storage. If a suitable extraction method for such homogenates could be found then stored tissues from a number of previous studies would become available for analysis of phospholipid hydroperoxides.

Initial experiments were conducted on tris buffer homogenates containing 50mg of rat liver in 1ml of 25mM tris buffer. These were spiked with 100μl of a 4 hour Rose Bengal irradiant of 1mg/ml phosphatidylcholine. 400μl aliquotes of these spiked reagents were extracted with 3mls of 2:1 chloroform/methanol, and shaken for 2 hours. The organic layer was removed, taken to dryness, and the residue was reconstituted with 200μl chloroform. The phospholipid was separated from the extract using the Kaluzny method described above (section 4.4.2). Phospholipid fractions were analysed by HPLC monitored by UV detection at 235nm and by chemiluminescence detection.

Results from homogenates were extremely variable with unspiked homogenates at times showing 3 times the recovery exhibited by spiked homogenates for chemiluminescent response with equally random results for conjugated diene measurement at 235nm. Recommended methods of antioxidation were applied, however the addition of either 50μg of butylated hydroxytoluene (BHT) (Christie, 1987; Hamilton et al, 1992) or 500μg of ethylenediamine tetra acetic acid (EDTA) (Gutteridge et al, 1991) to homogenates did not stabilise the lipids.

These results imply that dynamic oxidative changes were taking place in the phospholipid component of the homogenates during extraction. The results further indicate that substantial changes to the hydroperoxides are taking place under these conditions and
that, surprisingly, the addition of antioxidants (eg BHT) or metal chelating agents (eg EDTA) did not protect the hydroperoxide from further reaction in the homogenate. An additional problem was that recovery of spiked oxidised phospholipids from tris buffer was only in the order of 25% after extraction with 2:1 chloroform/methanol. This type of lipid extraction was clearly unsatisfactory for the analysis of lipid peroxidation products, by reason of unstability of analyte and poor extraction efficiencies.

4.9.2 Modified Folch Extractions of Methanol Homogenates

While the results on the buffer homogenates were disappointing in that it denied access to a source of stored material which might prove of interest, the data produced from these samples indicated that problems were present in the traditional techniques of antioxidant stabilisation of samples prior to lipid analysis. The use of antioxidants such as BHT to inhibit radical attack and subsequent deterioration of lipids is often described but evidence for the efficacy of the procedure rarely cited. It is even recommended in standard texts on lipid analysis (Christie, 1987; Hamilton et al, 1992). The problems of stabilisation and extraction are linked in that the development of rapid methods coupled with chemical stabilisation of the hydroperoxides required to reduce destructive losses of hydroperoxides is at variance with the often lengthy and exhaustive lipid extraction techniques usually applied to tissue samples. The strategy employed to overcome these problems was to investigate simplified forms of traditional solvent extraction techniques for the extraction of phospholipid hydroperoxides, and then develop suitable stabilisation techniques in tandem with them.
In order to develop a suitable extraction procedure for phospholipid hydroperoxides a more traditional tissue extraction procedure was investigated. Rat liver was homogenated in methanol to a concentration of 100mg wet liver to 1ml methanol. 500µl of homogenate was then mixed with 4.5ml of chloroform for 120 seconds and then back extracted for 60 seconds with 1ml of 0.15M sodium chloride. The lower organic layer was aspirated off, dried under oxygen free nitrogen, and reconstituted in methanol prior to HPLC analysis. Residual tissue in the saline back extraction was re-extracted with 10% methanol in chloroform to check extraction efficiency. HPLC analysis of these second organic extracts showed that recovery of total phospholipids by this method was in excess of 95% of total phospholipid as measured at 205nm. No recovery of 100µl aliquots of 4 hour Rose Bengal irradiants of 1mg/ml phosphatidylcholine spiked into the homogenate could be detected at this stage but the high recovery of phospholipid was promising.

Recovery of analytes from tissue extracts is difficult to assess, because it is impossible to add a marker compound to tissues in such a way as to ensure that its concentration is known or it is integrated with the matrix bound analyte. For example, the addition of a radiolabelled analyte to a tissue sample in vitro may allow the recovery of the label to be assessed, but offers no guarantee that matrix bound analyte is extracted with the same efficiency. Even the repeat extraction method discussed in the preceding paragraph assumes that the tissue residue has remained unaltered during the initial extraction step, and that the remaining lipids will behave in the same way as the lipid removed in the first extraction step. Some of the lipids may form close complexes with proteins, or other insoluble components of the tissue and not be recovered. There is certainly evidence that some phospholipids are tightly associated with membrane proteins (Driscoll and Bettger,
1992). While not giving absolutely accurate data on phospholipid recovery, re-extraction data should provide a close approximation as most phospholipids are only weakly associated with other tissue components (Christie, 1987).

In order to discover whether the losses of oxidised phospholipids were due to poor extraction recovery, or to breakdown during extraction, comparisons of recoveries from methanolic solutions of oxidised phospholipids and methanolic liver homogenates were conducted. The results of these experiments are given in Table 4.17. Recoveries of phospholipid oxidation products, particularly hydroperoxides, was very low from spiked liver homogenates. Recoveries were higher from methanolic solutions of oxidised phospholipids but discrepancies between conjugated diene recoveries and the recovery of hydroperoxides indicates that even in the absence of biological material some degradation of hydroperoxides is taking place. Of course, as discussed above, it is difficult to prove

### Table 4.17 Recovery of Oxidised Species from Methanol and Methanolic Liver Homogenates with Chloroform Extraction.

<table>
<thead>
<tr>
<th>% Methanol in MeOH/CH₂CL</th>
<th>% Recovery of Oxidised Phospholipid from Methanol</th>
<th>% Recovery of Oxidised Phospholipid from Liver Homogenates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LOOH</td>
<td>Diene</td>
</tr>
<tr>
<td>10</td>
<td>80</td>
<td>100</td>
</tr>
<tr>
<td>20</td>
<td>95</td>
<td>100</td>
</tr>
<tr>
<td>30</td>
<td>94</td>
<td>100</td>
</tr>
</tbody>
</table>

Homogenates contained 100mg liver/ml of methanol. LOOH = Phospholipid hydroperoxide as measured by chemiluminescent HPLC. Diene = conjugated diene as measured by UV response at 235nm of phosphatidylcholine HPLC peak.
that some of the losses of oxidised lipid are not due to poor extraction.

The use of BHT as a stabilising agent with this extraction protocol was examined. BHT was added to homogenates at 50μg/ml concentrations, and 500μl of homogenates were then extracted with 4.5ml of chloroform at timed periods, after spiking with 100μl of a 1mg.ml phosphatidylcholine solution which had been irradiated with Rose Bengal for four hours. The results of a comparison with untreated homogenates are shown in figures 4.28 and 4.29. BHT does not stabilise hydroperoxides during the homogenate extractions, as witnessed by the dramatic and progressive losses of hydroperoxides with time. More interesting are the results of the conjugated diene analysis. These compounds show a marked increase in concentration initially in homogenates that have not been treated with BHT. This effect seems to be delayed, but not terminated by the use of BHT. No increase in hydroperoxides or conjugated dienes were observed in extractions of blank homogenates. Clearly BHT is affecting the peroxidation stimulated by addition of hydroperoxides to the system, but does not offer any protection to the hydroperoxides themselves.

In order to test at what stage deterioration was occurring blank extracts of liver homogenates were performed. 0.5ml of 50mg/ml homogenate of rat liver in methanol was extracted by addition of 5ml of chloroform followed by two minutes of whirlimixing. Samples were back extracted with 0.5ml of 0.15M sodium chloride, centrifuged at 1000rpm for 10 minutes to separate the layers and the organic layer removed. The solvent extracts were then spiked with 200μg of phosphatidylcholine which had been oxidised with Rose Bengal for 4 hours. The spiked samples were incubated for various times prior
Figure 4.28 Effect of Addition of BHT to Stability of Phospholipid Hydroperoxides in Rat Liver Homogenates.

BHT added to a homogenate containing 50 mg/ml wet weight of rat liver per ml methanol. Homogenates were spiked with 100μl of a 4 hour Rose Bengal irradiant of 1 mg/ml phosphatidylcholine. Data points show mean and SD of 3 experiments.

to being taken to dryness under oxygen free nitrogen, reconstituted in methanol and then analysed by HPLC. A number of samples were left unspiked before reconstitution and then spiked with oxidised phospholipid at this stage to test stability of oxidised phospholipids after reconstitution. The results of these experiments are given in table 4.18. Slight losses of hydroperoxides were evident from the chloroform extracts over a two and a half hour incubation, but this was a considerable improvement over incubations
Figure 4.29 Effect of Addition of BHT to Stability of Phospholipid Conjugated Dienes in Rat Liver Homogenates.

BHT added to a homogenate containing 50 mg/ml wet weight of rat liver per ml methanol. Homogenates were spiked with 100μl of a 4 hour Rose Bengall irradiant of 1 mg/ml phosphatidylcholine. Data points show mean and SD of 3 experiments.

with homogenate. Recoveries of oxidised lipids was even higher with the reconstituted extracts. These results indicate that breakdown of peroxides is promoted by the non-extractable fraction of the tissues.

Instability of the hydroperoxides may be due to metal catalysed breakdown of the hydroperoxides, or to enzymatic alteration of the molecule either by reduction of the hydroperoxide group by glutathione peroxidase or by the removal of fatty acids containing

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hydroperoxide functional groups by the action of phospholipases. Control of these mechanisms offered a strategy for the stabilisation of hydroperoxides during homogenisation and extraction.

Table 4.18 Stability of Oxidised Phospholipids in Tissue Extracts.

<table>
<thead>
<tr>
<th>Time Post-Spiking</th>
<th>Sample Matrix</th>
<th>% Recovery Dienes</th>
<th>% Recovery LOOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>60 mins</td>
<td>CHCl₃ Homogenate Extract</td>
<td>97</td>
<td>89</td>
</tr>
<tr>
<td>90 mins</td>
<td>CHCl₃ Homogenate Extract</td>
<td>95</td>
<td>71</td>
</tr>
<tr>
<td>160 mins</td>
<td>CHCl₃ Homogenate Extract</td>
<td>95</td>
<td>82</td>
</tr>
<tr>
<td>4 hrs</td>
<td>Reconstituted MeOH Extract</td>
<td>110</td>
<td>103</td>
</tr>
<tr>
<td>40 hrs</td>
<td>Reconstituted MeOH Extract</td>
<td>108</td>
<td>100</td>
</tr>
<tr>
<td>75 hrs</td>
<td>Reconstituted MeOH Extract</td>
<td>105</td>
<td>97</td>
</tr>
</tbody>
</table>

0.5 ml of 50mg/ml rat liver in methanol extracted into 5ml of chloroform. Extracts dried and reconstituted in methanol prior to HPLC analysis. Samples spiked with 100µl of a 4 hour irradiant of 1mg/ml phosphatidylcholine. 'Dienes' indicates conjugated dienes measured by UV detection at 235nm. 'LOOH' indicates phospholipid hydroperoxides measured by chemiluminescence. Results are from single determinations.

Three control systems were therefore investigated;

a) control of glutathione peroxidase activity by depleting levels of reduced glutathione in the system

b) control of metal catalysis by chelation

c) control of enzyme activity by reducing extraction temperature.

Incubations of homogenates at -60 °C in a hexane-dry ice bath, with 20mg/ml EDTA or 100mg/ml diethylmaleate were performed, and timed from addition of oxidised
phospholipid to addition of chloroform for extraction. The results are summarised in figure 4.30.

Diethylmaleate has been widely used to reduce endogenous concentrations of reduced glutathione (GSH) \textit{in vivo} and is thought to reduce the endogenous levels of GSH \textit{in vitro} (Comport, 1987). The use of this compound did little to increase recovery of the oxidised species, and slightly stimulated the degradation of oxidised species.

The result for the chelating agent EDTA were more marked, with levels of hydroperoxides being reduced to less than 5% of the spike level almost immediately.

While EDTA is a good chelating agent, it has been shown that at certain concentrations the compound in common with other chelating agents has pro-oxidant properties, therefore stimulating lipid peroxidation (Gutteridge et al, 1979). Pro-oxidant effects of chelating agents may be due to mobilisation of iron and other transition elements, making them more available for catalysis of hydroperoxide breakdown and production of hydroxyl radicals via the Fenton reaction (Grootveld and Halliwell, 1986). While the levels of EDTA used in this experiment were well in excess of the concentrations reported as inhibiting peroxidation \textit{in vitro} (Gutteridge et al, 1979) stimulation of hydroperoxides was obvious. In addition dried extracts of the EDTA treated homogenates all showed slight brown residues indicative of possible iron contamination. This was not seen in any other extract, and supports the hypothesis that EDTA is mobilising iron \textit{in vitro}. 

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Figure 4.30 Effect of Stabilisation Strategies on Recovery of Oxidised Lipids from Rat Liver Homogenates.

0.5ml of 50mg/l liver homogenate in methanol extracted as detailed in table 4.18.
DEM: 100μg diethyl maleate/ml homogenate
EDTA: 20mg ethylenediamine tetra acetic acid/ml homogenate
Data points are mean of 3 experiments

The use of desferrioxamine, an iron chelating agent, was also investigated. This compound produced serious interference with the HPLC method and in consequence little quantitative data could be generated. However, the data obtained did indicate that stability of hydroperoxides was not improved using this chelator, even though it has been demonstrated to be able to reduce the catalytic function of iron in peroxidation reactions at all concentrations (Gutteridge et al, 1979). Such a result indicates that, while EDTA may stimulate peroxidation reactions in the homogenates, iron catalysis is not the only
mechanism at work.

The most promising results regarding hydroperoxide stabilisation were from the temperature control experiments. While significant losses did occur, levels of hydroperoxide were maintained over the incubation period. The most plausible interpretation of these results is that the integrity of the spiked homogenates is maintained at -60°C, but that losses occur during extraction and drying down as this step is performed at ambient temperature.

In conclusion, reduced temperature proved to be the most effective method of stabilisation of phospholipid hydroperoxides. Once extracted and removed from subcellular fractions the hydroperoxides were relatively stable. It would therefore be necessary to develop tissue extraction methods which would be amenable to temperature control and rapid separation of the organic lipid extract from tissue residues.

4.9.3 Methanol/Water Homogenisation with Rapid Filtration

The most rapid extraction technique possible is direct homogenisation followed by immediate filtration. Re-extraction experiments were conducted to ascertain whether high recoveries of total phospholipid could be obtained by this approach. Samples were homogenised in a variety of solvent systems, undissolved material pelleted by centrifugation at 2500rpm for 30mins, and the pellets re-extracted. The results are shown in table 4.19.
Table 4.19 Recovery of Phosphatidyl Choline from Rat Liver by Homogenisation in Organic Solvents.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>% Recovery of Phosphatidyl Choline</th>
</tr>
</thead>
<tbody>
<tr>
<td>2:1 MeOH:CHCl₃</td>
<td>95.8</td>
</tr>
<tr>
<td>MeOH</td>
<td>96</td>
</tr>
<tr>
<td>20:1, MeOH:H₂O</td>
<td>95.6</td>
</tr>
<tr>
<td>9:1, MeOH:H₂O</td>
<td>95.3</td>
</tr>
</tbody>
</table>

% recovery calculated as the percentage of the 205nm response for first extraction of tissue to total response obtained from two extractions. Rapid filtration of the homogenate was achieved by the use of luer syringes and 0.45μm PTFE Acrodisc filters.

Prior to use these filters were tested with solutions of oxidised phospholipid in 10% water in methanol. No loss of hydroperoxides occurred using this procedure.

The use of reduced temperatures to stabilise the hydroperoxides in homogenates was investigated with this method of extraction. The results are shown in figure 4.31. 100mg of rat liver was homogenised in 5ml of 10% water in methanol. Aliquots of this extract were equilibrated to the experimental temperature and timing was started from addition of the oxidised phospholipids. The results clearly show that at -30°C the hydroperoxides are conserved in liver homogenates for at least 15mins. A typical chromatogram from a filtered liver extraction is shown in figure 4.32.

While not as convenient as chemical stabilisation strategies, the only viable stabilisation for extraction of lipid hydroperoxides from animal tissue is temperature reduction.
Assessment of the inter and intra-assay variation of the method was conducted by spiking tissue with solutions of phosphatidyl choline hydroperoxide and conducting homogenisation at -30°C, followed by rapid filtration and subsequent HPLC as described.

Within assay variation was measured by adding 100 μl of methanol solutions of known concentration of phosphatidyl choline hydroperoxide (as measured by stabilised iodometric analysis) to 50 mg of rat liver and homogenising the tissue in 0.9 ml methanol at -30°C. The sample was then filtered using a 0.45 μm PTFE Acrodisc and 50 μl injected onto the HPLC. Concentrations of phospholipid hydroperoxide were calculated by comparison with pure phospholipid hydroperoxide standards subjected to HPLC - chemiluminescence analysis. Six replicates at two concentrations were performed at 4.0 and 15.0 nmol hydroperoxide per ml of homogenate.

Between assay variation was estimated in a similar manner except that spiking of homogenates was performed on the day of analysis. Six replicates were again performed at 4.0 and 15.0 nmol / hydroperoxide per ml homogenate. Fresh chemiluminescent reagent was used for each assay batch and each assay batch was independently calibrated. The phospholipid hydroperoxide content of the stock solution used for preparation of calibrant and spike dilutions was measured by stabilised iodometric analysis prior to the first assay batch and after the last assay batch and results were found to be within 5% of each other, indicating stability of the stock solutions. Results from these experiments are given in table 4.20.
Table 4.20 Intra and InterAssay Precision of HPLC Chemiluminescence Assay of Tissue Phosphatidyl Choline Hydroperoxide

<table>
<thead>
<tr>
<th>Phospholipid Hydroperoxide Concentrations (nmol/ml)</th>
<th>IntraAssay Precision</th>
<th>InterAssay Precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean Recovery</td>
<td>% CV</td>
</tr>
<tr>
<td>4.0</td>
<td>95.2%</td>
<td>23.2%</td>
</tr>
<tr>
<td>15.0</td>
<td>103%</td>
<td>6.4%</td>
</tr>
</tbody>
</table>

50 mg of rat liver spiked with 100 µl of methanolic solutions of phosphatidyl choline hydroperoxide. 900 µl of cold methanol was added and the sample homogenised at -30°C, followed by rapid filtration (0.45 µ PTFE Acrodisc) and injection of 50 µl of filtrate onto HPLC. n = 6 for both experiments.

Intraassay precision was acceptable, particularly at the 15.0 nmol/ml concentration level, however InterAssay precision was high at both concentration levels. This was possibly caused by intermittent blocking of the pump check valve with at least two assay batches, and illustrates the exceptional care that must be exercised with the use of the chemiluminescent reagents if precise determinations are to be achieved.

Recovery data from the precision experiment demonstrated linearity of the homogenate assay up to a concentration of 15 nmol phospholipid hydroperoxide per ml homogenate, however duplicate spikes at 30 nmol/ml were conducted during one assay batch with individual recoveries of 95.8% and 98.4%, demonstrating linearity up to this concentration in tissue samples.
Short term stability of phospholipid hydroperoxides in tissue preparations has been
demonstrated (figure 4.31). Long term stability checking poses particular problems. At
present this could only be done with spiked homogenates or by spiking onto frozen tissue, and
storing at an appropriate temperature (ie -80°C). Neither approach offers a valid way of
estimating stability in real tissue. Homogenisation results in destruction of the integrity of
tissue and cells and therefore will probably have a marked adverse effect on sample stability.
Liver samples required for analysis, if stored at all, would be better stored frozen whole by
freeze clamping.

Spiking onto frozen tissue is equally problematic. Not only is a non homogenous sample
being stored for analysis, the surface of the tissue will be composed of damaged and disrupted
cells likely to promote deterioration of hydroperoxides.

The method as described is appropriate for the analysis of fresh, freeze clamped tissue from
animal experiments, and samples have been shown to be stable over times consistent with sample
pretreatment and analysis. Longer term stability studies would be better performed on intact
tissues with elevated endogenous levels of phospholipid hydroperoxides.
Figure 4.31 Effect of Temperature on Recovery of Phospholipid Hydroperoxides from Liver Homogenates.

1ml of 50mg/ml homogenate of rat liver in methanol was spiked with 40μl of a 4 hour Rose Bengal irradiant of 1 mg/ml phosphatidylcholine. Experiments were timed from addition of spike until injection of sample onto HPLC. Samples were filtered prior to injection using a 0.45m PTFE Acrodisc. Data points are mean and SD of 3 Experiments.

4.9.4 Summary

Methods for the extraction of phospholipids from animal tissues, and for stabilisation of hydroperoxides in these extracts have been examined. Antioxidant and chelation strategies, commonly recommended to stabilise lipid extractions, were found to be of little value for the extraction of phospholipid hydroperoxides as substantial losses of these compounds occurred during extraction. Temperature reduction was found to be a reliable method of conserving hydroperoxides. The optimum method of extracting phospholipid hydroperoxides from tissues was to homogenise tissue at -30°C in 5% methanol in water, followed by rapid filtration using low volume acrodisc filters.
Figure 3.32 Chromatograph of Rat Liver Extract Spiked with Oxidised Phospholipid.

Extraction Conditions as figure 3.31. HPLC conditions: 25cm aminopropyl column, mobile phase 5% water in methanol at 1.0ml/min. A: phosphatidylcholine, B: sphingomyelin, C: phosphatidylethanolamine.
4.10 Conclusions

Normal phase HPLC using aminopropyl columns was shown to be the optimum technique for chromatographic analysis of oxidised phosphatidylcholine and phosphatidylethanolamine. Care must be taken with the chromatography as there is a possibility of resolution of oxidised from parent forms which might cause problems when quantitating oxidised forms by measurement of conjugated diene groups at 235nm. The use of aminopropyl columns with methanol/water/pentane eluents gave the best combination of efficiency and resolution.

Post column chemiluminescence detection offers a sensitive alternative which does not suffer from interference from parent phospholipids. Consequently this is the detection method of choice.

Tissue analysis of phospholipid hydroperoxides is hampered by problems of extraction. Traditional methods of stabilisation such as chelation or antioxidation proved to be ineffective, while temperature control at -30°C gave good recoveries of phospholipid hydroperoxide. The use of rapid homogenisation and filtration as a lipid extraction technique gave good recovery of phospholipid and should provide a suitable extraction technique for the extraction of phospholipid hydroperoxides from tissues when used in conjunction with temperature control.
CHAPTER 5: GENERAL DISCUSSION

5.1 Findings of the study

5.1.1 Chromatography of Lipid Peroxides

The comparison of normal phase and reversed phase columns showed the same general pattern of separation of phospholipids as that described by other authors, namely species resolution by reversed phase materials and the resolution of classes by normal phase materials. However, more detailed investigation of these separation techniques revealed characteristics not previously published, particularly in relation to normal phase chromatography.

Reversed phase chromatography of phospholipids departed from the results of Crawford et al in that C18 columns retained parent compounds for long periods using methanol/water eluents. The chromatography of these compounds on C8 columns was more successful but unfortunately overlap of groups of interest and partial resolution of species meant that this form of chromatography would not be useful in the analysis of complex lipids from tissues.

Normal phase separations, particularly with aminopropyl columns, proved more useful. Separation of phospholipid classes from each other, and from other lipids was achieved, however separation of oxidised from parent forms was observed under some conditions. Partial resolution of oxidised from parent phospholipids within classes on normal phase
materials has not been described by others attempting these type of separations (Yamamoto et al., 1987; Yamada et al., 1987; Matsushita and Terao, 1987) This may be because elutions were so similar under the conditions used by these authors as to make partial resolution less obvious, and perhaps these effects went unnoticed. Such partial resolution must be identified and controlled if reproducible quantitation of phospholipid hydroperoxides is to be achieved.

The highest efficiencies for phospholipid separations were seen with aminopropyl columns using methanol/water/pentane eluents. These eluents also provided the widest range of retentions without causing species separation. This eluent combination has not previously been reported, possibly because methanol and pentane are often believed to be immiscible. The solvent system is compatible with chemiluminescence detection and may be useful for phospholipid separations with a range of columns.

The separations described in this study were developed with a view to being compatible with sensitive detection systems. For this reason a number of possible improvements in chromatography were not pursued. However, in the current position of having a well established and sensitive detection system it should be possible to improve the efficiency of the chromatography. Theoretically this could be performed at once with the use of narrow bore columns and/or the use of mobile phase additives for pH control, or possibly even ion pairing reagents. However such changes may affect the chemiluminescence reaction and thus disrupt the detection system.

By far the best separations of phospholipid classes have been achieved using gradient
elution (Christie, 1987). This form of elution is theoretically very difficult to link to chemiluminescent detection as the response of the reagent will be affected by the composition of the eluent. In consequence the potential increase in sensitivity due to improved chromatographic efficiency may be offset by the loss in response that the mobile phase composition may cause.

While an increase in efficiency is desirable it is perhaps not appropriate in this instance to strive for the ultimate chromatographic efficiency when linked to chemiluminescence detection. The optimum sensitivity of the detector relies in part on a minimum detection window which allows the maximum signal to be captured. Any increase in efficiency which requires this window to be reduced would produce a loss of sensitivity, and at a certain point the reduction in sensitivity would outweigh the increase in sensitivity occasioned by presenting a smaller volume of more concentrated analyte to the detector. In addition higher efficiencies would be adversely affected by dispersion caused by mixing of the eluent with chemiluminescence reagents.

Although the chromatographic methods for separation of phospholipids that have been developed would benefit from some improvement, particularly in efficiency, the methods are suitable for the analysis of phospholipids from tissues and are compatible with chemiluminescence detection.
5.1.2 Detection

a) Chemiluminescence Detection

This study has shown the chemiluminescence detection system to be a reliable, robust system, sensitive enough to be applied to tissue analysis of phospholipid hydroperoxides and highly resistant to interference from parent phospholipids. The limits of detection described in this thesis are, however, not as low as described by Yamamoto et al. and Miyazawa et al., in spite of the clear improvement in response from pH 10 to pH 13 of the chemiluminescence reagent. The relative increase in sensitivity does not appear to be due to better chromatographic efficiency in either of the other authors systems.

The most likely reason for higher detection limits in the described system is the design and configuration of the flow cell on the detectors. The home made cell utilised in this project proved to be robust and detection was highly reproducible, however, the geometry of the cell did not maximise the return of emitted energy to the photomultiplier. The reflective surface of the original fluorometer cell would have returned some energy, but the optimum emission position was blocked by the fluorometer flow cell, which was left in place. Redesign of the cell would have increased the signal for a given concentration of analyte and thereby improved sensitivity.

b) Electrochemical Detection

The use of electrochemical detection was found to be impractical at an early stage,
because of interference from parent phospholipid. however, as a detection technique for organic peroxides the technique still has possibilities. Using column switching techniques to separate the hydroperoxides from parent phospholipids using short reversed phase columns, and then performing a normal phase class separation and quantifying the hydroperoxides using reductive mode electrochemistry is one possible protocol. Of course the presence of phospholipid hydroxides, cyclic endoperoxides, and aldehydes might still cause significant interference with the detection system. An alternative approach might be to reverse the protocol and heart cut one phospholipid class onto a reversed phase system for subsequent separation of species and quantitation of phospholipid hydroperoxides by electrochemical detection.

It may be that reversed phase separations using methanol/water/pentane eluents could provide the resolution of oxidised and parent classes of phospholipid that was strived for in initial work with reversed phase columns (see section 4.3.2). In such a circumstance the use of electrochemical detection for phospholipid peroxides would be available.

These hypothetical protocols all assume that the detection system could be developed for analysis without problem. This is unlikely to be the case. Many chromatographers avoid reductive mode electrochemical detection because of the problems of oxygen interference. Particular care would have to be exercised in order to develop an assay into the picamole range using this method of detection.
5.1.3 Tissue Extraction of Phospholipids

The clearest conclusion of this project as a whole was that serious problems exist in the methods used for extraction of oxidation products of phospholipids in general, and phospholipid hydroperoxides in particular. The assumptions usually made about extraction, particularly in relation to the stability of oxidised phospholipids, are often too general. Data regarding stability and reproducibility of extraction should be included in any paper that claims to report quantitation of oxidised phospholipids from biological samples. This is not generally the case.

In this work a range of potential stabilising agents for lipid hydroperoxides were investigated, and only one strategy gave acceptable results, namely extraction under reduced temperature. While not, perhaps, the most convenient technique given the temperatures required, namely at or less than -30°C, the means to perform such a procedure is readily to hand in most analytical laboratories. A hexane-dry ice bath will produce temperatures of -60°C.

Rapid separation from tissue debris by microfiltration has also been shown in this study to improve stability of phospholipid hydroperoxides. Again, this technique is readily available to analysts. Other methods of separation, such as centrifugation or liquid/liquid partition, are time consuming and will therefore lead to degeneration of hydroperoxides. The possibility of temperature controlling these procedures, particularly down to -30°C or below, would be technically very difficult. For all these reasons microfiltration is the preferred method for removal of tissue debris prior to analysis.
While providing a workable means to measure phospholipid hydroperoxides without loss, rapid extraction techniques as described in this study are relatively crude. Use of a sensitive and highly selective detection method, such as chemiluminescence, avoids problems of interference and thus avoids problems with quantitation. However, crude lipid extracts do have a damaging effect on the aminopropyl columns used for separation of phospholipid classes. Increased retention times, and gradually decreasing efficiency result from repeated analysis of these extracts.

5.1.4 Recommended Procedure for the Analysis of Phospholipid Hydroperoxides in Animal Tissues

From the conclusions made above regarding each stage of the analytical procedure, it is possible to design an optimum method for the determination of phospholipid hydroperoxides in tissue.

a) Sample extraction

Rapid homogenisation of tissue into 5% water in methanol, at or below -30°C, followed by rapid filtration immediately prior to chromatography.

b) Chromatography

Direct injection of filtrate onto a 25cm aminopropyl HPLC column eluted isocratically with 5% water in methanol at 1.0ml/min. This results in separation of phospholipid classes
from other interfering compounds and from each other, and in co-elution of oxidised and parent species.

c) Post Column Chemiluminescence Detection

Rapid mixing of HPLC eluent with a chemiluminescent reagent comprising 70% methanol and 30% 50mM sodium tetraborate containing 25mg/l microperoxidase, and 200mg/l isoluminol, all at pH13. Under these conditions a time delay of 8 seconds and a flow cell residence time of 3 seconds provided sensitivity with minimum loss of chromatographic efficiency. This form of detection could be used in conjunction with co-elution of parent phospholipids with no loss of detector response.

5.2 Possible Future Developments in Lipid Peroxide Analysis

While HPLC chemiluminescent detection is at present the method of choice for lipid hydroperoxides there are some developments in analytical science which may find application in the assay of oxidised lipids in future. The two principal approaches are HPLC-mass spectrometry and capillary electrophoresis.
5.2.1 HPLC-Mass Spectrometry (HPLC-MS)

The use of mass spectrometry as a chromatography detector has been widely applied in GC, where interfacing with the chromatograph is relatively straightforward. Such interfacing with HPLC, while theoretically possible, has always been more difficult because of the technical problems associated with introducing large volumes of liquids into the mass spectrometer (Evershed, 1992). One of the most common methods applied to lipids is direct liquid introduction (Kuksis, 1987), however, the splitting of the solvent severely reduces sensitivity by three or four orders of magnitude. Recent advances in atmospheric pressure vaporisation techniques may well open up the HPLC-MS system for lipid analysis and offer another sensitive and specific detection system for analysis of lipid peroxides.

A note of caution must be introduced. The specificity of LC-MS may not be entirely appropriate to studies of lipid peroxidation. As described in chapter 1, lipid peroxidation is a complex process, acting on an extremely heterogeneous group of compounds. For reasons of ease of interpretation it may be better to analyse for specific classes of peroxide rather than sensitively measuring individual compounds. Use of the mass spectrometer in scanning mode would address this problem, but would compromise sensitivity.

5.2.2 Capillary Zone Electrophoresis

One of the fastest growing areas in separation science is capillary zone electrophoresis (CZE). The technique works on the simple principle of differential migration of molecules
due to their charge across a potential. The use of fused silica capillaries means that extremely large plate numbers can be generated, of the order of $10^6$ plates (Xu, 1993; Perrett and Ross, 1992). Resolution from such columns are therefore extremely high. This alone would be enough to make the technique a candidate for any analysis, but CZE has also been coupled with electrochemical detection (Ewing et al., 1994). The possibility therefore exists of complete separation of all lipid species from each other, and the ability to selectively and sensitively detect hydroperoxide species by reductive mode electrochemical detection. The sensitivity of the technique has been claimed to be low enough as to be able to achieve results from single cell analysis. Electrochemical cell arrays have also been described for CZE, which will allow profiling of the contents of single cells (Ewing et al., 1994). How practical these approaches are for routine determinations remains to be seen. There is little at present in the literature on CZE analysis of lipids, however recent work on the separation of phospholipids indicates that resolutions are little better than those achievable by HPLC (Ingvardson et al., 1994)

5.2.3 Conclusions

While both HPLC-MS and CZE may have a promising future in lipid peroxidation analysis, at present they do not provide a viable alternative to HPLC-chemiluminescence. HPLC-MS because of its sensitivity and selectivity will continue to be widely adopted, but it is this feature which limits its applicability to peroxidation studies. CZE on the otherhand, is limited because of the lack of a high resolution method for phospholipids. Clearly, if the resolution described for some analytes can be found for lipid species, then the technique could become a powerful tool in the analysis of phospholipid
hydroperoxides. At present, however, HPLC chemiluminescence is the only technique which combines sensitivity with the ability to detect the whole range of phospholipid hydroperoxides which may be found in vivo.

It should also not be forgotten that all of the techniques discussed in this section rely on sample preparation, and can only provide valid data if a reliable sample preparation method is used.

5.3 Applications of the Chemiluminescence HPLC to Analysis of Lipid Hydroperoxides in Tissues

Hydroperoxides in Tissues

5.3.1 Immediate needs

Methods for the chemiluminescence HPLC analysis of phospholipid hydroperoxides in tissues have been reported (Yamamoto et al, 1987; Frei et al, 1988; Yoshida et al, 1990; Miyazawa et al, 1992), however, there is some controversy over the results obtained. Frei et al (1988) have challenged the levels of lipid hydroperoxides found in plasma by Miyazawa et al (1988), claiming they were artifacts of sample preparation. Certainly the methods used for extraction of phospholipid hydroperoxides from liver (Yoshida et al, 1990; Miyazawa et al, 1992) are similar to conditions under which significant losses occurred in this study, recoveries of phosphatidylcholine hydroperoxide and phosphatidylethanolamine hydroperoxide were quoted as 73 plus or minus 7% and 82 plus or minus 13% respectively, indicating that losses were indeed occuring.
In consequence the most obvious and important application of the assays described in this thesis remains the determination of phospholipid hydroperoxides in tissues. Chemiluminescence HPLC following rapid low temperature extraction provides a way of measuring phospholipid hydroperoxides in tissues. Chromatography and detection are direct and require no prior derivitisation, and the extraction protocol has been developed to ensure that losses are minimised.

Clearly the assay requires formal validation. While a considerable amount of work has been done on investigating and optimising the assay, a detailed validation of the assay for liver analysis is required. This validation program must examine the precision of the assay both within and between batch when analysing real samples and consider in detail other potential sources of interference, such as endogenous antioxidants. No negative peaks were detected during analysis of tissue homogenates, indicating that interference was not a problem, however a rigorous evaluation of potential assay interference is still required. Validation must also critically evaluate recovery of hydroperoxides from tissues. As discussed in section 4.9 above, determination of recovery has its difficulties, however the major problem with interpretation of all assays of lipid peroxidation products is the uncertainty over artifact production.

Homogenisation and filtration, while effective, is relatively crude and leads to rapid deterioration of the columns used. Data presented on the stability of the homogenate in methanol extracts indicates that at this stage more extensive sample preparation procedures are possible. Use of the solid phase extraction techniques described by Kaluzny et al (1985) and further developed in this study (see table 4.18) may offer a
viable method of clean up of tissue extracts. Separations of individual phospholipid classes using aminopropyl solid phase extraction have been reported (Pietsch and Lorenz, 1993) and depending on the elution of oxidised species, may extend the facility of solid phase extraction for these compounds.

5.3.2 Future Applications

The application of the method to direct tissue analysis alone has considerable possibilities. In particular the measurement of levels of phospholipid hydroperoxides present in arterial cell walls undergoing atherosclerotic changes will help to clarify some of the unanswered questions regarding atherosclerotic plaque formation and its links with oxidised low density lipoproteins. Material extracted from atherosclerotic plaques has been shown to stimulate lipid peroxidation (Smith et al, 1992) which indicates that peroxidation is occurring in these lesions. The measurement of lipid hydroperoxides in developing atherosclerotic lesions artificially induced in laboratory animals would allow the relationship of peroxidation with lesion progression to be clarified, and possibly provide a model system which would enable the affects of antioxidants on progression of atherosclerotic lesions to be directly studied (see section 1.6.2a).

Such techniques will also help in the understanding of the oxidative status of cancer cells. In particular they may help unravel the complex associations between antioxidant status and cell proliferation. While it is clear that cancer tissue has an altered antioxidant status, and that some tumours have much higher proportions of saturated fatty acids associated with their cell membranes (see section 1.6.2b), no direct measurement of tissue peroxide
species has yet been conducted. Tissue analysis of tumours and surrounding tissues for phospholipid hydroperoxide concentrations would help our understanding of the significance of such observations to carcinogenesis.

It would be particularly interesting if the techniques could be applied to subcellular fractions in order to widen the understanding of the role of compartmentalisation of the cell in control of peroxidation, and the role of peroxidation products in control of the cell. Application of existing cell fractionation techniques would probably cause many profound changes to the hydroperoxides, and it may be that a marriage of these techniques is unrealistic. However, such an approach would open up an enormously interesting and important area of research.
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