ASSAY AND CHARACTERISATION OF LIPID PEROXIDATION PRODUCTS IN MODEL SYSTEMS AND BODY FLUIDS FROM PATIENTS WITH MULTIPLE SCLEROSIS

by

KAREN L. HERBERT

A thesis submitted to the
University of St. Andrews in
application for the degree of
Master of Science.

University of St. Andrews,
Department of Biochemistry and Microbiology,
Irvine Building,
North Street,
St. Andrews,
Fife,
Scotland,
KY16 9AL.

September 1987

CERTIFICATE

I hereby declare that Karen Lesley Herbert has spent nine terms in research work under my supervision and that she has fulfilled the conditions of Ordinance No. 16 (St. Andrews) and that she is qualified to submit this thesis for the degree of Master of Science.

(Dr. M.I.S. Hunter)

DECLARATION

I hereby declare that the following thesis is based on work carried out by me, that the thesis is my own composition and that no part of it has been presented previously for a higher degree.

The research was carried out in the Department of Biochemistry and Microbiology of the University of St. Andrews under the direction of Dr. M.I.S. Hunter.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to all my colleagues and friends in St. Andrews especially:-

Dr. Ian Hunter, my supervisor, for his excellent advice, support and much appreciated personal encouragement throughout the past two years.

Mr. Jim Hunter for his invaluable technical assistance.

Dr. Duncan Davidson and his patients at Dundee Royal Infirmary for supplying the clinical samples necessary for this study.

Miss Margaret Wilson for typing this thesis.

Mr. Bill Blyth for his photographic work.

The Multiple Sclerosis Society for financial assistance.

DEDICATION

To my parents.

ABSTRACT

Free radical-mediated attack of polyunsaturated lipids resulting in their subsequent non-enzymic peroxidation, has been widely recognised as an important process in a diverse range of disease and toxicity states. The chemistry of lipid peroxidation (LPX) is extremely complicated and although several reactions have been characterised, the precise nature and identity of some of these products remains obscure and in some areas totally ambiguous. There has been considerable evidence supporting the role of free radicals in the pathogenesis of Multiple Sclerosis - a common neurological disease of unknown aetiology.

The aim of this work has been to investigate, identify and characterise the peroxidation products found in both plasma and CSF of Multiple Sclerosis patients with the view to developing a specific, sensitive assay for a clearly defined and chemically characterised end product of LPX thus forming the basis for a diagnostic marker of the disease.

LPX indices were measured using two of the most extensively employed methods:- fluorimetric assay of (malondialdehyde) MDA, - a bifunctional agent produced during the peroxidative process and fluorescent assay of lipid soluble end products which are thought in part to be derived from MDA cross-linking with the amino phospholipids phosphatidyl ethanolamine (PE) and phosphatidyl serine (PS). The products of the reaction between MDA and PE and PS were characterised in simple phospholipid systems under controlled physiological conditions using both TLC and HPLC methods to separate them. Partial characterisation of PE-MDA products

revealed that such adducts were not produced in significant quantities in *in vitro* biological systems (microsomes, erythrocytes, brain homogenate) and were not detected in M.S. or control body fluids. PS was found to be extremely unreactive *in vitro* towards MDA.

Extensive statistical analyses revealed no significant difference between M.S. and control plasma or CSF in terms of production of MDA or lipid soluble fluorescent pigments ($p > 0.1$). By stressing both M.S. and control haemolysates with various LPX inducers, we fail to report any significant difference in the susceptibility of MS erythrocytes towards oxidative challenge.

CONTENTS

INTRODUCTION

INTRODUCTION

1. MULTIPLE SCLEROSIS

The first description of Multiple Sclerosis (M.S.) is generally attributed to Charcot, (1) who in 1868 recognised the characteristic clinical and pathological features of the disease. Although his work has stimulated extensive experimental investigations and a wealth of literary data postulating many conflicting theories, a heuristic model applicable to the human situation has yet to be found. No preventative measures or definitive therapies exist. The fundamental cause and pathogenesis remains unknown.

1.1 MULTIPLE SCLEROSIS AS A DISEASE

Multiple Sclerosis or disseminated sclerosis is the most common human demyelinating disease (2) and indeed, after psychogenic disorders it is the most common disease to affect the nervous system in young and middle-aged adults (3). Even though the annual incidence of new cases is quite low, the chronicity of M.S. leads to a fairly high prevalence rate affecting 1 in 2000 of the population in England and about 1 in 800 in the Hebrides Scotland (4). It is rare or virtually absent in certain racial groups such as blacks of African origin and orientals (2 cases/100,000) (5). The commonest age of onset of M.S. is between the 20th and 40th year but it may occur up to the age of 50 (6). Among women the disease

commonly appears just before the age of 30 and in men rather later (7), females being more frequently affected than males in the proportion of 1.7:1 (2).

M.S. is a disease of the central nervous system (CNS) and symptoms include loss of motor control; including weakness, paralysis, spasticity and sensory dysfunction; with impaired vision and partial or complete blindness. The clinical course of M.S. varies markedly between individuals, although it is normally classified as progressive, acute or benign depending on the rapidity and extent of the neurological dysfunction (8). Approximately 60% of patients fall within the former class, where the disease is initially manifested by episodes of dysfunction in the brain and spinal cord which have a tendency to remit, leaving the patient with no obvious signs of disability. With the passage of time however, the severity of these attacks may increase, resulting in a less complete recovery of function after each exacerbation. In the acute form, neurologic dysfunction progresses over a few weeks or months with incomplete remissions of short duration followed by severe relapses and may be terminal within a few months. Unlike these severe forms, the benign form is characterised by only a few exacerbations often mild, followed by complete recovery.

1.2 THE DIAGNOSIS OF MULTIPLE SCLEROSIS

There is no absolute way to diagnose M.S. other than autopsy and despite many recent advances in laboratory methods, the diagnosis of the disease is made primarily on clinical grounds and can only be presumptive. At present

probably the most important aspect of diagnosis is a history of episodes of neurological disturbances scattered in time and space, thus confirming lesions present at more than one site in the CNS. Clinical assessment may also be supplemented by various electrophysiological techniques which provide objective evidence of clinically silent lesions, such as evoked potentials (visual, somatosensory, auditory) and N.M.R. imaging. However, the more fundamental and routinely performed laboratory tests are restricted to analysis of various parameters in cerebrospinal fluid (CSF):-

(i) Protein

A mild increase in total protein may be observed but it is rarely more than 1 g/litre (6).

(ii) Cytology

In 25% -30% of patients usually early in the disease, there is a leukocytosis of 5 - 50 cells/mm^3 and in a small proportion the cell count is > 100 cells/mm^3 (6). These cells are usually lymphocytes (predominantly mononuclear) and plasma cells.

(iii) Oligoclonal banding

Immunoelectrophoresis of CSF proteins identifies characteristic oligoclonal bands visible within the IgG region in up to 98% of patients with M.S. (9, 10).

(iv) Immunoglobulin G

In most laboratories standard practice is to estimate the levels of immunoglobulin G, (IgG) expressing it as a percentage of either the total protein or albumin. Levels > 15% of total protein are suggestive of M.S. Alternatively the IgG index can be derived from the formula:-

$$\text{IgG index} = \frac{\text{CSF IgG}}{\text{Serum IgG}} \times \frac{\text{Serum albumin}}{\text{CSF albumin}}$$

Ratios of > 0.66 are indicative of M.S. (6).

All of these tests identify abnormal IgG quantities in CSF suggesting the production of immunoglobulin within the CNS. However the problem of false 'positives' arises because those conditions which may clinically cause confusion e.g.: virus infections, neurosyphilis and other chronic inflammatory and infective conditions, may also result in high levels of gamma globulin in the CSF. Thus retrospectively what is clearly lacking in the field of M.S. diagnosis is a simple, relatively rapid and inexpensive test which is indicative of the disease.

1.3 THE CLASSIFICATION OF M.S.

A number of schemes have been formulated over the years (11, 12) in attempts to define diagnostic categories of varying certainty, all based on the clinico-pathological definition of the disease established by Charcot (1). While there is fairly good correspondence between the criteria for individual categories in different systems (11, 12) the nomenclature varies. The following summary is based on the publications of the Poser Committee (13, 14):-

(i) Clinically definite Multiple Sclerosis (CDMS)

(a) Two attacks and clinical evidence of two separate lesions.

(b) Two attacks, clinical evidence of one and paraclinical evidence of another separate lesion.

(ii) Laboratory supported definite Multiple Sclerosis (LSDMS)

(a) Two attacks, either clinical or paraclinical evidence of one lesion and CSF oligoclonal bands.

(b) One attack, clinical evidence of two separate lesions and CSF oligoclonal bands.

(c) One attack, clinical evidence of one and paraclinical evidence of another separate lesion and CSF oligoclonal bands.

(iii) Clinically probable Multiple Sclerosis (CPMS)

(a) Two attacks and clinical evidence of one lesion.

(b) One attack and clinical evidence of two separate lesions.

(c) One attack, clinical evidence of one lesion and paraclinical evidence of another separate lesion.

(iv) Laboratory supported probable Multiple Sclerosis (LSPMS)

(a) Two attacks and CSF oligoclonal bands.

Most clinical investigators adopt the Kurtzke disability scale and functional grading system for assessing the clinical state of M.S. Numerical values of between 1 and 10 are assigned to each patient in ascending order of disability, a score of 6 indicating the confinement of a patient to a wheelchair (15).

1.4 THE PATHOLOGY OF MULTIPLE SCLEROSIS

The primary pathology of this demyelinating disease is confined to the nervous system where macroscopic lesions ranging from 1 mm - 4 cm are scattered throughout the white matter (16). These lesions, or plaques, are characterised by

focal perivascular inflammation (infiltration of lymphocytes, plasma cells and macrophages), interstitial oedema, loss of myelin and oligodendroglia, proliferation of fibrous astrocytes and gliosis (17). The macrophages are responsible for ingesting fragments of myelin, initially stripping the axon of its outer lamellae and sparing the axon itself.

The CSF also contains lymphocytes, plasma cells, polymorphs, monocytes, eosinophils, macrophages which also possess intracellular myelin fragments; thus suggesting the possibility that some of the myelin destruction occurs extracellularly.

Increased leakage of the blood-brain-barrier has been reported in > 50% of cases following an acute attack and disappears spontaneously over the course of a few weeks to months (18). This could allow entry of immunocompetent cells, antibody, or infections which lead to myelin destruction. However it is important to note that this defect occurs as part of many infections and other inflammatory brain lesions, so it is equally possible that it represents a secondary phenomenon. Nevertheless its existence may add to a cascade of events which might otherwise be benign or self-limited.

1.5 THE EPIDEMIOLOGY OF MULTIPLE SCLEROSIS

Any theory that attempts to define the aetiology of M.S. must surely account for its striking epidemiology. By 1980 more than 220 studies of the geographic distribution of M.S. had been conducted (19). Whether one looks at the incidence, prevalence or mortality rate, in terms of distribution of the disease, the general pattern holds true

in that it is regional with a distinct predilection for temperate climates and for economically developed countries.

M.S. is seen with greater frequency as the distance from the equator is increased, (primarily in the Nothern hemisphere) with a prevalence rate of 30 - 80 cases/100,000 (20). In contrast, regions of low risk including Africa and Asia, have a prevalence of < 5 cases/100,000. Extensive epidemiological studies indicate that the disease may be acquired by environmental or genetic factors.

1.5.1 Environmental Factors

(i) Diet

Studies of various dietary components indicate that the increased incidence of M.S. at higher latitudes correlates well with a high animal fat diet compared with their mediterranean neighbours who favour a low animal fat diet rich in polyunsaturated fatty acids (PUFA's (21). Several mechanisms have been postulated whereby diet could influence M.S.:-

(a) A high intake of fat increaes vascular sludging thus producing hypoxia distal to plugged arterioles and results in perivascular demyelination (22). The perivascular distribution of plaques in M.S. is cited as support for such a mechanism (23).

(b) Diet may influence the composition of the myelin membranes, in that a high saturated fat intake characteristic of temperature regions where M.S. is common, could result in less stable myelin membranes and a greater risk of demyelination (24).

(c) Finally, diets rich in animal protein may result in a more competent immune responsiveness of the host in the individuals in temperate regions (25).

(ii) Sanitation

Attention focused on similarities between the geographical distribution of M.S. and poliomyelitis, suggested that an enteric virus like polio might cause the disease and demonstrated that enteric illness and other measures of water sanitation had high correlations with M.S. frequency (26).

(iii) Migration studies

Migrants from areas of high risk to areas of lower risk, acquire the lower risk provided that migration has occured before the age of 15 (19). The converse is also true.

(iv) Epidemics in isolated populations

The Orkney, Shetland and Faroe Islands have the highest rates of M.S. in the world, with an epidemic of the disease preceded by the occupation of the islands by British troops in the 1940-45 period (3). An epidemic of canine distemper was also reported to have lasted throughout the British occupation with no disease prior to 1940 nor after 1956 (27).

1.5.1 Genetic Factors

(i) Familial studies

Strong evidence that an exogenous or environmental "trigger" is required to produce M.S., is provided by genetic studies particularly on concordance of monozygotic (MZ) and dizygotic (DZ) twins. It has been reported that no more than 30% of MZ twins develop M.S. thus indicating that even when

the entire genotype of an M.S. patient is duplicated, the probability of developing the disease phenotype is still much less than 100% (3).

(ii) The human leukocyte-associated antigen system (HLA)

The antigens of the HLA system are expressed on the surface of most cells throughout the body but are particularly well represented on lymphocytes. HLA antigens are coded by a cluster of closely linked genes which reside on the short arm of chromosome six and their associations with M.S. have been demonstrated in population studies by comparing the frequency of individual antigens in patients and controls of the same ethnic group. For example the antigens HLA-A_3, HLA-B_7 and HLA-DW_2 occur substantially more commonly in patients with M.S. than in persons from the same area without the disease (28).

It is noteworthy that this system relates to the regulation of the immune system and antigen-antibody reactions and there have been many reports of increased levels of antibodies to rubella, measles and varicella zoster in serum (29, 30), CSF (31) and brain homogenate (32) of patients with M.S. It is possible that the HLA linked factor in M.S. is acting via an alteration in immune responsiveness, either as a response to a virus or via autoimmunity to a component in the CNS such as myelin (33).

1.6 THEORIES FOR THE PATHOGENESIS OF MULTIPLE SCLEROSIS

Embolism

It has been proposed by James (34) that a subacute embolism may be the cause of M.S. whereby fat may lodge in

the microcirculation of the nervous system causing distal perivenous oedema with subsequent loss of myelin from the axons. The main evidence supporting this theory is that most of the small acutely demyelinated areas surround a capillary, or more usually a small vein. James also suggested that depot fat is the major source of embolic fat in acute fat embolism and it may be hydrolysed by endothelial lipases to release a spectrum of toxic fatty acids, which in turn is a reflection of the diet. This theory however has been criticised by several workers (35) who have not detected fat embolism in their M.S. tissue bank, and also the theory does not explain the immunological findings in M.S.

Virus infections in the Central Nervous System

A pre-requisite for M.S. appears to be a persistent infection of the CNS (36) and several lines of evidence support the view that a virus-induced immune disease is involved in the primary pathogenesis of M.S. As mentioned previously, various epidemiological studies could permit the assumption that a viral agent "triggers" the onset of the disease. There are three possible mechanisms through which a virus might produce the lesions of M.S. (37):-

(i) Direct infection and damge of myelin-producing oligodendrocytes could result in myelin breakdown accompanied by a secondary inflammation.

(ii) An inflammatory immunological response against virus persisting in the CNS (i.e. a virus induced change in surface antigens in the CNS) could have a secondary demyelinating effect.

(iii) Viral infection results in an autoimmunisation against

some component of white matter, giving rise to immunological mediated inflammatory disease.

To summarise, the outstanding problem in M.S. remains the aetiology and pathogenesis of the disease and the many theories which have been advanced concerning its origin embrace viral, immunological, nutritional and metabolic considerations, alone or in various combinations. Evidence for the involvement of membrane lipids is as yet fragmentary, however the paramount role of lipids in cell membranes of all kinds suggests that whatever theory is favoured, they will enter into the argument at some stage.

1.7 THE LIPID BIOCHEMISTRY OF MULTIPLE SCLEROSIS

Structure of myelin

Degeneration of the myelin sheath in areas of the CNS is a characteristic feature of M.S. The lipids of myelin are like those of other membranes in their amphipathic nature. Purified myelin fractions of human brain contain approximately 70% of dry weight of lipid and the lipid composition in different areas of the CNS has in common cholesterol:phospholipid:galactolipid with a molar ratio of 2:2:1 (38). Whereas the high concentration of cholesterol in myelin is a characteristic feature of most other animal plasma membranes, myelin is notable for its high concentrations of glycosphingolipids, principally monogalactosylceramide (39). In addition to monogalactosylceramide, the other galactolipids in abundance are sulphatides. These contain sphingosine as normal, as well as hydroxy fatty acids with hydrocarbon chains of 4-5 carbon atoms longer than the mean chain length found in other

membranes, (40) thus conferring stability of the myelin sheath. The remaining 30% consists of equal proportions of an encephalitogenic basic protein and a lipid soluble proteolipid protein with small amounts of high molecular weight proteins and glycoproteins (e.g.: 2' 3' cyclic nucleotide 3' phosphodiesterase).

Abnormalities in M.S. myelin, plaques and white matter

The biochemical changes in plaque tissues from M.S. brain reflect the expected results of the demyelination process; loss of galactolipids from degenerating myelin and the formation of cholesterol esters (17, 41, 42). Davison and colleagues reported decreased levels of sphingomyelin (Sm) and plasmalogen in apparently normal cerebral white matter of M.S. patients (43) and other workers found decreased levels of phosphatidyl serine (PS), sulphatides and ethanolamine containing phosphoglycerides (44, 45). Arnetoli (46) reports that the ratio of saturated methyl esters:unsaturated methyl esters of C_{18} fatty acids of ethanolamine containing phospholipids are significantly elevated in M.S. He suggests that PE containing lipids may be affected earlier or to a greater extent than for example phosphatidyl choline (PC) in the disease and that ethanolamine-containing lyso compounds are formed during demyelination and may play a significant role in the pathological process.

Activities of acid proteinase and other acid hydrolases are significantly increased in plaque areas thus resulting in almost complete degeneration of myelin basic protein by these lysosomal enzymes (47, 48, 49). This degeneration process

may take months and in the latter stages infiltration of macrophages are largely responsible for the final dissolution of myelin.

Other abnormalities in Multiple Sclerosis

A. In Serum and Plasma

Possible abnormalities of serum and plasma components in M.S. patients have attracted much attention in terms of developing a diagnostic test for the disease and the possible therapeutic value of linoleate supplementation of the diet (50). Several researchers report decreases in the percentage of total serum fatty acids (particularly linoleic acid) in M.S. patients, especially in esterified cholesterol (51, 52), although these findings are extremely controversial (53, 54, 55). Furthermore there have been reports of decreased arachidonate levels in M.S. red cell lipids where most of the fatty acid is usually found in the acidic phospholipids PE and PS (56).

Platelet stickiness in M.S. has been shown to increase which correlates inversely to serum cholesterol linoleate concentrations (52, 57). Linoleic and arachidonic acids have been shown to effect electrophoretic mobility of erythrocytes and lymphocytes in M.S., thus distinguishing from other neurological diseases (56) although this too has been refuted (58).

B. In Cerebrospinal Fluid

It has been suggested that lipid changes in CSF may originate from lymphocytes and serum due to disfunction of the blood-brain-barrier, thus reflecting changes in erythrocytes or serum as opposed to a local change in CSF

composition. Perderson et al. (59) showed that there is a mean decrease in phospholipid concentrations in M.S., particularly PE, with an increase in esterified cholesterol.

C. In Lymphocytes

Much less work has been done on the fatty acid components of lymphocytes in M.S., however as in other blood fractions linoleate levels have been found to be significantly reduced (51).

2. MEMBRANE LIPID PEROXIDATION

2.1 GENERAL FEATURES

The presence of unsaturated lipids in cells is a potential threat because of the extreme lability of such lipids in the presence of oxygen and their widespread distribution in cell membranes (60). Rat liver mitochondrial membranes, lysosomal membranes and endoplasmic reticulum for example, contain polyunsaturated fatty acids (PUFA's) in considerable amounts (61). The presence of double bonds in these fatty acid side chains render the lipids highly susceptible to proton abstraction by any species have sufficient reactivity, thus resulting in the formation of a stable free radical (62). Once a free radical is formed in the lipids, in the presence of oxygen, the autocatalytic process of peroxidation is initiated and is followed by a complex series of propagative reactions resulting in the irreversible destruction of the original lipid (60). In cells this destruction could lead to the loss of integrity of the membranes, loss of membrane fluidity, decreased membrane potentials, increased ion permeability and eventual lysis releasing cell and organelle contents (62).

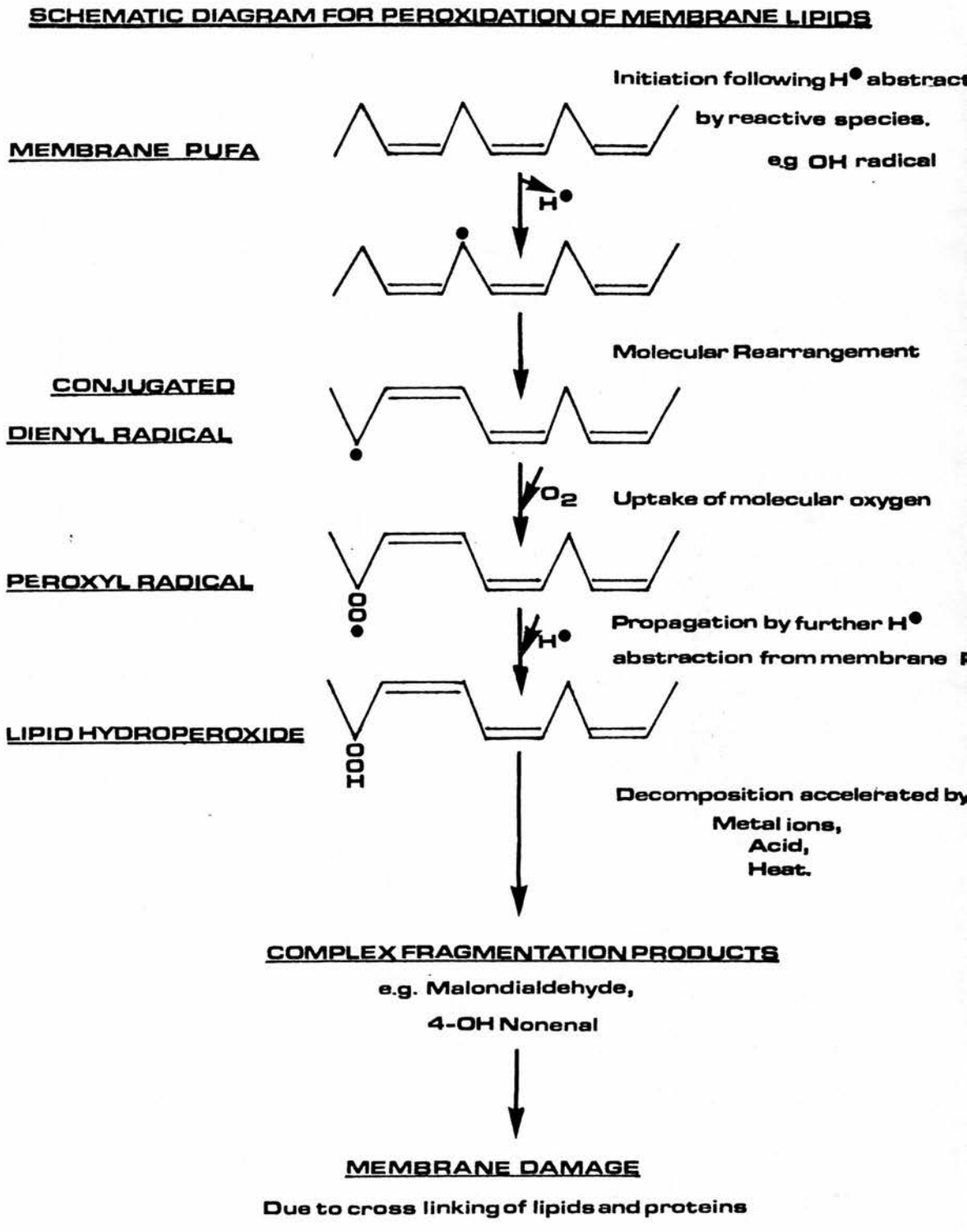
It is obvious, therefore, that the cells must have means for preventing these occurences which include (63):-

(i) The spatial separation between the various components required in the peroxidation reaction.

(ii) Low oxygen tension.

(iii) Metal chelation.

FIGURE 1


SCHEMATIC DIAGRAM FOR PEROXIDATION OF MEMBRANE LIPIDS
Initiation following H● abstract
by reactive species.
e.g OH radical
MEMBRANE PUFA
H●
Molecular Rearrangement
CONJUGATED
DIENYL RADICAL
O2
Uptake of molecular oxygen
PEROXYL RADICAL
OO●
Propagation by further H●
abstraction from membrane
H●
LIPID HYDROPEROXIDE
OOH
Decomposition accelerated by
Metal ions,
Acid,
Heat.
COMPLEX FRAGMENTATION PRODUCTS
e.g. Malondialdehyde,
4-OH Nonenal
MEMBRANE DAMAGE
Due to cross linking of lipids and proteins

(iv) The presence of effective protective mechanisms (these will be discussed in a later section).

Under normal physiological conditions, the extent of peroxidative damage is very low due to the operation of these efficient protective mechanisms, however under certain pathological conditions, this damage becomes elevated due either to an over-production of free radicals or to a deficiency in one or more of these preserving mechanisms (64).

2.2 THE CHEMISTRY OF LIPID PEROXIDATION

The peroxidation of PUFA's, especially 20:4 and 22:6 is a feature of many types of cell injury in which free radical intermediates are produced in excess of local defence mechanisms (65). The chemistry underlying LPX is extremely complex, and in some areas incompletely understood, although most of the reactions have been thoroughly characterised. A simplified scheme is shown in Figure 1. Basically the process may be divided into two phases, an initiation and a propagation phase. During initiation of LPX, a reactive free radical such as the hydroxyl radical ($OH^{\cdot}$), alkoxy radical ($RO^{\cdot}$), peroxy radical ($ROO^{\cdot}$) (but not O_2^{-}), abstracts a hydrogen atom from a PUFA side chain of the membrane lipid to form a carbon centred radical which is rapidly stabilised by molecular rearrangement to yield a conjugated dienyl radical. The conjugated diene will then react rapidly with molecular oxygen to form a peroxy radical which propagates the autocatalytic chain reaction by abstracting a further proton from another fatty acid. This chain reaction will continue until the substrate has been exhausted unless a chain-

breaking antioxidant is present to terminate the process (e.g. Vitamin E). The peroxy radical combines with the proton to give a lipid hydroperoxide which will decompose non-enzymically (catalysed by transition metal ions, heat and acid) to yield a variety of complex carbonyl-containing fragmentation products including hydrocarbon gases, ketones, epoxides, aldehydes (malondialdehyde (MDA), 4 hydroxynonenal (4-OH NA), 4,5 dihydroxydecenal) and other radicals capable of abstracting further protons from the PUFA's (66-70).

The aldehydic products, in particular, are extremely reactive and complex with many biologically important amines, including RNA, DNA and phospholipids PE and PS (71) to form stable end products of LPX. Since this heterogeneous mixture of end products accumulates in tissues their measurement is indicative of the degree of peroxidative damage that has occured there (see later section).

2.3 POTENTIAL SOURCES OF OXIDANTS

Although oxygen (O_2) is absolutely essential to the life of aerobic organisms, it has long been known to be toxic to them when supplied at concentrations only slightly greater than those present in normal air (70). In fact there is consideral evidence that even 21% O_2 has slowly-manifested damaging effects. Why then is oxygen toxic? (72)

A. It inactivates enzymes by oxidation of essential thiol groups.

B. It causes formation of hydrogen peroxide (H_2O_2).

$$O_2 + 2H^+ + 2e \xrightarrow[\text{oxidases}]{\text{cellular}} H_2O_2$$

C. It oxidises lipids to lipid peroxides.

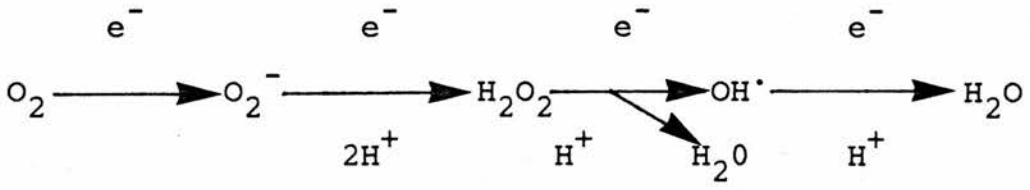
Under certain conditions, PUFA's of cell membrane lipids can undergo oxidation to produce lipid peroxides. Normally this process proceeds at a very slow rate but it is greatly accelerated by metal ions or haem compounds.

Normally in the cell efficient protective mechanisms exist (see later) whereby the toxic effects of oxygen are maintained at tolerable low levels. Several years ago it was proposed that many of the damaging effects of O_2 could be attributed to the formation of oxygen radicals and this hypothesis was developed by Fridovich (73) into a superoxide theory of oxygen toxicity which states that formation of superoxide radical in vivo plays a major role in the toxic effects of O_2.

General Properties and formation of activated oxygen species

A free radical may be defined as any species that has one or more unpaired electrons (70). This broad definition includes the H atom, most transition metals and molecular O_2 (or dioxygen) itself. However, due to its electronic configuration ($2e^-$ with same spin quantum number) O_2 itself is much less reactive as an oxidant in its ground state than one would otherwise expect. The reactivity of O_2 can be increased by overcoming its spin restriction and transition metals (found at the site of many oxidases and oxygenases) are well suited to this purpose because of their ability to accept and donate single electrons. The reactivity of O_2 can also be increased when pigments such as retinal, flavins or porphyrins are illuminated in its presence. Thus potential cellular oxidants are mostly derivatives of activated O_2:-

superoxide (O_2^-), peroxide (O_2^{2-}), singlet oxygen $^1O_2$) and the most reactive of all of these, the hydroxyl radical ($OH^\cdot$). The univalent reduction of molecular oxygen leading to the successive formation of O_2^-, H_2O_2, $OH^\cdot$ and H_2O is shown below (74):-



Superoxide Radical (O_2^-)

One electron reduction of O_2 produces O_2^-. It is formed in almost all aerobic cells (75), a major source being "leakage" of electrons onto O_2 from various components of the electron transport chain of mitochondria and the endoplasmic reticulum. It is also established that O_2^- is produced during the respiratory burst of phagocytic cells and plays a key role in the killing of toxic bacteria (76). The metal-ion-dependant oxidations of adrenaline, noradrenaline and thiol compounds also produce O_2^- and a few oxygen-utilizing enzymes produce O_2^- directly, including xanthine oxidase and tryptophan dioxygenase (77). Superoxide in aqueous solution, is not highly reactive (104) but it becomes converted into H_2O_2 by a dismutation reaction:-

$$2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

Hence systems generating O_2^- usually produce H_2O_2 as well. Despite the poor reactivity of O_2^- in aqueous solution, O_2^- generating systems have been observed to do a considerable degree of biological damage and evidence that O_2^- is a species worth removing *in vivo* comes from the fact that superoxide dismutases (SOD's) accelerate the above reaction

by 4 orders of magnitude, (75) even at the expense of forming H_2O_2. However it is worth mentioning here that <u>in vivo</u> SOD's work in conjunction with the H_2O_2 removing enzymes catalase and glutathione peroxidase (GPX).

<u>Hydrogen Peroxide</u>

Two electron reduction of O_2 produces H_2O_2 and like O_2^-, it has a low reactivity to most organic molecules (78). It is not a radical because it has no unpaired electrons, and unlike the charged O_2^- radical it can rapidly traverse cell membranes. O_2^- will only cross membranes if there is a specific "channel" for it to do so, and such a channel has only been demonstrated in the erythrocyte membrane (106). As mentioned above, systems generating O_2^- usually produce H_2O_2 as well. There is considerable evidence that in the presence of H_2O_2, O_2^- can give rise to the highly toxic OH radical (72, 73) and since Haber and Weiss proposed in 1934 that $H_2O_2$and O_2^- do react together, it was naturally proposed as the source of OH$^\cdot$

$$H_2O_2 + O_2^- \longrightarrow O_2 + OH^\cdot + OH^-$$

However, it is now generally accepted that this reaction does not occur at significant rates in biological systems (79) and that transition metal ions are required for catalysis (see next section).

<u>Hydroxyl radical</u>

The OH$^\cdot$ reacts with great speed with almost every molecule found in living cells including DNA, proteins and carbohydrates (77). It has also a potentially devastating effect upon the PUFA side chains of membrane lipids, thus initiating membrane LPX, the consequences of which have

already been detailed. The $OH^{\cdot}$ can be produced by the effect of heat or ionising radiation on H_2O_2 and also through the transition metal ion-catalysed decomposition of H_2O_2. The following reactions are referred to as Fenton reactions (70):-

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$$

Traces of Fe^{3+} can react further with O_2^{-} acting as the reductant:-

$$Fe^{3+} + O_2^{-} \longrightarrow Fe^{2+} + O_2$$

Unless some other reagent is added to intercept the $OH^{\cdot}$, many more radical reactions are possible, the net reaction being an iron catalysed decomposition of H_2O_2:-

$$2H_2O_2 \xrightarrow{\text{Fe salt}} 2H_2O + O_2$$

It can be seen therefore that a simple mixture of an iron salt and H_2O_2 can provoke a whole series of radical reactions. Other metal ions including Cu^{+}, Mn^{4+}, Ti^{3+} and Co^{2+} are also capable of catalysing the Fenton reaction in the same way as Fe^{2+}.

<u>In vivo</u> virtually all of the iron is present in a chelated form bound to enzymes or other proteins (e.g. haemoglobin, transferrin, lactoferrin, ferritin) and considerable controversy exists as to whether natural, sequestered forms of iron or possibly other transition metal ions are effective in catalysing the Fenton reaction (74). However Gutteridge (80) revealed in studies with metal-containing extracellular fluids that they do catalyse the formation of $OH^{\cdot}$ and it seems likely that the metal ion-catalysed Haber-Weiss and Fenton reaction are significant <u>in vivo</u>.

Singlet Oxygen

Two singlet states of O_2 exist. Ground state singlet O_2 ($^1O_2$) is the most important biological systems since the higher energised form usually decays to the ground state before it has time to react with anything. $^1O_2$ is not a radical since it has no unpaired electrons. It is produced when O_2 is excited in presence of several illuminated pigments (e.g. flavins) and therefore is produced where any pigmented system is exposed to light (e.g. lens of the eye) (70). It is an exceptionally reactive and oxidising form of oxygen (81). Excessive singlet O_2 formation can lead to several diseases e.g. porphyrias (70). There have also been claims that $^1O_2$ is formed by dismutation of O_2^- and during the respiratory burst of activated phagocytes, (70) however, this theory is not in general acceptance since evidence so far has proved inconclusive (82).

Finally, if these radicals formed inside cells can produce such a wide diversity of deleterious reactions, one might wonder who we survive at all. In fact we are in possession of normally very efficient and widely distributed protective mechanisms of various kinds, and it is only when these are overcome, either due to an overproduction of free radicals or a defect in one or more of these defences that damage results.

2.4 PHYSIOLOGICAL CONSEQUENCES OF FREE-RADICAL MEDIATED LIPID PEROXIDATION

The autocatalytic process of membrane lipid peroxidation has attracted much attention in recent years with the realisation that many toxic agents can be

metabolically activated within cells to free radical intermediates that can initiate the chain reaction and result in cell injury (61, 83). Membrane lipid peroxidation occurs in both normal physiological processes; e.g. cell aging (84-86), prostaglandin biosynthesis (87), phagocytosis (88), and in abnormal conditions including liver necrosis (89), chronic alcoholism (90), rheumatoid arthritis (91), Duchenne muscular dystrophy (92, 93), ceroid lipofuscinosis (94, 95), pancreatic disease (96), cervical pre-cancer (97), cerebral ischemia (98), some phases of atherosclerosis (99), and haemolytic anaemia (100, 101).

Extensive studies with model and in vitro biological systems have clearly shown that free radicals, highly reactive species occuring during the peroxidation process, are able to produce chemical modifications of, and damage to proteins, lipids, carbohydrates and nucleotides. Irradiation of tissue, cells, or cell organelles are capable of producing reactive free radicals in the same way as metabolically activated toxic agents. It has been determined that based on moles of enzyme inactivated per mole of free radical, that lipid peroxidation is one tenth as damaging as ionizing radiation (66). Although the nature and general properties of the free radicals formed from lipid molecules are the same as those formed from other classes of compounds (102), the nature of lipids will necessarily modify these properties. For example lipids are either hydrophobic or amphipathic (or intrinsic or extrinsic) leading to the formation of the radical centres in a nonaqueous environment. Secondly, the amphipathic lipids exist in an arrangement that may permit

maximum interaction of the individual molecules and may also contribute to maximum damage to the cell. Reactive free radicals may damage cells through a pathway dependant essentially on membrane damage (65):-

(i) By direct covalent binding of the free radical to membrane enzymes or receptors thereby modifying activities of membrane components.

(ii) By direct covalent binding of the free radical to membrane components thereby changing structure and producing effects on membrane function and/or antigenic character.

(iii) By disturbance of transport processes through covalent binding, thiol group oxidation, or change in PUFA's.

(iv) By initiation of LPX of PUFA's with direct effects on membrane structure, function, fluidity and cross-linking.

If the reactive free radical is formed close to DNA it may produce a change in structure resulting in a mutation or cytotoxicity (103, 104). Protein and non-protein thiol groups are readily oxidised by many free radicals thus leading to profound changes in enzyme activity.

Many secondary products resulting from LPX can produce a wide range of damaging effects outside of the local site of the reaction by diffusing in the plane of the membrane and reacting further, thereby spreading the biochemical lesion, e.g. lipid hydroperoxy radicals and lipid hydroperoxides (65). These secondary products can undergo degradation reactions leading to a complex variety of products including malondialdehyde (66, 67), 4,5-dihydroxydecenal (68) and

4-hydroxynonenal (69). These aldehydes are capable of inducing membrane damage, notably MDA which has two free amino groups (the latter two have only one) and can therefore both inter and intramolecular X-link amino group containing membrane phospholipids and proteins (105, 106). This reaction gives rise to a whole spectrum of complex and stable "end products" of lipid peroxidation. (The chemical nature and structure of these are discussed in a later section). It is noteworthy that the presence of these cross-linked fluorescent products is ubiquitous in biological tissues and they are now known to arise as a result of the normal ageing process (107), - collectively these are referred to as lipofuscin. However, accumulation of lipofuscin proceeds at a much slower rate during senescence as opposed to the onslaught of free radical-induced membrane lipid peroxidation (107).

2.5 PROTECTIVE MECHANISMS

2.5.1 Enzymic Defence Systems

Superoxide Dismutase (SOD)

Unlike other antioxidant enzymes, the SOD's use free radicals as their substrate (108) and their presence in virtually every aerobic tissue provides evidence that their substrate is widely generated. 4 different forms of SOD have been found to date, their classification based on the metal ions associated with them (109):-

(i) Contains Cu and Zn (Eukaryotic cytosol).

(ii), (iii) Contain Mn (Mitochondrial matrix of eukaryotes and prokaryotes).

(iv) Contains Fe (Prokaryotes).

SOD's catalyse the following reaction:-

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

Thus the enzymic dismutation of O_2^- into H_2O_2 and O_2 prevents the formation of highly oxidising singlet oxygen and also the possible transition metal ion-catalysed Haber-Weiss reaction of O_2^- with H_2O_2 yielding the $OH^\cdot$ radicals. Although the reaction generates potentially toxic H_2O_2, both catalase and glutathione peroxidase (GPX) destroy the H_2O_2 produced. In erythrocytes SOD and catalase are extracted in the same fraction as haemoglobin while GPX appears to be "loosely bound" to the cellular structure suggesting that catalase acts in series with SOD against bursts of oxygen radicals formed from oxyhaemoglobin, while GPX may protect the cell membrane against low H_2O_2 concentrations (110).

Catalase

As mentioned above, catalase is partially responsible for destroying H_2O_2 produced by the action of SOD:-

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

Catalase is found in the peroxisomes of most cells, but in erythrocytes it is found in the same compartment as SOD (cytocol). The Km of catalase for H_2O_2 is considerably higher and it has a greater catalytic capacity, than that of GPX and so catalase is better suited to dealing with sudden elevations in H_2O_2 rather than low steady state levels (111).

Glutathione Peroxidase

GPX, a tetramer (M.W. 84,000) is unusual for a peroxidase since it contains no haem of flavin prosthetic group. Tissue GPX activity is due both to a selenium containing enzyme (Se GPX) (112) and to a non-selenium

dependant enzyme (non Se GPX (112, 113). Se GPX catalyses the breakdown of H_2O_2 and organic hydroperoxides whereas non-Se GPX catalyses the breakdown of the latter, but has no effect on H_2O_2.

The reaction catalysed by GPX is:-

$$\underset{\text{Reduced Glutathione}}{2GSH} + H_2O_2 \text{ (or ROOH)} \underset{\text{Peroxy acid}}{} \longrightarrow \underset{\text{Oxidised Glutathione}}{GSSG} + H_2O \text{ (or ROH)} \underset{\text{Hydroxy acid}}{}$$

By scavenging H_2O_2, GPX prevents $OH^{\cdot}$ radical formation in the same way as catalase but has the added advantage of destroying lipid peroxides.

Glutathione Reductase (GR)

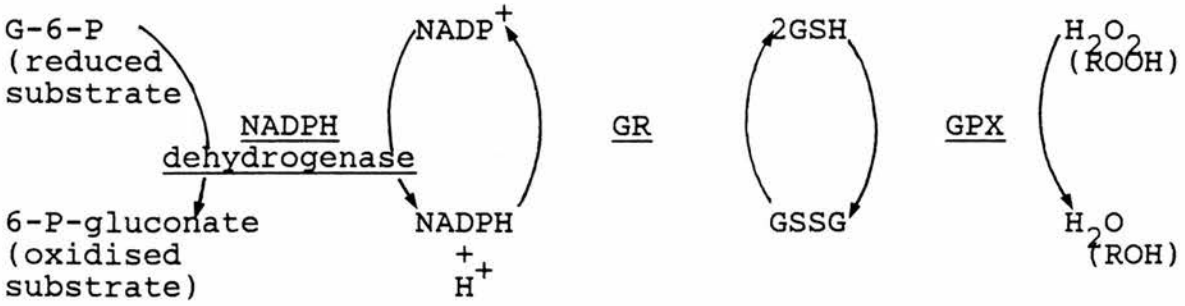
GR is responsible for recycling reduced glutathione which is converted to oxidised glutathione by the action of GPX. The reaction catalysed is:-

$$GSSG + NADPH + H^+ \longrightarrow 2GSH + NADP^+$$

NADPH generating dehydrogenases

As illustrated above, NADPH is essential for GR to regenerate GSH from the action of GPX. Most of the NADPH is regenerated for $NADP^+$ by the dehydrogenase enzymes of the pentose phosphate pathway i.e. Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (114).

The interrelationship linking GPX, GR and NADPH dehydrogenase is shown below (115).



2.5.2 Non Enzymic Defence Systems

Vitamin E (α-tocopherol)

α-tocopherol is the predominant form of Vitamin E in both plasma and red blood cells, γ -tocopherol being the only other significant contributor (116). Vitamin E is the only lipid soluble antioxidant and its lipophilic structure allows it to scavenge free radicals within the cell membrane. It inhibits the propagation process of LPX by acting as a free radical scavenger, by interacting with peroxy radicals preventing the propagation reaction since it forms the less reactive tocopheroxyl radical (72).

Vitamin C (ascorbate)

Vitamin C acts as a free radical scavenger in the water soluble phase quenching both $OH^{\cdot}$ and O_2^- radicals in addition to SOD (117). It is also responsible for the regeneration of vitamin E from tocopheroxyl radicals at the membrane/aqueous interface.

Transferrin

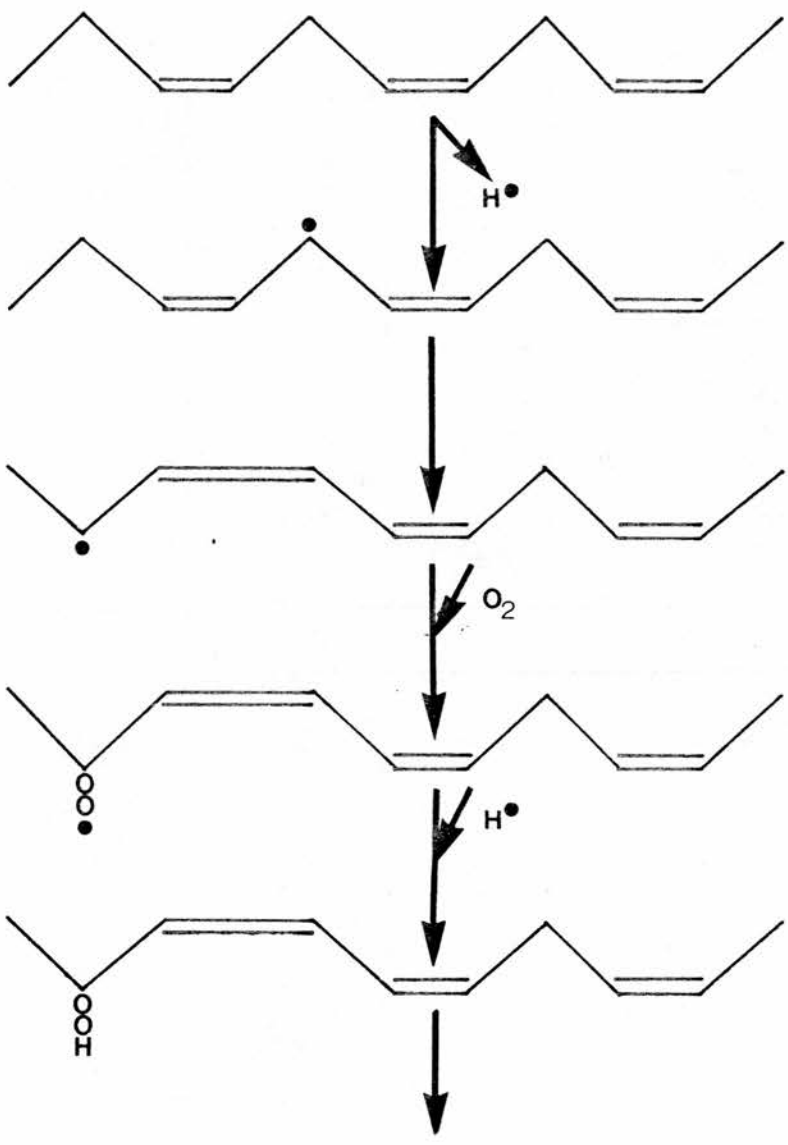
This protein scavenges extracellular Fe^{3+}. The Fe^{3+}-transferrin is then taken up intracellularly by receptor mediated endocytosis, thus making less free Fe^{2+} available for the Fenton reaction.

Caeruloplasmin (Cp)

The precise biological function of the copper containing plasma protein Cp remains uncertain, however its multifunctional role as a plasma transport protein, as an oxidase enzyme and as an antioxidant have been established (118-122). The ferroxidase activity of Cp (i.e. its ability to oxidise Fe^{2+} to Fe^{3+}) is important in allowing Cp to

FIGURE 2

MEASUREMENT OF LIPID PEROXIDATION



PUFA

SUBSTRATE LOSS BY GLC ANALYSI

CONJUGATED DIENES

UV ABSORBANCE 232 nm

MOLECULAR OXYGEN

UPTAKE USING O_2 ELECTRODE

LIPID HYDROPEROXIDES

CHEMICAL ASSAY

CARBONYLS

eg. MALONDIALDEHYDE

TBA-REACTIVE

UV ABSORBANCE 532 nm

SPECTROFLUORIMETRY 553 nm

LIPID SOLUBLE FLUORESCENT PIGMENTS

SPECTROFLUORIMETRIC ASSAY

340-370 nm EXCITATION

420-470 nm EMISSION

inhibit iron dependant radical reactions such as LPX (121) and thus allows the loading of Fe^{3+} onto transferrin. Like other α-globulins, Cp is an "acute phase" protein and is raised whenever there is active tissue damage and during inflammation it could act as an antioxidant by (122):-

(i) Preventing decompartmentalised iron acting as a free radical catalyst.

(ii) Directly inactivating free radicals produced by phagocytes which may escape into extracellular fluids (these do not contain SOD and catalase).

There are many other protective agents present *in vivo* to combat the toxic effects of membrane lipid peroxidation. These include free glutathione, uric acid, mannitol, albumin, haptoglobin, and hydroquinone. Very recently it has been proposed that the enzyme phospholipase A_2 (123) has an essential function in the detoxification of lipid peroxides formed during LPX.

2.6 DETECTION AND ASSAY OF LIPID PEROXIDATION

Lipid peroxidation is notoriously difficult to measure in biological systems due to the transient nature of the intermediates and the multiplicity of end products formed. Many techniques developed to date measure a different intermediate or end product and no one method may be used to give an accurate, absolute measure of LPX (62). The following section (and Figure 2) describes some of the methods used for detection of LPX.

(i) O_2 uptake and loss of unsaturated fatty acid

The O_2 uptake coupled to peroxidation can be readily followed using an O_2 electrode (61), however in biological

systems O_2 uptake is associated with a variety of enzymic steps and the percentage of total cellular O_2 caused by radical reactions can not be accurately determined. The loss of PUFA's may be determined by gas chromatography following extraction of membrane lipids (83), however extreme care must be taken to ensure minimal oxidative damage through handling.

(ii) Volatile hydrocarbons

Measurement of volatile hydrocarbons derived from the transition metal-ion catalysed breakdown of lipid hydroperoxides can be measured by GLC analysis e.g. ethane, pentane (124, 125, 126).

(iii) Lipid hydroperoxides

Measurement of lipid hydroperoxides as an indicator of LPX has not been so widely used as other methods since in practice they are easily broken down by transition metal ions, by their reduction with thiols and by their metabolism with peroxidases such as GPX. Chemical methods have been described based on iodometric titration and on peroxidase-catalysed reactions, (127) but these have been subject to interference and lack specificity.

(iv) Conjugated dienes

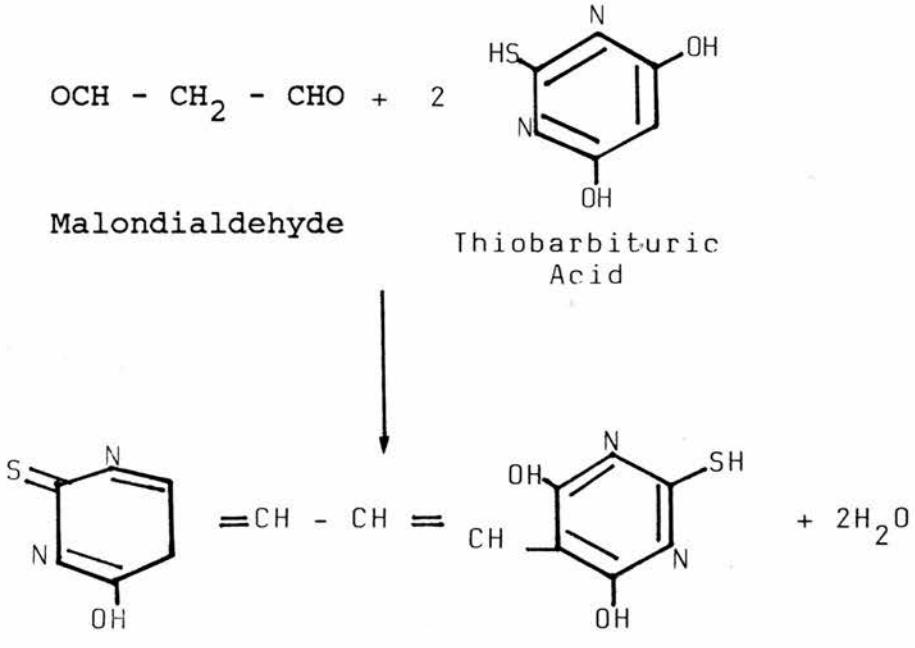
Like many other substances, naturally occuring lipids exhibit simple end absorption in ultraviolet light (U.V.) as the wavelength is lowered toward 200 nm (128). However oxidation of unsaturated fatty acid side-chains is accompanied by the formation of conjugated dienes which are characterised by intense absorption, the so-called K band in the 230-235 nm wavelength range. Their spectrophotometric measurement in biological materials may be carried out

following extraction procedures. Recently using HPLC analysis to detect these major UV absorbing products in human serum, bile and duodenal juice (129), it has been shown that these are not simple conjugates of the type illustrated in Figure 2 and that the predominant fatty acid conjugated diene *in vivo* is an isomerised $C_{18:2}$ compound (octadeca 9, 11-dienoic acid) esterified to cholesterol esters, triacyl-glycerols and phospholipids.

(v) Thiobarbituric Acid-Reactive Substances (TBARS)

The TBA test is the oldest, simplest and most frequently used test as a measure of lipid autoxidation both in the food industry and in biological research (130). The test depends on the reaction between malondialdehyde and thiobarbituric acid (TBA) under acidic conditions to form a red chromogen which can be measured spectrophotometrically at 532 nm.

Basically, MDA combines stoichiometrically with TBA (1:2) to form an adduct as shown below:-



2:1 TBA-MDA Adduct

Since this adduct is also fluorescent at 553 nm, a more sensitive assay may be achieved using spectrofluorimetry (131, 132).

The specificity of the TBA test has been a subject of controversy. The test itself is usually calibrated using MDA prepared by the hydrolysis of 1,1,3,3,-tetramethoxypropane (TMP) or 1,1,3,3,tetraethoxypropane (TEP) (133, 134, 135) and the results expressed in terms of the amount of MDA produced in a given time. However the test detects not only free MDA and much of the MDA detected in biological samples arises as a result of the breakdown of lipid peroxides under the conditions of the assay (heat, acid, metal ions). There are many modifications of this test adapted to eliminate interference from other biological molecules. For example TBA strongly reacts with sialic acid in trichloracetic acid solution but not in acetic acid (131). Water soluble substances which react with TBA to yield the same product as lipid peroxides in body fluids such as serum or plasma, may be removed by isolating the lipids by precipitation of serum protein and lipoprotein using phosphotungstic acid-sulphuric acid systems (132). (This method also eliminates TBARS liberated from platelet aggregates during sampling of blood).

More recently, several methods have been developed for assaying TBA-reactive MDA adducts in biological fluids using various HPLC systems (136-140).

(vi) Fluorescent pigment assay

As already described, LPX generates a heterogeneous mixture of lipid and water soluble fluorophores of uncertain structure. Direct measurement of the formation of

fluorescent products is an extremely sensitive assay of LPX and unlike the TBA assay which measures transient intermediates, the fluorescent pigment assay is reported to measure stable end products (even though only a small fraction of the total end-products are measured) (62). Whatever the chemical nature of these products (see later section) they are readily extracted from biological systems; if the amino donor is water soluble (e.g. RNAse) a polar fluorescent product is formed and if a phospholipid is involved a non-polar product is formed which can be extracted into chloroform-methanol.

Whether the lipid soluble fluorescent pigments are extracted from biological tissues (e.g. "age pigment") or from peroxidising polyunsaturated lipids, they exhibit characteristic emission maxima in the 420-470 nm range when excited at 340-370 nm (140, 141).

(vii) Oxidative stress test

This is a specialised test used by many workers to measure the extent of LPX in biological tissues, notably erythrocytes and haemolysates (64, 85, 143-147). Basically non-enzymic oxidative breakdown can be induced in red cells by exposure to H_2O_2 and the degree of LPX products generated (measured by the TBARS assay) is proportional to the susceptibility of the tissue to oxidative stress; i.e. the test back-titrates the total antioxidant defences against H_2O_2 concentrations.

Although much work has been published on methods for assaying LPX products, no single technique has proved to be entirely specific and each has its own drawbacks and

limitations. Within this context, a sensitive assay for a clearly defined, chemically characterised end product of LPX which is quantitatively important in biological systems has yet to be developed.

2.7 THE NATURE AND STRUCTURE OF FLUORESCENT PRODUCTS OF LIPID PEROXIDATION

As already described, polyunsaturated lipids readily undergo peroxidation to be transformed into hydroperoxides, which in turn degrade to a mixture of secondary products such as aldehydes, epoxides, ketones and other products. The ubiquitous, natural compound malondialdehyde (MDA) is produced in substantial quantities in mammalian tissues both as a side product of prostaglandin and thromboxane biosynthesis (148) and as one of the most important secondary products of peroxidation of PUFA's (149(. It is now fairly well established that this highly reactive aldehyde, once formed during LPX in biological systems, will lead to the formation of fluorescent chromolipids (150, 151) which have very similar characteristics to those in chloroform-methanol extracts of the age pigments: ceroid and lipofuscin (66, 141, 142). These stable end products of lipid peroxidation which accumulate in tissues (resist oxidation by tissue aldehyde oxidases (152)) are proposed to be derived by cross-linking reactions of MDA with a wide range of biological molecules containing free amino groups e.g. proteins (66), DNA (103) and phospholipids PE and PS (153). Several studies on the reaction between MDA and various biological molecules have been carried out in an attempt to elucidate the precise nature of these fluorescent pigments

FIGURE 3

FORMATION OF FLUORESCENT PIGMENT

PUFA

↓

LIPID PEROXIDES

↓

MALONDIALDEHYDE
(MDA)

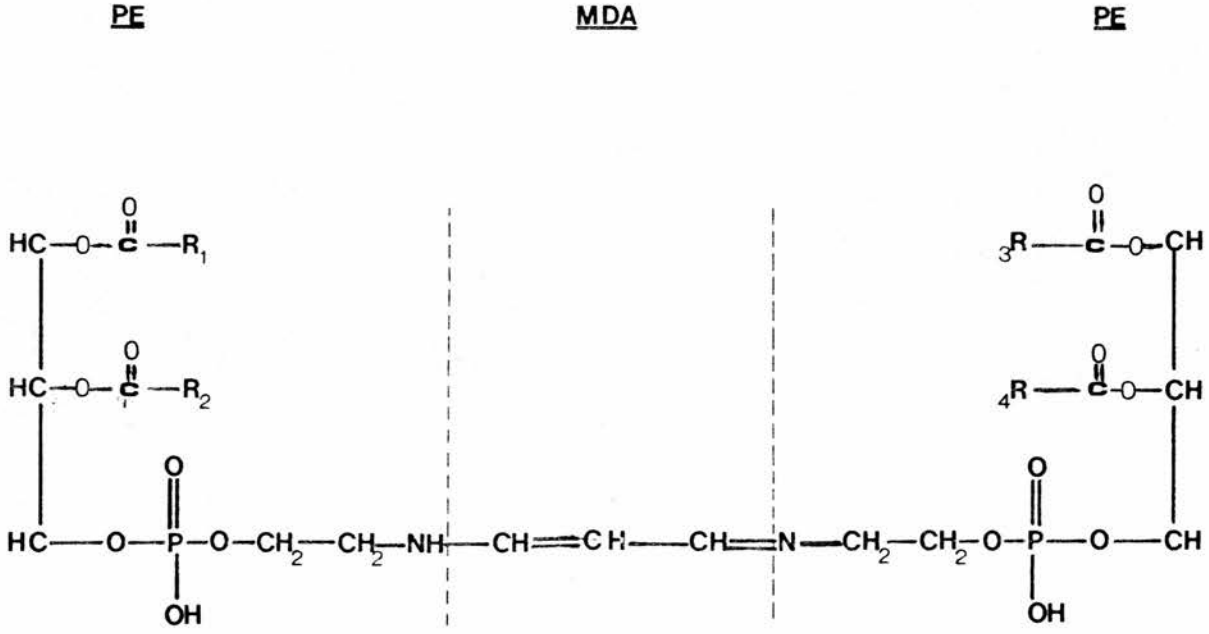
+

PHOSPHATIDYL ETHANOLAMINE
(PE)

↓

FLUORESCENT PIGMENT

e.g. PE–MDA HOMODIMER



and to clearly define the products which are quantitatively important in biological systems. The following section reviews the work published to date.

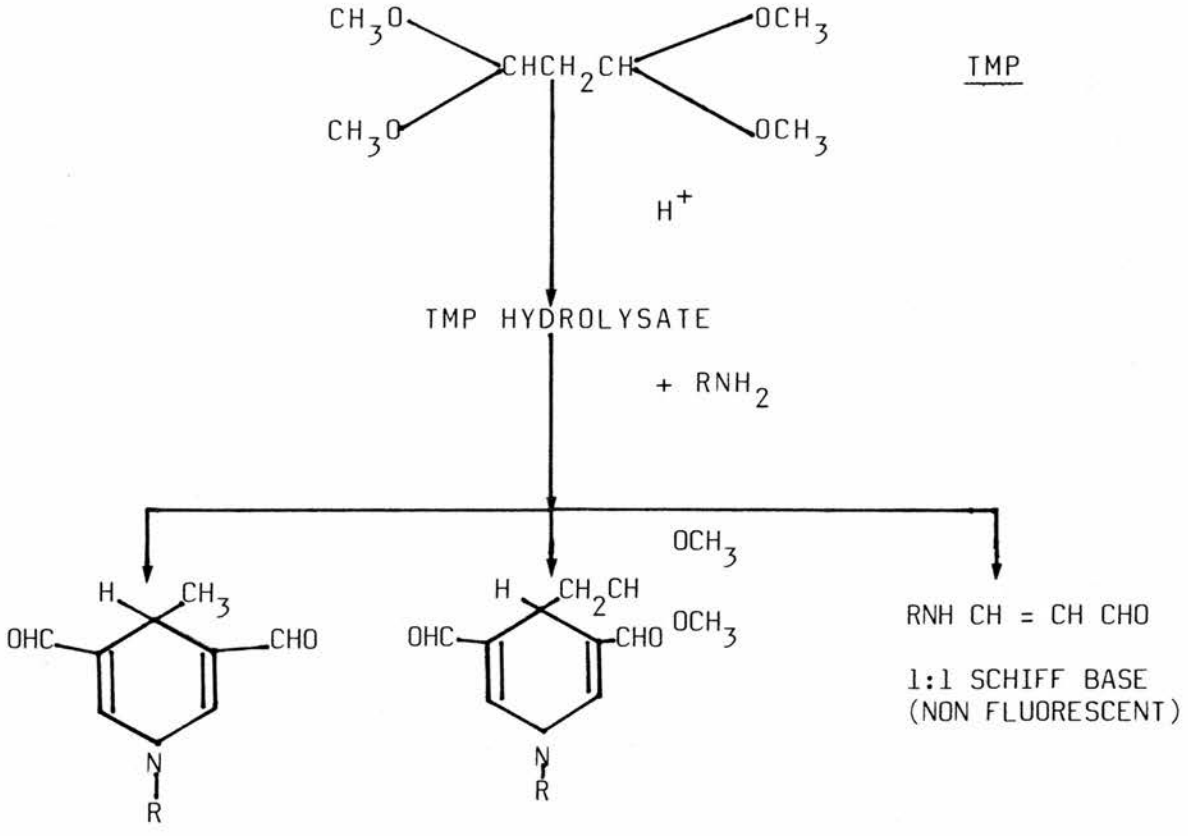
A. Synthetic studies

In the 1960's, the in vitro studies of Chio and Tappel between MDA and primary alphatic amines (66, 142) under strongly acidic conditions were shown to yield N,N'-disubstituted 1-amino-3 iminopropenes which are conjugated Schiff bases. The chromophoric system responsible for fluorescence is > N - C = C - C = N - and a proposed pathway for the formation of fluorescent pigment derived from PE and MDA is shown in Figure 3 (102).

These pigments were shown to have characteristic excitation and emission maxima in the range fo 340-370 and 420-470 nm respectively. Fluorescent product formation with similar but not identical characteristics has also been shown to occur in oxidising subcellular particles (150, 154), amino acid and phospholipid systems (153, 155), peroxidising arachidonic acid and DNA (103) and antioxidant-deficient mouse (156) etc. Because of the high sensitivity, fluorescence measurements are widely used as a parameter of LPX in vitro and in vivo (157-159).

Since the work of Chio et al. proposing that these fluorescent biological products are Schiff bases possessing the 1-amino-3-iminopropene structure, Nair et al. (148) have demonstrated that the reaction between MDA and amino acids under more physiological mildly acidic conditions, failed to give adducts with such properties. They suggested that non-fluorescent 1:1 Schiff bases were generated.

The very much more recent work of Kikugawa et al. (67, 106, 160, 161) also suggests that the compounds responsible for the fluorescence are very much different to those suggested by the earlier findings of Chio and Tappel. By analysing the products of the reaction of amines with MDA under the more physiological mildly acidic conditions, Kikugawa et al. proposed that the molecular species responsible for fluorescence are dihydropyridine derivatives. The major and complex products formed from the reaction between the methylamine and pure MDA and with acidic TMP hydrolysate are shown below:-



PRODUCT A

1,4-dimethyl-1,4, dihydropyridine,-3,5 dicarbaldehyde

PRODUCT B

1-methyl-4 (dimethoxyethyl) -1-4 dihydropyridine dicarbaldehyde

PRODUCT C

Mild reaction of methylamine and MDA produced a single fluorescent compound (A) and both (A) and (B) were produced with acidic TMP hydrolysate, the latter arising as a result of impurities in the TMP hydrolysate. However the major product of the acidic reaction (C) was a non fluorescent 1:1 Schiff base. Since the fluorescence spectra of (A) and (B) were not identical to those of lipofusin and $CHCl_3$-extractable fluorescence for peroxidised biological systems, it was suggested by these authors that it is highly unlikely that MDA alone contributes to the formation of fluorescent components in age-pigments or related biological fluorescent substances.

Recent interest in 1986 has focused upon the role played by other aldehydes in the generation of these fluorescent chromolipids with the advent of Esterbauers' work (69) with 4-hydroxynonenal (4-OH NA). This LPX product had been shown earlier to be formed in significant amounts during the LPX process (162, 163) and is most reactive to PE and PS leading to the formation of a fluorescent chromophore identical in the emission maximum (430 nm) and excitation maximum (360 nm) with the fluorophore formed in peroxidising microsomes and mitochondria (69). However, as yet structures have not been proposed for the products formed and their biological significance relies on precise characterisation of their origin.

B. Biological studies

As previously mentioned, much attention has been focused upon characterising fluorescent pigments of lipid peroxidation in biological tissues using various TLC and

spectrofluorimetric techniques following extraction of the lipids. The biological tissues investigated include notably: rat liver microsomes, human erythrocytes, brain homogenate, mitochondria and hepatocytes.

Dillard et al. (150) have shown that during non-enzymatic ascorbate induced LPX in microsomes that there is an increase in fluorescence due to formation of chromolipid and Koster et al. (151) report fluorescent products with identical characteristics in both microsomes and hepatocytes when subject to enzymatic NADPH dependant LPX. Other investigators (164) have shown that erythrocytes generate these fluorescent pigments when stressed with H_2O_2, causing specific loss of aminophospholipids. This suggests that these products are formed in the cross-linking or other modifications of PE and PS during the process of LPX of membranes (165). Jain et al. (85) have shown that addition of MDA to erythrocytes in vitro causes the formation of these chromolipids and the same products have been generated by Koster et al. (166) by stressing erythrocyte ghosts with TBH. Jain et al (100) examined lipid extracts from sickled-cell erythrocyte and MDA incubations by TLC analysis and identified a novel lipid spot chromatographing between PS and PE. This 'spot' was phosphorus positive and ninhydrin negative, fluoresced under U.V. light and its intensity was proportional to the concentration of MDA used. By incubating PE + PS (1:1) with MDA in vitro, an identical product was formed, and it was suggested that this spot was the heterologous (2:1) Schiff's base adduct formed by cross-linking amino groups of PE and PS with MDA.

Thus it can be summarised that a great deal of experimental research has taken place in attempts to characterise and structurally define the major products of LPX in biological systems *in vitro*. However as yet, the literature is full of conflicting observations and no single area of research has provided conclusive evidence regarding the chemical nature and precise origin of these fluorescent compounds.

3. EVIDENCE FOR THE ROLE OF LPX IN M.S.

Since the primary pathogenesis of M.S. is essentially degeneration of CNS myelin, and LPX is involved in other degenerative conditions, it seems possible that membrane LPX may play a role in the disease process, and that membrane damage may be reflected in other cell types, e.g. erythrocytes. There is much evidence to suggest that LPX is implicated in the pathology of M.S.:-

1. Membrane-associated changes in erythrocytes from M.S. patients have been reported including; increased osmotic (167-170) and mechanical (171) fragility; increased size and altered morphology (168, 172) and decreased electrophoretic mobility following incubation with linoleic acid (173).
2. Increased incidence of M.S. in populations consuming high proportions of animal fat have been reported (174). Since Vitamin E is predominantly associated with dietary plant lipids, its deficiency may be reflected in its antioxidant role and consequently more free radicals will be available for initiating the peroxidative process.
3. Abnormal activities of the antioxidant enzymes GPX (175-178) and SOD (179) have been reported in M.S. erythrocytes, lymphocytes and granulocytes.
4. Decreased proportions of phospholipid classes which have the highest PUFA content in M.S. myelin have been reported (17). These are the acidic phospholipids. However this may be due to the fact that before or

after subsequent extraction and analysis their peroxidisibility could lead to preferential losses.

5. M.S. tissues including platelets, serum, leukocytes, brain and erythrocytes have been found to contain decreased PUFA's, especially linoleic acid (52, 57, 179-181).

6. There have been reports of increased incidence of M.S. (and also nutritional muscular dystrophy in cattle) associated with deficiency of Vitamin E and Selenium in certain areas of Finland (182). Selenium is an essential cofactor for GPX.

7. Elevated MDA concentrations have been reported both in M.S. plasma at 1.5 - 2 X normal levels (183) and in CSF (184). Lipid-soluble fluorescent pigments were also shown to be increased in CSF (184).

Considerable attention has been directed towards the possible role of free radical intermediates in the inflammatory process and in particular the beneficial effects of SOD, serving to decrease concentrations of O_2^- radicals generated by stimulated white cells attracted to the site of damage by chemotactic agents (61). As discussed previously, O_2^- generates $OH^\cdot$ radicals which initiate LPX thus leading to cell membrane damage. In M.S., this could lead to the generation of antigens within the cell membrane and stimulate an immune response much in the same way as a viral infection is capable of producing a similar effect.

Free radical intermediates are also implicated in cell killing by "professional phagocytes". It has been shown that in the later stages of M.S., an infiltration of macrophages

into the CSF accompanies the inflammatory process (17) due to a leaky blood-brain-barrier (18). These activated cells display an "oxidative or metabolic burst" during ingestion of foreign antigens whereby a shower of reduced oxygen species is liberated (185). LPX may then be induced as discussed previously leading to degeneration of the myelin membrane. However this phenomenon also occurs as part of several other infections and inflammatory brain lesions, nevertheless its existance may play a significant, if only minor role in the aetiology of the disease. Elevated levels of LPX products seen in CSF and plasma could thus be evidence of phagocytic cell activity.

4. AIMS OF THIS WORK

This work was carried out for three main reasons:-

1. To characterise the products of the reaction between the amino-group containing phospholipids PE and PS, and the bi-functional agent MDA in synthetic in vitro reaction systems.

It was envisaged that thin layer chromatography (TLC), spectrofluorimetry and high performance liquid chromatography (HPLC) would be carried out to separate the products of the reaction and various chemical tests employed to establish their identities.

2. To establish whether these LPX products are generated in model biological systems.

It was intended to induce LPX in three biological tissues; rat liver microsomes, human erythrocytes and sheep brain homogenate, and to extract and compare these reaction products with those generated in the synthetic phospholipid systems.

3. To identify these LPX products in M.S. fluids.

After partially characterising the LPX products generated in biological systems derived from PE and PS, it was hoped to identify their presence in plasma

and/or CSF from Multiple Sclerosis patients, since pilot studies in our laboratory (184) have shown a significant increase in LPX indices in CSF from M.S. patients. It was hoped that by examining plasma and CSF from both M.S. patients and patients suffering other neurological diseases (controls); that an HPLC assay system could be developed, which might form the basis for a diagnostic test. It was also the intention to carefully correlate indices of LPX with age, sex and severity of disability. Finally it was intended to examine the susceptibility of M.S. haemolysates to oxidant stress induced by TBH and H_2O_2, and to compare this susceptibility with that from other neurological diseases, since pilot studies in our laboratory (64) had indicated that erythrocytes from M.S. patients were less susceptible to oxidative stress than controls.

MATERIALS AND METHODS

MATERIALS AND METHODS

1. GENERATION OF PRODUCTS FROM THE REACTION OF MALONDIALDEHYDE WITH PHOSPHATIDYL ETHANOLAMINE AND PHOSPHATIDYL SERINE

1.1 REACTION CONDITIONS

Since the major lipid soluble lipid peroxidation (LPX) products had been reported to consist of the amino containing phospholipids phosphatidyl ethanolamine (PE) and phosphatidyl serine (PS) crosslinked by malondialdehyde (MDA), an attempt to synthesise model LPX products derived from MDA and PE and PS was carried out using a modification of the method of Trombley and Tappel (105). This method required the preparation of MDA via acid hydrolysis of 1,1,3,3,tetramethoxypropane (TMP) without subsequent neutralisation. Previous work in our laboratory showed that these conditions clearly generated MDA adducts but also lyso PE and so by using neutralised TMP hydrolysate it was anticipated that a spectrum of products would arise under the more "physiological conditions".

1.1.1 Dipalmitoyl PE and MDA

1.1.1.1 Preparation of neutralised TMP hydrolysate (161)

1.64g of TMP was mixed with 0.9 mls 1M HCl at 40^{o}C in a shaking water bath until homogeneous. A 1M MDA solution was then prepared by making this solution up to 10.0 mls with distilled H_2O and incubating at 37^{o}C for 1 hour. The solution was neutralised to pH 7.0 with 5M NaOH.

1.1.1.2 Reaction systems

The method of Trombley and Tappel was further modified in that it was also decided to investigate the products formed from two differing molar ratios of MDA to PE namely:-

(i) PE-MDA 1:2

(ii) PE-MDA 4:1

and in two different dispersion mediums:-

(i) 0.1M phosphate buffer pH 7.0

(ii) $CHCl_3$/MeOH/H_2O (1:2:0.8)

The reaction systems and controls were prepared as shown in Table 1.

TABLE 1

PE (mg)	1M MDA (µl)	PE:MDA RATIO
25	9	4:1 Reaction
25	72	1:2 Reaction
0	9	4:1 MDA Control
0	72	1:2 MDA Control
25	0	PE CONTROL

Each reaction system was then dispersed in either phosphate buffer or $CHCl_3$/MeOH/H_2O as shown below:-

(i) Phosphate buffer

25 mg PE was mixed with 5 mls 0.1M phosphate buffer pH 7.0 in a scintillation vial and sonicated for 5 x 1 minute periods at 6 microns with 30 seconds cooling between each period. The vial was surrounded by an ice jacket during sonication. 0.6 mls Triton X-100 was then added to further disperse the mixture and the appropriate volume of 1M MDA solution added.

(ii) $\underline{CHCl_3/MeOH/H_20}$

25 mg PE was mixed with 1.4 mls distilled H_20 in a scintillation vial and sonicated excatly as described above. Finally 1.9 mls $CHCl_3$ and 3.8 mls MeOH was added together with the appropriate volume of 1M MDA solution. Thus a monophasic Bligh and Dyer (186) system was achieved with the $CHCl_3/MeOH/H_20$ ratio being 1:2:0.8.

Each reaction system or control was incubated at 37°C for up to 12 days. Aliquots were removed at daily intervals and the characteristic fluorescent excitation and emission maxima determined for each system. Organic systems were measured directly in, and any necessary dilutions made using, monophasic Bligh and Dyer reagent. Fluorescent lipids in the phosphate system were extracted according to the method of Kikugawa (161) by mixing 0.1 ml of sample with 100 mg NaCl and 0.15 ml 1N NaOH. 1 ml $CHCl_3$ was then added, mixed and centrifuged at 2000g for 15 minutes. The fluorescence of the lower $CHCl_3$ phase was then recorded and all results expressed as relative fluorescent units versus time. Finally the remaining total reaction mixtures were extracted accordingly, prior to further analysis of the products.

1.1.2 Egg Yolk PE and MDA

Reaction systems were prepared as described in Section 1.1.1 substituting egg yolk PE for dipalmitoyl PE, since the fatty acids esterified to the former PE are unsaturated and may therefore be easily monitored using U.V. detection on HPLC. Also, since the reaction products generated were found to be the same using ratios of PE-MDA 1:2 or 4:1 and that the yield was greater with the 1:2 ratio, it was decided to

prepare systems incorporating PE-MDA ratios of 1:2 only. Furthermore in reaction mixtures dispersed in phosphate buffer, the Triton X-100 was extracted into the organic phase and found to interfere with TLC analysis, hence the synthetic systems were dispersed in $CHCl_3$/MeOH/H_2O only.

1.1.3 Bovine brain PS or PS + PE and MDA

Reaction mixtures were prepared as described in Section 1.1.2 incorporating the only other amino group containing phospholipid PS, in order to compare the reaction products formed between the two different phospholipids. Also systems containing both PE and PS with MDA were prepared.

1.1.4 ^{14}C PE and MDA

From the various detection methods used to examine the thin-layer chromatograms of synthetic phospholipid systems, it was apparent that a complex mixture of fluorescent products was generated, many of which did not contain phosphorus. Some, if not all, of these may be derived from MDA by polymerisation. To distinguish these from products derived from PE, it was decided to prepare a synthetic system incorporating ^{14}C PE. (L-3-phosphatidyl[2-C14] ethan-1-ol-2-amine, dioleoyl in toluene/ethanol solution. 50-60 mCi/mM, 376 K Bq). A reaction system was set up exactly as detained in Section 1.1.2 incorporating 4 µCi of the labelled PE together with a PE control. The reaction products were then extracted exactly as described previously in Section 1.1.1.2 except that prior to extraction, a small aliquot of both $CHCl_3$ and MeOH/H_2O phases was retained for liquid scintillation counting.

1.2 TLC ANALYSIS OF LIPID PEROXIDATION PRODUCTS

1.2.1 TLC Plates and Adsorbent

TLC plates 20 cm x 20 cm in size, coated with silica gel 60 were either obtained commercially or laboratory prepared. Commercial plates were of 0.25 mm thickness and laboratory prepared plates were of 0.5 mm or 1 mm thickness. The silica adsorbent was pre-washed in an appropriate solvent immediately prior to preparing the laboratory manufactured plates. Immediately prior to chromatography, all plates were activated in a 110^{o}C oven for 1.5 hours.

1.2.2 Unidimensional TLC

1.2.2.1 Analytical TLC

Up to 100 µl of lipid extracts containing up to 50 µg of lipid per spot were applied to commercially prepared plates and up to 100 µg for 0.5 mm laboratory prepared plates. Samples were spotted 1 cm from the base line and approximately 1 cm apart. A space of 1 cm was left at each edge of the plate and as many as 14 samples could be spotted on a single 20 cm x 20 cm plate. The plates were then developed immediately at 4^{o}C. Standard phospholipids PC, PS, PE, PI and Sm were also run in order to identify their presence in synthetic, biological and clinical samples. In all cases tanks were lined with chromatography paper, pre-equilibrated with solvent for at least 1 hour and sealed with adhesive tape to ensure minimal loss of solvent through evaporation. The following solvent systems were selected and used after considerable development work to optimise separation, as shown in Table 2.

TABLE 2

	SOLVENT SYSTEM	APPLICATION
A.	$CHCl_3$/MeOH/NH_4OH (aq. 35%) (16:8.5:1 v/v)	To separate major phospholipid classes in biological samples.
B.	n-propanol/H_2O (3:1 v/v)	To separate fluorescent LPX products in biological samples.
C.	Propan-1-ol/NH_4OH (aq. 35%)/H_2O (6:1:0.75 v/v)	To separate fluorescent lipids in biological fluids. (CSF and plasma).

TLC solvent B was adapted from Levis et al. (187).

1.2.2.2 Preparative TLC

Up to 500 µl of lipid extracts containing up to 3 mg of lipid was strip loaded at the origin of 1 mm plates. These were then developed as described previously in Section 1.2.2.1 in an appropriate solvent system detailed in Table 2.

1.2.2.3 2-Dimensional TLC

Up to 100 µl of lipid extracts containing up to 100 µg of lipid were applied to the right hand corner of the plate approximately 1 cm from each edge. The plates were then developed in the first dimensional solvent until the solvent

front was within 1 cm of the top of the plate, then dried thoroughly using warm air, rotated clockwise through 90° and developed in the second solvent.

The following solvent systems were finally used routinely after experimenting with the ratios of the components in order to achieve optimum separation:-

FIRST SOLVENT	$CHCl_3$/MeOH/NH_4OH (aq. 35%) (16:8.5:1 v/v)
SECOND SOLVENT	$CHCl_3$/MeOH/HAc (4.2:2.3:1 v/v)
APPLICATION	To separate major phospholipid classes and LPX products.

1.3 DETECTION OF LIPID PEROXIDATION PRODUCTS

1.3.1 Autofluorescence

In order to detect any autofluorescent products, the plates were examined under a U.V. transilluminator (300-500 nm band range) and photographed using FP4 type film of medium contrast. Photographic exposure time varied between each chromatogram, although the camera setting of f 5.6 remained constant.

1.3.2 I_2 Staining

The plates were placed in a tank saturated with I_2 vapour which non-specifically stains any lipid material present and then photographed.

1.3.3 Detection of Free Primary Amino Groups

The detection reagent was prepared by dissolving 0.25% ninhydrin in acetone-lutidine solution (9:1 v/v). Plates were sprayed with this reagent until evenly wet and allowed to dry at room temperature. After 1-2 hours purple/mauve

spots appear identifying primary amino containing lipids.

1.3.4 Detection of Phosphorus

A. Primary solutions -

(i) 5N H_2SO_4.

(ii) Antimony potassium tartrate solution.

1.37g K(SbO) $C_4H_4O_6 \cdot \frac{1}{2}H_2O$ dissolved in 500 ml distilled H_2O and stored refrigerated in a dark bottle at 4°C.

(iii) Ammonium molybdate solution.

20g $(NH_4)_6$ $Mo_7O_{24} \cdot 4H_2O$ dissolved in 500 ml distilled H_2O and stored refrigerated at 4°C.

(iv) Ascorbic acid solution.

1.76g ascorbic acid dissolved in 100 mls distilled H_2O. This was stored in a refrigerator and made up freshly each week.

B. Combined reagent -

H_2SO_4 Solution	25.0 ml
Antimony potassium tartate solution	2.5 ml
Ammonium molybdate solution	7.5 ml
Ascorbic acid solution	15.0 ml

C. Detection reagent -

Combined reagent	8 ml
Iso-propyl alcohol	1 ml
Distilled H_2O	13 ml

TLC plates were sprayed with the detection reagent until evenly wet and then allowed to dry at room temperature. After 1-2 hours, blue spots appear identifying any phosphorus containing lipids.

1.3.5 Autoradiography

After TLC the plates were thoroughly dried and exposed to a sheet of Fuji X-ray film type N.I.F., RX, (20 cm x 20 cm) in a dark room. The film was sandwiched between the TLC plate and a clear glass plate, secured using adhesive tape and then inserted into a double black light-proof envelope for up to 7 days. After the required exposure period, the film was developed and dark spots were visible indicating the incorporation of ^{14}C PE into the LPX products generated.

1.4 ELUTION OF LIPIDS FROM TLC PLATES

Lipids were eluted by scraping off the fluorescent bands into centrifuge tubes together with a region from the bottom of the plate serving as a control. 15 mls of $CHCl_3$/MeOH/H_2O 1:2:0.8 v/v (monophasic Bligh and Dyer reagent) was pipetted into each tube and the mixture vortexed for 2 minutes. The tubes were centrifuged at 1000g for 5 minutes and the supernatant decanted. This extraction was repeated at least twice more to ensure that all of the lipids had been eluted. The total eluant was made up to 55 mls with monophasic Bligh and Dyer reagent and equal volumes (12.5 mls) of $CHCl_3$ and H_20 added to achieve a biphasic system. After mixing, the tubes were centrifuged at 1000g for 5 minutes and the lower organic layer filtered through 0.2 µM millipore filter to remove any traces of silica. The samples were then reduced to dryness under a stream of N_2 and redissolved in a known amount of solvent for phosphate estimation and HPLC analysis.

1.5 PHOSPHORUS ESTIMATION Rouser et al. (188)

When comparing the relative intensity of any

fluorescent adducts generated during induced LPX of microsomes, erythrocytes and brain homogenate, it was necessary to ensure that equivalent masses of phospholipid were loaded onto TLC plates for both induced and control samples. Loadings were thus based on the percentage of lipid phosphorus in the sample determined using the following method.

Appropriate aliquots of eluted lipids were transferred to kjeldahl tubes and refluxed on an electrically heated digestion rack for 30 minutes with 0.9 ml of 72% perchloric acid in order to convert all organic phosphorus to inorganic phosphate. The tubes were then allowed to cool and 7 mls distilled $H_2 0$ added. 1.0 ml 2.5% w/v ammonium molybdate was then added to complex with the inorganic phosphate and the addition of 1.0 ml 10% w/v ascorbate reduced the complex to give a characteristic blue compound which was measured spectrophotometrically. The contents were transferred to test tubes and boiled at 100°C for 5 minutes to ensure maximal colour development. After cooling, the optical density was recorded at 820 nm versus a reagent blank.

A standard curve ranging from 0-10 µg of phosphorus in the form of aqueous $Na_2 H_2 PO_4$ (5 µg P/ml) was prepared. The standard curve was linear and unknown sample sizes were adjusted to ensure that all readings fell within the range of this curve.

1.6 HPLC ANALYSIS OF LIPID PEROXIDATION PRODUCTS

1.6.1 Instrumentation

The lipid samples were further studied by HPLC gradient elution analysis. Our HPLC system consisted of two Gilson

model 303 pumps with a Rheodyne injection system and gradient elution was controlled by an Apple lle computer. The column used was a 25 cm Zorbax Sil column (5 µm silica) supplied by Du Pont Instruments and the guard column was packed with 15-25 µ silica. Detection of the lipids at 206nm was via a Gilson 303 U.V. detector and is primarily dependant on the degree of unsaturation of the fatty acid side chains of the lipids, together with functional groups such as carbonyl, carboxyl, phosphate, amino and quaternary ammonium of each molecule (Van Kessel et al., 189). Fluorescence detection was monitored via a Gilson 121 fluorometer using excitation and emission filters with a band pass range of 305-395nm and 420-650nm respectively. Retention times and quantitation by integration of peak areas was given on an automatic readout using a Shimadzu C-RIB integrator/plotter.

1.6.2 Preparation of Standard Phospholipids

A standard phospholipid mixture used throughout all HPLC analyses consisted of the following standard phospholipids, the concentrations of which gave approximately equal peak areas on detection at 206nm:-

PS (bovine brain) - 1 mg/ml
PE (egg yolk) - 1 mg/ml
★PI (soya bean) - 5 mg/ml
PC (egg yolk) - 2.5 mg/ml
Sm (bovine brain) - 10 mg/ml

★ Phosphatidyl Inositol

20 µl of this standard mixture was injected daily in order to ensure that the HPLC system was operating correctly and to detect any slight differences in retention times resulting from minor changes in conditions such as fresh batches of solvents, temperature and pressure.

1.6.3 Preparation of Solvents and Development of Gradient Systems

All solvents were of HPLC grade and prior to use they were filtered through a 0.2 µM millipore filtration apparatus and degassed by sonication for 5 minutes. Preliminary work employed the use of the gradient system developed by Patton et al. (190) for the separation of the standard phospholipids. However, after a considerable amount of development work a final solvent system was developed which resulted in the optimal separation of phospholipids and LPX products present in both model and biological systems as monitored by U.V. and fluorescence. The two solvent mixtures developed for gradient elution analysis are detailed below:-

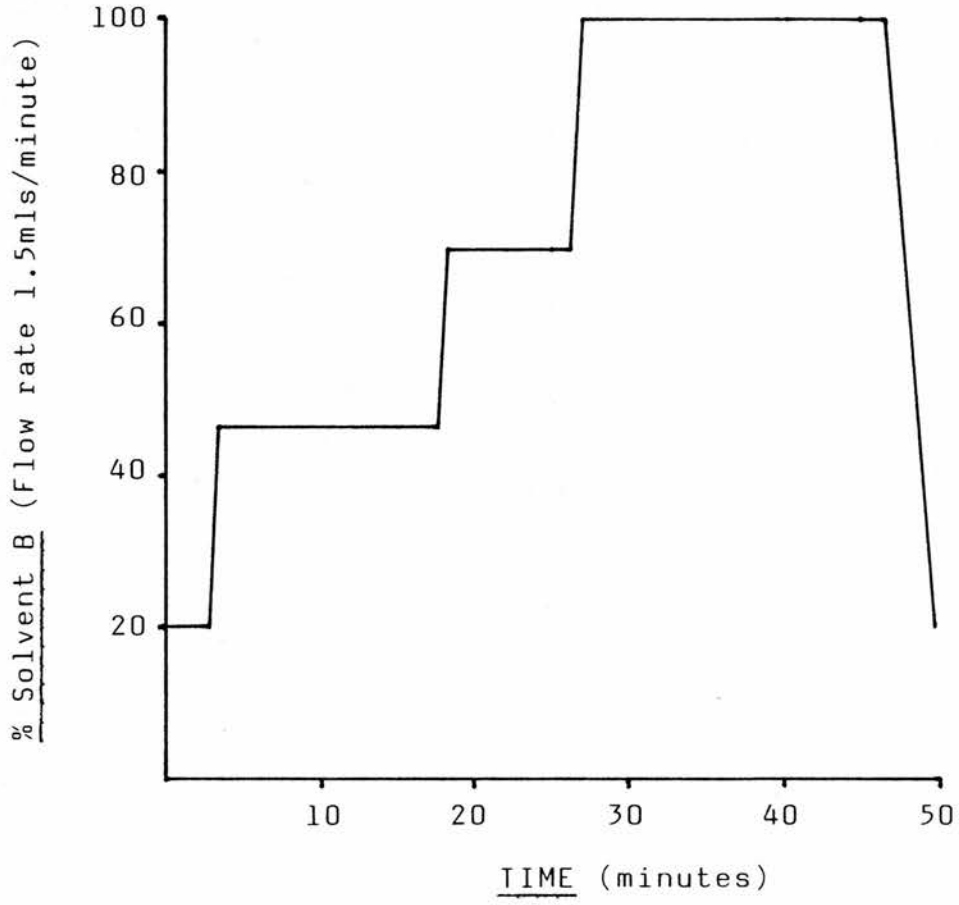
	HPLC SOLVENT A	HPLC SOLVENT B
ISOPROPANOL	245 ml	245 ml
HEXANE	195 ml	185 ml
WATER	15 ml	45 ml
ETHANOL	50 ml	50 ml
H_3PO_4	700 µl/litre	700 µl/litre

The inclusion of minor amounts of H_3PO_4 greatly improved the peak shape and resolution of the acidic phospholipids.

The two chromatographic systems developed and used routinely throughout this study are shown in Figure 4, one being a sophisticated 4 step gradient system and the other an isocratic system:-

FIGURE 4

HPLC SYSTEM 1



TIME (minutes)	% SOLVENT B
0	20
2.38	20
2.50	45
17.50	45
18.75	70
26.25	70
27.50	100
47.50	100
50	20

HPLC SYSTEM 2

This consisted of 20% solvent B introduced at a constant flow rate of 1.5 mls/minute.

1.6.4 Fraction Collection

Fractions were collected into glass test tubes at 0.3 minute intervals using a Gilson model 201-202 fraction collector. Thus 0.45 ml fractions were collected and diluted 1 in 3 with HPLC solvent B and the fluorescence emission intensity measured using a spectrofluorimeter at the characteristic excitation and emission maxima versus a solvent blank. Results were expressed as relative fluorescence units versus time/fraction number. Fractions corresponding to separate peaks were then combined, the solvent evaporated off and the radioactivity of each peak determined by liquid scintillation counting.

1.6.5 Liquid Scintillation Counting

Scintillation fluid was prepared by dissolving P.O.P.[1] (0.1 g/L) and P.P.O.[2] (5 g/L) in toluene/Triton X-100 (2:1 v/v). 5 mls of this fluid was added to the dried samples and counted for 10 minutes in a Intertechnique SL30 L.S. Spectrometer.

1 1,4-bis(5-phenyl-2-oxazolyl)-benzene

2 2,5-diphenyloxazole

2. GENERATION OF LIPID PEROXIDATION PRODUCTS IN BIOLOGICAL SYSTEMS

Having synthesised and partially characterised LPX products from synthetic phospholipids, it was important to determine whether these compounds were produced by LPX in biological systems. 3 systems were investigated, namely rat liver microsomes, human erythrocytes and sheep brain homogenate.

2.1 RAT LIVER MICROSOMES

2.1.1 Preparation of Microsomal Fraction Slater et al. (191)

The liver was removed from a freshly killed laboratory rat and immediately placed on ice. The weighed liver was then cut into small pieces and homogenised using a Potter homogeniser with ice cold 0.32M sucrose to give a 10% w/v suspension. The homogenate was centrifuged at 11,700g at 2°C for 10 minutes in a M.S.C. centrifuge. The supernatant (crude microsomal fraction containing microsomes and cytosol) was then centrifuged at 157,000g for 1 hour at 2°C. After discarding the supernatant, the pellet was washed twice with ice cold 0.15M KCl. The purified microsomal fraction was then resuspended in ice cold 0.15M KCl (1g liver/ml KCl) and frozen at -15°C until required. A small aliquot was retained for protein estimation.

2.1.2 Protein Estimation

The Lowry method (192) was used in estimating protein concentrations.

Folin-Ciocalteau (phosphomolybdic phosphotungstate) reagent was prepared by freshly diluting the commercial

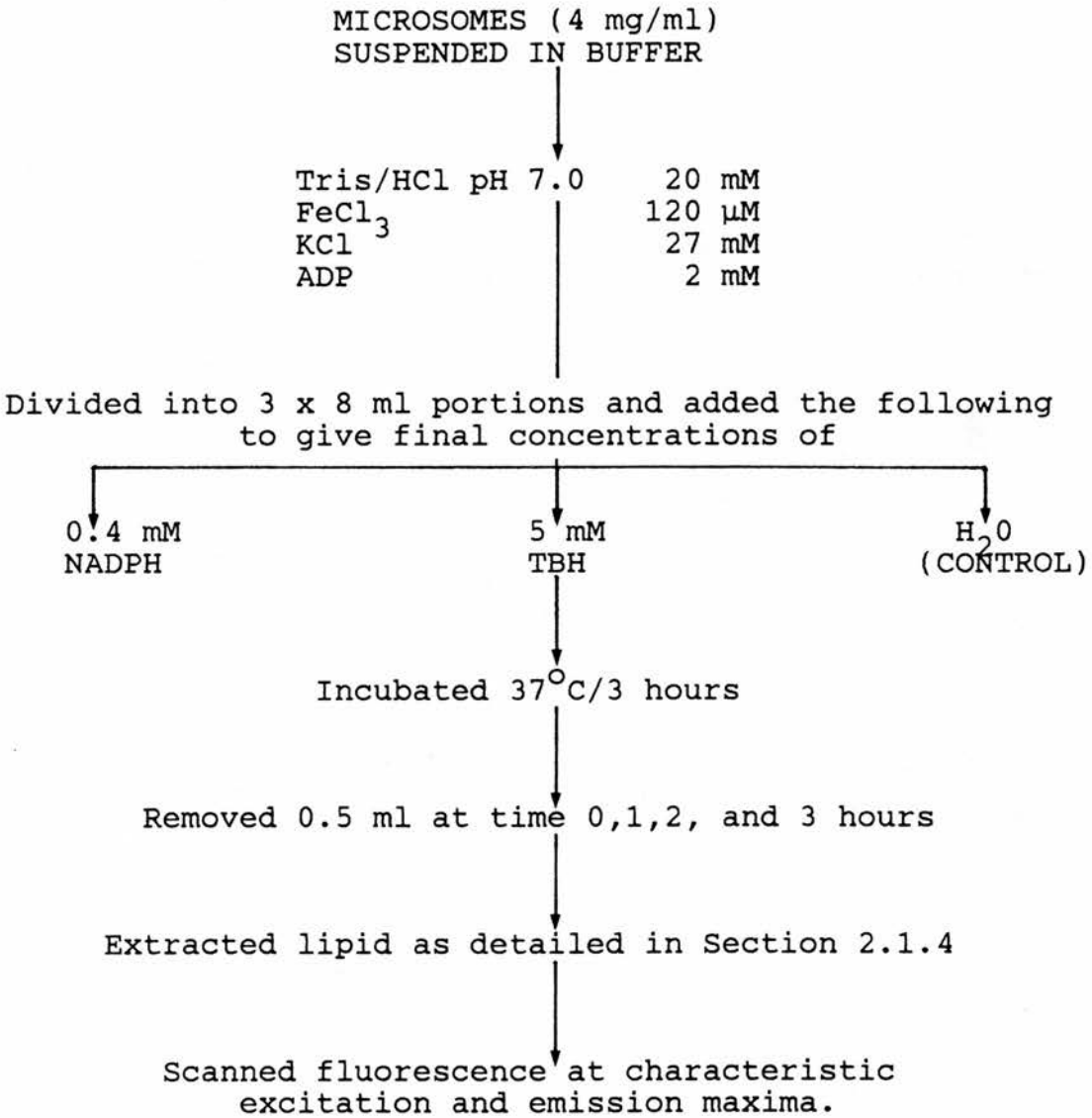
concentration 1:1 with distilled H_2O. A 0.5% w/v stock solution of alkaline copper sulphate was freshly prepared by dissolving the copper sulphate in 0.1M NaOH containing 1% tri-sodium citrate and 2% Na_2CO_3. An albumin standard curve was prepared from stock albumin solution (4 mg/ml) ranging from 0-100 µg/ml. The microsomal fraction was diluted 1 in 100 prior to assay.

0.5 ml of sample was mixed with 3 mls of alkaline copper sulphate solution and allowed to stand for 10 minutes at room temperature. 0.3 ml of the diluted Folin-Ciocalteau reagent was then added and after further mixing, the contents were incubated for 1 hour at room temperature. The absorbance of standards and samples was determined at 540 nm versus a reagent blank.

2.1.3 Induction of Lipid Peroxidation

The microsomal extract was divided into 3 aliquots and suspended in tris/HCl buffer, 20 mM, pH 7.0, to give a final protein concentration of 4 mg/ml. Each aliquot also contained $FeCl_3$ and ADP as shown below. Incubations were carried out using NADPH or t-butyl hydroperoxide (TBH) to stimulate lipid peroxidation and a suitable control sample was set up omitting these inducers as shown in Figure 5.

FIGURE 5



2.1.4 EXTRACTION OF MICROSOMAL LIPIDS Koster et al. (151)

0.5 ml of microsomal suspension was rigorously mixed with 1.875 mls $CHCl_3$/MeOH 1:2 v/v, left to stand at room temperature for 10 minutes and centrifuged at 2000g for 5 minutes to pellet any precipitate. Equal volumes (0.675 ml) of $CHCl_3$ and MeOH were mixed with the supernatant and centrifuged at 2000g for 5 minutes to form a biphasic system. 1.0 ml of the lower organic layer was then carefully removed and mixed with 0.1 ml MeOH. The fluorescence spectrum was

recorded and results expressed as relative emission maxima versus time of incubation. Finally the remainder of the $CHCl_3$ extractable lipids were isolated as above, concentrated and redissolved in $CHCl_3$/MeOH 10:1 v/v prior to TLC and HPLC analysis.

2.2 ERYTHROCYTES

2.2.1 Preparation of Erythrocytes

10 mls of venous blood was collected from an antecubital vein into precoated lithium heparin tubes and mixed by gentle shaking. The blood was centrifuged at 1500g for 10 minutes and the packed red cells obtained by removing the plasma and buffy coat (white cells and platelet aggregates).

2.2.2 Generation of Lipid Peroxidation Products

(i) H_2O_2 treatment

The combined erythrocytes were resuspended to 5% P.C.V. in 0.5% physiological saline with 2 mM azide inhibit catalase. An equal volume of 20 mM H_2O_2 in 0.5% saline was added, mixed and incubated in a shaking water bath at 37°C for 2 hours.

(ii) MDA treatment

The combined erythrocytes were resuspended to 5% P.C.V. in 0.5% physiological saline containing 2% glucose and incubated with 2 mM MDA at 37°C in a shaking water bath for 24 hours.

(iii) TBH treatment

The combined erythrocytes were resuspended to 10% P.C.V. in 0.5% physiological saline containing 2 mM azide and incubated with 5 mM TBH in a shaking water bath at 37°C for 2 hours.

2.2.3 Extraction of Erythrocyte Lipids

Following incubation under the various conditions detailed above, the lipids were extracted. For every 0.5 ml of packed erythrocytes, 3 mls of $CHCl_3$/MeOH 2:1 v/v and 3 mls of H_20 was added. The mixture was vortexed and centrifuged at 1000g for 15 minutes to form a biphasic system. The lower $CHCl_3$ phase was removed, concentrated and redissolved in $CHCL_3$/MeOH 10:1 v/v prior to TLC and HPLC analysis.

2.3 SHEEP BRAIN HOMOGENATE

2.3.1 Preparation of Homogenate Stocks et al. (193)

A sheep brain was stripped of its meninges and all blood clots washed off using ice-cold 0.15M NaCl. The tissue was then chopped into small pieces and homogenised for 2 minutes in a M.S.E. automix in 4 times its weight of ice cold phosphate-saline buffer (40 mM KH_2PO_4/K_2HPO_4, pH 7.4 in 0.142M NaCl). The homogenate was centrifuged for 15 minutes at 1000g and the supernatant transferred to 50 ml containers and stored at -15°C.

2.3.2 Autoxidation of Brain Homogenate

A sample of the stock brain homogenate (6 mls) was thawed at room temperature and diluted 1 in 4 with ice cold phosphate-saline buffer. $FeCl_3$ was then added to give a final concentration of 0.1 mM. The homogenate was then divided into two equal aliquots. One aliquot was incubated for 4 hours at 37°C to induce autoxidation and the second aliquot incubated at room temperature (19°) for 4 hours. 0.5 ml aliquots were removed at time 0 and at 30 minute intervals thereafter up to a period of 4 hours and the lipids extracted as detailed below in Section 2.3.3. Autoxidation was

monitored by fluorescence at the appropriate excitation and emission maxima and results expressed as relative fluorescent units versus time of incubation. The remaining homogenate was then extracted after the time period corresponding to the maximum yield of fluorescent products.

2.3.3 Extraction of Brain Homogenate Lipids

Brain lipids were extracted using the method of Koster et al. as described previously in Section 2.1.4. The $CHCl_3$ phases were removed, concentrated and redissolved in $CHCl_3$/MeOH 10:1 v/v prior to TLC and HPLC analysis.

2.4 TLC ANALYSIS OF BIOLOGICAL SAMPLES

Both 1D, 2D and preparative TLC was carried out on microsomal, erythrocyte and brain homogenate lipid extracts as described for synthetic phospholipid studies detailed in Section 1.2.

2.5 HPLC ANALYSIS OF BIOLOGICAL SAMPLES

The biological samples were further analysed using HPLC gradient elution analysis as detailed in Section 1.6. Both U.V. (206 nm) and fluorescent profiles were carefully examined in order to identify and characterise any LPX products generated in each tissue. In particular, it was attempted to correlate any adducts with those present in synthetic reaction systems.

3. LIPID PEROXIDATION PRODUCTS FROM PATIENTS WITH MULTIPLE SCLEROSIS AND OTHER NEUROLOGICAL DISEASES

Having identified and partially characterised LPX products generated in biological systems, it was decided to investigate whether these adducts could be identified in tissues from M.S. patients, since pilot studies in our laboratory (184) suggested an increase in peroxidative damage in these patients. Thus, in addition to repeating the assays previously carried out for thiobarbituric acid-reactive substances (TBARS) and lipid-soluble fluorescent pigments (LSFP), the lipid extracts used for the latter were examined chromatographically by both TLC and HPLC.

3.1 CSF AND PLASMA STUDIES

3.1.1 Patient Details

All CSF and plasma samples were obtained from the Department of Neurology, Dundee Royal Infirmary with the exception of those used for method development. In the majority of cases CSF and plasma samples were taken on the same day and were patient matched, however some data refers to plasma without the corresponding CSF and vice-versa.

Of the 15 M.S. patients, 6 were male and 9 were female with ages ranging from 16 to 66 years. Patients were diagnosed as having either:-

Clinically definite M.S. (CDMS)

Laboratory supported definite M.S. (LSDMS)

or Clinically probable M.S. (CPMS)

The patients were also rated for severity of the disease using the Kurtzke Ranking System.

Of the 25 control samples from patients having other neurological disease (OND) than M.S., 17 were male and 8 were female with ages ranging from 16 to 83 years. Patients were diagnosed as having either:-

Degenerative diseases (DEG) e.g., Parkinsons Disease, Cerebellar Degeneration.

Demyelinating diseases (DEM) e.g., Sub-sclerosing Pan-encephalitis, Freidrich's Ataxia.

or Demyelinating/degenerative diseases (DEMG) e.g., Guillain Barré Syndrome, Degenerative Vitamin B_{12} deficiency.

Table 3 summarises the patient details for all M.S. and OND patients:-

3.1.2 Processing of Plasma and CSF Samples

10 mls of venous blood was removed from an ante-cubital vein and collected into lithium-heparin tubes. The plasma fraction was separated from the red blood cells via centrifugation at 1500g for 10 minutes and then carefully removed using a pasteur pipette.

1.5 ml aliquots of plasma and CSF were frozen in plastic eppendorf tubes at -70^{o}C and stored for up to 12 months. The effect of freezing and thawing of the samples was investigated together with the effect of centrifugation prior to sampling. Both inter and intra assay coefficients of variation were determined for each assay method used in this study and each assay was performed in triplicate.

TABLE 3

PATIENT NUMBER	AGE	SEX	DIAGNOSIS	KURTZKE SCALE
1	36	M	CPMS	3-6
2	46	M	CPMS	1-2
3	57	M	CPMS	1-2
4	33	F	CPMS	1-2
5	45	F	CPMS	1-2
6	49	F	CPMS	1-2
7	20	M	CDMS	1-2
8	37	F	CDMS	1-2
9	39	F	CDMS	1-2
10	29	M	LSDMS	1-2
11	57	M	LSDMS	7-9
12	16	F	LSDMS	3-6
13	46	F	LSDMS	1-2
14	47	F	LSDMS	1-2
15	53	F	LSDMS	1-2
16	19	M	DEM	-
17	20	M	DEM	-
18	29	M	DEM	-
19	35	M	DEM	-
20	36	M	DEM	-
21	56	M	DEM	-
22	75	M	DEM	-
23	80	M	DEM	-
24	81	M	DEM	-
25	16	F	DEM	-
26	29	F	DEM	-
27	42	F	DEM	-
28	62	F	DEM	-
29	68	F	DEM	-
30	83	F	DEM	-
31	46	M	DEG	-
32	53	M	DEG	-
33	62	M	DEG	-
34	65	M	DEG	-
35	69	M	DEG	-
36	73	M	DEG	-
37	48	M	DEMG	-
38	57	M	DEMG	-
39	39	F	DEMG	-
40	66	F	DEMG	-

3.2 ASSAY FOR THIOBARBITURIC ACID - REACTIVE SUBSTANCES (TBARS)

TBARS were measured using both colorimetric and fluorimetric methods of analysis for both plasma and CSF samples, in order to compare the sensitivities of both assay methods. The TBARS assay is relatively unspecific in that free MDA is measured together with lipid peroxides or intermediate products which will decompose to yield MDA under the assay conditions.

3.2.1 Preparation of Malondialdehyde Standard

A 10 mM stock solution of 1,1,3,3,tetraethoxypropane (TEP) was prepared feshly each day by dissolving 220 mg (237 µl) of TEP in distilled H_2O and making up to 100.0 mls. This was further diluted x 100 to yield a solution containing 100 nmoles/ml MDA, since under the conditions of the test, TEP will hydrolyse quantitatively to yield MDA. Aliquots of this stock were diluted in distilled H_2O to give a final concentration range of MDA between 0-10 nmoles/assay.

3.2.2 Colorimetric Assay Satoh et al. (194)

0.5 ml plasma, CSF, or standard containing 0 to 20 nmoles/ml MDA was mixed with 0.5 ml 35% w/v TCA and 0.5 ml Tris/HCl buffer (50 mM, pH 7.4), vortex mixed and incubated at room temperature for 10 minutes. 1.0 ml TBA reagent was added (0.75% TBA in 2M Na_2SO_4) and boiled at 100°C for 45 minutes. After cooling, 1.0 ml 70% TCA was added and then centrifuged at 950g for 10 minutes. The chromophore was then extracted into 2.0 mls butanol and the absorbance measured at 535 nm. The final MDA concentration was calculated using $E^{1\%}_{1cm}$ = 13,700.

3.2.3 Fluorimetric Method Yagi (131)

3.2.3.1 Principle of the test

The colorimetric MDA method can be subject to interference from other plasma components. Since the chromophore formed by the reaction of MDA and TBA is also fluorescent (unlike the interfering components), a more sensitive and specific assay can be achieved using spectrofluorimetry. The principle of Yagi's method is that lipids in association with plasma lipoproteins are isolated by precipitation with serum protein using phosphotungstic acid (PTA) followed by the TBA reaction. Hence the method assays only lipid peroxides in association with serum lipoproteins and any free MDA as well as interfering TBARS (e.g. sialic acid) are removed. The original intention in the present study was to assay both total TBARS and protein-associated TBARS. The fluorophore is measured at 515 nm excitation, 553 nm emission using a scanning fluorimeter, calibrated daily with 0.1 µg/ml quinine sulphate in 0.1N H_2SO_4. Optimum settings for excitation and emission slit widths and the photomultiplier tube were found to be 3, 1 and 1 respectively.

3.2.3.2 Method of Yagi

0.5 ml of plasma was mixed with 4.0 mls N/12 H_2SO_4 and shaken gently. 0.5 ml 10% PTA was added and after standing at room temperature for 5 minutes, the mixture was centrifuged at 2000g for 10 minutes. The supernatant was discarded and the sediment mixed with 2.0 mls N/12 H_2SO_4 and 0.3 ml 10% w/v PTA. After centrifuging at 2000g for 10 minutes, the sediment was resuspended in 4.0 mls distilled

H_2O and 1.0 ml TBA reagent (equal volumes of 0.67% TBA aqueous solution and glacial acetic acid). The reaction mixture was heated for 60 minutes at 95°C in a glycerol bath.

After cooling, 5.0 mls n-butanol was added and the mixture shaken vigorously. The n-butanol layer was taken for fluorimetric measurement at 553 nm with 515 nm excitation following centrifugation at 2000g for 15 minutes. The lipid peroxide concentration was calculated by comparison with standard MDA as prepared in Section 3.2.1.

Since extensive repetitions and minor modifications of Yagi's method failed to produce linear results, it was proposed to:-

(a) Modify the method of precipitation.

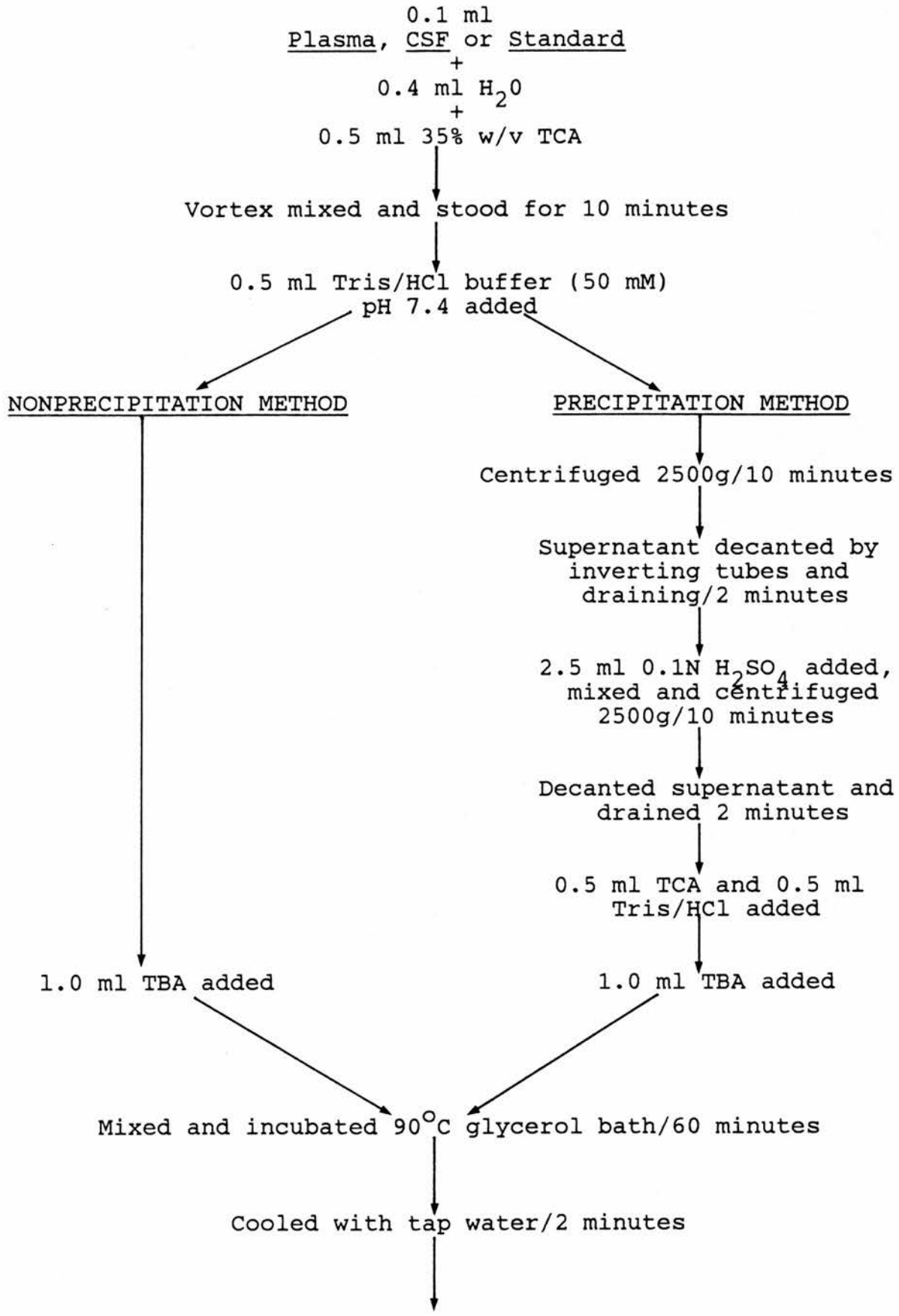
(b) To also assay plasma samples using a method which did not require a precipitation step.

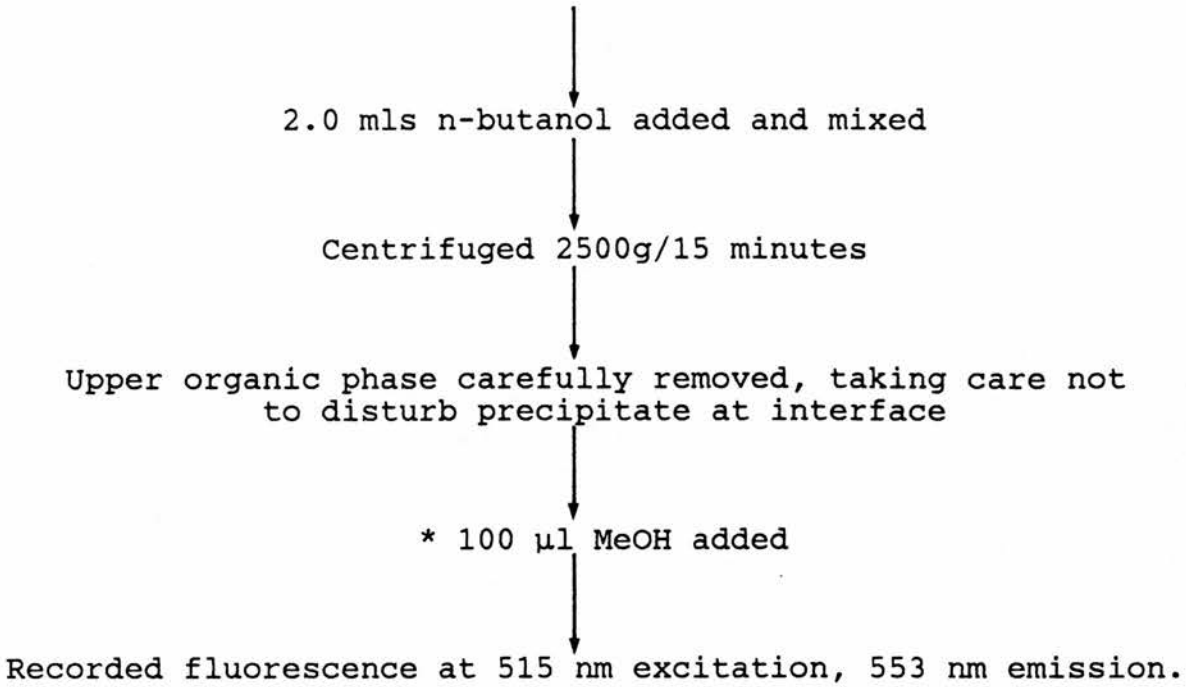
The precipitation method was not carried out on CSF since there is approximately 200 x less protein present in CSF compared to plasma and therefore no precipitate was visible.

3.2.3.3 Final method adopted

Frozen samples (-70°C) were thawed at room temperature and then incubated at 37°C for 10 minutes. The samples were then centrifuged at 2000g for 10 minutes immediately prior to analysis as described in Figure 6.

FIGURE 6


0.1 ml
Plasma, CSF or Standard
+
0.4 ml H_2O
+
0.5 ml 35% w/v TCA
Vortex mixed and stood for 10 minutes
0.5 ml Tris/HCl buffer (50 mM) pH 7.4 added
NONPRECIPITATION METHOD
PRECIPITATION METHOD
Centrifuged 2500g/10 minutes
Supernatant decanted by inverting tubes and draining/2 minutes
2.5 ml 0.1N H_2SO_4 added, mixed and centrifuged 2500g/10 minutes
Decanted supernatant and drained 2 minutes
0.5 ml TCA and 0.5 ml Tris/HCl added
1.0 ml TBA added
1.0 ml TBA added
Mixed and incubated 90°C glycerol bath/60 minutes
Cooled with tap water/2 minutes



* A small volume of MeOH was added at this stage to help remove any slight turbidity which would otherwise interfere with the fluorescence measurements.

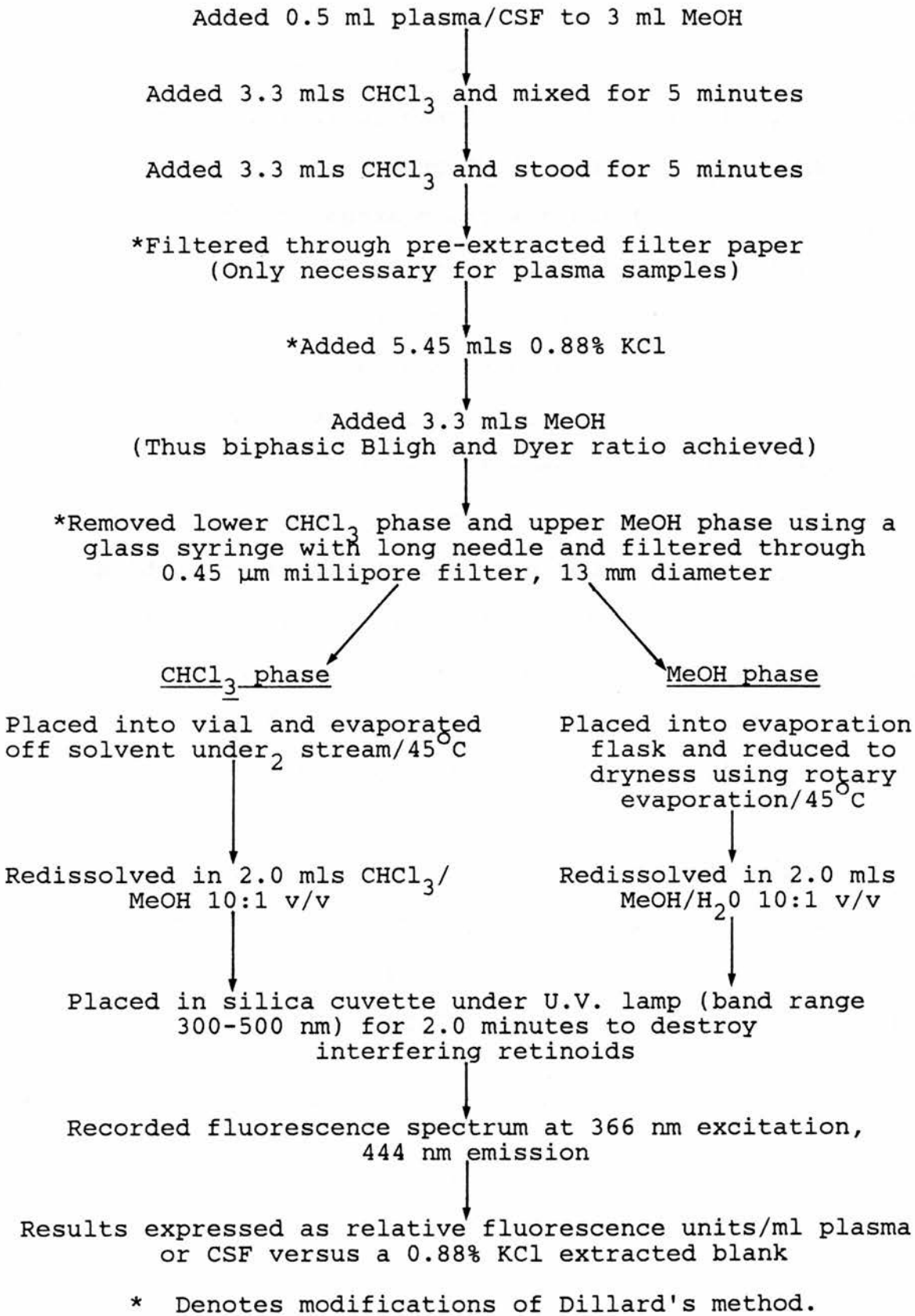
3.3 FLUORESCENT PIGMENT ASSAY

MDA is reported (141) to react with amino group containing phospholipids and proteins to yield N,N'-disubstituted 1-amino-3-iminopropenes, which are fluorescent conjugated Schiff bases. Extraction of tissues/body fluids using $CHCl_3$/MeOH (Bligh and Dyer) is thought to concentrate the phospholipid-derived products in the $CHCl_3$ phase and the protein-derived products in the upper MeOH/H_2O phase. Whatever the chemical nature of these compounds, the measurement of total fluorescence correlates well with other indices of LPX and has been widely used as a sensitive indicator of the process in biological as well as synthetic systems.

The assay was carried out on both lipid soluble ($CHCl_3$ phase) and water soluble (MeOH/H_2O phase) extracts of plasma and CSF samples as shown in Figure 7.

FIGURE 7

Extraction of Fluorescent Pigments
Modified from Dillard et al. (141)



* Denotes modifications of Dillard's method.

In order to obtain reproducible results, a considerable amount of method development using the basic method of Dillard et al. (141) was carried out, thus resulting in the final method adopted as indicated in Figure 7. The various modifications are described below:-

A. Prior to extracting plasma into biphasic Bligh and Dyer reagent thus separating $CHCl_3$ and $MeOH/H_2O$ soluble lipids, the monophasic extract was filtered through pre-extracted filter paper since the extraction procedure precipitation large amounts of plasma protein thus making isolation of the lower $CHCl_3$ phase (in a pure state), extremely difficult.

B. 0.88% KCl was used in preference to water for the Bligh and Dyer extraction, since this minimises losses of acidic, polar lipids into the $MeOH/H_2O$ phase.

C. A final filtration step of the $CHCl_3$ extract was carried out using a 0.45 μm millipore filter, since the method was found to be extremely sensitive to interference from very small particles which induced light scattering.

D. The $MeOH/H_2O$ phase was also filtered as in C and its fluorescence spectrum examined for polar LPX products not extractable into the $CHCl_3$ phase.

3.4 SPECTROPHOTOMETRIC DETECTION OF LIPID CONJUGATED DIENES

Recknagel et al. (128)

Naturally occuring lipids exhibit end absorption in U.V. light as the wavelength is lowered towards 200 nm. The spectra of molecules containing conjugated dienes however are characterised by intense absorption, the so called K band

from 215-250 nm. Peroxidising lipids are characterised by an intense K band at 233 nm and so this method may be used to detect conjugated dienes in lipid samples.

Conjugated dienes were measured in $CHCl_3$ extracted lipid from CSF samples by their U.V. absorption at 233 nm. Lipids were extracted into $CHCl_3$ as described in Seciton 3.3 and dried down under a stream of N_2. The samples were then redissolved in 3.0 mls cyclohexane and their U.V. absorbance measured relative to a cyclohexane blank. Cyclohexane is used as the solvent for this method as it is relatively transparent to U.V. light at 233 nm, whereas $CHCl_3$ absorbs strongly.

3.5 TLC ANALYSIS OF PLASMA AND CSF LIPIDS

The $CHCl_3$ and $MeOH/H_2O$ extracted lipids used for the fluorescent pigment assay (Section 3.3), were prepared for TLC analysis by reducing to dryness under N_2 on a sample concentrator at 45°C. Each sample representing lipid extracted from 0.5 ml of plasma/CSF, was redissolved in 1.0 ml of $CHCl_3$/MeOH 2:1 v/v, warmed to ensure solubilisation of the lipids and transferred to a 1.5 ml sample vial. This was then reduced to dryness again and redissolved in 100 µl $CHCl_3$/MeOH 2:1 v/v. 1D TLC analysis was carried out using appropriate solvent systems described in Section 1.2.

3.6 HPLC ANALYSIS OF PLASMA AND CSF LIPIDS

(a) $CHCl_3$ phases

Lipid was extracted from 0.5 ml plasma and CSF as detailed in Section 3.3. The total $CHCl_3$ phase was evaporated under N_2 at 45°C and redissolved in 0.5 ml $CHCl_3$/MeOH 10:1 v/v. Aliquots of 20 µl were taken for HPLC

analysis as described in Section 1.6.

(b) MeOH/$H_2$0 phases

Lipid was extracted from 0.5 ml plasma and CSF as detailed in Section 3.3. The total MeOH/$H_2$0 phase was evaporated on a rotary evaporator at 45°C and redissolved in 0.5 ml MeOH. Aliquots of 20 µl were taken for HPLC analysis as described in Section 1.6.

3.7 INDUCTION OF LIPID PEROXIDATION IN HAEMOLYSATES

3.7.1 Patient Details

All M.S. and OND samples were obtained from the Department of Neurology, Dundee Royal Infirmary. Of the 10 M.S. patients, 2 were male and 8 were female with ages ranging from 30 to 68 years. 9 OND samples were obtained from 5 male and 4 female patients with ages ranging from 24 to 68 years. Samples from healthy volunteers within the Department of Biochemistry, University of St. Andrews were used in developing the method and also as normal controls. Patient details are summarised in Table 4.

TABLE 4

PATIENT NUMBER	AGE	SEX	DIAGNOSIS
1	30	M	CDMS
2	57	M	CDMS
3	39	F	CDMS
4	41	F	CDMS
5	54	F	CDMS
6	55	F	CDMS
7	59	F	CDMS
8	59	F	CDMS
9	68	F	CDMS
10	-	F	CDMS
11	24	M	OND
12	26	M	OND
13	36	M	OND
14	68	M	OND
15	-	M	OND
16	24	F	OND
17	43	F	OND
18	50	F	OND
19	64	F	OND

3.7.1.1 Isolation of erythrocytes

10 mls of blood was collected from M.S. patients and OND controls by venepuncture from an ante-cubital vein into lithium-heparin precoated tubes. The contents were mixed by gently shaking.

The blood was immediately centrifuged at 1500g for 10 minutes and the plasma aspirated off. The packed erythrocytes (approximately 3.5 ml) were then transferred to 30 ml glass stoppered centrifuge tubes and resuspended in an equal volume of buffered isotonic saline. (0.87% NaCl, 0.25 mM $MgCl_2$, 0.25 mM $CaCl_2$, adjusted to pH 7.4 with 0.05M Tris). The cells were centrifuged at 1500g for 5 minutes and the buffy coat carefully removed. The packed erythrocytes were then washed twice more with fresh aliquots of the same buffer.

3.7.1.2 Estimation of haemoglobin Dacie and Lewis (195)

Since the concentration of haemoglobin (Hb) will affect the degree of LPX possibly due to some protective role of Hb, (143) it was necessary to accurately measure and adjust the concentration in each sample to 10g/100 mls by diluting with an appropriate volume of isotonic saline.

Hb reagent was prepared by dissolving the following in 1 litre of distilled water:-

200 mg - potassium ferricyanide

50 mg - potassium cyanide

140 mg - potassium dihydrogen phosphate

1 ml - nonidet P40

10 µl of packed erythrocytes were diluted 200 x with the above reagent and the optical density recorded at 540 nm against a reagent blank. The Hb concentration was calculated using the following equation:-

$$\text{Hb (Mg/L)} = \frac{\text{O.D.}_{540} \times \text{M.W. Hb} \times \text{dilution factor}}{\text{Molar extinction coefficient} \times d}$$

Where:-

Molecular weight Hb = 64,500

dilution factor = 200

Molar extinction coefficient = 44

Path length (d) = 1

After adjusting the Hb levels to 10g/100 mls, the samples were frozen in 1 ml aliquots at -70°C.

3.7.2 Stress Test

The recent method of Kobayashi et al. (147) was used to assess the susceptibility of the red cells to LPX. This

method, unlike that of Stocks and Dormandy (145), has the advantage in that it makes use of frozen haemolysates. The advantage lies in the fact that:-

(a) Samples may be stored and do not need to be tested immediately.

(b) Fe^{3+} is used as the stressor in preference to H_2O_2 and the haemolysates are thus subjected to a more physiological inducer of LPX.

However, the published method of Kobayashi proved to be unsuccessful in that Fe^{3+} did not appear to stimulate LPX and consequently the following parameters were investigated with a view to developing a modified method:-

(i) Sonication time

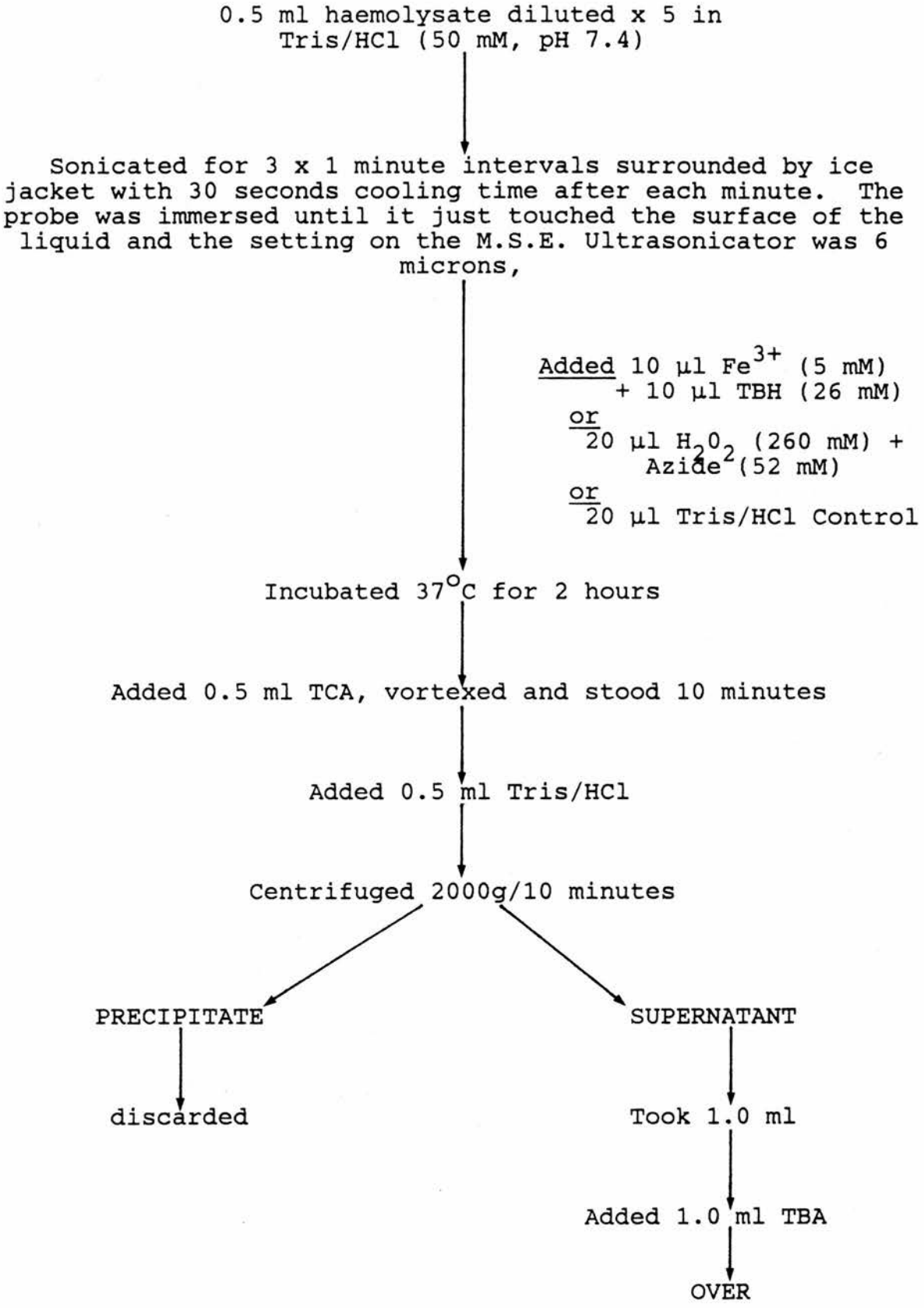
(ii) Effect of Azide

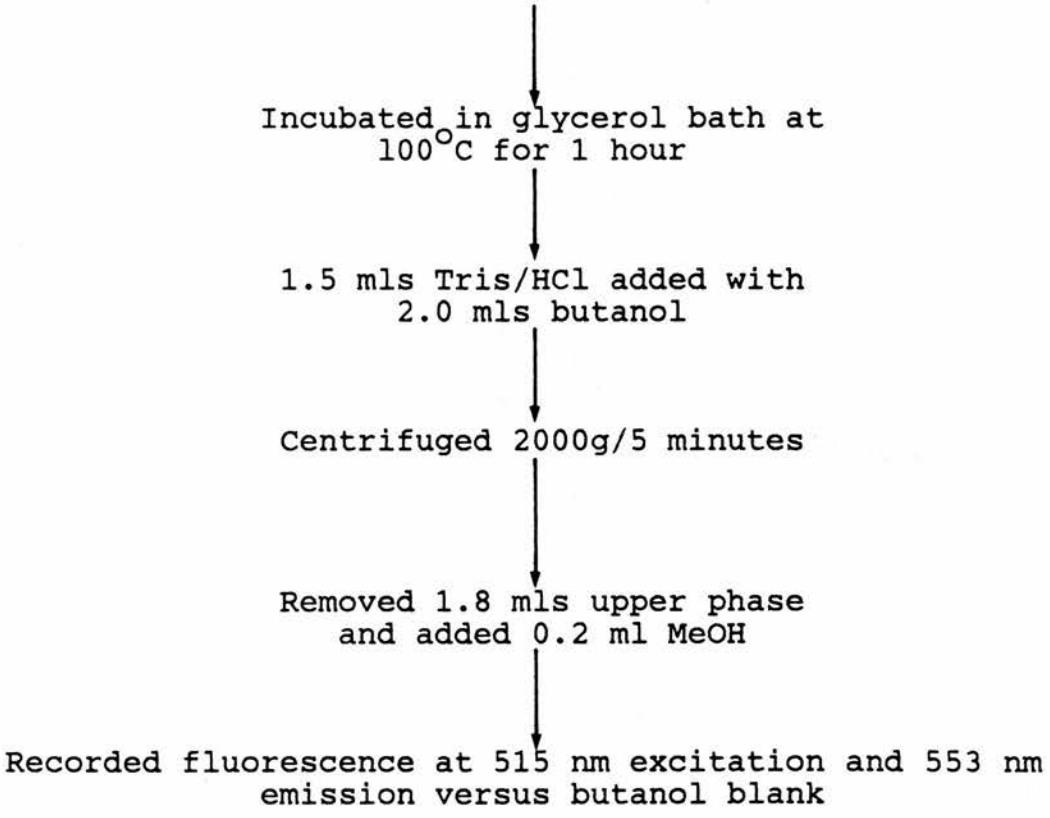
(iii) Use of T.B.H. and H_2O_2 as an alternative stressors.

3.7.2.1 Final method adopted

A series of standard MDA solutions were prepared by dilution of stock MDA (Section 3.2.1) in 0.5 mls Tris/HCl (50 mM, pH 7.4), to give a final concentration ranging from 0-10 nmoles per assay. 20 µl of Tris/HCl was then added to each standard and treated exactly as the samples undergoing 2 hour incubations as shown below in Figure 8.

FIGURE 8


0.5 ml haemolysate diluted x 5 in Tris/HCl (50 mM, pH 7.4)
Sonicated for 3 x 1 minute intervals surrounded by ice jacket with 30 seconds cooling time after each minute. The probe was immersed until it just touched the surface of the liquid and the setting on the M.S.E. Ultrasonicator was 6 microns,
Added 10 µl Fe3+ (5 mM) + 10 µl TBH (26 mM)
or
20 µl H2O2 (260 mM) + Azide (52 mM)
or
20 µl Tris/HCl Control
Incubated 37°C for 2 hours
Added 0.5 ml TCA, vortexed and stood 10 minutes
Added 0.5 ml Tris/HCl
Centrifuged 2000g/10 minutes
PRECIPITATE
discarded
SUPERNATANT
Took 1.0 ml
Added 1.0 ml TBA
OVER


Incubated in glycerol bath at
100°C for 1 hour
1.5 mls Tris/HCl added with
2.0 mls butanol
Centrifuged 2000g/5 minutes
Removed 1.8 mls upper phase
and added 0.2 ml MeOH
Recorded fluorescence at 515 nm excitation and 553 nm
emission versus butanol blank

4. STATISTICAL METHODS

4.1 DETERMINATION OF COEFFICIENT OF VARIATION

n = Number of results

X = An individual result

Σ = Sum of

$\bar{x}$ = Mean of a set of results

S.D. = Standard deviation

$$\text{MEAN} \quad \bar{x} = \frac{\Sigma x}{n}$$

$$\text{STANDARD DEVIATION S.D.} = \sqrt{\frac{\Sigma(x)^2 - \frac{(\Sigma x)^2}{n}}{n - 1}}$$

$$\text{COEFFICIENT OF VARIATION C.V.} = \frac{\text{S.D.}}{x} \times 100$$

4.2 THE MANN-WHITNEY TEST (U)

Like most non-parametric tests the rationale behind the Mann-Whitney test is extremely simple. In this study for example it allows us to determine the probability that a given separation between the ranks of M.S. patients or a specific class of control could have arisen by chance. If this probability is low, (i.e. $p < 0.05$) one could reject the null hypothesis that the ordering ranks is purely random, in favour of the alternative hypothesis that the independent variable has produced a difference in the values measured for the two groups. As with a t-test, a given difference between the two sets of rankings is regarded as more significant if the direction of the difference was predicted before the

experiment (one-tailed test), than if it was not (two-tailed test).

The Mann-Whitney and standard deviation values were determined using a statistical programme developed for a B.B.C. microcomputer (196).

n_1 = size of smaller group of results

n_2 = size of larger group of results

R = sum of results for smaller group of results

When $n_2 < 20$

$$U = n_1 n_2 + \frac{n_1 (n_1 + 1)}{2} - R$$

When $n_2 > 20$

$$Z = \frac{U - \frac{n_1 n_2}{2}}{\sqrt{\frac{n_1 \, n_2 \, (n_1 + n_2 + 1)}{12}}}$$

Both U and Z values were looked up in statistical tables to reveal the probability that the observed difference between two sets of data could have arisen by chance (two-tailed test).

RESULTS

RESULTS

1. SYNTHETIC PHOSPHOLIPID STUDIES

1.1 DEVELOPMENT OF FLUORESCENCE IN SYNTHETIC PHOSPHOLIPID REACTION MIXTURES

As mentioned previously in the method section 1.1, synthetic reaction mixtures described in this section were dispersed in MeOH/$H_2$0 and not in phosphate buffer, since the addition of Triton X-100 required in this latter system to disperse the phospholipid, was extractable into organic phases and thus interfered with chromatographic separations.

1.1.1 Dipalmitoyl PE and MDA

As shown in Figure 9, the fluorescent adducts generated from the reaction of dipalmitoyl PE (DPPE) and MDA, reached a maximum level around 7 to 8 days with little further increase over the 8 to 12 day period. On this basis a new reaction system was prepared and all the products extracted following a 7 day incubation period. There was no significant generation of fluorescent material in either PE or MDA control incubations. The total fluorescent yield was slightly higher in the PE-MDA 1:2 reaction systems compared with the PE-MDA 4:1 systems.

1.1.2 Egg Yolk PE and MDA

As shown in Figure 10, egg yolk PE (EYPE) and MDA followed a similar pattern as that of DPPE and MDA in that maximum fluorescence was reached following a 4 day incubation period and remained constant up to 7 days. Although the

FIGURES 9 AND 10

Development of Fluorescence in Synthetic Phospholipid Reaction Mixtures

● PE-MDA 4:1

★ PE-MDA 1:2

● Control 4:1

★ Control 1:2

FIGURE 9

Dipalmitoyl PE + MDA

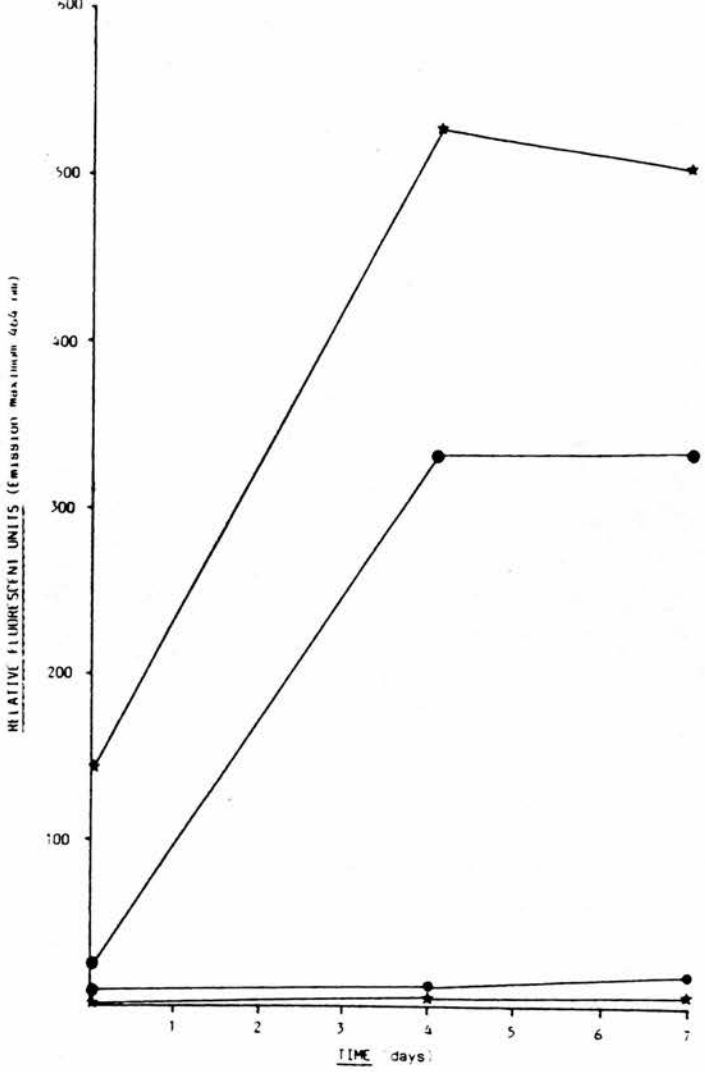
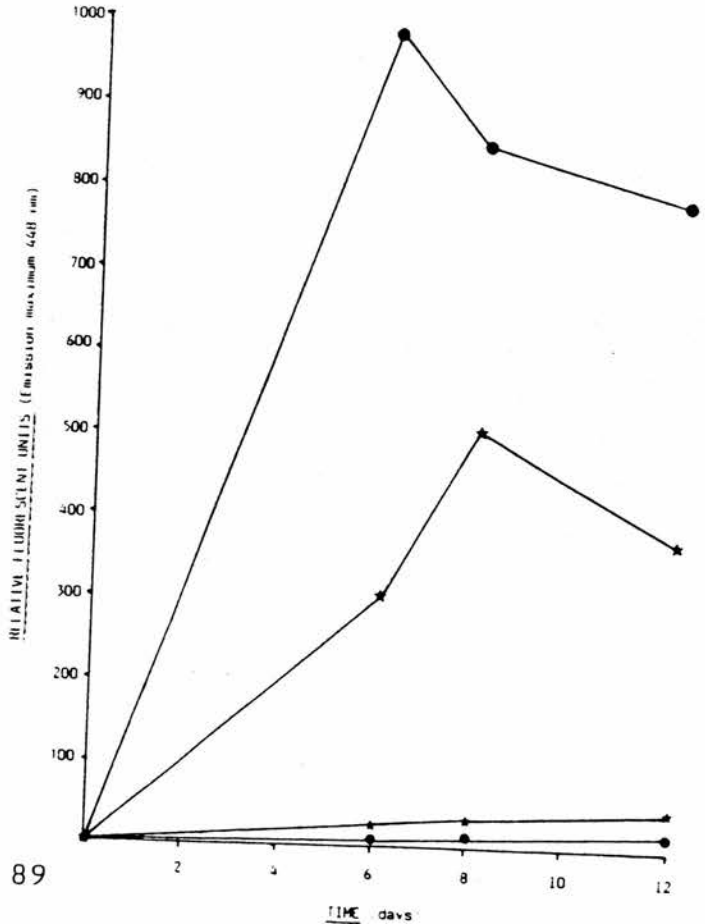


FIGURE 10

Egg Yolk PE + MDA



PE-MDA 4:1 reaction mixture appeared to generate more total fluorescent material than the PE-MDA 1:2 mixture, a much greater yield of the major fluorescent lipid product using the latter system was visible by TLC analysis (Figures 13 and 14) and thus it was decided to prepare all subsequent reaction mixtures using PE-MDA ratios of 1:2 only.

1.1.3 PS and MDA

Data is not shown for this system since the total fluorescent yield was considerably smaller than systems incorporating DPPE and EYPE. Subsequent TLC and HPLC analysis (sections 1.2.1 and 1.2.2) also demonstrated a lack of significant reaction products.

1.2 CHROMATOGRAPHIC CHARACTERISATION OF REACTION PRODUCTS

1.2.1 TLC

1.2.1.1 1D Analytical TLC

1.2.1.1.1 Dipalmitoyl and egg yolk PE-MDA

Figures 11, 12, 13 and 14 show the products generated in DPPE-MDA mixtures at ratios of 1:2 and 4:1 and that the yield of the major fluorescent product is greater in the 1:2 reaction system. This major product (spot A, Figures 11, 12, 13 and 14) is strongly autofluorescent and stains with both I_2 and ninhydrin. Two minor products can also be detected (spots B and C), the least polar of which stains with I_2 and contains phosphorus whilst the more polar product is autofluorescent. As one would expect both PE and lyso PE (spots D and E) contain phosphorus and stain with I_2 and ninhydrin. Both also showed a small degree of autofluorescence. Reaction mixtures containing EYPE-MDA show a more complex picture than DPPE-MDA in that the strongly

1D TLC Chromatograms Depicting PS-MDA and DPPE-MDA Reaction Mixtures 4:1 and 1:2 with Controls

Sample Identification:-

1 PS

2 PS Control

3 PS-MDA 4:1

4 PS-MDA 1:2

5 PE

6 PE Control

7 PE-MDA 4:1

8 PE-MDA 1:2

Solvent System

$CHCl_3$/MeOH/NH_4OH

(16:8.5:1 v/v)

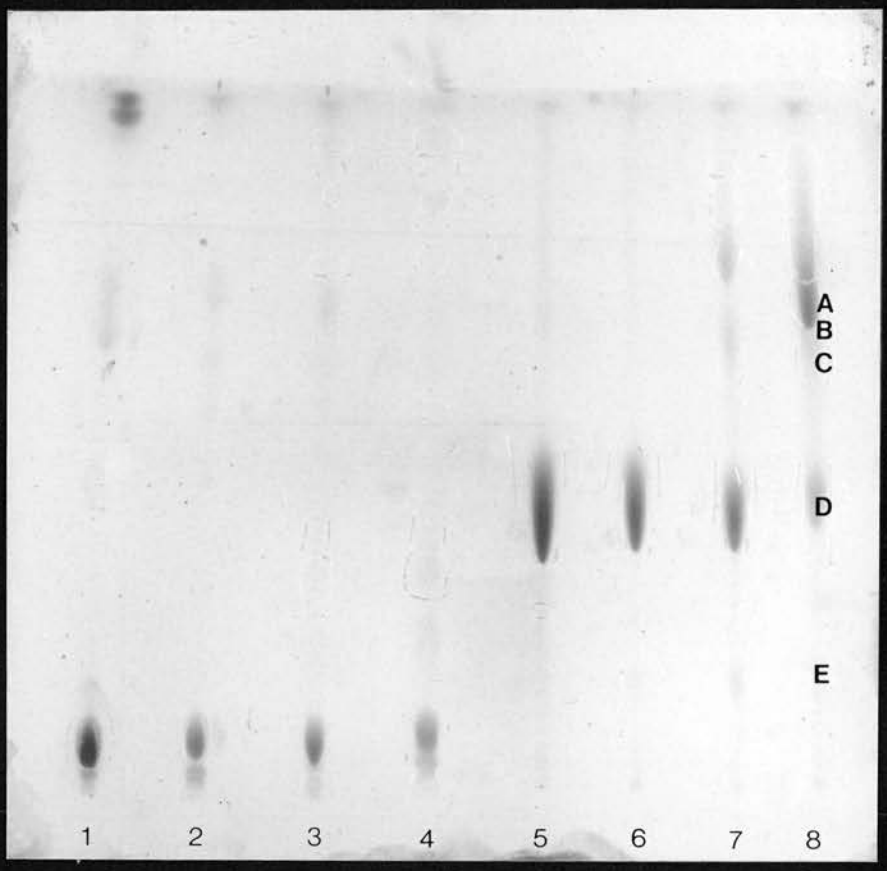
Loading

50 µg lipid/spot

Detection

I_2 staining

FIGURE 11



Identification of Spots

AF = Autofluorescent; I = Iodine Staining

N = Ninhydrin Staining

A	AF, I_2, N
B	I_2
C	AF
D	AF, I_2, N
E	AF, I_2, N

1D TLC Chromatograms Depicting DPPE-MDA 4:1 and PE/PS Reaction Mixtures with Controls

Sample Identification

1 PE-MDA 1:2

2 PE-MDA 4:1

3 MDA 1:2 control

4 MDA 4:1 control

5 PE control

6 PE/PS-MDA

7 PE/PS control

Solvent System

$CHCl_3$/MeOH/NH_4OH

(16:8.5:1 v/v)

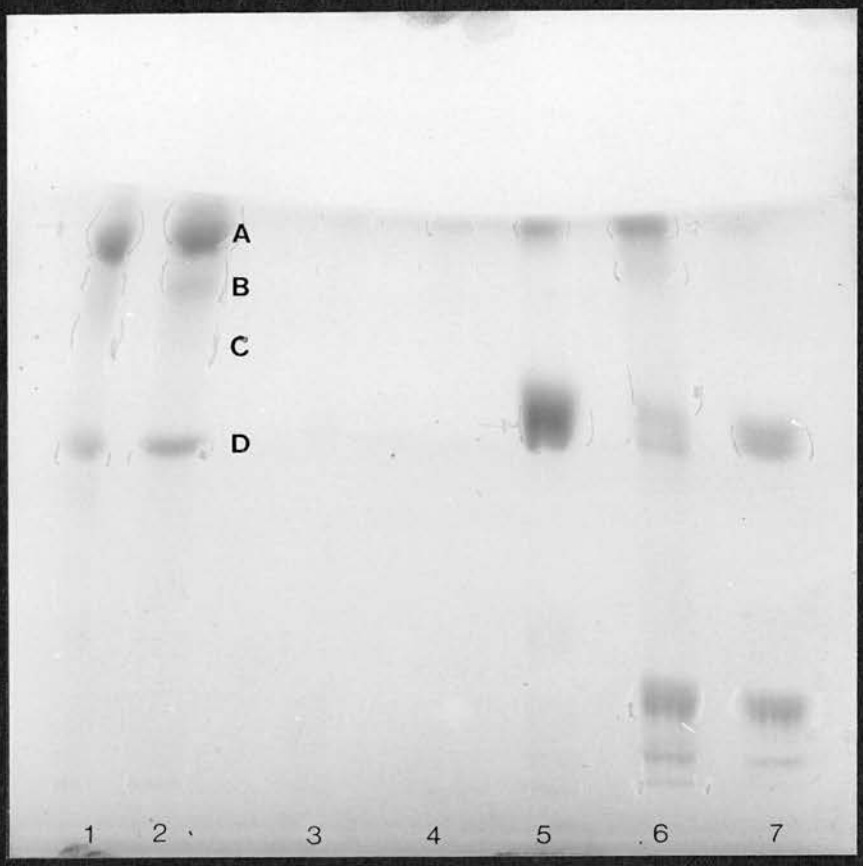
Loading

50 µg lipid/spot

Detection

I_2 staining

FIGURE 12



Identification of Spots

AF = Autofluorescent; I = Iodine Staining

N = Ninhydrin Staining

A	AF, I_2, N
B	I_2
C	AF
D	AF, I_2, N

1D Preparative TLC of DPPE-MDA Reaction Mixtures

FIGURE 13 DPPE-MDA 1:2

FIGURE 14 DPPE-MDA 4:1

Solvent System

$CHCl_3/MeOH/NH_4OH$

(16:8.5:1 v/v)

Loading

750 µg lipid/streak

Identification of Spots

AF = Autofluorescent; I = Iodine Staining

N = Ninhydrin Staining

A AF, I_2, N

B I_2

C AF

D AF, I_2, N

E AF, I_2, N

FIGURE 13

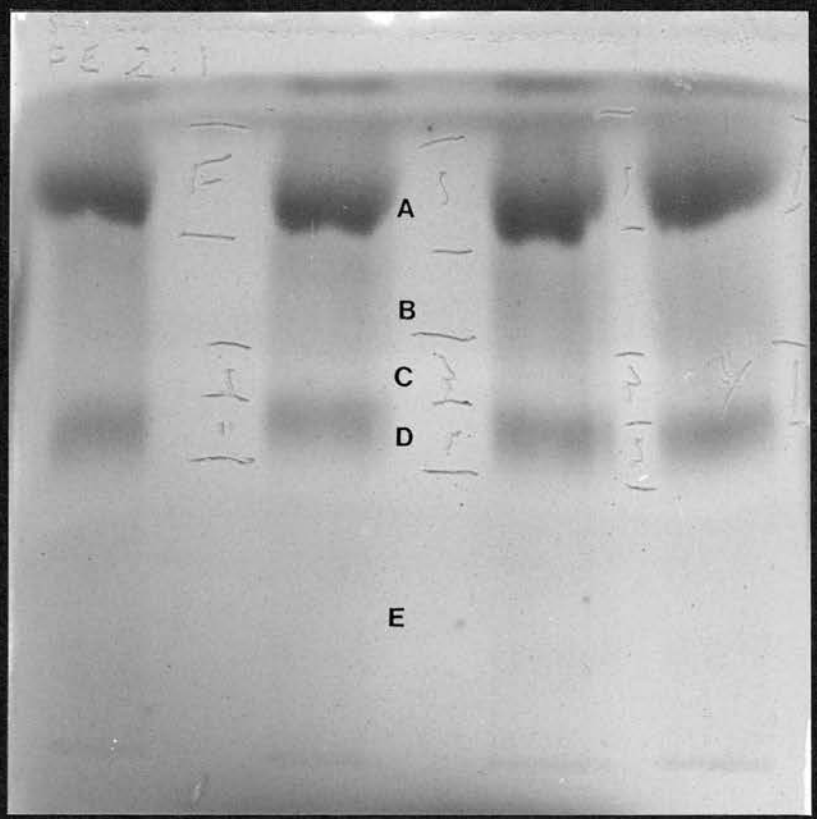
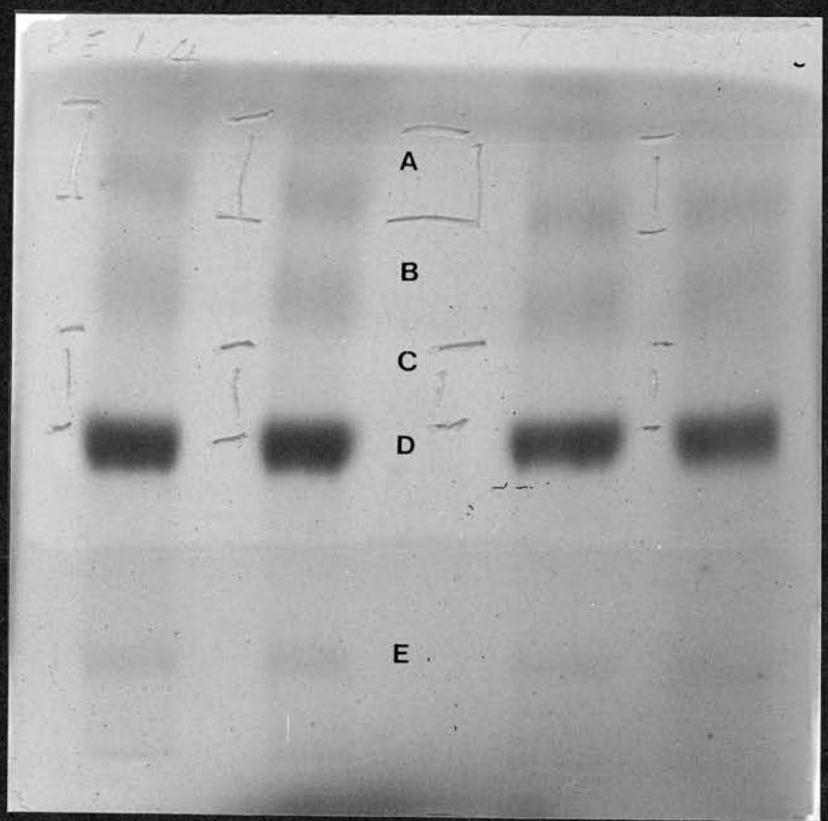


FIGURE 14



fluorescent (Figure 15b) and I_2 containing (Figure 15c) spot clearly contains more than 1 component and autoradiography (Figure 15a) also indicates the presence of 2 other components associated with this major spot. Only the most polar of these three components (X) which contains the bulk of the radioactivity from ^{14}C PE and also stains most strongly with I_2 is ninhydrin positive, although this could simply be a problem of the sensitivity of the detection reagents. The other two spots (Y and Z) are less fluorescent and stain for phosphate but not with ninhydrin. These components also contain significant amounts of radioactivity as shown by autoradiography (Figure 15a) and stain strongly with I_2 (Figure 15c). Controls containing PE or MDA only showed no unexpected spots except that the MDA control produced some weakly fluorescent components towards the solvent front (Figure 12, lanes 3 and 4).

1.2.1.1.2 PS and MDA

Figure 11 shows that with both PS-MDA ratios of 1:4 and 2:1 (lanes 3 and 4 respectively), no significant I_2 staining of fluorescent products were formed during the reaction in that virtually no difference was observed in comparison to the PS control (lane 2). The major fluorescent components which were unique to the PS-MDA reaction mixtures did not stain with I_2 and are probably derived from MDA alone.

1.2.1.1.3 PS/PE-MDA

Figure 12 shows that in comparison with the control (lane 7), the only additional I_2 staining products visible in the PE/PS-MDA reaction mixture (lane 6) are also observed in the PE-MDA mixtures (lanes 1 and 2) suggesting that no

FIGURE 15

1D TLC Chromatograms of ^{14}C PE-MDA

1:2 Reaction Mixture

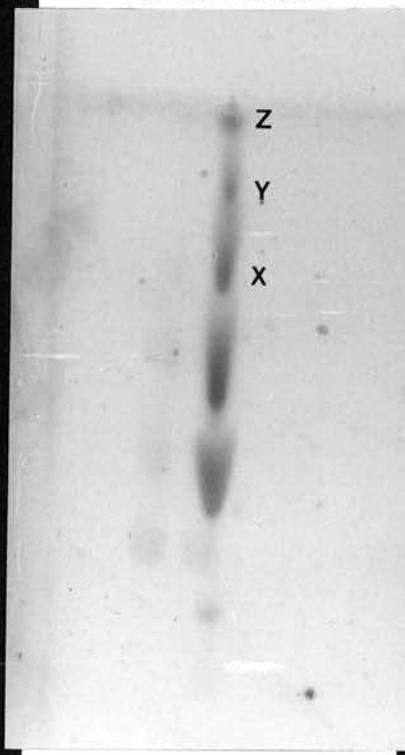
FIGURE 15a

Autoradiogram

FIGURE 15b

Autofluorescence

FIGURE 15c



Iodine Staining

Solvent System

$CHCl_3$/MeOH/NH_4OH

(16:8.5:1 v/v)

Loading

50 µg lipid/spot.

Autoradiogram

24 hours exposure, 0.04 µ Ci total loading.

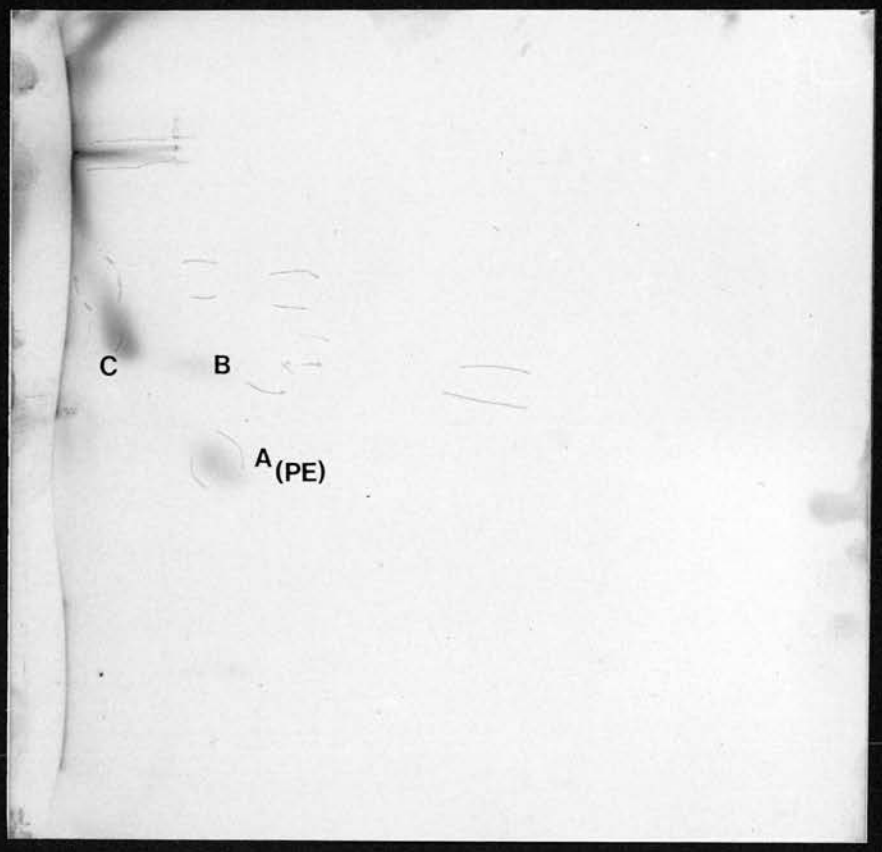
reaction products are formed due to the conjugation of both PE and PS with MDA, unless these happen to overlap with other components.

1.2.1.2 2D TLC

2D TLC analysis of DPPE-MDA reaction products gave better resolution of the fluorescent material as shown in Figure 16, but the findings were still in agreement with the 1D TLC in that only 3 I_2 containing spots (A, B, C) including PE were clearly resolved, although as with 1D TLC there is an impression of heterogeneity in the major spot (C).

EYPE reaction mixtures showed a much more complex profile as disclosed by autoradiography (Figure 17a), autofluorescence (Figure 18a) and by I_2 staining (Figure 19a). The autofluorescence photograph reveals at least 4 major fluorescent products (A, B, C, D) other than PE and lyso PE which also autofluoresce, one of which (A) is closely associated with PE itself. All of these products stain with I_2 and contain ^{14}C PE. Autoradiography and I_2 staining also reveal at least 3 other minor products (not strikingly apparent on these photographs) thus derived from PE, however these are difficult to identify on the fluorescence photograph due to streaking. It is also noteworthy that the visible colour of the fluorescence of the many components ranged from orange to deep purple/violet in colour, with the major components being bright yellow. Fluorescent material close to the solvent front was not detected by autoradiography and is thus probably derived from polymerised MDA. In each case controls are shown in Figure 17b, 18b and 19b and these show that the major products are absent and therefore only formed in the presence of both PE and MDA.

FIGURE 16



2D TLC Chromatogram of DPPE-MDA Reaction Mixture

Solvent System

1st Dimension $CHCl_3$/MeOH/NH_4OH
(16:8.5:1 v/v)

2nd Dimension $CHCl_3$/MeOH/HAc
(4.2:2.3:1 v/v)

Loading

50 µg lipid/plate

2D TLC Chromatograms Showing ^{14}C EYPE-MDA 1:2 Reaction Mixture + PE Control

Figure 17a and b

Reaction Mixture + Control Autoradiogram

Figure 18a and b

Reaction Mixture + Control Autofluorescence

Figure 19a and b

Reaction Mixture + Control I_2 Staining

Solvent System

1st Dimension	$CHCl_3$/MeOH/NH_4OH (16:8.5:1 v/v)
2nd Dimension	$CHCl_3$/MeOH/HAc (4.2:2.3:1 v/v)

Loading

50 µg lipid/plate

Autoradiography

24 hours exposure/0.04 µCi total loading

FIGURE 17a

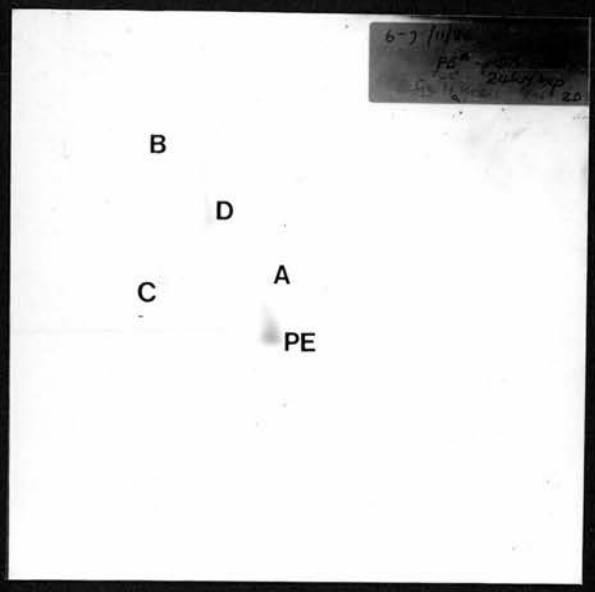


FIGURE 17b

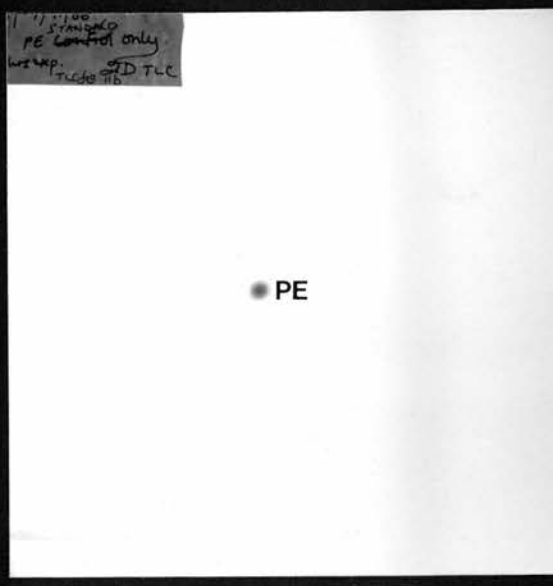


FIGURE 18a

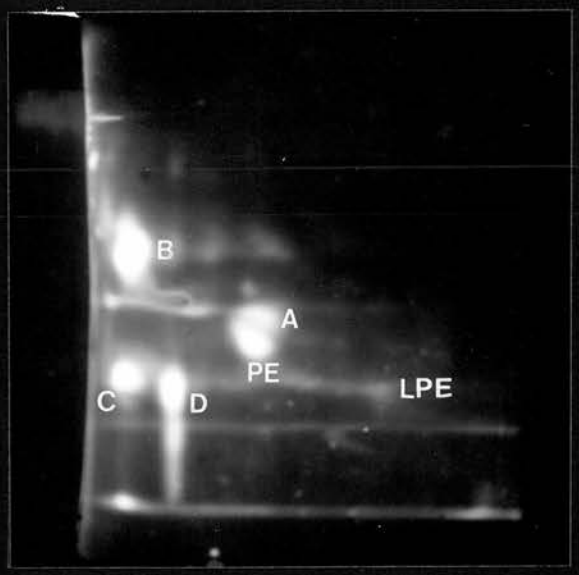


FIGURE 18b

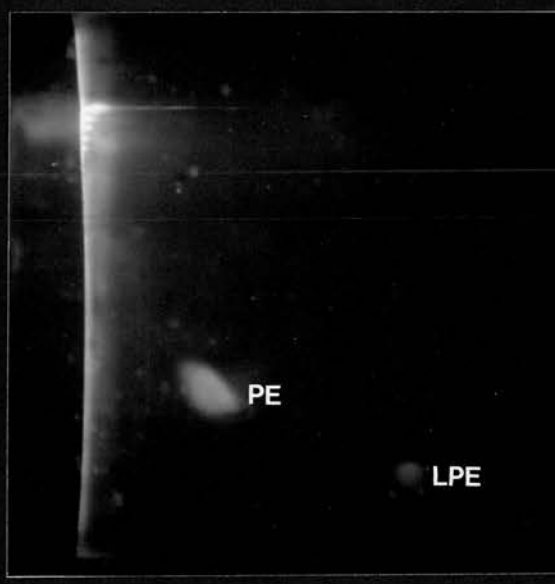


FIGURE 19a

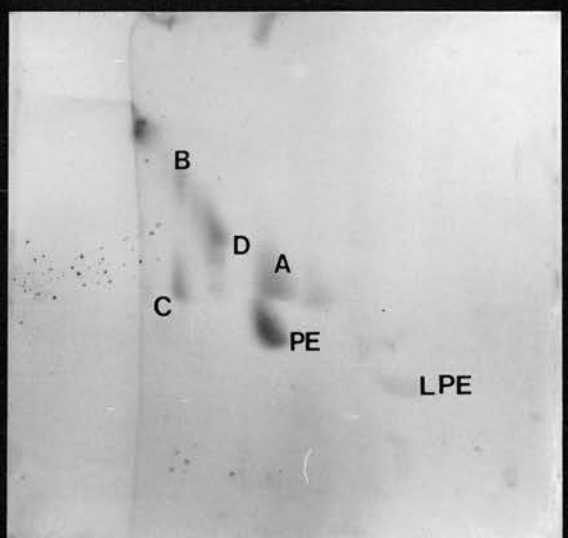
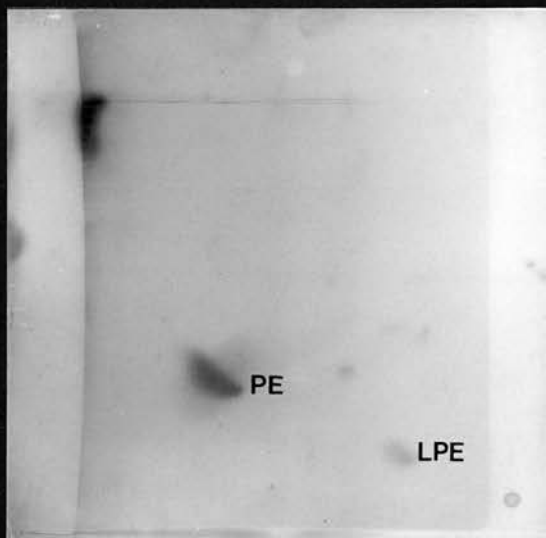


FIGURE 19b



1.2.1.3 Preparative TLC

In an attempt to further characterise the reaction products, preparative TLC was carried out on the EYPE-MDA reaction system as shown in Figure 20a and 20b. The fluorescence spectra of each fraction were determined and, as shown in Table 6, were found to exhibit different excitation and emission maxima. The least polar fraction corresponding to the major product was shown quantitatively to account for the most fluorescence (46%) and also contained the bulk of the lipid phosphorus (73%). The relative proportion of the total radioactivity in each fraction showed good agreement with the phosphorus data, indicating an 86% conversion of ^{14}C PE into this major reaction product. It is significant that although both phosphorus estimations and radioactivity experiments showed residual PE (Fraction 3) to be a minor component nevertheless this region of the chromatogram showed only marginally less fluorescence than the major product (fraction 1).

1.2.2 HPLC

The gradient system was developed in this study in order to achieve optimum resolution of both phospholipid standards and also fluorescent reaction products. A typical chromatogram showing separation of the standard phospholipids PS, PE, PI, PC and Sm is shown in Figure 21.

1.2.2.1 HPLC of total egg yolk PE-MDA reaction mixture

HPLC analysis of the total PE-MDA reaction mixture by U.V. detection as shown in Figure 22a, resolves 3 minor peaks (ABC) and 4 major peaks (DEFG) including PE. Unreacted PE

FIGURES 20a and 20b

Solvent System

$CHCl_3/MeOH/NH_4OH$

(16:8.5:1 v/v)

Loading

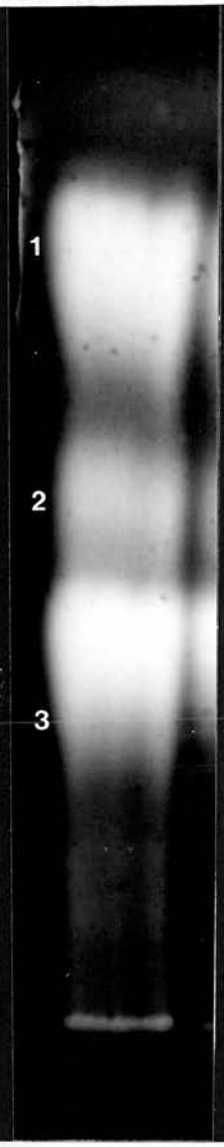
3 mg lipid/plate

Autoradiography

4 day exposure/1.2 µCi total loading

1D Preparative TLC of ^{14}C PE-MDA 1:2 Reaction Mixture

FIGURE 20a



Autofluorescence

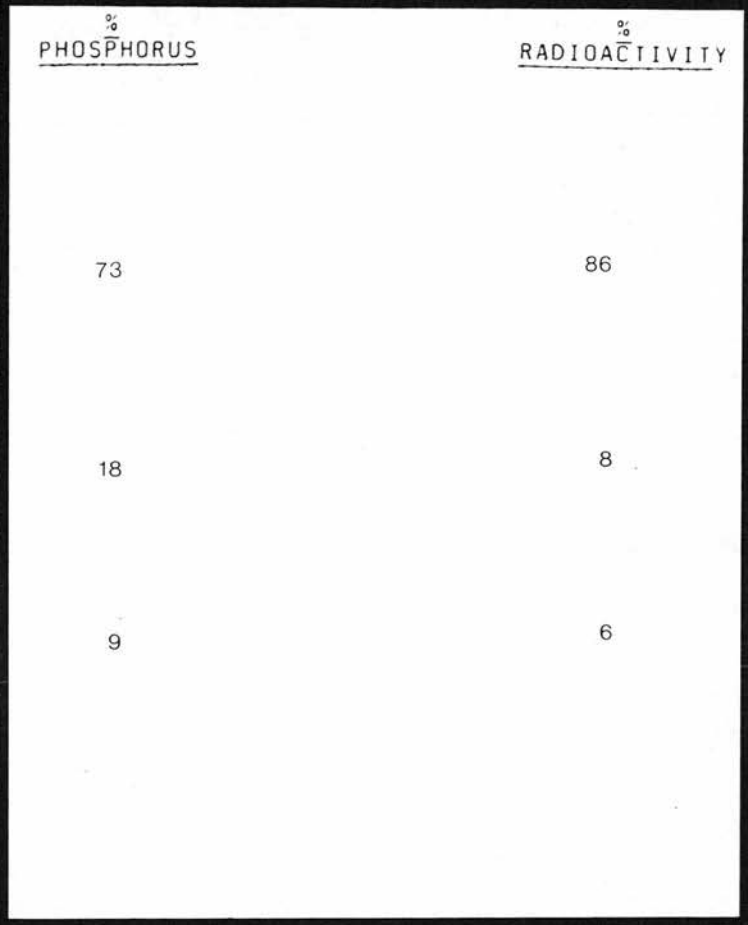


FIGURE 20b

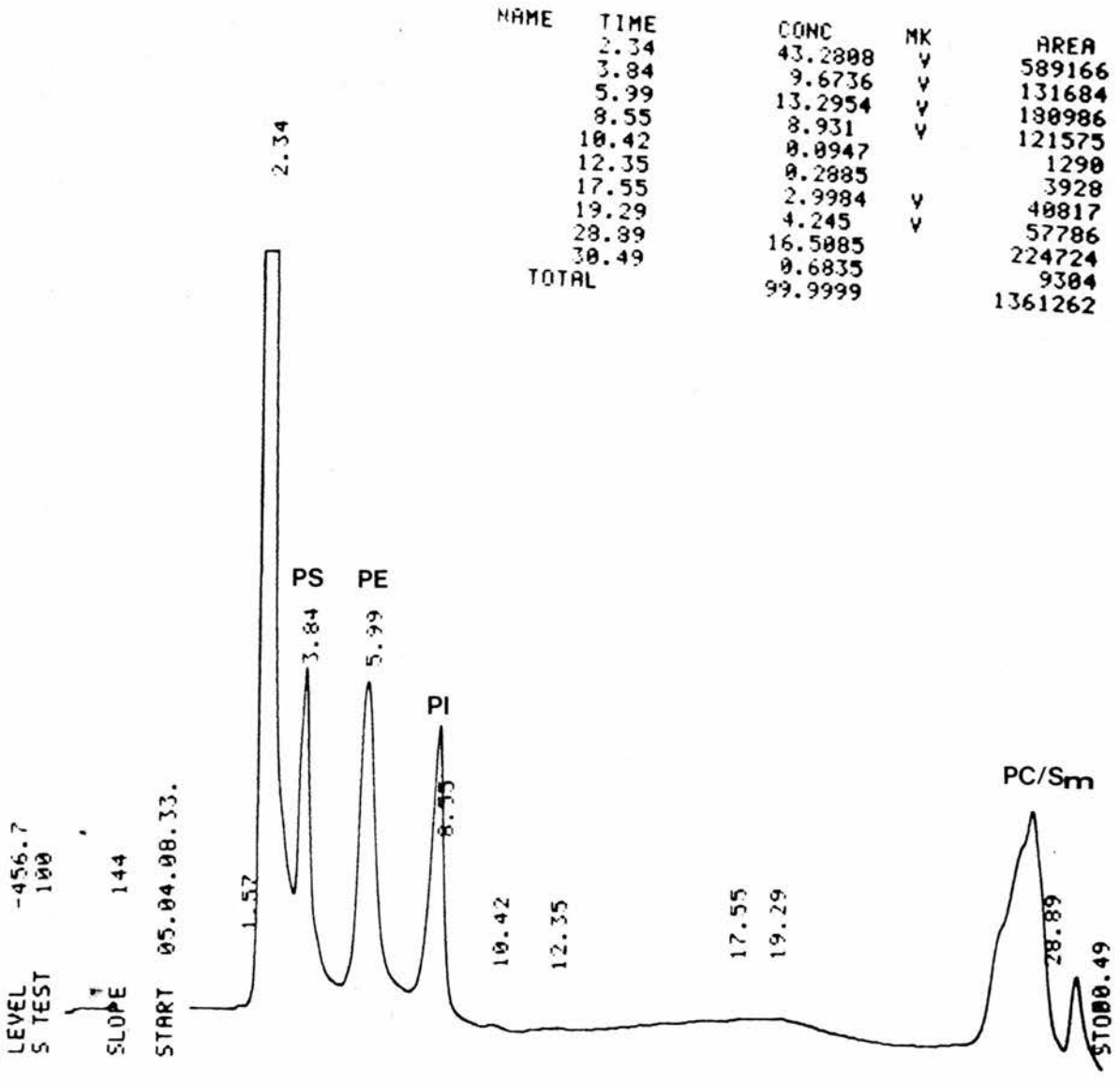
Autoradiogram

TABLE 6

FRACTION NUMBER	FLUORESCENT MAXIMA (nm)		% FLUORESCENCE
	Excitation	Emission	
1	374	444	46
2	380	450	22
3	392	464	33

FIGURE 21

Typical Standard Phospholipid Profile (206 nm) using HPLC Gradient System 1



Chromatographic Conditions

Flow Rate	-	1.5 mls/minute
Range	-	0.5
Detection	-	U.V. 206 nm

HPLC Chromatograms Depicting U.V. and Fluorescence Profiles of EYPE-MDA 1:2 Reaction Mixture

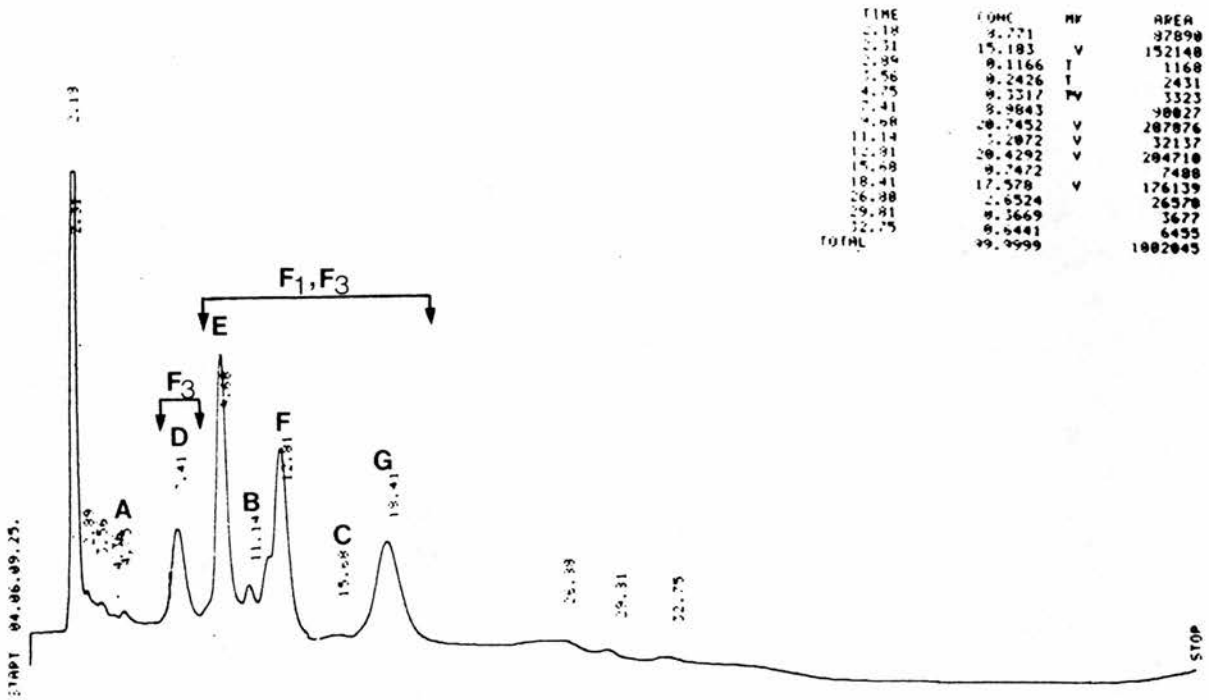
(HPLC Gradient System 1)

Figure 22a U.V. detection 206 nm.

Figure 22b Fluorescence detection.

Note: Preparative TLC fractions are indicated on each chromatogram.

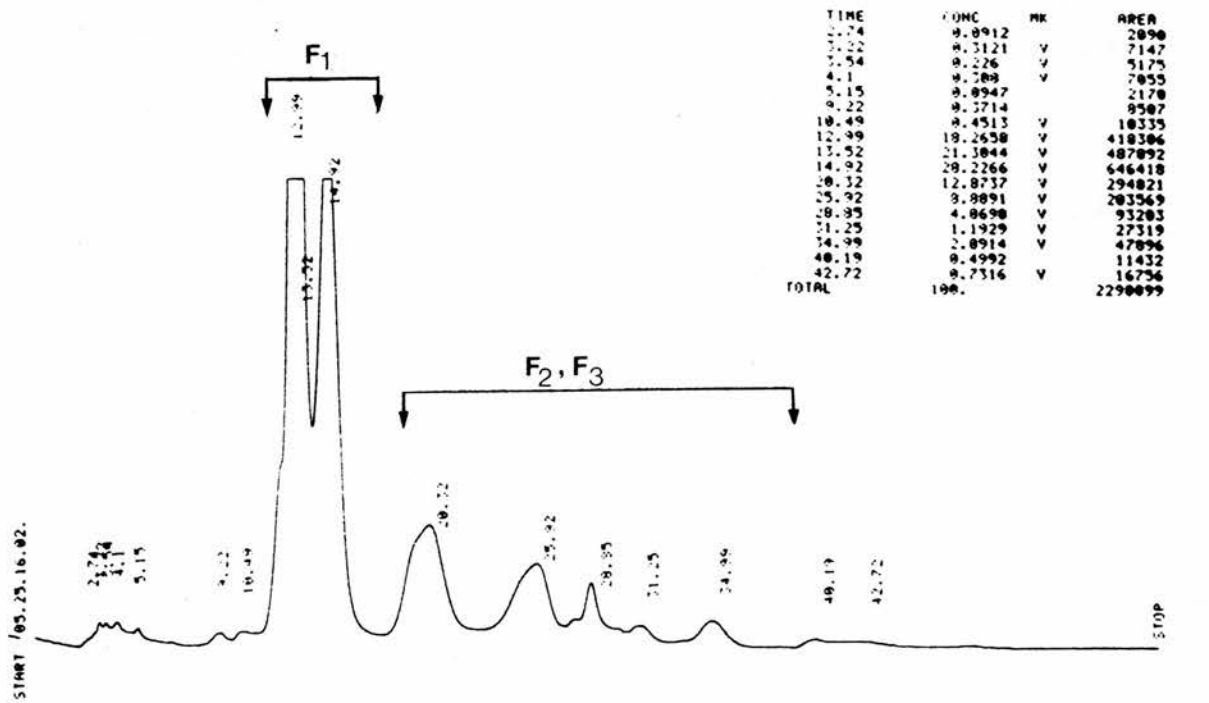
FIGURE 22a



TIME	CONC	MK	AREA
2.13	4.771		87890
2.31	15.183	V	152148
2.84	0.1166	T	1168
3.56	0.2426	T	2431
4.75	0.3317	TV	3323
7.41	8.9843		90027
9.68	20.7452	V	207876
11.14	3.2072	V	32137
12.81	20.4292	V	204710
15.68	0.7472		7488
18.41	17.578	V	176139
26.88	2.6524		26578
29.81	0.3669		3677
32.75	0.6441		6455
TOTAL	99.9999		1002045

(20 µl neat, Range 1)

FIGURE 22b



TIME	CONC	MK	AREA
2.74	0.0912		2090
3.22	0.3121	V	7147
3.54	0.226	V	5175
4.1	0.308	V	7055
5.15	0.0947		2170
9.22	0.3714		8507
10.49	0.4513	V	10335
12.99	18.2658	V	418386
13.52	21.3044	V	487892
14.92	28.2266	V	646418
20.32	12.8737	V	294821
25.92	8.8891	V	203569
28.85	4.0698	V	93203
31.25	1.1929	V	27319
34.99	2.0914	V	47896
40.19	0.4992		11432
42.72	0.7316	V	16756
TOTAL	100.		2290099

(16 µl 1 in 4 dilution, Range 1)

constituted approximately 9% of the total U.V. absorbing material, the other 3 major peaks containing approximately equivalent amounts (20% each) of reaction product on the basis of absorbance at 206 nm. Figure 22b indicates that the major fluorescent products do not correspond to the major U.V. absorbing products as shown by differing retention times. Three major fluorescent peaks were detected with retention times of 12.99, 13.52 and 14.92 minutes, constituting approximately 18%, 21% and 28% respectively of the total fluorescent product generated. Five or six minor and more polar fluorescent compounds were also detected as shown.

1.2.2.2 HPLC of fractions from preparative TLC

It was intended to effect partial purification of the reaction products by preparative TLC and this process was monitored by examining each fraction by HPLC. The HPLC chromatograms shown in Figures 22a and 22b also illustrate in which TLC fractions the major U.V. and fluorescent components were localised. The 3 major components detectable by fluorescence were clearly concentrated in fraction 1, (see Figure 20 for TLC fractions) with minor amounts of the more polar products in fractions 2 and 3. U.V. detection at 206 nm indicates as expected, that PE is contained within fraction 3, with amounts of the other 3-4 major products detectable in all 3 fractions.

1.2.2.3 Distribution of radioactivity in components resolved by HPLC

Aliquots of PE-MDA reaction mixture prepared using ^{14}C-labelled PE, were subjected to HPLC analysis with both

U.V. and fluorescence detection. Fractions constituting the major detectable fluorescent peaks were combined and the radioactivity determined for each as detailed in the methods section 1.6.5. A composite elution profile showing U.V. absorbance at 206 nm, fluorescence and radioactivity (corrected for background) is shown in Figure 23. Using the more sensitive procedure of fraction collection and monitoring by spectrofluorimetry, it is clear that the profile of fluorescent products which also contain significant amounts of radioactivity and are thus derived from PE, is extremely complex. There are also radioactive peaks which, although not corresponding to the fluorescent peaks, do correspond to the U.V. absorbing peaks, the most obvious of these being PE itself. (Retention time 7.41 mins.). Also it is noteable that the U.V. absorbing peak eluting just after PE at 9.68 minutes contains relatively high amounts of radioactivity although it shows little fluroescence either by HPLC detection or spectrofluorimetry. The major fluroescent components (Retention times 12.99 and 13.52 mins.) also coincide with the maximum radioactivity, although it is noteworthy that these only account for approximately 15% of the total counts, the remainder being widely distributed over minor and more polar components which do not absorb at 206 nm.

1.3 SUMMARY OF SYNTHETIC STUDIES

1D TLC resolves one major fluorescent, PE derived component (as detected by I_2 staining, phosphate estimation and autoradiography) for PE-MDA reaction mixtures. However, 1D TLC does suggest heterogeneity in this component although

Composite Fractionation Profile of

^{14}C PE-MDA Reaction Mixture Monitored by:-

FIGURE 23a — HPLC Fluorescence Detection and

⊖—⊖ Spectrofluorimetry (of collected fractions)

FIGURE 23b Radioactivity Determination

FIGURE 23c HPLC U.V. Detection 206 nm

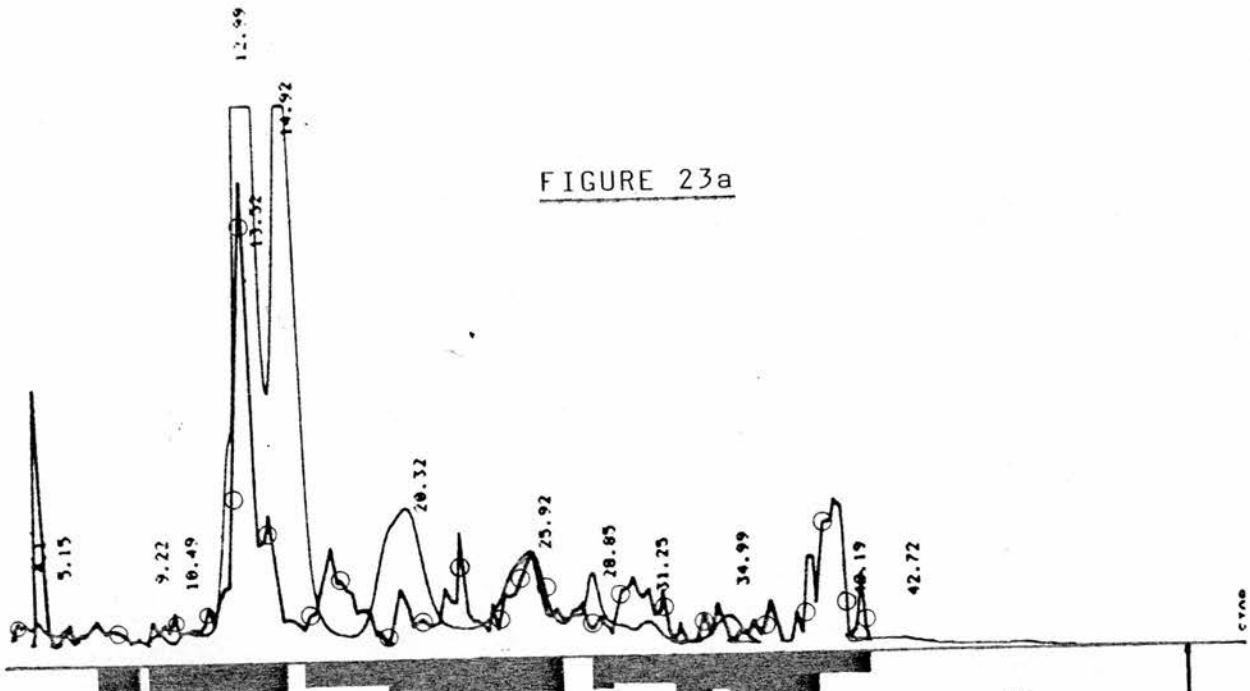


FIGURE 23a

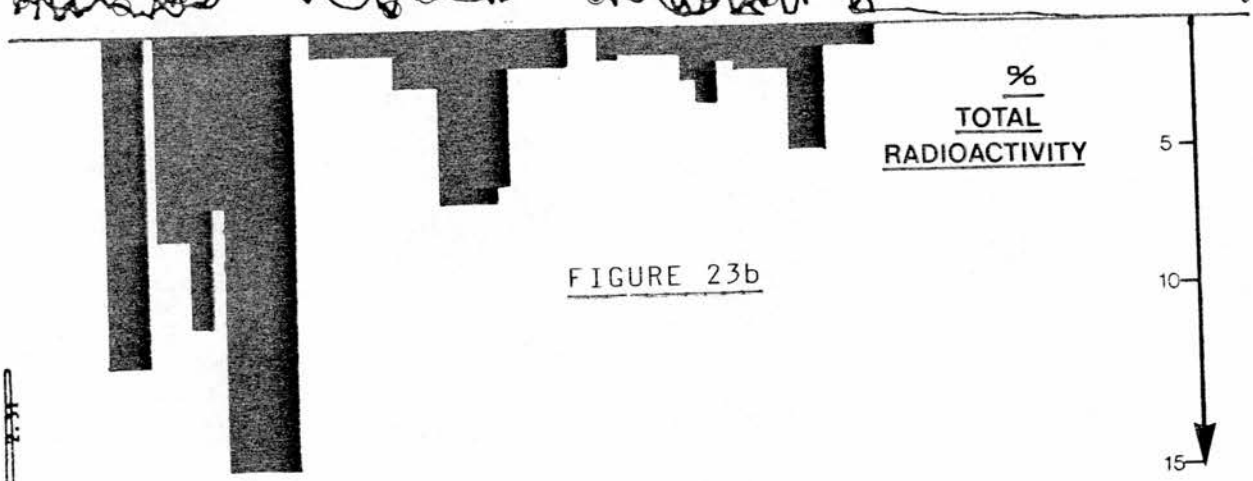


FIGURE 23b

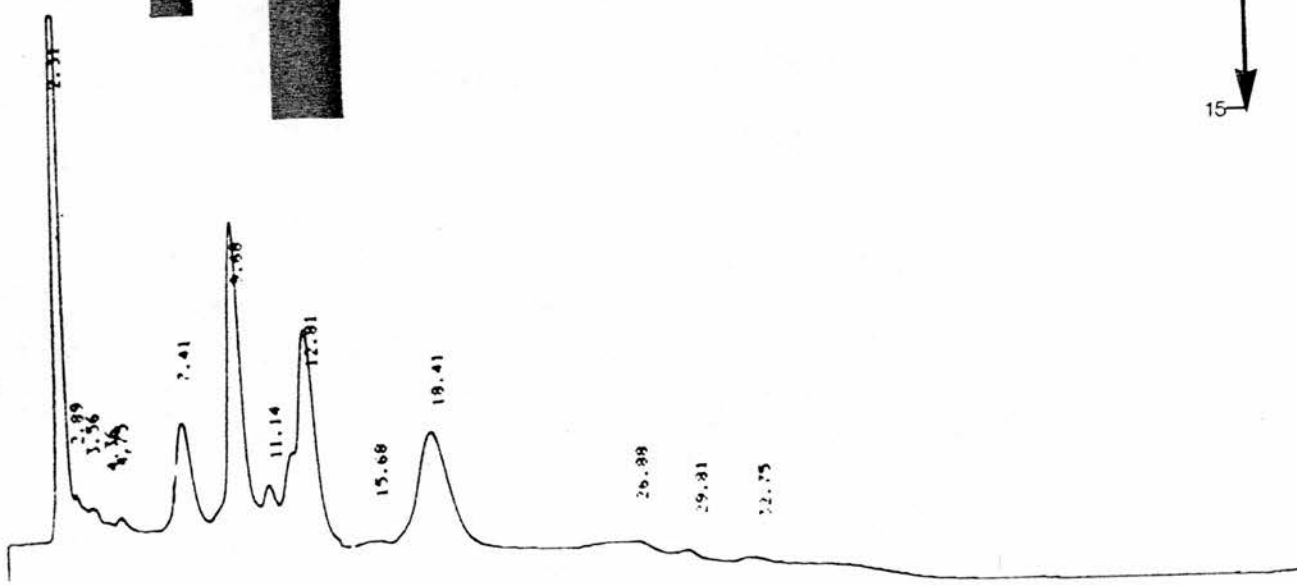


FIGURE 23c

resolution is not achieved. 2D TLC, (as detected by I_2 staining, autofluorescence and autoradiography) amplifies this heterogeneity and allows resolution of particularly the fluorescent components. Some of these contain little ^{14}C-PE and are therefore most likely derived from MDA polymerisation.

HPLC of the total reaction mixture and of fraction 1 purified by preparative TLC, confirms the complexity of this major product which is resolvable into a large number of components as detected by fluorescence, U.V. absorbance and by radioactive labelling.

2. BIOLOGICAL SYSTEM STUDIES

2.1 DEVELOPMENT OF FLUORESCENCE IN BIOLOGICAL REACTION MIXTURES

2.1.1 Rat Liver Microsomes

Incubations of rat liver microsomes with NADPH as the inducer of LPX, resulted in little increase in fluorescent material over a 3 hour incubation period in comparison to a 7-8 fold increase using TBH (Figure 24). Consequently the reaction products from the TBH system were extracted after a 3 hour incubation period whilst the NADPH system was continued for a total of 24 hours. This resulted in a 3 fold increase in total fluorescence and the products were then extracted. A negligible increase was observed in the unstimulated control.

2.1.2 Sheep Brain Homogenate

As expected, autoxidation of the brain homogenate at the higher, physiological temperature of 37^{o}C resulted in a 100% increase in fluorescence compared to the 19^{o}C incubation which resulted in oly a 50% increase (Figure 25). Maximal autoxidation was achieved following a 1 hour incubation and thereafter declined almost linearly up to 4 hours. As a result of these findings, a second incubation was carried out and the reaction products extracted after 1 hour. (Note - hereafter the autoxidised homogenate at the lower temperature (19^{o}C) is referred to as the reaction control).

FIGURE 24

Fluorescence Development in Rat Liver Microsomes

Induced by TBH and NADPH

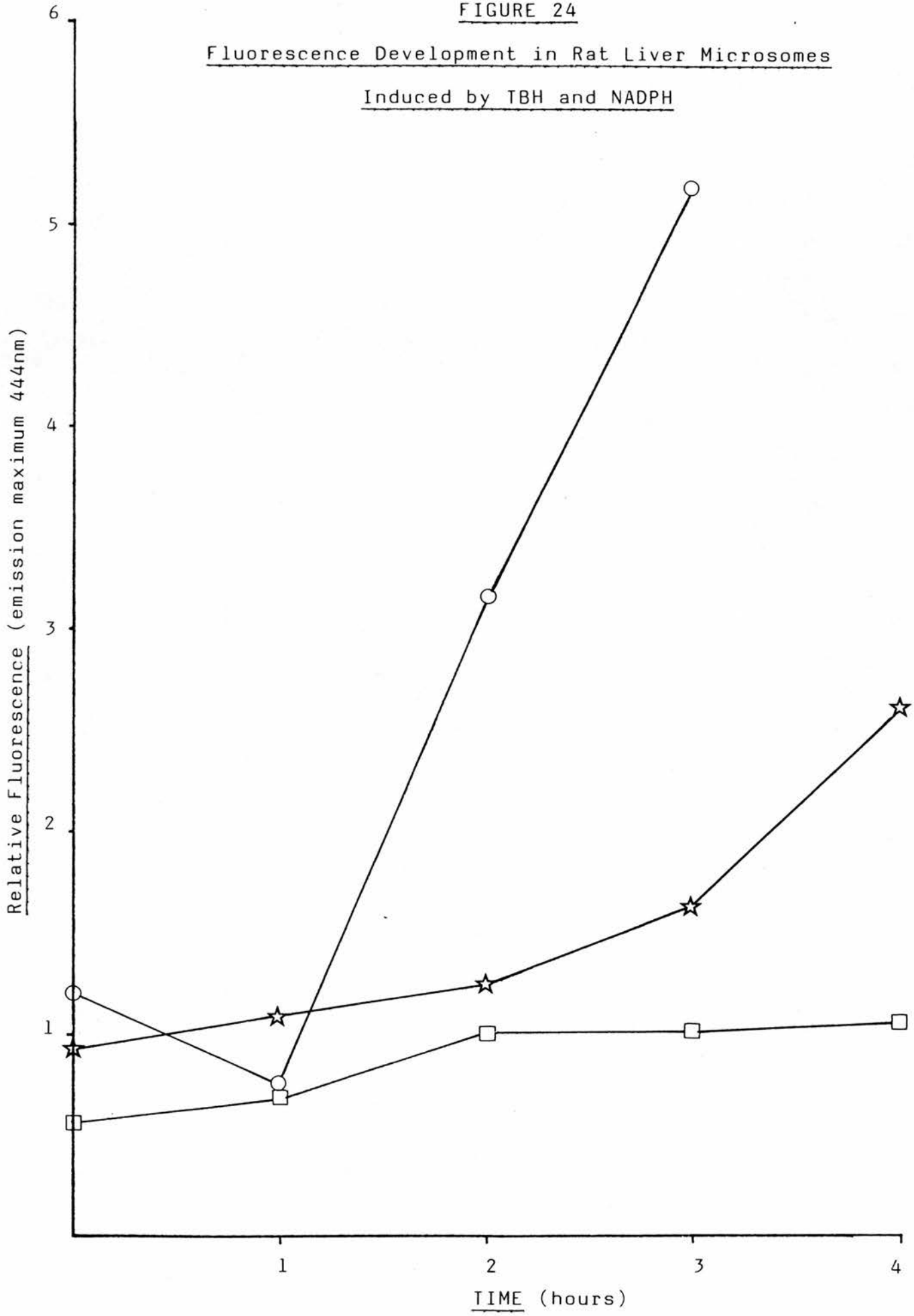
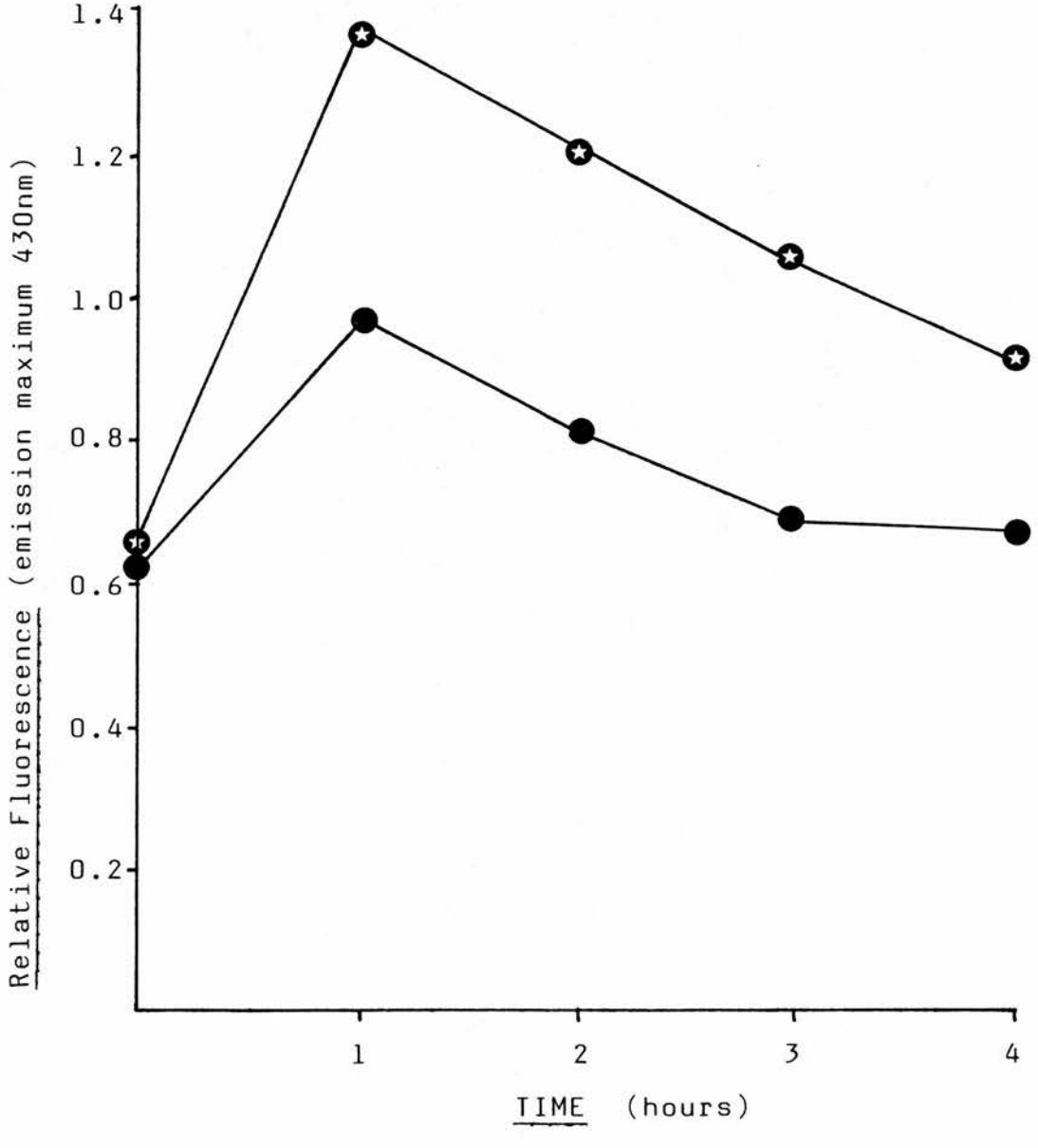


FIGURE 25

Fluorescence Development in Sheep Brain Homogenate

Autoxidised at 37°C and 19°C



2.2 CHROMATOGRAPHIC CHARACTERISATION OF REACTION PRODUCTS

2.2.1 Rat Liver Microsomes

2.2.1.1 TLC

2.2.1.1.1 1D TLC

As shown in Figure 26 there appeared to be little qualitative difference between the stimulated microsomes (lane 4) and the control microsomes, (lane 3) except for the presence of a fluorescent spot (X) in the former system which appeared to have a similar mobility to the major fluorescent product generated in the synthetic PE-MDA 1:2 system (Figures 13 and 15a). The total autofluorescence in the stimulated microsomes, particularly surrounding the PE region, was greater than that observed in the control. Although the illustrated chromatograms depict TBH stimulated microsomes only, there was no discernable difference between these and NADPH stimulated samples.

2.2.1.1.2 2D TLC

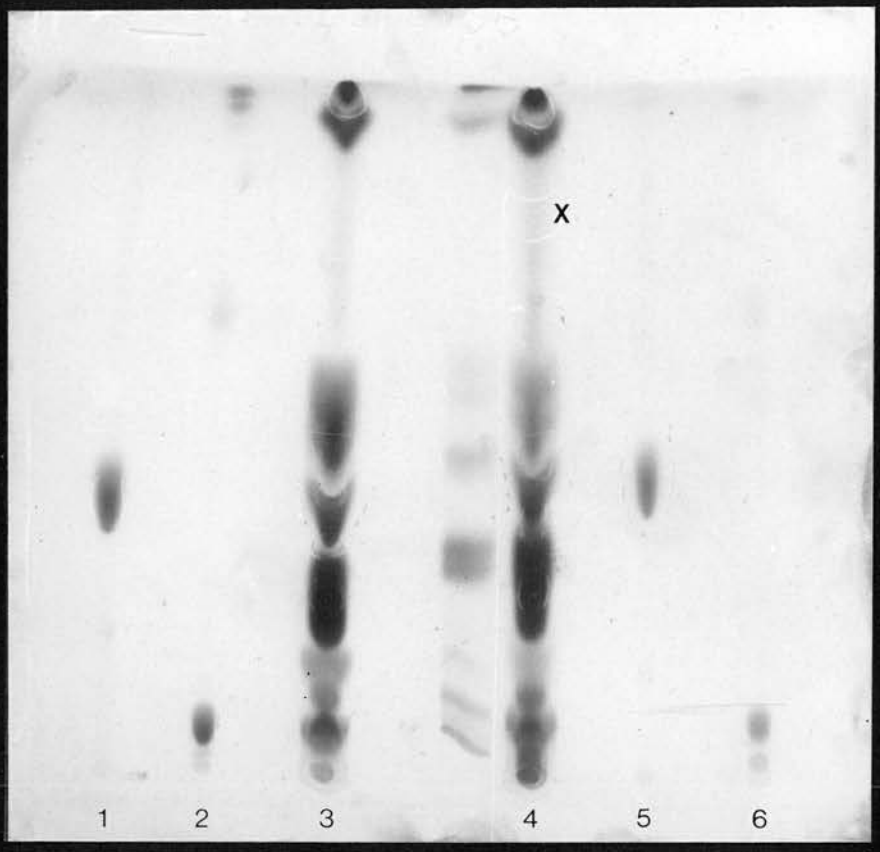
The lack of qualitative changes indicated with 1D TLC was further emphasised by 2D TLC in that the only clear difference between control and stimulated microsomes was a fluorescent, non I_2 staining spot (X) in the latter system. In addition there seemed to be more autoflurorescent, phosphate containing I_2 staining material at the origin from extracts of stimulated microsomes (Figure 27a and 27b). Also there was a visible increase in total fluorescence intensity particularly in the PE region of the stimulated sample.

2.2.1.2 HPLC

HPLC analysis using silica adsorbent was in agreement with the TLC findings in that few qualitative differences

FIGURE 26

1D TLC Chromatogram of TBH Induced Rat Liver Microsomes + Controls



Sample Identification:-

1 PE Standard

2 PS Standard

3 Control Microsomes

4 TBH Microsomes

5 PE Control

6 PS Control

Solvent System $CHCl_3$/MeOH/NH_4OH (16:8.5:1 v/v)

Loading 50 µg lipid/spot

Detection I_2 staining

2D TLC Chromatograms of TBH Induced Rat Liver Microsomes + Control

FIGURE 27a TBH Induced Microsomes

FIGURE 27b Control Microsomes

Key to Spots

0	Origin
1	Lyso PC
2	Sm
3	PC
4	Lyso PE
5	PS
6	PA
7	PE

Solvent System

1st Dimension	$CHCl_3$/MeOH/NH_4OH (16:8.5:1 v/v)
2nd Dimension	$CHCl_3$/MeOH/HAc (4.2:2.3:1 v/v)

Loading 50 µg lipid/plate

Detection I_2 staining

FIGURE 27a

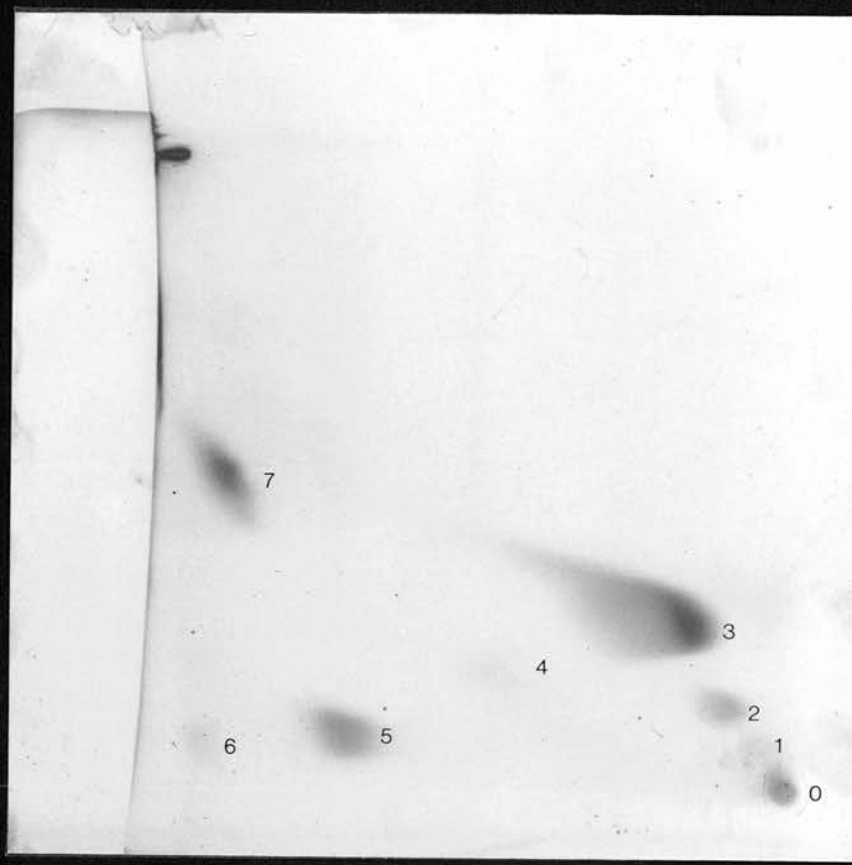


FIGURE 27b

were observed between the induced and control microsomes using either U.V. or fluorescence detection. Figures 28a, b and c show the HPLC chromatograms as monitored by U.V. and fluorescence detection. Table 7 indicates that there is little difference between the relative proportions of the major phospholipid classes (in terms of their absorbance at 206 nm), however it is noteworthy that two unidentified components with retention times of 12.9 and 13.5 minutes, appear to be diminished in the TBH induced microsomes compared with the control. The major fluorescent peaks present in both induced and control microsomes do not correspond to any of the major phospholipid classes, yet do appear to correspond with the major peak visible in the synthetic PE-MDA reaction systems (see Figure 22).

2.2.2 Human Erythrocytes

2.2.2.1 1D TLC

2.2.2.1.1 TBH induced erythrocytes

Figure 29a and 29b, (lanes 6 and 1 respectively) indicate the presence of an additional I_2 staining spot (X) visible between PS and the origin in the TBH treated erythrocytes compared to the controls (lanes 7 and 3 respectively). Also an extra autofluorescent spot (Y) was observed in the induced erythrocytes, situated slightly above the PE spot.

2.2.2.1.2 MDA treated erythrocytes

Figures 29a and 29b, (lanes 10 and 5 respectively) indicate little qualitative difference between MDA treated erythrocytes compared with the controls (lanes 7 and 3 respectively). The TBH controls were used again for

HPLC Chromatograms Depicting TBH Induced Rat Liver Microsomes + Control as Monitored by Fluorescence and U.V. Detection (HPLC Gradient System 1)

FIGURE 28a — TBH Induced Microsomes - U.V. detection (20 µl, R = 0.5)

FIGURE 28b — Control Microsomes - U.V. detection (20 µl, R = 0.5)

FIGURE 28c — TBH Induced and Control Microsomes - Fluorescence detection (20 µl, R = 1)

FIGURE 28a

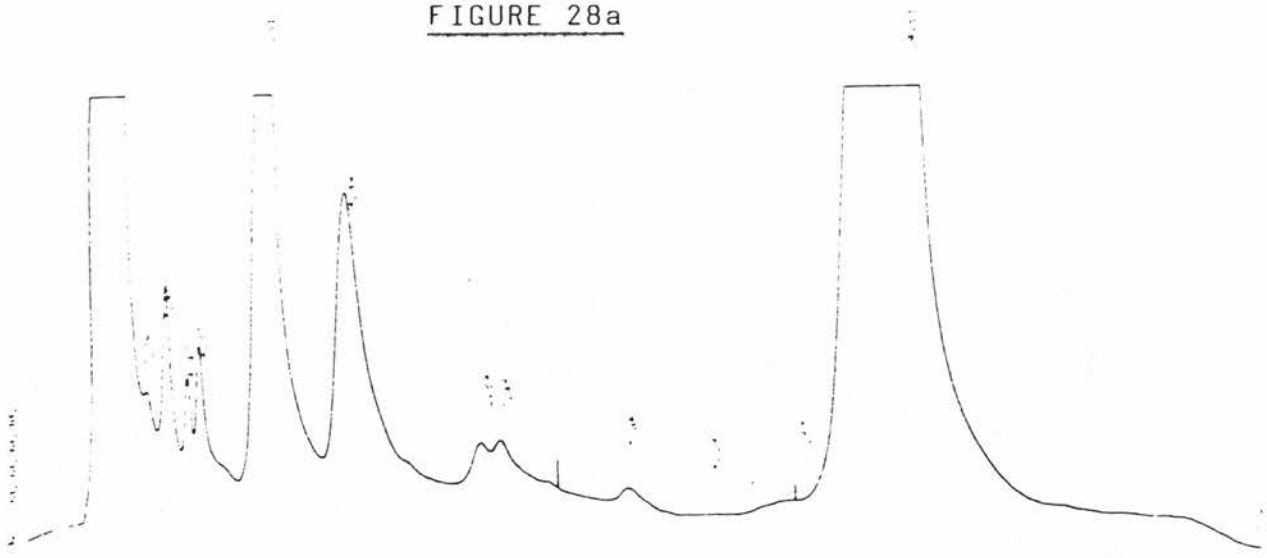


FIGURE 28b

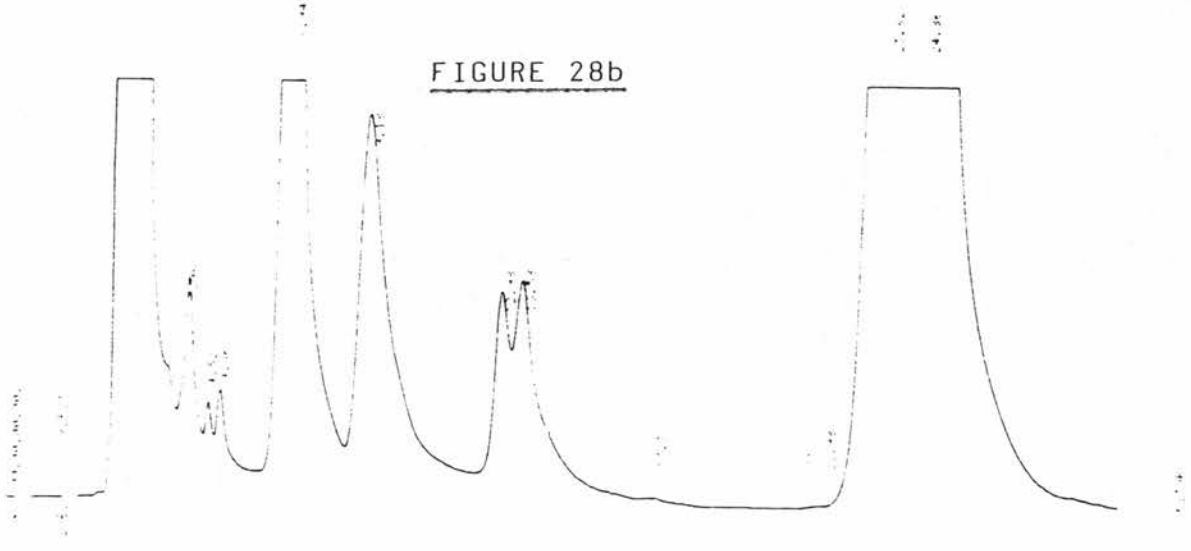


FIGURE 28c

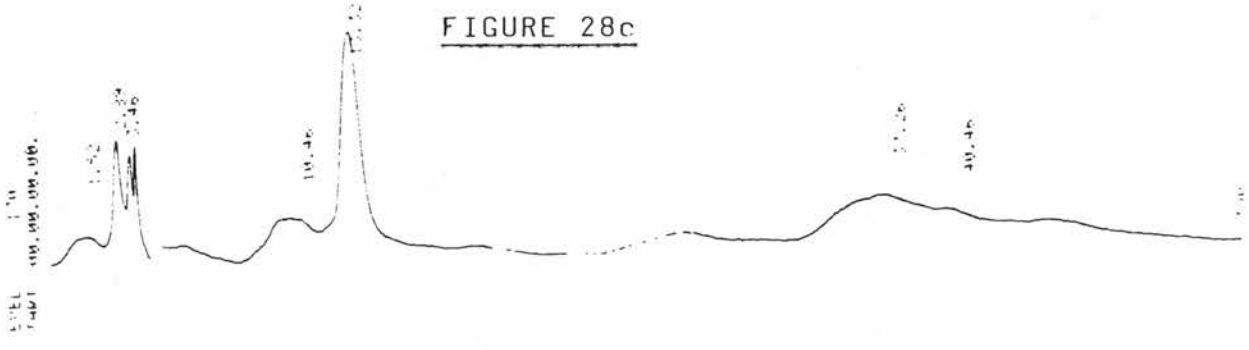


TABLE 7

Results Showing Percentage Phospholipid Class in TBH Induced and Control Microsomes as Determined by HPLC Analysis

(U.V. detection 206 nm)

	% ABSORBANCE 206nm	
Phospholipid Class	**Induced Microsomes**	**Control Microsomes**
PS	9·6	7·7
PE	16·8	15·5
PI	2·2	1·8
PC/Sm	72·3	75·9

1D TLC Chromatograms Depicting TBH and MDA Treated Erythrocytes + Controls

Key to Figures 29a and 29b

29a

1 PE standard
2 PS standard
3 PC standard
4 Sm standard
5 PE-MDA reaction 4:1
6 TBH microsomes ($CHCl_3$)
7 TBH control ($CHCl_3$)
8 TBH microsomes (MeOH)
9 TBH control (MeOH)
10 MDA microsomes ($CHCl_3$)
11 MDA microsomes (MeOH)
12 MDA control ($CHCl_3$)
13 MDA control (MeOH)
14 PE-MDA reaction 1:2

29b

1 TBH microsomes ($CHCl_3$)
2 TBH microsomes (MeOH)
3 TBH control ($CHCl_3$)
4 TBH control (MeOH)
5 MDA microsomes ($CHCl_3$)
6 MDA microsomes (MeOH)
7 MDA control ($CHCl_3$)
8 MDA control (MeOH)

Solvent system $CHCl_3$/MeOH/NH_4OH (16:8.5:1 v/v)

Loading 50 µg lipid/spot

Detection Iodine staining

FIGURE 29a

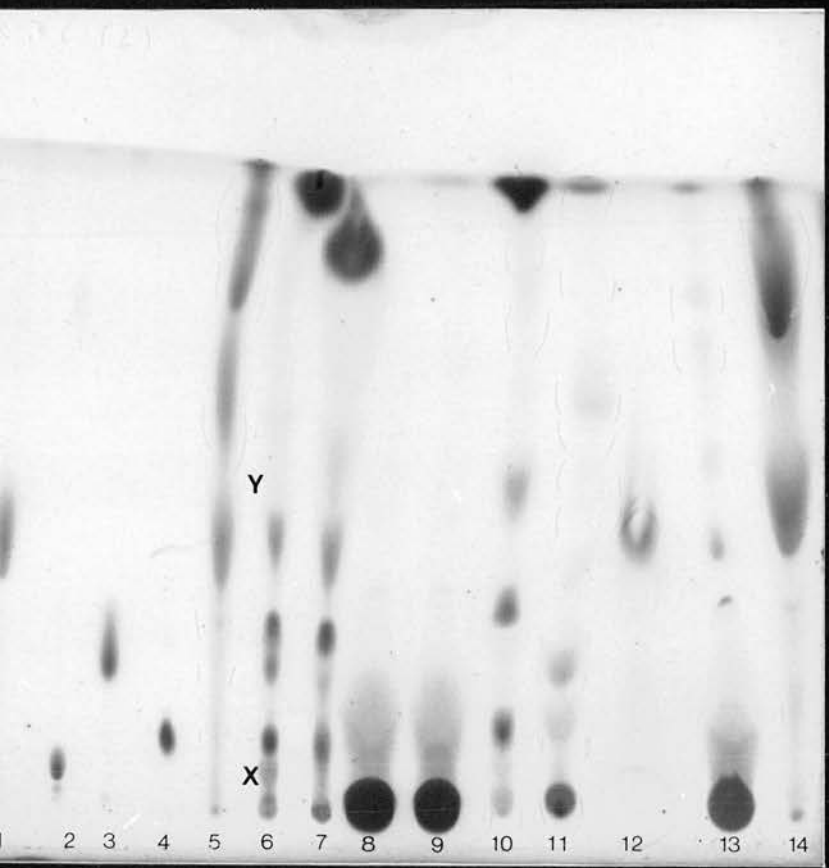
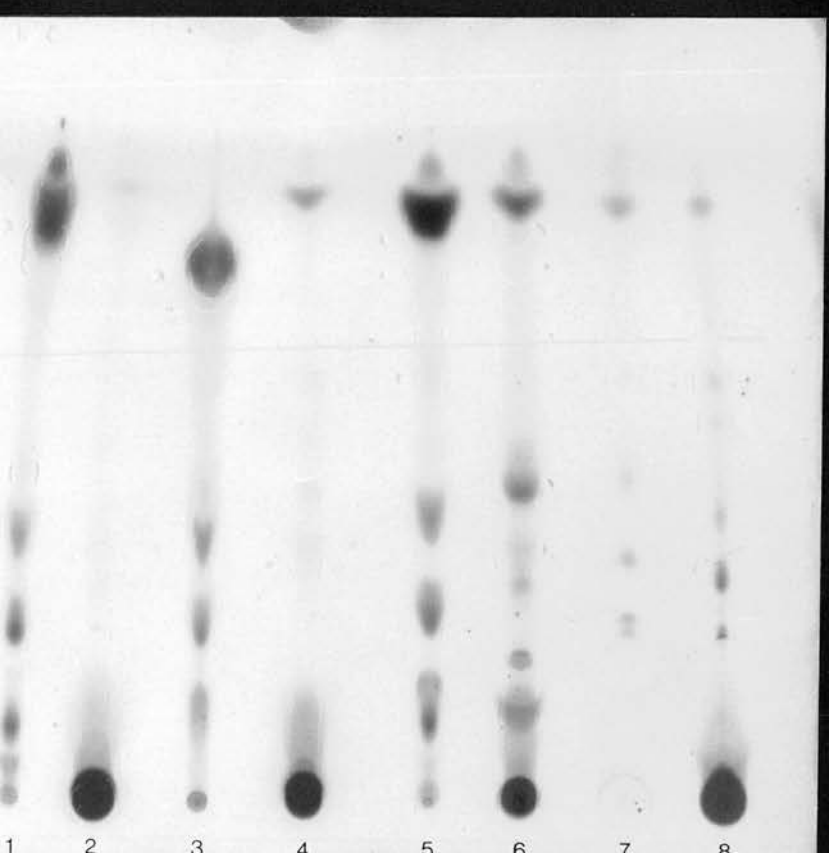


FIGURE 29b



comparison here since the appropriate control for MDA treated erythrocytes was accidently destroyed.

2.2.3 Sheep Brain Homogenate

In this later work, it will be clear from the I_2 stained plates and the autofluorescence photographs, that the newly developed 1D solvent system, while not giving complete resolution of the major phospholipids, did achieve superior resolution of the fluorescent components and was thus used for this purpose.

2.2.1.1 TLC

2.2.3.1.1 1D TLC

As discovered in the other biological systems, there was no evidence for the formation of major novel components in the autoxidised homogenate compared with the control detected by autofluorescence (Figure 30a, lanes 1 and 2) and I_2 staining (Figure 30b, lanes 1 and 2). However in both control and autoxidised homogenate, there are several fluorescent components, one of which (lanes 1 and 2, spot Y) has comparable mobility to the major fluorescent product (Z) formed during in vitro incubations of EYPE and MDA (lanes 4 and 5). It is also noteworthy that there is more fluorescent material in total but also in the region of PE and at the origin of the 37°C peroxidised brain extract compared with the 19°C incubation control.

2.2.3.1.2 2D TLC

The preliminary findings of 1D TLC analysis were further substantiated by 2D TLC in that no components unique to the autoxidised homogenate were visible, although there was a marked increase in fluorescence particularly

1D TLC Chromatograms Depicting 19°C and 37°C Autoxidised Brain Homogenate and Synthetic PE-MDA 1:2 Reaction Mixture + Controls

Key to Figures 30a and 30b

1 37°C Autoxidised Brain

2 19°C Autoxidised Brain

3 PE Standard

4 PE-MDA 1:2 Reaction Mixture (20 µl)

5 PE-MDA 1:2 Reaction Mixture (5 µl)

Solvent System

n-propanol/water

(3:1 v/v)

Loading

20 µg lipid/spot

FIGURE 30a

Autofluorescence

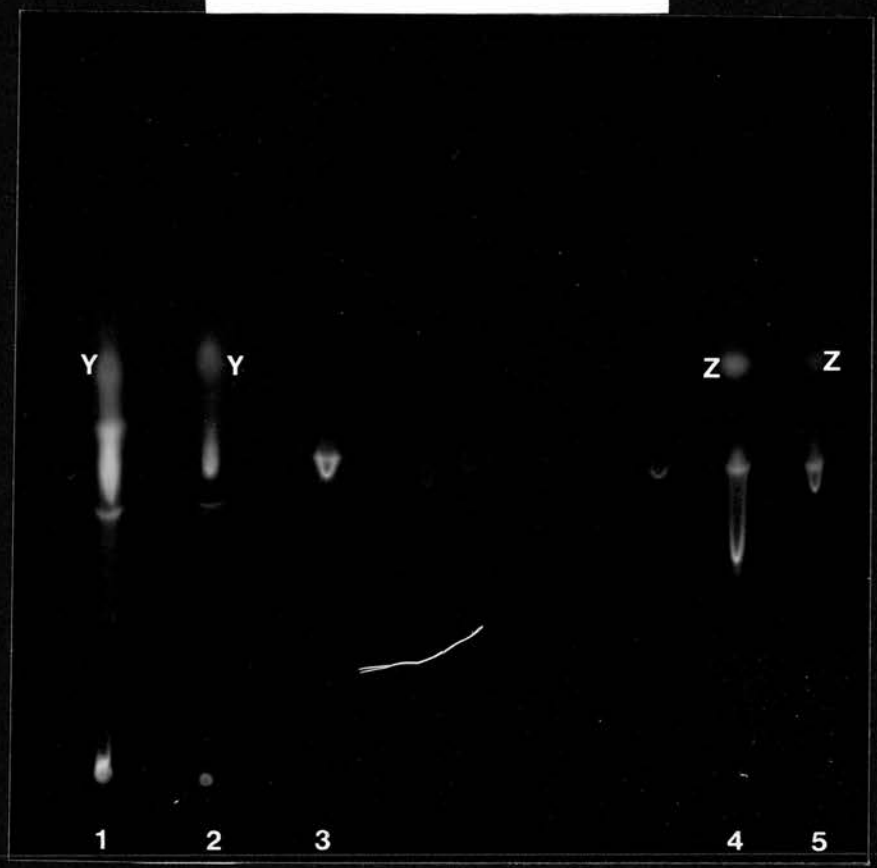
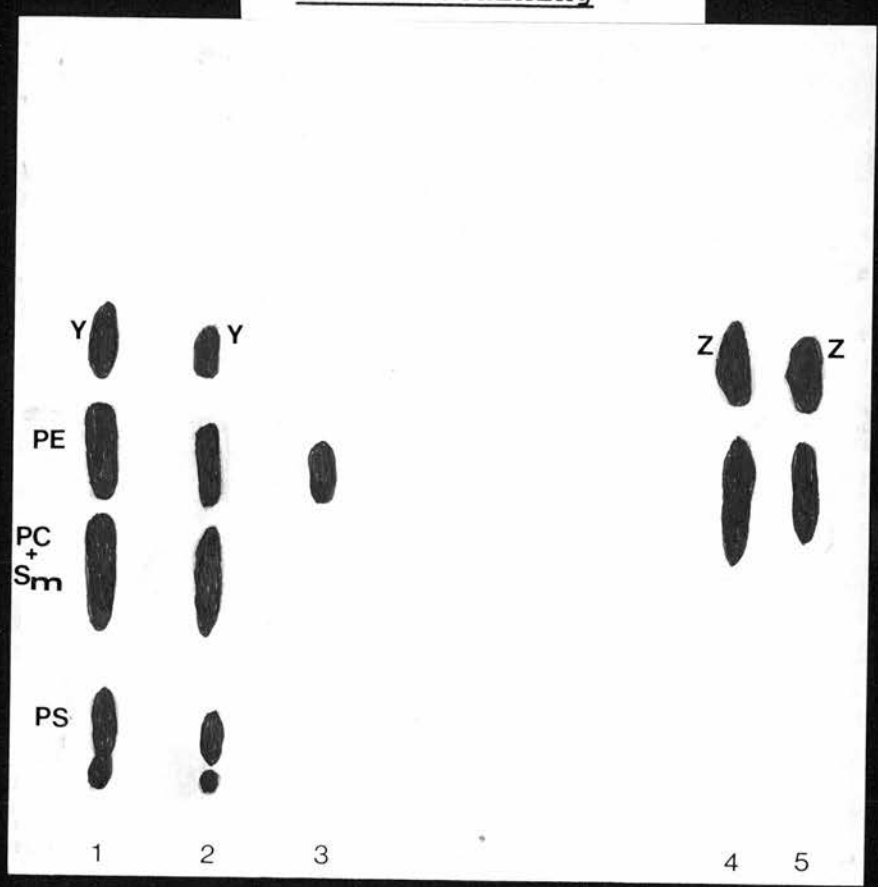


FIGURE 30b

Iodine Staining



surrounding the PE region (Figures 31a, b and 32a, b). Quantitative analysis of the major phospholipid classes by colorimetric phosphate determinations following 2D TLC, indicated a minor increase in PE and PS (relative to the other major phospholipid classes) in the 37°C autoxidised homogenate as shown in Table 8. This is consistent with the findings from HPLC analysis of peroxidised microsomal lipids (Table 7).

2.2.3.1.3 Preparative TLC

Preparative TLC was carried out in an attempt to effect partial purification of the major fluorescent components. Figures 33a and 34a of chromatograms loaded with identical amounts of total lipid phosphorus, clearly show a much greater amount of total autofluorescence in the autoxidised homogenate compared with the 19°C control (Figure 33b and 34b), although the difference was even more pronounced to the human eye than is apparent from the fluorescence photographs. Each band was then eluted and analysed for the following:-

Fluorescence spectrum,

Relative fluorescence at appropriate λ maxima.

Table 9 summarised the results obtained. In comparison with the control, fraction 1 of the autoxidised sample contained much more fluorescent material. Fraction 4 of the control homogenate was not the same as the autoxidised sample since in the latter it represented a very broad and diffuse band suggesting that it contained peroxidised PE or at least PE whose resolution was influenced by the presence of PE autoxidised derivatives (see Figure 33 and 34). Whereas fraction 4, together with fraction 3 (PE) in the autoxidised

2D TLC Chromatograms Depicting 19°C and 37°C Autoxidised Brain Homogenate

Fibure 31a

37°C Autoxidised Brain

Figure 31b

37°C Autoxidised Brain

Figure 32a

19°C Autoxidised Brain

Figure 32b

19°C Autoxidised Brain

Solvent System

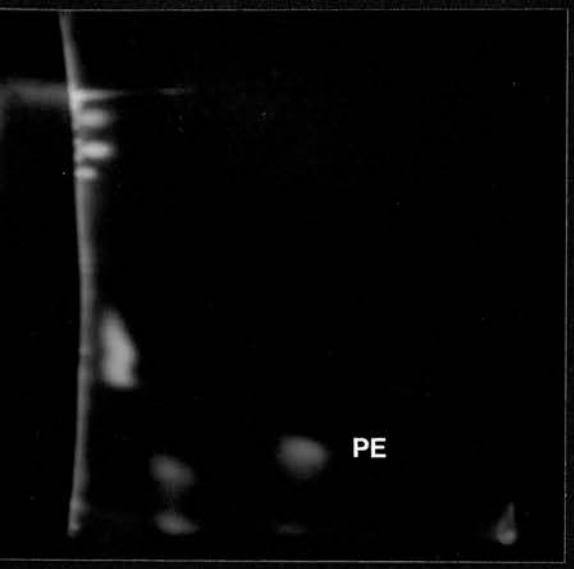
1st Dimension	$CHCl_3$/MeOH/NH_4OH (16:8.5:1 v/v)
2nd Dimension	$CHCl_3$/MeOH/HAc (4.2:2.3:1 v/v)

Loading

50 µg lipid/plate

FIGURE 31a

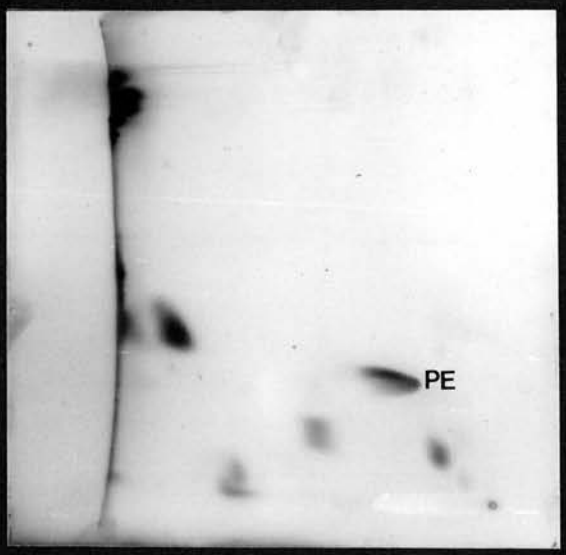
FIGURE 32b

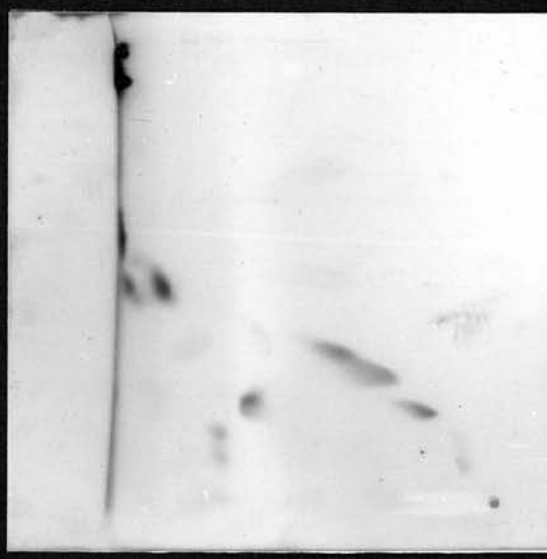


Autofluorescence

FIGURE 31b

FIGURE 32a





Iodine Staining

TABLE 8

Results Showing Percentage Phospholipid Class in 19°C and 37°C Autoxidised Brain Homogenate as Estimated by Colorimetric Phosphorus Assay

	% PHOSPHORUS		
Phospholipid Class	37°C Homogenate	?19°C 37°C Homogenate	Scott et al (197)
LPC	8.6	14.4	-
Sm	17.4	13.6	12.8
PC	32.0	34.7	37.3
PI/PS	17.0	13.6	11.3
PA	5.6	5.9	2.6
PE	19.4	17.8	7.7
Plasmalogen	-	-	16.5
Other	-	-	11.8

Preparative TLC of 19°C and 37°C Autoxidised Brain Homogenate

Figure 33a

37°C Autoxidised Brain

Figure 33b

19°C Autoxidised Brain

Figure 34a

37°C Autoxidised Brain

Figure 34b

19°C Autoxidised Brain

Solvent System

n-propanol/water

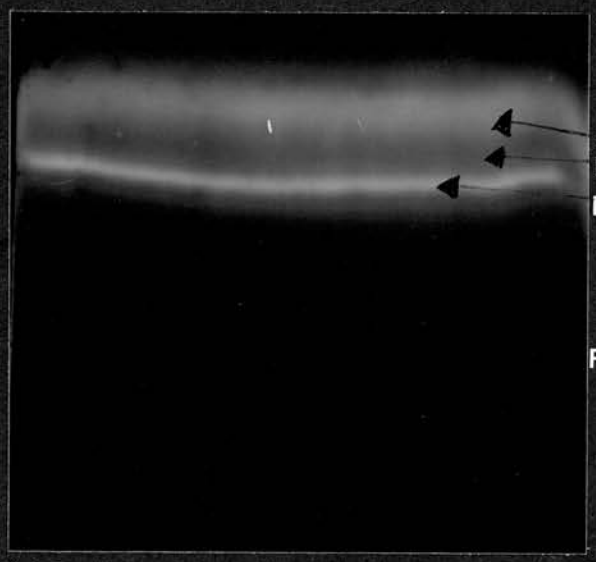
(3:1 v/v)

Loading

3 mg lipid/plate

FIGURE 33a

FIGURE 33b



Autofluorescence

FIGURE 34a

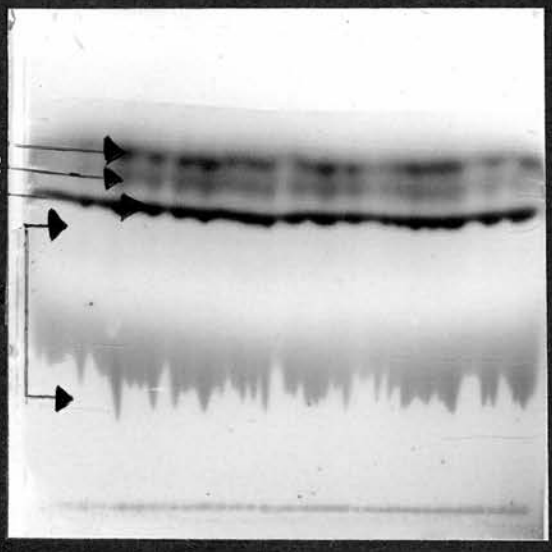
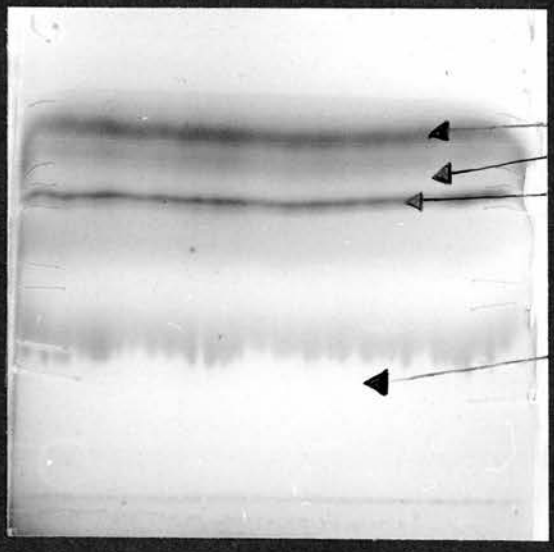


FIGURE 34b



Iodine Staining

TABLE 9

Fluorescent Properties and Phosphate Content of 19°C and 37°C Autoxidised Brain Homogenate Fractionated by Preparative TLC

	37°C Homogenate			19°C Homogenate		
Fraction	% Fluorescence	Excitation Maxima	Emission Maxima	% Fluorescence	Excitation Maxima	Emission Maxima
1	21	376	456	15	380	460
2	9	368	444	13	374	446
3	33	376	446	55	376	444
4	37	384	454	18	366	438

homogenate contained the bulk of the fluorescent material (70%), only fraction 3 of the control sample contained relatively significant amounts of fluorescent material (55%), suggesting that this was in fact unreacted PE.

2.2.3.2 HPLC

As shown in Figures 35a and b, there was no major difference between autoxidised brain homogenate and the control by U.V. detection. However a minor peak with a retention time of 5.21 minutes was visible in the control and absent in the autoxidised sample. Table 10 shows the relative differences in peak areas of the standard phospholipids. There was an apparent relative decrease in both PS and PI in the autoxidised homogenate but an increase in PE as judged by the absorbance at 206 nm.

Although the loadings of both autoxidised and control homogenate were the same for fluorescent HPLC analysis, the autoxidised sample was chromatographed at a less sensitive setting as shown in Figures 36a and b. This confirms the TLC observations since clearly the total amount of fluorescence in the autoxidised sample is much greater than that observed in the control. The chromatograms show a complex mixture of components emerging before PE as well as some with longer retention times. The three least polar components with retention times of 4.04, 4.74 and 5.34 minutes appear to be unique to the autoxidised sample.

HPLC chromatograms showing various fractions of autoxidised and control brain homogenate isolated by preparative TLC and detected using fluorescence are illustrated in Figure 37a-h. These results indicate that the

HPLC Chromatograms Depicting 19°C and 37°C Autoxidised Brain Homogenate as Monitored by U.V. Detection 206nm (HPLC Gradient System 1)

Figure 35a 37°C Autoxidised Homogenate (20 µl, Range 0.5)

Figure 35b 19°C Autoxidised Homogenate (20 µl, Range 0.5)

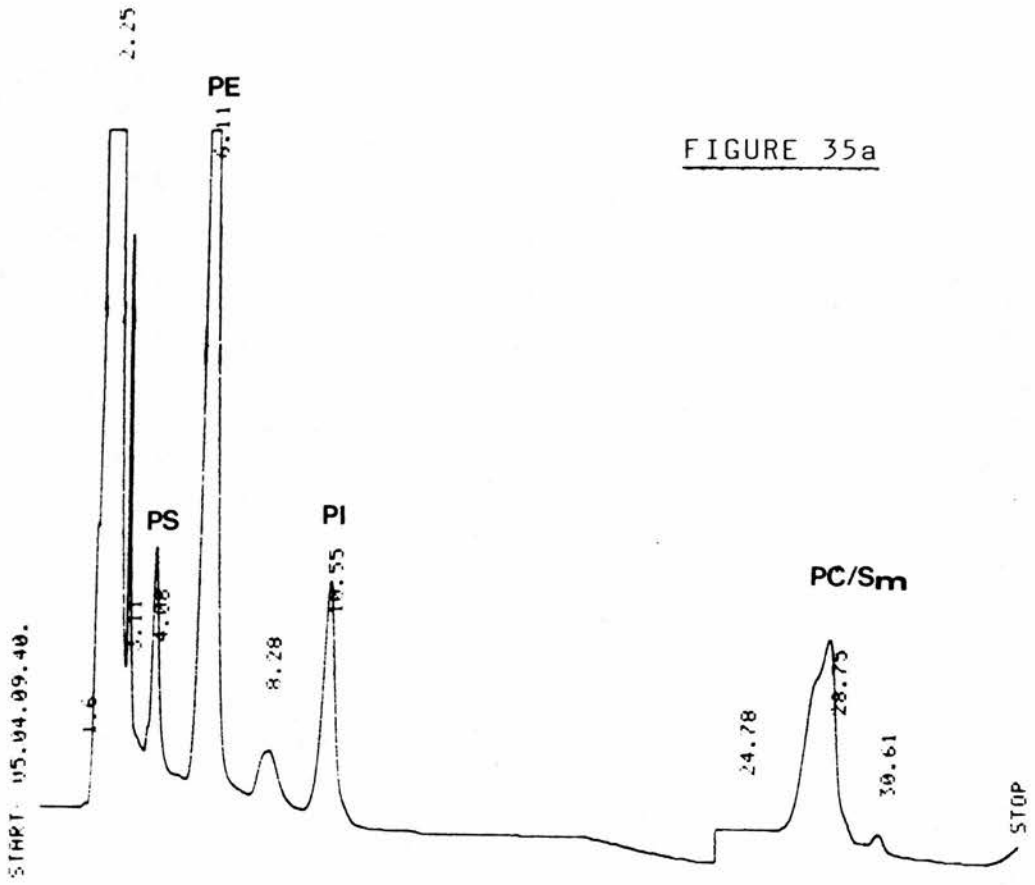


FIGURE 35a

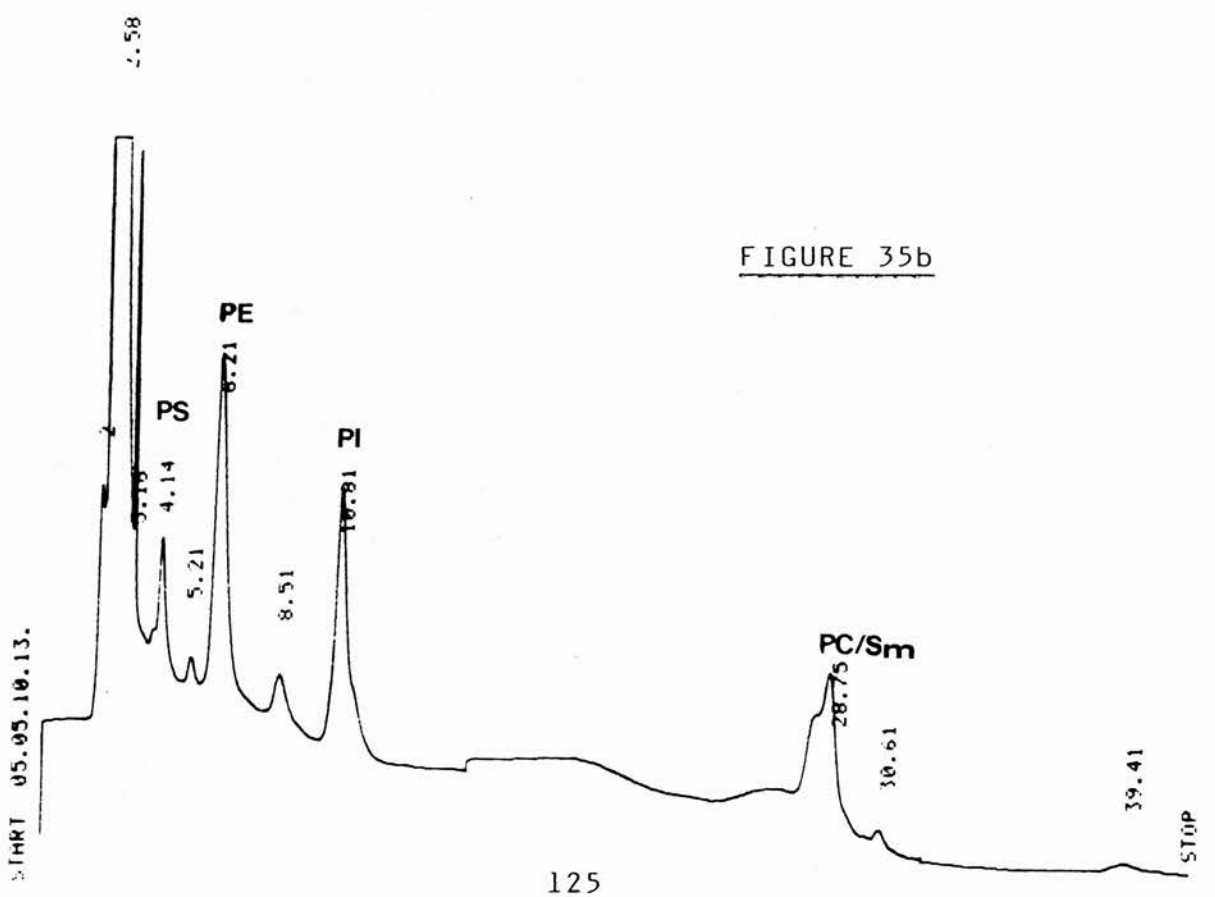


FIGURE 35b

TABLE 10

Results Showing Percentage Phospholipid Class in 19°C and 37°C Autoxidised Brain Homogenate as Determined by HPLC Analysis (U.V. detection 206 nm)

	% ABSORBANCE 206nm	
Phospholipid Class	**Autoxidised Homogenate**	**Control Homogenate**
PS	11·33	16·40
PE	54·20	39·00
PI	12·30	23·30
PC/Sm	22·17	21·30

HPLC Chromatograms Depicting 19°C and 37°C Autoxidised Brain Homogenate as Monitored by Fluorescence Detection (HPLC Gradient System 1)

FIGURE 36a

37°C Autoxidised Homogenate
(20 µl, Range 0.05)

FIGURE 36b

19°C Autoxidised Homogenate
(20 µl, Range 0.02)

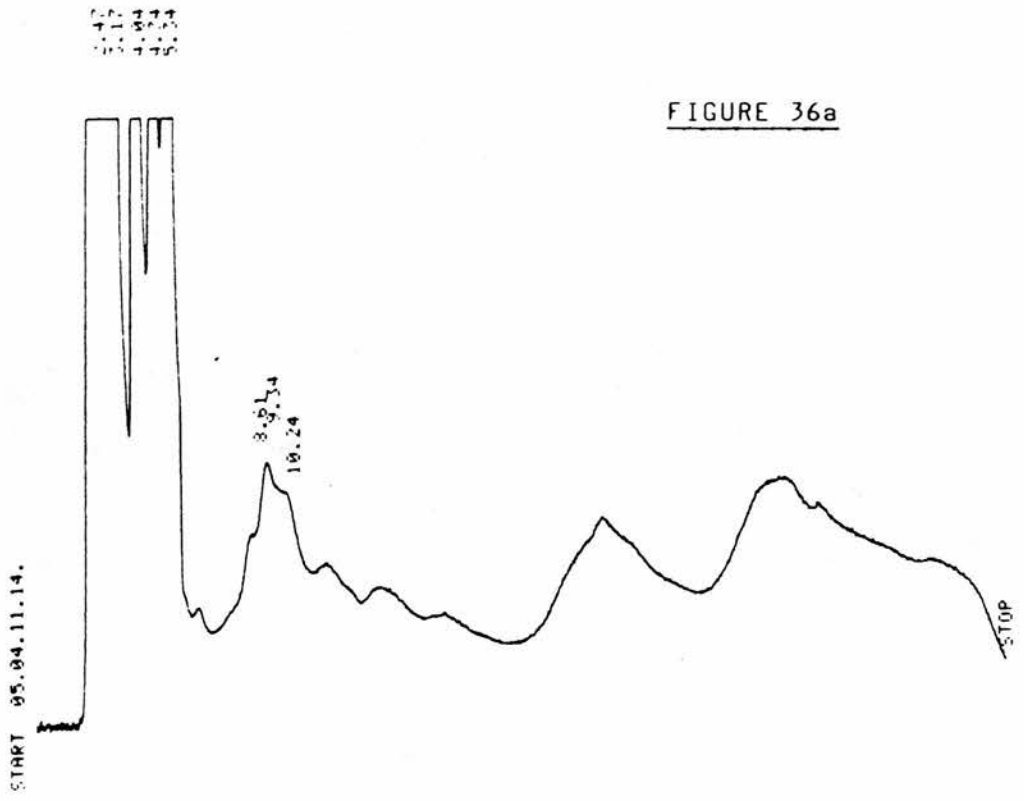


FIGURE 36a

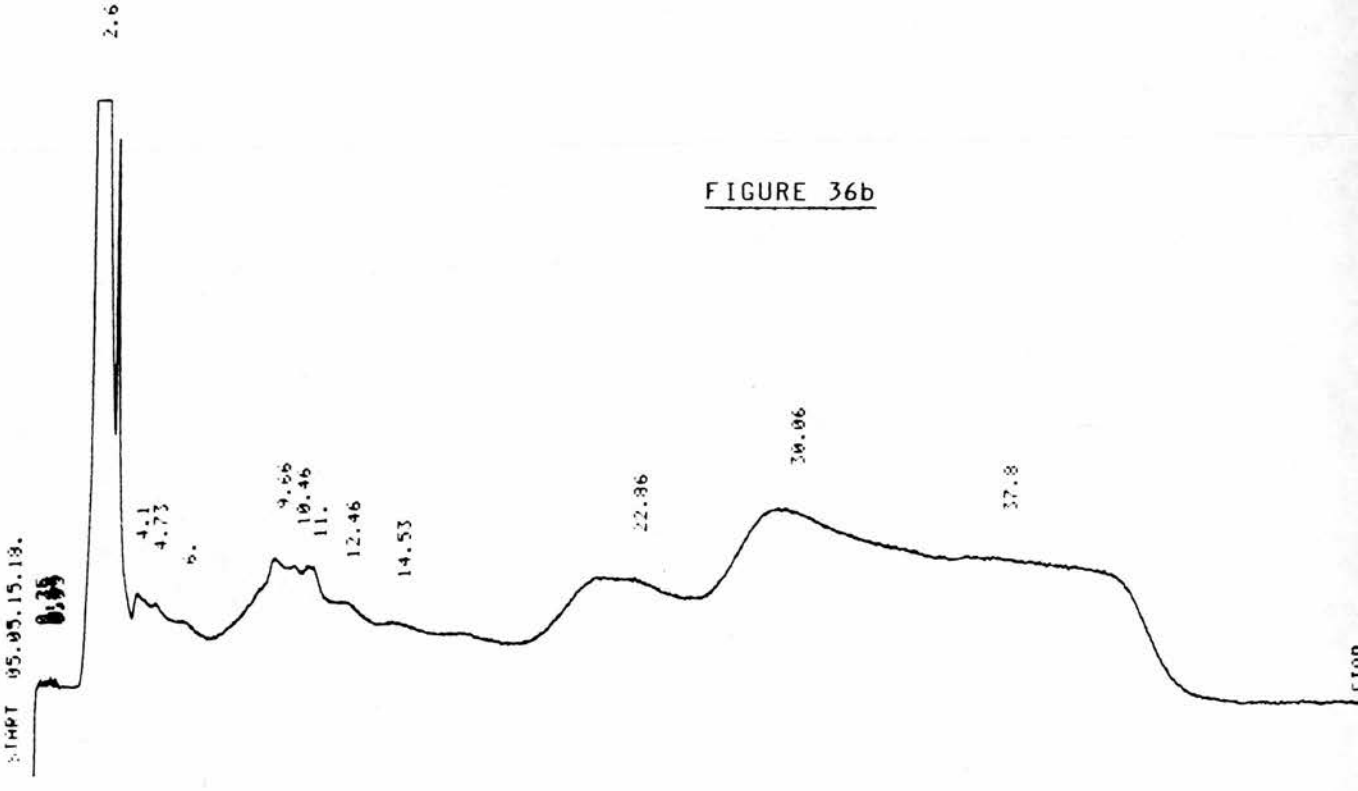


FIGURE 36b

FIGURE 37a-h

HPLC Chromatograms Depicting Fractionation of 19°C and 37°C Autoxidised Brain Homogenate from Preparative TLC as Monitored by Fluorescence

(HPLC Gradient System 1)

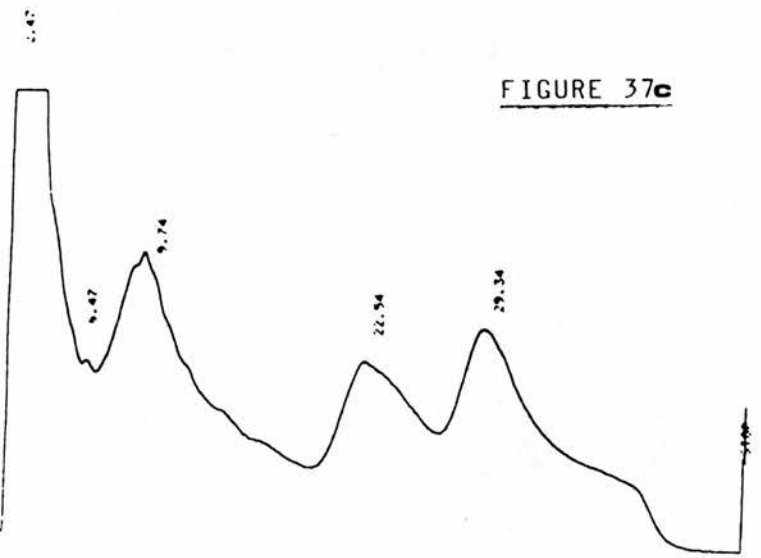
FIGURES 37a-d

37°C Autoxidised Homogenate, fractions 1-4.

FIGURES 37e-h

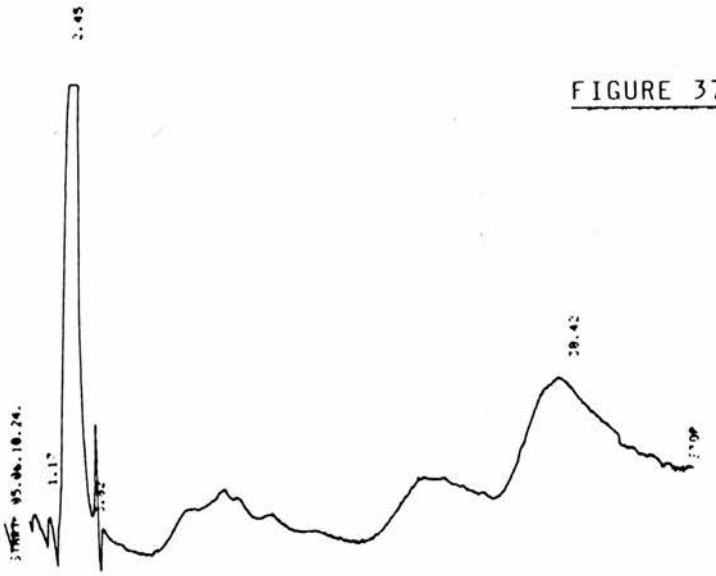
19°C Autoxidised Homogenate, fractions 1-4.

FIGURE 37c



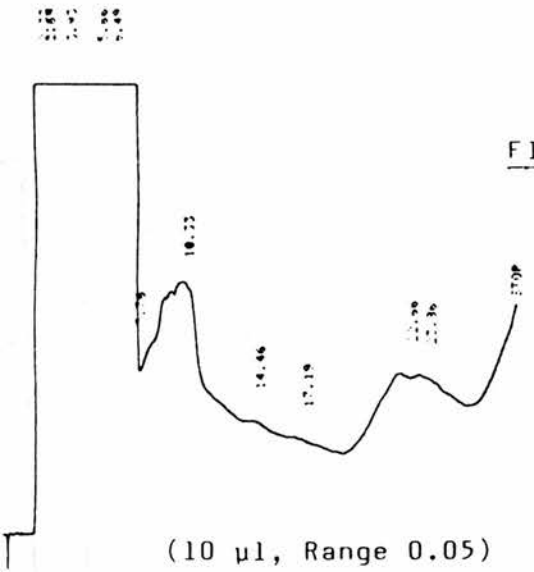
(10 µl, Range 0.02)

FIGURE 37g



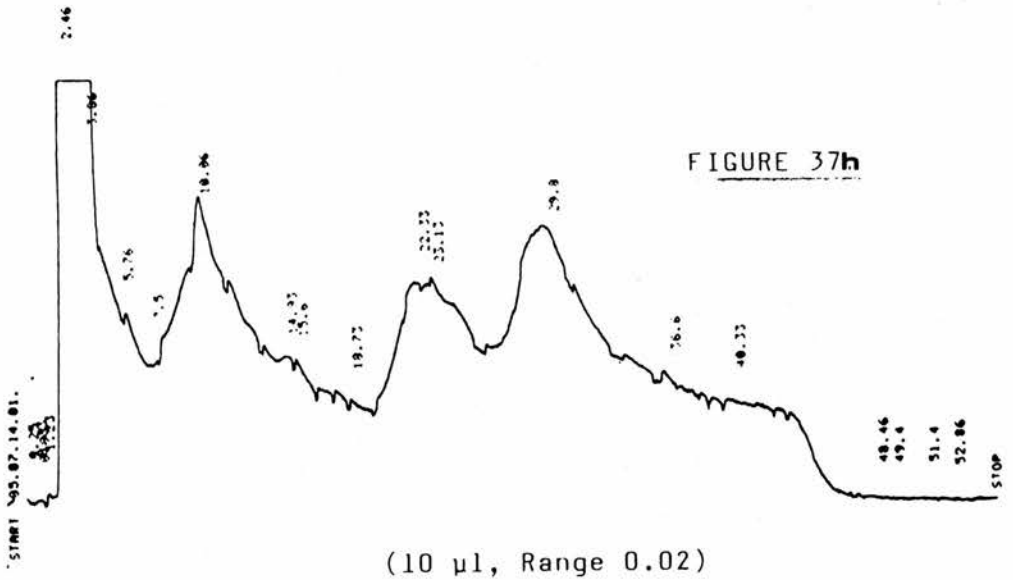
(20 µl, Range 0.02)

FIGURE 37d



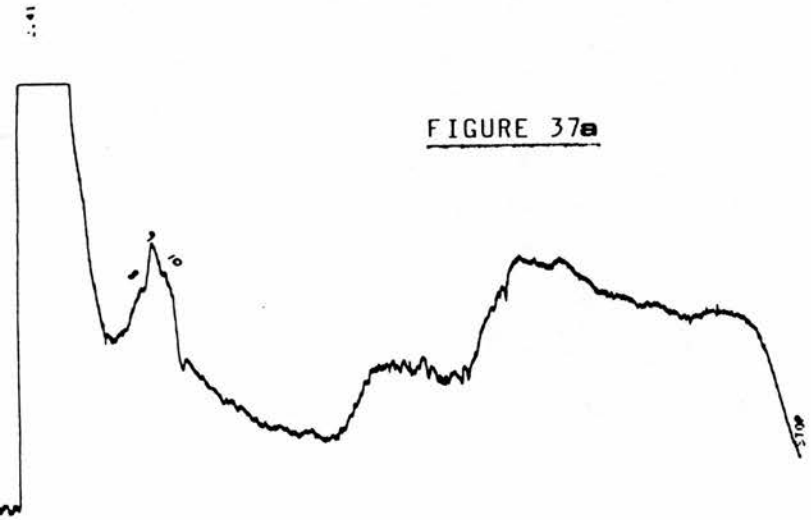
(10 µl, Range 0.05)

FIGURE 37h



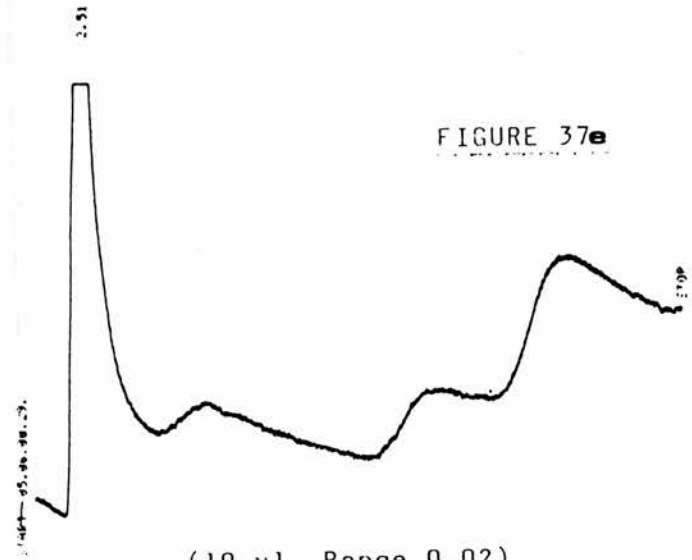
(10 µl, Range 0.02)

FIGURE 37a



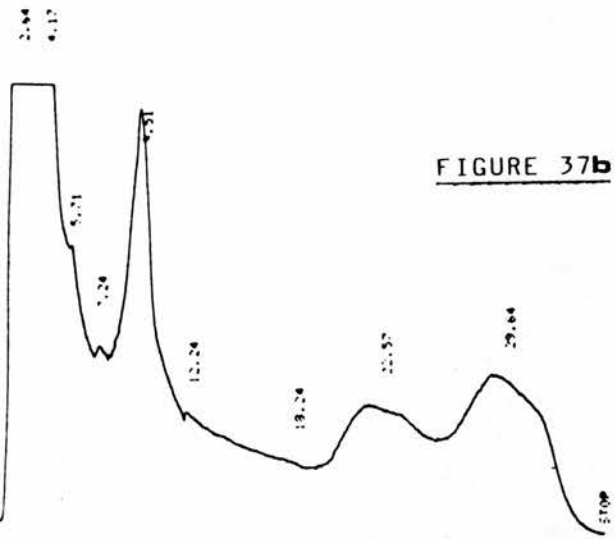
(10 µl, Range 0.1)

FIGURE 37e



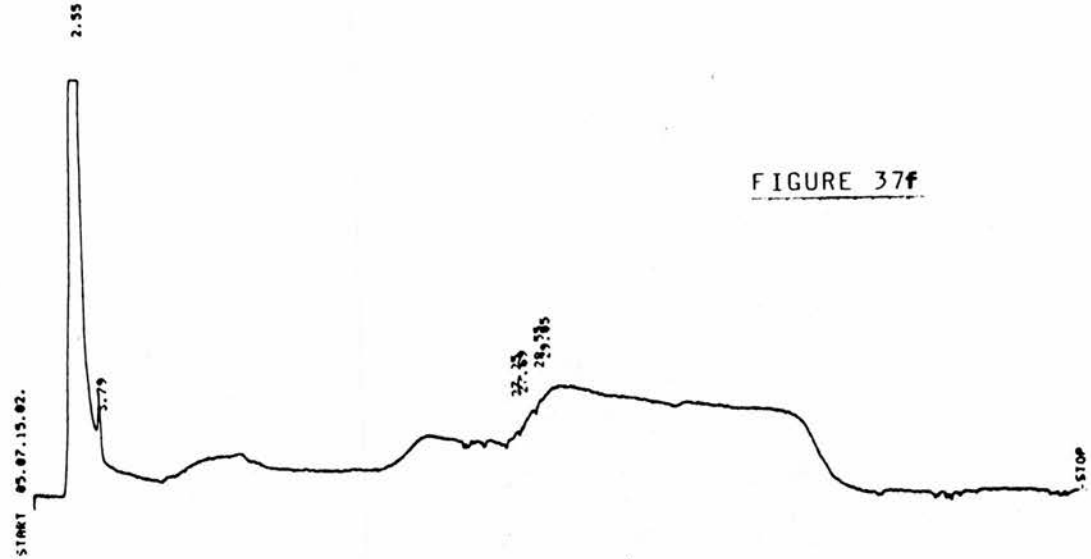
(10 µl, Range 0.02)

FIGURE 37b



(20 µl, Range 0.2)

FIGURE 37f



(20 µl, Range 0.05)

three most polar components are associated with the major fluorescent band fraction 4, (see Figure 33 and 34) with the less polar components distributed between all 4 fractions, the majority being present in fraction 2.

2.3 SUMMARY OF BIOLOGICAL STUDIES

In liver microsomes and brain homogenate in which LPX is induced, there is an increase in total lipid soluble fluorescence. Analysis of lipid extracts from these systems and of TBH treated erythrocytes, show that this fluorescence can be located on thin layer chromatograms:-

(1) In microsomes chiefly at the origin and in a spot with similar mobility to the major fluorescent component found from dipalmitoyl PE/EYPE and MDA reaction systems;

(2) In brain homogenate chiefly in the region of the PE spot, again as found in the phospholipid-MDA incubations.

HPLC analysis revealed a major fluorescent componet with an identical retention time to that found in the EYPE-MDA reaction, but only in the microsomes and not in the brain homogenate. The bulk of the fluorescent lipid detectable by HPLC for brain homogenate had retention times comparable with, but less than, PE.

Lipid phosphorus analysis demonstrated a small relative increase in both PE and PS compared with the other phospholipid classes in brain homogenate. HPLC analysis as gratified by U.V. absorbance at 206 nm, showed similar trends

(small increases in PE and PS) in the microsomes but a striking increase in PS when brain homogenate lipids were estimated by HPLC.

3. PLASMA AND CSF STUDIES

3.1 TBARS ASSAY

3.1.1 Colorimetric Assay

Since the maximum volume of CSF obtained from some patients was considerably smaller than originally anticipated, (less than 0.5 ml) it was decided to measure TBARS using only the more sensitive fluorimetric method of analysis (approximately 100 X more sensitive (150)) for both plasma and CSF samples. Sufficient material was then available in order to perform the fluorescent pigment assay, followed by TLC and HPLC analysis of this extract for each patient's plasma and CSF sample. However, the precision of the colorimetric method was evaluated for plasma and was found to give an intra assay coefficient of variation of 7.4% and 1.5% for 250 µl of plasma respectively ($n = 6$, μ ($\pm$ S.D.) = 0.05 ± 0.004).

3.1.2 Fluorimetric Assay

It had originally been intended to measure both total (lipid peroxide bound MDA + free MDA) and precipitable (lipid peroxide bound MDA only) TBARS. However, the original method of Yagi proved extremely unreliable and although much time was spent in attempting to develop an alternative means of precipitation, for example using TCA, acceptable precision with any precipitation method was never obtained. Consequently all data refers to total TBARS present in plasma and CSF. Coefficients of variation for the non precipitation method are shown below:-

	n	μ (± S.D.	C.V.
Intra-assay evaluation	6	4.6 ± 0.02	0.45%
Inter-assay evaluation	4	4.1 ± 0.61	15%

A typical calibration graph for the method is shown in Figure 38 and demonstrates that a linear response is achieved up to 2.5 nmoles of MDA per assay.

The linearity of the method for both plasma and CSF was also determined and Figure 39 and 40 show that for both body fluids a linear response was achieved up to 500 μl of sample volume.

3.1.2.1 Effect of freezing and thawing

One possible source of error and irreproducibility in the fluorimetric method was considered to be the effect of freezing and thawing of the samples, since freezing of both plasma and CSF produced precipitates which could not be readily redissolved upon thawing. A series of experiments was carried out on successive days in order to investigate the effect of different sample handling on the outcome of the fluorimetric TBARS assay. Pooled samples analysed on successive days were treated in 3 different ways:-

A. Frozen samples stored at -70°C were thawed and then incubated at 37°C for 10 minutes in order to redissolve as much of the cryoprecipitate as possible. These were then centrifuged at 2000g for 10 minutes and the supernatant stored at 4°C. Analysis was carried out on aliquots of this supernatant without any further centrifugation.

FIGURE 38

Standard Calibration Graph Showing nmoles MDA Versus Relative Fluorescence

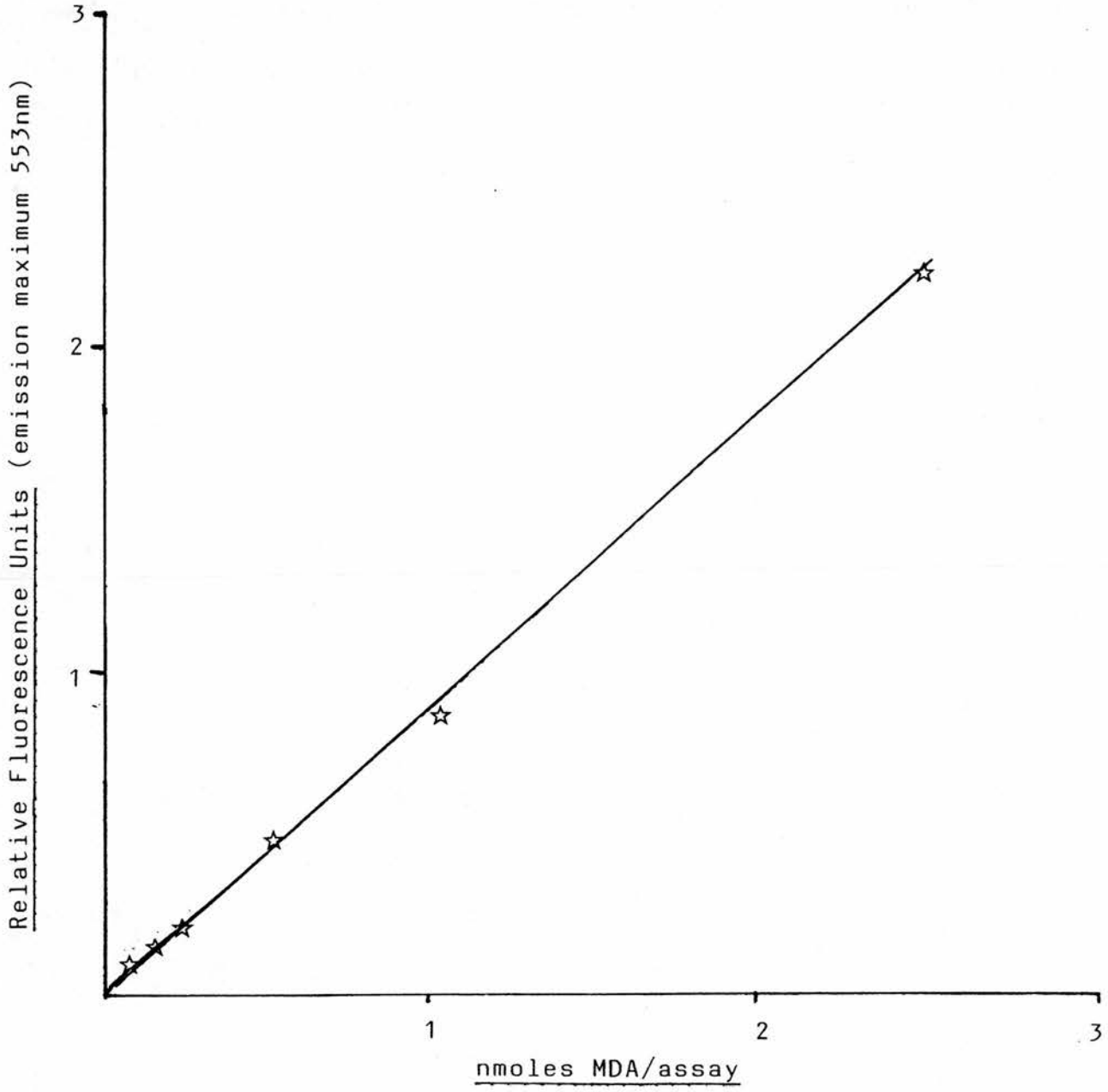
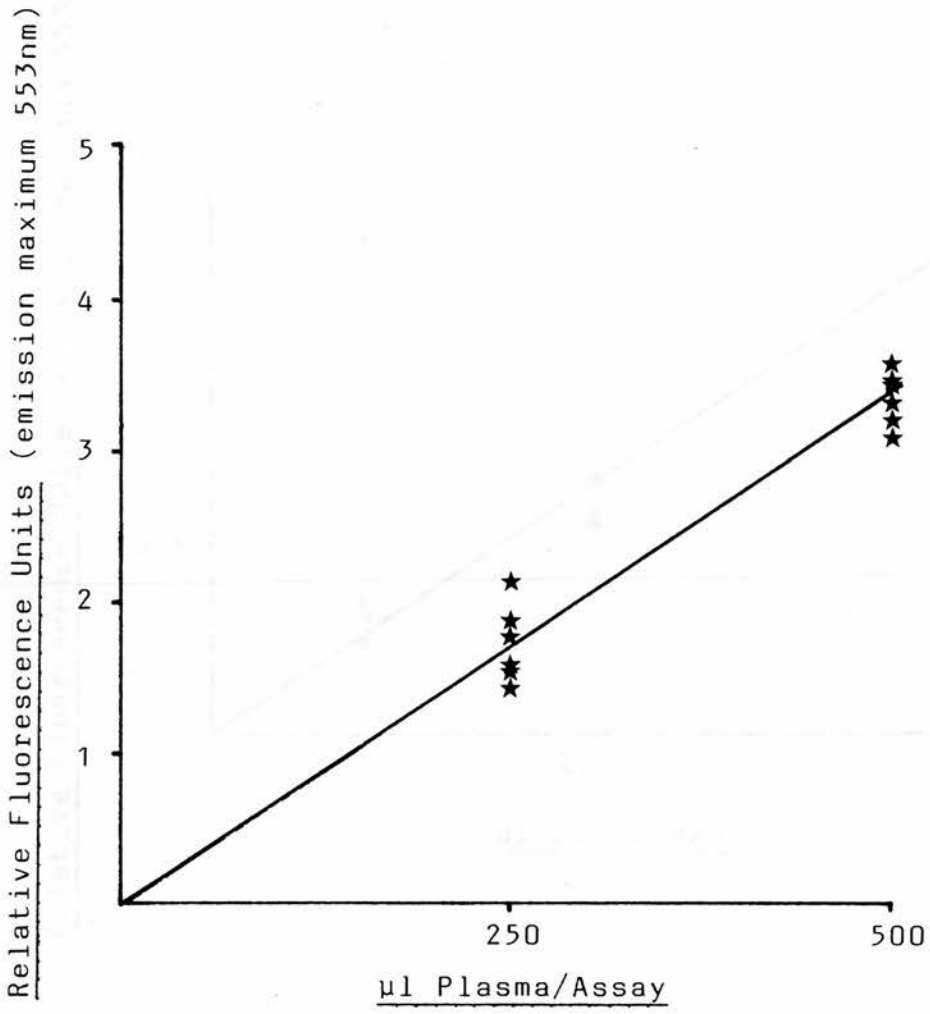


FIGURE 39

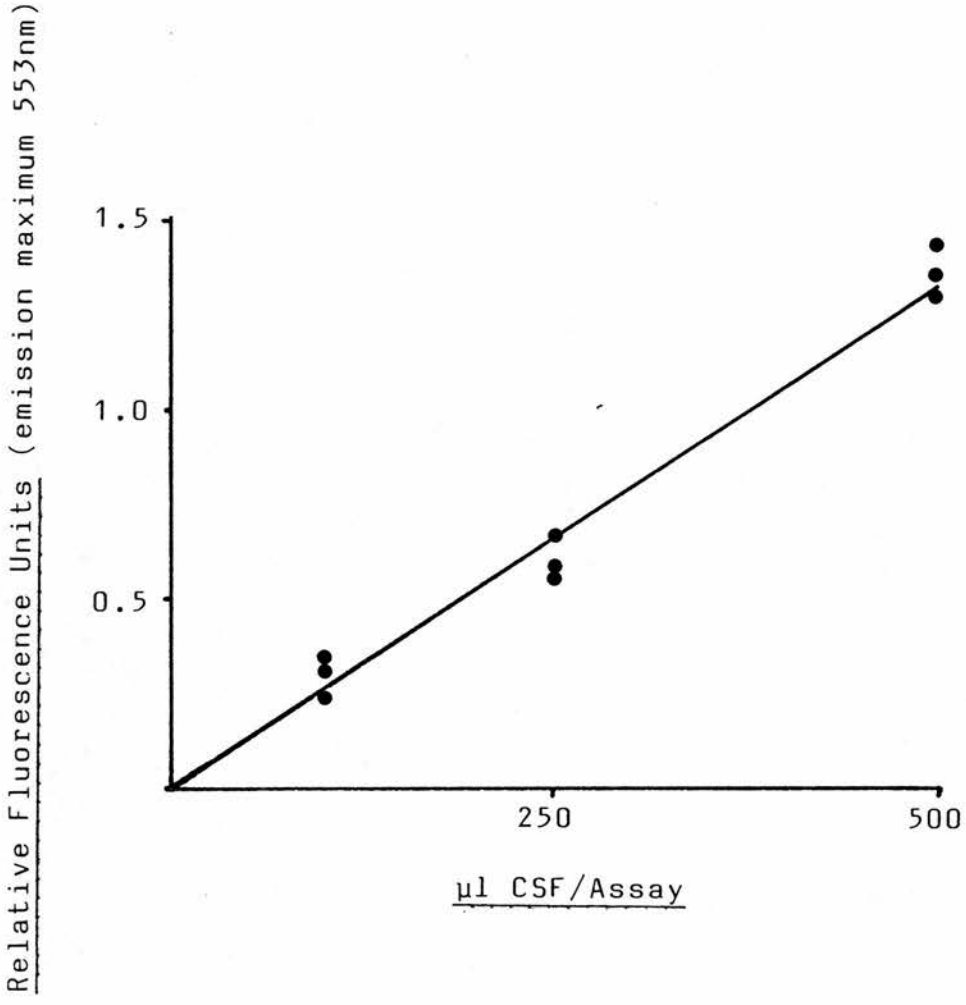
Graph Showing Linear Relationship Between Plasma Volume and Relative Fluorescence



Note Each point represents 1 assay (performed in triplicate).

FIGURE 40

Graph Showing Linear Relationship Between CSF Volume and Relative Fluorescence



B. Other samples were treated exactly as detailed in A except that the supernatant was stored at -70°C and when thawed prior to analysis, no further centrifugation step was carried out.

C. Frozen samples were treated exactly as detailed in B except that each thawed supernatant was centrifuged immediately prior to analysis. Table 11 shows the mean, standard deviation and coefficient of variation of each 100 µl sample assayed in triplicate over a 5 day period:-

TABLE 11

	METHOD A	METHOD B	METHOD C
Mean (±S.D.)	3.47 ± 0.64	3.45 ± 0.96	4.10 ± 0.61
C.V.	18.41	27.70	14.94

On the basis of these results, method C gave the best precision and hence all samples were stored at -70°C, thawed and incubated at 37°C for 10 minutes, then centrifuged at 2000g for 10 minutes immediately prior to analysis.

The results for the fluorimetric TBARS assay are shown in Table 12 and are expressed in a number of different ways. For both plasma and CSF the TBARS (expressed as nmoles MDA) are related to volume of body fluid, unit protein and IgG content of CSF and also to the CSF albumin/IgG ratio. The rationale for presenting the plasma data in relationship to these CSF parameters is that these values may be indicative

TABLE 12

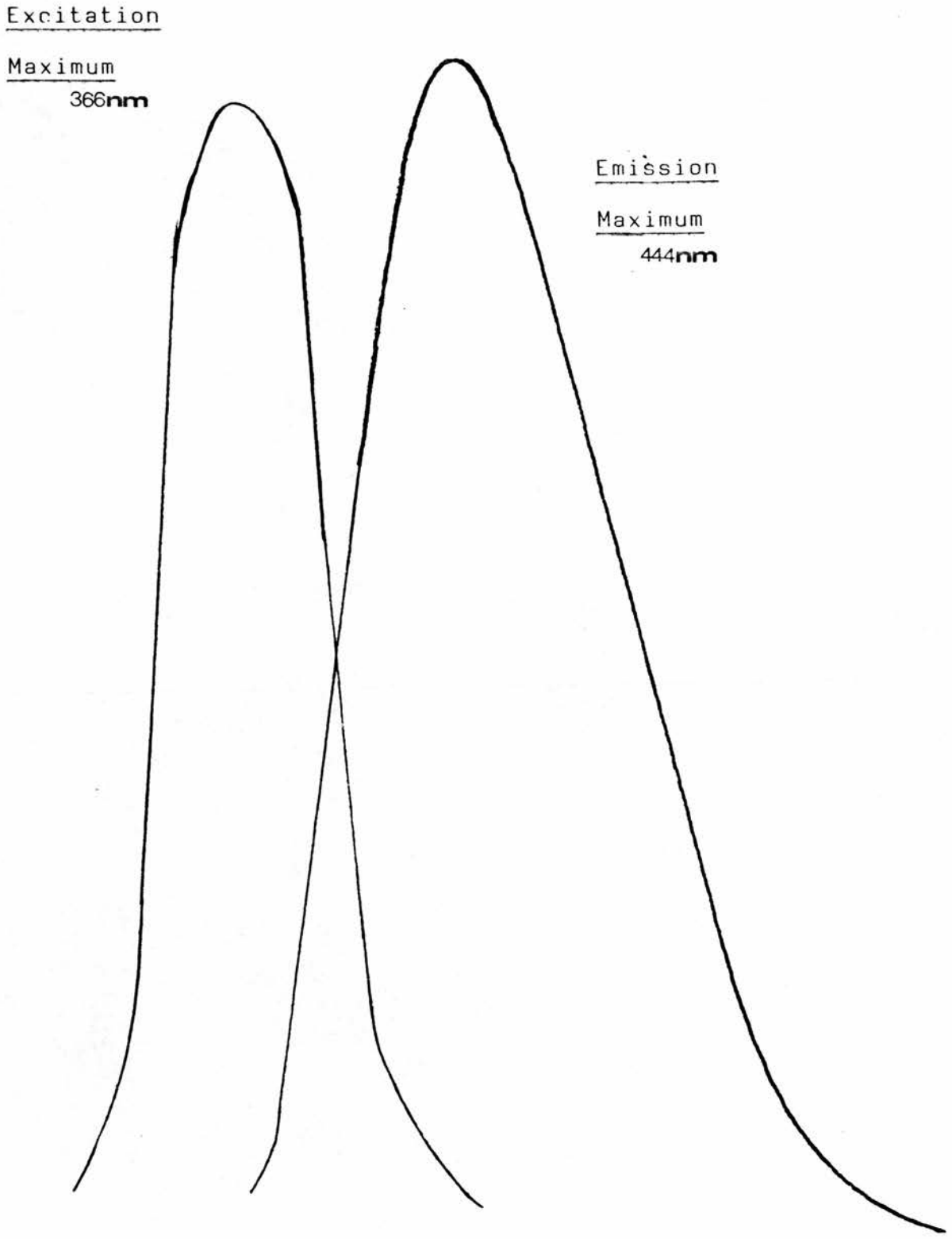
	DIAGNOSIS					
	M.S.	DEM	DEG	DEMG	ALL CONTROLS	NORMAL CONTROLS
Number of Samples	15	15	6	4	25	18
Plasma MDA/ml	5.63 (±1.7)	4.85 (±1.5)	6.03 (±1.6)	5.56 (±1.8)	5.25 (±1.6)	5.63 (±1.5)
Plasma MDA/µg CSF IgG	0.12 (±0.06)	0.13 (±0.10)	0.20 (±0.08)	0.11 (±0.09)	0.14 (±0.10)	0.21 (±0.09)
CSF MDA/ml	0.74 (±0.3)	0.84 (±0.5)	0.81 (±0.3)	0.72 (±0.4)	0.80 (±0.4)	1.06 (±0.4)
CSF MDA/mg Protein	1.98 (±1.1)	1.42 (±0.6)	2.02 (±0.7)	1.11(±0.2)	1.54 (±0.7)	2.78 (±1.0)
CSF MDA/µg IgG	0.01 (±0.006)	0.02 (±0.01)	0.03 (±0.01)	0.008 (±0.004)	0.02 (±0.01)	0.04 (±0.02)

of blood-brain-barrier damage. The blood-brain-barrier will influence the rate at which TBARS originating in the central nervous system appear in the plasma. Irrespective of the mode of expression of the results, a two tailed Mann-Whitney test showed no significant difference ($p > 0.2$) between M.S. patient data and any of the control groups (including normal controls), either individually or in total. However in all cases, values obtained for normal controls (healthy individuals with no neurological diseases/complications) were relatively higher than those patients suffering neurological disease including M.S.

3.2 FLUORESCENT PIGMENT ASSAY

A typical fluorescence spectrum illustrating both excitation (366 nm) and emission (444 nm) maximum is shown in Figure 41. The linearity of the method was also tested and as shown in Figure 42, was found to be linear up to a plasma volume of 1 ml. The results for the fluorescent pigment assay are shown in Table 13 and the relative fluorescence units are expressed in a number of different ways for both plasma and CSF as detailed in Section 3.1.2.1. Irrespective of the mode of expression of the results, a two-tailed Mann-Whitney test showed no significant difference ($p > 0.2$) between M.S. patient data and any of the control groups, either individually or in total. One may conclude therefore that there is no significant difference in levels of fluorescent pigements extractable into either $CHCl_3$ or $MeOH/H_2O$ between M.S. patients and normal or OND controls.

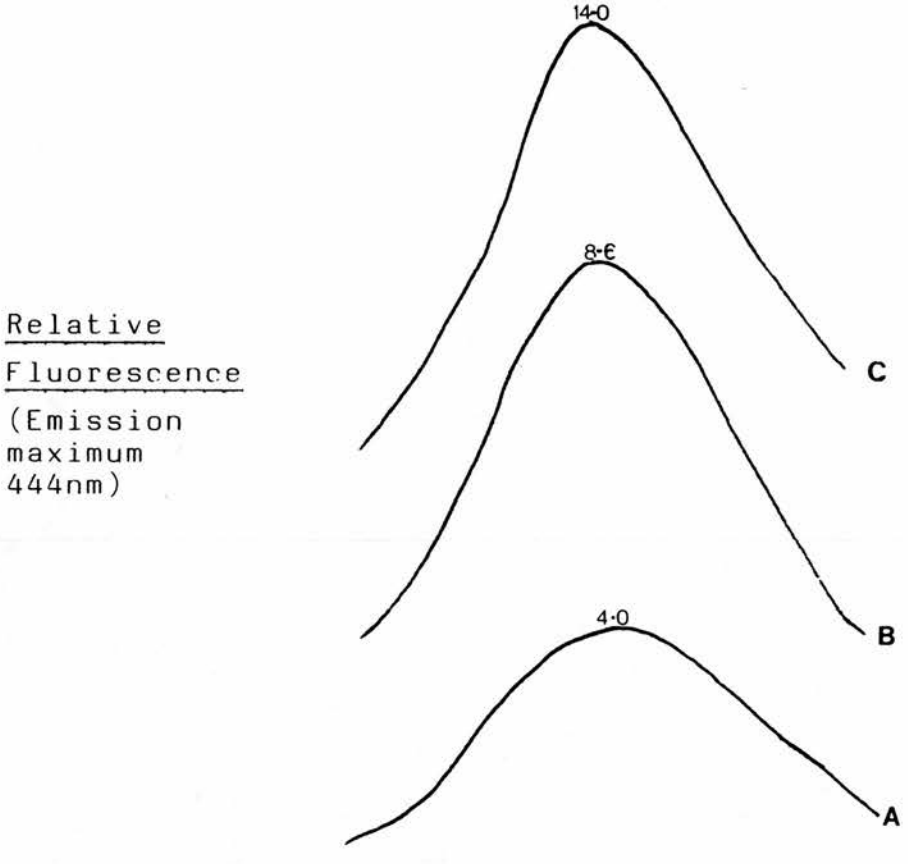
FIGURE 41



Typical Fluorescence Scans Illustrating
Excitation and Emission Maxima

FIGURE 42

Linear Response Between Plasma Volume and Relative Fluorescence (Fluorescent Pigment Assay)



Where:-

A 250 µl plasma
B 500 µl plasma
C 1000 µl plasma

TABLE 13

	DIAGNOSIS					
	M.S.	DEM	DEG	DEMG	ALL CONTROLS	NORMAL CONTROLS
Number of Samples	15	15	6	4	25	18
Plasma FPC/ml	10.92 (±14.11)	35.68 (±86)	11.94 (±9.9)	3.43 (±4.5)	23.1 (±65)	3.49 (±4.7)
Plasma FPC/mg CSF Protein	24.33 (±31)	60.53 (±131.3)	29.76 (±22.5)	6.23 (±6.01)	43.86 (±100)	12.26 (±17.8)
Plasma FPC/µg CSF IgG	0.34 (±0.75)	0.89 (±1.8)	0.34 (±0.28)	0.05 (±0.04)	0.23 (0.38)	0.11 (±0.13)
CSF FPC/ml	0.36 (±0.25)	0.37 (±0.1)	0.28 (±0.2)	0.34 (±0.1)	0.34 (±0.1)	0.40 (±0.2)
CSF FPC/mg Protein	1.05 (±0.9)	0.71 (±0.4)	0.85 (±0.6)	0.65 (±0.4)	0.73 (±0.5)	1.11 (±0.8)
CSF FPC/µg IgG	0.008 (±0.008)	0.01 (±0.01)	0.01 (±0.01)	0.008 (±0.005)	0.009 (±0.009)	0.02 (±0.01)
CSF FPM/ml	4.7 (±3.0)	5.7 (±2.3)	4.0 (±0.8)	4.3 (±1.1)	5.1 (±2.0)	4.7 (±1.5)
CSF FPM/mg Protein	11.5 (±9.6)	11.8 (±6.2)	12.4 (±5.7	10.1 (±8.7	11.7 (±6.1)	12.3 (±5.2)
CSF FPM/µg IgG	0.09 (±0.06)	0.15 (±0.12)	0.13 (±0.05)	0.14 (±0.11)	0.14 (±0.1)	0.19 (±0.1)

Where:- FPC - $CHCl_3$ extractable fluorescent pigments

FPM - $MeOH/H_2O$ extractable fluorescent pigments

Values denote Mean (± S.D.)

3.3 CONJUGATED DIENE ASSAY

The filtered lipid extracts used for the fluorescent pigment assay were reduced to dryness, redissolved in cyclohexane and the absorbance spectrum between 200-250 nm determined.

This assay was only carried out on CSF samples and the results are shown in Table 14, the relative absorption units being expressed in a number of different ways as detailed in Section 3.1.2.1.

Once again irrespective of the mode of expression of the results, a two tailed Mann-Whitney test showed no significant difference ($p > 0.2$) between M.S. patient data and any of the control groups either individually or in total. Thus one may conclude that there is no significant difference in levels of conjugated dienes found in CSF from M.S. patients and OND and normal controls.

3.4 CHROMATOGRAPHIC STUDIES

3.4.1 TLC

3.4.1.1 CSF

Both $CHCl_3$ and $MeOH/H_2O$ extracts from CSF of M.S. patients and controls were concentrated and subjected to TLC analysis. Plates were examined both for autofluorescence and I_2 staining and although a complex pattern of components was revealed for most samples, there were no obvious consistant differences between the M.S. patient and control groups. Figure 43 shows I_2 staining of the $MeOH/H_2O$ phases from the CSF samples. ($CHCl_3$ phases are not shown, since little was visible on these plates due to the small amount of extractable lipid).

TABLE 14

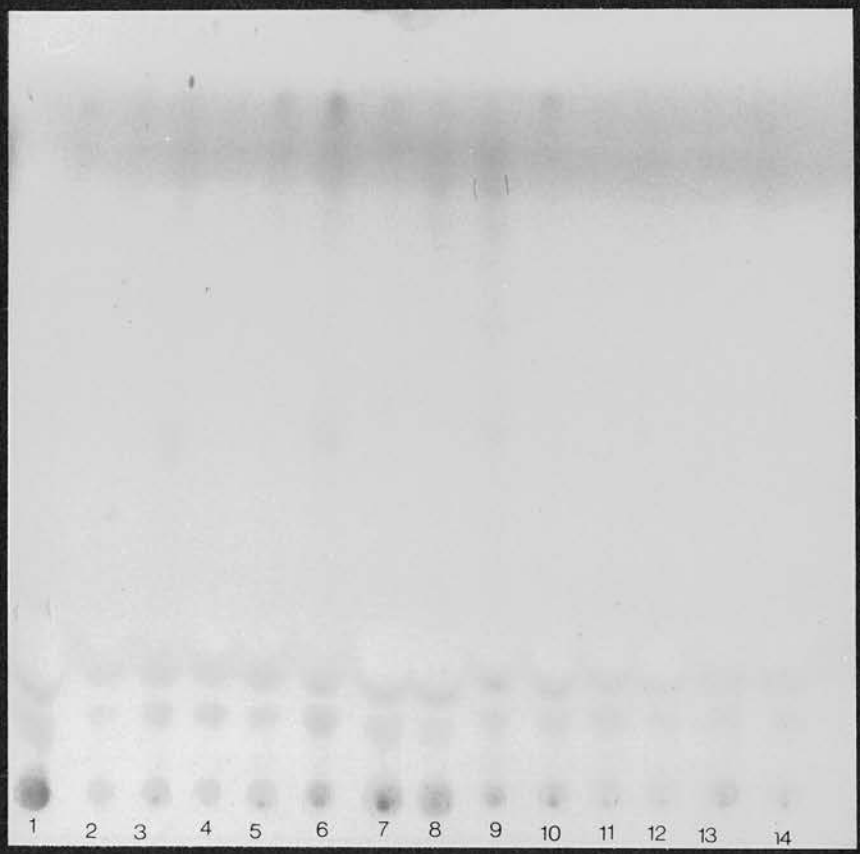
	DIAGNOSIS					
	M.S.	DEM	DEG	DEMG	ALL CONTROLS	NORMAL CONTROLS
Number of Samples						
CSF CD/ml	0.71 (±0.8)	0.77 (±0.8)	0.29 (±0.2)	0.40 (±0.1)	0.53 (±0.5)	0.55 (±0.4)
CSF CD/mg protein	1.78 (±2.4)	1.44 (±1.0)	0.81 (±0.8)	0.86 (±0.8)	1.17 (±0.9)	1.42 (±1.2)
CSF CD/µg IgG	0.01 (±0.02)	0.02 (±0.01)	0.01 (±0.01)	0.01 (±0.01)	0.02 (±0.01)	0.02 (±0.02)

Where:- CD - Conjugated Dienes

Values denote Mean (± S.D.)

FIGURE 43

1D TLC Chromatogram Depicting MeOH/H_2O Lipid Extracts from M.S. and Control CSF



Where:-

2,4	DEM Controls
8	DEMG Control
6,10,12	Normal Controls
1,3,5,7,9,11,13,14	M.S.

Solvent System

$CHCl_3$/MeOH/NH_4OH

(16:8.5:1 v/v)

Loading

50 µg lipid/spot

Detection

I_2 staining

3.4.1.2 Plasma

$CHCl_3$ phases only, from plasma fo M.S. patients and controls were subjected to TLC analysis as described in Section 3.4.1.1. Once again no obvious differences were seen between the two groups. Figures 44a, 45a, and 44b, 45b show autofluorescence and I_2 stained chromatograms respectively.

3.4.2 HPLC

Despite the disappointing outcome of the results revealed by TLC analysis, it was hoped that the superior resolving power of HPLC would reveal differences between MS and control samples. Both $CHCl_3$ and $MeOH/H_2O$ extracts from plasma and CSF samples were subjected to HPLC analysis using the isocratic HPLC system 2 (20% HPLC solvent B) with both U.V. and fluorescence detection. This system was selected in preference to the gradient system because all of the material eluting from the column was extremely non-polar and using the latter system, was eluting together with the solvent front. By experimenting with various ratios of the two solvents A and B, the isocratic system giving optimum resolution of these non polar compounds was found to be 20% B.

Fluorescence detection of CSF lipid extracts proved to be completely unsuccessful due to the lack of sensitivity of our HPLC system and plasma lipid extracts revealed a single unidentified peak eluting around 12 minutes in some but not all M.S. and control samples (Figure 46). Sensitivity problems were also encountered with U.V. detection since extremely small amounts of lipid were present in each sample. However U.V. detection of the $CHCl_3$ extracts revealed a fairly simple profile with the identification of PE and PS

FIGURES 44a and 44b

1D TLC Chromatograms depicting $CHCl_3$

Lipid Extracts from M.S. Plasma

Solvent System	Propan-1-ol/NH_4OH/H_2O (6 : 1 : 0.75 v/v)
Loading	50 µg lipid/spot

FIGURE 44a

Autofluorescence

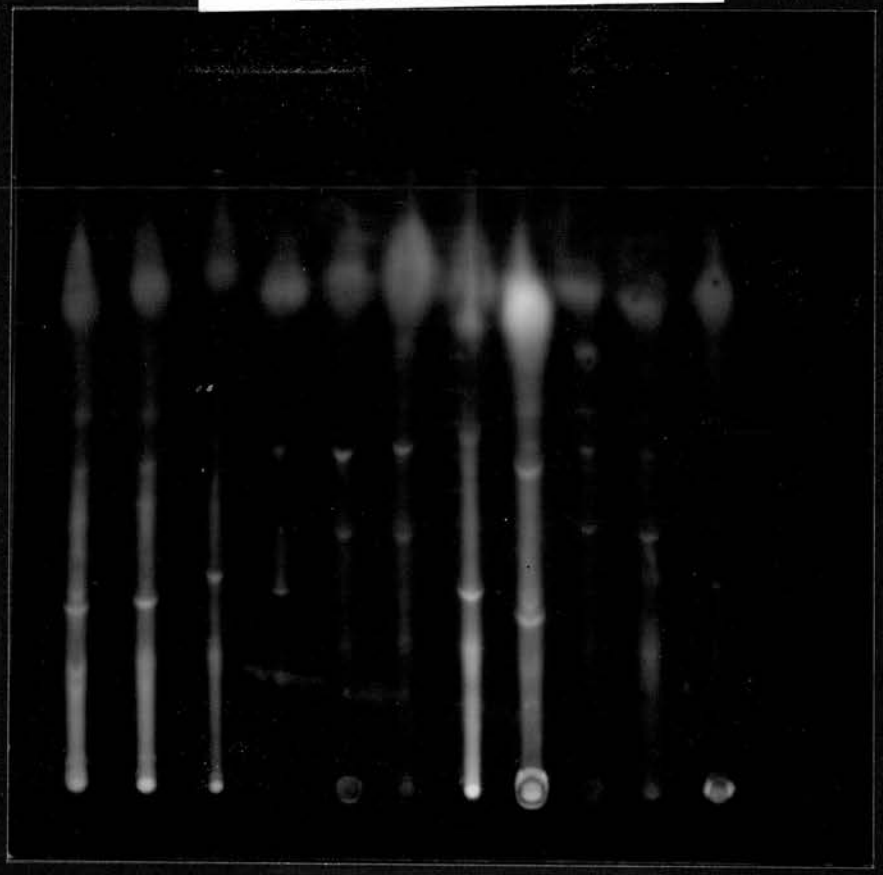
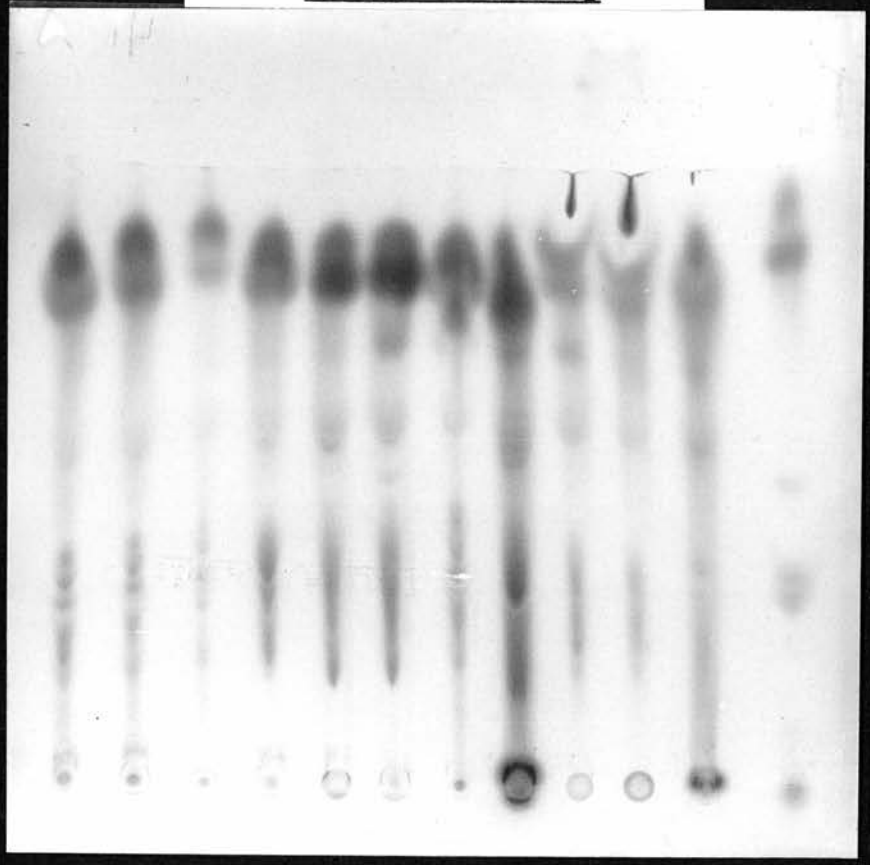


FIGURE 44b

Iodine Staining



FIGURES 45a and 45b

1D TLC Chromatograms Depicting $CHCl_3$ Lipid Extracts from Control Plasma

Where:-

7,8,9,10	DEM Controls
1,2,3	DEG Controls
4	DEMG Control
5,6	Normal Controls
11	PE Standard

Solvent System	Propan-1-ol/NH_4OH/H_2O (6 : 1 : 0.75 v/v)
Loading	50 µg lipid/spot

FIGURE 45a

Autofluorescence

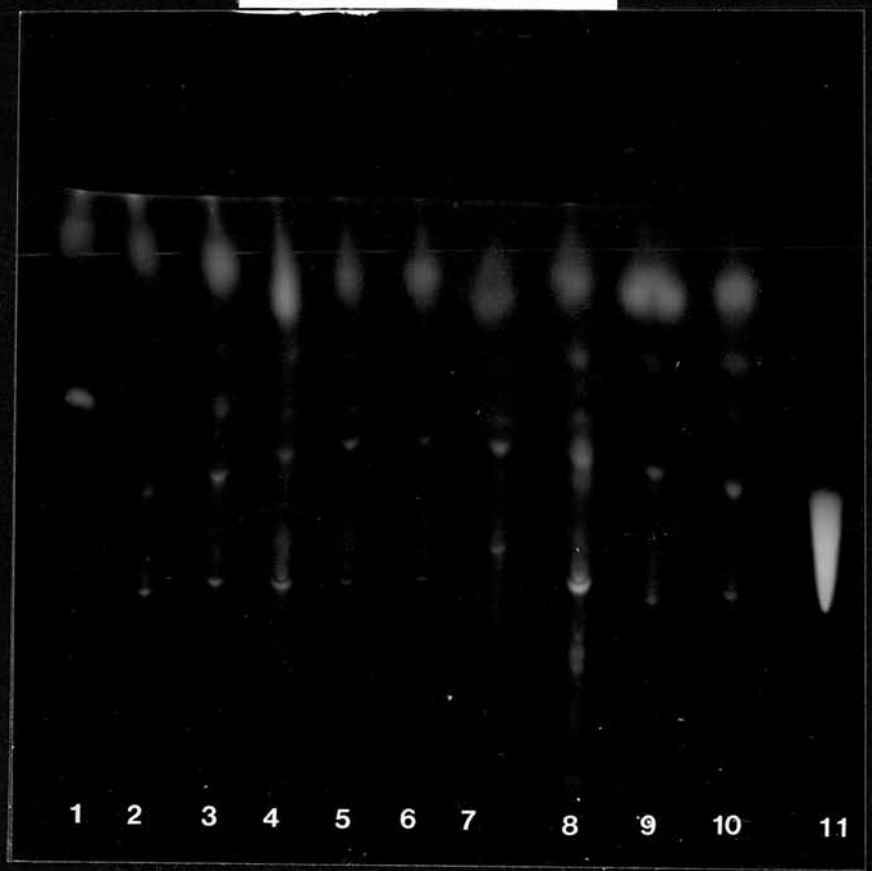


FIGURE 45b

Iodine Staining

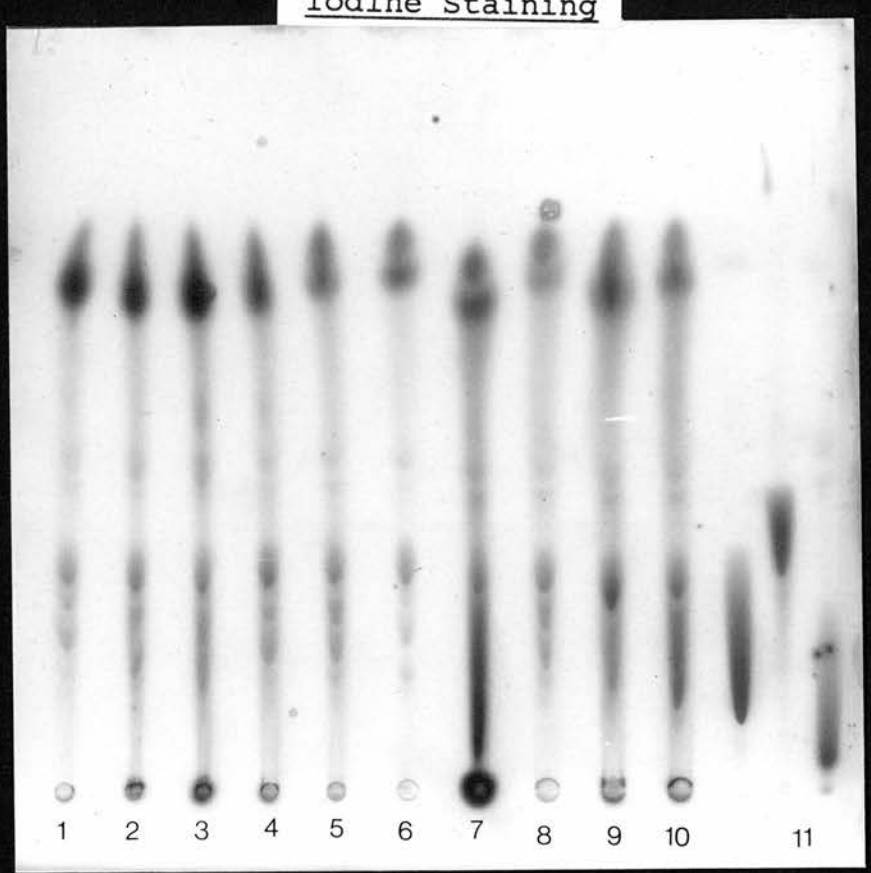


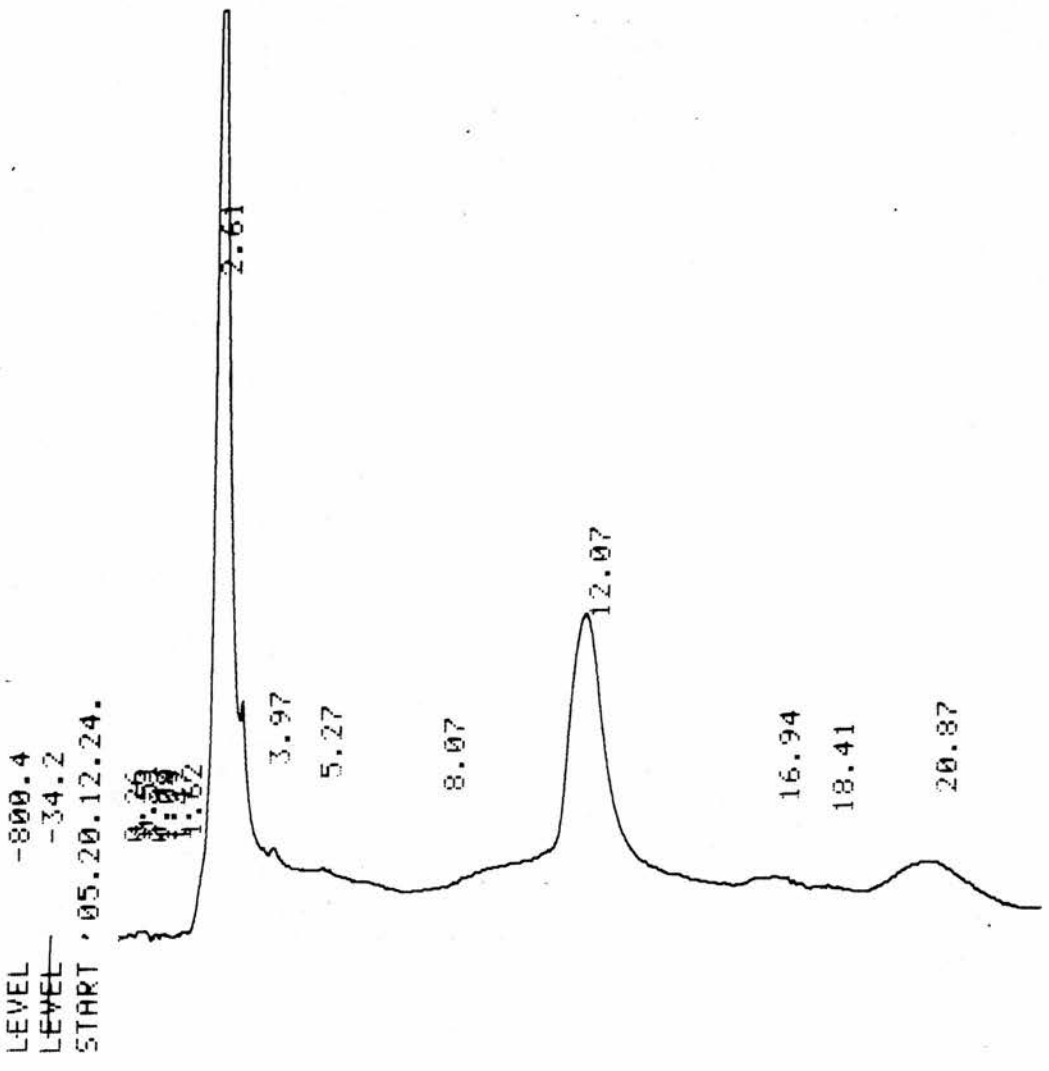
FIGURE 46

HPLC Chromatogram Depicting Plasma $CHCl_3$ Lipid Extract

Using HPLC System 1 with Fluorescence Detection

Loading

20 µl (corresponding to 0.5 ml plasma), Range 0.05.



but with the bulk of the U.V. absorbing material being eluted off the column prior to these phospholipids. Typical chromatograms of CSF $CHCl_3$ and $MeOH/H_2O$ extracts are shown in Figures 47 and 48 respectively.

Very careful analysis of all chromatograms considering both major and minor components, failed to reveal any obvious differences between M.S. and control samples and no components detectable in these samples (by TLC or HPLC) compared with those detected in the EYPE-MDA reaction mixture.

3.5 PEROXIDE STRESS TEST

Preliminary experiments employing the original method of Kobayashi et al. (147), showed that although MDA was measurable using Fe^{3+} as the stimulator of LPX, little difference was observed relative to the controls. Several repetitions failed to induce stimulation and therefore it was decided to investigate various modifications to the method.

3.5.1 Effect of Alternative Stimulators of LPX

Different stimulators of LPX could lead to a greater degree of LPX or may be important in that they could interact with a different segment of the LPX reaction pathway and thus reveal a biochemical defect in M.S. The Kobayashi method was used as previously described in the methods section 3.7.2.1 except that Fe^{3+} (5 mM) was replaced by;

(a) Fe^{3+} and Tertiary-butyl hydroperoxide (TBH)

10 µl of $FeCl_3$ (5 mM) and 10 µl of TBH (26 mM) were added to 0.5 ml of sonicate to give final concentrations of 0.1 mM $FeCl_3$ and 0.5 mM TBH. 20 µl of 17 mM N-2-hydroxy-ethylpiperazine-N'-2-ethanesulphonic acid (HEPES) was used as

HPLC Chromatograms Depicting CSF Lipid Extracts Using HPLC Isocratic System 2 (20% Solvent B) with U.V. Detection 206 nm

Figure 47

$CHCl_3$ extract

Figure 48

$MeOH/H_2O$ extract

Loading

20 µl (corresponding to 0.5 ml CSF), Range 0.5

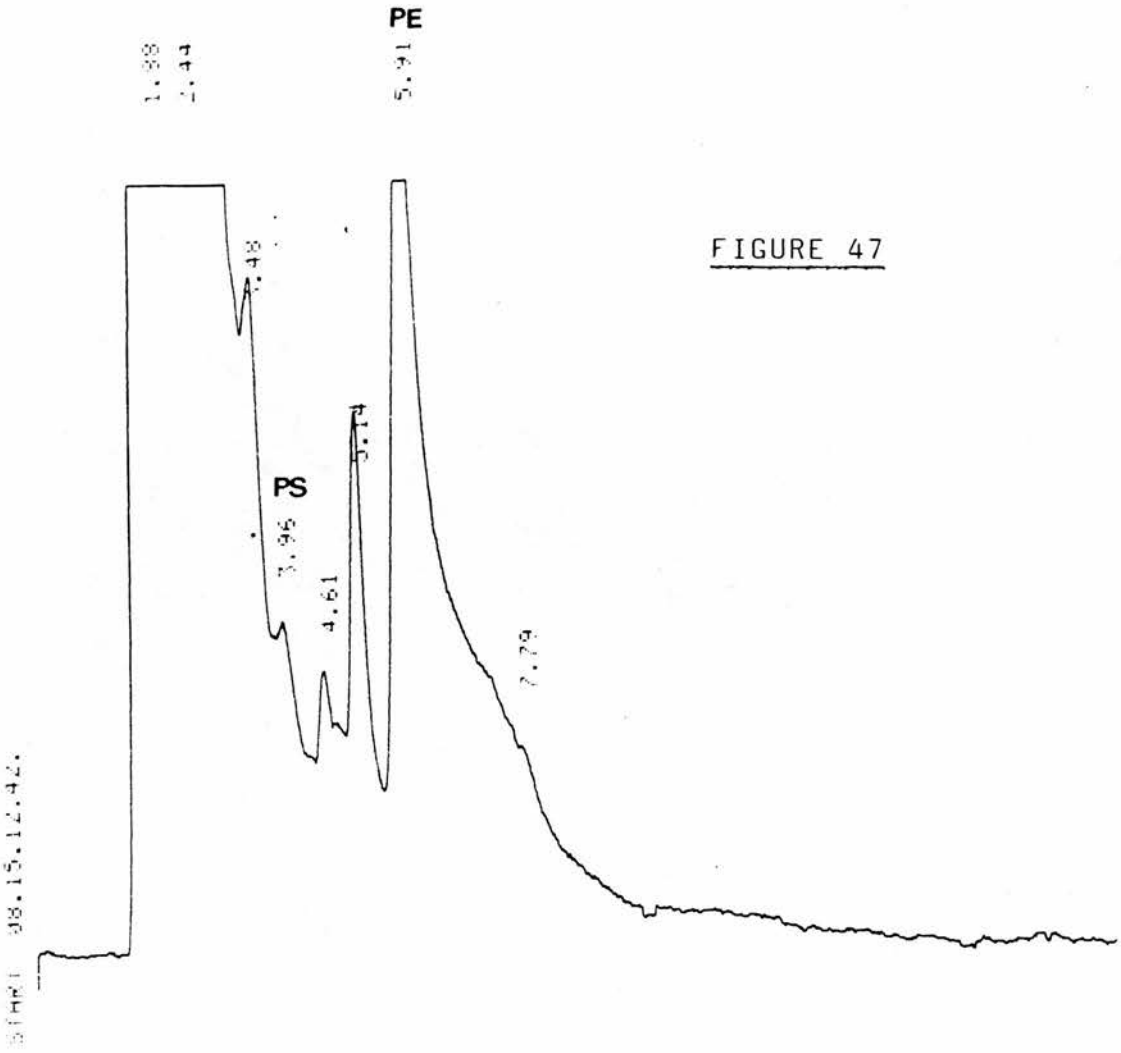


FIGURE 47

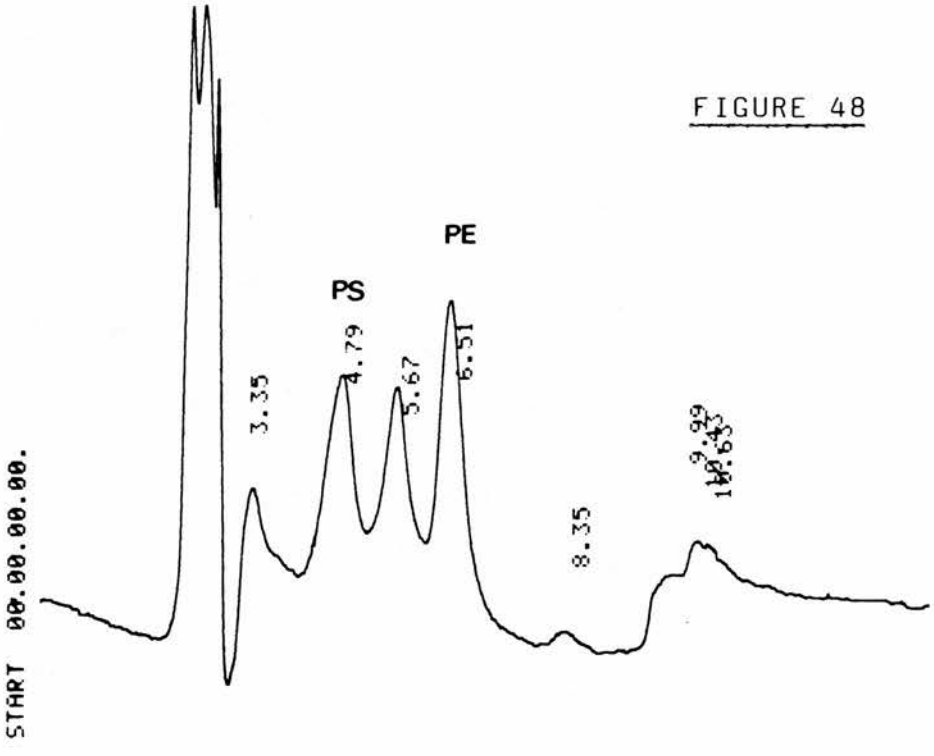


FIGURE 48

the control.

(b) ADP and Fe^{3+}

10 µl of ADP (83.2 mM) and 10 µl of $FeSO_4$ (0.936 mM) were added to 0.5 ml of sonicate to give final concentrations of 1.6 mM and 0.018 mM respectively. 20 µl of HEPES was used as the control.

(c) H_2O_2 and NaN_3

20 µl of a solution containing 52 nM NaN_3 and 260 mM H_2O_2 was added to 0.5 ml of sonicate to give final concentrations of 2 mM and 10 mM respectively. NaN_3 was added to inhibit catalase thus eliminating inter sample variability in catalase activity contributing to different values of TBARS. For the controls, 20 µl of HEPES was added to 0.5 ml of haemolysate. Incubation was carried out for 2 hours as suggested by Stocks and Dormandy (145).

For conditions described in (a), (b) and (c), a sonication time course was also carried out to see if the relationship between LPX and sonication time is specific to the mechanism by which LPX is initiated.

An experiment was carried out to investigate the effect of azide upon the standard curve and since azide presence has virtually no effect as shown in Figure 49, it was decided that a single standard curve was adequate for all experiments regardless of the LPX initiator used.

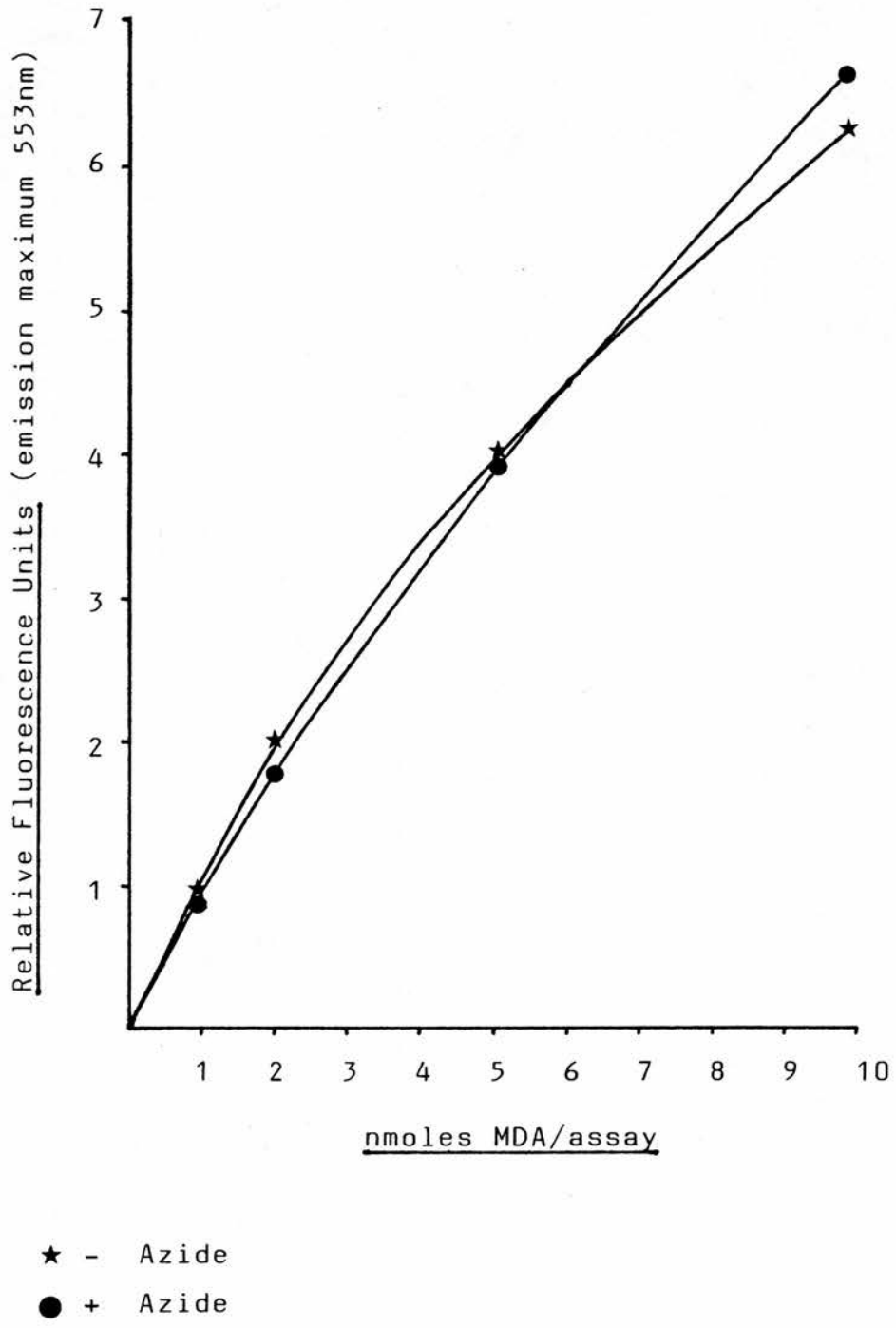
3.5.2 Effect of Sonication

A high degree of variability was observed between triplicate values and the sonication procedure seemed a likely source of both interassay and intra-assay variance.

Heating effects were kept minimum and constant by

FIGURE 49

MDA Standard Curves in Presence and Absence of Azide



submerging only the tip of the probe into 1 ml aliquots of the haemolysate diluted 1 in 5 with HEPES in a glass vessel surrounded by an ice jacket. Sonication was carried out at 6 μ for 30 seconds and the probe immersed in ice for 30 seconds between each sonication period. 5 ml aliquots of the haemolysate were sonicated for up to 4 minutes sonication time and the results using Fe^{3+} and TBH as the LPX inducers are shown in Figure 50. Surprisingly no sonication favours the greatest degree of LPX and yet Kobayashi et al. found sonication to be essential in inducing LPX. It was also interesting to discover that sonication time had little effect on the control incubations. A sonication time of 3 minutes was decided upon for all subsequent experiments since this gave the best replicate values.

Figure 51 shows the sonication time course for LPX induced by Fe^{3+} only and ADP/Fe^{2+}. By comparing the maximal relative fluorescence units obtained with those seen in Figure 50, it can be seen that both Fe^{3+} and ADP/Fe^{2+} are poor initiators of LPX compared with Fe^{3+}/TBH.

The sonication time course for H_2O_2 (+ azide) is shown in Figure 52 and indicates that under the conditions of the assay, H_2O_2 is a better stimulator of LPX than Fe^{3+}/TBH.

The finally selected stimulators of LPX-Fe^{3+}/TBH and H_2O_2/azide, were chosen on the basis that they induced LPX to a significant extent compared with the controls, thus minimising relative experimental error and allowing a legitimate comparison of results between both M.S. and control groups.

The linearity of the final method was tested and as

FIGURE 50

Sonication Time Course for LPX Using Fe^{3+}/TBH Inducers

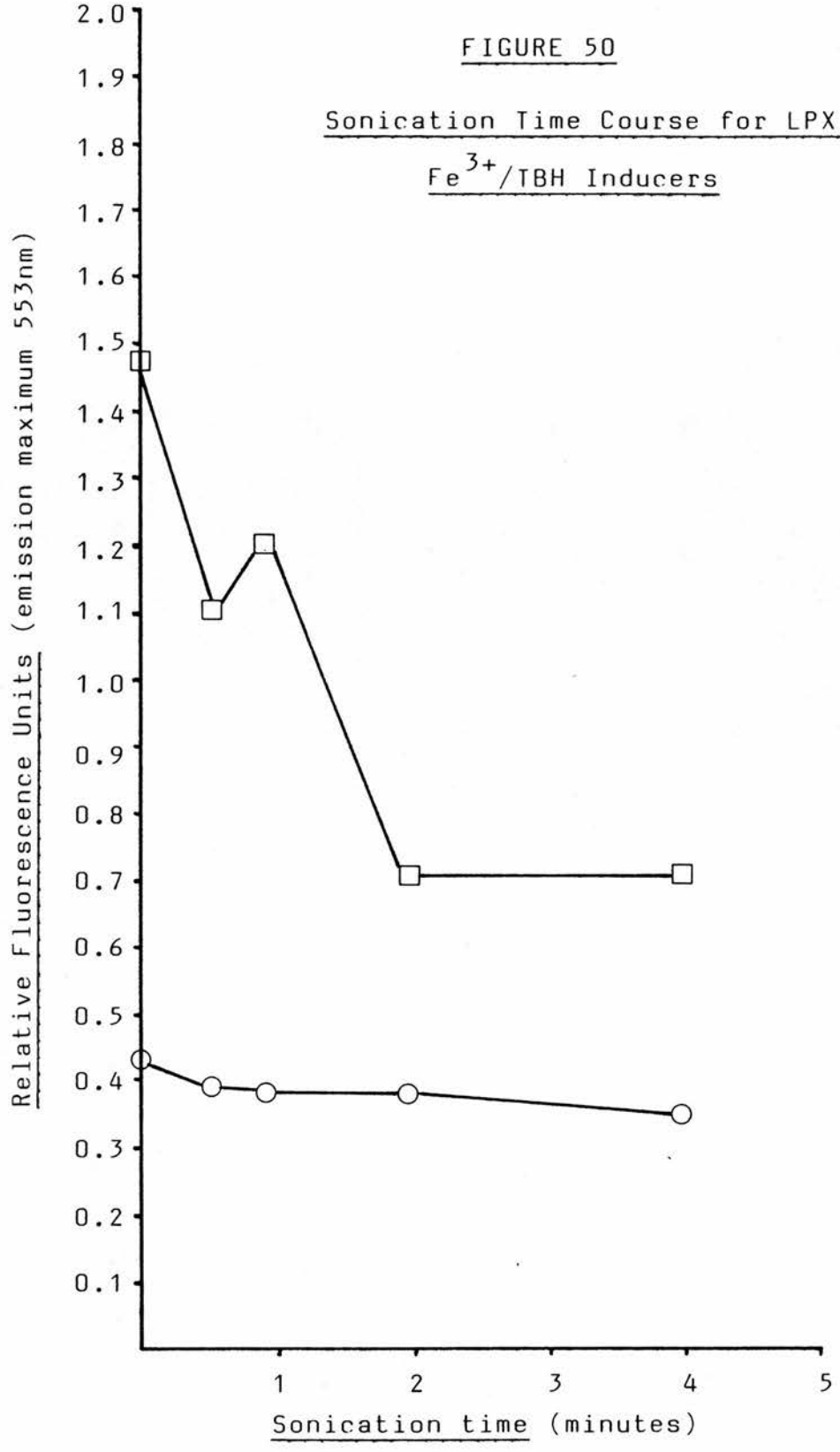
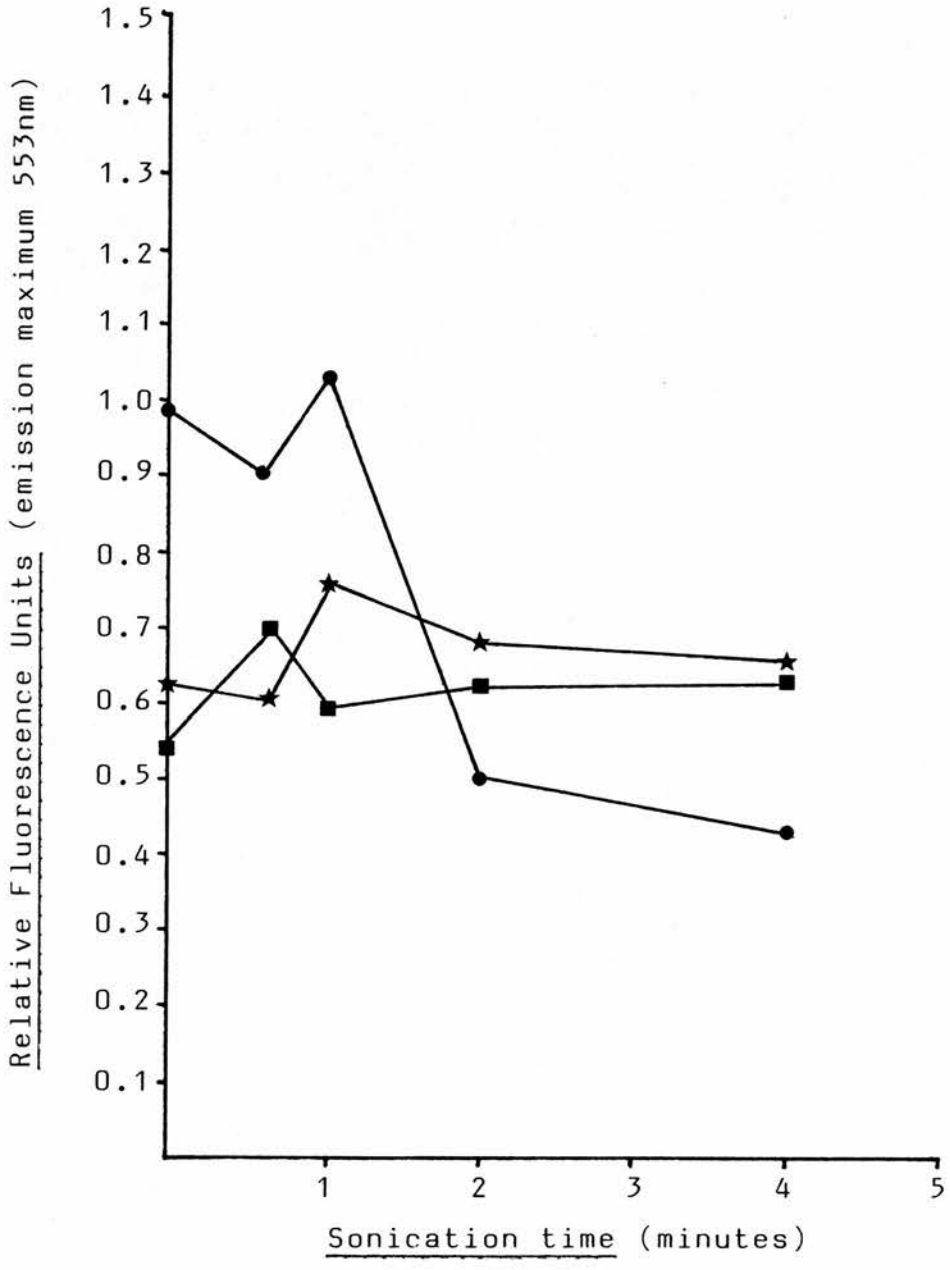


FIGURE 51

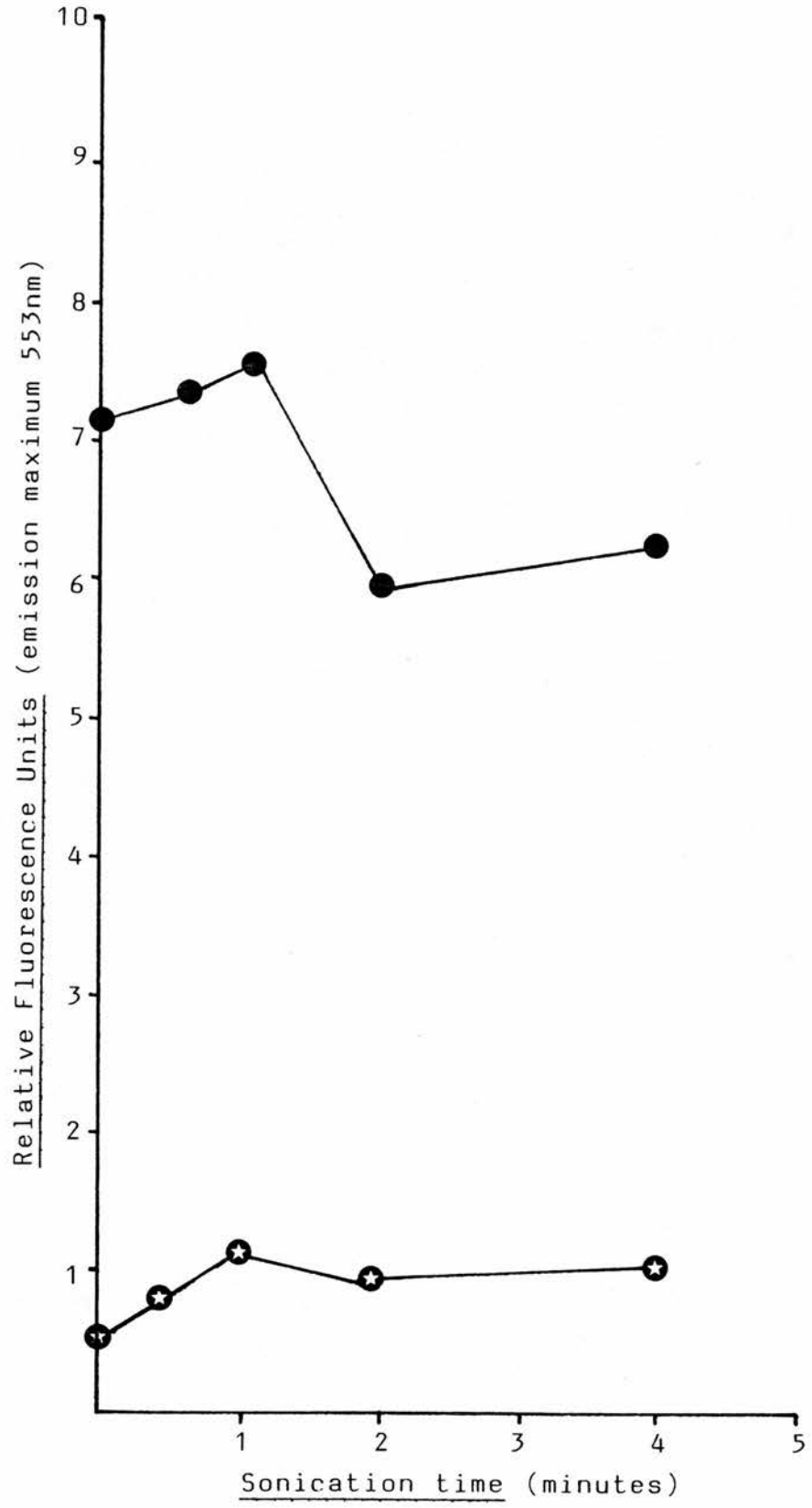
Sonication Time Course for LPX Induced by Fe^{3+} only and ADP/Fe^{2+}



- ● ADP/Fe^{2+}
- ★ Fe^{3+} only
- ■ Control

FIGURE 52

Sonication Time Course for LPX Induced by H_2O_2/azide



● H_2O_2/azide

✪ Control

shown in Figure 53, was quantitative and linear up to at least 0.5 ml of diluted haemolysate. The intra and interassay coefficients of variation were determined by assaying three different samples over a 5 day period and the results are shown below in Table 15.

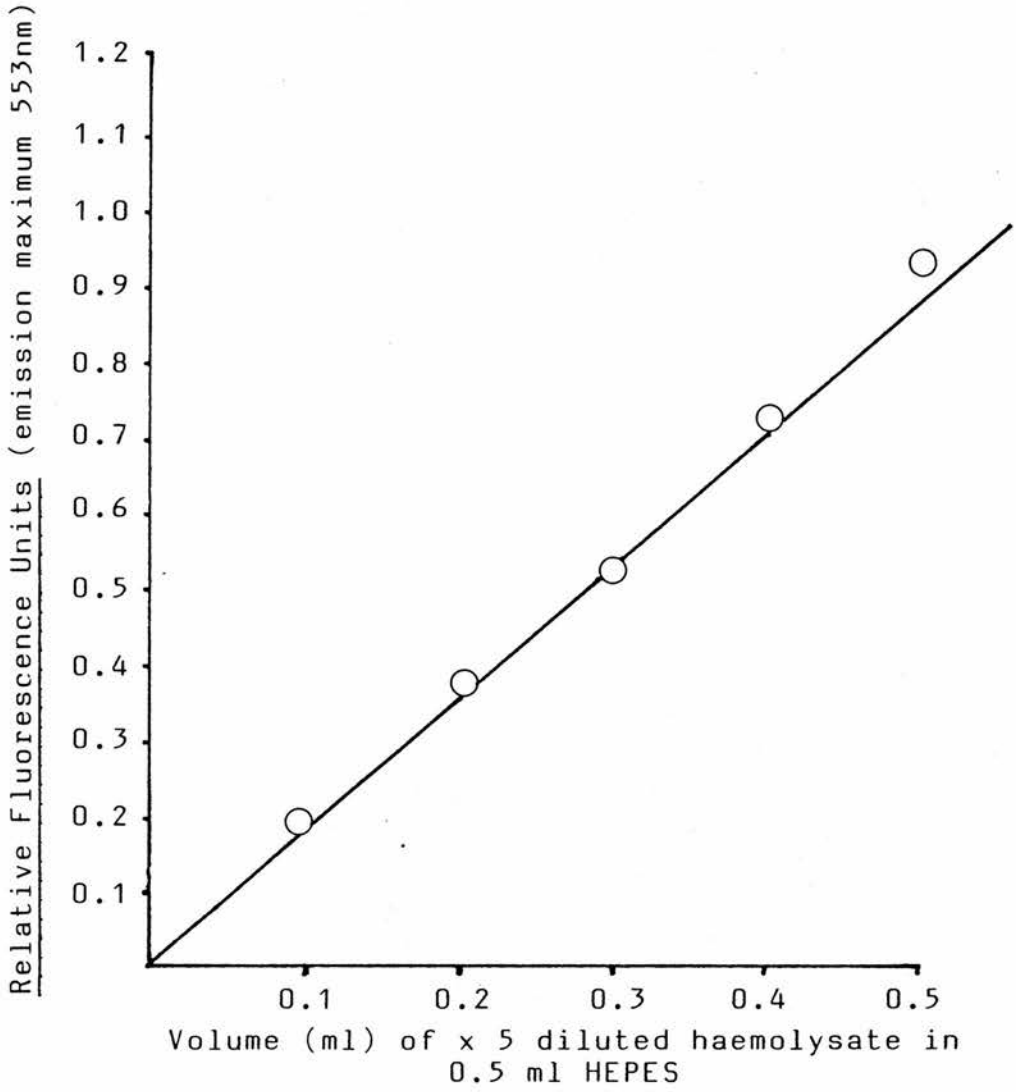
TABLE 15

Stimulant	Intra-assay C.V.	Mean ± S.D.	Inter-assay C.V.	Mean ± S.D.
Fe^{3+}/TBH	6.23%	4.41 ± 0.3	10.30%	4.61 ± 0.5
H_2O_2/azide	6.13%	6.6 ± 0.3	13.80%	5.6 ± 0.3

The results of the method applied to patient samples are shown in Figure 54 and 55. Application of a two tailed Mann-Whitney test to the results using Fe^{3+}/TBH stimulation showed no significant difference in the susceptibility of haemolysates for M.S. patients compared with OND controls to Fe^{3+}/TBH induced LPX ($u = 30$, $p > 0.1$). Simiarly there was no significant difference between haemolysates from M.S. patients and OND controls in their susceptibility to H_2O_2/azide induced LPX ($u = 23$, $p > 0.1$). Nevertheless the mean values for both Fe^{3+}/TBH and H_2O_2/azide induction of LPX, were slightly higher in OND samples compared with M.S. samples.

FIGURE 53

The Relationship Between Haemolysate Concentration and TBARS



TBA-Reactive Substances in M.S. and OND Patients

Following Induction of LPX

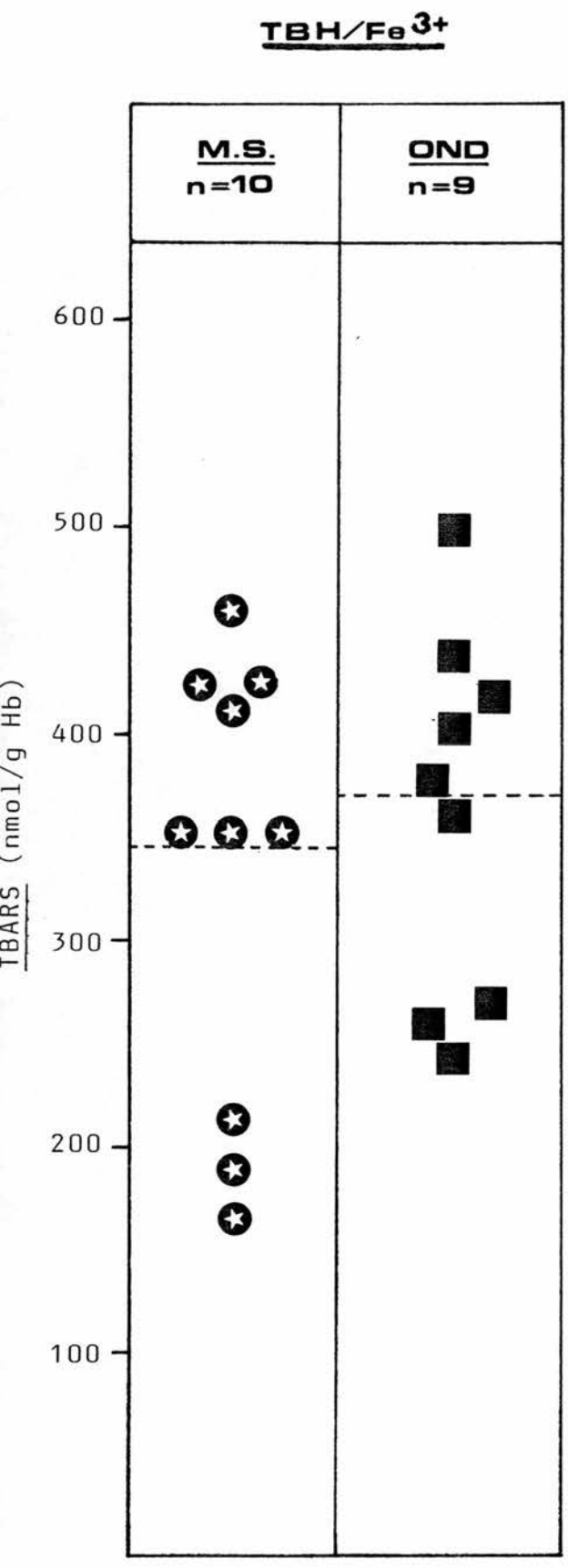


FIGURE 54

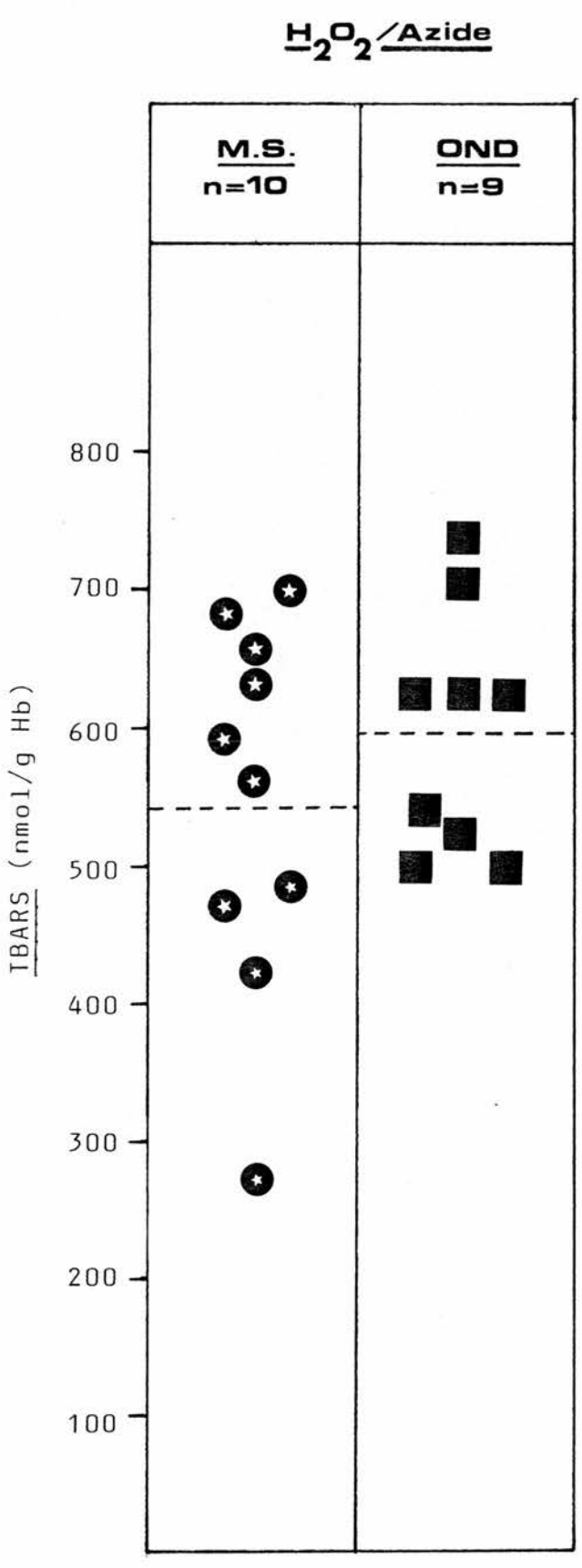


FIGURE 55

3.6 SUMMARY OF CLINICAL STUDIES

All assays of indices of LPX:TBARS, $CHCl_3$ and $MeOH/H_2O$ soluble fluorescent pigments and conjugated dienes, for both CSF and plasma, and irrespective of mode of expression of results, failed to show a significant difference between M.S. patients and controls. This was true for both the two groups of subjects in total and comparing subgroups.

Similarly, chromatography of the lipid extracts from these samples by TLC and HPLC, failed to reveal any consistent qualitative or quantitative differences between M.S. patients and controls. CSF studies were hampered by detection, constantly due to very small amounts of lipid material being present. CSF samples contained PS + PE as well as several unidentified, less polar components. PI, PC and SM were not detected. Streaking of the plasma lipid samples on TLC made it impossible to determine whether M.S. patients or controls exhibited the presence of autofluorescent components corresponding to the major product from EYPE-MDA reaction mixtures.

Modification of the stress test of Kobayashi *et al.* using H_2O_2/azide or Fe^{3+}/TBH as stressor, produced mean levels of TBARS which were lower for M.S. patients than controls, but this difference was not statistically significant.

DISCUSSION

DISCUSSION

It is now well established that LPX of biological membranes leads to the formation of a number of aldehydic degradation products such as MDA, 4-OH NA, n-alkanals and 2-alkenals (150, 162, 163). It has been reported that the increase in fluorescence in the lipid extract of peroxidised biological samples and model phospholipids is due to modification of phospholipids by MDA (153, 159). Pilot studies carried out in our laboratory suggested that LPX may be implicated in the pathogenesis of M.S., in that LPX products (measured as TBARS and LSFP's) were found to be significantly elevated in CSF from these patients when compared to OND controls (184). This work was carried out then, in order to substantiate these findings with M.S. patients and to characterise the components responsible for the fluorescent and TBA-reacting properties of M.S. CSF with a possible view to the application of LPX products as diagnostic indices of the disease.

It was decided in the first instance, to investigate the formation of fluorescent products of the reaction between MDA and the aminophospholipids PE and PS in model systems.

Synthetic Studies

Incubations at physiological temperature ($37^{o}C$) of differing molar ratios of MDA (produced by neutralised TMP^{+} hydrolysate pH 7.4) with both DPPE and EYPE in $MeOH/H_2O$ dispersion medium, generated fluorescent adducts (as monitored by spectrofluorimetry) reaching a maximal level around 7 and 4 days respectively. The characteristic

excitation and emission maxima of 404 nm and 448-464 nm respectively were very similar to the properties of the conjugated Schiff-bases described by Dillard and Tappel (155), derived from the reaction of oxidised fatty acids with PE; to the 1,4,dihydropyridine-3,5-dicarbaldehydes described by Kikugawa and Ido (161), derived from the reaction of MDA with primary amines; and to those of lipofuscin pigments (157). In both phospholipid systems, the reaction was favoured by a PE-MDA ratio of 1:2 (rather than 4:1) as judged by the yield of product detected by 1D TLC analysis (Figures 13 and 14). This is probably due to the fact that an excess of MDA will favour the generation of MDA polymerisation products which may co-chromatograph with the lipid derived products. It is well known that studies of the chemistry of this aldehyde are complicated by its tendency to undergo self-condensation reactions (133). Both TLC and HPLC systems developed throughout these model studies were of great value in extending the fluorescence analysis of LPX products to the level of the individual fluorescent compounds. The fluorescent adducts were resolved by preparative TLC into 3 distinctly defined fluorescent fractions, (including PE) (Figure 20), the spectral characteristics of which conformed with those described for conjugated Schiff bases (105). The major and least polar fraction stained with I_2 vapour, ninhydrin and phosphate spray; fluoresced an intense turquoise blue and contained 73% of total lipid phosphorus and 86% of total radioactivity recovered from ^{14}C-PE-MDA incubations. 2D TLC, autoradiography and HPLC analysis further revealed the heterogeneity and complexity of this

major fraction in that it was composed of at least 3 components, the major and least polar of which possessed the staining and fluorescence characteristics of the total fraction obtained by preparative TLC. The other two components were less fluorescent, contained significant amounts of radioactivity and stained for phosphorus but not with ninhydrin, - although as mentioned in the results section this could be due to the sensitivity of the ninhydrin reagent. 2D TLC analysis in conjunction with autoradiography also revealed several other minor components generated in the synthetic reaction system in addition to PE and lyso PE; however these were difficult to identify on the fluorescence photographs due to streaking and obviously the development of a more suitable solvent system could greatly improve their resolution. Of particular interest in the fluorescence photograph (Figure 18a) was the presence of a major region of fluorescence closely associated with, although visibly distinct from, the PE spot.

Preparative TLC followed by HPLC analysis using a 4 step multi-gradient system proved successful in further resolving the fluorescent adducts. Three large fluorescent peaks were detected in the major fluorescent fraction obtained by preparative TLC (Figure 22b). However, it is important to note that peak heights cannot be taken as a measure of the relative amounts of each component but are merely an indication of the intensity of fluorescence of a particular species when excited. Nevertheless, these findings were in agreement with 1D analytical TLC and autoradiography in that the major "reaction product" was

composed of three different fluorescent species. One of the major fluorescent products resolvable by HPLC, also corresponded to one of the 4 major U.V. absorbing peaks (Figures 23a, b, c) and supported the probability that it was derived from PE and MDA, since the U.V. absorbing properties of phospholipids are largely based on the presence of PUFA side chains.

In summary, TLC, both 1 and 2D suggests there are 3 major fluorescent products formed from EYPE, which migrate well ahead of PE itself. Three major components can also be resolved by HPLC when preparative TLC fractions from this region are examined. However, particularly on 2D TLC, a fluorescent component which has a similar, though not identical, mobility to PE is also apparent. It is difficult to account for this particular component on HPLC. HPLC of fraction 3 from preparative TLC (which is the band associated with PE) contains a number of polar (eluting much later than PE) fluorescent components. This suggests that either the chromatographic behaviour of this material is markedly different on TLC compared with HPLC or that these fluorescent component(s) seen on TLC are unstable and breakdown to give more polar fragments during subsequent recovery and/or chromatography. It is conceivable, for example that the (albeit small) amount of phosphoric acid in the HPLC solvent may encourage such decomposition.

Considerable instability of the major fluorescent components derived from EYPE-MDA is also suggested by the discrepancy between the yield of product estimated, (i) by colorimetric phosphorus assay and radioactivity recovery

after 1D TLC (86% yield) and (ii) from recovery of radioactivity after HPLC (16% yield). In the latter experiment the remainder of the radioactivity was found located in PE (11%) and spread over a wide range of more polar components, detectable by fluorimetry. This data also suggests that the HPLC conditions cause degradation of at least some of the fluorescent compounds.

Considering the HPLC analysis of the ^{14}C PE-MDA reaction products in more detail, it can be seen that the single most radioactive peak at 12.8 - 12.9 minutes, is also one of the major fluorescent components which also has a hgh U.V. extinction. The main purpose of the labelling experiment was to distinguish PE-derived fluorescent products from those formed from MDA alone by polymerisation and other side-reactions. However, both 2D TLC and HPLC demonstrate that few fluorescent components resolvable by these techniques do not also have radioactivity associated with them. This either means that almost all the fluorescent material is PE-derived or that MDA polymers/products overlap with PE derivatives. It is noteworthy that peaks of radioactivity at 7.4 and 9.7 minutes, although not corresponding to fluorescent components, do have matching U.V. peaks, one of which (7.4 minutes) is PE, the other of which is unknown. It should be emphasised that the ^{14}C PE used for these experiments was labelled on the 2-carbon of the glycerol backbone. This means that labelled components must at least contain the intact backbone of the original phospholipid and are not derived from deacylated fatty acyl substituents. The possibility exists of course that they may

represent glyceride derivatives in which there has been at least partial deacylation, or in which the fatty acid side chains have been modified.

On the basis of these results, it is apparent that the reaction between PE and MDA at physiological temperature in vitro, is extremely complex and generates a whole spectrum of fluorescent (and non fluorescent) adducts with similar spectral properties, albeit with very different chemical natures. From the evidence presented here, it would be entirely speculative to suggest structures for the major adducts formed in this reaction, however the simplest possible conjugated Schiff base type adducts which could theoretically be formed are shown below:-

PE-MDA-PE	Lyso PE_1-MDA-Lyso PE_2
PE-MDA-Lyso PE_1	Lyso PE_1-MDA-Lyso PE_1
PE-MDA-Lyso PE_2	Lyso PE_2-MDA-Lyso PE_2

It is also conceivable that MDA polymers may cross-link with phospholipids i.e. PE-MDA (n) giving rise to a vast range of theoretically possible adducts. Also glycerophosphorylethanolamine derivatives may be formed by the complete deacylation of PE. However under our controlled conditions of physiological pH and temperature it was assumed that breakdown of PE would be minimised. This was confirmed by chromatography, since although MDA polymerisation products were detected, (fluorescent material which did not contain significant amounts of radioactivity by TLC (Figures 17 and 18) and HPLC (Figure 23) analysis) there were only traces of lyso PE visible even after prolonged incubation.

On the basis of spectrofluorimetric studies, autoradiography and TLC and HPLC analysis, the major non-polar fluorescent was clearly derived from PE and MDA in that, (i) it contained phospholipid as revealed by I_2 staining, phosphorus estimation, incorporation of radioactive carbon and U.V. absorbtion at 206 nm and (ii) it had the fluorescent properties consistent with a conjugated Schiff base as revealed by spectrofluorimetry. This fraction contained 3 separate species resolved by TLC and HPLC analysis, the major one of which stained with ninhydrin. Since this reagent only stains free amino groups, we suggest that the major adduct is not the 2:1 PE-MDA conjugated Schiff base described by Trombly and Tappel (105), since the PE amino groups would be involved in bonding with MDA and not react with ninhydrin, although the fluorescent properties do agree with their findings. Jain et al. (100) also claimed to detect a highly fluorescent phospholipid adduct formed when incubating MDA with erythrocytes and which chromatographed between PE and PS on TLC. This component was analysed by them and found to be the heterologous PE-MDA-PS adduct. They also suggested that it was likely that homologous adducts (PE-MDA-PE or PS-MDA-PS) would also have been formed, but state that their TLC system failed to separate these adducts from their parent compound. Their analytical data however are also consistent with the existance of equimolar proportions of the homologous PE-MDA + PS-MDA adducts making up this TLC component. In our synthetic systems using similar solvents, we did not detect any adduct of greater polarity than PE itself. Furthermore, we found PS to be

extremely unreactive towards MDA. This may mean that their conditions, perhaps the micro-environment of the PS in the erythrocyte membrane, were more favourable to PS-MDA conjugation. Our findings are also inconsistent with Kikugawas' (106, 161) theory that the major adduct is a 1:1 non fluorescent Schiff base, since our adduct was highly fluorescent and also stained with ninhydrin. It is also unlikely that our major fluorescent product is the heterocyclic structure proposed by Kikugawa et al., since this would also not stain with ninhydrin.

In conclusion we propose that the major fluorescent adducts formed from the reaction of PE with MDA, are not conjugated Schiff bases of the kind originally described by Dillard and Tappel, (155) although the major adduct is highly fluorescent and does possess the fluorescent characteristics of such products. Neither is it likely to correspond to the heterocyclic dihydropyridine derivative described by Kikugawa et al. (161) since this too would not stain with ninhydrin. If the primary amino group of PE is not involved in binding with MDA, what then could be the reactive substituent? It is conceivable that MDA may react under the conditions employed in these experiments with the phosphate group on the phospholipid molecule to yield a fluorescent adduct with a free, unmodified primary amino function. It is nevertheless possible that a number of our more minor products, which did not stain with ninhydrin, might be either Schiff bases or dihydropyridine derivatives.

Finally, reaction mixtures with PS and MDA did not generate any fluorescent, I_2 staining adducts, and those

adducts generated from PE/PS and MDA were only unique to PE and MDA reaction mixtures and are thus only derived from PE. Contrary to the suggestion in several reports that both amino group containing phospholipids PE and PS are highly reactive with MDA (69, 100), surprisingly we found PS to be extremely unreactive. This could simply be a reflection of the pK differences between the primary amine functions of the two phospholipids, since a greater proportion of that in PE may exist in the unprotonated form thus increasing its reactivity. Also the carboxyl group of PS may impose a form of steric hindrance thus restricting the access of MDA (or MDA polymers) to the free amino group. These findings are consistant with the reactivity of PS with glucose in vitro, in that it is considerably easier to glycosylate PE than PS (198).

Biological Studies

Using the various chromatographic techniques developed in the synthetic phospholipid studies, the major reaction products generated in vitro biological systems by inducers of LPX were investigated.

Incubations of rat liver microsomes with TBH and NADPH as inducers of LPX resulted in a 7-8 fold (after 3 hours) and 3 fold (after 24 hours) increase in lipid soluble fluorescent material respectively, with excitation and emission maxima within the 360 - 374 nm and 430 - 444 nm range respectively. These spectral characteristics are similar, although not identical particularly with respect to excitation maxima, to those of the major fluorescent adducts generated in our synthetic phospholipid systems. The slow increase in the

fluorescence of the control is probably due to endogenous LPX of the microsomes on standing as defence mechanisms are exhausted. The wide discrepancy in levels of fluorescent material generated between the two initiation systems may be explained, at least in part, by the nature of the inducer. TBH is a relativley non-polar molecule and thus would be readily inter-calated into the lipid bi-layer matrix where it can decompose to form a reactive radical capable of rapidly initiating LPX. On the other hand, the poorly understood NADPH dependant reaction involving radical generation by cytochrome reductase may procede at a much slower rate since enzymatic radical generation would primarily commence in the aqueous phase at a more limited number of sites and may therefore require much higher concentrations of initiator and/or longer incubation periods in order to generate significantly detectable levels of fluorescent products.

1D TLC analysis (Figure 26) of lipid extracts demonstrated no striking differences between control and stimulated microsomes when incubated with TBH. However, a fluorescent post (X) present only in stimulated microsomes co-chromatographed with the major fluroescent adduct generated in the synthetic PE-MDA reaction systems (Figures 11, 12, 13 and 14, spot A). This component in the microsomes did not stain significanlty with I_2 and was therefore either a very minor component relative to the other lipid material, or a fluorescent compound <u>not</u> identical with our major synthetic product. 2D TLC anlaysis (Figure 27) revealed a considerably greater amount of fluorescent material present in the stimulated sample, both at the origin but particularly

surrounding the PE spot. HPLC analysis revealed little difference in the relative proportions of the standard phospholipid classes, although PE and PS were slightly raised. Using fluorescence detection, the major peak present in both control and induced microsomes (Figure 28c) corresponded with one of the major fluorescent peaks in the synthetic reaction mixtures (Figure 23a). U.V. detection revealed two peaks with very similar retention times to be slightly diminished in the stimulated sample. It is difficult to interpret these findings in comparison to those of the synthetic phospholipid studies, particularly since the high levels of fluorescence in the region of PE (in stimulated as opposed to control microsomes) revealed by TLC analysis, are not reflected in the HPLC chromatograms with fluorescence detection. One would expect the polar material visible at the origin of TLC analyses, to elute later in the HPLC system, although no difference whatsoever was observed during fluorescent analysis. One can only speculate that our HPLC conditions are inappropriate for the analysis and detection of these particular fluorescent compounds. For example even our most polar solvent B at 100% may be insufficient to elute these compounds from the column. In view of our earlier discussions, the stability of some of these compounds on HPLC should be examined closely.

1D TLC analysis of TBH-treated erythrocytes indicated the presence of an additional I_2 staining spot chromatographing between PS and the origin (Figures 29a and b, spot X), and an autofluorescent spot (Y) closely associated with the PE region. In contrast, erythrocytes

treated with MDA produced no qualitative differences between induced and control erythrocytes. This means that we were unable to repeat the findings of Jain et al. (100) despite using identical conditions. It is also therefore, fairly unlikely that the new components seen after TBH treatment are simple MDA adducts. No further work was done to characterise these components. In vivo, a wide range of other amino group containing macromolecules are present which could theoretically cross-link with MDA, notably proteins and nucleic acids. The fluorescent adducts formed during the reaction between such molecules and MDA, would be extractable into the MeOH/H_2O soluble phase, and TLC analysis (Figure 29b, lanes 2, 4, 6 and 8) of such extracts obtained from erythrocytes illustrated a great deal of polar, I_2 staining and highly fluorescent material situated at the origin, which was not resolved using our solvent system. Detailed analysis and characterisation of fluorescent adducts extractable into aqueous phases was beyond the scope of this project (although TLC and HPLC analysis was carried out on the aqueous phase from several of our synthetic and biological systems) since time did not permit the development of alternative solvent systems in order to resolve the apparent complexity of components produced. However, phosphorus estimations on both total aqueous and organic phases from erythrocyte incubations did reveal an approximate 15% loss of lipid phosphorus into the aqueous phase after peroxidation, which may represent phospholipids cross-linked with proteins.

Autoxidation of sheep brain homogenate at 37^{o}C with Fe^{3+} produced a two fold increase in fluorescent material in

comparison with the 22°C control. Unfortunately Fe^{3+} was also added to the 22°C autoxidised homogenate in error and therefore only relative differences between the two samples can be expected due entirely to the temperature difference. A new 1D TLC solvent system was developed at this stage in order to give superior resolution of the fluorescent components, although at the expense of incomplete resolution of the major phospholipid classes (other than PE or PS). As with the other biological systems, there was no evidence for the formation of major novel components using TLC analysis (Figure 30). However in both 22°C and 37°C homogenate, one fluorescent component (Figure 30a, spot Y) is of comparable mobility to the major adduct formed in in vitro PE-MDA incubations. The levels of Y were greater in the 37°C homogenate. Also in agreement with the findings in other biological systems, the total fluroescence surrounding the PE region was markedly greater in the 37°C sample, and a minor increase in PE and PS (as in microsomes) in 37°C compared with 22°C samples was revealed by quantitative phosphorus estimations. Preparative TLC further amplified the greater total fluorescence of the 37°C homogenate (Figures 33 and 34 and Table 9). The distribution of this fluorescence between the fractions was also different: in the 22°C sample, the bulk of the fluorescence was associated with the PE band (Fraction 3) whereas in the 37°C sample more was found in the least polar band fraction 1 and also in the most polar band fraction 4. It is noteworthy that the excitation and emission maxima of all 4 fractions were very similar both to each other and with the corresponding maxima detected in rat

liver microsome extracts. HPLC analysis of these fractions revealed no striking qualitative differences between the two homogenate preparations although in the total 37°C sample, there was a decrease in the acidic phospholipids (PS and PI) with an incrase in PE, as monitored by U.V. absorption at 206 nm. It is conceivable that this is not a true quantitative estimate of native PE (especially since this is inconsistent with phosphate determinations after TLC), but perhaps represents a molecular species of PE with a high E_{206} due to a modified fatty acid side chain, but which elutes during HPLC. 3 non-polar compounds eluting prior to PE (Figures 36a and b) appeared to be unique to the 37°C homogenate and these clearly originated from the major fluorescent fraction 4 (Figure 37d). The less polar components were distributed between all 4 fractions.

To summarise then, it is clear that the lipid soluble fluorescent products of LPX are extremely complex both in biological systems and in synthetic systems. The TLC and HPLC methods employed in this were successful in resolving these components from their parent compounds using both fluorescence and spectrophotometric detection methods. It proved impossible to achieve optimum separation of both phospholipid classes and fluorescent products by TLC, but this was achieved by HPLC although the stability of, at least the synthetic, products was questionable. Model studies confirmed that MDA reacts with PE to form fluorescent products having spectral characteristics (excitation 404 nm, emission 464 nm) which are only broadly similar to the fluorescence characteristics of lipids extracted from

peroxidised microsomes (excitation 384 nm, emission 454 nm). Although the spectral characteristics of the fluorophores generated in our biological systems are very similar and therefore, by this alone, would appear to be independant of the type of system studied (i.e. different initiatiors), nevertheless, the chromatographic characteristics of the major products are different. This clearly illustrates the pit-falls of past workers in the field of LPX who have claimed similarity or even identity of products from different sources solely on the basis of similar or identical fluorescence maxima. In microsomes the fluorescence is associated chiefly with the origin and in a spot with similar mobility to the major product generated in PE-MDA incubations, and in brain homogenate chiefly in the region of the PE spot. In both systems as monitored by HPLC with U.V. detection at 206 nm small increases in PE and PS were observed, PS being strikingly increased in brain homogenate lipids. These findings do not agree with our original expectations in that during LPX, MDA generated will cross-link to these phospholipids with concomittant loss of the original phospholipid. However, it is highly likely that apparent increases in such phospholipid classes represent slightly modified components (by HPLC analysis at 206 nm). One would not necessarily expect the products generated between reactive phospholipids and MDA to be identical in different biological systems. The differing structural organisation and composition of various cell membranes (notably the amount and type of lipid present), will lead to profound differences in their properties and in particular to

their susceptibility to free radical attack and subsequent oxidative degradation. This appears to hold true for our findings with erythrocytes and microsomes in that TLC analysis revealed very different adducts generated. The fact that we found PS in solution in organic solvents to be extremely unreactive towards MDA in vitro does not necessarily mean that it is unreactive towards this aldehyde in in vitro incubations of biological membranes. It has been known for some time that 4-OH NA is formed in large amounts during LPX (162, 163), and it has recently been shown that this highly reactive aldehyde reacts with PE and PS leading to the formation of a fluorophore with identical emission (430 nm) and excitation maxima (360 nm) to the fluorophore found in peroxidising microsomes and mitochondria (69). The authors point out that these maxima are significantly different from those of MDA adducts or model Schiff base conjugates - a fact conveniently overlooked by previous authors. The spectral difference between our PE-MDA fluorophore and the fluorophore detected in our brain homogenate and microsome preparations should also not be neglected, and in fact rather indicates that substances other than MDA may be involved in their formation. Nevertheless TLC and HPLC analysis did reveal that a component of comparable chromatographic mobility to the major product generated in our synthetic systems was detectable in biological systems. The biological significance of other alternative alternative aldehydes produced by LPX such as 4-OH NA should be investigated more fully in order to determine whether they generate fluorescent adducts

possessing the same chromatographic characteristics as those derived from biological systems before proposing that 4-OH NA adducts and not MDA-derived products are of physiological significance.

Plasma and CSF Studies

Measurement of LPX indices in both plasma and CSF (TBARS, conjugated dienes and $CHCl_3$ and $MeOH/H_2O$ soluble fluorescent pigments), failed to show a significant difference in M.S.; either expressing the results in comparison to control groups in total, or within various sub-groups. This more exhaustive study failed to confirm earlier findings in our laboratory that LPX indices were raised in CSF from M.S. patients. Much time was spent on developing and refining existing methods for the quantification of these LPX indices in biological fluids, notably CSF.

It was not entirely surprising that we were unable to detect total TBARS (free MDA and lipoprotein bound MDA) in CSF using a modification of Satohs' spectrophotometric method (194) since the protein concentration in CSF is very small (15 - 40 mg/100 ml (199)) and lipid is normally absent. Even if a leaky blood-brain barrier resulted in such molecules originating from the blood leaking into CSF, the sensitivity of the method (100 x less sensitive than spectrofluorimetry (150)) coupled with the fact that very small volumes of sample (< 1 ml) were available to us did not allow such LPX indices to be detected in CSF. Severe problems were also encountered using the more sensitive spectrofluorimetric assay of Yagi (131). Colour development in the TBA assay

depends critically on several factors including type and concentration of the acid, and heating time. Although we did not find absolute sensitivity to be a problem, considerable discrepancies occured due to irreproducibility of results resulting in unacceptably high inter-assay coefficients of variation. Eventually a successful non-precipitation method was developed for assay of total TBARS in plasma and CSF. The presence of phosphotungstic or trichloracetic acids required for the precipitation method in assaying TBARS bound to lipoproteins, were found to cause irreproducibility of results and after much investigation this method was abandoned. Both the heating time and cooling time of the samples were found to be critical in attaining good reproducibility of the non precipitation method, as was the treatment of the samples following thawing from -70°C. Freezing of both plasma and CSF produced precipitates which could not be readily redissolved on thawing. As a result the samples were centrifuged prior to freezing and immediately prior to analysis, since if plasma samples were stored at 4°C for several hours prior to analysis visible precipitation (thought to be the cryoprecipitate of factor VIII) resulted in inhomogeneity of the sample and thus irreproducibility of results. We suggest the fact that we were unable to repeat the earlier findings in our laboratory where TBARS and fluorescent pigments were found to be significantly elevated in CSF from M.S. patients is due to different treatment of the CSF prior to storage in the two studies. In the present study, CSF was centrifuged prior to storage at a speed sufficient to remove leukocytes and cell debris (since it is

well known that a leukocytosis accompanies M.S.). During the earlier pilot studies, CSF samples were not centrifuged prior to analysis and the levels of TBARS measured were probably a reflection of these LPX products associated with the leukocytes. The fluorescent pigment assay of Dillard et al. (141) was also refined since the extraction procedure precipitated large amounts of plasma protein thus making isolation of a purely organic phase extremely difficult. The sensitivity of the spectrofluorimetric method of analysis of the lipid extracts was such that the presence of small particulate impurities induced light scattering. These were removed in the modified method by incorporating an additional millipore filtration stage.

The lack of quantitative difference in LPX indices between M.S. and control biological fluids was further proven by chromatographic studies on both lipid soluble and MeOH/H_2O extracts. TLC analysis in conjunction with I_2 staining and autofluorescence detection generally revealed a complex pattern of components (Figures 43a, b, 44a, b, 45a, b) although the expected major phospholipid classes were identified, with the help of standards, in $CHCl_3$ phases extracted from plasma. Sensitivity was again a problem in both CSF and plasma analysis due to the small amount of extractable lipid. Even the superior resolving power of our HPLC system revealed little material in the samples using either U.V. or fluorescence detection. However U.V. detection of $CHCl_3$ extracts from plasma revealed that most of the lipids were extremely non polar eluting prior to PE and PS and PI was not detected. U.V. detection of CSF $CHCl_3$

extracts revealed PE and PS with several unidentified less polar lipids and PI, PC and Sm were not detected. This is in contrast with the work of Pederson (59) who detected PC and Sm in M.S. CSF lipid extracts. Fluorescence detection even at the highest sensitivity setting proved to be highly unsuccessful for CSF analysis whereas in some but not all M.S. and control plasma samples, a single unidentified peak eluting around 12 minutes was visible. These HPLC chromatograms clearly indicate that the major LPX products generated in synthetic phospholipid reaction systems and in model biological systems are not present in biological fluids obtained from patients suffering neurological diseases.

In conclusion, these findings have revealed the failure of these assays of LPX indices (TBARS, FP assay, TLC and HPLC studies) as diagnostic indicators of M.S. The earlier findings in our laboratory that LPX indices were elevated in M.S. CSF have not been substantiated and in contrast to other workers (183) we confirm that MDA levels in M.S. plasma are in fact normal. However in view of our findings with biological and synthetic studies that considerable amounts of PE-associated fluorescent material were detected, the development of more suitable/precise chromatographic solvent systems (possibly in conjunction with reverse phase HPLC) could reveal a clearer picture of the nature and origin of these fluorescent components. It is still questionable as to whether or not membrane LPX plays a significant (albeit secondary) role in the pathogenesis of M.S. Even though our studies support the suggestion of Esterbauer et al. (69) that MDA does not contribute significantly to the generation of

LSFP's in biological systems *in vitro*, we feel that this work is still worth pursuing in view of the fact that other toxic aldehydes (e.g. 4-OH NA) formed during the peroxidative process may be significantly generated *in vivo* and be involved in M.S. pathogenesis.

Erythrocytes from M.S. patients and controls with other neurological diseases, when incubated with H_2O_2, TBH and ADP/Fe^{2+} as a model for oxidative stress, failed to show any significant difference in susceptibility to membrane LPX. Initial problems encountered using the method of Kobayashi *et al.* (147) in using Fe^{3+} as a stimulator, were overcome with the adoption of a final modified method which was found to be sensitive and reliable. Surprisingly unlike Kobayashi *et al.*, we were unable to initiate significant LPX using Fe^{3+} and experiments carried out (200) using increased concentrations of Fe^{3+} (to optimise the Fe^{3+} requirement) and increased washing of the erythrocytes prior to haemolysis (to remove residual traces of transferrin), still proved unsuccessful in terms of MDA production. The absence of significant MDA formation may suggest that the levels of lipid hydroperoxides pre-formed within the cell membrane are extremely low and that their Fe^{3+} catalysed breakdown is not sufficiently effective in initiating LPX to a significant extent, but this still does not account for the discrepancy in the findings of Kobayashi *et al.* Recently however, Braughler *et al.* (201) have shown that peroxidation of brain synaptosomes by Fe^{3+} is dependant upon traces of Fe^{2+} and that optimal ratios of Fe^{3+} to Fe^{2+} is the primary determining factor for the initiation of LPX reactions and

hence an imbalance of metal ions in our system could be the reason for our lack of success with Kobayashi's method. Incidentally this theory of Braughlers conflicts with that of Kobayashi et al. in that the latter suggests the presence of both ions (Fe^{2+} and Fe^{3+}) results in the complete abolition of any enhancing effect on the rate of LPX, than individually they might have achieved.

Considerable time was spent investigating the effects of alternative stimulators of LPX. H_2O_2/azide (in agreement with Van der Zee experiments with erythrocyte stressors (202)), was found to be a more potent initiator of LPX than Fe^{3+}/TBH; and Fe^{3+}/TBH much better than Fe^{2+}/ADP or Fe^{3} alone and consequently the former two systems were selected in order to oxidatively challenge the haemolysates. One could expect the two chosen inducing systems to invoke different responses in haemolysates since they operate on different segments of the LPX pathway: the likely effect of TBH is caused by its Fe^{2+} catalysed breakdown to produce free radicals (RO$^{\cdot}$ and OH$^{\cdot}$) which are capable of acting as initiators both within the aqueous and lipid soluble phases of the membrane (since TBH is amphipathic). However, H_2O_2, a polar molecule, interacts with O_2^{-} radicals to produce OH$^{\cdot}$ within the aqueous membrane phase. The nature of these stresssors will influence the type of protective mechanism interacting in order to combat the possible deleterious effects of LPX (e.g. GPX, catalase (aqueous phase) and Vitamin E (hydrophobic phase)) and could explain any difference in results obtained using the two stressors. The length of time of sonication was found to be critical in

attaining good reproducible results. Contrary to Kobayashi et al., surprisingly no sonication produced the highest rate of LPX (Figure 50) in Fe^{3+}/TBH treated haemolysates. A possible explanation relates to the access of GPX to the stressor. In the absence of sonication, most GPX is within the red blood cells and therefore unable to react with TBH, and LPX proceeds at a maximal rate. On disruption of the membrane, GPX is released variably (dependant upon sonication time), until the inactivation of TBH by GPX is maximal and constant leading to the kind of data shown in Figure 50. As TBH can rapidly diffuse into the membrane matrix (unlike Fe^{3+} in Kobayashi's experiments), sonication is not necessary in order to achieve access to the cell and even extracellular TBH initiates LPX directly in the presence of Fe^{3+}.

In summary our results showed no significant difference between M.S. erythrocytes and controls whether stressed with H_2O_2/azide or Fe^{3+}/TBH. Although activities of various antioxidant enzymes have been shown to be abnormal in M.S. erythrocytes (i.e. GPX, SOD and GR (175-179)), this was clearly not reflected in abnormal stress test results. These findings disagree with previous work in our laboratory that reported M.S. erythrocytes being less susceptible to peroxidative stress than healthy controls. (Both plasma and red cell Vitamin E content were normal in M.S. thus indicating that patients abnormal red cell peroxidisability did not arise as a result of increased α-tocopherol (203). The results in this study may however be expected to differ from previous work since on this occasion haemolysates were used in preference to whole, washed erythrocytes. We

conclude that this test (as it stands) is of no value as a diagnostic indicator for M.S., however if, as previously mentioned, 4-OH NA is more significant than MDA in causing LPX damage, then a specific assay method for 4-OH NA (as opposed to total TBARS), may be of great value together with employing the principle of this stress test as a measure of peroxidisability potential. (HPLC methods have been developed to specifically assay 4-OH NA).

Finally despite the theoretical possibility that the two stressors (TBH/Fe^{3+} and H_2O_2/azide) may probe different areas of the LPX pathway, infact the relative response of the M.S. and control samples was similar, although the absolute response was different.

SUMMARY

SUMMARY

With direct reference to our aims, the following questions can be answered in conclusion to this work:-

(i) What are the major products of the reaction in vitro between MDA and PE or PS?

1D, 2D and preparative TLC in conjunction with autoradiography, fluorescence properties and extensive HPLC analysis using both U.V. and fluorescence detection have shown that the major product of the reaction cannot be a Schiff base or a dihydropyridine derivative. PS was shown to be extremely unreactive towards MDA in vitro.

(ii) Are these products generated to a significant extent in biological systems?

We have shown that the fluorescence excitation and emission spectra measured for lipid soluble fluorescent pigments in brain homogenate, erythrocytes and microsomes do not agree precisely with those fluorophores generated between the reaction of PE with MDA in vitro. Further chromatographic analysis confirms the fact that those products generated in synthetic phospholipid systems in vitro are not generated to a significant extent in in vitro biological systems.

(iii) Are these products increased in M.S. body fluids in comparison to other neurological diseases thus forming the basis of a discriminatory test for the disease?

LPX indices (as measured by LSFP and MDA formation), are not significantly elevated in M.S., CSF and plasma in comparison to other neurological diseases. Furthermore,

extensive TLC and HPLC analysis failed to confirm any conclusive abnormalities in the lipid profiles between M.S., control or normal body fluids.

In view of these results it is tempting to suggest that LPX does not play a significant role in the pathogenesis of M.S. However, with the ever increasing knowledge of the role of free radicals being implicated in disease processes and considering our findings in that the spectrum of products generated during membrane LPX is even more complex than originally anticipated, we feel that this area of research is worth pursuing for two main reasons:-

(1) Improved resolution of membrane LPX products could be achieved using better developed chromatographic systems with subsequent mass spectrometry in order to determine the precise structure of such adducts.

(2) Recently another aldehyde, 4-OH NA has been shown to produce a product in in vitro incubations with PE and PS, whose fluorescent characteristics are almost exactly the same as those exhibited in lipid extracts of peroxidised biological tissues. Perhaps the characterisation of such products will cast further enlightenment as to the precise origin of such LPX products in vivo and lead to a better understanding of this rather controversial and poorly understood area of research.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. CHARCOT, J.M. (1868) Gaz. Hosp. (Paris) 41: 554-556.
2. ADAMS, C.W.M. (1972) "Research on Multiple Sclerosis". Pub: Thomas, Illinois. Ed. I.N. Kugelmass.
3. GONZALEZ-SCARANO, F., SPIELMAN, R.S., and NATHANSON, N. (1986) "Multiple Sclerosis". Pub: Butterworth, London. Ed: W.I. McDonald and D.H. Silderberg, pp. 37-55.
4. DAVISON, A.N. (1978) Biochem. Soc. Trans. 6: 443-447.
5. OFFICE OF HEALTH ECONOMICS (1975) "Multiple Sclerosis".
6. BATES, D. (1986) Hosp. Update 12: 771-788.
7. ACHESON, E.D. (1985) "McAlpines Multiple Sclerosis". Pub: Churchill Livingstone, Edinburgh. Ed: W.B. Matthews, pp. 3-46.
8. McFARLIN, D.E. and McFARLAND, H.F. (1982) N. Engl. J. Med. 307, 19: 1183-1188.
9. GLYNN, P., GILBERT, H.M., NEWCOMBE, J. and CUZNER, M.L. (1982) Clin. Exp. Immunol. 48: 102-110.
10. KOSTULAS, V.K. and LINK, H. (1982) J. Neurol. Sci. 54: 117-127.
11. McALPINE, D. (1972) "Multiple Sclerosis: A Reappraisal". Pub: Churchill Livingstone, Edinburgh. Ed: D. McAlpine, C.E. Lumsden and E.D. Acheson (2nd Ed.), pp. 224-257.
12. SCHUMACHER, G.A., BEEBE, G.W. and KIBLER, R.F. (1965) Ann. N.Y. Acad. Sci., 122: 552-568.
13. POSER, C.M., PATY, D.W. and SCHEINBERG, L. (1983) Ann. Neurol. 13: 227-231.

14. POSER, C.M. (1984) Ann. N.Y. Acad. Sci. 436: 233-245.

15. SMITH, T. (1986) "M.S. News" 130, 7-10. Ed. J. Walford.

16. ADAMS, C.W.M. (1977) Brit. Med. Bull. 33, 1: 15-20.

17. YU, R.K., UENO, K., GLASER, G.H. and TOURTELLOTTE, W.W. (1982) J. Neurochem. 39, 1: 464-473.

18. EBERS, G.C., VINUELA, F.V., FEASBY, T. and BASS, B. (1984) Neurology 34: 341-346.

19. KURTZKE, J.F. (1980) Acta Neurol. Scand. 62: 65-80.

20. McFARLIN, D.E. and McFARLAND, H.F. (1982) N. Engl. J. Med. 307, 20: 1246-1251.

21. ALTER, M., LEIBOWITZ, U. and SPEER, J. (1966) Arch. Neurol. 15: 234-237.

22. FIELD, E.J. and CASPARY, E.A. (1964) Lancet, 2: 876-880.

23. LUMSDEN, C.E. (1972) "Multiple Sclerosis: A Reappraisal". Pub: Churchill Livingstone, Edinburgh. Ed. D. McAlpine, C.E. Lumsden and E.D. Acheson (2nd Ed.), pp. 512-621.

24. GALLI, C., WHITE, H.B.Jr. and PAOLETTI, R. (1970) J. Neurochem. 17: 347.

25. LISAK, R.P. (1975) Ann. Clin. Lab. Sci. 5: 324.

26. ALTER, M. (1977) "Multiple Sclerosis: A Critical Conspectus". Pub: MTP Press Ltd., Lancaster. Ed: E.J. Field, pp. 35-82.

27. COOK, S.D., DOWLING, P.C. and RUSSELL, W.C. (1978) Lancet, 1: 605-606.

28. JERSILD, C., DUPONT, B., FOG, T., PLATZ, P.J. and SVENJGAARD, A. (1975) Transplant Rev. 22: 148-163.

29. LAMOUREUX, G., GIARD, N., JOLICOEUR, R., TOUGHLIAN, V. and DESROSIERS, M. (1976) Brit. Med. J. 1: 183-186.

30. ILONEN, J., REUNANEN, A., SALMI, A. and TIILIKAINEN, A. (1981) J. Neurol. Sci. 49: 117-133.

31. BROWN, P., CATHALA, D., GAJDUSEK, D.C. and GIBBS, J.R., C.J. (1971) Proc. Soc. Exp. Biol. Med. 137: 956-961.

32. UTERMOHLEN, V. and ZABRISKIE, J.B. (1973) Lancet 2: 1147-1148.

33. STEWART, G.J. and KIRK, R.L. (1983) "Multiple Sclerosis". Pub: Chapman and Hall Ltd., London. Ed: J.F. Hallpike, C.W.M. Adams and W.W. Tourtellotte, pp. 97-128.

34. JAMES, P.B. (1982) Lancet 1: 380-385.

35. ADAMS, C.W.M. and HIGH, O.B. (1982) Lancet, 1: 621.

36. SIMON, J. (1982) IRCS Med. Sci. 10: 433-435.

37 WAKSMAN, B.H. and REINGOLD, S.C. (1986) Trends Neurol. Sci. 10: 388-391.

38. RUMSBY, M.G. (1978) Biochem. Soc. Trans. 6: 448-460.

39. GREGSON, N.A. (1983) "Multiple Sclerosis". Pub: Chapman and Hall Ltd., London. Ed: J. Hallpike, C.W.M. Adams, and W.W. Tourtellotte, pp. 1-28.

40. DAVISON, A.N. and CUZNER, M.L. (1977) B. Med. Bull. 33, 1: 60-66.

41. SHAH, S.N. and JOHNSON, R.C. (1980) Exp. Neurol. 68: 601-605.

42. CLAUSEN, J.C. and HANSEN, I.B. (1970) Acta Neurol. Scand. 46: 1-17.

43. DAVISON, A.N. and WAJDA, M. (1962) J. Neurochem. 9: 427-432.

44. GERSTL, B., ENG, L.F., TAVASTSTJERNA, M., SMITH, J.K. and KRUSE, S.L. (1970) J. Neurochem. 17: 677-689.

45. ALLING, C., VANIER, M.T. and SVENNERHOLM, L. (1971) Brain Res. 35: 325-336.

46. ARNETTOLI, G., PAZZAGLI, A. and AMADUCCI, L. (1969) J. Neurochem. 16: 461-463.

47. CUZNER, M.L. and DAVISON, A.N. (1973) J. Neurol. Sciences 19: 29-36.

48. EINSTEIN, E.R., CSEJTEY, J., DALAL, K.B., ADAMS, C.W.M., BAYLISS, O.B. and HALLPIKE, J.F. (1972) J. Neurochem. 18: 653-662.

49. HIRSCH, H.E., DUQUETTE, P. and PARKS, M.E. (1976) J. Neurochem. 26: 505-512.

50. DWORKIN, R.H., BATES, D., MILLAR, J.H.D. and PATY, D.W. (1984) Neurology 34: 1441-1445.

51. BELIN, J., PETTET, N., SMITH, A.D., THOMPSON, R.H.S. and ZILKHA, K.J. (1971) J. Neurol. Neurosurg. Psychiat. 34: 25-29.

52. BAKER, R.W.R., THOMPSON, R.H.S. and ZILKHA, K.J. (1964) J. Neurol. Neurosurg. Psychiat. 27: 408-414.

53. KARLSSON, I., ALLING, C. and SVENNERHOLM, L. (1971) Acta Neurol. Scand. 47: 403-412.

54. FEWSTER, M.E., HIRONO, H. and MEAD, J.F. (1976) J. Neurol. 213: 119-131.

55. WOLFGRAM, F., MYERS, L., ELLISON, G. and KNIPPRATH, W. (1975) Neurology 25, 8: 786-788.

56. FIELD, E.J. (1977) "Multiple Sclerosis: A Critical Conspectus". Pub: MTP Press Ltd., Lancaster. Ed.: E.J. Field, pp. 245-261.

57. SANDERS, H., THOMPSON, R.H.J., WRIGHT, H.P. and ZILKA, K.J. (1968) J. Neurol. Neurosurg. Psychiat. 31: 321-325.

58. LOVE, W.C. (1973) Biochem. Soc. Trans. 1: 141-143.

59. PEDERSON, H.E. (1974) Acta Neurol. Scand. 50: 171-182.

60. BARBER, A.A. and BERNHEIM, F. (1967) Adv. Gerontol. Res. 2: 355-403.

61. SLATER, T.F. (1972) "Free Radical Mechanisms in Tissue Injury". Pub: Pion Press, London.

62. HALLIWELL, B. and GUTTERIDGE, J.M.C. (1985) "Mol. Asp. Med." 8, 2: 119-133.

63. TAPPEL, A.L. (1978) Adv. Exp. Med. Biol. 97: 111-131.

64. HUNTER, M.I.S., LAO, M.S., BURTLES, S.S. and DAVIDSON, D.L.W. (1984) Neurochem. Res. 9, 4: 507-516.

65. SLATER, T.F. (1984) Biochem. J. 222: 1-15.

66. CHIO, K.S. and TAPPEL, A.L. (1969) Biochemistry 8, 7: 2827-2832.

67. KIKUGAWA, K., MACHIDA, Y., KIDA, M. and KURECHI, T. (1981) Chem. Pharm. Bull. 29, 10: 3003-3011.

68. BENEDETTI, A., COMPORTI, M., FULCERI, R. and ESTERBAUER, H. (1984) Biochim. Biophys. Acta 792: 172-181.

69. ESTERBAUER, H., KOLLER, E., SLEE, G. and KOSTER, J.F. (1986) Biochem. J. 239: 405-409.

70. HALLIWELL, B. and GUTTERIDGE, J.M.C. (1984) Biochem. J. 219: 1-14.

71. TAPPEL, A.L. (1973) Fed. Proc. 32, 8: 1870-1874.

72. HALLIWELL, B. (1978) Cell Biol. Int. Rep. 2, 2: 113-119.

73. FRIDOVICH, I. (1975) Ann. Rev. Biochem. 44: 147-159.

74. YOUNGMAN, R.J. (1984) Trends Biochem. Sci. 6: 280-283.

75. HALLIWELL, B. and GUTTERIDGE, J.M.C. (1985) "Mol. Asp. Med." 8, 2: 93-109.

76. TAUBER, A.I. (1982) Trends Biochem. Sci., 411-414.

77. HALLIWELL, B. and GUTTERIDGE, J.M.C. (1985) Trends Neurol. Sci.: 22-26.

78. ELSTNER, E.F. (1982) Ann. Rev. Plant Physiol. 33: 73-96.

79. HALLIWELL, B. (1978) FEBS Lett. 92, 2: 321-326.

80. GUTTERIDGE, J.M.C., ROWLEY, D.A. and HALLIWELL, B. (1982) Biochem. J. 206: 605-609.

81. FOOTE, C.S. (1982) "Pathology of Oxygen". Pub: N. York Academic Press. Ed.: A.P. Autor, pp. 21-44.

82. ARUDI, R.L., BIELSKI, B.J.J. and ALLEN, A.O. (1984) Photochem. Photobiol. 39: 703-706.

83. SLATER, T.F. (1984) "Meth. Enzymol." 105: 283-293.

84. CHIA, L.S., THOMPSON, J.E. and MOSCARELLO (1983) FEBS Lett. 157, 1: 155-158.

85. JAIN, S.K. and HOCHSTEIN, P. (1980) Biochem. Biophys. Res. Comm. 92, 1: 247-254.

86. GUTTERIDGE, J.M.C. (1985) Biochim. Biophys. Acta, 834: 144-148.

87. PARANTAINEN, J. (1982) "Proceedings of Symposium on Aspects of Free Radicals in Cell Aging". Ed. P. Holmberg.

88. HALLIWELL, B. (1982) Cell Biol. Int. Rep. 6: 529-542.

89. McCAY, P. (1981) Fed. Proc. 40, 2: 173.

90. FINK, R., MARJOT, D.H., CAWOOD, P., IVERSEN, S.A., CLEMENS, M.R., PATSALOS, P., NORDEN, A.G. and DORMANDY, T.L. (1985) Lancet, 2: 291-294.

91. GUTTERIDGE, J.M.C., HILL, C. and BLAKE, D.R. (1984) Clin. Chim. Acta 139: 85-90.

92. HUNTER, M.I.S. and MOHAMED, J.B. (1986) Clin. Chim. Acta 155: 123-132.

93. MURPHY, M.E. and KEHRER, J.P. (1986) Life Sci. 39: 2271.

94. GUTTERIDGE, J.M.C., ROWLEY, D.A., HALLIWELL, B. and WESTERMARCK, T. (1982) Lancet, 2: 459-460.

95. WESTERMARCK, T., GUTTERIDGE, J.M.C., HEISKALA, H., SANTAVUORI, P., RAHOLA, T. and SUOMELA, M. (1984) Life Chem. Rep. Sup. 2: 325-331.

96. BRAGANZA, J. (1987) Unpublished abstracts of the XIIIth Conference of the Society of Free Radical Research held at St. Andrews, Scotland.

97. SINGER, A. (1987) Unpublished abstracts of the XIIIth Conference of the Society of Free Radical Research held at St. Andrews, Scotland.

98. CHAN, P.H., SCHMIDLEY, J.W., FISHMAN, R.A. and LONGAR, S.M. (1984) Neurology 34: 315-320.

99. YAGI, K. (1984) BioEssays 1, 2: 58-60.

100. JAIN, S.K. and SOHET, S.B. (1984) Blood 63, 2: 362-367.

101. BERETTA, L., GERLI, G.C., FERRARESI, R., AGOSTONI, A., GUALANDRI, V. and ORSINI, G.B. (1983) Acta Haematol. 70: 194-197.

102. MEAD, J.F. (1980) "Free Radicals in Biology" 1: 51-67. Pub: N. York Academic Press, Ed: W.A. Prior.

103. REISS, U. and TAPPEL, A.L. (1973) Lipids 8, 4: 199-202.

104. PARSHAD, R., TAYLOR, W.G., SANFORD, K.K., CAMALIER, F., GANTT, R. and TARONE, R.E. (1980) Mut. Res. 73: 115-124.

105. TROMBLY, R. and TAPPEL, A.L. (1975) Lipids 10, 8: 441-447.

106. KIKUGAWA, K. and SUGIMURA, Y. (1986) Chem. Pharm. Bull. 34, 4: 1794-1800.

107. MIQUEL, J., ORO, J., BENSCH, K.G. and JOHNSON, Jr., J.E. (1980) "Free Radicals in Biology". 3. Pub: N. York Academic Press. Ed.: W.A. Pryor.

108. DORMANDY, T.L. (1983) Lancet, 2: 1010-1014.

109. VANELLA, A., PINTURO, R., GRIMALDI, R., TIRIOLO, P., SILVESTRO, I., GRASSO, M., D'URSO, G. and GEREMIA, E. (1981) IRCS Med. Sci. 9: 144-145.

110. BOZZI, A., MAVELLI, I., AGRO, A.F., STROM, R., WOLF, A.M., MONDOVI, B. and ROTILIO, G. (1976) Mol. Cell. Biochem. 10: 11-16.

111. COHEN, G., and HOCHSTEIN, P. (1963) Biochemistry 2: 1420.

112. FLOHE, L. and GUNZLER, W. (1974) Proceedings of the 16th Conference of the German Society for Biological chemistry: 132-145. Ed. Flohe, L. Pub: Thieme, Stuttgart.

113. JENKINSON, S.G. (1983) Tox. App. Pharm. 68: 399-404.

114. AEBI, H. and SUTER, H. (1974) Proceedings of the 16th Conference of the German Society for Biological Chemistry: 192. Ed: Flohe, L. Pub: Thieme, Stuttgart.

115. BAST, A. and HAENEN, G.R.M.M. (1984) Trends Biochem. Sci. 12: 510-513.

116. CHOW, C.K. (1975) Am. J. Clin. Nut. 28: 756-760.

117. SASAKI, R., KUROKAWA, T. and TERO-KUBOTA, S. (1983) J. Gerontol. 38, 1: 26-30.

118. AL-TIMINI, D.J. and DORMANDY, T.L. (1977) Biochem. J. 168: 283-288.

119. GUTTERIDGE, J.M.C., RICHMOND, R. and HALLIWELL, B. (1980) FEBS Lett. 112, 2: 269-272.

120. GUTTERIDGE, J.M.C. (1983) FEBS Lett. 157, 1: 37-40.

121. GUTTERIDGE, J.M.C., WINYARD, P.G., BLAKE, D.R., LUNEC, J., BRAILSFORD, S. and HALLIWELL, B. (1985) Biochem. J. 230: 517-523.

122. GUTTERIDGE, J.M.C. (1978) Ann. Clin. Biochem. 15: 293-296.

123. VAN KUIJK, F.J.G.M., SEVANIAN, A., HANDELMAN, G.J. and DRATZ, E.A. (1987) Trends Neurol. Sci. 12: 31-34.

124. TAPPEL, A.L. and DILLARD, C.J. (1981) Fed. Proc. 40, 3: 174-178.

125. KOSTRUCHA, J. and KAPPUS, H. (1986) Biochim. Biophys. Acta 879: 120-125.

126. LAWRENCE, G.D. and COHEN, G. (1984) "Meth. Enzymol." 105: 305-310. Pub: London Academic Press. Ed.: L. Packer.

127. PRYOR, W.A. and CASTLE, L. (1984) "Meth. Enzymol." 105: 293-298. Pub: London Academic Press. Ed: L. Packer.

128. RECKNAGEL, R.O. and GLENDE, E.A. (1984) "Meth. Enzymol." 105: 331-336. Pub: London Academic Press. Ed.: L. Packer.

129. CAWOOD, P., WICKENS, D.G., IVERSEN, S.A., BRAGANZA, J.M. and DORMANDY, T.L. (1983) FEBS Lett. 162, 2: 239-243.

130. GUTTERIDGE, J.M.C., STOCKS, J. and DORMANDY, T.L. (1974) Anal. Chimica. Acta. 70: 107-111.

131. YAGI, K. (1984) "Meth. Enzymol." 105: 328-330.

132. YAGI, K. (1976) Biochem. Med. 15: 212-216.

133. GUTTERIDGE, J.M.C. (1975) Anal. Biochem. 69: 518-526.

134. UCHIYAMA, M. and MIHARA, M. (1978) Anal. Biochem. 86: 271-278.

135. GUTTERIDGE, J.M.C. and TICKNER, T.R. (1978) Anal. Biochem. 91: 250-257.

136. WADE, C.R., JACKSON, P.G. and RIJ, A.M.V. (1985) Biochem. Med. 33: 291-296.

137. BULL, A.W. and MARNETT, L.J. (1985) Anal. Biochem. 149: 284-290.

138. YU, L.W., LATRIANO, L., DUNCAN, S., HARTWICK, R.A. and WITZ, G. (1986) Anal. Biochem. 156: 326-333.

139. BIRD, R.P., HUNG, S.S.O., HADLEY, M. and DRAPER, H.H. (1983) Anal. Biochem. 128: 240-244.

140. ESTERBAUER, H., LANG, J., ZADRAVEC, S. and SLATER, T.F. (1984) "Meth. Enzymol." 105: 319-327. Pub: London Academic Press. Ed: L. Packer.

141. DILLARD, C.J. and TAPPEL, A.L. (1984) "Meth. Enzymol." 105: 337-341. Pub: London Academic Press. Ed: L. Packer.

142. CHIO, K.S. and TAPPEL, A.L. (1969) Biochemistry 8, 7: 2821-2827.

143. RICE-EVANS, C., BAYSAL, E., PASHBY, D.P. and HOCHSTEIN, P. (1985) Biochim. Biophys. Acta 815: 426-432.

144. STOCKS, J., KEMP, M. and DORMANDY, T.L. (1971) Lancet 1: 266-269.

145. STOCKS, J. and DORMANDY, T.L. (1971) Brit. J. Haematol. 20: 95-112.

146. YAMAMOTO, Y., NIKI, E., EGUCHI, J., KAMIYA, Y. and SHIMASAKI, H. (1985) Biochim. Biophys. Acta 819: 29-36.

147. KOBAYASHI, Y., YOSHIMITSU, T. and USUI, T. (1983) J. Immunol. Meths. 64: 17-23.

148. NAIR, V., COOPER, C.S., VIETTI, D.E. and TURNER, G.A. (1986) Lipids 21, 1: 6-10.

149. GARDNER, W.H. (1979) J. Agr. Food Chem. 27: 220.

150. DILLARD, C.J. and TAPPEL, A.L. (1971) Lipids 6, 10: 715-721.

151. KOSTER, J.H. and SLEE, R.G. (1980) Biochim. Biophys. Acta 620: 489-499.

152. GUTTERIDGE, J.M.C. (1982) "Ceroid-lipofuscinosis". Pub: Elsevier Biomedical Press. Ed: D. Armstrong, N. Koppang and J.A. Rider.

153. BIDLACK, W.R. and TAPPEL, A.L. (1973) Lipids 8, 4: 203-207.

154. BIDLACK, W.R. and TAPPEL, A.L. (1973) Lipids 8, 4: 183-189.

155. DILLARD, C.J. and TAPPEL, A.L. (1973) Lipids 8, 4: 183-189.

156. TAPPEL, A.L. (1973) J. Gerontol. 28, 4: 415-424.

157. FLETCHER, B.L., DILLARD, C.J. and TAPPEL, A.L. (1973) Anal. Biochem. 52: 1-9.

158. TSUCHIDA, M., MIURA, T., MIZUTANI, K. and AIBARA, K. (1985) Biochim. Biophys. Acta 834: 196-204.

159. TAPPEL, A.L. (1980) "Free Radicals in Biology" 4: 2-47. Pub: N. York Academic Press. Ed: W.A. Pryor.

160. KIKUGAWA, K., TSUKUDA, K. and KURECHI, T. (1980) Chem. Pharm. Bull. 28, 11: 3323-3331.

161. KIKUGAWA, K. and IDO, Y. (1984) Lipids 19, 8 600-608.

162. POLI, G., DIANZANI, M.U., CHEESEMAN, K.H., SLATER, T.F., LANG, J. and ESTERBAUER, H. (1985) Biochem. J. 227: 629-638.

163. ESTERBAUER, H., CHEESEMAN, K.H., DIANZANI, M.U., POLI, G. and SLATER, T.F. (1982) Biochem. J. 208: 129-140.

164. JAIN, S.K. (1981) Biochim. Biophys. Acta 648: 46-54.

165. SHIMASAKI, H., UETA, N., MOWRI, H. and INOUE, K. (1984) Biochim. Biophys. Acta 792: 123-129.

166. KOSTER, J.F. and SLEE, R.G. (1983) Biochim. Biophys. Acta 752: 233-239.

167. SULLIVAN, S.G. (1983) Biochem. Pharm. 32, 19: 2891-2902.

168. KURANSTIN-MILLS, J., SAMJI, N., MOSCARELLO, M.A. and BOGGS, J.M. (1982) Neurochem. Res. 7, 12: 1523-1540.

169. CASPARY, E.A., SEWELL, F. and FIELD, E.J. (1967) Brit. Med. J., June 3rd, 610-611.

170. STASIW, D.M., ROSATO, S., MAZZA, J. and CERNY, L.C. (1977) J. Lab. Clin. Med. 89, 2: 409-413.

171. SCHAUF, C.F., FRISCHER, H. and DAVIS, F.A. (1980) Neurology 30: 323-325.

172. PRINEAS, J. (1968) Acta Neurol. Scand. 44: 81-90.

173. FIELD, E.J. (1976) Lancet, 2: 367-368.

174. MERTIN, J. and MEADE, C.J. (1977) Brit. Med. Bull. 33, 1: 67-71.

175. JENSEN, G.E. and CLAUSEN, J. (1984) J. Neurol. Sci. 63: 45-53.

176. SHUKLA, V.K.S., JENSEN, G.E. and CLAUSEN, J. (1977) Acta Neurol. Scand. 56: 542-550.

177. SZEINBERG, A., GOLAN, R., EZZER, J.B., PINHAS, I.S., SADEH, M. and BRAHAM, J. (1979) Acta Neurol. Scand. 60, 5: 265-271.

178. POLIDORO, G., ILIO, C.D., ARDUINI, A., ROVERE, G. and FEDERICI, G. (1984) Int. J. Biochem. 16, 5: 505-509.

179. GUL, S., SMITH, A.D., THOMPSON, R.H.S., WRIGHT, H.P. and ZILKHA, K.J. (1970) J. Neurol. Neurosurg. Psychiat. 33: 506-510.

180. THOMPSON, R.H.S. (1975) "Multiple Sclerosis Research". Ed: A. Davison, J. Humphrey, A. Liversedge, W.McDonald and J. Porterfield, pp. 184-197.

181. CHERAYIL, G.D. (1984) J. Neurol. Sci. 63: 1-10.

182. WIKSTROM, J., WESTERMARCK, T. and PALO, J. (1976) Acta Neurol. Scand. 54: 287-290.

183. ROGONIVA, N.I. and KOKLOV, A.P. (1980) Zh. Neuropatol. Paikhiatr. Im. S.S. Korasakova 80: 696-700.

184. HUNTER, M.I.S., NLEMADIN, B.C. and DAVIDSON, D.L.W. (1985) Neurochem. Res. 10, 12: 1645-1652.

185. MICHELL, B. (1983) Trends Biochem. Sci. 8, 4: 117-118.

186. BLIGH, E.G. and DYER, W.J. (1959) Can. J. Biochem. Physiol. 37, 8: 911-817.

187. LEVIS, G.M., KARLI, J.N. and MOULOPOULOS, S.D. (1979) Lipids 14, 1: 9-14.

188. RAUSER, G., FLEISCHER, S. and YAMOTO, A. (1969) Lipids 5: 494-496.

189. VAN KESSEL, W.S.M.G., HAX, W.M.A., DEMEL, R.A. and GIER, J.D. (1977) Biochim. Biophys. Acta 486: 524-530.

190. PATTON, G., FASULO, D. and ROBINS, S. (1982) J. Lipid Res. 23.

191. SLATER, T.F. and SAWYER, B. (1971) Biochemistry 123: 26.

192. LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. and RANDALL, R.J. (1951) J. Biol. Chem. 193: 265-275.

193. STOCKS, J., GUTTERIDGE, J.M.C., SHARP, R.J. and DORMANDY, T.L. (1974) Clin. Sci. Mol. Med. 47: 215-222.

194. SATOH, K. (1978) Clin. Chim. Acta 90: 37-43.

195. DACIE, J.V. and LEWIS, S.M. (1975) "Practical Haematology". Pub: Longman Group Limited, Edinburgh, Ed.: J.V. Dacie and S.M. Lewis, pp. 21-67.

196. KEMP, G. (Personal Communication).

197. SCOTT, B.J. (1967) Biochim. Biophys. Acta 104: 1040.

198. ORAGUI, E. (Personal Communication).

199. WHITE, A., HANDLER, P., SMITH, E.L., HILL, R.L. and LEHMAN, I.R. "Principles of Biochemistry". Pub: McGraw-Hill Kogakusha Limited. Ed.: White, A., Handler, P., Smith, E.L., Hill, R.L. and Lehman, I.R., pp. 1045.

200. FARMER, S. (Personal Communication).

201. BRAUGHLER, J.M., DUNCAN, L.A. and CHASE, R.L. (1986) J. Biol. Chem. 261, 22: 10282-10289.

202. VAN DER ZEE, J., DUBBELMAN, T.M.A.R. and VAN STEVENINCK, J. (1985) Biochim. Biophys. Acta 818: 38-44.

203. HUNTER, M.I.S. (Personal Communication).