The Effect of Pathological Concentrations of Phytanic Acid on the Fatty Acid Composition, Vitamin E Concentration and Function of Membranes of Cultured Mammalian Retinal Cells

by

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ABSTRACT

The purpose of this study was to investigate possible mechanisms for the retinal degeneration seen in adult Refsum disease. This is an inherited disorder in which phytanic acid, a dietary branched chain fatty acid, cannot be catabolised and as a result it accumulates in tissues and serum. Phytanic acid has the same structure as the side chain of the tocopherols (vitamin E), and some of the signs and symptoms seen in patients with severe and chronic vitamin E deficiency are similar to those seen in adult Refsum disease. For these reasons it has been suggested that an accumulation of phytanic acid in membranes may interfere with vitamin E function. Alternatively phytanic acid may exert its pathological effect through alteration of membrane composition and structure, thereby affecting membrane functions. These hypotheses were investigated by studying the effect of modulating phytanic acid and α-tocopherol concentrations on the lipid composition and certain functional parameters of retinal cell lines in culture. Methods were established and validated for (i) the supplementation of the cell lines with reproducible concentrations of phytanic acid and α-tocopherol, (ii) the quantification of various membrane components of interest, (iii) the measurement of membrane fluidity and the membrane transport of choline, and (iv) the determination of the susceptibility of altered membranes to in vitro lipid peroxidation. Results showed a) the phospholipid fraction of retinal cells readily incorporated phytanic acid resulting in an altered membrane fatty acid composition b) there was no competition in uptake between phytanic acid and α-tocopherol, c) the incorporation of phytanic acid increased membrane fluidity and the transport of choline, and d) the incorporation of phytanic acid did not appear to affect the susceptibility of membranes to in vitro lipid peroxidation. Taken together these results suggested that phytanic acid did not interfere with the incorporation and function of α-tocopherol in retinal membranes but directly affected retinal membrane composition, structure and function.
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<th>Description</th>
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<tbody>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>ARD</td>
<td>Adult Refsum disease</td>
</tr>
<tr>
<td>BHT</td>
<td>Butylated hydroxytoluene</td>
</tr>
<tr>
<td>CM</td>
<td>Chylomicrons</td>
</tr>
<tr>
<td>c.v.</td>
<td>Coefficient of variation</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>DPH</td>
<td>Diphenylhexatriene</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential scanning calorimetry</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
</tr>
<tr>
<td>ES</td>
<td>External standard</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>faf-BSA</td>
<td>Fatty acid free-bovine serum albumin</td>
</tr>
<tr>
<td>FAME</td>
<td>Fatty acid methyl ester</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FID</td>
<td>Flame ionisation detection</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>HDL</td>
<td>High density lipoproteins</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>IDL</td>
<td>Intermediate density lipoproteins</td>
</tr>
<tr>
<td>IS</td>
<td>Internal standard</td>
</tr>
<tr>
<td>LDL</td>
<td>Low density lipoproteins</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PVC</td>
<td>Packed cell volume</td>
</tr>
<tr>
<td>PTS</td>
<td>Peroxisomal targeting sequences</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
</tr>
<tr>
<td>rbc</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RCDP</td>
<td>Rhizomelic chondrodysplasia punctata</td>
</tr>
<tr>
<td>RPE</td>
<td>Retinal pigment epithelial</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VLCFA</td>
<td>Very long chain fatty acids</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very low density lipoproteins</td>
</tr>
</tbody>
</table>
1. INTRODUCTION

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1.1 Introduction

Classical or adult Refsum disease (ARD) is an autosomal recessive metabolic disorder in which degenerative changes in the retina, and central and peripheral nervous systems are the major features. The disease is caused by an isolated deficiency of the enzyme, phytanic acid α-hydroxylase, which catalyses the first step in the degradative pathway of the exogenous fatty acid, phytanic acid. ARD has been classified as a peroxisomal disorder as the pathway of phytanic acid degradation is generally believed to occur in the peroxisome. The resulting accumulation of phytanic acid in the serum and tissues of adult Refsum patients is thought to be responsible for the clinical features of this condition. However, the underlying mechanism(s) as to how this occurs has not been elucidated. Several hypotheses have been proposed, but relatively little progress towards our understanding of the mechanism(s) of this disease has been made. The following study was designed to investigate the pathogenesis of the retinal abnormalities in ARD. Cultured mammalian retinal cell lines supplemented with phytanic acid in the medium were used as an in vitro model for ARD. The effect of modulating phytanic acid concentrations on membrane fatty acid composition and function were studied.

In this introduction I will briefly consider peroxisomal disorders including ARD, phytanic acid metabolism and possible mechanisms for the pathogenesis of the retinal abnormalities in ARD.

1.2 Peroxisomes

Peroxisomes are subcellular organelles that perform a variety of metabolic functions and were first characterised by De Duve and Baudhuin in 1966. These organelles are thought to be ubiquitous amongst cell types except the mature erythrocyte and are particularly abundant in the liver and kidney. They are bounded by a single membrane and vary in morphology, size and abundance. Peroxisomes are usually spherical but may in some cell types be interconnected forming an intracellular compartment referred to as a peroxisomal reticulum. They are formed by division of pre-existing peroxisomes and have a half life of about 1.5 to 2 days. Peroxisomes were so named due to the peroxide based respiration that occurs within them. A number of compounds such as D- and L-
amino acids, oxalate and polyamines are oxidised by peroxisomal enzymes with the
concomitant production of hydrogen peroxide. Catalase then converts hydrogen
peroxide to water and molecular oxygen or the hydrogen peroxide is used to peroxidise
substrates including ethanol, methanol, nitrites, quinone, and formate.

Both anabolic and catabolic functions are carried out by peroxisomes (Lazarow and
Moser, 1995). For example, peroxisomes are involved in the biosynthesis of the
plasmalogens (phospholipids which have a 1,2 unsaturated alcohol in ether linkage at
the Sn1 position on the glycerol backbone), cholesterol (in addition to the cholesterol
biosynthesis that occurs in the endoplasmic reticulum) and bile acids. They also
contribute to the gluconeogenic pathway by serving as the site of deamination of some
amino acids. Peroxisomes possess a β-oxidation system which shortens the cholesterol
side chain during the biosynthesis of bile acids and is thought to be involved in the
biosynthesis of the polyunsaturated fatty acids, docosahexaenoic acid (22:6n3) and
docosapentaenoic acid (22:5n6) (Sprecher et al., 1995). Peroxisomal β-oxidation also
contributes to the degradation of fatty acids and eicosanoids, overlapping in its
specificity for fatty acids with the mitochondrial β-oxidation system. However very
long chain fatty acids (VLCFA) (> 22 carbons in length) and long-chain dicarboxylic
acids are preferentially catabolised by the peroxisomal system. There is also evidence
that α-substituted branched chain fatty acids such as pristanic acid are degraded
principally by this β-oxidation system (ten Brink et al., 1992a; Singh et al., 1992). The
peroxisome is most probably the site of α-oxidation of the β-substituted fatty acid,
phytanic acid. The exact location of the α-oxidation pathway has been a matter of some
debate and is discussed in more detail in section 1.5.3.

1.3 Peroxisomal Disorders
The important metabolic role played by peroxisomes is evident from the devastating
effects that occur when one or more peroxisomal functions are impaired. The
peroxisomal disorders are a heterogeneous group of inherited disorders and are
classically divided into three categories (see table 1.1). For a review see Lazarow and
Moser (1995). The first group comprises the disorders of peroxisomal biogenesis. The
failure to form peroxisomes results in a multiple deficiency of peroxisomal functions.
### Classification of Peroxisomal Disorders

<table>
<thead>
<tr>
<th>Type 1</th>
<th>Disorders of peroxisomal biogenesis, characterised by a generalised deficiency of peroxisomal functions and an apparent absence of peroxisomes.</th>
<th>Diseases</th>
<th>Biochemical Abnormalities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zellweger syndrome</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Infantile Refsum disease</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Neonatal adrenoleukodystrophy</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hyperpipecolic acidaemia</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Raised tissue and plasma concentrations of very long chain fatty acids, phytanic acid and pipecolic acid. Bile acid intermediates can be detected in plasma and urine. Deficiency of plasmalogens.</td>
<td></td>
<td></td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Type 2</th>
<th>Peroxisomes are present but a subset of peroxisomal functions are deficient.</th>
<th>Diseases</th>
<th>Biochemical Abnormalities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rhizomelic chondrodysplasia punctata</td>
<td></td>
<td>Deficiency of plasmalogens and raised phytanic acid concentration. 3-oxoacyl CoA thiolase is present in the unprocessed form but (\beta)-oxidation is normal.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Type 3</th>
<th>Isolated peroxisomal enzyme deficiencies when intact peroxisomes are present.</th>
<th>Diseases</th>
<th>Biochemical Abnormalities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acatalassaemia</td>
<td></td>
<td>A variety of isolated biochemical abnormalities including defective peroxisomal (\beta)-oxidation of VLCFA and bile acids, (\alpha)-oxidation of phytanic acid and deficiency of catalase.</td>
</tr>
</tbody>
</table>

|        | X-linked adrenoleukodystrophy                                               |          |                            |
|        | Adult Refsum disease                                                        |          |                            |
|        | Pseudo-Zellweger syndrome                                                   |          |                            |
|        | Hyperoxaluria type 1.                                                        |          |                            |

### Table 1-1: Summary of the peroxisomal disorders.
Included in this group are Zellweger syndrome, infantile Refsum disease, neonatal adrenoleukodystrophy and hyperpipecolic acidaemia. Zellweger syndrome represents the most severe form of peroxisomal disorders and patients with this disease usually die within the first year of life. Symptoms of this syndrome include severe hypotonia, severe retardation, characteristic dysmorphic features, neonatal seizures, hepatomegaly, renal cysts and visual abnormalities including retinitis pigmentosa-like symptoms. Biochemical abnormalities include an accumulation of VLCFA, intermediates of bile acid biosynthesis, phytic acid and pipecolic acid, the latter being an intermediate in lysine catabolism. Plasmalogens are deficient and there is a mislocalisation of catalase to the cytoplasm. The spectrum of symptoms and biochemical defects is similar in the other diseases within this group, although they tend to be milder.

In recent years insights into the mechanisms involved in the disorders of peroxisomal biogenesis have been made (reviewed in Subramani, 1997). The demonstration of peroxisomal membrane "ghosts" in cells from patients suggested that there may be a defect in the targeting of peroxisomal proteins, all of which are encoded by the nucleus, to the peroxisomal matrix. Peroxisomal proteins are imported into the peroxisome post-translationally and are targeted by integral signals within the protein structure that are not cleaved after translocation. Two peroxisomal targeting sequences (PTS) that are responsible for directing proteins into the peroxisomal matrix have been identified and both are highly conserved (Subramani, 1997). PTS1 is the tripeptide serine-lysine-leucine and is found at the C-terminal of proteins destined for the peroxisome. The majority of peroxisomal matrix proteins carry this signal (or variants of this). PTS2, the polypeptide (arginine/lysine)-(leucine/valine/isoleucine)-X5-(histidine/glutamine)-(leucine/alanine) is found at the N-terminal end of a small number of peroxisomal matrix proteins. It seems that the import of PTS1 carrying proteins alone or in combination with PTS2 proteins are affected in type 1 peroxisomal disorders. The genes involved are those that encode the peroxisomal membrane receptors for these signals.

Defective import of PTS2 proteins alone occurs in rhizomelic chondrodysplasia punctata (RCDP). This disease constitutes the second class of peroxisomal disorders in which peroxisomes are present but a subset of peroxisomal functions are deficient,
including plasmalogen biosynthesis and phytic acid degradation. RCDP is phenotypically different from the type one disorders most noticeably in the shortening of proximal limbs.

The third type of peroxisomal disorders are those caused by the deficiency of a single peroxisomal enzyme. Acyl CoA oxidase, bifunctional enzyme and 3-oxoacyl CoA thiolase are enzymes in the peroxisomal β-oxidation pathway. Isolated deficiency of any of these enzymes has been found to lead to disorders that are clinically similar to Zellweger syndrome and have thus been previously designated as “pseudo-Zellweger syndrome”. Other diseases assigned to this category are acatalassaemia, X-linked adrenoleukodystrophy, hyperoxaluria type 1 and most probably ARD (see below).

1.4 Adult Refsum Disease (ARD)

1.4.1 Clinical Findings
Adult or classical Refsum disease, originally termed heredopathia atactica polyneuritiformis, is a rare autosomal recessive metabolic disorder first identified by Sigvald Refsum in 1946 and has been reviewed by Steinberg (1995). The metabolic defect is an isolated deficiency of phytanic acid α-hydroxylase activity (ten Brink et al., 1992b; Pahan et al., 1996) (see 1.5.2). As a consequence of this enzymatic deficiency there is a gradual accumulation of phytanic acid in the tissues and blood of patients, leading to the onset of symptoms and a progressive deterioration of their condition. The age of onset is variable and may occur in childhood or much later in the fourth or fifth decades of life. Night blindness is often the first manifestation of the disease which goes on to develop into retinitis pigmentosa-like symptoms (see section 1.7.2). Retinitis pigmentosa along with peripheral neuropathy, cerebellar ataxia and an elevated cerebrospinal fluid protein concentration in the absence of pleocytosis (abnormal cell count), are the defining clinical features of this disease. The neuropathy is usually symmetrical with distal lower limb regions being mainly affected. Muscles of the lower limbs become weak and atrophied. Motor and sensory nerve conduction velocities are reduced and evidence of denervation has been shown by electromyography. Other features of the peripheral neuropathy include a reduction or loss of vibration sense, proprioception (position sense) and of reflexes, initially starting with the ankle reflex
Refsum et al., 1984). The pigmentary retinopathy is atypical with the pigmentation having a 'salt and pepper' appearance rather than the more usual 'bony spicule' appearance seen in other forms of retinitis pigmentosa (Refsum et al., 1984). Pigmentation usually occurs in the periphery of the retina. Other ophthalmologic changes have also been observed such as optic atrophy resulting in blindness, cataracts and vitreous opacities. Electoretinographic findings have demonstrated a loss of rod and cone responses (Refsum et al., 1984).

The pathological abnormalities in the eyes of a patient with ARD have been described (Toussaint and Danis, 1971). An accumulation of lipid was noted in the sclera, trabecular meshwork, iris muscles and in the RPE. There was an absence of rods and cones, the outer nuclear and plexiform layers were atrophied, the inner nuclear layer was thinned and the ganglion cells were reduced in number. Retinal vessels were narrowed and occluded. In many areas of the posterior pole, the RPE was absent. The optic nerve showed mild demyelination.

A range of other symptoms may also occur in ARD including anosmia (loss of the sense of smell), an ichthyosis-like dry scaling skin condition, nerve deafness, pupillary abnormalities, nystagmus (a rapid involuntary oscillation of the eyeballs) and electrocardiographic abnormalities. Skeletal abnormalities are also observed and include a shortening of the fourth metatarsal, syndactyly (a union of two or more digits), hammer toes, pes cavus (an exaggeration of the normal arch of the foot), and osteochondritis dissecans (displaced fragment of cartilage and bone in a joint cavity, usually in the knee). The range of symptoms seen at the time of diagnosis of the disease is variable and children often present with an incomplete syndrome (Steinberg, 1993). The clinical course can be that of a gradual or a rapid progressive deterioration of the patient's condition. Sudden death has occurred in a number of cases, thought to have been caused by cardiac complications or respiratory failure, although renal failure was reported in one patient (Refsum et al., 1984). A marked worsening of the condition of some patients has been known to occur under conditions of stress such as during a serious illness, pregnancy, following rapid weight loss or after surgery. Mobilisation of fat stores as a result of stress probably increases the plasma phytanic acid concentration.
and causes the clinical deterioration. Very high concentrations of phytanic acid in the serum (>100 mg/dl compared to normal concentrations of < 0.5 mg/dl) can result in toxic symptoms including weight loss, failure to thrive, and fatigue in both humans and experimental animals (Refsum et al., 1984). Serum concentrations of phytanic acid of 200-250 mg/dl have been observed to have fatal consequences (Refsum et al., 1984). Periods of exacerbation are usually followed by remission where the progress of the disease appears to be interrupted for some time (Refsum et al., 1984; Steinberg, 1995).

1.4.2 Neuropathology in Adult Refsum Disease

The neuropathy seen in ARD is characterised by a chronic distal symmetrical sensorimotor neuropathy. Histopathological and electron microscopic studies have revealed interstitial hypertrophic changes in the peripheral nerves of Refsum patients (Refsum et al., 1984). The nerve hypertrophy is maximal proximally and most evident in the limb girdle plexuses. Spinal nerve roots and sensory ganglia may also be affected (Thomas et al., 1997). Nerve hypertrophy is caused by repeated demyelination and remyelination of nerve axons. A proliferation of Schwann cells occurs in response to demyelination and those cells not involved in remyelinating the axon form a concentric arrangement around the nerve fibre. Such 'onion bulb' formations in Refsum disease have been noted to contain many unmyelinated axons and collagen bundles are densely packed both inside and around the whorls (Refsum et al., 1984). A reduction in the number of myelinated nerve fibres and a segmental demyelination of teased-fibre preparations are also found. These changes are suggestive of a primary defect in Schwann cell function. Schwann cells have been found to contain osmiophilic bodies that are probably lipid granules and these are sometimes associated with typical lipofuscin granules (Refsum et al., 1984). Fat deposits in the central nervous system, the choroid plexuses and in the soft membranes enveloping the brain and spinal cord (leptomeninges) have also been found. Large crystalline inclusions have been observed in Schwann cell cytoplasmic processes with a structure suggestive of an intramitochondrial origin (Refsum et al., 1984). Axonal and hepatic liver mitochondrial abnormalities have been reported (Refsum et al., 1984).

Kuntzer et al (1993) performed a longitudinal study of nerve conduction with quantitative electromyographical analysis on a Refsum patient over a 21 year period.
Motor and sensory conduction velocities were reduced in a non-uniform manner which supports the histopathological evidence of a sensorimotor demyelinating neuropathy with segmental demyelination.

1.4.3 Diagnosis of Adult Refsum Disease

Confirmation of the diagnosis of ARD may be made by measuring the concentration of phytanic acid in plasma, which in normal subjects is usually below 0.5 mg/dl (16 µmol/l) but can be elevated to 5-100 mg/dl (160-3200 µmol/l) in untreated patients. In one of the original studies, phytanic acid accounted for 5-30 % of the total serum lipid fatty acids in nine patients (Refsum et al., 1984). In addition to measuring the plasma phytanic acid concentration, the phytanic acid oxidative capacity of cultured skin fibroblasts may be measured. Fibroblasts from patients with ARD oxidise phytanic acid at about 5 % the rate of fibroblasts from unaffected individuals and heterozygotic fibroblasts oxidise at approximately 50 % the normal rate (Steinberg, 1995). Heterozygotes may sometimes show a slight accumulation of phytanic acid in their serum, but remain asymptomatic (Steinberg, 1995).

1.4.4 Occurrence and Genetics of Adult Refsum Disease

The occurrence of ARD is very rare; Steinberg has estimated that the number of confirmed cases in the literature is about 150 (Steinberg, 1995). Most cases have been reported from Northern Europe. Very recently the gene encoding an enzyme with phytanoyl-CoA α-hydroxylase activity was identified (Jansen et al., 1997; Mihalik et al., 1997). In the ARD patients which have been studied, a number of inactivating mutations in this gene have been found which confirms the biochemical evidence for a deficiency of phytanoyl-CoA α-hydroxylase activity as the metabolic defect in ARD (Jansen et al., 1997; Mihalik et al., 1997). This gene has been localised to chromosome 10p (Mihalik et al., 1997) which is the same region where the gene responsible for an atypical form of the disease, in which phytanic acid accumulation occurs alongside an accumulation of pipecolic acid, is found (Nadal et al., 1995).
1.4.5 Treatment

ARD can be treated by dietary management. Phytanic acid is believed to be entirely of exogenous origin as studies investigating whether endogenous synthesis takes place have yielded negative results (Steinberg, 1995). Thus by limiting the intake of phytanic acid and its precursor phytol, phytanic acid accumulation can be halted. There is evidence that patients either retain some residual phytanic acid oxidation activity or are able to excrete the unchanged acid. There is often a delay in the response to treatment, probably due to mobilisation of phytanic acid from fat stores, but eventually a much reduced plasma concentration can be achieved. This may be aided in the initial stages of the treatment by plasmapheresis. Circulating concentrations very rarely return to normal, but remain slightly elevated at levels of between 10-30 mg/dl (Steinberg, 1995). Treatment in this way halts the progression of the disease and appears to prevent relapses. An improvement in many of the symptoms such as the peripheral neuropathy, ichthyosis, muscle strength and gait is seen (Refsum et al., 1984; Steinberg, 1995), whereas retinal and auditory function, while stabilised do not appear to regress (Steinberg, 1995).

1.5 Phytanic Acid

1.5.1 Dietary Origins Of Phytanic Acid

Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) is a saturated, multi-branched fatty acid composed of four isoprenoid units (figure 1-1). It is derived from the phytol side chain of chlorophyll and is present in our diet in a number of food sources (see below). Degradation of chlorophyll releases phytol which, as the free alcohol, can be converted to phytanic acid (Hansen, 1980). This process can occur in animals including man after free phytol has been absorbed from the diet (Steinberg, 1995). The enzymatic conversion of phytol to phytanic acid has been demonstrated in rat liver mitochondrial and microsomal fractions (Muralidharan and Muralidharan, 1986). The conversion of free phytol to phytanic acid is also thought to occur in the intestinal tract of ruminants through microbial action (Hansen, 1980). This results in an increase in the amount of preformed phytanic acid absorbed by these animals. Hence the major sources of preformed phytanic acid in the human diet are foods derived from ruminants such as fat,
Figure 1-1: Structure of an isoprene unit and some examples of isoprene compounds derived from this unit.
meat, and dairy products. The plasma concentration of phytanic acid in cattle is also influenced by their method of feeding. Cattle fed grass-silage have a higher plasma phytanic acid concentration than pasture fed animals. Hansen (1980) suggested this could be due to an increase in the amount of liberated phytol as a result of the mildly acidic environment of silage. In non-ruminant animals, intestinal conversion of phytol to phytanic acid is thought to be of little significance. Phytanic acid and phytenic acid, an intermediate in the conversion of phytol to phytanic acid, have been shown to accumulate in the liver of germ free rats fed phytol (Steinberg et al., 1966). Intake of preformed phytanic acid in man has been estimated to be between 50 to 100 mg/day. The amount of total phytol (esterified to chlorophyll or as the free alcohol) in the diet represents only 10% of the amount of preformed phytanic acid. In its esterified form phytol is poorly absorbed and is lost in the faeces, but both free phytol and phytanic acid are efficiently absorbed (Hansen, 1980; Steinberg, 1995).

1.5.2 Metabolism of Phytanic Acid - The α-Oxidation Pathway

Normally only trace amounts of phytanic acid are detected in the plasma of humans, indicating that it is efficiently catabolised. Phytanic acid has a methyl group in the β position which prevents it from being degraded by the β-oxidation pathways in mitochondria and peroxisomes. Instead it undergoes an initial α-oxidation step, resulting in the removal of a 1-carbon unit and the formation of pristanic acid (Steinberg, 1995). Pristanic acid is then further degraded by cycles of β-oxidation. The exact sequence of the α-oxidation pathway and its intracellular location have been subjects of much debate and controversy. A proposed scheme of the pathway based on the evidence to date and involving at least four steps is shown in figure 1-2.

1) Activation to Phytanoyl-CoA

Evidence to date suggests that the conversion of phytanic acid to its coenzyme A derivative is the initial step in the pathway. Phytanoyl-CoA ligase activity has been detected in peroxisomal, mitochondrial, and endoplasmic reticulum fractions of human and rat liver and skin fibroblasts (Pahan and Singh, 1993; Singh et al., 1994). In peroxisomes this activity was found to be distinct from lignoceroyl and palmitoyl CoA ligase activities (Pahan and Singh, 1993) and has been localised to the cytoplasmic side.
Figure 1-2: One possible pathway for the \( \alpha \)-oxidation of phytanic acid based on current evidence.
of the peroxisomal membrane (Pahan and Singh, 1995a). A requirement for the activation of phytanic acid to phytanoyl-CoA in the α-oxidation pathway has been implicated by the results from a number of studies (for example Muralidharan and Muralidharan, 1987; Singh et al., 1990; Watkins et al., 1994; Mihalik et al., 1995). Watkins and co-workers (1994) demonstrated using isolated rat liver peroxisomes that the release of [2,3-^H]phytanic acid required the presence of phytanoyl-CoA synthetase activity. Croes et al. (1996) demonstrated that the α-oxidation of 3-methyl-substituted fatty acids in permeabilised rat hepatocytes (where intracellular organelles and membranes remained intact) required the presence of CoA, ATP, and Mg^{2+} indicating the involvement of an acyl-CoA synthetase in this pathway. However there are conflicting reports concerning the subsequent fate of phytanoyl-CoA. Both phytanoyl-CoA and α-hydroxyphytanoyl-CoA (thought to be the next intermediate in the pathway) were detected by high performance liquid chromatographic (HPLC) analysis of the products formed following α-oxidation of phytanic acid in rat liver peroxisomes (Mihalik et al., 1995). Also 3-methylpalmitoyl-CoA and 2-hydroxy-3-methylpalmitoyl-CoA were identified as intermediates of the α-oxidation of 3-methylpalmitate (as a substitute substrate for phytanic acid) by permeabilised rat hepatocytes, rat liver homogenates and isolated peroxisomes (Croes et al., 1996). This suggests that phytanoyl-CoA is the substrate for the second step in the pathway (α-hydroxylation) which would result in the production of α-hydroxyphytanoyl-CoA. However Pahan and Singh (1993) have reported that it is the free acid and not phytanoyl-CoA that is the substrate for this second step. They showed using an isolated peroxisomal matrix or permeabilised peroxisomes that α-oxidation activity did not require cofactors for the activation of fatty acids. In addition activity was unaffected by an acyl-CoA ligase inhibitor, naproxen. They also showed that phytanoyl-CoA is quickly hydrolysed after transportation into the peroxisomal matrix.

2) α-Hydroxylation

The second step in the α-oxidation pathway, which is the step that is impaired in ARD, is the α-hydroxylation of phytanoyl-CoA to 2-hydroxyphytanoyl-CoA in the matrix of the peroxisome (Pahan and Singh, 1993). The evidence for this step comes from a number of sources. The presence of 2-hydroxyphytanic acid in the plasma of healthy
humans and in patients suffering from certain types of peroxisomal disorders in which defective phytanic acid degradation occurs has been demonstrated (ten Brink et al., 1992a). Formation of 2-hydroxyphytanic acid from phytanic acid has also been shown in vivo in human subjects. ten Brink et al (1992b) detected [1-13C]2-hydroxyphytanic acid in the plasma of control subjects administered a dose of [1-13C]phytanic. In the plasma of ARD patients taking part in the same study this metabolite was not detected. Muralidharan and Muralidharan (1987) have characterised the α-hydroxylation of [1-14C]phytanic acid in rat liver and showed there was a time dependent increase in the amount of [1-14C]2-hydroxyphytanic acid formed. Mihalik et al (1995) have demonstrated the enzyme that catalysed the α-hydroxylation of phytanic acid exhibits co-factor requirements (2-oxoglutarate, Fe2+ and ascorbate) which they reported as being consistent with a dioxygenase reaction mechanism. These co-factors were also shown to be required for the α-oxidation of 3-methyl-substituted fatty acids in rat liver homogenates (Croes et al., 1996). Pahan et al (1995b) showed that the α-hydroxylation of phytanic acid in peroxisomes isolated from human liver and fibroblasts and Hep G2 cells could be inhibited by imidazole antimycotics suggesting that this step is mediated by a cytochrome P-450 containing enzyme.

3) α-Oxidation
The next step in the α-oxidation of phytanic acid is an oxidative decarboxylation of 2-hydroxyphytanic acid resulting in the formation of pristanic acid and CO2. Evidence that this step involves more than one reaction was provided by Draye et al (1987) who demonstrated the production of 2-oxophytanic acid from L-2-hydroxyphytanic acid in rat kidney cortex. 2-hydroxyphytanic acid oxidase activity has also been detected in rat and human liver (Wanders et al., 1994), and an H2O2 producing enzyme with this activity has been localised to the peroxisome in human liver (Wanders et al., 1994; Wanders et al., 1995). The conversion of an α-hydroxyl group to an α-keto group is analogous to the mechanism of degradation of other α-hydroxyl acids (Wanders and van Roermund, 1993), for instance the conversion of lactate to pyruvate and acetyl-CoA. However ten Brink et al (1992a) failed to detect 2-oxophytanic acid in the plasma of healthy humans and patients with peroxisomal disorders, even in those conditions where 2-hydroxyphytanic acid and pristanic acid were both elevated (as occurs in the disorders
of peroxisomal biogenesis). It is possible that 2-oxophytanic acid is rapidly decarboxylated as it is formed, thus preventing its detection.

4) Decarboxylation

Carbon dioxide and pristanic acid are usually cited as the products of phytanic acid α-oxidation. However, a number of studies have provided evidence that the decarboxylation of 2-oxophytanic acid occurs via the formation of formic acid which is then converted to CO$_2$. Poulos et al. (1993) first identified [14C]formic acid as the major water-soluble product formed from [1-14C]phytanic acid and [1-14C]3-methylhexadecanoic acid degradation by human skin fibroblasts. The authors speculated that CO$_2$ was produced from formic acid and not vice versa, as formic acid production was in excess of CO$_2$ production. Mihalik et al. (1995) reported similar results in α-oxidation experiments with [1-14C]phytanic acid in rat liver peroxisomes. These findings are supported by the work of Croes and colleagues who demonstrated that formic acid was a primary product of α-oxidation in intact rat hepatocytes (Croes et al., 1996) and in peroxisomal enriched fractions from human liver (Casteels et al., 1997). Croes et al. (1997) have also reported that in addition to formate, α-oxidation of 3-methyl substituted acids results in the production of an aldehyde which can be converted to an acid. This suggests that pristanal would be the major product of α-oxidation of phytanic acid which would then be converted to pristanic acid.

In summary, phytanic acid is converted to pristanic acid by an α-oxidation pathway in the first stage of its degradation. Research into this pathway has revealed that at least 4 steps may be involved; activation of phytanic acid to phytanoyl-CoA (possibly followed by post-transport hydrolysis to the free acid), α-hydroxylation to the 2-hydroxy-derivative, oxidation to the 2-oxo-derivative, and decarboxylation to pristanoyl-CoA (or pristanic acid), possibly via the formation of pristanal, with the concomitant production of formate which is then converted to carbon dioxide.

1.5.3 Subcellular Localisation of Phytanic Acid α-Oxidation

Phytanic acid accumulation occurs in a number of the peroxisomal disorders (Lazarow and Moser, 1995) (see table 1.1) which suggests that phytanic acid degradation may be
located in the peroxisome. However, studies on the subcellular localization of the α-oxidation pathway have produced conflicting results. The presence of α-oxidation activity has been demonstrated in peroxisomes (for example Singh et al., 1993a; Singh et al., 1993b; Wanders et al., 1994; Croes et al., 1996; Casteels et al., 1997), in mitochondria (Tsai et al., 1969; Muralidharan and Kishimoto, 1984; Skjeldal and Stokke, 1987; Watkins and Mihalik, 1990; Wanders et al., 1991; Singh et al., 1993b; Wanders and van Roermund, 1993) and in the endoplasmic reticulum (Huang et al., 1992; Singh et al., 1993a). In type 1 peroxisomal disorders, mitochondria with abnormal structure and function have been observed in some cases, and thus a mitochondrial localization is feasible.

Most investigations have employed rat and human tissues, usually skin fibroblasts and liver, using methods that either measured individual steps or the entire α-oxidation pathway. For example, $^3$H release from [2,3-3H]phytanic acid was used to measure the α-hydroxylation step and this activity was found to be localized to peroxisomes isolated from rat liver (Watkins et al., 1994; Mihalik et al., 1995). The formation of 2-ketophytanic acid from 2-hydroxyphytanic acid has been used as a measure of 2-hydroxyphytanic acid oxidase activity. This was found to be associated with the distribution of the peroxisomal enzyme, catalase, in fractions of rat kidney cortex (Draye et al., 1987) and it was localized to peroxisomes in rat liver (Wanders et al., 1994). The release of $^{14}$CO$_2$ from [1-14C]phytanic acid is a measure of the entire α-oxidation pathway. For example, using this technique Singh et al. (1993b) demonstrated that in humans the subcellular location of this pathway was predominantly peroxisomal whereas in rats it was predominantly mitochondrial. When $^{14}$CO$_2$ release was measured in control human skin fibroblast fractions, activity was found in peroxisomal, mitochondrial and ER fractions, with predominant activity in the peroxisomal fraction (Singh et al., 1993a). In subcellular fractions obtained from fibroblasts from ARD patients, activities in the mitochondrial and ER fractions were comparable to controls, whereas peroxisomal activity was deficient (Singh et al., 1993a). The low levels of α-oxidation activity normally found in the endoplasmic reticulum and mitochondria may explain the low levels of residual activity in Refsum patients (Steinberg, 1995). However, the finding that formate is a primary product of α-oxidation which is then
converted to CO$_2$ (Poulos et al., 1993; Mihalik et al., 1995; Croes et al., 1996; Casteels et al., 1997) suggests that using $^{14}$CO$_2$ release from [1-$^{14}$C]phytanic acid as a measure of \( \alpha \)-oxidation activity may give misleading results. This is because formate may be degraded to CO$_2$ by at least two pathways; a folate dependent pathway in the cytosol and mitochondria, and a catalase dependent peroxidative pathway in peroxisomes (Mihalik et al., 1995) and this may explain why activity has been detected in different organelles. In addition, Casteels et al (1997) suggested that the low levels of activity reported in many studies could be due to the fact that CO$_2$ production is not a direct measure of \( \alpha \)-oxidation.

Other evidence for the subcellular localisation of the \( \alpha \)-oxidation pathway comes from the concentrations of intermediates of this pathway in the serum of patients with peroxisomal disorders. Serum concentrations of 2-hydroxyphytanic acid are raised in patients with disorders of peroxisomal biogenesis who suffer from global peroxisomal function deficiencies. This suggests that the conversion of 2-hydroxyphytanic acid to pristanic acid is a peroxisomal function (ten Brink et al., 1992a). It also suggests that \( \alpha \)-hydroxylation of phytanic acid could, however, occur at a site other than the peroxisome. Thus it has been suggested that the \( \alpha \)-oxidation pathway may be split between the mitochondria and the peroxisome rather than being exclusive to one organelle (ten Brink et al., 1992a). This concept is supported by the observation of Wanders and van Roermund (1993) that intact rat hepatocytes had higher \( \alpha \)-oxidation activity than whole homogenates and postnuclear supernatant.

The studies mentioned above are a few examples of the investigations on the subcellular location of \( \alpha \)-oxidation of phytanic acid that have been reported in the literature. Overall, it is difficult to draw a general consensus from these studies and a number of factors may have contributed to the differences in results. For instance, where enzyme markers were not used the purity of the cellular fractions are unknown (Tsai et al., 1969; Muralidharan and Kishimoto, 1984). The recoveries of activity after cell fractionation have not been reported in some studies (Tsai et al., 1969; Muralidharan and Kishimoto, 1984; Watkins and Mihalik, 1990). Nycodenz, a substance commonly used for the subcellular fractionation of peroxisomes has been shown to inhibited \( \alpha \)-oxidation
activity (Singh et al., 1993b), although activity can be recovered following its removal by dialysis. Therefore, in studies where Nycodenz had not been removed, peroxisomal activity may have been inhibited (Watkins and Mihalik, 1990; Wanders et al., 1991). The necessary co-factors for α-oxidation activity (as determined by Mihalik et al (1995) and Croes et al (1996)) were not included in many studies.

In summary, although the site of subcellular localisation of phytanic acid α-oxidation has not been unequivocally proven, it seems very likely that at least part of the pathway occurs in peroxisomes and that activity is not probably confined to a single organelle.

Two papers describing the identification of the gene for phytanoyl-CoA hydroxylase (Jansen et al., 1997; Mihalik et al., 1997) have recently been published. These two studies have shown that the protein encoded by this gene contains a type-2 peroxisomal targeting sequence in its N-terminus (see section 1.3) (Jansen et al., 1997; Mihalik et al., 1997) and that it interacts with a peroxisomal receptor for this sequence (Mihalik et al., 1997). Thus ARD can now be unequivocally classified as a type 3 peroxisomal disorder and the α-hydroxylation step of the α-oxidation pathway as a peroxisomal function.

1.6 Possible Mechanisms for the Pathogenesis in Adult Refsum Disease

It is evident from the clinical observations of ARD and results from animal studies that a build up of phytanic acid in tissues and serum has toxic consequences. In humans it has been demonstrated that phytanic acid accumulates in the peripheral and central nervous system (MacBrinn and O'Brien, 1968) and it could therefore be responsible for the observed neurological symptoms. However, the neurological symptoms have not been reproduced in animals fed large doses of phytol or phytanic acid. Steinberg et al. (1966) attempted to create an animal model of ARD using the mouse, rat, rabbit and chinchilla. In order to cause an accumulation of phytanic acid in tissues, it was necessary to feed the animals phytol or phytanic acid in amounts that exceeded the capacity of the animal to metabolise them. Between 2 - 5 % by weight in the diet of phytol or phytanic acid were administered over a period of up to 2 months. Excessive amounts of phytanic acid accumulated in the liver and serum and fatty deposits were observed in the liver. Growth of the animals was inhibited and at the higher dosages
caused death. The retina and peripheral and central nervous systems appeared to be unaffected. However due to the necessity of feeding large doses of phytol or phytanic acid, which proved to be toxic, only short term studies could be carried out. The amount of phytanic acid which accumulated in the lumbar plexes and sciatic nerves of rats in this study was 0.1 mg/g. This is much lower than that reported in the sciatic nerve of an ARD patient (approximately 8 mg/g). The polecat is an animal with a low α-oxidation capacity. It has been attempted to reproduce the neurological symptoms of ARD in this animal in a long term feeding experiment over two generations in an attempt to mimic the chronic nature of ARD (Refsum et al., 1984). Again large amounts of phytanic acid accumulated in peripheral tissues, but only trace amounts were detected in brain lipids and neurological lesions did not develop. The results from these studies suggest that phytanic acid accumulates in the nervous system of humans more easily than it does in other animals, but do not rule out the idea that phytanic acid could cause the neurological symptoms seen in ARD.

The biochemical mechanism(s) whereby phytanic acid accumulation leads to the spectrum of signs and symptoms observed in ARD has not been elucidated. A number of hypotheses have been suggested and are discussed by Steinberg (1993; 1995) and by Stokke and Eldjarn (in Refsum et al., 1984). A brief outline of the main hypotheses is given below.

1.6.1 The Antimetabolite Hypothesis

Phytanic acid is structurally similar to a number of molecules which, like phytanic acid, are derived from the isoprenoid unit, a five carbon structure. Examples of such molecules include the fat soluble vitamins E, K and A (see figure 1-1), ubiquinone, an electron carrier in the inner mitochondrial membrane, dolichol, a lipid found of the endoplasmic reticulum membrane involved in the glycosylation of proteins and the prenyl groups that serve as lipid anchors, positioning proteins in membranes (see section 1.6.1.2). It has been suggested that an accumulation of phytanic acid may interfere with the function of structurally similar molecules (Steinberg, 1995) and the strongest evidence for this is in the case of vitamin E.
1.6.1.1 *Interference with the Function of Vitamin E*

Vitamin E is closely related to phytanic acid in structure as it possesses a phytyl side chain which serves to anchor it in membranes. Vitamin E is considered to be the major lipid soluble chain-breaking antioxidant (Burton and Traber, 1990) and thus plays an important role in protecting cellular membranes from lipid peroxidation. A severe deficiency of this vitamin in humans occurs in a number of disorders involving fat absorption or transport (Harding, 1987; Sokol, 1993) and results in a spectrum of symptoms similar to those observed in ARD. This includes peripheral and central neuropathy, retinal degeneration and muscle weakness (Müller and Goss-Sampson, 1990). It is possible that an accumulation of phytanic acid in tissues and cellular membranes in some way perturbs the function of vitamin E and so induces a vitamin E deficiency-like state. This hypothesis is further discussed in chapter 6.

1.6.1.2 *Interference with Protein Prénylation*

An interference with the function of membrane proteins that are modified with farnesyl and geranylgeranyl (prenyl) groups has been suggested (Steinberg, 1995). An example of such a protein is ras, a GTP-binding protein that plays a central role in receptor tyrosine kinase signal transduction pathways which are stimulated by growth factors and some hormones such as insulin. Prenylation targets ras to the plasma membrane and without this association the protein does not function. High concentrations of phytanic acid may interfere with the prenylation of proteins or the placement of these proteins in membranes. In support of this hypothesis is the discovery that choroideremia, a form of retinal degeneration similar to that found in ARD, is caused by a defect in a gene encoding a protein with homology to the component A of rat geranylgeranyl transferase (Seabra et al., 1993). A deficiency of component A activity in lymphoblasts of patients with choroideremia has been demonstrated (Seabra et al., 1993).

1.6.1.3 *Interference with the Regeneration of 11-cis Retinol in the RPE*

An interference with the normal metabolism of retinol has been suggested as a possible mechanism for the retinitis pigmentosa seen in ARD (Refsum et al., 1984). Bernstein and co-workers investigated the ability of cell membranes from cultured foetal bovine retinal pigment epithelial cells treated with 200 μmol/l phytanic acid to esterify retinol and isomerise all-trans retinoids to 11-cis retinoids (Bernstein et al., 1992). These processes are essential in the regeneration of retinol, bleached in the process of
phototransduction, by the retinal pigment epithelial cells. No difference between phytanic acid treated cells and controls were observed.

1.6.2 The Molecular Distortion Hypothesis

The accumulation of phytanic acid in tissues in ARD is non-specific and widespread. Infiltration of cellular lipid pools by this exogenous fatty acid will result in its incorporation into membrane lipid structures at the expense of other fatty acids. Such an alteration of membrane structure will affect the physicochemical properties of those membranes and in turn may have an effect on membrane associated functions. Thus it is possible that a disturbance of membrane functions underlies the pathogenesis of the disease. It has been suggested that the demyelination that occurs in Refsum disease is a result of myelin destabilisation through incorporation of phytanic acid into its structure (Steinberg, 1995). See chapter 9 for further discussion.

1.6.3 Induced Essential Fatty Acid Deficiency

It has been suggested that phytanic acid accumulation in membranes may displace other fatty acids and thus induce an essential fatty acid deficiency state in Refsum patients. A deficiency of linoleic acid (18:2n6) in man is characterised by a number of symptoms including ichthyosis (Gurr and Harwood, 1991) which is also a symptom of Refsum disease. Low levels of this fatty acid in tissues of Refsum patients have been observed (refs. within Steinberg, 1995).

1.7 Retinal Degeneration in Adult Refsum Disease

The earliest clinical manifestation of Refsum disease is usually night blindness which suggests that the retina is more sensitive to phytanic acid accumulation than other tissues. It is possible that due to the high turnover of photoreceptor outer segment membranes (see section 1.7.1), phytanic acid is incorporated into these structures at a higher rate than into other membranes, which may explain the relative vulnerability of the retina. The vulnerability of the photoreceptors within the retina to alterations or deficits in structural components of photoreceptor membranes and in components of the visual transduction cascade is evident from the many different forms of photoreceptor degeneration which are seen in clinical practice and which are collectively known as
retinitis pigmentosa. During membrane turnover of the photoreceptors, components of these membranes are recycled within the retina. Disturbances in membrane turnover and recycling will therefore have a detrimental effect on photoreceptor function and may affect their viability. Phytanic acid accumulation in the retina may directly interfere with photoreceptor membrane function by displacing fatty acids from membrane lipids (molecular distortion hypothesis) or by indirectly altering membrane fatty acid composition by competing with vitamin E and rendering the photoreceptor membranes more susceptible to lipid peroxidation (antimetabolite hypothesis). It may also be interfering in some way with the retinal recycling of photoreceptor membrane components.

In view of the susceptibility of the retina to phytanic acid accumulation, this tissue was chosen to study the mechanism(s) of pathogenesis of ARD. In the following section an overview of retinal structure, the various forms of retinitis pigmentosa and what is known about the underlying causes of this group of retinal diseases is presented. The unusual fatty acid composition of the photoreceptor membranes and how alterations in composition may affect visual function is also described.

1.7.1 The Structure of the Retina

The retina is the thin layer of nervous tissue that lines the posterior half of the eye cup which is responsible for the conversion of light stimulus into nervous excitations (see figure 1-3a). Partial processing of the sensory information occurs in the retina before it is relayed to the visual cortex in the brain, via the optic nerve, resulting in the perception of vision. The retina is composed of a number of cell types and can be divided into several layers (see figure 1-3b). The outer most layer is the retinal pigment epithelium (RPE), a single layer of hexagonal cells containing melanin pigments. The RPE forms part of the blood-retinal barrier lying in close proximity to the choriocapillaries which provide the blood supply to the outer layers of the retina. The RPE performs a number of functions that are important for the maintenance of the health and normal functioning of the photoreceptors. This layer of cells mediates the passage of nutrients and metabolites between the choriocapillaries and the photoreceptors and also acts as a store and recycling centre for components of the photoreceptors. Projections from the apical
Figure 1-3: Diagramatic representation of:
a) the human eye, b) the human retina.
The photoreceptor cell layer lies anterior to the RPE and consists of rod and cone cells. In the peripheral areas of the retina, the rod cells predominate, whereas the cone cells are concentrated in the central region called the macula. The photoreceptors are specialised nerve cells with elongated structures extending out from the cell body towards the rear of the retina. The long axis of these cells is aligned in the same direction as that of light entering the retina. The photoreceptors are structurally divided into two parts, the inner and outer segments (see figure 1-4). The inner segment contains the cellular organelles including numerous and large mitochondria and is connected to the outer segment by a ciliary stalk. The outer segment contains a series of stacked flattened double membrane discs or sacs (between 500-2000 in rod cells) that are regularly aligned parallel to one another, enclosed within the plasma membrane. These disc structures house the photopigments. Rod cells contain the photopigment rhodopsin which is sensitive to dim light. In human cone cells, three opsins have been identified that are sensitive to light at wavelengths corresponding to blue, green and red light. Cone cells are responsible for the perception of colour and for central vision. As already mentioned there is a continual turnover of the outer segments which are shed at the tips and renewed from the base. New discs are formed by invagination of the photoreceptor plasma membrane at the junction of the inner and outer segments. The interior of the disks are initially open to the extracellular space. As the disks move away from the base of the outer segment towards the tip, the membrane pinches off to form an enclosed
Figure 1-4: Structure of a rod photoreceptor cell.
structure. The inner layers of the retina are composed of a network of nerve cells including the horizontal and bipolar cells which synapse with the photoreceptors. Amacrine, retinal interneurons and ganglion cells are other neurons within this network. This complex array of neurons partially processes the nervous impulses arising from the photoreceptors and passes the information onto the brain. The axons of the ganglion cells make up the inner most layer of the retina, the nerve fibre layer. These axons converge at the optic disc, the point from which they leave the retina and form the optic nerve. There is one type of glial cell in the retina known as Müller cells which are responsible for the maintenance of retinal neurons.

1.7.2 Retinitis Pigmentosa

Retinitis pigmentosa is the name given to a set of hereditary conditions where there is a progressive degeneration of the photoreceptor cells. The earlier clinical changes of this condition include impaired adaptation (the adjustment of the retina to varying degrees of illumination), night blindness and a narrowing of the visual field. As the disease progresses central vision is eventually lost. In later stages of the disease a bony-spicule pigmentation is observed on examination of the fundus (interior of the eyeball around the posterior pole) which is distributed circumferentially in the mid periphery where rod photoreceptors are concentrated. In addition, retinal vessels become attenuated and the optic disc appears pale (Berson, 1993). Cataracts, cystoid macular oedema (fluid retention in the macular region), and refractive errors including myopia and astigmatism may also occur (Berson, 1993). Retinitis pigmentosa can occur as part of the syndrome of a disease, as in the case of ARD, or it can be the sole manifestation of a genetic disease affecting some component of the retina. The different forms of isolated retinitis pigmentosa vary widely in their mode of inheritance and in the rate of photoreceptor loss.

1.7.2.1 Mutations of Genes Expressed in the Photoreceptor Cell in Retinitis Pigmentosa

A number of the genes responsible for the isolated forms of retinitis pigmentosa have been identified (see Dryja and Li, 1995, for a review). These include some that encode photoreceptor proteins involved in the phototransduction cascade including rhodopsin, the \( \alpha \) and \( \beta \) subunits of the cGMP phosphodiesterase, and the \( \alpha \) subunit of the cGMP-
gated channel. Disruption of the phosphodiesterase γ subunit gene in mice has also been shown to cause a retinal degeneration resembling retinitis pigmentosa (Tsang et al., 1996). Null mutations of the genes for rhodopsin, phosphodiesterase or the cGMP-gated channel demonstrate that the presence of functional forms of these proteins is necessary for photoreceptor viability (Dryja and Li, 1995). For dominant mutations of rhodopsin there is evidence suggesting an interference with rhodopsin processing or its delivery to the plasma membrane may be the cause of the photoreceptor degeneration (Dryja and Li, 1995; Lisman and Fain, 1995). Mutations of two genes encoding integral membrane proteins of the photoreceptor outer segment discs have been discovered; the retinal degeneration slow (RDS) gene encoding peripherin and the gene for the rod outer segment membrane protein 1 (ROM1). Peripherin is thought to have a structural role in the outer segment discs which is supported by the fact that mutations in this protein disrupts disc formation (Dryja and Li, 1995). Thus failure to produce outer segment discs appears to lead to photoreceptor degeneration. In the most common form of X-linked retinitis pigmentosa, RP3, the genetic defect has been localised to a gene which is expressed in the retina and RPE. This gene shows homology to a guanine nucleotide exchange factor (a GTPase regulator), and has in the C-terminal region a potential isoprenylation anchorage site (Meindl et al., 1996). It has been suggested that the protein may be involved in the regulation of membrane transport or trafficking. Usher’s syndrome is a form of retinitis pigmentosa that occurs in association with hearing loss. The gene responsible for the 1B form of this syndrome encodes a myosin protein, myosin VIIA (Weil et al., 1995). The function of this protein is not known but a functional role in the photoreceptor ciliary stalk or in the microvilli of the apical membranes of the RPE has been suggested (Weil et al., 1995).

The mechanisms of retinal degeneration in the different forms of retinitis pigmentosa are not well understood. One suggestion that has been proposed for some mutations in genes involved in phototransduction is the equivalent light hypothesis. This hypothesis suggests that some mutations result in a physiological state within the photoreceptor that is equivalent to that caused by continuous exposure of the retina to light (Lisman and Fain, 1995). The net result of the activation of rhodopsin by light is a closure of the cGMP-gated ion channels in the plasma membrane. The plasma membrane then
becomes hyperpolarised and this in turn inhibits glutamate release by the photoreceptor into the photoreceptor synapse. Some rhodopsin mutations have been shown to be constitutively active in vitro (Lisman and Fain, 1995) and null mutations of cGMP ion channels are likely to result in a continuous hyperpolarisation of the photoreceptor plasma membrane thereby producing an equivalent light effect. Exposure of the retina to continuous light for long periods of time results in a retinal degeneration similar to that occurring in retinitis pigmentosa. Lisman and Fain suggested (1995) that retrograde signals regulating photoreceptor growth and differentiation or circadian processes involved in outer segment membrane renewal could be disrupted by permanent activation of the phototransduction cascade.

There is evidence that photoreceptor degeneration as a result of some genetic defects in phototransduction proteins (Lolley et al., 1994; Steele, 1997) or in response to light exposure (Hafezi et al., 1997) occur through apoptotic mechanisms and not by passive cell disruption. Thus in mice carrying a mutation in the gene encoding the β-subunit of cGMP phosphodiesterase DNA fragmentation, which is characteristic of apoptosis, was observed in the retina. Chromosomal condensation in pycnotic nuclei and a rapid clearance of DNA indicating rapid removal of degenerative cells were other indications of apoptosis (Lolley et al., 1994). It has also been shown that light-induced apoptotic photoreceptor cell death could be prevented in mice lacking c-fos, a mediator of apoptosis (Hafezi et al., 1997).

The different forms of retinitis pigmentosa indicate that a disturbance in the process of phototransduction or the formation and renewal of photoreceptor structures can lead to retinal degeneration. It may be possible, in some cases, that this degeneration is caused by a disruption of circadian processes involved in the maintenance of photoreceptor structures and/or a triggering of apoptotic mechanisms leading to cell death. How this may relate to the retinitis pigmentosa observed in ARD is discussed in section 1.7.7.

1.7.3 Fatty Acid Composition of Rod Outer Segment Membranes

Rod outer segment membranes have an unusual fatty acid composition being highly enriched in polyunsaturated fatty acids (PUFAs), particularly docosahexaenoic acid (DHA) (22:6n3) (Daemen, 1973). The amount of DHA in highly purified bovine, frog,
and rat rod outer segment membranes was found to be between 40 - 50 mol % of total fatty acids (Stone et al., 1979). In the human retina, the peripheral regions have been shown to contain a higher amount of DHA than the macula, 22% compared to 16% of total fatty acids respectively (van Kuijk and Buck, 1992). Enrichment of DHA also occurs in nerve synaptic membranes (Cotman et al., 1969; Breckenridge et al., 1971; Sun and Sun, 1972). DHA is the most polyunsaturated fatty acid found in significant quantities in membranes. It is derived from the essential fatty acid, linolenic acid (18:3n3), by a series of elongation and desaturation reactions (see figure 8-4). Thus it may be expected that the level of DHA in the retina is influenced by the n-3 fatty acid content of the diet. It has been shown however that whereas many organs and tissues are depleted of DHA during n-3 fatty acid dietary deficiency, the retinal DHA concentrations are conserved for a longer period (Neuringer and Conner, 1986). For instance it has been shown that the level of DHA in the rods of albino rats fed on fat-free diets for 10-12 weeks did not change whereas in other tissues it was depleted (Anderson et al., 1974). The conservation of DHA by the retina suggests that this fatty acid is important for its normal functioning. Concentrations of DHA in the retina can be depleted but only after a prolonged n-3 deficiency. For example, in the albino rat it was possible to reduce the amount of DHA in rod outer segments after two generations had been raised on a fat-free diet (19.0 mol % in second generation fat deprived rats compared to 45.2 mol % in controls) (Anderson et al., 1974). As the degree of turnover of rod outer segment membranes is high, the retina must recycle DHA for it to be conserved during n-3 deficiency. A number of studies have provided evidence that the RPE is actively involved in the conservation of DHA, recycling DHA back to the photoreceptors from degraded outer segments (Chen and Anderson, 1993). A depletion of DHA from retinal tissue has a deleterious effect on visual function (see below).

1.7.4 The Effect of a Dietary Deficiency of n-3 Fatty Acids on Visual Function

In animals and humans a dietary deficiency of the n-3 fatty acids has been associated with visual abnormalities. The effect of n-3 fatty acid deficiency on visual function has been studied in primates (Connor et al., 1992). An n-3 deficiency state was achieved in rhesus monkeys by rearing two generations on an n-3-deficient diet. Plasma, red blood cell, brain and retinal DHA levels were all reduced compared to controls. A
compensatory increase in n-6 fatty acids, particularly the highly unsaturated 22:5n6 was observed. The n-6 family of fatty acids is a second group of PUFAs derived from an essential fatty acid, linoleic acid, 18:2n6. In this study visual function was assessed using visual acuity tests which are a measure of the integrity of the neural pathway between the retina and the occipital cortex. Rod and cone responses to a light stimulus were measured using electroretinography. In the n-3 deficient rhesus monkeys the visual acuity was reduced and the electroretinogram was abnormal. In addition polydipsia (chronic excessive thirst) was also exhibited by these animals. In human infants, it has been reported that n-3 fatty acid deficiency is associated with abnormal visual function and peripheral neuropathy (Holman et al., 1982). Feeding studies have demonstrated that new born infants who are not breast fed are at risk of n-3 deficiency if linolenic acid and its elongation and desaturation products are not supplied by milk formulas (Birch et al., 1992; Hoffman et al., 1993a; Uauy-Dagach et al., 1994). Preterm infants are more vulnerable having less opportunity to accumulate fat stores during the last trimester of pregnancy. Preterm infants fed a formula with a low n-3 content had lower red blood cell DHA concentrations and reduced rod responses compared to infants fed n-3 enriched formulas or those who were breast fed (Hoffman et al., 1993a). Visual acuity tests demonstrated poorer visual function in the n-3 deficient preterm infants (Hoffman et al., 1993a).

1.7.5 Deficiency of Docosahexaenoic Acid in Retinitis Pigmentosa

A deficiency of DHA, may be involved in some forms of retinitis pigmentosa. For example low levels of n-3 fatty acids in plasma and red blood cells have been associated with some forms of X-linked retinitis pigmentosa (Gong et al., 1992; Hoffman et al., 1995). In addition, the concentrations of n-3 and n-6 long chain PUFAs in the red cells of patients with autosomal dominant retinitis pigmentosa (ADRP) have been reported to be significantly lower than control levels (Hoffman et al., 1993). In these patients the plasma levels of n-6 PUFAs were significantly raised. There was also a trend towards an increase in plasma n-3 PUFAs compared to control concentrations, although this was not statistically significant. The authors suggested that in these patients the delivery of essential fatty acid elongation products, formed in the liver, from the plasma to tissues including red blood cells and the retina may be impaired. The fatty acid content of red
blood cells has been previously correlated to the fatty acid composition of retinal and neural tissues in rats and non-human primates (Carlson et al., 1986; Connor et al., 1993). Thus red cell essential fatty acid status has been used as an indicator of the level of these fatty acids in neural tissues. The results of the plasma and red blood cell fatty acid analysis in these sub-populations of patients with retinitis pigmentosa therefore suggest that a deficiency of DHA in the retina may result in retinal degeneration.

Further evidence comes from animal studies. It has been shown that n-3 and n-6 fatty acid deficiency in rats interferes with photoreceptor disc renewal (Anderson et al., 1974). In these rats rhodopsin was synthesised by photoreceptors but was not incorporated into new discs. This disturbance in outer segment disc renewal is similar to the effect of peripherin mutations on the retina. In the rd mouse model of retinal degeneration, DHA retinal levels are reduced compared to controls suggesting its metabolism is altered (Bazan et al., 1984a; Scott et al., 1988). Bush et al (1994) investigated the effect of n-3 fatty acid deficiency on rhodopsin content and function in rats. They showed that rhodopsin had a reduced capacity to absorb photons and this may explain the reduced sensitivity of photoreceptors to light observed in n-3 fatty acid deficiency. The reduced photon-catching ability of rhodopsin was attributed mainly to a slower rate of rhodopsin regeneration indicating some disturbance in the visual cycle.

Low levels of the essential fatty acids have also been implicated in other forms of retinal degeneration. Bazan et al (1986) measured the plasma concentration of DHA and arachidonic acid (AA) (20:4n6) in subjects with Usher’s syndrome from a large kindred in Canada. They found that DHA and AA concentrations were significantly reduced in the phospholipid fraction but were normal in the triglyceride fraction, suggesting that the metabolism of the essential fatty acids may be altered in this disease. In some peroxisomal disorders where retinitis pigmentosa is also a feature, including Zellweger syndrome and neonatal adrenoleukodystrophy, the levels of DHA in the brain, retina and other tissues are significantly reduced (Martinez, 1989; Martinez, 1990; Martinez, 1992). The biochemical pathway for the elongation and desaturation of 18:2n6 and 18:3n3 to 22:5n6 and 22:6n3, respectively, has recently been revised and is now believed to involve a β-oxidation step probably located in the peroxisome (Sprecher et
al., 1995) (see figure 8-4). Thus in the peroxisomal disorders, reduced levels of DHA could possibly be due to a deficiency of \( \beta \)-oxidation activity.

### 1.7.6 Function of Docosahexaenoic Acid in the Retina

The function of the high levels of docosahexaenoic acid found in rod outer segment membranes is not known. It is however likely to be related to the physical effect that such a high concentration of a PUFA would impose on the properties of those membranes. Brown (1994) proposed that high levels of DHA in rod outer segment membranes energetically favour the phototransitions of rhodopsin by increasing the curvature elastic stress at the lipid/water interface (see section 9.1.3).

A specific biochemical role for DHA in rod outer segment membranes was implicated in a recent report by Reinboth and co-workers (1996). They showed that DHA was released from phospholipids of isolated rat retina in response to light exposure and that this was partly mediated by phospholipase A\(_2\). It was postulated that the release of DHA from membrane phospholipids may influence the production of eicosanoids from AA. AA is the precursor of the 2-series of prostanoids (prostaglandins and thromboxanes) and the 4-series of leukotrienes. These products are local mediators that regulate a number of processes. For example, thromboxane A\(_2\) is a potent platelet aggregator and vasoconstrictor. Leukotriene B\(_4\) is an inducer of inflammation and a powerful inducer of leukocyte chemotaxis and adherence. Eicosanoids may also be produced from the n-3 fatty acids, eicosapentaenoic acid (EPA) (20:5n3) and DHA (through retroconversion to EPA). EPA is the precursor of the 3-series of prostanoids and the 5-series of leukotrienes and competes with AA for the cyclooxygenase and lipoxygenase activities that are responsible for the production of the prostanoids and leukotrienes. The EPA derived eicosanoids differ in their biological activity to those derived from AA. For instance, thromboxane A\(_3\) is a weak platelet aggregator and weak vasoconstrictor and prostacyclin PGI\(_3\) is a vasodilator and inhibitor of platelet aggregation. Leukotriene B\(_5\) is a weak inducer of inflammation and a weak chemotactic agent. An increase in competition between EPA and AA as the substrate for cyclooxygenase activity reduces the production of the more potent AA-derived eicosanoids and hence lessens the inflammatory response. Thus retroconversion of released DHA to EPA may modulate
the inflammatory response to light lesions within the retina. DHA itself has been shown to be a substrate for lipoxygenase activity in intact canine retinas (Bazan et al., 1984b).

Whatever the role of DHA in the rod outer segments, it is evident from animal and human studies that a deficiency of n-3 fatty acids leads to visual abnormalities. The compensatory increase in rod outer segment membranes of the n-6 fatty acid docosapentaenoic acid (22:5n6) during n-3 fatty acid deficiency does not prevent visual dysfunction (Connor et al., 1992). Docosapentaenoic acid is very similar in structure to DHA differing only in the number of double bonds. DHA has one extra double bond which is closer to the terminal carbon at the methyl end of the acyl chain and is therefore positioned in the innermost region of the membrane lipid bilayer. Hence the requirement for DHA in retinal membranes appears to be specific.

1.7.7 Phytanic Acid and Retinal Degeneration

The mechanism of retinal degeneration in ARD is not known. However considering what is known about other forms of retinal dysfunction and degeneration one can hypothesise as to how an accumulation of phytanic acid may exert a detrimental effect on the health of the retina.

PUFAs such as DHA are substrates for lipid peroxidation and as large amounts of these fatty acids occur in the retina this tissue is vulnerable to membrane damage from oxidative stress. Thus antioxidants such as vitamin E play an important protective role in the retina. An interference with the function of vitamin E, as proposed by the antimetabolite hypothesis, is therefore likely to manifest itself in the retina. An interference with its function might occur in the PUFA enriched photoreceptor outer segment membranes or in the RPE which recycles the components of the outer segments including DHA. The possible interference of vitamin E by phytanic acid is discussed in more detail in chapter 6.

The molecular distortion hypothesis proposes that membrane function could be adversely affected by the incorporation of significant amounts of phytanic acid into membrane structures. As discussed above (1.7.4 to 1.7.6) retinal function is sensitive to
a deficiency of DHA in its membranes. Displacement of DHA by phytanic acid may thus affect the function of photoreceptor outer segment membranes and in other forms of retinitis pigmentosa a disturbance of photoreceptor function leads to retinal degeneration. Although phytanic acid containing phospholipids have been shown in vitro to favour native photochemical behaviour of rhodopsin and to be able to replace DHA in this respect (Brown, 1994), (see 9.1.3), it has not been shown whether phytanic acid can be incorporated into outer segment membranes to produce stable and functioning membranes in vivo.

Phytanic acid taken up by the retina will first enter the RPE before reaching the photoreceptors. It is therefore possible that phytanic acid accumulation could disturb the functions carried out by the RPE. The Royal College of Surgeons strain of rats is an autosomal recessive animal model of retinal degeneration. The defect has been identified as a defective ingestion of outer segment debris by the RPE (Chaitin and Hall, 1983). Just as phytanic acid may perturb the function of photoreceptor membranes, incorporation into the RPE apical membranes may also have a detrimental effect. Studies on the metabolism of DHA by the RPE have shown that DHA is released from ingested outer segment lipids and incorporated into RPE lipids, primarily triglycerides (Chen and Anderson, 1993). It has been postulated that RPE triglycerides represent a temporary store of DHA due to be recycled back to the photoreceptors. Phytanic acid may thus displace DHA from triglycerides or prevent its incorporation into these lipids.

1.8 Plan of Investigation

The ideal approach to investigating the mechanism(s) of pathogenesis in ARD would be to create an animal model and study the effect of phytanic acid accumulation on various cellular functions. Unfortunately attempts to do this have failed to create a model where phytanic acid concentrations in neural tissue are sufficiently increased. An alternative approach is to use in vitro models based on cell culture systems. The advantage of using cell culture systems is that phytanic acid can be directly administered to the cell type of interest and thus the amount taken up by that cell can be controlled. The disadvantage is that this type of model is more removed from the disease process occurring in patients.
The aims of this project were to investigate the effect of pathological concentrations of phytanic acid on vitamin E status and function (antimetabolite hypothesis) and on the fatty acid profiles and membrane function (molecular distortion hypothesis) of retinal cell lines. Retinal pigment epithelial cells are amenable to cell culture and were therefore one choice for an in vitro model. A second cell line chosen for this project was a commercially available retinoblastoma cell line, Y79. This cell line is believed to be derived from neuroectodermal tissue in the retina and can be enriched with DHA by supplementation with 18:3n3 (see section 8.2). Thus these two cell lines were used to establish an in vitro model of ARD by supplementation of the culture medium with varying concentrations of phytanic acid. Once the cell culture models had been established and characterised, the effect of phytanic acid uptake by these cell types on the following parameters was investigated:

1) the fatty acid composition of the different lipid fractions in both cell lines,
2) the uptake of vitamin E by the RPE cells,
3) the susceptibility of n-3 and vitamin E enriched Y79 cells to lipid peroxidation,
4) the fluidity of membranes from both cell lines as an indicator of an alteration of a membrane physical property and
5) the uptake of $[^3H\text{-methyl}]$choline by Y79 cells, a membrane mediated function.
2. THE CULTURE OF RETINAL DERIVED CELL LINES

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2.1 Introduction

A primary objective of this project was to establish a suitable model with which to investigate possible mechanisms for the pathogenesis of ARD in the retina (see section 1.6). Previous attempts at establishing an in vivo model of the neurological abnormalities in ARD have been unsuccessful due to the resistance exhibited by various laboratory animals to the accumulation of phytanic acid in their nervous and retinal tissues (see section 1.6). An alternative approach is to study the effect of phytanic acid on individual cell types in culture. Two in vitro models of ARD using tissue and cell cultures have been previously reported. Dubois-Dalcq et al (1972) used rodent organotypic spinal ganglia cultures exposed to phytanic acid to model ARD in the nervous system. Bernstein et al (1992) used cultured human and bovine retinal pigment epithelial (RPE) cells treated with 200 µmol/l phytanic acid as an in vitro model of ARD in the retina.

The membrane lipid composition of cultured cells can be manipulated by the addition or omission of various lipids to the culture medium (Spector and Yorek, 1985). Phospholipid profiles, fatty acid profiles and cholesterol content can all be influenced in this way. By using this approach it is possible to study the effect of lipid modification on specific cell types. It allows a certain degree of influence over bilayer composition whilst being able to assess the effects of such manipulations on cellular processes. This contrasts to the use of a purely physical system such as liposomes (artificial phospholipid bilayers of specific composition), which are useful for providing information on the effect of lipids on specific physical properties of lipid bilayers and on other bilayer components, but do not provide information on cellular processes.

As discussed in Chapter 1 (see section 1.7), phytanic acid accumulation in the retina may have several effects on the functions and viability of the photoreceptors and/or the RPE. Thus these two cell types would be suitable candidates for an in vitro model of ARD in the retina. It is possible to culture cells from both the neural layers of the retina and from the pigmented epithelium. The majority of examples of photoreceptor and other retinal neuronal cultures have been derived from embryonic or immature retinal
tissue. Adult derived neuronal cultures depend upon the presence of feeder layers, or
certain explant conditions for survival (Hicks et al., 1994). RPE cells are more amenable
to cell culture than retinal neuronal cells and cultures have been established from a
variety of species including human, monkey, rat, bovine, frog and fish. Therefore this
cell type was chosen for the development of a cell culture model. Bovine RPE were
initially the cell of choice due to the availability of bovine eyes as a tissue source and
the relative ease with which RPE may be released from the bovine eyecup compared, for
example, to RPE from the rat. Bovine RPE cultures were used in the initial phytic
acid supplementation studies (see chapter 4). Subsequent to attempts at establishing
primary cultures of bovine RPE cells, an immortalised rat RPE cell line became
available (see 2.4) and this cell line was used for the majority of studies. It offered the
advantage of good growth characteristics and a continuous supply of cells, a large
number of which were required for this study. The RPE cell lines were used to
investigate both the antimetabolite and molecular distortion hypotheses by examining
phytanic acid and α-tocopherol uptake and undertaking membrane fluidity studies.

In addition to the use of RPE cell lines, a retinoblastoma cell line (Y79) was used to
study specific aspects of both hypotheses. This cell line is commercially available and
has the ability to elongate and desaturate 18:3n3 provided in the medium into longer
chain PUFAs, including DHA, to a degree not demonstrated by other retinoblastoma
cell lines (Hyman and Spector, 1981; Yorek et al., 1985). It is therefore possible to
enrich the membranes of these cells with DHA by supplementation with 18:3n3 and in
this way obtain a cell culture model for the PUFA enriched photoreceptor outer
segments.

2.2 Materials
Dulbecco’s Modified Eagles Medium (DMEM), RPMI-1640, L-glutamine, (Gibco
Plc.); γ-irradiated foetal bovine serum (FBS) (Imperial Laboratories, Andover,
Hampshire, UK); gentamicin, amphotericin B, penicillin/streptomycin, Hams F10 (20
mM Hepes, without Na₂HCO₃ or L-glutamine), fatty acid free-bovine serum albumin
fraction V, Na₂HCO₃ solution (7.5% K⁺ poly- L-lysine, insulin (bovine pancreas), apo-
transferrin (human), putrescine, L- thyroxine, triiodothyronine, progesterone, sodium
selenite, trypsin/EDTA in Ca\(^{2+}\)/Mg\(^{2+}\) free Hanks balanced salt solution, (Sigma-Aldrich Company Ltd., Poole, Dorset, UK); bovine serum albumin path-o-cyte 4 (ICN Pharmaceuticals Ltd. Thame, Oxon); Betadine\(^{®}\) antiseptic solution (aqueous Povidone-Iodine USP 10% w/v) (NAPP Laboratories, Cambridge).

2.3 Bovine Retinal Pigment Epithelial (RPE) Cells

2.3.1 Preparation of Primary Bovine RPE Cell Cultures
Bovine eyes were obtained from an abattoir within 1 to 2 hours after enucleation. During transport and preparation procedures the eyes were kept on ice. Fatty tissue and external muscle were removed from the eyeball. The exterior of the eyes was then sterilised by soaking in 70%/\(\text{v/v}\) ethanol (1 minute), and in an iodine antiseptic solution (Betadine\(^{®}\)) (2 minutes). The iodine was removed by washing the eye in sterile sodium thiosulphate (0.1% \(\text{v/v}\) for 30 seconds). The eyes were then rinsed twice in sterile PBS (pH 7.4) and soaked in amphotericin B/ gentamicin (0.025% & 0.005% (w/v) respectively) on ice for 20 minutes. The sterilised eyes were placed on sterilised filter paper in a petri dish. An incision was made just below the sera orrata and the eye cut horizontally around the circumference. The anterior of the eye (lens and cornea) and the vitreous humour were removed. The neural retina was peeled away from the back of the eyecup using microforceps and the interior of the eyecup was washed three times with sterile PBS and then incubated with trypsin/EDTA (0.25% & 0.02% (w/v) respectively) at 37\(^\circ\)C for approximately 30 minutes. The optic disc was protected from the enzyme solution by covering it with a sterile plastic container which was weighted down. The enzyme solution was gently triturated with a sterile Pasteur pipette over the surface of the eyecup and the resulting cell suspension transferred to a centrifuge tube. The cell suspension was centrifuged for 5 minutes at 1000 g and the pellet resuspended in 1 ml medium (see below) and plated onto poly-L-lysine coated 25 cm\(^2\) tissue culture flasks. The cells were allowed to attach for approximately 1 hour before the addition of a further 3 ml medium. Cells were incubated at 37\(^\circ\)C in a 5%/\(\text{v/v}\) \(\text{CO}_2\) humidified atmosphere until required.
2.3.2 Bovine RPE Culture Medium

Bovine derived RPE cell lines were cultured in a chemically defined medium, SATO with 1\% (v/v) retinal extract (see below). The extra growth factors, hormones and nutrients added to this medium allows cells to be cultured in a serum free environment. It was originally intended to supplement cells with phytanic acid using a serum free medium to limit the uptake of other exogenous fatty acids. When primary cultures were initiated, 10\% FBS was added to this chemically defined medium as an extra nutrient source to aid cell recovery and growth. Subsequent sub-culturing of the cells was continued in the defined medium, with 1\% retinal extract and 10\% FBS. When it became apparent that the presence of serum had no unwanted effects on the uptake of phytanic acid, the use of a chemically defined medium became superfluous to the needs of this project. Nevertheless the use of the defined medium with 1\% retinal extract and 10\% FBS was continued for all studies using these cells.

2.3.2.1 Preparation of SATO Defined Medium

SATO medium without FBS, but with retinal extract (see below) was prepared by mixing 93 ml DMEM, 2 ml 0.05 \% (w/v) insulin in 10 mM HCl, 1 ml 1 \% (w/v) transferrin in deionised H$_2$O (dH$_2$O), 1 ml 100 mM glutamine, 0.1 ml 5 \% (w/v) gentamicin, 1 ml retinal extract, and 2.2 ml SATO mix. When 10\% FBS was added, this replaced 10 ml DMEM. The SATO mix was prepared from 206 ml BSA path-o-cyte (0.98 \% (w/v) in PBS), 200 ml putrescine (0.16 \% (w/v) in dH$_2$O), 20 ml thyroxine (T4) (0.04\% (w/v) in ethanol), 20 ml tri-iodo-thyronine (T3) (0.03 \% (w/v) in ethanol), 2 ml progesterone (0.06 \% (w/v) in ethanol), and 2 ml selenium (0.04 \% (w/v) in PBS). Aliquots of SATO mix were stored at -20°C.

2.3.2.2 Preparation of Retinal Extract

Retinal extract was prepared by homogenising 12 fresh bovine retinas in 100 ml Ca$^{2+}$, Mg$^{2+}$-free balanced salt solution, followed by centrifugation at 17000 g for 20 minutes. The supernatant was filter sterilised and stored in aliquots at -20°C.

2.3.3 Maintenance of Bovine RPE Cultures

Bovine retinal cultures were maintained with twice weekly changes of medium until they reached confluency. Confluent cells were passaged by removing the medium, washing with sterile PBS and incubating at room temperature for 5-10 minutes with
0.05 % trypsin solution in PBS (2 ml for a 75 cm² flask) until the cells lifted off. Trypsin activity was inhibited by the addition of 5 ml serum containing medium and cells were collected by centrifugation at 1000 g and reseeded in fresh medium at a dilution of 1:3. Once the cells had attached to the culture vessel surface, the remaining medium was added (total of 10 ml for a 75 cm² flask).

2.3.4 Identity of the Bovine RPE Cultures
A number of cultures attained the cobblestone morphology associated with epithelial cells and these were used in the initial phytanic acid uptake studies. However the establishment of primary cultures was not always successful. Difficulties also arose concerning the logistics of obtaining bovine eyes from an abattoir and completing the lengthy preparation procedure within the limited time in which living cells could be isolated from this tissue. In order to confirm the identity of RPE cells, immunostaining for the epithelial marker, cytokeratin, may be used. However attempts at this technique were unsuccessful and the unequivocal identity of the cultures derived from bovine eyes as RPE was not confirmed. Thus where they were used in the phytanic acid uptake studies, they are referred to as bovine retinal cells. Eventually the use of the bovine RPE cultures was discontinued in favour of either the transformed rat RPE cell line or the Y79 retinoblastoma cell line.

2.4 Immortalised Rat Retinal Pigment Epithelial Cell Line
Two rat RPE cell lines that had been immortalised using the SV40 large T antigen, were the generous gift of Dr. John Greenwood, The Institute of Ophthalmology, London. This included a parent cell line (LD7) and a clone generated from the parent cell line (LD7.4). The development and characterisation of these cells have been described (Greenwood et al., 1996). The cloned cell line (LD7.4) was shown to be morphologically similar to primary cultures of rat RPE, although it had lost its pigmentation. This cell line was shown to be positive for cytokeratins and the rat RPE antigen RET-PE2 (Greenwood et al., 1996).

2.4.1 Maintenance of the Rat RPE Cell Lines
The immortalised rat RPE cell lines were cultured in Hams F10 with 20 mmol/l HEPES, 20 % FBS, 0.09 % Na₂CO₃, 1 mM glutamate, and penicillin (100 IU)/streptomycin
(100 μg/ml) in a 5% CO₂ humidified atmosphere at 37°C. The medium was replaced twice/week. Confluent cells were passaged and split 1:3 weekly, by treatment with 0.05% trypsin/EDTA in Ca²⁺/Mg²⁺-free Hanks balanced salt solution at room temperature for about 5 minutes. The cells were rinsed with PBS (pH 7.4) and with PBS: trypsin/EDTA solution (3:1 (v/v)) for 10 seconds prior to trypsinisation. The cells were then trypsinised with trypsin/EDTA at room temperature until detachment of cells from the flask surface had occurred. The brief exposure of cells to the PBS: trypsin/EDTA solution prior to trypsinisation improved cell-cell detachment and helped prevent the formation of cell clumps during trypsinisation. The inhibition of trypsin activity and the reseeding conditions were as those described for the bovine RPE cells (see 2.3.3).

2.5 Y79 Retinoblastoma Cell Line

Y79 is an established human retinoblastoma cell line that grows in suspension and was obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, Wiltshire). The characteristics of this cell line were documented by Reid et al (1974). Y79 cells are derived from those cells that separated from a spindle-cell matrix growing from the edges of the original explant and grew as clusters in suspension. Of the different morphologic cell types observed during the characterisation of this cell line, the predominant type was a small, round and undifferentiated cell. After 36 months in culture the established cell line retained ultrastructural and biochemical characteristics of the cells of the original tumour. In some retinoblastoma cell lines a certain degree of photoreceptor differentiation has been observed and it has therefore been proposed that retinoblastomas are derived from this cell type (Ts'O et al., 1969; Ts'O et al., 1970). However, Kyritsis et al (1984) showed that Y79 can be made to differentiate into neuronal and glial-like cells depending upon which growth factors were used. The authors therefore suggested that retinoblastoma in general originate from primitive multipotential neuroectodermal cells.

2.5.1 Maintenance of the Y79 Retinoblastoma Cell Line

Y79 cells were maintained in RPMI-1640 medium with 10% FBS, 2 mM glutamine, and penicillin (100 IU)/streptomycin (100 μg/ml), at a density of between 4-9 x 10⁵ cells/ml. The cells were incubated at 37°C in humidified 5% CO₂ air. Fresh medium was
added to the cultures twice a week, replacing 1/3 of the suspension which was either reseeded or discarded.

2.6 Storage of Cells in Liquid Nitrogen

Retinal cell lines utilised for all studies were of passage number 1 to 25 after either establishment of the bovine retinal cell line or after receipt of the rat RPE and Y79 cell lines. Stores of cells of low passage number were kept in liquid nitrogen and regenerated when necessary.

Anchorage dependent cell lines, i.e. the bovine and rat RPE cells were detached from culture flasks by trypsinisation and resuspended in medium containing 7.5 % (v/v) dimethylsulphoxide (DMSO) and 20% FBS. Y79 cells were stored in medium containing 10% DMSO and 25% FBS. The resuspended cells were frozen at -20°C for 1 hour and then at -70°C overnight. Cells were then left in N₂ vapour at the top of the liquid N₂ tank for 3 hours before being placed in the liquid nitrogen. Cells from one confluent 75 cm² flask of RPE or one 175 cm² flask of Y79 cells were frozen in 2 x 1 ml aliquots.

Frozen cells were regenerated by quickly thawing in a water bath at 37°C (2 minutes) followed by the addition of 1 ml of medium (at 37°C). The cell suspension was then added dropwise into 10 ml medium (at 37°C). The cells were collected by centrifugation at 1000 g and resuspended and plated in fresh medium. RPE cells were plated in a minimum amount of medium to encourage cell adherence to the culture vessel surface. The cells usually adhered within 4 - 6 hours.

2.7 Supplementation of Retinal Cell Lines with α-Tocopherol and Fatty Acids

The development of an in vitro model of ARD using retinal cell lines required the establishment of a protocol for the supplementation of the cultured cells with phytanic acid. In addition, Y79 cells were supplemented with linolenic acid (18:3n3) and α-tocopherol supplementation was required for investigations of the antimetabolite hypothesis. Medium containing FBS has low concentrations of phytanic and linolenic
acid and undetectable amounts of α-tocopherol. In order to supplement cell cultures with these fatty acids and α-tocopherol it was necessary to provide these compounds in a form that could be taken up by the cells in a reproducible and dose dependent manner. A description of the supplementation protocols used and the preparation of supplemented cells for fatty acid and α-tocopherol analysis is given below.

2.7.1 Supplementation of Medium With Fatty Acids
The method used for the supplementation of medium with fatty acids is based on that of Calder et al (1990). Medium was supplemented with phytanic acid and 18:3n3 complexed to fatty acid free-bovine serum albumin (faf-BSA). Potassium salts of the fatty acids were prepared by incubating equi-molar ratios of fatty acid and KOH at 70°C for 1 hour. Typically 250 μmol of phytanic acid (78 mg) or 18:3n3 (70 mg) and 5 ml of 0.05 M KOH were used. After cooling, the K⁺ salt was added to medium containing faf-BSA such that the final faf-BSA concentration was 5 % (w/v) and final volume was 50 ml. This mixture was rotated for 2 hours at room temperature. The pH was adjusted to 7.4 with 1 M KOH and the solution was sterilised through a 0.22 μm filter (Falcon) and stored at -20°C. The concentration of the fatty acids in this medium was determined using gas chromatography with flame ionisation detection (see section 3.3). This stock medium was diluted with unsupplemented medium to achieve the desired concentration of fatty acid.

The supplementation of all the retinal cell lines with phytanic acid is described in chapter 4 and the supplementation of Y79 cells with linolenic acid is described in section 8.2. The results from these studies demonstrated the reproducible and dose dependent uptake of these fatty acids.

2.7.2 Supplementation of Medium with α-Tocopherol
Various methods have been used to supplement cell culture media with different forms of vitamin E. The phosphate ester and the analogue trolox c, which lacks the phytol side chain, are water soluble forms that can be used. The free tocopherols are not water soluble and have been added either directly to media dissolved in a solvent such as ethanol (Amano et al., 1994), or solubilised using polyethylene glycol (Suzuke et al., 1993). The protocol used in these studies is based on the method of Tran and Chan
(1992) who dispersed \( \alpha- \) and \( \gamma- \) tocopherol in FCS before its addition to the media. The all-rac form of \( \alpha- \)tocopherol was used in these studies.

2.7.2.1 Protocol for the Supplementation of Medium with \( \alpha- \)Tocopherol

80 \( \mu l \) (or less depending upon the final concentration required) of 500 mM \( \alpha- \)tocopherol in acetone was added to 30 ml FBS warmed in a water bath at 37\( ^{\circ} \)C for at least 15 minutes. Nitrogen was briefly blown over the surface of the FBS to facilitate removal of the acetone before mixing by rotation at room temperature for 2 hours. The FBS was then sterilised by passing through a 0.22 \( \mu m \) filter. 3 x 50 \( \mu l \) aliquots of the filtrate were extracted and the concentration of \( \alpha- \)tocopherol in the FBS determined (see section 5.3). It was necessary to determine the concentration of \( \alpha- \)tocopherol after each filter sterilisation as this procedure resulted in a variable reduction in concentration, probably due to the \( \alpha- \)tocopherol adhering to the filter. The modified FBS was added to basal medium to achieve the desired concentration of \( \alpha- \)tocopherol. The final concentration of FBS in the medium was always that required for the particular cell type, the difference being made up with untreated FBS.

\( \alpha- \)Tocopherol supplemented medium was prepared fresh on the day of each feed. In the initial uptake studies described in chapter 5, medium was replaced on alternate days. In later studies, adequate and reproducible uptake of \( \alpha- \)tocopherol was shown to occur when the medium was replaced on the third day of a 7 day supplementation period (see chapter 7).

2.7.2.2 The Effect of Serum on the Dispersion of \( \alpha- \)Tocopherol

The dispersion of \( \alpha- \)tocopherol in FBS prior to its addition to medium was more effective than adding \( \alpha- \)tocopherol directly to medium containing FBS followed by mixing. When the latter approach was used, oil films were observed on the surface of the medium when it was left to stand overnight at 37\( ^{\circ} \)C. \( \alpha- \)Tocopherol is a very hydrophobic molecule and it is probable that serum aids the dispersion of \( \alpha- \)tocopherol by providing a concentrated source of lipoproteins and serum proteins such as albumin to which it can bind.
A preliminary experiment on the effect of adding α-tocopherol to medium with or without serum is shown in figure 2-1. α-tocopherol in acetone was added to 10 ml DMEM with or without 6% FBS and mixed by vortexing. Nominal final concentrations ranged from 10 to 200 μmol/l. The concentration of α-tocopherol was determined (see 5.3) and the results were expressed as a % of the expected concentration. Without serum, the % of nominal concentration obtained decreased from 100 to 10 % when the nominal concentration increased from 10 to 100 μmol/l. With serum present the concentration measured remained above 80 % up to a nominal concentration of 100 μmol/l and fell to 58 % at a concentration of 200 μmol/l.

The adequate dispersion of α-tocopherol in FBS after rotation for two hours was shown by the reproducibility of α-tocopherol measurements of the supplemented FBS alone and after it had been mixed with the basal medium (figure 2-2).

2.7.3 Harvesting of Cells Supplemented with Fatty Acid and/or α-Tocopherol

It was necessary to ensure the complete removal of the phytanic acid, linolenic acid or α-tocopherol containing medium from supplemented cells prior to the analysis of the cellular contents of fatty acids and α-tocopherol. The efficiency of different procedures for removing external α-tocopherol and phytanic acid containing medium from the cells was therefore assessed.

2.7.3.1 Efficiency of Removal of α-Tocopherol Supplemented Media

Confluent bovine retinal derived cells were exposed to media supplemented with 100 μmol/l α-tocopherol for 5 minutes at 37°C. Harvested cells were washed after trypsinisation with 3 x 10 ml warmed PBS (37°C) alone (wash 1) or with 5 ml unsupplemented warmed FBS (37°C) followed by 3 x 10 ml warmed PBS (37°C) (wash 2). A third group of unexposed cells were subjected to the same procedure as wash 2 (control). Crude membrane pellets were then prepared as described in 3.3.2.1 and α-tocopherol concentrations were determined as described in 5.3. The results are shown in figure 2-3. The mean α-tocopherol concentrations were 0.55 nmol/mg protein (wash 1), 0.24 nmol/mg protein (wash 2) and 0.17 nmol/mg protein (control). These results
Figure 2-1: The effect of serum on the dispersion of α-tocopherol in medium. α-tocopherol in acetone was added to medium ± 6%FBS.

The nominal concentration ranged from 10 to 200 µmol/l.

The measured α-tocopherol concentration was expressed as a % of the nominal concentration.

n = 1 for each point.
Figure 2-2: Reproducibility of α-tocopherol measurements in supplemented FBS alone (■) and in medium mixed with supplemented FBS (9:1, v/v) (●).

The results of three separate preparations are shown.

Measurements of each preparation were made in triplicate and individual measurements are shown.
Washing Efficiency of Cells Exposed to α-Tocopherol

Figure 2-3: α-Tocopherol concentrations in post-wash crude membranes from bovine derived retinal cells exposed to 100 μmol/l α-tocopherol supplemented medium for 5 minutes at 37°C.

Wash 1 = 3 x 10 ml warmed PBS (37°C).

Wash 2 = 5 ml unsupplemented warmed FBS (37°C) followed by 3 x 10 ml warmed PBS (37°C).

Control = unexposed cells washed with 5 ml unsupplemented warmed FBS (37°C) and 3 x 10 ml warmed PBS (37°C).

Bars show mean values (n = 3).
indicate that inclusion of FBS in the wash procedure aids the removal of external α-tocopherol.

2.7.3.2 Efficiency of Removal of Phytanic Acid Supplemented Media
Confluent bovine retinal derived cells were exposed to 1.25 mmol/l phytanic acid supplemented medium for 5 minutes at 37°C. Harvested cells were then washed with 5 ml unsupplemented warmed FBS (37°C) followed by 3 x 10 ml warmed PBS (37°C) and the phytanic acid concentration from crude membrane preparations was determined as described in chapter 3. The concentration was compared to that of unexposed cells washed using the same procedure and the results are shown in figure 2-4. The mean phytanic acid concentration in the phytanic acid exposed group was 1.6 % total fatty acid peak area compared to 0.8 % in the control group. This indicated that there was some carry over of phytanic acid from the medium. However the concentration used in this study was > 4 times that used in the uptake studies (maximum of 300 μmol/l). The extent of carry over is therefore unlikely to influence interpretation of the uptake studies. It is very unlikely that the higher concentration of phytanic acid observed in the exposed group after 5 minutes was due to uptake of phytanic acid by these cells.

2.7.3.3 Conclusion
On the basis of these results, α-tocopherol and phytanic acid supplemented cells were routinely harvested by trypsinisation and washed with warmed FCS (37°C) (1x) and then with warmed phosphate buffered saline (37°C) (3x).
Washing Efficiency of Cells Exposed to Phytanic Acid

Exposed Cells = cells incubated with 1.25 mmol/l phytanic acid supplemented medium for 5 minutes at 37°C and washed with 5 ml unsupplemented warmed FBS (37°C) and 3 x 10 ml warmed PBS (37°C).

Controls = unexposed cells washed with 5 ml unsupplemented warmed FBS (37°C) and 3 x 10 ml warmed PBS (37°C).

Bars show mean values (n = 3).

Figure 2-4: Phytanic acid concentrations in post-wash crude membranes from bovine derived retinal cells.
3. FATTY ACID ANALYSIS AND SYNTHESIS

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3.1 Introduction

Retinal cell lines exposed to phytanic acid at concentrations which represented the pathological levels observed in ARD were used as an *in vitro* model of this disease. The Y79 cell line was also supplemented with linolenic acid (18:3n3) to increase the unsaturation index of Y79 membranes (see section 8.2). The fatty acid analysis of cellular lipids was of central importance to this study in order to assess the uptake of phytanic acid by retinal cells under different supplementation conditions and to determine the concentrations of phytanic acid and linolenic acid in supplemented medium. In addition, fatty acid analysis was used to study the effect of phytanic acid uptake on the fatty acid profiles of cellular lipids and the effect of phytanic acid supplementation on the uptake of α-tocopherol by retinal cells. The methods used to analyse fatty acids are described in this chapter and the uptake of phytanic acid by retinal cells is described in chapter 4.

The direct one-step transesterification method of Lepage and Roy (1986), as modified by Liebich et al (1991), was used to prepare total fatty acid methyl esters (FAMEs) from stock medium and lipid fractions separated by thin layer chromatography (TLC), and was modified for the preparation of FAMEs from crude membrane preparations. It was originally intended to analyse the fatty acid profiles and α-tocopherol concentrations of the red blood cells of patients with disorders of peroxisomal biogenesis. A protocol was therefore developed for the analysis of FAMEs from red cell ghosts. The method of Lepage and Roy (1986) was chosen as it had already been established for the analysis of plasma fatty acids. FAMEs were analysed by gas chromatography (GC) with flame ionisation detection (FID) but also on occasions with mass spectrometry (MS) for additional confirmation of fatty acid identity.

3.2 Materials

Toluene, Analar grade (BDH, Poole, England); hexane and methanol, HPLC grade, (Rathburn Chemicals Ltd, Walkerburn, Scotland); fatty acid standards, acetyl chloride, (Sigma-Aldrich Co. Ltd., Poole, Dorset); potassium carbonate anhydrous, (BDH Laboratory Supplies, Poole, Dorset).
3.3 Preparation of Fatty Acid Methyl Esters (FAMEs) from Culture Medium, Crude Membrane Preparations and Lipid Fractions

3.3.1 Standards
An aliquot of the appropriate standard solution (see section 3.5) was added to a borosilicate tube (10 x 13 mm) and the solvent was removed under N$_2$. 100 µl dH$_2$O$_{0.019}%$ BHT was added followed by the addition of 2 ml methanol:toluene 4:1 (v/v) whilst vortexing. Samples were cooled in an ice bath for 2 minutes before the dropwise addition of 200 µl acetyl chloride whilst vortexing. The borosilicate tubes were tightly capped with teflon lined lids and incubated at 100°C for 2 hours, mixing after 1 hour. The samples were then cooled and 5 ml 0.25 M potassium carbonate was slowly added. The phases were separated by centrifugation at 2000 g for 5 minutes and the upper toluene phase was taken for the analysis of total FAMEs. The toluene phase was usually taken to dryness under N$_2$ and reconstituted in a smaller volume of hexane for analysis by GC.

Typically 100 µl of the standard mix was derivatised (see section 3.5) and amounts ranging from approximately 60 to 400 ng were injected onto the column.

3.3.2 Medium
2 ml methanol:toluene 4:1 (v/v), with 0.01% butylated hydroxytoluene (BHT) was added to 100 µl stock medium in borosilicate tubes (10 x 13 mm) whilst vortexing. The remaining procedure is as described in section 3.3.1.

3.3.3 Cultured retinal cell membranes

3.3.3.1 Crude Membrane Preparation
Cell suspensions were sonicated with 3 x 20 seconds bursts at 10 amps using a probe sonicator. The suspensions were cooled on ice between each burst to minimise over heating. Sonicates were then transferred to 6 ml Beckman ultracentrifuge tubes and centrifuged at 100 000g for 1 hour at 4°C in a fixed angle Beckman rotor (70 Ti). After removing the supernatant, the membrane pellet was washed with 3 x 1 ml dH$_2$O.
3.3.3.2 **FAME Preparation**
The preparation and extraction of FAMEs from the crude membrane preparations followed essentially the same protocol as that for the medium. Membrane pellets were homogenised (using a hand held 0.1 ml glass homogeniser) and suspended in 1.6 ml methanol. 0.4 ml of toluene (with 0.05% BHT) was then added to this suspension whilst vortexing. The separate addition of methanol prior to that of toluene facilitated the solubilisation of membranes and prevented the formation of insoluble clumps. The addition of acetyl chloride and the remaining procedure was as described above for the medium.

3.3.4 **Red Cell Ghosts**
In their original paper Lepage and Roy (1986) assessed the effect of aqueous sample volume on the extraction efficiency of their method. It was shown that aqueous sample volumes greater than 100 µl affected the recoveries of cholesterol ester and sphingomyelin standards when 2 ml methanol:toluene 4:1 (v/v) was used for the extraction. Therefore for this study, the red blood cell membrane pellet was prepared in a volume no greater than 100 µl.

3.3.4.1 **Ghost Preparation**
Whole blood, collected in EDTA or lithium/heparin tubes, was centrifuged at 2000 g for 5 minutes at 4°C. The plasma and buffy layer were removed and the red blood cells were washed three times in isotonic saline pH 7.4 (150 mM NaCl, 5 mM Na⁺ ascorbate, 1 mM EDTA disodium salt, adjusted to pH 7.4 with 150 mM Na₂HPO₄), pelleting the cells at 2000 g for 5 minutes at 4°C after each wash. A final red cell suspension was prepared with a haematocrit (HCT) of approximately 0.10. 1 ml of the suspension was then lysed in 20 ml 5 mM phosphate buffer pH 8.0. The lysed cells were centrifuged at 15000 g in a Sorvall centrifuge at 4°C for 15 minutes. The supernatant was removed and the pellet washed in 20 ml 2.5 mM phosphate buffer, pH 8.0, centrifuging at 15000 g at 4°C for 15 minutes. The red cell ghosts were transferred to a 1 ml eppendorf tube using 1.25 mM phosphate buffer pH 8.0 and were centrifuged in a microfuge at 17000 g for 5 minutes. It was found that a 1 ml sample of red cell suspension with HCT of approximately 0.1 resulted in a pellet of approximately 50 µl. This method of washing...
red cell ghosts in increasingly hypotonic buffer resulted in red cell membrane creamy-coloured ghosts free of contaminating haemoglobin (Burton et al., 1981).

3.3.4.2 Transesterification
1.6 ml of methanol was used to transfer the pellet to the borosilicate tube with minimum loss of sample. 400 µl toluene (with 0.05% BHT) was then added whilst vortexing and samples were treated as described in section 3.3.1.

3.3.5 Lipid Fractions
To prepare FAMEs from lipid fractions separated on silica plates by thin layer chromatography (see section 4.3), 2 ml methanol:toluene, (4:1 (v/v) with 0.01% BHT) was added to bands of silica scraped from the plates whilst vortexing thoroughly. The samples were then treated as previously described (section 3.3.1). The mass of silica in the incubations mixtures never exceeded 200 mg. This quantity of silica was shown by Lepage and Roy (1986) not to interfere with the transesterification reaction or recoveries using the volumes indicated in section 3.3.1.

3.4 Analysis of FAMEs by Gas Chromatography

3.4.1 GC-FID
FAMEs were analysed on a Pye Unicam 4550 gas chromatograph with flame ionisation detection and were separated using a SP2330 fused silica capillary column (30 m, 0.32 mm internal diameter, 0.2 µm film thickness) supplied by Supelco, UK. This stationary phase contains bis-cyanopropyl and phenyl groups which provides both polar and polarisable characteristics. It has been used previously by others for the separation of FAMEs and is particularly suitable for PUFA separation. The flow rate of the helium carrier gas was 20 cm/second at a pressure of 0.5 kg/cm². Manual injections of typically 1 µl were made in split mode with a split ratio of 2:1. The injector port temperature was 220°C, and the detector 240°C. FAMEs were separated using a temperature program starting at 140°C, ramped to 180°C at 4°C/minute, and then to 210°C at 1.5°C/minute and finally held at 210°C for 5 minutes. The sample run time was 35 minutes. Data was collected on a SP4770 integrator (Spectra Physics Ltd.).
3.4.2 GC-MS
The identity of fatty acids in a number of representative samples including a standard mix, was determined using GC-MS to confirm the identification made by GC-FID. The separation was carried out on a Hewlett Packard 5890 Series II gas chromatograph with a Hewlett Packard 7673 auto-injector. The same column and temperature program as that described for the GC-FID analysis in section 3.4.1 were used. 0.5 µl injections were made in split mode with a split ratio of 10:1. A triple focusing magnetic sector VG Autospec mass spectrometer (Fisons UK) coupled to the GC was used for the mass spectrometric analysis. Mass spectrometric analysis was made using electron impact ionisation (70eV) and total ion current monitoring between a mass to charge ratio (m/z) of 50 to 700 at 1 scan per second.

3.4.3 Confirmation of Identification of FAMEs
The mass spectrum for each peak in the chromatograms was examined and identification of fatty acids was determined on the basis of molecular ion mass and fragmentation pattern. The spectra were compared to library matched data to aid identification. Once spectra had been characterised for the standard fatty acid peaks, this information was used in the identification of peaks of cellular fatty acid profiles. FAME preparations from unsupplemented cell lines, RPE supplemented with phytanic acid and Y79 supplemented with phytanic acid and 18:3n3 were analysed (data not shown) and this information was used for the confident interpretation of chromatograms obtained later using GC-FID.

3.4.3.1 Fragmentation Patterns of FAMEs
The mass spectra of 16:0, 20:4n6 and phytanic acid methyl esters are shown in figures 3-1 to 3-3. These were chosen to illustrate the fragmentation patterns of a long chain saturated, a polyunsaturated and a branched chain FAME. The mass spectra of straight chain saturated FAMEs are characterised by the presence of the ion m/z 74 which is formed by the transfer of a γ-hydrogen to the carbonyl oxygen followed by cleavage of the bond between the α and β carbons (figure 3-4a). This is known as the McLafferty rearrangement. The m/z 74 is the most abundant ion in the spectra. Other prominent ions present include the molecular ion (M⁺) and the molecular ion minus 31 mass units (M-31) which arises from the loss of a methoxy group (CH₃O) (figure 3-4b). A series of
Figure 3-1: Mass spectrum of 16:0 methyl ester.
Figure 3-3: Mass spectrum of phytanic acid methyl ester.
Figure 3-4: Examples of the fragmentation of fatty acids methyl esters.

a) McLafferty rearrangement resulting in m/z of 74.

b) Loss of CH$_3$O (m/z = 31).
carbomethoxy ions \((\text{CH}_3\text{OCO(CH}_2)_{n}\text{)}^{+}\) are also present and are thought to arise from a complex rearrangement process (see Murphy, 1993, for a more detailed discussion). The ion m/z 87 belongs to this series and is the next most abundant ion of straight chain fatty acid methyl esters after m/z 74. The ions m/z 143 and 199 are also members of this series and are enhanced for fatty acids of chain length greater than 16 carbons.

In the mass spectra of unsaturated fatty acid methyl esters the McLafferty rearrangement ion m/z 74 is less abundant. Major ions in these spectra belong to a hydrocarbon series corresponding to \(C_nH_{2n-1}\), \(C_nH_{2n-3}\), or \(C_nH_{2n-5}\) for fatty acids with 1, 2 or 3 double bonds respectively. The molecular ions of polyunsaturated FAMEs formed by electron impact ionisation are unstable and have low abundances. Docosahexaenoic acid (22:6n3) and docosapentaenoic acid (22:5n3) did not produce a detectable molecular ion. This problem could have been overcome by using chemical ionisation with a protonating reagent gas such as methane or isobutane which leads to stable and hence abundant molecular ions \([M + H]^+\).

The fragmentation behaviour of branched chain FAMEs is similar to that of straight chain FAMEs. An abundant McLafferty rearrangement ion and the ions of the carbomethoxy series are present although with different m/z values, depending upon the position and size of the alkyl branches. Phytanic acid is a 16 carbon chain fatty acid with methyl groups substituted at positions 3, 7, 11 and 15. Hence the McLafferty rearrangement ion for this molecule has a m/z value of 74, and prominent ions in the carbomethoxy series are m/z 101 and 171.

3.5 Standards and Quantitation

3.5.1 Fatty Acid Standards

Standard fatty acids were prepared as individual solutions in chloroform:methanol 1:2 (v/v), unsaturated fatty acids being protected with 0.01% \(\text{BHT}\). The concentrations used and choice of fatty acid were based on published values of red cell fatty acid concentrations (Martinez, 1989) (see table 3-1). The elution order of a mixture of these standards was determined from the retention times of individually analysed standards.
<table>
<thead>
<tr>
<th>Fatty Acids in Standard mix</th>
<th>Concentration</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μg/ml</td>
<td>μmol/l</td>
</tr>
<tr>
<td>14:0</td>
<td>65.2</td>
<td>285.5</td>
</tr>
<tr>
<td>16:0</td>
<td>410.8</td>
<td>1603.4</td>
</tr>
<tr>
<td>16:1n7</td>
<td>79.5</td>
<td>312.8</td>
</tr>
<tr>
<td>18:0</td>
<td>299.5</td>
<td>1053.5</td>
</tr>
<tr>
<td>18:1n9</td>
<td>218.0</td>
<td>772.2</td>
</tr>
<tr>
<td>18:2n6</td>
<td>156.0</td>
<td>556.8</td>
</tr>
<tr>
<td>20:0</td>
<td>40.0</td>
<td>128.0</td>
</tr>
<tr>
<td>20:4n6</td>
<td>250.0</td>
<td>821.8</td>
</tr>
<tr>
<td>22:0</td>
<td>60.2</td>
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</tr>
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</tr>
<tr>
<td>22:6n3</td>
<td>103.0</td>
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</tr>
<tr>
<td>24:0</td>
<td>101.6</td>
<td>275.8</td>
</tr>
<tr>
<td>24:1n9</td>
<td>90.5</td>
<td>247.0</td>
</tr>
<tr>
<td>26:0</td>
<td>61.2</td>
<td>154.3</td>
</tr>
</tbody>
</table>

Table 3-1: Fatty acid standards used for the identification of fatty acids from red blood cell and retinal cell extracts. The concentrations used are based on published values of red blood cell fatty acid concentrations.
In addition to this mixture of standards, separate preparations of the following fatty acids, phytanic acid, 18:3n3, 20:5n3, and 22:5n3 were also used (concentrations ranging from 0.75 to 9.25 mmol/l). Examples of the separation of FAME standards and fatty acid extracts from control and phytanic acid supplemented transformed rat RPE cells (LD7) and control and 18:3n3 supplemented Y79 cells are shown in figures 3-5 to 3-9. In the fatty acid standard mixture, 16:1n7 and 18:1n9 were used. However in the FAME profiles of retinal cells 16:1n9 and 18:1n7 were also present and were incompletely separated from 16:1n7 and 18:1n9 respectively. This was confirmed by comparison to previously published separation profiles of FAMEs using the same column and GC temperature program (Martinez, 1990). The areas of the incompletely separated n-7 and n-9 peaks of 16:1 and also 18:1 were combined and reported as a single species.

Heneicosanoic acid (21:0) was chosen as an internal standard as it eluted in a relatively uncrowded part of a typical red cell and retinal cell FAME chromatogram and did not co-elute with any other peak (see figure 3-5). Between 20 -50 μl of 0.5 mg/ml (1.6 mmol/l) heneicosanoic acid in chloroform:methanol, 1:2 (v/v) was typically used when extracting/derivatising 100 μl standard mix, crude membrane preparations derived from cells grown in a 75 cm² flask, silica bands from TLC plates and fatty acid supplemented medium.

3.5.2 Quantitation of Fatty Acids in Supplemented Culture Medium
The concentration of fatty acids in supplemented medium preparations was quantified using a three point calibration curve using the relative peak area ratio method. Equal amounts of internal standard (IS) were added to the external standard (ES) and test samples. Peak area ratios of the ES fatty acid to IS were plotted against ES concentration to generate the calibration curve. The concentrations of fatty acids in the test samples were calculated from the calibration curves using the peak area ratios of these samples. The use of an internal standard compensated for variations in sample preparation, the amount injected onto the column and chromatographic conditions.
Figure 3-5: Fatty acid standards (standard mix and phytanic acid) analysed by GC-FID.
Figure 3-6: Fatty acid profile of rat RPE cells cultured in control medium, analysed by GC-FID.
Figure 3-7: Fatty acid profile of rat RPE cells supplemented with 100 μmol/l phytanic acid for 7 days, analysed by GC-FID.
Figure 3-8: Fatty acid profile of Y79 cells cultured in control medium, analysed by GC-FID
Figure 3-9: Fatty acid profile of Y79 cell crude membranes supplemented with 50 µmol/l 18:3n3 for 7 days, analysed by GC-FID.
3.5.2.1 Linearity of Fatty Acid Standard Curves

The linearity of fatty acid standard curves was evaluated using concentration ranges based on the fatty acid concentrations expected from FAME analysis of 100 μl packed red blood cells. Concentrated methyl esters of the standard fatty acid mix, phytanic acid, and 21:0 were prepared separately. A series of mixtures were made with increasing concentrations of standards and a constant concentration of 21:0. These mixtures were analysed by GC-FID and the peak area ratios (ES/IS) were determined and plotted against fatty acid concentration. Linear regression was performed using Graphpad Prism version 2 software (California, USA). The standard curves of phytanic acid, 18:0 and 22:6n3, are shown in figure 3-10 as representative curves of a branched chain, a saturated straight chain and a polyunsaturated fatty acid. A linear relationship between the peak area ratios and standard concentration was seen for all fatty acids analysed (data not shown). The Pearson (or linear) correlation coefficient ranged from 0.968 for 14:0 to 0.999 for 22:6n3 and 20:0).

3.5.3 Quantitation of Membrane Fatty Acids

The concentrations of fatty acids in red cell and retinal cell crude membrane preparations and lipid fractions separated by TLC, were expressed as a percentage of total peak area. This is the simplest method of quantitation and assumes equal response factors for all analytes. A response factor is defined as;

\[
\text{Response Factor (RF)} = \frac{A(C)}{A(IS)} \times \frac{W(IS)}{W(C)}
\]  
(Eq. 3-1)

Where \( A = \) peak area, \( W = \) mass of sample, \( C = \) compound of interest, \( IS = \) internal standard

Up to 18 fatty acids were analysed in each membrane extract. Quantitation of these samples using the internal/external standardisation method described in 3.5.2 would have been difficult. In addition quantifying fatty acids relative to each other eliminated the need for quantitative preparation of crude membranes and of FAMEs.
Figure 3-10: Standard curves of phytanic acid, 18:0 and 22:6n3.
3.5.3.1 Comparison of Fatty Acid Response Factors

Using the calibration curve data from 3.5.2.1, response factors for each concentration point were calculated using equation 3-1. The results are shown in figure 3-11. The response factors were found to vary for the different fatty acid standards; ranging from 1.56 for 14:0 to 0.58 for 22:4n6. The response factors decreased with increasing chain length and may therefore be related to the retention times of the fatty acids. These results show that the expression of the results as a % of total peak area is an approximation of the true relative concentrations. However for the purposes of this study, expression of results in this way was considered to be acceptable as the fatty acid profiles of phytanic acid supplemented cells were compared to control profiles and trends in changes in the relative proportions of the different fatty acids could still be determined.

3.6 Reproducibility of FAME analysis of Red Cell Ghosts

The reproducibility of FAME analysis of membranes was determined using red cell ghosts. A red cell suspension was prepared from fresh blood from a normal subject as described in 3.3.4 and 6 replicate samples were prepared for FAME analysis. Fatty acids were determined as a % of total peak area and the coefficient of variation (c.v.) for the major fatty acids calculated. The results are shown in figure 3-12. The c.v. varied from 3.2 for arachidonic acid (20:4n6) to 11.8 for lignoceric acid (24:0). A c.v. value of 5 % or less is normally considered representative of a reproducible assay. Variations in the reproducibility could possibly result from differences in extraction and methylation efficiencies between the fatty acids.

3.7 Synthesis of Phytanic Acid

Phytanic acid is available commercially (from Sigma-Aldrich Company Ltd, Poole, Dorset, UK), but because of its expense and the amounts required for these studies, it was synthesised from phytol using the method of Jellum et al (1966). Phytol, an unsaturated multi-branched alcohol is hydrogenated at room temperature and atmospheric pressure over a Raney-nickel catalyst to produce dihydrophytol. Dihydrophytol is then converted to dihydrophytol-phytanate ester by oxidation with acidified chromic oxide and hydrolysis of the ester produces phytanic acid and dihyrophytol which can be separated by differential extraction.
Figure 3-11: Response factors of fatty acid standards. Each point is the individual response factor for a concentration point on the standard curve. The bar represents the mean.
Figure 3-12: Coefficient of variation of replicate measurements of fatty acids in red blood cell ghosts. Fatty acids were determined as a % of total peak area. n = 6
3.7.1 Materials
Raney-nickel catalyst, practical grade phytol (approximately 60% pure), silica gel 60
(from Sigma-Aldrich Company Ltd, Poole, Dorset, UK); BSTFA + 1 % TMCS, (Pierce,
Rockford, Illinois, USA).

3.7.2 Hydrogenation
20 g of phytol was dissolved in 240 ml ethanol and approximately 2 g of Raney-nickel
catalyst was added. A stabilised borohydride solution was prepared by dissolving 7.71 g
sodium borohydride in 200 ml 0.1 M NaOH. This was added dropwise to 20 ml glacial
acetic acid whilst stirring to produce hydrogen which was passed into a second reaction
vessel which contained the phytol solution and catalyst. This mixture was stirred for 4
hours in the presence of the hydrogen atmosphere. To confirm hydrogenation to
dihydrophytol, 10 µl of the reaction mixture was taken and the solvent removed under
nitrogen. The residue was then derivatised by heating at 70°C for 5 minutes with 100 µl
of bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 1 % trimethylchlorosilane
(TMCS):acetonitrile (1:1, v/v) and analysed using GC-MS. The injector temperature
was 280°C; the initial oven temperature was 160°C which was held for 1 minute and
then ramped at 10°C/minute to 290°C and held for 5 minutes. Analytes were detected by
scanning from m/z 350 to 400 . Completion of the reaction was confirmed by the
presence of the trimethylsilyl ethers of dihydrophytol (m/z = 370.7) and the absence of
phytol (m/z = 368.7).

3.7.3 Oxidation
The hydrogenation reaction mixture was filtered through a sintered glass funnel to
remove the catalyst and the ethanol was removed by rotary evaporation. 10g of chromic
oxide in 125 ml 80% acetic acid saturated with KHSO₄ was added dropwise to the
dihydrophytol in glacial acetic acid (130 ml) over 30 minutes. Stirring was continued
for another 90 minutes after which time 270 ml dH₂O was added. The solution was then
extracted twice with an equal volume of petroleum ether (b.p 40° - 60°).

3.7.4 Hydrolysis
After the removal of the solvent from the combined extracts by rotary evaporation the
dihydrophytyl-phytanate ester remained as a yellow oil. The ester was hydrolysed by
refluxing for 90 minutes with 0.5 M ethanolic KOH (250 ml). 250 ml dH₂O was added and the hydrolysed dihydrophytol was extracted twice with an equal volume of petroleum ether (b.p 40° - 60°). The potassium salt of phytanic acid remained in the aqueous phase. 21 ml of concentrated H₂SO₄ was added to the aqueous phase and the phytanic acid was extracted into petroleum ether as above. The petroleum ether extracts were filtered and combined and the solvent removed by rotary evaporation. 10 g of activated carbon was added and after 10 minutes of stirring was removed by filtration. Approximately 13 g of crude phytanic acid was obtained at this stage.

3.7.5 Purification

A 100 g silica gel 60 column (370 mm x 20 mm, particle size 0.063 - 0.200 mm, mesh 70-230), prepared in 30 ml hexane, was used to remove impurities from the crude phytanic acid. The crude phytanic acid was dissolved in 2 %ethyl acetate in hexane (150 ml) and was applied to the column. 6 %ethyl acetate was used to wash phytanic acid through the column which could be seen as a yellow band. The first 170 ml of effluent was discarded and the remainder was collected as 20 ml fractions. 5 μl aliquots of the odd numbered fractions were taken for analysis by GC-MS. The solvent was removed under N₂ and the samples were derivatised with 100 μl BTSTFA + 1 % (v/v) TMCS:acetonitrile (1:1 v/v) at 70°C for 5 minutes. The GC-MS conditions were as above except that the scanning range was m/z = 50 to 500. The results of the GC-MS analysis showed that the majority of the phytanic acid (m/z = 384.4 of trimethylsilyl ester) eluted in the first 17 fractions which were combined and the solvent removed by rotary evaporation. Some residual dihydrophytol (m/z = 370.7 of trimethylsilyl ether) and other impurities were also present in these fractions. The extraction step was therefore repeated to try and purify the phytanic acid even further. The final extract was rotary evaporated and the phytanic acid methylated (as described in section 3.3.1) and analysed by GC as described in section 3.4.1. The purity was calculated as the % of total peak area and the results showed that the synthesised phytanic acid was 96.4 % pure.
4. UPTAKE OF PHYTANIC ACID BY RETINAL CELL LINES

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4.6 DISCUSSION .............................................................................................................. 115
4.1 Introduction
In this chapter a series of experiments are described in which the uptake of phytanic acid was studied in the bovine retinal derived cell line, the immortalised rat RPE cell lines, and the Y79 cell line. Cells were supplemented with phytanic acid added to the culture medium as a complex to BSA. The uptake of phytanic acid was assessed by measuring the concentration of phytanic acid in crude membrane preparations as a % of total fatty acid peak area using FAME analysis. The effect of phytanic acid supplementation concentration, length of time of exposure to phytanic acid, and stage of cell growth on the uptake characteristics were investigated. Using this data, the supplementation parameters were established for a number of studies designed to assess the effect of phytanic acid uptake on the fatty acid profiles of total lipids and lipid fractions (described in this chapter) and the effect of phytanic acid on the uptake of α-tocopherol by RPE cells (chapter 7).

4.2 The Uptake of Phytanic Acid by Retinal Cell Lines

4.2.1 Demonstration of the Uptake of Phytanic Acid by Bovine Retinal Cells
The bovine derived retinal cell line was used in the initial studies on phytanic uptake prior to obtaining the immortalised rat RPE cell lines.

4.2.1.1 Exposure of Confluent Bovine Retinal Cells to 200 μmol/l Phytanic Acid
Confluent bovine retinal derived cells were exposed to 200 μmol/l phytanic acid supplemented medium for 7 days with one change of medium. This concentration is comparable to the serum concentrations observed in untreated ARD (see 1.4.3). It was also the concentration used by Bernstein et al (1992) in a RPE cell culture model of ARD. In the current study it was observed that supplemented cells developed a black grainy appearance and sloughing of cells into the medium occurred within 3 days. FAME analysis of crude membrane preparations from cells harvested by trypsinisation after 7 days, was carried out and the results are shown in figure 4-1. The phytanic acid concentration of exposed cells was 28.3 % of total peak area (n = 3 separate flasks) compared to a concentration of 0.8 % (n = 2) in control cells fed unsupplemented medium. This experiment demonstrated that phytanic acid was taken up by the bovine
Incorporation of Phytanic Acid Into Confluent Bovine Retinal Cells

Figure 4-1: Fatty acid profile of confluent bovine derived retinal cells supplemented with 200 µmol/l phytanic acid over 7 days. Controls, n = 2; + 200 µmol/l phytanic acid, n = 3
retinal cells when exposed to medium supplemented with 200 μmol/l phytanic acid. The sloughing of cells into the medium indicated that 200 μmol/l phytanic acid was toxic to these cells. The grainy appearance of the cells was later shown to be due to an accumulation of lipid droplets in the cytoplasm (see section 4.4). A decrease in the relative concentration of 18:0, 18:1 (n9 and n7 combined), 18:2n6, 20:4n6 and 22:6n3 was observed in the phytanic acid treated cells.

4.2.1.2 Exposure of Non-Confluent Bovine Retinal Cells to 50 μmol/l Phytanic Acid
A second study was conducted to determine whether phytanic acid would be incorporated into actively dividing cells using a lower supplementation concentration. 24 hours after passage, bovine retinal cells were supplemented with 50 μmol/l phytanic acid for 7 days with one change of medium. It was observed that cells grew to confluency in the presence of 50 μmol/l phytanic acid and FAME analysis demonstrated the uptake of phytanic acid by these cells as shown in figure 4-2. The concentration of phytanic acid in the supplemented cells was 17.4 % of total peak area (n = 3) compared to 0.8 % in control cells (n=3). Cells also developed a “grainy” appearance, but to a lesser extent than that observed using 200 μmol/l supplementation. The fatty acid profiles of control and phytanic acid supplemented cells were similar to those obtained in the previous study.

4.2.1.3 A Comparison of Uptake of Phytanic Acid with Time by Confluent and Non-Confluent Bovine Retinal Cells Exposed to 50 μmol/l Phytanic Acid
A time course study on the uptake of phytanic acid by confluent and non-confluent (growing) bovine retinal cells was undertaken. Confluent cells were supplemented with 50 μmol/l phytanic acid over a period of 9 days with two changes of medium. Non-confluent cells were supplemented with 50 μmol/l phytanic acid 24 hours after passage, for up to 8 days with two changes of medium. Cells reached confluency within 5 days. Cells from each group were harvested at various time points during the exposure period. Suspensions of washed cells in PBS were stored at -20°C under N₂ and FAME preparation and analysis were carried out when all the samples had been collected. It was only possible to collect data from day 4 onwards in the non-confluent time course study, because cell numbers were too low to provide enough material for FAME analysis in the early stages. The results are shown in figure 4-3. Phytanic acid uptake by confluent cells increased steadily from a concentration of 0.6 % total peak area at day 1.
Figure 4-2: Fatty acid profile of bovine derived retinal cells supplemented with 50 µmol/l phytanic acid, whilst growing, over 7 days. Controls, n = 3; + 50 µmol/l phytanic acid, n = 3
Figure 4-3: Uptake of phytanic acid with time by bovine retinal cells. Confluent cells were supplemented with 50 μmol/l phytanic acid over 9 days. Non-confluent cells were supplemented with 50 μmol/l phytanic acid 24 hours after seeding and grown in this medium for up to 8 days. n = 1 for each time point.
(no exposure) to 24.0 % after 7 days exposure. After this time there was no further increase. The uptake of phytanic acid by the growing cells was comparable to that of the confluent cells from the time point measured. There was an increase from 17.5 % total peak area at day 4 to 28.1 % at day 8.

The results from these experiments demonstrated that growing and confluent bovine retinal cells are able to take up phytanic acid bound to BSA in the medium. Whilst 50 μmol/l was tolerated by the cells, 200 μmol/l appeared to be toxic. The uptake of phytanic acid was similar in cells that were supplemented in an initial non-confluent state compared to those supplemented from an initial state of confluency. An increase in phytanic acid uptake was observed with increasing exposure time. After 7 days the concentration of phytanic acid in cells appeared to reach a maximum. The highest concentration of phytanic acid observed in the cells was 28 % total peak area after exposure of confluent cells to 200 μmol/l phytanic acid over 7 days and also after exposure of growing cells to 50 μmol/l over 8 days.

4.2.2 The Uptake of Phytanic Acid by the Immortalised Rat RPE Cell Lines
The time and dose dependency of phytanic acid uptake by confluent rat RPE cells were investigated. Both the parent (LD7) and cloned cells (LD7.4) were used for these studies.

4.2.2.1 The Effect of Time on the Uptake of Phytanic Acid Uptake by the Rat RPE Parent Cell Line (LD7)
Confluent LD7 cells were supplemented with 50 μmol/l phytanic acid over a period of 10 days with 2 changes of medium. Cells were harvested at several time points during this time and stored at -20°C under N₂ for up to 2 weeks before FAME analysis was carried out on crude membrane preparations. The results are shown in figure 4-4. The concentration of phytanic acid increased in these cells from 0.5 % total peak area at 0 days exposure to 7.7 % at 10 days. The concentration rose sharply over the first 2 days to 5.7 % after which time the increase was more gradual. These results demonstrated the uptake of phytanic acid from the medium by the immortalised parent rat RPE cell line.

The degree of uptake of phytanic acid by this cell line was lower than that of the bovine retinal cell line (6.8 % compared to 24.0% respectively) after 7 days of supplementation.
Figure 4-4: The uptake of phytanic acid by the immortalised rat retinal pigment epithelial cell line, LD7, supplemented with 50 μmol/l phytanic acid over a period of 10 days. n = 1 for each time point.
with 50 μmol/l. From these results, a supplementation period of 7 days with one change of medium was considered to be sufficient to achieve an adequate uptake of phytanic acid by the RPE cell line.

4.2.2 Effect of Concentration on the Uptake of Phytanic Acid Uptake by the Rat RPE Cloned Cell Line, LD7.4

Confluent LD7.4 cells were exposed to a range of phytanic acid concentrations of 0 to 300 μmol/l over 7 days with one change of medium. Cells were harvested and FAME analysis was carried out on crude membrane preparations. The results of phytanic acid uptake are shown in figure 4-5. The concentration of phytanic acid in the crude membrane preparations increased from 0.67 % at 0 μmol/l phytanic acid supplementation to 40.8 % at 300 μmol/l. A linear relationship was observed up to 200 μmol/l supplementation. At 300 μmol/l the curve began to plateau. At 50 μmol/l supplementation the phytanic acid crude membrane concentration was 7.7 %. This was comparable to the concentration obtained (6.8 %) when the parent cell line was supplemented with 50 μmol/l for 7 days suggesting that the uptake characteristics in both cell lines were similar.

From these results, a concentration range of between 0 to 200 μmol/l was considered to be suitable for further studies on the uptake of phytanic acid by the rat RPE cell lines. It was observed that phytanic acid supplemented RPE cell lines, like the bovine retinal cell line, also accumulated “granular” material in the cytoplasm.

4.2.3 Uptake of Phytanic Acid by Y79 Cell Line

To demonstrate that Y79 cells could also incorporate phytanic acid in a dose dependent manner, a dose response study was carried out. Y79 cells were seeded at 4.5 x 10^5 cells/ml in medium supplemented with between 0 to 200 μmol/l phytanic acid and were cultured for 7 days, with one addition of fresh medium during this time. The results are shown in figure 4-6. A dose dependent uptake of phytanic acid by the cells was observed. The crude membrane phytanic acid concentration increased from 1.3 % at 0 μmol/l to 53.0 % total peak area at 200 μmol/l. The greatest rise from 1.3 to 42.9 % was observed between 0 to 90 μmol/l. Phytanic acid concentrations above this caused less of an increase and the curve appeared to plateau. These results demonstrated that a supplementation concentration range of 0 to 200 μmol/l was suitable for uptake studies.
Effect of Concentration on the Uptake of Phytanic Acid by Rat RPE Cells

Figure 4-5: Uptake of phytanic acid by the immortalised rat retinal pigment epithelial cell line, LD7.4, supplemented with 0 to 300 μmol/l phytanic acid over a period of 7 days.

n = 1 for each concentration point, except at 50 μmol/l (n = 2) and 150 μmol/l (n = 3).

Min - max values are shown for 150 μmol/l (range too small to be shown for 50 μmol/l).
Effect of Concentration on the Uptake of Phytanic Acid by Y79 Cells

Figure 4-6: Uptake of phytanic acid by Y79 cells grown in 0 to 200 µmol/l phytanic acid supplemented medium for 7 days.

n = 1 for each concentration point except at 50 and 110 µmol/l where n = 3.
Min - max is shown for 110 µmol/l (range too small to be shown for 50 µmol/l).
of phytanic acid by the Y79 cell line. The degree of uptake of phytanic acid by Y79 cells was greater than that observed in the parent and cloned RPE cell lines. This may result from the differences in growth characteristics between the cell lines. The Y79 cell line grows in suspension with the cells constantly in a growth phase whereas the RPE cell cultures were exposed to phytanic acid when confluent and therefore when growth had ceased.

4.3 The Distribution of Phytanic Acid in Cellular Lipid Fractions

The above studies demonstrated that the uptake of phytanic acid by cultured retinal cells took place in a time and dose dependent manner. In order to investigate the fate of phytanic acid taken up by the cells, the relative distribution of total cellular phytanic acid in the different lipid fractions was determined as described below. Thin layer chromatography (TLC) was used to separate neutral lipids of whole cell lipid extracts and FAME analysis of the separated lipid fractions was carried out as described in section 3.3.5.

4.3.1 Methods

4.3.1.1 Materials
L-α-phosphatidylcholine, linoleic acid, cholesterol linoleate, 1,2-dilinoleoyl-3-oleoyl-rac-glycerol, 1,3-dilinolein, 1-monolinoleoyl-rac-glycerol, (Sigma-Aldrich Co. Ltd., Poole, Dorset); diethyl ether, acetic acid, chloroform, AnalAr grade (BDH Laboratory Supplies, Poole, Dorset).

4.3.1.2 Extraction of Lipids
Lipids were extracted from cell and membrane preparations using the method of Folch et al (1957). Harvested cells were resuspended in 300 μl PBS (pH 7.4) and were disrupted using a probe sonicator (Soniprep 150 MSE) with three 20 second bursts at 10 amps, cooling on ice between each burst. Crude membranes, prepared as described in 3.3.3, were homogenised in 500 μl PBS using a 0.1 ml glass homogeniser. 20 volumes of chloroform:methanol (2:1, v/v) was added to the sonicate (for whole cell extraction) or membrane suspension (for membrane lipid extraction) in glass tubes and vortexed. After leaving to stand for 30 minutes, samples were centrifuged at 2000 g for 5 minutes to pellet the denatured protein. The extracts were transferred to clean tubes and phase
separation was achieved by adding 1/5 volume of 0.05 M CaCl₂ and centrifuging at 2000g. The lipid containing lower phase was collected, the solvent removed under a stream of nitrogen and the lipid extract reconstituted in approximately 40 μl chloroform:methanol (1:1, v/v).

4.3.1.3 Separation of Neutral Lipids
Lipids were separated on 20 by 20 cm glass silica gel 60 Merk TLC plates (Supelco, UK), using a solvent system of hexane:diethyl ether:acetic acid, 60:40:2 (solvent system A), or 80:20:1 (solvent system B). Plates were activated by heating at 100°C for 1 hour and cooled before use. The developing chamber was lined with Whatman filter paper and allowed to equilibrate with fresh solvent, approximately 1 cm deep, for at least 1 hour before use. Lipid extracts (typically 20 μl) were applied to the plates as 2 cm bands at the origin, using a 5 μl syringe. Plates were developed until the solvent front was approximately 2 cm from the top of the plate. After drying at room temperature, lipid fractions were visualised by exposure of the plate to iodine vapour.

4.3.1.4 Standards
Table 4-1 lists the standards used to identify bands separated by the TLC system described above. Unsaturated lipids were chosen as standards so that they could be visualised by the iodine vapour. The standards were initially run as single bands in individual lanes and their relative positions determined. A mixture of the standards was prepared and this was then used for each TLC separation to identify the different lipid classes in the whole cell and crude membrane extracts by comparison of the retardation factors, (Rf), of the standard and analyte bands.

4.3.1.5 Separation Profiles
Separation of neutral lipids using solvent system A was initially used as this resulted in the complete separation of all the standards. Solvent system B failed to resolve monoacylglycerols from phospholipids and cholesterol from diacylglycerols. A representative separation of standard lipids by the two solvent systems is shown in figure 4-7 and an example of Rf values obtained from a typical separation is given in table 4-2. A large amount of triacylglycerol was found to be present in the phytanic acid supplemented cells, and solvent system A did not resolve the triacylglycerol band from the cholesterol ester band of these lipid extracts. Solvent system B was therefore used to
### Lipid Class Standard Concentration (mg/ml)

<table>
<thead>
<tr>
<th>Lipid Class</th>
<th>Standard</th>
<th>Concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospholipid</td>
<td>( \text{L-} \alpha\text{-phosphatidylcholine} )</td>
<td>1.25</td>
</tr>
<tr>
<td>Free Fatty Acid</td>
<td>Linoleic acid</td>
<td>1.50</td>
</tr>
<tr>
<td>Cholesterol Ester</td>
<td>Cholesterol linoleate</td>
<td>1.50</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>Cholesterol</td>
<td>1.25</td>
</tr>
<tr>
<td>Triacylglycerol</td>
<td>1, 2-dilinoleoyl-3-oleoyl-rac-glycerol</td>
<td>1.25</td>
</tr>
<tr>
<td>Diacylglycerol</td>
<td>1,3-dil[(cis, cis)-9,12-octadecadienoyl]-rac-glycerol</td>
<td>1.25</td>
</tr>
<tr>
<td>Monoacylglycerol</td>
<td>1-monolinoleoyl-rac-glycerol</td>
<td>1.25</td>
</tr>
</tbody>
</table>

Table 4-1: TLC standards used for identification of fractions separated from whole cell and crude membrane lipid extracts.

### Lipid \( R_f \) Value

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Solvent System</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol Ester</td>
<td>( R_f ) Value</td>
<td>0.723</td>
<td>0.671</td>
</tr>
<tr>
<td>Triacylglycerol</td>
<td>( R_f ) Value</td>
<td>0.626</td>
<td>0.383</td>
</tr>
<tr>
<td>Free Fatty Acid</td>
<td>( R_f ) Value</td>
<td>0.335</td>
<td>0.201</td>
</tr>
<tr>
<td>Diacylglycerol</td>
<td>( R_f ) Value</td>
<td>0.219</td>
<td>0.067</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>( R_f ) Value</td>
<td>0.197</td>
<td>0.067</td>
</tr>
<tr>
<td>Monoacylglycerol</td>
<td>( R_f ) Value</td>
<td>0.032</td>
<td>0.000</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>( R_f ) Value</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Distance travelled by solvent front (cm)</td>
<td></td>
<td>15.5</td>
<td>14.9</td>
</tr>
</tbody>
</table>

Table 4-2: Comparison of \( R_f \) values of lipid standards separated by

i) solvent system A = hexane:diethyl ether:acetic acid (60:40:2)

ii) solvent system B = hexane:diethyl ether:acetic acid (80:20:1)
Figure 4-7: Representative separation profiles of lipid standards using hexane:diethyl ether:acetic acid.

solvent system A = 60:40:2 and solvent system B = 80:20:1

PL = phospholipid, MAG = monoacylglycerol, C = cholesterol,
DAG = diacylglycerol, FFA = free fatty acid, TG = triacylglycerol,
CE = cholesterol ester.
separate these two bands and estimate the relative amounts of phytanic acid within each. The amount of monoacylglycerols present in whole cell extracts and the percentage of phytanic acid incorporated into this band was minor. Thus its contribution to the phospholipid phytanic acid content was considered to be insignificant.

4.3.1.6 Determination of the Relative Proportions of Phytanic Acid in Cellular Lipid Fractions by FAME Analysis

A known volume of whole cell lipid extract was transmethylated together with 25 μl of 21:0 (0.5 mg/ml) internal standard. Known volumes of the whole cell extract were separated by TLC as described above. FAMEs of lipid fractions were prepared with the same volume of internal standard and the phytanic acid concentrations were calculated from the peak area ratio to the internal standard peak area. The distribution of phytanic acid in the different lipid fractions was then determined as the percentage of phytanic acid in the whole cell lipid extract.

4.3.2 Distribution of Phytanic Acid in Total Lipids of RPE Cells

Confluent rat RPE cells (LD7) were supplemented with 100 μmol/l phytanic acid over 7 days, with one change of medium. Whole cell total lipids were extracted and separated by TLC and FAME analysis of the total lipid extract and separated lipid fractions was carried out. The relative proportions of phytanic acid in the different lipid fractions are shown in table 4-3, column 1. No monoacylglycerol or diacylglycerol bands were detected. Two unidentified bands were present (termed “others combined” in the table). The triacylglycerol band was broad and was not fully resolved from the cholesterol ester fraction. These bands were combined and analysed together. The total recovery of phytanic acid extracted from the lipid bands was approximately 82% with over 50% of the phytanic acid recovered from the combined triacylglycerol and cholesterol ester fraction. The phospholipid fraction contained approximately 18% and the free fatty acid fraction 9% of the phytanic acid. Less than 2% was recovered in the unknown bands.

4.3.3 Comparison of the Distribution of Phytanic Acid in RPE Whole Cell and Crude Membrane Lipid Fractions

A second study was carried out with confluent rat RPE cells (LD7) supplemented with 100 μmol/l phytanic acid over 7 days, with one change of medium. Whole cell and crude membranes lipids were separated by TLC and lipid fractions were analysed. The
<table>
<thead>
<tr>
<th>Lipid</th>
<th>% of Total Phytanic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Whole Cell Extract</td>
</tr>
<tr>
<td>Cholesterol Ester and Triacylglycerol</td>
<td>54.1</td>
</tr>
<tr>
<td>Free Fatty Acid</td>
<td>8.6</td>
</tr>
<tr>
<td>Diacylglycerol</td>
<td>No Band Detected</td>
</tr>
<tr>
<td>Monoacylglycerol</td>
<td>No Band Detected</td>
</tr>
<tr>
<td>Phospholipid</td>
<td>17.6</td>
</tr>
<tr>
<td>Others combined</td>
<td>1.4</td>
</tr>
<tr>
<td>Total Recovered</td>
<td>81.7</td>
</tr>
</tbody>
</table>

Table 4-3: The % of phytanic acid recovered from lipid fractions separated from whole cell and crude membrane lipid extracts of rat RPE LD7 cells supplemented with 100 µmol/l phytanic acid for 7 days.

The results of two studies are shown.
results are shown in table 4-3, column 2. Comparing the whole cell lipid extract of this study to that of the previous study, a similar proportion of phytanic acid was found in the major lipid fractions, with 58% in the triacylglycerol and cholesterol ester fraction, 19% in the phospholipid fraction and 8% in the free fatty acid fraction. A diacylglycerol band was detected containing 1.5% total phytanic acid. The unidentified bands ("others combined") contained a higher amount of phytanic acid than in the previous study (7%). The percentage recovery of phytanic acid applied to the plate for both the whole cell and membrane lipid extracts was higher than that obtained in the previous study, being 94% and 96% respectively.

Triacylglycerol (incompletely separated from cholesterol ester) was also found to be present in the crude membrane lipid extract. This result was unexpected as triacylglycerol is not a membrane lipid. Thus its presence in crude membrane lipid extracts suggested an inadequate separation of cytoplasmic triacylglycerol from membranes during the preparation procedure. The distribution of phytanic acid in the lipid fractions from the membrane extract varied slightly from the whole cell results. The relative amount of phytanic acid in the triacylglycerol/cholesterol ester fraction was higher, 66% compared to 58%. There was less in the free fatty acid fraction, 1% compared to 8%. A similar percentage was found in the phospholipid fraction, 21% compared to 19%. Diacylglycerol and monoacylglycerol bands were detected and small amounts of phytanic acid were also present in these fractions (2% and 1% respectively). Phytanic acid was found to be present in the unidentified bands (6%) ("others combined").

4.3.4 Separation of Triacylglycerol From Cholesterol Ester and Estimation of the Relative Proportion of Phytanic Acid in These Fractions

The excess of triacylglycerol present in the phytanic acid supplemented cell lipid extracts resulted in the incomplete separation of triacylglycerol from cholesterol ester using solvent system A. Separation of whole cell extract from cells obtained in the first study (4.3.6) was repeated using solvent system B. Cholesterol ester was completely resolved from the broad triacylglycerol band. The staining intensity of the triacylglycerol band was not uniform throughout and appeared to be composed of a series of bands. This is probably due to a variety of triacylglycerol species present with
varying partition coefficients (see discussion, 4.6). FAME analysis of the triacylglycerol and cholesterol ester bands was performed and the amount of phytanic acid determined relative to a fixed amount of internal standard (25μl 21:0, 0.5 mg/ml). Of the total phytanic acid recovered from these two fractions, 86% was found in the triacylglycerol fraction. It can therefore be concluded that the triacylglycerol component contributed to the majority of the phytanic acid measured in the combined triacylglycerol/cholesterol ester bands in the studies described above.

4.3.5 Distribution of Phytanic Acid in Total Lipids of Y79 Cells

Y79 cells were seeded at 4.5 x 10^5 cells/ml in medium supplemented with 100 μmol/l phytanic acid and were cultured for 7 days, with one addition of fresh medium during this time. Whole cell lipids were extracted and separated by TLC using solvent system B. FAME analysis of the total lipid extract and separated lipid fractions was carried out as above. The separation profile of total cell lipids was similar to that of phytanic acid supplemented RPE cells with an extended triacylglycerol band. The cholesterol ester band was fully resolved from the triacylglycerol band by the solvent system used. The relative proportion of phytanic acid in the major lipid fractions was determined and was found to be comparable to the distribution of phytanic acid in RPE lipids with 60.3% of the phytanic acid present in the triacylglycerol fraction and 20.4% in the phospholipid fraction. Only 0.63% was present in the free fatty acid fraction and 1.4% in the cholesterol ester fraction which was lower than that observed for RPE cells. The percentage recovery of phytanic acid was 81%. These results indicated that phytanic acid taken up by the two retinal cell lines was treated in a similar manner, with a significant proportion incorporated into phospholipids, but the majority stored in the triacylglycerol fraction.

4.4 Neutral Lipid Staining

The granular like appearance developed by the retinal cell lines supplemented with phytanic acid was suggestive of an accumulation of lipid droplets in the cytoplasm. The results from the TLC analysis of lipid extracts from these cells indicated that the lipid droplets were likely to be composed of triacylglycerol. In order to verify the presence of neutral lipid within the cytoplasm, phytanic acid supplemented RPE and Y79 cells were
stained with Oil Red O, by the Histopathology Department at Great Ormond Street Hospital for Children NHS Trust (GOS).

Confluent RPE cells (LD7) were supplemented with ± 200 μmol/l phytanic acid for 7 days with one change of medium. Y79 cells, seeded at 4.5 x 10⁵ cells/ml, were supplemented with ± 100 μmol/l phytanic acid for 7 days with the addition of fresh medium after 3 days. Cells were harvested and washed with warmed FBS at 37°C (1x) and with PBS (3x) and were resuspended in PBS. Cytospin preparations of the cells were made and the cells were fixed for 1 to 2 minutes in formol-calcium acetate. After rinsing with tap water, the cells were stained in Oil Red O solution at room temperature for 1 hour. The slides were then briefly rinsed in 70 %ethanol, washed in tap water and counter stained with Carazzi’s Haematoxylin for 4-5 minutes before mounting in glycerin jelly.

The results of the neutral lipid staining are shown in figures 4-8 and 4-9. The RPE and Y79 cells supplemented with 200 μmol/l and 100 μmol/l phytanic acid respectively over 7 days stained strongly for neutral lipid, confirming its presence in the cytoplasm of these cells. There were a few cells within the population of the RPE control cells which stained for neutral lipid. These cells could have been macrophages present in the parent RPE cell line. Control Y79 cells showed minimal staining.

4.5 Uptake of Phytanic Acid into the Phospholipid Fraction of RPE Cells and Comparison to Uptake into the Total Cellular Lipid Pool

The uptake of phytanic acid into the phospholipid fraction of RPE cells as a marker for the uptake of phytanic acid into cellular membranes was investigated. A dose response study was carried out in which confluent parent RPE cells (LD7) were supplemented with 0 to 200 μmol/l phytanic acid for 7 days. Lipid extracts from whole cell preparations were separated using solvent system A and FAME analysis of the phospholipid band and of the total lipid extract was carried out for each concentration point. The amount of phytanic acid in the phospholipid fraction and total lipid extract relative to other fatty acids was determined as a % of total fatty acid peak area (figure 4-10). The relative concentration of phytanic acid in both the phospholipid and total lipid fractions increased with increasing phytanic acid supplementation. In the phospholipid
Figure 4-8: Oil Red O staining of neutral lipid in rat RPE cells.
a) Rat RPE cells (LD7) supplemented with 200 μmol/l phytanic acid.
b) Rat RPE cells (LD7) grown in control medium.
Figure 4-9: Oil Red O staining of neutral lipid in Y79 cells.
a) Y79 cells supplemented with 100 µmol/l phytanic acid.
b) Y79 cells grown in control medium.
Uptake of Phytanic Acid into RPE Whole Cells and Phospholipid Fractions

Figure 4-10: Uptake of phytanic acid by rat RPE cells (LD7) supplemented with 0 to 200 μmol/l phytanic acid over 7 days. Comparison of phytanic acid incorporation into the total lipid pool and phospholipid fractions.

n = 1 for each concentration point.
fraction phytanic acid increased from 0.3 % (at 0 μmol/l supplementation) to 15.3 % (at 200 μmol/l), whereas in the total lipid fraction the increase was from 0.6 % to 32.8 % at the same supplementation concentrations. Thus the increase in the relative concentration of phytanic acid in the phospholipid fraction was approximately half of that of the total lipid pool. The uptake of phytanic acid into the total lipid pool was similar to that obtained in crude membranes prepared from phytanic acid supplemented RPE cells (figure 4-5).

4.5.1 Comparison of Phospholipid and Total Lipid Fatty Acid Profiles
Figure 4-11 compares the fatty acid profiles of the RPE total lipid and phospholipid fraction following 0, 100 and 200 μmol/l phytanic acid supplementation The overall changes were similar for these two lipid fractions. An increase in the relative amount of phytanic acid was accompanied by a decrease in % of all the fatty acids analysed (16:1 (n7 and n9), 18:0, 18:1 (n7 and n9), 18:2n6, 20:4n6, 20:5n3 ,22:5n3 and 22:6n3) except 14:0 in the total lipid extract (an increase from 1.2 to 3.3 %), and 14:0 (from 0.7 to 1.2%) and 16:0 (20.8 % to 29.9 %) in the phospholipid fraction. In the total lipid extract, the change in 16:0 followed no significant trend.

The fatty acid profiles of the total lipids and the phospholipid fraction of Y79 cells following 100 μmol/l phytanic acid supplementation are shown in figure 4-12. Again the change in the relative amounts of fatty acids in the total lipid fraction was comparable to that seen in the phospholipid fraction and were also similar to those seen in the RPE cells. The increase in phytanic acid in the total lipid fraction was from 0.3 to 45.6 %. This was comparable to the increase in phytanic acid observed in crude membrane preparations from Y79 cells supplemented with 90 μmol/l phytanic acid (section 4.2.3). In the phospholipid fraction, phytanic acid increased from 2.8 % to 31.1 %, which was greater than that observed for RPE phospholipids, suggesting Y79 membranes can incorporate a greater amount of phytanic acid into their membrane lipids. A decrease in 16:1 (n7 and n9), 18:0, 20:4 n6 and 22:6n3 and an increase in 14:0 and 16:0 was found for total lipids and phospholipids of the phytanic acid supplemented Y79 cells compared to the controls.

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Figure 4-11: Fatty acid profiles of (a) total lipids and (b) phospholipids extracted from confluent rat RPE cells supplemented with 0, 100 and 200 μmol/l phytanic acid for 7 days. n = 1
Figure 4-12: Fatty acid profiles of (a) total lipids and (b) phospholipids extracted from Y79 cells supplemented with 0 and 100 µmol/l phytanic acid for 7 days. n = 1
4.6 Discussion

The alteration of membrane fatty acid composition of mammalian cell lines by supplementation of the culture medium with specific fatty acids is a well established technique. The lipids provided by the medium are utilised by cultured cells for the synthesis of membranes and suppress the endogenous biosynthesis of fatty acids and cholesterol by the cells (Spector and Yorek, 1985). The results of the studies described in this chapter have demonstrated that phospholipids (the major lipid component of cellular membranes) of cultured retinal cell lines can be enriched with phytanic acid using this approach which therefore provides an in vitro model for ARD in the retina. Cell and tissue cultures exposed to phytanic acid in the medium have previously been used for in vitro models of ARD. Bernstein et al (1992) supplemented bovine retinal pigment epithelial cells with phytanic acid as a model of the disease in the retina and Dubois-Dalcq et al (1972) used tissue culture of rodent spinal ganglia to model the disease in the nervous system. In neither of these two studies was the extent of phytanic acid uptake reported.

The present studies have shown that the uptake of phytanic acid by the bovine retinal derived cell line and the immortalised rat RPE cell line was time dependent, reaching a maximum after approximately 7 days. There appeared to be no difference in uptake of phytanic acid between confluent and non-confluent bovine retinal cells. A dose dependent uptake was also demonstrated in the rat RPE and Y79 cell lines. The maximum uptake by the Y79 cells occurred at a lower supplementation concentration than the rat RPE cells (approximately 100 µmol/l compared to 200 µmol/l respectively). A similar study was not undertaken for the bovine cell line. The concentration of phytanic acid incorporated into the phospholipid fraction of rat RPE cells relative to other fatty acids ranged from 0.3 % to 15.3 % and in total cellular lipids from 0.6 % to 32.8 % of the total peak area with 0 to 200 µmol/l supplementation over 7 days (see section 4.5). The levels achieved with the higher supplementation concentrations of phytanic acid are comparable to the concentrations of phytanic acid in the retina of two ARD patients which was reported to be 35.4 % and 9.6 % of total fatty acids (Levy, 1970). In these patients, the retinal phytanic acid concentration exceeded that in nerve
and brain tissue samples analysed, which ranged from 0.5% of total fatty acids in the cerebral cortex of one patient to 3.7% of total fatty acids in the sciatic nerve of the other. However, the greatest concentration of phytanic acid was in the liver (43.5%, 29.0%) and the heart (37.0%, 31.0%) of both these patients. Other reports on the phytanic acid concentrations in tissues of patients with ARD demonstrate the varying amounts to which phytanic acid accumulates between patients (Hansen, 1965; MacBrinn and O’Brien, 1968). From the limited data available, the retina does appear to accumulate relatively large amounts of phytanic acid compared to other tissues of the nervous system and hence may explain the earlier manifestation of the disease in this tissue.

In total cell lipid extracts the majority of phytanic acid was found in the triacylglycerol fraction (approximately 50%). This fraction appeared to be acting as a “sink” for the storage of excess phytanic acid taken up by the RPE cells even at low supplementation concentrations. The presence of a small amount of lipid droplets dispersed in the cytoplasm of most (if not all) cell types is a normal phenomenon. When however RPE cells were supplemented with phytanic acid, they developed a grainy appearance which was more pronounced at higher supplementation concentrations. An accumulation of neutral lipid droplets was demonstrated by histochemical techniques in the cytoplasm of supplemented RPE and Y79 cells. It therefore appears that these cell lines have a large capacity to store excess phytanic acid in the cytoplasm. The accumulation of triacylglycerol by cells supplemented with fatty acids in the culture medium has also been reported by others (refs. within Spector and Yorek, 1985) and thus appears to be a normal response to this form of treatment. Additionally, the accumulation of lipid droplets within the cytoplasm of RPE cells in vivo occurs as part of the normal functioning of these cells. Oil droplets in the cytoplasm of RPE cells from dark and light adapted rats have been observed (Baker et al., 1986) and were found to be composed principally of triacylglycerols with small amounts of retinol esters also present. These droplets were thought to be formed as a result of the clearance of phagosomes during the phagocytosis and recycling of shed photoreceptor outer segments by the RPE. Thus this cell type may be especially disposed to the storage of fatty acids taken up from the medium in the form of triacylglycerol.
The triacylglyceride that accumulated in the phytanic acid supplemented RPE and Y79 cells remained associated with crude membranes prepared by the ultracentrifugation of cell sonicates. The isolation of membranes free of triacylglyceride contamination would have required further work using a more sophisticated protocol, but this was not attempted in the present study. Other workers have developed methods for the isolation of membranes from fat-containing cells. For example, Belsham et al (1980) prepared plasma membranes from rat fat-cells by Percoll density gradient centrifugation and Moskalewski (1985) removed large fat droplets from chondrocytes by exposing the cells to cytochalasin B to disrupt actin filaments prior to Percoll centrifugation.

Yao and Dyck (1987) reported the presence of two additional bands (termed TG₁ and TG₂) that moved just ahead of the major triacylglycerol fraction (TGₐ) in a TLC separation of plasma nonpolar lipids of ARD patients. These bands corresponded to triacylglycerols containing one and two branched chain fatty acids respectively. In the study described in this chapter, the separation of neutral lipids of total cell lipid extracts of rat RPE cells supplemented with phytanic acid resulted in an extended TG band which appeared to be composed of more than one band. It is very likely that the extended band was composed of TG species containing varying numbers of phytanyl side chains. Yao and Dyck (1987) also reported the presence of triunsaturated phytanic acid in the plasma and erythrocytes of three ARD patients. This fatty acid was associated with the TGₐ and TG₁ fractions whilst saturated phytanic acid was associated with the TG₁ and TG₂ fractions. A monounsaturated phytanic acid species has also been reported in the serum and urine of ARD patients (Yao and Dyck, 1987). The origin of these unsaturated phytanic acid species is unknown. The presence of these different phytanic acid species was not investigated in this study.

The next largest proportion of phytanic acid was found in the phospholipid fraction (approximately 20 %). Phospholipids are the major lipid components of membranes and this result indicated that a substantial proportion of the phytanic acid was incorporated into cellular membranes. Such an alteration of membrane lipid structure might be expected to influence the function of those membranes. The lipid composition of nervous tissue from an ARD patient was described by McBrinn and O’Brien (1968). Phytanic acid was found to be present in the glycerophospholipids of all tissues.
examined (cerebral myelin, cerebral grey and white matter, spinal cord myelin and sciatic nerve). The greatest accumulation of phytanic acid was in the choline glycerophospholipids and it was established that phytanic acid was esterified exclusively in the Sn1 position of these phospholipids. It would thus be expected that phytanic acid incorporation into glycerophospholipids would displace the straight chain fatty acids that are normally found in the Sn1 position; 16:0 and 18:0 being the most common. However, the increase in the relative amount of phytanic acid in the phospholipid fraction of rat RPE and Y79 supplemented cells was accompanied by a decrease in the % of all the major long chain saturated and unsaturated fatty acids, except for 16:0 which increased. The medium chain fatty acid, 14:0, was also slightly increased. This implies that in this cell culture system phytanic acid may not be limited to incorporation into the Sn1 position. The relative increase in the % of 16:0 and 14:0 may occur to counterbalance the effect on membrane stability that the incorporation of a bulky multibranched fatty acid such as phytanic acid would have.

Some phytanic acid was found as free fatty acids (9 % in whole cell total lipids). Within cells there is a constant mobile population of fatty acids. Fatty acids are transported within cells bound to fatty acid binding proteins to sites of membrane renewal and repair. It is also possible that some fatty acids are freed from lipids by the action of lipases released during the preparation procedure.
5. THE ANALYSIS AND UPTAKE OF \(\alpha\)-TOCOPHEROL BY RETINAL CELL LINES

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5.1 Introduction
Investigations into the antimetabolite hypothesis involved the supplementation of retinal cell lines with α-tocopherol and the determination of α-tocopherol in the supplemented medium and retinal cell membranes. This chapter describes the method used for the analysis of α-tocopherol and its uptake of α-tocopherol by the RPE and Y79 cell lines. Initially protocols were developed for the analysis of α-tocopherol in red cell ghosts and were then modified for the analysis of α-tocopherol in crude membrane preparations of retinal cells. The analysis of α-tocopherol was performed using a normal phase high performance liquid chromatographic (HPLC) system with fluorimetric detection by a modification (Metcalf et al., 1989) of the method of Buttriss and Diplock (1983). This method had previously been established and validated for the analysis of serum and tissue α-tocopherol concentrations in the laboratories of the Biochemistry Unit, Institute of Child Health. This method has the advantage of using the same solvent for the mobile phase as used to extract α-tocopherol from biological samples. Thus extracts can be directly injected onto the HPLC column.

5.2 Materials
L-ascorbic acid (sodium salt), sodium chloride, all-rac-α-tocopherol, sodium dodecyl sulphate, di-sodium hydrogen orthophosphate-2-hydrate, sodium dihydrogen orthophosphate, bicinchoninic acid, bovine albumin protein standards, CuSO₄·5H₂O, (Sigma-Aldrich Co.); hexane and methanol of HPLC grade (Rathburn Chemicals Ltd. Walkerburn, Scotland); absolute ethanol A.R quality (Hayman Ltd. Witham, Essex).

5.3 Analysis of α-Tocopherol
5.3.1 Extraction Of α-Tocopherol
α-tocopherol was extracted from modified foetal bovine serum (FBS) and medium using the method for the extraction of serum samples (Buttriss and Diplock, 1983; Metcalfe et al., 1989). Extraction from red cell ghosts and retinal cell crude membrane preparations was based on the method of Burton et al (1985) involving solubilisation of membranes using sodium dodecyl sulphate (SDS).
5.3.1.1 Extraction from Biological Fluids
200 μl of α-tocopherol supplemented FBS or medium (see section 2.7.2) was vortexed with 1 ml of 75% ethanol for 40 seconds. The supplemented FBS was diluted 1 in 4 with dH2O before extraction. After addition of 1 ml hexane, the samples were vortexed for another 40 seconds. Phase separation was achieved by centrifugation at 2000 g and aliquots of the upper hexane phase were injected directly onto the HPLC column.

5.3.1.2 Extraction from Red Blood Cell Ghosts
Whole blood was centrifuged at 500 g for 5 minutes and the serum and buffy coat removed. The red blood cells (rbc) were washed 3 x with 150 mmol/l NaCl / 5 mM ascorbate solution, adjusted to pH 7.4 with 150 mmol/l Na2HPO4. When red cell concentrate was used (obtained from the blood bank service, GOS) the rbc were similarly washed. The rbc were resuspended in the same buffer, (approximately 1:1 rbc: PBS, v/v) and the haematocrit was measured using a Coulter Max M (Haemotology service, GOS). If cell suspensions were to be stored at -20°C, 1 mM EDTA was added to the buffered saline solution and the pH was adjusted to pH 7.4 with 150 mmol/l Na2HPO4. Cell lysis was achieved by the dropwise addition of 1 ml of the red cell suspension to 9 ml of 5 mM sodium phosphate buffer, pH 8.0. Samples were vortexed and then centrifuged at 2000 g for 15 minutes. The majority of the supernatant was removed without disturbing the pellet. 1 ml of 5 mmol/l sodium phosphate buffer with 5 mmol/l ascorbate, pH 7.4, was added, followed by 1 ml 80 mmol/l SDS, 2 ml of absolute ethanol and 1 ml hexane. Samples were vortexed for 40 seconds between each addition. Phase separation was achieved by centrifugation at 2000 g. Results were expressed as pmol/1 packed cell volume (pcv).

5.3.1.2.1 Reproducibility of Extraction of α-Tocopherol From Red Blood Cell Ghosts
The reproducibility of the extraction of α-tocopherol from rbc was determined. Normal rbc, obtained from the blood bank, were washed and resuspended in buffer as described above. 10 x 1 ml aliquots of the red cell suspension were extracted and α-tocopherol concentrations determined as described below (section 5.4). The mean rbc α-tocopherol concentrations in two separate studies were 3.6 and 5.6 μmol/l pcv with a coefficient of variation of 6.7 and 5.3 % respectively (n = 10 for both studies). These results
demonstrated that this method is reproducible for the determination of \( \alpha \)-tocopherol in fresh rbc.

5.3.1.3 *Extraction from Crude Membranes Pellets*
Crude membranes from cultured RPE and Y79 cells were prepared as described in 3.3.3. The protein concentration of the cell sonicate was determined using the bicinchoninic acid protein assay (see 5.3.2) prior to ultracentrifugation. Known volumes of sonicate were taken for ultracentrifugation. 1 ml 60 \( \mu \)M SDS was used to transfer the membrane pellet from the ultracentrifuge tube to 10 ml glass tubes. Samples were vortexed for 3 to 4 minutes to solubilise the membranes. 1 ml \( \text{dH}_2\text{O} \), 2 ml absolute ethanol and 1 ml hexane were added in succession. After each addition the samples were vortexed for 40 seconds. Phase separation was achieved by centrifugation at 2000 g. Results were expressed as nmol/mg total cell protein.

5.3.2 *Bicinchoninic Acid Protein Assay*
The bicinchoninic acid protein assay was based on the method of Smith et al (1985). An aliquot of sonicate (5 or 10 \( \mu \)l) was made up to 50 \( \mu \)l with \( \text{dH}_2\text{O} \) and 1 ml of bicinchoninic acid was added. The solution was mixed and incubated at 37°C for 10 minutes. 20 \( \mu \)l of 4 % (w/v) copper sulphate solution was added and the solution was further incubated at 37°C for 20 minutes. The absorbance at 562 nm was determined using a Cecil CE2040 2000 series spectrophotometer. The protein concentration was calculated using a standard calibration curve between the concentration range of 0 and 1 mg/ml using BSA as a standard.

5.3.3 *Analysis of \( \alpha \)-Tocopherol by HPLC*
\( \alpha \)-tocopherol extracts were chromatographed on a 3 \( \mu \) Apex silica column, 4.6 mm internal diameter, 15 cm length (Jones Chromatography Ltd.). The mobile phase was 1% methanol in hexane which was run at a flow rate of 1 ml/minute (pressure of 30 kPSI). 4A molecular sieves were used to remove \( \text{H}_2\text{O} \) from the mobile phase. \( \alpha \)-tocopherol was detected fluorimetrically (\( \lambda_{ex} = 290 \) nm, \( \lambda_{em} = 325 \) nm) using a Shimadzu RF-535 Fluorescence HPLC monitor and data was collected using a Spectra Physics SP4290 integrator. Injection volumes ranged from 5 to 20 \( \mu \)l.
5.3.4 Standardisation
\( \alpha \)-Tocopherol was quantified using all-rac-\( \alpha \)-tocopherol in hexane as the external standard. Single point calibrations were used, the concentrations varying from 5 to 100 \( \mu \text{mol/l} \) depending upon what was being measured.

5.3.4.1 Linearity of Standard Curve
A series of all-rac-\( \alpha \)-tocopherol standards (5 to 100 \( \mu \text{mol/l} \)) were analysed and the peak areas were plotted against concentration. Different attenuation settings on the fluorescence detector were used to accommodate the different standard concentrations (128 at 5 \( \mu \text{mol/l} \), 512 at 20 \( \mu \text{mol/l} \) and 1024 at 50 and 100 \( \mu \text{mol/l} \)). The peak areas were adjusted accordingly. Each standard was measured in duplicate. A typical standard curve is shown in figure 5-1 and was linear over the concentration range used.

5.3.5 Storage of Red Blood Cells for \( \alpha \)-Tocopherol Analysis
A study on the effect of storage at \(-20^\circ\text{C}\) on the concentration of \( \alpha \)-tocopherol in red cells was undertaken to determine storage conditions under which \( \alpha \)-tocopherol would be stable. Cells were initially stored as a suspension in phosphate buffered saline with 5 mmol/l ascorbate, but \( \alpha \)-tocopherol concentrations were found to significantly decrease after 8 days of storage from 5.6 to 2.3 \( \mu \text{mol/l} \) pcv (\( p < 0.001 \)) (figure 5-2a). The addition of 1 mM EDTA to the buffer resulted in consistent concentrations for at least 39 days (figure 5-2b). A fall in the rbc \( \alpha \)-tocopherol concentration was observed after 65 days from a starting concentration, at day 0, of 2.0 \( \mu \text{mol/l} \) pcv to 1.7 \( \mu \text{mol/l} \) pcv (\( p = 0.03 \)). EDTA is probably protecting \( \alpha \)-tocopherol by chelating iron ions from haemoglobin and thereby preventing limiting the production of free radical species through Fenton chemistry (see section 6.6.2).
Figure 5-1: Standard curve of all-rac-α-tocopherol between 5 and 100 µmol/l. 

\( n = 2 \) for each concentration point.
Figure 5-2:
a) α-tocopherol concentration (mean ± SEM) in red blood cell ghosts stored at -20°C in PBS with 5 mmol/l ascorbate over 8 days. n = 10 for each point.
b) α-tocopherol concentration (mean ± SEM) in red blood cell ghosts stored at -20°C in PBS with 5 mmol/l ascorbate and 1 mmol/l EDTA over 65 days. n = 10 for each point.
5.4 Uptake of α-Tocopherol by Retinal Cell Lines

The uptake of α-tocopherol by retinal cell lines was studied in order to determine the time and dose dependency characteristics of this process. The results from these studies were then used as a basis for the design of experiments investigating the effect of phytanic acid on the uptake of α-tocopherol described in chapter 7.

5.4.1 A Pilot Study

Uptake of α-tocopherol by cells exposed to α-tocopherol supplemented medium was assessed in a pilot study using the bovine retinal derived cell line. 3 x 75 cm² confluent flasks were grown in α-tocopherol supplemented medium prepared as outlined in section 2.7.2.1. Cells were fed every other day with freshly prepared supplemented medium and harvested on the 7th day after the initial feed. The mean α-tocopherol supplementation concentration over the three feeds was 90 μmol/l. 3 control flasks seeded from the same pool of cells were grown and maintained in unsupplemented medium over the same period of time, with changes of medium every other day. The α-tocopherol concentration in crude membrane preparations was determined as described in section 5.3. α-tocopherol measurements were calibrated using a 100 μmol/l standard and the results were expressed as nmol/flask. Results, shown in figure 5-3, indicated that cells readily incorporated α-tocopherol and values were similar between flasks. The mean α-tocopherol concentration in the supplemented group was 52.6 nmol/flask and in the control group, 0.1 nmol/flask.

5.4.2 Time Course Study of α-Tocopherol Uptake by Rat RPE (LD7)

Confluent rat RPE cells, LD7, were supplemented with α-tocopherol over a period of 13 days (mean concentration of 92 μmol/l). Medium was replaced on alternate days and cells were harvested at various time points during the study. Harvested cells were stored at -20°C under N₂ in PBS and crude membrane α-tocopherol concentrations were determined when all the samples had been collected. The results are shown in figure 5-4. The crude membrane α-tocopherol concentration increased steadily with increase in exposure time. At 0 days exposure, α-tocopherol was undetectable in the crude membrane extracts. After 1 day exposure the concentration had risen to 9 nmol/mg protein and after 7 days the concentration was 30 nmol/mg protein, rising to 46
Figure 5-3: Pilot study on the uptake of α-tocopherol by bovine retinal derived cells supplemented with 90 µmol/l α-tocopherol in the medium over a period of 7 days.
Figure 5-4: Uptake of α-tocopherol by confluent rat RPE cells (LD7) with time. Cells were supplemented with a mean α-tocopherol concentration of 92 μmol/l over 14 days and the medium was replaced on alternate days.

n = 1 for each time point.
nmol/mg protein after 13 days. From these results it was concluded that a 7 day exposure time would be adequate for studying the uptake of α-tocopherol by this cell line.

5.4.3 Effect of Increasing Concentrations of α-Tocopherol on its Uptake by the Rat RPE and Y79 Cell Lines

5.4.3.1 Rat RPE Cell Line (LD7)
Confluent rat RPE cells (LD7) were fed α-tocopherol supplemented medium (0 to 200 μmol/l) for 7 days and medium was replaced on alternate days. The results are shown in figure 5-5. Trace concentrations of α-tocopherol were detected in the crude membranes of cells cultured in the unsupplemented medium (mean concentration of 0.03 nmol/mg protein). An increase in the concentration of α-tocopherol in crude membrane preparations was observed with increasing supplementation concentration. At 85 μmol/l supplementation the mean α-tocopherol concentration was 29.5 nmol/mg protein. The curve began to plateau at this point. Triplicate flasks were grown at supplementation concentrations of 0, 21 and 85 μmol/l and gave reproducible results.

5.4.3.2 Y79 Cell Line
Y79 cells were seeded at 4.5 x 10^5 cells/ml in 8 ml α-tocopherol supplemented medium (0 to 150 μmol/l). Cells were grown for 7 days with the addition of a further 8 ml of fresh supplemented medium on day 4. Triplicate flasks were grown at 50 and 100 μmol/l supplementation concentrations. The results, shown in figure 5-6, demonstrated a dose dependent uptake of α-tocopherol by the Y79 cell line. The curve is a different shape from that of the RPE cell line (figure 5-5). The increase in uptake was linear between 0 and 75 μmol/l supplementation increasing from 0.0 to 8.4 nmol/mg protein. At 100 μmol/l there was a sharp increase in the crude membrane α-tocopherol concentration to 33.5 nmol/mg protein. The α-tocopherol concentration continued to rise linearly up to 46.3 nmol/mg protein at 150 μmol/l supplementation and a plateau was not reached.

5.4.4 Discussion
The method for α-tocopherol determination was initially developed using rbc ghosts as a model membrane system. It had originally been intended to study rbc from patients
Figure 5-5: The uptake of \( \alpha \)-tocopherol by the parent rat RPE cell line (LD7) with increasing supplementation concentrations. Cells were supplemented for 7 days with 0 to 183 \( \mu \text{mol/l} \) \( \alpha \)-tocopherol in the medium which was replaced on alternate days.

\( n = 1 \) for each concentration point except at 0, 21 and 85 \( \mu \text{mol/l} \) where \( n = 3 \). Mean value with min-max are shown for these points.
Figure 5-6: The uptake of α-tocopherol by the Y79 cell line with increasing supplementation concentrations. Cells were supplemented for 7 days with 0 to 150 μmol/l α-tocopherol in the medium with the addition of fresh medium on the 4th day.

n = 1 for each concentration point except at 50 μmol/l (n = 3) and 100 μmol/l (n = 2) and the mean value and min-max are shown for these points.
with peroxisomal disorders. The reproducible extraction and determination of \( \alpha \)-tocopherol from rbc ghosts was demonstrated and a protocol was established for the storage of whole rbc in which the \( \alpha \)-tocopherol concentration remained stable for at least 39 days. Stable storage conditions was achieved by the inclusion of EDTA in the storage buffer, demonstrating the importance of chelating metal ions in the protection of \( \alpha \)-tocopherol against oxidative stress.

The extraction protocol was modified for \( \alpha \)-tocopherol determination in cultured retinal cells. A time dependent uptake of \( \alpha \)-tocopherol by the rat RPE cell line and a dose dependent uptake by both the rat RPE and Y79 cell lines were demonstrated by the studies described above. The uptake of \( \alpha \)-tocopherol has previously been characterised by Kelley et al (1995) in a non-anchorage dependent lymphoblastic leukaemia cell line, L1210. The concentration of \( \alpha \)-tocopherol in unsupplemented cells was reported to be 0.2 ng/\( \mu \)g protein (0.46 nmols/mg protein). In the present study similar very low concentrations of \( \alpha \)-tocopherol were detected in unsupplemented rat RPE cells (0.03 nmol/ mg protein) whereas \( \alpha \)-tocopherol could not be detected in the control Y79 cells. These low levels are due to the low endogenous concentration of \( \alpha \)-tocopherol in normal culture medium (Kelley et al., 1995) which were below the detection limits of the present study.

The uptake of \( \alpha \)-tocopherol by confluent rat RPE cells plateaued after a supplementation concentration of 75 \( \mu \)mol/l over 7 days, whereas the uptake by the Y79 cell line did not plateau at the highest supplementation concentration of 150 \( \mu \)mol/l. This difference is probably due to the differences in the types of cells and the supplementation protocols used. The rat RPE cell line was supplemented with \( \alpha \)-tocopherol when confluent and the medium was replaced on alternate days. The Y79 cells was seeded in supplemented medium at a density that was chosen for good growth of the cells. After 4 days, the density of the Y79 cells was reduced with the addition of fresh supplemented medium. Hence these cells were maintained in a condition that favoured growth and division. In contrast to the uptake of \( \alpha \)-tocopherol by the Y79 cells, the uptake by the L1210 cells, reported by Kelley et al (1995), plateaued at 100 \( \mu \)mol/l supplementation. It should be noted however that the supplementation conditions
used by these workers were different to the ones in this study. The L1210 cells were supplemented with tocopherol acetate over a 24 hour period, the time point which resulted in maximum uptake.

The time course study using the rat RPE cell line demonstrated that the uptake of α-tocopherol by these cells was still increasing after 14 days of supplementation, with the greatest increase occurring over the first 6 days. It was therefore decided that for future studies a 7 day supplementation period with α-tocopherol would be adequate for this cell line which was also an appropriate time period for the study of phytanic acid uptake by the rat RPE and Y79 cell lines.
6. THE ANTIMETABOLITE HYPOTHESIS

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6.1 Introduction
The antimetabolite hypothesis is one of a number of proposals for the possible mechanism of pathogenesis in adult Refsum disease (see section 1.4). This hypothesis suggests that phytanic acid accumulation in cellular membranes may interfere with the function of vitamin E. Vitamin E plays an important part in the body's antioxidant defence systems and is believed to be the principal fat soluble secondary antioxidant in vivo thereby protecting cellular membranes against oxidative damage. In certain diseases, defective absorption or transport of vitamin E can lead to a prolonged and severe deficiency. A progressive degenerative disease ensues if the deficiency state is left untreated. The tissues primarily affected by severe vitamin E deficiency are the peripheral and central nervous system, the retina and skeletal muscles. The prominent symptoms observed are cerebellar ataxia, peripheral neuropathy and retinitis pigmentosa which are also three of the four main features of ARD (see 1.3.1.1). The symptoms of severe vitamin E deficiency are believed to be caused by a loss of its antioxidant protection. Vitamin E is anchored in membranes by its phytyl side chain and in order to serve as a membrane antioxidant, vitamin E needs to be positioned within the vicinity of polyunsaturated fatty acids, which are the substrate for lipid peroxidation. It is conceivable that the incorporation of phytanic acid into membrane phospholipids may interfere with the positioning or uptake of vitamin E into membranes resulting in an induced vitamin E deficiency state.

6.2 Vitamin E

6.2.1 The Vitamin E Chemical Family
Vitamin E is the common name given to a group of related lipophilic molecules that exhibit the biological activity of RRR-α-tocopherol. Members of this chemical family possess a chromanol ring structure and a saturated or unsaturated phytyl side chain giving rise to the tocopherol or tocotrienol subgroups respectively (see figure 6-1). There are four major types of tocopherol or tocotrienol (termed α-, β-, γ-, and δ-) which differ in the pattern of methyl group substitution on the chromanol ring. In addition eight stereoisomeric forms are possible for each tocopherol species due to the presence of three chiral centres at the 2-position on the chromanol ring and the 4’ and 8’ positions.
Figure 6-1: The structures of the tocopherol and tocotrienol forms of vitamin E.
of the side chain. The natural form of α-tocopherol is the RRR stereoisomer, whereas synthetic α-tocopherol consists of an equal mixture of all eight stereoisomers. The tocotrienols have a chiral centre at the 2-position giving rise to two possible stereoisomers and geometric isomerisation is possible at the 3' and 7' double bonds of the side chain.

6.2.2 Biological Activity, Absorption, Transport and Turnover of Vitamin E
Variable biological activity is exhibited by the different members of the vitamin E family and their stereoisomers. The biological activity of vitamin E is classically measured using the rat foetal resorption assay. In pregnant female rats a deficiency of vitamin E results in resorption of the foetus into the lining of the womb and hence a failure to produce live young. The foetal resorption assay measures the ability of a test compound administered to pregnant rats reared on a vitamin E-free diet to reverse this effect of vitamin E deficiency. The activities of the compounds tested are compared to that of RRR-α-tocopherol which exhibits the greatest biological activity. Examples of the relative activity of different forms of vitamin E are given in table 6-1.

The in vitro antioxidant activity of the different forms of vitamin E does not always appear to correlate with their biological activity. For instance, γ-tocopherol has been shown to have greater antioxidant activity than α-tocopherol in vitro whereas in vivo it is the α-form that has the greater activity as determined by the rat foetal resorption assay (Tran and Chan, 1992). The in vitro antioxidant activity of vitamin E is determined by the chromanol ring and is not influenced by the structure of the phytol tail (Burton and Traber, 1990). The difference in the biological activities in vivo compared to in vitro antioxidant activity of the different forms of vitamin E and their stereoisomers may be explained by selective differences in their transport to and retention by tissues. This difference is partly determined by the structure of the phytol side chain.

Studies on the absorption, transport, and turnover of vitamin E in vivo have been carried out using large, unphysiological doses of unlabeled vitamin E, radiolabeled tocopherols (the use of which is restricted to animals) and deuterated tocopherols. Deuterated tocopherols have been particularly useful for the study of the transport and turnover of different isomers of α-tocopherol in humans as well as in animals using physiological
<table>
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<th>Activity Compared to RRR-α-Tocopherol</th>
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<td>90%</td>
</tr>
<tr>
<td>SRS-α-tocopherol</td>
<td>37%</td>
</tr>
</tbody>
</table>

Table 6-1: Examples of the biological activity of different forms of vitamin E relative to RRR-α-tocopherol as determined by the rat resorption assay (taken from VERIS vitamin E abstracts, 1993).
concentrations (Burton and Traber, 1990). Taken together, the results from the various studies have shown that the intestinal absorption and secretion into chylomicrons of α- and γ- tocopherol in humans (Traber and Kayden, 1989) and of the 2R- and 2S- isomers of α-tocopherol in humans (Traber et al., 1990a) and rats (Kiyose et al., 1995) are comparable. However it has been shown that the liver preferentially secretes RRR-α-tocopherol over other forms of vitamin E as a component of very low density lipoproteins (VLDL) (Traber et al., 1990a; Traber et al., 1990b) and consequently plasma and thus tissues become enriched in this form of vitamin E. The preferential incorporation of RRR-α-tocopherol into VLDL is attributed to the selective activity of the hepatic α-tocopherol transfer protein. This discrimination by the liver may explain the greater biological activity exhibited by RRR-α-tocopherol compared to other forms of vitamin E (as determined by the rat resorption assay).

In addition to an enrichment in the plasma of RRR-α-tocopherol, there is some evidence that the turnover of the different forms of vitamin E in tissues varies. For instance Cheng et al (1987) demonstrated that rat red blood cell membranes preferentially retain RRR-α-tocopherol compared to the SRR form. Better cellular retention of α-tocopherol compared to γ-tocopherol by human endothelial cells was also suggested by results of an in vitro study (Tran and Chan, 1992). Studies in rats and guinea pigs using deuterated tocopherols have shown that there is also a difference in turnover of RRR-α-tocopherol between tissues (Burton and Traber, 1990). For example, plasma, red blood cells, liver and spleen have a higher rate of turnover than the heart, testis, muscle (biceps femoris), with neural tissues (brain and spinal cord) having the slowest turnover.

6.3 Antioxidant Function of Vitamin E

As mentioned above, vitamin E plays an important role as a component of the biological antioxidant defence systems. Living organisms require protection from the highly reactive oxygen derived free radicals, such as superoxide (O$_2^-$) and hydroxyl radicals (OH$^-$), that are constantly being produced as a result of aerobic metabolism. For instance, the electron transport systems of the mitochondria (involved in oxidative phosphorylation) and the endoplasmic reticulum (cytochrome P$_{450}$) produce free radical intermediates, including superoxide, during the reduction of molecular oxygen to water.
by the consecutive addition of single electrons. Oxygen derived free radicals are also produced by macrophages during their attack on phagocytosed pathogens such as bacteria. Leakage of free radicals from their site of production may lead to cellular damage. Cells contain a host of antioxidants that either prevent the formation of oxygen derived free radicals, or, like vitamin E, convert free radicals into less reactive species.

6.3.1 Lipid Peroxidation
Lipid peroxidation is a chain reaction (see figure 6-2) initiated when fatty acids containing two or more double bonds are attacked by free radical species such as hydroxyl radicals (OH⁻), that are capable of abstracting a hydrogen from a methylene group (-CH₂-) adjacent to a carbon-carbon double bond. The resulting fatty acyl radicals that are formed can react with molecular oxygen and produce lipid peroxyl free radicals. Lipid peroxyl free radicals are sufficiently reactive to abstract further hydrogen atoms from other polyunsaturated acyl chains. This results in the formation of another lipid peroxyl free radical and a stable lipid hydroperoxide. This is the propagation stage of the chain reaction. Termination will occur if two free radicals react to form stable products or if the lipid peroxyl radicals are converted to stable peroxides by the donation of a hydrogen atom by some other compound (an antioxidant) without the further production of lipid peroxyl radicals.

Vitamin E (T-OH) acts by scavenging fatty acid peroxyl free radicals (LOO⁻) and thereby terminating the chain reaction of lipid peroxidation. It does this through the donation of the phenolic hydrogen atom from the chromanol ring to the peroxyl radical, forming a lipid hydroperoxide (LOOH) and in the process it is itself converted into a free radical (T-O⁻).

\[
\text{LOO}^- \ + \ T-OH \ \rightarrow \ \text{LOOH} \ + \ T-O^- \quad \text{(Eq. 6-1)}
\]

Vitamin E is a very efficient radical trap as;
a) the scavenging of lipid peroxyl radicals by vitamin E occurs more readily than the abstraction of hydrogens from polyunsaturated acyl chains by the peroxyl radical in the propagation stage of lipid peroxidation and
Abstraction of a hydrogen initiates lipid peroxidation

Rearrangement of the fatty acyl radical results in the formation of a conjugated diene

Reaction with molecular oxygen produces a lipid peroxyl free radical

Abstraction of a hydrogen from a neighbouring polyunsaturated acyl chain propagates the chain reaction

Alternatively, donation of a hydrogen by vitamin E terminates the chain reaction. In both cases a lipid hydroperoxide is produced.

Figure 6-2: Lipid peroxidation.
b) the vitamin E free radical is relatively stable due to delocalisation of the unpaired electron into the aromatic ring structure and is unable to abstract hydrogens from neighbouring polyunsaturated fatty acids.

There are several fates that the vitamin E free radical can undergo. It can spontaneously rearrange to form a quinone or it can react further with a lipid peroxyl radical to form a non-radical product (equation 2).

\[
\text{LOO}^* + \text{T-O}^* \rightarrow \text{non-radical product} \quad \text{(Eq. 6-2)}
\]

It has also been hypothesised that vitamin E may be regenerated from its free radical by reduction with cytosolic antioxidants. The main candidate for this action is vitamin C (ascorbic acid) and whilst evidence has been provided \textit{in vitro} for a sparing effect of vitamin E by vitamin C, convincing evidence for it occurring \textit{in vivo} is yet to be forthcoming. The regeneration of vitamin E by vitamin C was first proposed by Tappel (1968) and in 1979 Packer et al provided the first evidence for this \textit{in vitro} with the report that tocopheroxyl radicals in organic solvent decayed rapidly in the presence of ascorbic acid. Further \textit{in vitro} studies involving the use of liposomes or microsomes have demonstrated synergistic antioxidant protection by vitamins E and C (see Reed, 1993 for a review). It has also been shown that in human platelet homogenates, oxidation of tocopherol induced by arachidonic acid could be reversed by both exogenous vitamin C and glutathione (Chan, 1993). Studies \textit{in vivo} have looked at the effect of dietary vitamin C on plasma and tissue vitamin E levels in the rat and guinea pig and on the symptoms of vitamin E deficiency in premature infants (refs within Burton et al., 1990). The results from these studies have not however provided conclusive evidence, with both synergistic and antagonistic effects being reported (Burton et al., 1990). Burton et al (1990) studied the rate of turnover of deuterated RRR-\alpha-tocopherol in the tissues of guinea pigs fed different amounts of vitamin C and deuterated RRR-\alpha-tocopherol over an 8 week period. The results from this study failed to produce evidence of a sparing effect of vitamin C on vitamin E as the turnover rates were comparable between the groups.
Other antioxidant molecules and systems have also been implicated in the regeneration of vitamin E by a number of _in vitro_ studies. These include glutathione and ubiquinones, and NADH and NADPH dependent electron transport enzymes (Packer and Kagan, 1993; Fuchs and Packer, 1993). The relevance of these observations to the regeneration of vitamin E _in vivo_ remains to be determined.

### 6.4 Other Functions of Vitamin E

The principal role of vitamin E is thought to be that of a lipid antioxidant maintaining the structural integrity of membranes by protecting phospholipid bound PUFAs from lipid peroxidation and additionally, protecting membrane proteins from damage by lipid peroxides. However, in addition to this role, other functions for this vitamin have been suggested which may or may not involve its antioxidant activity. For instance, it has been proposed that vitamin E regulates membrane fluidity and hence membrane stability, influences the synthesis of prostanoids, and modulates the activity of protein kinase C. A brief overview of these three proposed actions is given below.

#### 6.4.1 Regulator of Membrane Fluidity

The proposed role of vitamin E as a stabiliser of membrane structures has been investigated by studying the effect of vitamin E on the fluidity of membranes. Membrane fluidity is a term that refers to the ease of movement within membranes of lipids and proteins and is related to the structure and interactions of these components. There may be several different modes of movement of individual membrane components and techniques that measure "membrane fluidity" are usually specific to one particular type of motion. Membrane fluidity will be discussed in greater detail in chapter 9.

The effect of vitamin E on membrane fluidity has been studied using a number of different membrane systems including liposomes and isolated plasma and intracellular membranes. The results from these studies have been reviewed by Zimmer et al (1993). It appears that vitamin E affects the fluidity of membranes in two ways. The first is through a physical interaction with neighbouring phospholipid acyl chains. Phospholipid bilayers can exist in a gel-like or ordered state or, as the temperature is raised, in a more disordered or liquid-crystalline state. The results of studies using
liposomes have shown that like cholesterol, α-tocopherol has a disruptive effect on phospholipid bilayers when the lipids are in a gel-like or ordered state. When however the lipids are in a liquid-crystalline state, α-tocopherol acts like a wedge or a space filler and reduces fluidity. However, as pointed out by Zimmer et al (1993), the concentrations of vitamin E used in these studies are higher than those observed in tissues and the physiological significance of these findings are therefore questionable. A membrane stabilising effect of α-tocopherol has been demonstrated in isolated frog rod outer segments and liposomes prepared from lipids from these membranes using fluorescence anisotropy techniques with the probe diphenylhexatriene (DPH) (see section 9.2) (Moran et al., 1987). Thus vitamin E may have a dual role in photoreceptor outer segments firstly as an antioxidant and secondly as a membrane stabiliser.

The second way in which vitamin E affects membrane fluidity is through the protection of unsaturated acyl chains from lipid peroxidation. It has been shown for instance that when microsomes are exposed to oxidative stress there is a decrease in the concentration of arachidonic (20:4n6) and docosahexaenoic (22:6n3) acid, and an increase in membrane order (i.e. a decrease in membrane fluidity). This effect on both PUFA loss and membrane order was suppressed by the pre-treatment of microsomes with α-tocopherol (Zimmer et al., 1993). Thus if the unsaturated lipids are protected from peroxidation by the action of vitamin E, the physical state of the membrane, including fluidity, is maintained.

The physical and the antioxidant influences of vitamin E on membrane fluidity could explain the discrepancy in results from isolated plasma and organelle membrane studies where both increases and decreases in membrane fluidity have been reported. Many of these studies have looked at the effect of vitamin E on the fluidity of membranes that have been exposed to oxidative stress. Zimmer et al (1993) suggested that the decrease in membrane fluidity observed in some studies was due to the ordering effect of high, nonphysiological concentrations of vitamin E. At lower, physiological concentrations the effect of vitamin E on the fluidity of membranes exposed to oxidative stress (an increase in fluidity) appeared to be due to its antioxidant activity.
6.4.2 Modulator of Prostanoid Production

Prostanoids are local cell mediators that are synthesised by cyclooxygenases from arachidonic acid (AA) (20:4n6) after it is released from membrane phospholipids by phospholipase A2. It has been shown that the synthesis of prostanoids can be modulated by oxidants which promote the action of phospholipase A2 and also either stimulate or inhibit the activity of cyclooxygenase (Cornwell and Panganamala, 1993). In particular, lipid hydroperoxides have been shown to activate the cyclooxygenase enzyme complex (Cornwell and Panganamala, 1993). The action of oxidants on cyclooxygenase activity is dependent on a number of variables such as the concentration of oxidants and antioxidants in the incubation mixture and the length of exposure time to the oxidant.

As oxidants have been shown to modulate prostanoid synthesis it can be expected that antioxidants would also exert an influence. Under certain conditions antioxidants may inhibit synthesis by reducing the concentration of oxidant species, but under other conditions they may enhance synthesis by protecting cyclooxygenase from oxidative inactivation to which it is susceptible (Cornwell and Panganamala, 1993).

Investigations have been carried out to determine the effect of vitamin E on the synthesis of prostanoids and the results of these studies have been reviewed by Cornwall and Panganamala (1993). It has been shown using in vitro studies, that the effect of vitamin E varies, from having no effect on prostanoid synthesis in isolated microsomes and smooth muscle cells, to having an inhibitory effect in platelets and a stimulatory effect in endothelial cells. Cornwall and Panganamala (1993) have proposed that lipid peroxide as a component of lipoproteins is able to stimulate prostanoid synthesis, whereas membrane bound lipid peroxides are unlikely to exert an effect. Small amounts of mildly oxidised lipoproteins were shown to stimulate prostanoid synthesis in smooth muscle cells in culture, whereas high amounts had an inhibitory effect. The degree of lipoprotein oxidation is partly determined by the lipoprotein vitamin E concentration. Thus the modulation of prostanoid synthesis by vitamin E is probably mediated through its antioxidant activity and its protection of lipoprotein lipids from peroxidation.

6.4.3 Modulator of Protein Kinase C Activity

Protein kinase C (PKC) is an enzyme involved in the signal transduction pathways within cells that govern events such as growth, differentiation and secretion. The role of
cell proliferation in diseases such as atherosclerosis and hypertension and the possible protective effect of antioxidants in these diseases has led to interest on the effect of antioxidants on cell proliferation. Azzi and co-workers have investigated the effect of vitamin E on PKC activity (Azzi et al., 1993). They found that at physiological concentrations, α-tocopherol inhibited the activity of PKC isolated from rat brain. This effect was not seen with trolox, a water soluble analogue of vitamin E, or the lipid soluble anti-oxidant butylated hydroxytoluene. At similar concentrations, α-tocopherol was also shown to inhibit the proliferation of vascular smooth muscle cells in vitro and this effect correlated with the inhibition of PKC. The inhibition of PKC by α-tocopherol was observed only when certain mitogens were added to serum free medium to stimulate cell growth. This suggests that α-tocopherol inhibits specific pathways that are necessary for proliferation.

The modulation of PKC activity by α-tocopherol does not appear to be due to its free radical scavenging activity. RRR-β-tocopherol, in contrast to RRR-α-tocopherol has been shown to have no effect on vascular smooth muscle cell proliferation, although its uptake by cells was comparable and it has similar antioxidant activities (Tasinato et al., 1995). The inhibition of proliferation by RRR-α-tocopherol could be prevented by co-incubation of the cells with RRR-α-tocopherol together with RRR-β-tocopherol. Thus the inhibitory effect of α-tocopherol on proliferation is more likely to involve a site-directed recognition mechanism rather than antioxidant activity.

6.5 Vitamin E Deficiency in Man

Vitamin E, like other fat constituents of the diet, is solubilised into mixed micelles by bile acids and absorbed by enterocytes lining the small intestinal lumen. It is then incorporated into chylomicrons (CM) and transported to the liver via the mesenteric lymphatic system and the systemic circulation (Sokol, 1993). In the liver vitamin E is incorporated into VLDL which are secreted into the bloodstream. Lipoprotein lipase (LPL) is an enzyme bound to the endothelial lining of capillary walls that hydrolyses triglycerides in CM and VLDL. This enzyme has been shown in vitro to mediate the transfer of vitamin E from chylomicrons to cells in culture (Burton and Traber, 1990) and thus may represent a mechanism whereby vitamin E is delivered to tissues in vivo.
As CM and VLDL triglycerides (which are located in the core of the particle) are hydrolysed, excess material from the surface of the particle is transferred to high density lipoproteins (HDL). Some vitamin E is probably transferred to HDL along with this material. Vitamin E may also transfer to HDL by spontaneous exchange. This mechanism has been shown to occur between different lipoproteins and between lipoproteins and erythrocytes. The products of VLDL metabolism are intermediate and low density lipoproteins (IDL and LDL respectively). Vitamin E, as a component of IDL and LDL, may be taken up by peripheral tissues via the high affinity apolipoprotein B/E receptor mediated pathway (Muller and Goss-Sampson, 1990).

In man, defects in the absorption and transport processes of vitamin E can result in a deficiency state. Deficiency due to malnutrition is rare as vitamin E is found in a variety of dietary sources such as grains and vegetable and animal fats. The diseases in which vitamin E deficiency has been identified as a secondary effect of fat malabsorption or impaired transport are abetalipoproteinaemia, chronic cholestatic hepatobiliary diseases, cystic fibrosis, and other fat malabsorption disorders involving the small intestine such as short bowel syndrome (Harding, 1987; Sokol, 1993). A condition with an isolated vitamin E deficiency in the absence of generalised fat malabsorption has also been identified (Harding, 1987). The severity of vitamin E deficiency and the clinical manifestations in these conditions may vary. The signs and symptoms associated with severe (i.e. undetectable or trace plasma concentrations) and prolonged vitamin E deficiency include a progressive cerebellar ataxia, areflexia, a distal loss of proprioception (position sense), loss of vibration sense, ophthalmoplegia (paralysis of one or more of the optic muscles), ptosis (drooping of the upper eyelids), pes cavus (an exaggeration of the normal arch of the foot), scoliosis (lateral curvature of the spine), a pigmentary retinopathy and a generalised myopathy (Muller and Goss-Sampson, 1990).

Abetalipoproteinaemia is a rare autosomal recessive disease in which production of apolipoprotein B-containing lipoproteins (including CM, VLDL and LDL) by the intestine and liver is impaired, leading to defective transport of lipids. The defective gene involved has been identified as that encoding a microsomal triglyceride transfer protein (Sharp et al., 1993; Shoulders et al., 1993) which is involved in the assembly of
lipoproteins. In untreated cases, vitamin E serum and tissue levels are undetectable. Neurological symptoms usually develop in the second decade of life (Sokol, 1993). This is also the case for hypolipoproteinaemia, an autosomal dominant disease in which homozygotes develop an identical neurological syndrome to that seen in abetalipoproteinaemia (Harding, 1987) and is caused by defects in the gene encoding apolipoprotein B (Collins et al., 1988; Young et al., 1988). Similarly, in children with chronic cholestatic hepatobiliary diseases, where bile flow is reduced, similar neurological symptoms appear within the first few years of life. Appropriate treatment with vitamin E can prevent the onset of all signs and symptoms if implemented early enough. If supplementation is commenced after the onset of the characteristic features, progression is invariably halted and in some cases reversed.

In cystic fibrosis, a deficiency of pancreatic enzyme secretion causes fat malabsorption, but although reduced levels of vitamin E are common in unsupplemented patients, a severe deficiency and neurological symptoms usually occur only in conjunction with liver dysfunction or small bowel resection (Sokol, 1993). Vitamin E deficiency acquired in adulthood has generally occurred as a result of resection of the small bowel in the treatment of gastrointestinal diseases such as Crohn's disease (Harding, 1987).

Ataxia with isolated vitamin E deficiency (AVED) is an autosomal recessive neurological disorder that occurs as a result of an impaired ability to incorporate α-tocopherol into hepatically derived lipoproteins. In this disease gastrointestinal absorption of vitamin E is normal, but vitamin E reaching the liver is not resecreted into the blood and circulating concentrations are low. Mutations in the gene for the hepatic α-tocopherol transfer protein, which mediates the incorporation of α-tocopherol into VLDL, have been identified as the underlying cause of the disease (Gotoda et al., 1995; Ouahchi et al., 1995). The neurological disorder and response to treatment is similar to that seen in the other vitamin E deficiency states.

In addition to the evidence from human disease, numerous animal studies have also demonstrated that nervous, retinal, and muscular tissues are affected by a deficiency of vitamin E, the neuropathy being similar to that observed in man (Diplock, 1985; Muller
and Goss-Sampson, 1990). A number of other symptoms of vitamin E deficiency are observed in some species of animals that are not seen in humans such as muscular dystrophy (Diplock, 1985).

6.6 Comparison of Severe Vitamin E Deficiency to Adult Refsum Disease

The main clinical features shared by severe vitamin E deficiency and ARD are a peripheral neuropathy, cerebellar ataxia, muscle weakness and retinitis pigmentosa-like changes of the retina. Both diseases are progressive and the peripheral neuropathy appears to be similar with loss of reflexes, vibration sense and proprioception. Pes cavus, one of the minor symptoms of adult Refsum disease also occurs in severe vitamin E deficiency. The overlap of symptoms in the two disease states is not complete, for instance ichthyosis is not seen in severe vitamin E deficiency.

6.6.1 Neuropathology in Severe Vitamin E Deficiency

The neuropathological changes that occur in severe vitamin E deficiency are that of a distal dying back axonopathy (Muller and Goss-Sampson, 1990; Sokol, 1993). It is characterised by swollen dystrophic axons and an accumulation of lipofuscin in dorsal sensory neurons and in the cytoplasm of Schwann cells in peripheral nerves. The distal portions of axons suffer greater degenerative changes than regions more proximal to the nerve cell body. The demyelination that is observed is thought to be secondary to a primary axonopathy. Sensory neurons are more affected than motor neurons and the central nervous system more so than the peripheral. In the central nervous system the axonopathy was most prominent in the fasciculus cuneatus and fasciculus gracilis of the posterior column of the spinal cord and particularly in the cervical region. In the peripheral nervous system a selective loss of large calibre myelinated sensory axons was observed.

The exact mechanism for this neuronal degeneration has not been determined but there is evidence that an impairment of axonal transport is involved. The supply of cytoplasmic organelles and substances to the axon from the nerve cell body and the return of materials for recycling or disposal is carried out by anterograde and retrograde
transport processes respectfully. An accumulation of material such as abnormal and normal cytoplasmic organelles and multivesicular bodies has been observed in the sensory axon terminals of vitamin E-deficient rats (Muller and Goss-Sampson, 1990). In addition, axonal transport was shown to be reduced in rats after 50 weeks of vitamin E deficiency (Muller and Goss-Sampson, 1990). It has been hypothesised that the impairment of neuronal axonal transport observed in severe vitamin E deficiency may result from impaired mitochondrial function (MacEvilly and Muller, 1996). Mitochondrial membranes are normally enriched in vitamin E and contain relatively large amounts of polyunsaturated fatty acids. Mitochondria may be subjected to increased oxidative stress due to the production of oxygen-derived free radicals during oxidative phosphorylation. Thus the lack of lipid antioxidant protection in mitochondria, as would occur during vitamin E deficiency, would render mitochondria more vulnerable to lipid peroxidation. An impairment in the production of ATP by mitochondria would disrupt ATP dependent processes including the anterograde and retrograde transport systems. This could cause an accumulation of organelles etc. at the terminal end of the axon and result in “plugging” of the axons. This would isolate the terminal axons from the cell body and account for the distal dying back of neurons observed in vitamin E deficiency. It has been shown that the organelles, including mitochondria, isolated from myelinated nerves of vitamin E deficient rats are particularly sensitive to in vitro oxidative stress (MacEvilly and Muller, 1996). It has also been observed that mitochondria from vitamin E deficient rats show signs of membrane damage together with a specific reduction in the activity of the respiratory chain complexes I and IV (Thomas et al., 1993).

Thus although on a macroscopic scale the neuropathology in severe vitamin E deficiency appears to be similar to that occurring in ARD, the underlying events are different. As detailed earlier (see section 1.4.2), the neurodegenerative changes in ARD suggest a primary defect in Schwann cell function resulting in demyelination. In ARD, sensory and motor nerve conduction velocities are reduced (Refsum et al., 1984) whereas in vitamin E deficiency conduction velocities in peripheral sensory nerves are usually normal but action potential amplitudes are diminished (Muller and Goss-Sampson, 1990; Sokol, 1989).
6.6.2 Retinal Degeneration in Vitamin E Deficiency

The vertebrate retina including the RPE is a tissue that is particularly susceptible to oxidative stress for several reasons (Handelman and Dratz, 1986). Firstly, the photoreceptor outer segment membranes are highly polyunsaturated and are thus more susceptible to lipid peroxidation (see section 1.7.3). In addition the vertebrate retina has been shown to have a high oxygen consumption in comparison to other tissues. It is likely that a high oxygen tension in the retina is necessary to meet the metabolic demand of photoreceptor inner segments which contain abundant mitochondria. A high oxidative metabolic activity in the retina increases the risk of leakage of oxygen-derived free radicals produced by oxidative phosphorylation. In the human retina 70 - 80% of oxygen that reaches the neural retina passes through the RPE. As the RPE phagocytose and recycle the highly unsaturated photoreceptor outer segment membranes, this layer of cells may also be particularly vulnerable to oxidative stress. A third stress factor is the exposure of the retina to light. It has been suggested that light reaching the retina may increase the production of free radical species. In the eye of mature humans the wavelength of light reaching the retina is ≥ 400 nm, ultraviolet light being filtered out by the lens. Light of ≥ 400 nm cannot break covalent bonds but may act as a photosensitiser producing singlet oxygen or superoxide anion, or produce damage through thermal mechanisms (Handelman and Dratz, 1986).

The retina and RPE, like all other tissues contain a host of antioxidant defence mechanisms that protect against oxidative damage. For instance glutathione peroxidase, superoxide dismutase and catalase activity has been demonstrated in the retina of various animals (Handelman and Dratz, 1986). These enzymes are involved in the removal of various oxidant species. Glutathione peroxidase catalyses the reduction of peroxides to alcohols using glutathione (GSH) as the reductant.

\[
\text{e.g. } \quad \text{H}_2\text{O}_2 + 2\text{GSH} \rightarrow \text{GSSG} + 2\text{H}_2\text{O} \quad \text{(Eq. 6-3)}
\]

Superoxide dismutase (SOD) converts the reactive superoxide radical \((O_2^-)\) to hydrogen peroxide.

\[
2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad \text{(Eq. 6-4)}
\]
There are two forms of this enzyme. Reports on SOD activity in the retina (bovine and
dog) indicate that only the Cu-Zn form, which is situated in the cytoplasm, is present,
and not the mitochondrial Mn SOD (Handelman and Dratz, 1986).

Catalase, like glutathione peroxidase, removes hydrogen peroxide from cells.

\[
2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2 \quad \text{(Eq. 6-5)}
\]

Hydrogen peroxide is potentially harmful to cells as it can be converted to the highly
reactive hydroxyl (\(\text{OH}^*\)) radical by, for example, reacting with \(\text{Fe}^{2+}\) in what is known as
the Fenton reaction.

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^* + \text{OH}^- \quad \text{(Eq. 6-6)}
\]

Vitamin E is also found in both the retina and RPE and has been shown to be necessary
for the maintenance of a healthy retina in a number of animal models. Damage to the
retina occurs in the vitamin E-deficient rat, monkey and dog (Handelman and Dratz,
1986; Trevithick et al., 1993) in which degeneration of the central photoreceptor outer
segments is an early event (Anderson et al., 1984). In man, severe vitamin E deficiency
is also associated with retinal degeneration. Untreated patients with fat malabsorption
disorders suffer from a generalised deficiency of the fat soluble components of the diet.
Thus the symptoms observed in these diseases may not be due to the deficiency of a
single component. Indeed a combined deficiency of the fat soluble vitamins E and A,
appears to exacerbate the retinal degeneration (Muller and Goss-Sampson, 1990). A
deficiency of vitamin A alone can also cause photoreceptor degeneration (Trevithick et
al., 1993). A direct role of vitamin E deficiency in retinal degeneration has been shown
in patients who develop retinal abnormalities despite being supplemented with and
having normal levels of vitamin A, but are vitamin E deficient (Muller and Goss-
Sampson, 1990). It has also been reported that the treatment of a patient with fat
malabsorption, with large doses of vitamin E resulted in an improvement in retinal
function (Muller and Goss-Sampson, 1990). Vitamin E treatment in abetalipoproteinaemia has also been shown to halt the progression of, or prevent, retinal degeneration (Trevithick et al., 1993). Additional evidence for a specific role of vitamin E comes from patients with isolated vitamin E deficiency in whom retinitis pigmentosa (Yokota et al., 1997) and retinopathy (Rayner et al., 1993) have been reported.

It is generally thought that the retinal degeneration that occurs in vitamin E deficiency is caused through the loss of its antioxidant protection. There are several lines of evidence to suggest this. Firstly, lipofuscin has been shown to accumulate in the RPE of vitamin E deficient rats (Handelman and Dratz, 1986). This substance is believed to be an indigestible end product of lipid peroxidation and in this case may represent peroxidised outer segment membranes phagocytosed by the RPE. In vitamin E and selenium (the cofactor for glutathione peroxidase) deficient rats an accumulation of lipofuscin in the RPE occurred alongside a loss of PUFAs in the outer segments and RPE (Trevithick et al., 1993). Secondly, it has been shown that retinal degeneration occurs when lipid peroxidation is induced in the retina of experimental animals. Thus experimentally induced lipid peroxidation in the retina of albino rats through constant illumination, and in the frog by injection of ferrous sulphate into the vitreous, has been shown to result in retinal degeneration with initial changes occurring in the rod outer segments (Anderson et al., 1984). The RPE of the albino rat model was shown to accumulate rod outer segment debris. In addition, a loss of docosahexaenoic acid (22:6n3) and an increase in lipid hydroperoxides occurred in the rod outer segment membranes in both models. In another study, the injection of lipid peroxides into the vitreous of albino and pigmented rabbits also caused retinal degeneration, which was demonstrated by a progressive decrease in the electroretinogram response over a 12 day period (Armstrong et al., 1982).

The retinal degeneration that occurs in severe vitamin E deficiency and in ARD are both described by the term "retinitis pigmentosa". As discussed in section 1.7.2, this pattern of retinal degeneration can be caused by a whole range of underlying defects in retinal lipid and protein components. There is the possibility that in these two disease states a
similar mechanism of retinal degeneration operates and that this involves a perturbation of vitamin E antioxidant activity.

6.7 Plan of Investigation

In addressing the possibility that phytanic acid accumulation in cellular membranes may interfere with the function of vitamin E, the following questions were posed; Firstly, does phytanic acid interfere with the uptake of vitamin E into retinal cell membranes and secondly does phytanic acid perturb the antioxidant function of vitamin E? In an attempt to answer these questions, the effect of phytanic acid on a) the uptake of vitamin E and b) the susceptibility of retinal cells to in vitro oxidative stress was investigated. These studies were carried out using an in vitro retinal cell culture system and are described in chapters 7 and 8.
7. THE EFFECT OF PHYTANIC ACID ON THE UPTAKE OF \( \alpha \)-TOCOPHEROL (AND VICE VERSA) BY RAT RPE CELLS

7.1 INTRODUCTION

7.2 A PILOT STUDY ON THE EFFECT OF PHYTANIC ACID ON THE UPTAKE OF \( \alpha \)-TOCOPHEROL

7.3 THE EFFECT OF A RANGE OF PHYTANIC ACID CONCENTRATIONS ON \( \alpha \)-TOCOPHEROL UPTAKE BY RAT RPE CELLS

7.4 THE EFFECT OF PHYTANIC ACID ON THE \( \alpha \)-TOCOPHEROL CONCENTRATION OF RAT RPE CELLS PRESUPPLEMENTED WITH \( \alpha \)-TOCOPHEROL

7.5 THE EFFECT OF \( \alpha \)-TOCOPHEROL ON THE UPTAKE OF PHYTANIC ACID

7.5.1 Effect of \( \alpha \)-Tocopherol on the Incorporation of Phytanic Acid into the Lipid Fractions of RPE Cells

7.6 DISCUSSION
7.1 Introduction

In this chapter, the first of two series of investigations relating to the antimetabolite hypothesis (see chapter 6) is described. The aim of these studies was to address the possibility that vitamin E incorporation into membranes may be affected by the incorporation of large amounts of phytanic acid into membrane lipids. Therefore the effect of the combined exposure of rat RPE cells to phytanic acid and α-tocopherol on the uptake of both these compounds was assessed. Confluent parent RPE cells were exposed to α-tocopherol and phytanic acid supplemented medium of varying concentrations and combinations over a period of 7 days after which phytanic acid and α-tocopherol uptake were determined. For each study, cells were seeded from the same pool of cells and grown until just confluent, (3 to 5 days) before proceeding with the supplementation.

7.2 A Pilot Study on the Effect of Phytanic Acid on the Uptake of α-Tocopherol

The aim of this pilot study was to determine whether exposure of RPE cells to phytanic acid affects the uptake of α-tocopherol by these cells. Four groups of confluent cells (3 flasks per group) were supplemented as follows:

(i) with 120 μmol/l α-tocopherol, (group A);
(ii) with 120 μmol/l α-tocopherol and 110 μmol/l phytanic acid, (group B);
(iii) with 110 μmol/l phytanic acid, (group C);
(iv) with unsupplemented medium, (group D).

Medium was changed twice over the 7 day period at days 3 and 5 after the initial feed. Cells were harvested and washed as described in section 2.7.3, and fatty acid and α-tocopherol analyses of crude membrane preparations from each flask were carried out as in sections 3.3.3 and 5.3.1.3. The uptake of α-tocopherol by groups A and B is shown in figure 7-1. Group A had a mean α-tocopherol concentration of 37.5 and group B of 43.2 nmol/mg protein. The similarity in the uptake of α-tocopherol in both groups indicated that phytanic acid did not affect α-tocopherol uptake by RPE cells in culture at the concentrations used. α-tocopherol was not detected in groups C and D. Figure 7-2 shows the degree of uptake of phytanic acid in groups B and C. Group B (supplemented with phytanic acid and α-tocopherol) had a mean phytanic acid value of 26.4 % total
Uptake of α-Tocopherol by RPE Cells in the Presence of Phytanic Acid

![Graph showing the uptake of α-tocopherol and phytanic acid.]

Figure 7-1: α-tocopherol uptake by confluent rat RPE cells supplemented with α-tocopherol (120 µmol/l) (■) or α-tocopherol (120 µmol/l) and phytanic acid (110 µmol/l) (▲) for 7 days.

Uptake of Phytanic Acid by RPE Cells in the Presence of α-Tocopherol

![Graph showing the uptake of phytanic acid and α-tocopherol.]

Figure 7-2: Uptake of phytanic acid by confluent rat RPE cells supplemented with phytanic acid (110 µmol/l) (■) or α-tocopherol (120 µmol/l) and phytanic acid (110 µmol/l) (▲) for 7 days.
peak area, compared to 17.4 % in group C (supplemented with phytanic acid only). This result suggested that α-tocopherol was enhancing the uptake of phytanic acid by RPE in this cell culture system. Phytanic acid was not detected in groups A and D.

**7.3 The Effect of a Range of Phytanic Acid Concentrations on α-Tocopherol Uptake by Rat RPE Cells**

A second study was carried out using a lower concentration of α-tocopherol and a range of phytanic acid concentrations. In the previous study, the concentration of α-tocopherol used (120 μmol/l) is at the upper end of the α-tocopherol uptake dose response curve where uptake is approaching saturation (see figure 5-5). If phytanic acid does interfere with α-tocopherol uptake, it is more likely to be observed at lower α-tocopherol concentrations. Confluent cells were therefore supplemented with 50 μmol/l α-tocopherol, a concentration at which α-tocopherol uptake is approximately 50% of the saturation value (see figure 5-5). Cells were also supplemented with 0 - 200 μmol/l phytanic acid and the medium was changed once over the 7 day period. α-tocopherol uptake by the cells at the end of this period was assessed and results are shown in figure 7-3. Exposure of RPE to increasing concentrations of phytanic acid appeared to have no significant effect on α-tocopherol concentrations. A mean concentration of 19.2 nmol/mg protein was obtained over the different phytanic acid concentrations. This concentration is comparable to that found for 50 μmol/l α-tocopherol supplementation in the α-tocopherol uptake study (figure 5-5).

This study was repeated, using a phytanic acid concentration range of 0 - 250 μmol/l. In addition to α-tocopherol uptake, phytanic acid uptake was demonstrated by fatty acid analysis of crude membranes. The degree of uptake of α-tocopherol with increase in the relative amount of phytanic acid in RPE fatty acid profiles is shown in figure 7-4a. As in the previous study there appeared to be no consistent effect of phytanic acid exposure on the uptake of tocopherol by the cells. The mean α-tocopherol RPE concentration in this study was 16.0 nmol/mg protein. The relative amount of phytanic acid in the RPE membranes increased from 0.1 to 45.7 % total peak area with increasing phytanic acid concentration from 0 to 250 μmol/l in the medium (figure 7-4b). These values are similar to those obtained in the study on the effect of increasing phytanic acid concentration on its uptake by rat RPE cells (see figure 4-5). These results indicated that
Figure 7-3: The uptake of α-tocopherol by rat RPE when supplemented with 50 μmol/l α-tocopherol in the presence of 0 - 200 μmol/l phytanic acid over 7 days. 

n = 1 for each point.
Figure 7-4:

a) The uptake of α-tocopherol by rat RPE when supplemented with 50 μmol/l α-tocopherol and 0 - 250 μmol/l phytanic acid over 7 days.

b) The corresponding uptake of phytanic acid.

n =1 for each point.
the exposure of cultured RPE cells to pathological concentrations of phytanic acid had
no influence on their uptake of α-tocopherol.

7.4 The Effect of Phytanic Acid on the α-Tocopherol Concentration
of Rat RPE Cells Presupplemented with α-Tocopherol

A study was carried out to determine whether phytanic acid supplementation would
displace α-tocopherol already present in rat RPE cells which had been presupplemented
with α-tocopherol. Confluent rat RPE cells (LD7) were supplemented with 150 μmol/l
α-tocopherol over 7 days with two changes of medium on the third and fifth day. This
concentration of α-tocopherol was chosen because at this level of supplementation the
uptake of α-tocopherol reaches saturation point (see figure 5-5). An initial high
concentration of α-tocopherol was desirable as the α-tocopherol supplementation was to
be withdrawn in the second phase of the experiment. On the 7th day of α-tocopherol
supplementation, the concentration of α-tocopherol of crude membrane preparations
from three flasks was determined (day 0). For the second phase of the experiment the
remaining flasks were supplemented with or without 150 μmol/l phytanic acid and no
α-tocopherol over the next 7 days with one change of medium on the 4th day. Cells
from the two groups (three flasks per group) were harvested on the 3rd and 7th day after
starting the phytanic acid supplementation. The α-tocopherol and phytanic acid
concentrations from the crude membrane preparations of these cells were determined.

The results are shown in figure 7-5. The mean α-tocopherol concentration of RPE crude
membrane preparations after 7 days of supplementation with 150 μmol/l α-tocopherol
was 34.8 nmol/mg protein. After withdrawal of α-tocopherol supplementation and 3
days of phytanic acid supplementation the mean α-tocopherol concentration had fallen
to 23.3 nmol/mg protein. In the corresponding control group (no phytanic acid
supplementation) the mean concentration was 25.1 nmol/mg protein. After 7 days of
phytanic acid supplementation the mean α-tocopherol concentration had dropped even
further to 16.2 nmol/mg protein and to 15.9 nmol/mg protein in the control group. The
phytanic acid concentration after three days of supplementation was 17.8 % total peak
area (control = 0.19 %) and after 7 days 19.1 % (control = 0.19 %). The decline in RPE
α-tocopherol concentrations after withdrawal of the α-tocopherol supplemented media
The Effect of Phytanic Acid on Rat RPE Cell
α-Tocopherol Concentration After
Presupplementation with α-Tocopherol

Figure 7-5: The effect of phytanic acid supplementation on the α-tocopherol concentration of rat RPE cells over 7 days.

Cells were supplemented with 150 μmol/l α-tocopherol for 7 days (○) and then with 150 μmol/l phytanic acid (▲) or with unsupplemented media (■) for a further 3 or 7 days.
was therefore similar in both the phytanic acid supplemented and unsupplemented groups.

7.5 The Effect of α-Tocopherol on the Uptake of Phytanic Acid

The results from the pilot study suggested that rather than phytanic acid influencing α-tocopherol uptake, exposure of RPE to phytanic acid in the presence of α-tocopherol increased the uptake of phytanic acid. This possibility was further investigated in the following studies.

Confluent cells were exposed to 100 μmol/l phytanic acid and between 0 - 170 μmol/l of α-tocopherol, for 7 days with one change of medium. Cells were harvested and phytanic acid and α-tocopherol analyses of crude membrane preparations were performed as before. This study was performed in triplicate, with three flasks for every concentration of α-tocopherol. The increase in RPE α-tocopherol concentration from 0.0 to a mean of 49.4 nmol/mg protein with exposure to increasing medium α-tocopherol concentration of 0 to 170 μmol/l is shown in figure 7-6b. These results are comparable to those obtained when examining the effect of increasing α-tocopherol concentrations on its uptake (see figure 5-5). The uptake of phytanic acid is shown in figure 7-6a. The mean % of phytanic acid increased from 14.1 to 21.0 % total peak area when the medium α-tocopherol concentration was raised from 0 to 100 μmol/l (corresponding to a RPE α-tocopherol concentration increase of 0.0 to 36.4 nmol/mg protein) and then remained constant.

The above study was repeated using an increased α-tocopherol concentration range of 0-250 μmol/l. This study was not done in triplicate although more concentration points were used. RPE α-tocopherol uptake increased with exposure to increasing medium α-tocopherol concentration from 0.06 to 114.1 nmol/mg protein at 0 and 250 μmol/l medium α-tocopherol concentration respectively (see figure 7-7 b). A similar increase in the % phytanic acid in the RPE cells to that of the previous study was observed (figure 7-7a). The relative amount of phytanic acid in fatty acid profiles increased from 12.7 to 20.0 % with an increase in α-tocopherol medium concentrations from 0 to 80 μmol/l respectively, after which point there was no further increase.
a) Effect of α-Tocopherol on Phytanic Acid Uptake by RPE Cells

![Graph showing the effect of α-tocopherol on phytanic acid uptake by RPE cells.](image)

b) Uptake of α-Tocopherol by RPE Cells

![Graph showing the uptake of α-tocopherol by RPE cells.](image)

Figure 7-6:

a) The uptake of phytanic acid in the presence of α-tocopherol by rat RPE supplemented with 100 µmol/l phytanic acid and 0 - 170 µmol/l α-tocopherol over 7 days.

b) The corresponding increase in RPE cell crude membrane α-tocopherol concentration.

n = 3 for each point and the mean and min-max is shown.
Figure 7-7:
a) The uptake of phytanic acid in the presence of \( \alpha \)-tocopherol by rat RPE cells supplemented with 100 \( \mu \text{mol/l} \) phytanic acid and 0 - 250 \( \mu \text{mol/l} \) \( \alpha \)-tocopherol over 7 days.

b) The corresponding increase in RPE cell crude membrane \( \alpha \)-tocopherol concentration.

\( n = 1 \) for each point.
The results from these studies confirm that co-exposure of rat RPE cells to phytanic acid and α-tocopherol resulted in an increased uptake of phytanic acid by these cells. RPE cells exposed to phytanic acid concentrations as low as 25 μmol/l over 7 days, store a significant proportion (> 40%) of the phytanic acid as triacylglyceride (see section 4.3). In the above studies, the concentration of phytanic acid was measured in the crude membrane preparations from these cells. These preparations have been shown to be contaminated with triacylglyceride (see section 4.3.3). It was therefore of interest to know in which lipid fractions the phytanic acid was appearing. An increase of phytanic acid in the phospholipid fraction would correspond to an increase in uptake into cellular membranes whereas an increase in the triacylglyceride fraction might suggest that α-tocopherol was also associated with the lipid droplets in the cytoplasm that accumulate during phytanic acid supplementation. The following study describes the change in phytanic acid concentration in lipid fractions from cells supplemented with phytanic acid and a range of α-tocopherol concentrations over 7 days.

7.5.1 Effect of α-Tocopherol on the Incorporation of Phytanic Acid into the Lipid Fractions of RPE Cells

Rat RPE cells (10 LD7) were exposed to 100 μmol/l phytanic acid and 0 - 210 μmol/l α-tocopherol over a seven day period with one change of medium. Total lipid extracts from treated cells were separated by TLC using solvent system B, which is able to separate triacylglycerides from cholesterol esters from phytanic acid supplemented cells, (see section 4.3). The fatty acid profiles of the total lipids and lipid fractions were then analysed. Figure 7-8a demonstrates the relative increase in phytanic acid in both phospholipids and total lipids when RPE are simultaneously exposed to phytanic acid and increasing levels of α-tocopherol in the medium. In both the phospholipid and total lipid fractions there was a small but discernible increase in the % of phytanic acid. The phytanic acid in the phospholipid fraction increased from 5.8 to 8.4 % total peak area, and in total lipids from 12.9 to 16.8 %, with an increase in the medium α-tocopherol concentration from 0 to 100 μmol/l. At higher α-tocopherol supplementation concentrations there was no further increase. Figure 7-8b shows the change in the % phytanic acid in the triacylglyceride, cholesterol ester and free fatty acid lipid fractions. Unlike the phospholipid and total lipid fractions there was no increase in the % of phytanic acid.
a) Effect of α-Tocopherol on the Uptake of Phytanic Acid Into Lipid Fractions

![Graph showing the effect of α-Tocopherol on the uptake of phytanic acid into lipid fractions.]

b) Medium α-Tocopherol (pmol/l)

![Graph showing the change in the phytanic acid concentration in the lipid fractions of rat RPE cells over 7 days.]

Figure 7-8: Change in the phytanic acid concentration in the lipid fractions of rat RPE cells when exposed to 100 μmol/l phytanic acid and 0 - 210 μmol/l α-tocopherol over 7 days.

a) the % phytanic acid in the total lipid extract and the phospholipid fraction.

b) the % phytanic acid in the triacylglyceride (TG), cholesterol ester (CE), and free fatty acid (FFA) fractions.

n = 1 for each point.
It therefore appeared from these results that the increase in rat RPE crude membrane phytanic acid concentration with increasing $\alpha$-tocopherol medium concentration corresponded to an increase in uptake into the phospholipid fraction. There seems to be no consistent effect on the % phytanic acid in other lipid fractions including the triacylglycerides, the major storage form of phytanic acid in supplemented rat RPE cells.

7.6 Discussion

These studies investigated the antimetabolite hypothesis for the pathogenesis of ARD which proposes that because of similarities in structure, phytanic acid may interfere with $\alpha$-tocopherol incorporation into cells. The results presented above have demonstrated that the simultaneous exposure of rat RPE cells to $\alpha$-tocopherol and increasing concentrations of phytanic acid (up to 250 $\mu$mol/l) in the culture medium had no effect on the uptake of $\alpha$-tocopherol by those cells. This indicates that phytanic acid and $\alpha$-tocopherol do not compete for the same uptake mechanisms by the RPE cells in this culture system. In vivo, free fatty acids are transported in the blood to tissues bound to albumin, whereas $\alpha$-tocopherol is transported as a component of lipoproteins. The mechanism(s) of transport of long chain free fatty acids across plasma membranes is not yet fully understood. It is thought that the uptake of long chain free fatty acids released from albumin or lipoproteins by cells could occur by both a passive diffusion and a carrier mediated mechanism involving membrane associated fatty acid-binding proteins (FABP) and translocases or membrane channels (Glatz et al., 1997). Fatty acids that are translocated across the plasma membrane are then bound to cytoplasmic FABP which facilitate their intracellular trafficking (Glatz et al., 1997). The major route for the uptake of $\alpha$-tocopherol by cells is thought to be via the low density apolipoprotein B/E receptor mediated pathway (Muller and Goss-Sampson, 1990). In the culture medium in these studies phytanic acid was bound to albumin and $\alpha$-tocopherol was “dispersed” in the foetal calf serum, most probably associated with lipoprotein complexes. It is therefore likely that the uptake of $\alpha$-tocopherol and phytanic acid occurred via different pathways.

Cells supplemented with both $\alpha$-tocopherol and phytanic acid accumulated phytanic acid in a dose dependent manner. The phytanic acid was incorporated principally into
intracellular triacylglycerides and membrane phospholipids (see section 4.3) whereas the α-tocopherol was likely to be incorporated into membrane structures. Thus the presence of phytanic acid containing phospholipids in membranes did not appear to hinder the incorporation of α-tocopherol.

Phytanic acid supplementation did not significantly affect the rate of decline of α-tocopherol in rat RPE cells pre-loaded with α-tocopherol. Once α-tocopherol supplementation was withdrawn from the cells, it would be expected that excess α-tocopherol would be lost from the cells or the α-tocopherol would be consumed as a result of oxidative processes. The fact that a similar decline was observed in the non phytanic acid supplemented control groups indicated that the presence of phytanic acid had no effect on the loss or oxidation of α-tocopherol.

An interesting and unexpected result from these studies was the enhanced uptake of phytanic acid by RPE cells when co-supplemented with α-tocopherol. This increase in phytanic acid occurred only in the phospholipid fraction. This effect may be explained by the physical effect of α-tocopherol on membrane structure. α-Tocopherol decreases the fluidity of membranes when membrane phospholipids are in the liquid crystalline state, i.e. when phospholipids are above the phase transition temperature. The incorporation of phytanic acid into membranes appears to have a destabilising effect on membrane structure. Evidence for this is presented in chapter 9 and has previously been shown in studies using liposomal systems (see chapter 9 for a more detailed discussion). α-Tocopherol, through its membrane stabilising effect may counter this effect of phytanic acid and thereby allow a greater amount to be incorporated into membrane phospholipids. A modulating effect of α-tocopherol on the phospholipid acyl composition in cultured cells has previously been shown. Giasuddin and Diplock (1981) demonstrated that supplementation of fibroblasts with α-tocopherol in the presence of linoleic acid (18:2n6) resulted in a change in phospholipid fatty acid profiles, with an increase in arachidonic acid content, whereas no effect was found in the fatty acyl composition of triacylglycerols or cholesterol esters.
8. THE EFFECT OF PHYTANIC ACID ON THE ANTIOXIDANT FUNCTION OF α-TOCOPHEROL

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8.1 Introduction

In the second series of studies to investigate the antimetabolite hypothesis, the possibility that phytanic acid may be interfering with the antioxidant function of α-tocopherol was explored. The Y79 cell line supplemented with 18:3n3 was used in these studies as a model of a PUFA enriched cell type. Supplementation of Y79 cells with 18:3n3 results in an accumulation of this fatty acid and its elongation and desaturation products including DHA (22:6n3) (Hyman and Spector, 1981). An in vitro peroxidative stress system was then used to promote lipid peroxidation in preparations from 18:3n3 supplemented Y79 cells that were also co-supplemented with α-tocopherol and phytanic acid. The extent of lipid peroxidation was assessed by measuring the increase in free malondialdehyde concentration by HPLC.

There are a number of methods available for the promotion of lipid peroxidation in vitro. For example, exposure of lipid peroxides already present in biological samples to transition metals such as copper and iron, can stimulate the lipid peroxidation process. This occurs through the decomposition of existing lipid peroxides in the sample to alkoxy and peroxyl free radicals. For instance, lipid peroxides (ROOH) can react with Fe$^{2+}$ and Fe$^{3+}$ complexes to form alkoxy (RO') and peroxyl radicals (ROO') respectively (Halliwell and Gutteridge, 1989) (equations 1 and 2).

\[
R\text{-}OOH + \text{Fe}^{2+} \text{- complex} \rightarrow \text{Fe}^{3+} \text{- complex} + \text{OH}^- + R\text{-}O' \quad (\text{Eq. 8-1})
\]

\[
R\text{-}OOH + \text{Fe}^{3+} \text{- complex} \rightarrow \text{Fe}^{2+} \text{- complex} + \text{H}^+ + R\text{-}OO' \quad (\text{Eq. 8-2})
\]

The alkoxy and peroxyl free radicals generated are capable of abstracting a hydrogen atom from a polyunsaturated fatty acid and thus propagate the lipid peroxidation process. Fe$^{3+}$-ADP/ascorbate is one example of an in vitro system that stimulates lipid peroxidation through this mechanism. The inclusion of ascorbate in this reaction mixture results in the reduction of Fe$^{3+}$ to Fe$^{2+}$ which is much more reactive towards hydroperoxides than Fe$^{3+}$ (Halliwell and Gutteridge, 1989).
Alternatively, lipid peroxidation may be promoted \textit{in vitro} by systems that directly generate free radicals that are capable of abstracting hydrogen atoms from PUFAs (such as the hydroxyl radical), thus initiating lipid peroxidation. The Cu$^{2+}$/H$_2$O$_2$ free radical generating system is an example of such a system and was used in this study. This method had previously been established in our laboratory to investigate the effect of \(\alpha\)-tocopherol deficiency in rats on the susceptibility of various tissues to \textit{in vitro} peroxidative stress (MacEvilly and Muller, 1996). Reduced transition metals such as Fe$^{2+}$ and Cu$^{+}$ react with H$_2$O$_2$ to produce the hydroxyl radical by the Fenton reaction (equation 8-3). For this reaction to occur, the Cu$^{2+}$ in the free radical generating system needs to be reduced before it can produce the hydroxyl radical. As no exogenous reductants were added to the system, this reduction is dependent upon the action of endogenous reductants in the incubation mixture.

\[
\text{Cu}^{+} + \text{H}_2\text{O}_2 \rightarrow \text{Cu}^{2+} + \text{OH}^- + \text{OH}^-(\text{Eq. 8-3})
\]

The extent of lipid peroxidation can be assessed using a variety of methods. In this study it was followed measuring the change in free malondialdehyde (MDA) concentration. MDA is an end product of lipid peroxidation and occurs in its free form, or bound to a variety of molecules such as proteins and DNA through Schiff linkage. MDA is one of a number of secondary products of lipid peroxidation formed by the decomposition of lipid peroxides and is frequently used as an index of lipid peroxidation. MDA is formed from lipid peroxides of fatty acids with 3 or more double bonds and is thought to proceed via cyclisation of the lipid peroxyl radical to form an oxybridged radical, followed by the formation of cyclic peroxides (see figure 8-1).

Methods available for MDA determination include those that assay it directly and those in which a derivative of MDA is measured. A commonly used method utilising the latter approach is the reaction of MDA with thiobarbituric acid and the measurement of thiobarbituric acid reactive substances (TBARS). MDA is reacted with thiobarbituric acid at low pH and at elevated temperatures (80-90°C) to form a fluorescent and pigmented adduct (TBA)$_2$-MDA. This assay is relatively simple and straightforward.
Figure 8-1: Formation of malondialdehyde from a lipid peroxyl radical via a cyclic endoperoxide
However a major disadvantage of this method is that it is non-specific as other low molecular weight aldehydes can also react with thiobarbituric acid. In addition, the incubation conditions of the reaction stimulate the degradation of lipid peroxides into MDA which in effect amplifies the peroxidation process. This method does not therefore measure the concentration of free MDA in the starting reaction mixture.

In this study a direct and specific assay for MDA using HPLC with UV detection was used based on the method of Esterbauer et al (1984). This method exploits the fact that in aqueous solution at pH > 7.0, MDA exists as an enolate anion and can therefore be separated on an aminophase HPLC column.

The studies involving the effect of 18:3n3 supplementation on the fatty acid profile of Y79 cells are described in Part I of this chapter. This is followed by a description of the in vitro peroxidative studies of 18:3n3 enriched Y79 cells also supplemented with α-tocopherol and phytanic acid in Part II.

**Part I**

### 8.2 Supplementation of Y79 Cells with Linolenic Acid (18:3n3)

The unusual ability of the Y79 retinoblastoma cell line to accumulate elongation and desaturation products of 18:3n3 when supplemented with this essential fatty acid was reported by Hyman and Spector in 1981. Their results showed that Y79 incorporated 18:3n3 mainly into the phospholipid fraction, with a greater enrichment in the ethanolamine glycerophospholipids compared to other phospholipid species. The degree of 18:3n3 uptake and incorporation into phospholipids was greater than that observed for 18:2n6. Pulse chase experiments using [1-14C]18:3n3 showed that initially (1.5 hours after uptake), very little metabolism of 18:3n3 to its longer chain PUFA derivatives occurred. However after 72 hours an appreciable amount of the labelled 18:3n3 was converted to 20:5n3, 22:5n3 and 22:6n3 and further enrichment of 22:6n3 occurred after longer incubation periods. The extent to which Y79 cells incorporate and convert 18:3n3 into 22:6n3 is greater than that of other cell lines studied (Hyman and
Spector, 1981), including another retinoblastoma cell line, WERI Rb-1 (Yorek et al., 1985). For the purposes of this series of investigations, the enrichment of Y79 cells with n-3 fatty acids by supplementation with 18:3n3 was used as a model of a PUFA enriched cell type.

### 8.2.1 The Effect of 18:3n3 Supplementation Concentration on the Fatty Acid Profile of Y79 Cells

A dose response study was undertaken to establish an 18:3n3 supplementation protocol that would result in the satisfactory enrichment of Y79 cells with n-3 fatty acids. It has previously been shown that supplementation with 10 - 100 μmol/l 18:3n3 had only a slight inhibitory effect on cell growth (Hyman and Spector, 1981). A supplementation concentration range of 0 to 160 μmol/l was used in this study.

Y79 cells were seeded at 5 x 10⁵ cells/ml in 20 ml medium supplemented with 0 to 160 μmol/l 18:3n3 (Sigma-Aldrich Co. Ltd., Poole) (for details of tissue culture see chapter 2). After 3 days a further 15 ml of medium was added to the flasks. The cells were harvested at the end of 7 days and the fatty acid profiles of crude membrane preparations were determined.

The changes in the relative concentrations of 18:3n3 and 20:5n3, 22:5n3 and 22:6n3, which were the major n-3 fatty acids identified in the fatty acid profile of supplemented cells (see figure 3-9 for an example), are shown in figures 8-2a and 8-2b. 18:3n3 was not detected in the control group which received no 18:3n3 supplementation. There was little increase in the % of 18:3n3 over the supplementation concentration range of 0-75 μmol/l; the relative concentration was 2.3 % total peak area at 75 μmol/l. However at higher supplementation concentrations there was a sharp increase in 18:3n3, reaching a relative concentration of 26.4 % total peak area at 160 μmol/l. With no 18:3n3 supplementation, very little 20:5n3 and 22:5n3 were present, both with a relative concentration of 0.2 % total peak area. There was an increase in both these fatty acids with increasing 18:3n3 supplementation concentration. 20:5n3 reached a maximum of 8.3 % total peak area at 75 μmol/l. The increase in 22:5n3 was less than that of 20:5n3, reaching a maximum of 3.3 % total peak area at 95 μmol/l supplementation. 22:6n3 increased from 4.6 % with no supplementation, to a maximum of 12.3 % total peak area.
Figure 8-2: The effect of supplementation of Y79 cells with 18:3n3 (0 - 160 µmol/l) over 7 days on fatty acid profiles of crude membranes.

a) Change in the relative amount of 18:3n3.

b) Change in the relative amounts of the elongation and desaturation products of 18:3n3. i.e. 20:5n3, 22:5n3 and 22:6n3.

n = 1 at all concentration points except for 0, 30 and 95 µmol/l where n = 3 (mean and min - max are shown).
at 75 μmol/l 18:3n3. In contrast to 20:5n3 and 22:5n3, a steady decline in 22:6n3 was observed at higher supplementation concentrations. At 160 μmol/l the relative concentration reached a value of 2.3 % total peak area, which was lower than that observed in the control group. The relative concentrations of the major fatty acids (14:0, 16:0, 18:2n6, 20:0, 20:4n6) remained relatively constant except for 18:1 (combined n7 and n9 species) and 16:1 (combined n7 and n9 species) which decreased with increasing 18:3n3 supplementation concentrations (see figure 8-3). The concentration of 18:1 decreased from 28.8 to 10.5 % and 16:1 from 14.5 to 3.4 % total peak area between 0 to 160 μmol/l supplementation. 18:0 remained unchanged with increasing 18:3n3 supplementation concentration until 160 μmol/l when the relative concentration dropped from 13.1 % (at 95 μmol/l supplementation) to 8.8 % total peak area.

8.2.2 Discussion

The fatty acid composition of 18:3n3 supplemented Y79 cells determined in this study was similar to that reported by Hyman and Spector (1981) under comparable supplementation conditions. Supplementation of Y79 cells with 18:3n3 (0 to 160 μmol/l) increased the relative concentration of n-3 fatty acids in these cells. 22:6n3 was the major n-3 fatty acid in the Y79 cell fatty acid profile up to 75 μmol/l 18:3n3. At higher 18:3n3 medium concentrations a decrease in the relative concentration of 22:6n3 was observed and this was accompanied by an increase in 18:3n3. This result suggested that the exposure of Y79 cells to these concentrations of 18:3n3 resulted in an inhibition of the conversion of 18:3n3 to 22:6n3. There was little change in the relative concentrations of the intermediates 20:5n3 and 22:5n3 at these supplementation concentrations suggesting that the conversion of 22:5n3 to 22:6n3 was particularly sensitive to inhibition by 18:3n3 and there was a reduced flux from 18:3n3 to 20:5n3. 18:3n3 is metabolised to 22:6n3 by a series of elongation, desaturation and partial β-oxidation steps and a scheme of this pathway as proposed by Sprecher et al (1995) is presented in figure 8-4. 22:5n3 is converted to 22:6n3 by three steps; elongation to 24:5n3, desaturation to 24:6n3 and finally β-oxidation to 22:6n3. There is evidence to suggest that the β-oxidation step is carried out by the peroxisomal β-oxidation system (Sprecher et al., 1995). This system can degrade long chain and very long chain fatty acids and is generally more active towards unsaturated than saturated fatty acids (Lazarow and Moser, 1995). It is possible that the accumulation of 18:3n3 results in a
Supplementation of Y79 Cells with 18:3n3

Figure 8-3: The effect of supplementation of Y79 cells with 18:3n3 (0 - 160 μmol/l) over 7 days on the relative concentrations of 18:0, 16:1 (n-7 and n-9 combined) and 18:1 (n-7 and n-9 combined).

n = 1 at each concentration point except for 0, 30 and 95 μmol/l where n = 3 (mean and min - max are shown).
Metabolism of Linolenic Acid to Docosahexaenoic Acid

9, 12, 15 - 18:3n3
\[ \Delta^6 \text{ desaturation} \]

6, 9, 12, 15 - 18:4n3
\[ \text{elongation} \]

8, 11, 14, 17 - 20:4n3
\[ \Delta^5 \text{ desaturation} \]

5, 8, 11, 14, 17 - 20:5n3
\[ \text{elongation} \]

7, 10, 13, 16, 19 - 22:5n3
\[ \text{elongation} \]

9, 12, 15, 18, 21 - 24:5n3
\[ \Delta^6 \text{ desaturation} \]

6, 9, 12, 15, 18, 21 - 24:6n3
\[ \beta\text{-oxidation} \]

4, 7, 10, 13, 16, 19 - 22:6n3

Figure 8-4: The pathway of the biosynthesis of docosahexaenoic acid (22:6n3) from the essential fatty acid linolenic acid (18:3n3), as proposed by Sprecher et al (1995).
competitive inhibition of the β-oxidation of 24:6n3 to 22:6n3 as the cell attempts to
degrad excess 18:3n3 using the same β-oxidation enzyme system. Inhibition of de
novo synthesis of fatty acids in cultured rat hepatocytes when supplemented with 0.5
mmol/1 PUFAs has previously been shown (Mikkelsen et al., 1993). This inhibition was
correlated with increased cytotoxicity and lipid peroxidation and could be prevented by
the addition of antioxidants to the culture medium. Other studies have shown that
supplementation of cultured cells with PUFAs increases the extent of lipid peroxidation
and cellular injury, both in untreated cells and in cells exposed to in vitro peroxidative
stress (for example, Hart et al., 1991; Wey et al., 1993; Furuno and Sugihara, 1994). It is
therefore possible that the changes in the fatty acid profile of Y79 cells observed at
supplementation concentrations of 18:3n3 greater than 75 μmol/l, are caused by an
increase in lipid peroxidation and related cytotoxic events.

The most noticeable change in the fatty acid profiles, apart from those of the n-3 fatty
acids, was a decrease in the percentage of 16:1 and 18:1 with increasing medium
concentrations of 18:3n3. A significant decrease in 18:1 in cultured pulmonary artery
endothelial cell membranes in response to supplementation and uptake of 18:2n6 has
previously been reported by Sekharam et al (1990). The decrease in these
monounsaturated fatty acids may represent an active response by the supplemented cells
to compensate for the increase in n-3 polyunsaturated fatty acids and the effect this will
have on membrane fluidity. Fatty acids with one or more double bonds have a disruptive
effect on the packing order of membrane lipids thus increasing the ease of movement of
phospholipid acyl chains (i.e. increased membrane fluidity). It is thought that one way
in which cells regulate membrane fluidity is by altering the unsaturation status of
phospholipid acyl chains through a process known as homeoviscous adaptation (Spector
and Yorek, 1985).

The investigation into the effect of phytic acid on the susceptibility of cell
preparations to in vitro peroxidative stress, involved the co-supplementation of Y79
cells with 18:3n3, phytic acid and α-tocopherol. The concentration of 18:3n3 used in
these experiments was 100 μmol/l. A concentration higher than the optimal for 22:6n3
and 20:5n3 enrichment (i.e. 75 μmol/l) was chosen as some degree of competition in
uptake and incorporation into cellular lipids between 18:3n3 and phytic acid would be
expected. The incubation time was shortened from 7 days to 5 days (although the same
feeding volumes and times were used) in order to reduce the length of the study.

Part II

8.3 The Effect of Phytanic Acid and α-Tocopherol on In Vitro Lipid Peroxidation of 18:3n3 Supplemented Y79 Cells

The effect of phytanic acid and α-tocopherol on the susceptibility of 18:3n3
supplemented Y79 cells to in vitro peroxidative stress was investigated. In these studies
Y79 cells were co-supplemented with 100 µmol/l 18:3n3 and a range of α-tocopherol
and phytanic acid concentrations. Y79 cells were seeded at 4.9 x 10^5 cells/ml in 175
cm² flasks in 20 ml of medium. A further 15 ml of medium was added on the third day
after seeding and cells were harvested on the fifth day. Y79 cells were collected and
washed using the same procedure as that for the fatty acid and α-tocopherol assays
(section 2.7.3.).

8.3.1 Materials

1,1,3,3-tetramethoxypropane, Trizma® base (Tris[hydroxymethylaminomethane]),
Trizma® HCl, hydrogen peroxide (30 % solution, w/w), copper sulphate, sulphuric
acid (Sigma-Aldrich Co. Ltd. Poole, Dorset); acetonitrile (Rathburn Chemicals Ltd.
Walkerburn, Scotland).

8.3.2 The In Vitro Lipid Peroxidation Assay

Harvested Y79 cells were resuspended and sonicated in 40 mmol/l trizma buffer, pH
7.4. 5 µl aliquots were taken for protein measurement using the BCA protein assay (see
section 5.3.2). Sonicates were incubated with hydrogen peroxide and copper sulphate
solutions prepared in 40 mmol/l trizma, pH 7.4 (sonicate:H₂O₂:CuSO₄: 5:1:1 (v/v/v)).
Final concentrations of H₂O₂ and CuSO₄ are given when describing individual
experiments. Sonicates were first added to ice cold H₂O₂ and the reaction was started by
the addition of the CuSO₄, followed by brief vortexing. The samples were incubated in a
water bath at 37°C for varying lengths of time. The reaction was stopped by the addition
of an equal volume of chilled acetonitrile to that of the incubation mixture, followed by vortexing for 15 seconds. The protein precipitate was pelleted by centrifugation in a microfuge at 13000 g for 5 minutes. Samples were kept on ice and the free MDA was analysed within 3 hours.

Measurement of free MDA concentrations in pre-stress, i.e. control samples, involved the addition of the sonicate to the chilled acetonitrile prior to the addition of H$_2$O$_2$ and CuSO$_4$.

### 8.3.3 Measurement of Malondialdehyde by High Performance Liquid Chromatography

Samples were analysed using HPLC (LC-10AS Liquid Chromatograph, Shimadzu) with UV detection (LDC Analytical Spectromonitor 3100). Samples were separated on a Spherisorb S5NH$_2$ column (Phase Separations Ltd.), 25 cm x 4.6 mm with a mobile phase of 0.03M trizma pH 7.4/acetonitrile (21:79, v/v) and a flow rate of 2 ml/min. MDA was detected at 267 nm. Chromatograms were recorded using Class-VP Data System software (version 4.0) (Shimadzu). Typically 50 μl of the supernatant or standard MDA solution (see section 8.4.3) was injected onto the column using a SIL-9A Autoinjector (Shimadzu).

### 8.3.4 Standardisation

MDA standards were prepared by hydrolysis of 1,1,3,3-tetramethoxypropane. 1,1,3,3-tetramethoxypropane was diluted 1 in 500 (v/v) with 1% (v/v) sulphuric acid and left at room temperature for two hours. A stock standard solution was prepared by further dilution of this initial solution, 1 in 100, (v/v) with 1% sulphuric acid. The precise concentration of the stock standard was determined spectrophotometrically (ε = 13750 at 245 nm) using a Unikon spectrophotometer (Kontron Systems). The concentration of the stock standard solution was normally approximately 100 μmol/l. Stock standard solutions were prepared weekly and kept at 4°C. Working standards were prepared daily by dilution of the stock standard with 0.1 mol/l trizma pH 7.4/acetonitrile (50:50 v/v). Calibrations were made using a three point standard curve with a concentration range of 0.5 to 3.0 μmol/l.
An example of the linear relationship of peak area and MDA concentration from 0.1 to 3.0 μmol/l is shown in figure 8-5.

8.3.5 Validation and Reproducibility of MDA Measurements and the *In Vitro* Peroxidation Assay

The reproducibility of:

a) MDA measurements by the HPLC system,

b) the extraction of free MDA, and

c) the *in vitro* lipid peroxidation assay

were assessed. The results are shown in table 8-1.

a) The reproducibility of MDA measurements by the HPLC system was determined by calculating the coefficient of variation (c.v.) of replicate measurements (n = 8) of 50 μl of 1.11 μmol/l MDA standard. The c.v. was found to be 3.5 % which indicated that MDA measurement by this system was reproducible.

b) The reproducibility of the extraction of MDA was assessed by calculating the variability of replicate MDA extractions (n = 9). A cell preparation of Y79 cells supplemented with a mean concentration of 60 μmol/l 18:3n3 over 1-2 weeks was used. This gave a mean MDA concentration of 0.24 μmol/l with a c.v. of 5.3 %. This c.v. represented the combined variation of the extraction procedure and MDA determination by HPLC and indicated that both these procedures were reproducible. As expected, the variation was greater than following measurement alone.

c) The reproducibility of the complete *in vitro* lipid peroxidation procedure including oxidative stress, extraction and measurement of MDA was assessed. The cell preparation used for the replicate MDA extractions in (b) was exposed to 10 mmol/l H₂O₂ and 0.04 mmol/l Cu²⁺ for 1 hour at 37°C. The mean MDA concentration was 2.42 μmol/l with a c.v. of 7.8 %. These results validated the reproducibility of the *in vitro* peroxidation procedure and free MDA extraction and determination.

8.3.6 Substrate/Product Relationship

The relationship between the concentration of substrate used for oxidative stress and the concentration of free MDA produced was investigated. A series of dilutions of a cell preparation from Y79 cells supplemented with 100 μmol/l 18:3n3 for 7 days was
Figure 8-5: MDA standard curve from 0.1 to 3.1 μmol/l MDA.

$r^2 = 0.992$.

$n = 1$ for each point.
<table>
<thead>
<tr>
<th></th>
<th>Mean Conc. (μmol/l)</th>
<th>n</th>
<th>Standard Deviation</th>
<th>Coefficient of Variation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a) Replicate injections (50 μl of 1.11 μmol/l)</td>
<td>0.97</td>
<td>8</td>
<td>0.034</td>
<td>3.5</td>
</tr>
<tr>
<td>b) Replicate pre-stress MDA extraction and determination</td>
<td>0.24</td>
<td>9</td>
<td>0.013</td>
<td>5.3</td>
</tr>
<tr>
<td>c) Replicate post-stress MDA extraction and determination</td>
<td>2.42</td>
<td>7</td>
<td>0.188</td>
<td>7.8</td>
</tr>
</tbody>
</table>

Table 8-1: Reproducibility of:

a) MDA determination by HPLC,

b) determination and extraction of free MDA from Y79 cell sonicates,

c) determination and extraction of free MDA following oxidative stress using a H$_2$O$_2$/Cu$^{2+}$ in vitro lipid peroxidation system.
prepared using 40 mmol/l trizma buffer, pH 7.4. These dilutions were then exposed to 10 mmol/l H$_2$O$_2$ and 0.2 mmol/l Cu$^{2+}$ for 1 hour and the concentration of free MDA was determined. The results are shown in figure 8-6. A linear relationship was observed between the number of cells (expressed as a protein concentration) and the concentration of free MDA produced. Thus results from cell preparations with protein concentrations ranging between 2.5 and 12.5 mg/ml can be directly compared when MDA concentrations are expressed as a ratio to protein concentration.

### 8.3.7 The Effect of α-Tocopherol Supplementation on the Production of MDA

The following studies describe the effect on the production of free MDA after exposure to the free radical generating system of supplementing Y79 cells with α-tocopherol in combination with 18:3n3.

In a preliminary experiment, Y79 cells were supplemented with 100 μmol/l 18:3n3 and ± 100 μmol/l α-tocopherol for 5 days. The protein concentrations of the cell sonicates prepared from each group were adjusted to 5 mg/ml with 40 mmol/l trizma buffer, pH 7.4. Sonicates were incubated for up to 3 hours at 37°C with 10 mmol/l H$_2$O$_2$ and 0.2 mmol/l CuSO$_4$. 50 μl aliquots were taken at various time points, the reaction stopped and the concentration of free MDA was determined. The results are shown in figure 8-7. A ten fold increase in free MDA was detected in the control group supplemented with 100 μmol/l 18:3n3 over a 3 hour period (from 0.09 nmol/mg protein at 0 minutes to 0.9 nmol/mg protein after 3 hours). The increase in free MDA followed a linear relationship with time of exposure to the free radical generating system up to approximately 2 hours. The concentration of free MDA produced did not increase after 2 hours, indicating an exhaustion of substrate after this time. The concentration of free MDA in the α-tocopherol supplemented group was measured at 1 and 3 hours. After 1 hour the concentration of MDA was 0.05 nmol/mg protein which increased to 0.15 nmol/mg protein after 3 hours. The relatively small increase in MDA production in the α-tocopherol supplemented group compared to the control group demonstrated that supplementation of Y79 cells with 100 μmol/l α-tocopherol had a protective effect against in vitro peroxidative stress.
Figure 8-6: The change in free MDA production when various dilutions of a Y79 cell preparation (expressed as protein concentration) were exposed to 10 mmol/l H₂O₂ and 0.2 mmol/l CuSO₄ for 1 hour.

n = 1 for each point

A linear relationship was observed over the substrate concentration range assessed (r² = 0.975).
In Vitro Peroxidation of Y79 Cell Sonicates

![Graph showing MDA production over time](image)

Figure 8-7: Increase in free MDA production with time in Y79 cell sonicates exposed to in vitro peroxidative stress. Y79 cells were supplemented with 100 μmol/l 18:3n3 and with (▲) or without (■) 100 μmol/l α-tocopherol for 5 days. Cells were then sonicated and incubated with 10 mmol/l H₂O₂ and 0.2 mmol/l CuSO₄ at 37°C for up to 3 hours.

n = 1 for each point.

MDA production in the control group appeared to be linear with time over the first 2 hours as shown by the dashed regression line (r² = 0.954).
It was necessary to determine the range of concentrations of $\alpha$-tocopherol supplementation that would produce a measurable differential protective effect on the production of free MDA by this system. It would then be possible to choose a concentration of $\alpha$-tocopherol supplementation suitable to investigate whether phytanic acid supplementation perturbed the protective effect of $\alpha$-tocopherol. The following two studies investigated the effect of a range of concentrations of $\alpha$-tocopherol supplementation on free MDA production.

Y79 cells were supplemented with 100 $\mu$mol/l 18:3n3 and 0 to 50 $\mu$mol/l $\alpha$-tocopherol for 5 days. Cell sonicates were adjusted to a protein concentration of 5 mg/ml with 40 mmol/l trizma buffer (pH 7.4) and were exposed to 10 mmol/l $\text{H}_2\text{O}_2$ and 0.2 mmol/l CuSO$_4$ for 3 hours. Free MDA concentrations before and after oxidative stress were determined. The results from this study are shown in figure 8-8. The post-stress MDA concentration without $\alpha$-tocopherol supplementation was 0.96 nmol/mg protein. With 5 $\mu$mol/l $\alpha$-tocopherol supplementation the post-stress concentration decreased to 0.29 nmol/mg protein. Supplementation with higher concentrations of $\alpha$-tocopherol did not appear to further significantly influence the post-stress MDA concentrations. The pre-stress MDA concentrations were comparable at all concentrations of $\alpha$-tocopherol supplementation (mean of 0.16 nmol/mg protein). The free MDA concentrations before exposure to in vitro oxidative stress were lower than that after stress for all concentrations of $\alpha$-tocopherol. It can be concluded from these results that 5 $\mu$mol/l $\alpha$-tocopherol supplementation gave maximum protection of the Y79 cell sonicates from in vitro peroxidative stress imposed by the Cu$^{2+}$/H$_2$O$_2$ free radical generating system.

The above study was repeated using a lower concentration range of $\alpha$-tocopherol supplementation (0 - 5 $\mu$mol/l). The results are shown in figure 8-9. Triplicate measurements were made for control cell sonicates (unsupplemented Y79 cells) pre- and post-stress and at 1 $\mu$mol/l $\alpha$-tocopherol supplementation post-stress. The post-stress free MDA concentration in the control sonicates was 1.07 nmol/mg protein which decreased to 0.58 nmol/mg protein with 0.25 $\mu$mol/l $\alpha$-tocopherol supplementation. At higher $\alpha$-tocopherol concentrations there was no apparent further significant decrease in the concentration of free MDA produced. An apparent decrease in free MDA was observed with increasing $\alpha$-tocopherol supplementation concentrations in the pre-stress
The Effect of α-Tocopherol on *In Vitro* Peroxidation of Y79 Cell Sonicates

![Graph showing the effect of medium α-Tocopherol concentration on MDA production.]

**Figure 8-8:** MDA production in cell preparations of Y79 cells supplemented with 100 μmol/l 18:3n3 and between 0-50 μmol/l α-tocopherol for 5 days.

- (●) Free MDA following exposure of cell sonicates to 10 mmol/l H₂O₂ and 0.2 mmol/l Cu²⁺ for 3 hours.
- (■) Free MDA without exposure of sonicates to oxidative stress.

n=1 for each point.
The Effect of α-Tocopherol on *In Vitro* Peroxidation of Y79 Cell Sonicates

![Graph showing MDA production in Y79 cell preparations with and without exposure to H₂O₂ and Cu²⁺.](image)

Figure 8-9: MDA production in Y79 cell preparations with (●) and without (■) exposure to 10 mmol/l H₂O₂ and 0.2 mmol/l Cu²⁺ for 3 hours. Y79 cells were supplemented with 100 μmol/l 18:3n3 and between 0 - 5 μmol/l α-tocopherol for 5 days prior to harvesting.

n = 1 for each point except for 0 μmol/l α-tocopherol pre- and post-stress and 1 μmol/l post-stress where n = 3 (mean and range shown).
samples which was not observed in the previous study. The pre-stress free MDA concentration showed a gradual decrease from 0.53 to 0.31 nmol/ mg protein from 0 to 1.0 μmol/l α-tocopherol supplementation. No further decrease was seen at higher α-tocopherol supplementation concentrations. This decrease may have occurred as a result of an increase in antioxidant protection corresponding to the increasing α-tocopherol supplementation concentrations. This effect was not seen in the previous experiment (figure 8-8) but only a single determination was carried out for the control (no α-tocopherol supplementation). Again, as in the previous study, the concentration of free MDA produced after exposure to the free radical generating system did not fall to the pre-stress levels.

Increasing α-tocopherol supplementation concentrations above the lowest concentration used (i.e. 0.25 μmol/l) had no effect on MDA production following oxidative stress. Therefore 0.25 μmol/l α-tocopherol was chosen for the studies to investigate whether phytanic acid could perturb the production of free MDA by this system.

8.3.8 The Effect of Phytanic Acid and α-Tocopherol Supplementation on the Production of MDA

An initial study on the effect of phytanic acid on the production of free MDA in α-tocopherol/18:3n3 supplemented Y79 cells was carried out. Y79 cells were supplemented with 100 μmol/l 18:3n3, ± 100 μmol/l phytanic acid and ± 0.25 μmol/l α-tocopherol over 5 days. Cell sonicates were adjusted to a protein concentration of 5 mg/ml and were exposed to 10 mmol/l H₂O₂ and 0.2 mmol/l Cu²⁺ for 3 hours. Pre-stress and post-stress free MDA concentrations were determined in triplicate. The results are shown in figure 8-10. Similar pre-stress MDA concentrations were observed in all groups (± α-tocopherol, ± phytanic acid). The greatest increase in free MDA after exposure to the free radical generating system was seen in the control group supplemented with only 18:3n3. In the phytanic acid supplemented group, a smaller increase was observed. This was probably due to a reduction in the concentration of polyunsaturated fatty acids as a result of supplementation of cells with phytanic acid in addition to 18:3n3. Supplementation with α-tocopherol resulted in a similar reduction of the concentration of post-stress MDA ± phytanic acid. The increase in free MDA (pre to post-stress) of the 18:3n3/phytanic acid/α-tocopherol supplemented group was 0.13
The Effect of Phytanic Acid and α-Tocopherol on Free MDA Production

![Figure 8-10](image)

Figure 8-10: Pre (■) and post (●) stress MDA concentrations of Y79 cell sonicates from cells supplemented with 100 μmol/l 18:3n3 and ±100 μmol/l phytanic acid (phyt) and ± 0.25 μmol/l α-tocopherol (α-T) for 5 days. Cells were incubated with 10 mmol/l \( \text{H}_2\text{O}_2 \) and 0.2 mmol/l \( \text{Cu}^{2+} \) for 3 hours.

\( n = 3 \) for each group and the bar shows the mean.
nmol/mg protein (from 0.38 to 0.51 nmol/mg protein) and was 0.11 nmol/mg protein
(from 0.42 to 0.53 nmol/mg protein) in the 18:3n3/α-tocopherol group. Thus the
addition of phytanic acid did not appear to affect the production of free MDA under the
conditions of in vitro peroxidative stress used.

The experiment was repeated using a range of phytanic acid concentrations. Y79 cells
were supplemented with 100 μmol/l 18:3n3, ± 0.25 μmol/l α-tocopherol and 0 to 100
μmol/l phytanic acid. Protein concentrations of the cell preparations were found to be
lower than usual and a final concentration of 2 mg/ml was used for this assay. Post­
stress free MDA concentrations were determined in triplicate for the 0, 50 and 100
μmol/l phytanic acid ± 0.25 μmol/l α-tocopherol supplemented groups. The complete
results are shown in figure 8-11 and the 0 and 100 μmol/l phytanic acid ± 0.25 μmol/l
α-tocopherol results (pre and post-stress) are also presented in figure 8-12 for
comparison with figure 8-10. The concentration of pre and post-stress free MDA tended
to be higher than that in the previous studies. The mean pre-stress concentrations of
MDA were 0.55 and 0.51 nmol/mg protein for groups without and with α-tocopherol
supplementation respectively. Little difference was observed in the pre-stress free MDA
concentrations with increasing phytanic acid supplementation and ± α-tocopherol. A
decrease in post-stress free MDA concentration from 3.1 to 1.8 nmol/ mg protein with
an increase in phytanic acid supplementation from 0 to 75 μmol/l was observed in the
absence of α-tocopherol. There appeared to be no further decrease after this
concentration. This finding is in agreement with the previous study and is further
evidence that co-supplementation of Y79 cells with 18:3n3 and phytanic acid decreases
the concentration of peroxidisable substrate (n-3 fatty acids) compared to
supplementation with 18:3n3 alone. Supplementation with α-tocopherol reduced the
post stress free MDA concentration and a reduction was also observed with increasing
phytanic acid supplementation in this group. Free MDA decreased from 1.9 to 1.1
nmol/mg protein between 0 to 25 μmol/l phytanic acid supplementation, after which
there was no further decrease. This difference in post-stress MDA concentration in the
α-tocopherol supplemented group between the 0 and 100 μmol/l phytanic acid samples
was greater than that observed in the previous study (0.50 compared to 0.02
respectively) (compare data sets 3 and 4 in figures 8-10 and 8-12).
Figure 8-11: Free MDA concentrations (pre- and post stress) in cell sonicates of Y79 cells supplemented with 100 μmol/l 18:3n3, ± 0.25 μmol/l α-tocopherol and 0 to 100 μmol/l phytanic acid over 5 days. Sonicates were exposed to 10 mmol/l H₂O₂ and 0.2 mmol/l Cu²⁺ for 3 hours. n = 1 for 25 and 75 μmol/l phytanic acid. n = 3 for 0, 50 and 100 μmol/l phytanic acid where mean and ranges are shown (unless smaller than the data point).

- ■ 100 μmol/l 18:3n3 (post stress)
- ▲ 100 μmol/l 18:3n3, 0.25 μmol/l α-tocopherol (post stress)
- ● 100 μmol/l 18:3n3 (pre stress)
- ★ 100 μmol/l 18:3n3, 0.25 μmol/l α-tocopherol (pre stress)
The Effect of Phytanic Acid and α-Tocopherol on Free MDA Production

Figure 8-12: Presentation of the results from figure 8-11 showing only the pre (■) and post (●) stress MDA concentrations of Y79 cell sonicates from cells supplemented with 100 μmol/l 18:3n3 and ± 100 μmol/l phytanic acid (phyt) and ± 0.25 μmol/l α-tocopherol (α-T) for 5 days.

n = 3 for each group and the bar shows the mean.
Figure 8-13 shows the difference in post-stress MDA concentrations between the α-tocopherol unsupplemented and supplemented cells with increasing phytanic acid concentration. The difference in free MDA concentration between the two groups was reduced from 1.2 at 0 μmol/l phytanic acid supplementation to 0.5 nmol/mg protein at 75 μmol/l, with no further decrease at 100 μmol/l. This reduction was due to a greater decrease in the post-stress free MDA concentration in the α-tocopherol unsupplemented group compared to the supplemented group with increasing phytanic acid supplementation.

8.3.9 Discussion

As mentioned in the introduction of this chapter, there are a variety of methods available for both the promotion and determination of in vitro lipid peroxidation. The methods chosen i.e. the Cu^{2+}/H_{2}O_{2} free radical generating system and the determination of MDA by HPLC had previously been established in this laboratory and shown to produce reproducible results. The measurement of MDA by HPLC had the advantage of being a direct and specific measurement for a secondary product of lipid peroxidation that required little sample work up. Lipid peroxidation can also be assessed by methods that measure a decrease in the concentration of the substrate (PUFAs) or the production of the primary products (lipid peroxides). However lipid peroxides are unstable and are thus more difficult to assay than their breakdown products. Lipid peroxides can break down into a variety of products such as hydrocarbons and carbonyl compounds. The amount and type of secondary products formed can be influenced by the fatty acid substrate composition, the temperature, pH and exposure to transition metals. Ideally, more than one method should be employed when investigating lipid peroxidation as any one method usually tends to give an incomplete picture of the whole process.

The exposure of sonicates from Y79 cells supplemented with 100 μmol/l 18:3n3 to 10 mmol/l H_{2}O_{2} and 0.2 mmol/l Cu^{2+} resulted in a measurable increase in the concentration of free MDA that was dependent upon incubation time and sonicate concentration. In this study, the concentrations of H_{2}O_{2} and Cu^{2+} used were 10 x greater than that used by MacEvilly and Muller (1996). Relatively strong peroxidation conditions were chosen to ensure a measurable increase in lipid peroxidation. The concentrations of free MDA produced from the Y79 cell sonicates after in vitro peroxidative stress were lower than
Figure 8-13: The difference in free MDA concentration between sonicates of Y79 cells supplemented with or without 0.25 μmol/l α-tocopherol and 0 to 100 μmol/l phytanic acid for 5 days. All cells were supplemented with 100 μmol/l 18:3n3. Sonicates were exposed to 10 mmol/l H₂O₂ and 0.2 mmol/l Cu²⁺ for 3 hours.
that reported for rat brain homogenates (control and vitamin E deficient) but similar to that in myelin (MacEvilly and Muller, 1996). Incubation of Y79 sonicates from cells supplemented with 100 \( \mu \text{mol/l} \) 18:3n3 to 10 mmol/l \( \text{H}_2\text{O}_2 \) and 0.2 mmol/l \( \text{Cu}^{2+} \) for 60 minutes resulted in a free MDA concentration of 0.35 nmol/mg protein. In comparison, the concentration of free MDA obtained after exposure to 1 mmol/l \( \text{H}_2\text{O}_2 \) and 0.02 mmol/l \( \text{Cu}^{2+} \) for 1 hour ranged from 10 - 30 nmol/mg protein for different brain regions and 0.66 nmol/mg protein for myelin under similar conditions (MacEvilly and Muller, 1996). These differences are most likely attributable to the amount of peroxidisable substrate and the antioxidant status of the various substrates.

Under the strong peroxidising conditions used in the current study it is likely that only a gross perturbation of the antioxidant function of \( \alpha \)-tocopherol by phytanic acid would result in a significant difference in free MDA production. Similarly under very weak oxidising conditions, it is also unlikely that a slight perturbation of \( \alpha \)-tocopherol antioxidant function would be detected due to excess antioxidant capacity in the system. It may be possible however to detect a small difference under oxidising conditions that just exceeded the antioxidant capacity. It would therefore have been interesting to study the effect of a range of concentrations of \( \text{H}_2\text{O}_2 \) and \( \text{Cu}^{2+} \) on free MDA production. In addition, rate studies of MDA production under different oxidising conditions may have provided further insight on the antioxidant capacity of phytanic acid supplemented cells.

Co-supplementation of Y79 cells with 18:3n3 and \( \alpha \)-tocopherol at concentrations as low as 0.25 \( \mu \text{mol/l} \) reduced the concentration of free MDA produced after \textit{in vitro} peroxidative stress. Thus supplementation with \( \alpha \)-tocopherol protected cell sonicates against lipid peroxidation stimulated by the \( \text{Cu}^{2+}/\text{H}_2\text{O}_2 \) free radical generation system. No further discernible decrease in post-stress free MDA concentration was observed with concentrations of \( \alpha \)-tocopherol greater than 0.25 \( \mu \text{mol/l} \). It therefore appeared that supplementation with 0.25 \( \mu \text{mol/l} \) \( \alpha \)-tocopherol resulted in maximal protection against lipid peroxidation by this antioxidant. The concentration of post-stress free MDA was greater than the pre-stress free MDA concentration at all \( \alpha \)-tocopherol supplementation concentrations used. This implied that \( \alpha \)-tocopherol was unable to protect cell sonicates completely against \textit{in vitro} lipid peroxidation. Alternatively, it is possible that under the incubation conditions of the oxidative stress (up to 3 hours at 37°C), some degree of
breakdown of pre-existing lipid peroxides occurred. It is likely that Cu$^{2+}$ in the incubation mixture would have stimulated the decomposition of lipid peroxides (see section 8.1). Copper salts have been shown to stimulate the peroxidative process in liposomes prepared from bovine brain lipids and in linolenic (18:3n3) micelles (Gutteridge, 1984). Thus the difference in free MDA observed between the pre- and post stress samples may have been caused by either increased lipid peroxidation and/or increased breakdown of pre-existing peroxides.

Co-supplementation of Y79 cells with 18:3n3 and phytanic acid resulted in a reduced concentration of post-stress free MDA with increasing phytanic acid supplementation concentrations. This was probably due to a reduced incorporation of n-3 PUFAs into Y79 cell membranes when co-supplemented with phytanic acid and suggested that phytanic acid incorporation into membranes protected against lipid peroxidation. One of the major problems with the cell culture system used in this study was that the cellular fatty acid profile and hence the concentration of peroxidisable substrate in the various groups would be altered by the different supplementation conditions. In addition to the probable competition between phytanic acid and 18:3n3, co-supplementation of cultured cells with α-tocopherol may also alter fatty acid profiles. α-tocopherol was shown to enhance the uptake of phytanic acid by rat RPE cells (see section 7.5) and hence may have also affected the uptake of other fatty acids including 18:3n3. Obviously, more studies are required to investigate the effect of co-supplementation of 18:3n3, phytanic acid, and α-tocopherol on the fatty acid profile of Y79 cells. It would also have been of interest to have carried out parallel studies with a liposomal system where the concentration of different fatty acids and α-tocopherol could have been more precisely controlled.

The co-supplementation of 18:3n3 enriched Y79 cells with 100 µmol/l phytanic acid and 0.25 µmol/l α-tocopherol did not produce an observable difference in the amount of free MDA produced after oxidative stress compared to supplementation with α-tocopherol alone (figure 8-10). However in the study where a range of phytanic acid concentrations was used (figures 8-11 and 8-12) increasing the phytanic acid concentration did appear to result in a decrease in the post-stress free MDA concentration in the α-tocopherol supplemented group (figure 8-12, data sets 3 and 4).
The reasons for this difference between the two studies are unclear. In both studies the difference between the $\alpha$-tocopherol unsupplemented and supplemented post-stress free MDA concentrations was less at higher phytanic acid supplementation concentrations (see figures 8-10 and 8-13). Thus at higher phytanic acid concentrations there appeared to be less of a difference in the susceptibility between the vitamin E unsupplemented and supplemented cell preparations to \emph{in vitro} peroxidative stress. This was most likely related to changes in the fatty acid profile and hence to the peroxidisable substrate concentration, as a result of the different supplementation conditions.

These studies did not provide any direct evidence that phytanic acid consistently interfered with the antioxidant function of $\alpha$-tocopherol in Y79 cell sonicates. The antimetabolite hypothesis assumed that interference was possible due to the similarity in structure between phytanic acid and the tocopherol side chain. This could occur if either phytanic acid, as a free acid, competed with $\alpha$-tocopherol for similar positions in the membrane, or as a phospholipid acyl chain, stearically hindered the interaction of tocopherol molecules with lipid peroxyl radicals. In the studies on the distribution of phytanic acid in various lipid fractions from whole cell lipid extracts (see chapter 4), phytanic acid was found in both the phospholipid and free fatty acid fractions. In membranes, however, it is more likely that the majority of phytanic acid will be bound up in phospholipids. The precise location of tocopherol molecules in membranes, their interactions with membrane components and the effect these have on the tocopherol antioxidant function are not entirely understood. A number of studies have investigated the positioning of tocopherol molecules in membranes and the effect of tocopherol on the physical properties of artificial phospholipid bilayers and biological membranes. These have provided some insights on tocopherol - phospholipid interactions.

Localisation studies, using techniques such as $^{13}$C-NMR and fluorescence quenching, have provided evidence that $\alpha$-tocopherol is aligned in membranes with the chromanol moiety near to the lipid/water interface and the phytanyl chains buried in the hydrophobic interior (Perly et al., 1985; Gomez-Fernandez et al., 1993). The localisation of the hydroxyl group near the lipid/water interface would be consistent with the proposal that the tocopheryl radical is recycled back to tocopherol by a water soluble reductant (see section 6.3.1). With regards to its position relative to lipid peroxyl radicals, it has been
proposed that the peroxyl radical, formed in the hydrophobic interior of membranes, would move towards the lipid/aqueous interface due to the large dipole moment of the peroxyl radical centre (Perly et al., 1985). Thus the tocopherol hydroxyl group would also be ideally situated to interact with peroxyl radicals. At the lipid/aqueous interface the chromanol hydroxyl group is positioned in the vicinity of the polar head groups of phospholipids. It has been shown by Fourier-transform infrared spectroscopy that hydrogen bonding occurs between the hydroxyl group of α-tocopherol and the carbonyl and phosphate groups of phosphatidylethanolamine but not of phosphatidylcholine, the major phospholipid species in membranes (Gomez-Fernandez et al., 1993). However, studies using differential scanning calorimetry (DSC) have indicated that the hydroxyl group is in some way involved in interactions between α-tocopherol and phosphatidylcholine. For example, DSC studies have demonstrated that α-tocopherol affected the phase transition from gel to liquid crystalline in liposomes composed from fully saturated phosphatidylcholine species (Gomez-Fernandez et al., 1993). α-tocopherol decreased the phase transition temperature, broadened the range over which it occurred, and decreased the enthalpy of the transition. In contrast, α-tocopheryl acetate had a lesser effect on the phase transition indicating that the hydroxyl group of α-tocopherol was in some way involved with its interaction with phosphatidylcholine (Gomez-Fernandez et al., 1993).

The effect of the structure of the phytyl side chain of the tocopherols on antioxidant activity and membrane stabilising property (decreasing membrane fluidity in the liquid crystalline phase, see section 6.4.1) has also been investigated. Using the rat curative myopathy, plasma pyruvate kinase assay, Ingold et al. (1990) showed that whilst the length of the side chain appeared to influence the in vivo antioxidant function, methyl branching at the 4’, 8’ and 12’ positions of the side chain had no effect. Similarly, the methyl branches did not appear to have any effect on the ability of α-tocopherol to decrease membrane fluidity (Urano et al., 1988). Analogues of α-tocopherol were incorporated into dipalmitoylphosphatidylcholine liposomes containing unsaturated fatty acids and the effect on the steady state fluorescence anisotropy of the membrane probe diphenylhexatriene was measured. The length of the phytyl chain and the methyl substitution pattern of the chromanol ring were shown to exert an effect. The lack of an effect by the methyl branches of the phytyl side chain suggested that these groups were
not necessary for the interaction of tocopherol with phospholipid polyunsaturated acyl groups. This result is contrary to the suggestion by Diplock et al (1977) that the tocopherol methyl branches were associated with z pockets along the chains of PUFAs. If the methyl branches are unimportant in tocopherol/phospholipid interactions, it is unlikely that phytanic acid would inhibit tocopherol function by stearic hindrance of the tocopherol isoprenoid side chain.

Although no consistent effect on the antioxidant function of α-tocopherol was shown in the studies presented in this chapter, there are numerous possibilities for exploring the antimetabolite hypothesis using different approaches. For example, it would be useful to determine whether phytanic acid affected the interactions between tocopherol and phospholipids using studies similar to those described in the discussion above. Artificial bilayers would serve as a simple membrane model in which the effect of free phytanic acid or phytanoylphospholipid species on specific aspects of tocopherol interactions in membranes could be investigated. For example, it would be of particular interest to investigate whether phytanic acid could perturb the interactions of the tocopherol chromanol hydroxyl group with the polar head groups of bilayer phospholipids. As indicated by the studies described above, interactions in the polar region of the bilayer appear to be important for the effect exerted by tocopherols on the physical properties of membranes. If such a perturbation by phytanic acid could be shown, then it would be conceivable that phytanic acid may also affect the interaction of α-tocopherol with lipid peroxyl radicals which has been postulated to occur in the same region.
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9.4 SUMMARY
9.1 Introduction

The simplest hypothesis discussed by Steinberg to explain the pathogenesis of ARD is the molecular distortion hypothesis (Steinberg, 1995) (see section 1.6). This hypothesis proposes that the incorporation of phytanic acid into tissue lipids may be directly responsible for the pathogenesis of the disease by altering the lipid composition of membranes. More specifically, displacement of membrane lipid acyl chains with phytanic acid may perturb the function and/or integrity of those membranes. In ARD, non-specific accumulation of phytanic acid in tissues occurs as the disease progresses. The extent of phytanic acid uptake by cells, its conversion to CoA esters and the rate of turnover of membrane lipids will influence the degree of incorporation of phytanic acid into membranes. The sensitivity of cellular functions to such alterations in membrane fatty acyl composition is likely to vary and could at least partly explain the difference in susceptibility of different tissues to phytanic acid accumulation. This would therefore imply that the retina and nervous tissues, the principal tissues affected in ARD, are particularly susceptible to changes in membrane composition. The detrimental effect of deficiencies or changes in the normal components of photoreceptor membranes on the normal function and viability of retinal cells has already been discussed in chapter 1.

The neurological abnormalities observed in ARD are associated with what is thought to be a primary demyelinating process leading to neural degeneration (see section 1.4.2). Myelin is a specialised membrane formed by the extension of the plasma membrane of Schwann cells. The myelin wraps around the axon of neurons resulting in a compact multilamellar sheath that acts as an insulator and this specialised function is reflected in its unusual structure. The myelin membrane is rich in lipid which accounts for approximately 70-80% of its dry weight (Dewille and Horrocks, 1992). The main lipid species are cholesterol, sphingomyelin, glycolipid, ethanolamine plasmalogen, and phosphatidylinositol 4,5-bisphosphate. A number of membrane proteins such as the peripheral protein and myelin basic protein are involved in the stabilisation and compaction of the myelin layers. A detailed discussion of the possible mechanisms triggering demyelination in ARD is beyond the scope of this thesis. It is possible that an accumulation of phytanic acid in Schwann cells may in some way affect the stability or
turnover of myelin. It is known that dietary fatty acids contribute towards the overall fatty acid profile of myelin lipids (Dewille and Horrocks, 1992). Thus the incorporation of phytanic acid into lipids synthesised by Schwann cells may prevent the proper formation of myelin membranes.

In order to consider the molecular distortion hypothesis in more detail, membrane structure and its relationship to membrane function will be discussed.

9.1.1 Membrane Structure
The fluid mosaic model of biological membranes, proposed by Singer and Nicholson in 1972, serves as a basis for the generally accepted concept of membrane structure. Membranes were known to be composed of a bilayer of lipids in which protein molecules could reside in their native conformation. The fluid mosaic model proposed that membrane proteins were arranged in a tightly packed mosaic with membrane lipids and that water was excluded from this structure. In addition, membrane lipid and protein molecules were considered to be able to freely diffuse within the plane of the bilayer. Since it was first proposed, this model has been revised and modified with a greater appreciation of the complexities of the mobility and arrangement of membrane lipids and proteins. A simplified representation of a membrane bilayer is shown in figure 9-1.

Membrane Lipids
Phospholipids are the main class of lipid from which membranes are formed. They are amphipathic molecules derived from glycerol or sphingosine bonded to hydrophobic acyl chains and phosphate containing polar head groups (see figure 9-2). In glycerophospholipids, the acyl chains are ester bonded at the Sn1 and Sn2 positions of the glycerol backbone. At the Sn3 position, polar head groups are linked to the backbone via a phosphate group. The common head groups of glycerophospholipids found in membranes are choline, ethanolamine, serine and inositol. Plasmalogens are glycerophospholipids which have an unsaturated alcohol in ether linkage at the Sn1 position in place of an acyl group. In sphingophospholipids, the acyl group is bound to sphingosine (a base with a hydrocarbon tail) by an amide linkage. Sphingomyelins are the main class of sphingophospholipids and have phosphocholine as the head group moiety. Phospholipid molecules are roughly cylindrical in shape, with the hydrophobic
Figure 9-1: Cross-section of a biological membrane showing some of the main components. A bilayer is formed from phospholipids and other lipids, including cholesterol and vitamin E, are intercalated between the phospholipid molecules. Integral proteins can span the bilayer or be partially embedded and peripheral proteins are associated at the lipid/aqueous interface.
Figure 9-2: Structures of

a) phospholipids with examples of the common polar head groups

b) sphingomyelin.
acyl groups aligned parallel to each other. In aqueous solution they spontaneously form bilayers with the hydrophobic tails facing inwards and the polar head groups forming a lipid/aqueous interface. The formation of phospholipid bilayers is driven by hydrophobic forces that thermodynamically favour the aggregation of the apolar acyl chains of the phospholipids with the exclusion of water molecules (Houslay and Stanley, 1990).

The composition and structure of membranes are determined by the combined effects of the enzyme systems that synthesise and retailor membrane lipids and by the availability of fatty acid species endogenously synthesised or obtained from the diet. Membrane lipids are constantly being replaced and degraded, and restructuring of membrane phospholipids can also occur in situ by the replacement of individual acyl chains. The molecular species of glycerolipids synthesised by cells are partly determined by the specificity of the enzymes involved in their synthesis. For instance, saturated fatty acids tend to be incorporated at the Sn1 position of glycerol-3-phosphate and unsaturated fatty acids at the Sn2 position. Dietary influences on membrane fatty acid composition are exerted through changes in the make up of the free fatty acid pool (Lynch and Thompson, 1988; Clandinin et al., 1991). This cellular pool is small and has been estimated to be between 2 - 10 μM in mammalian cells (Lynch and Thompson, 1988). Fatty acids are rapidly cycled through this pool and excess is stored as triacylglycerols, the production of which is almost unlimited. The conversion of fatty acids to CoA esters provides substrates for acylation reactions involved in phospholipid synthesis. The fatty acids 18:2n6 and 18:3n3 cannot be synthesised de novo by animals and have to be obtained from the diet. Thus the concentrations of these fatty acids and their elongation and desaturation products in membranes are strongly influenced by dietary composition.

Other lipid species are found intercalated in membrane phospholipid bilayers such as cholesterol and vitamin E. A single hydroxyl group in the structure of both these molecules confers upon them a slight amphipathic nature which affects their orientation in membranes. Cholesterol esters, dolichols, mono-, di, and triacylglycerols, and free fatty acids are minor lipid species that may also exist in some membranes (Benga and Holmes, 1984).
Membrane Proteins

Membrane proteins can be classified as integral or peripheral. An integral protein is defined as one which requires organic solvents or detergents for its extraction from a membrane (Houslay and Stanley, 1990). Peripheral proteins are those which can be removed by high or low ionic strength buffers (Houslay and Stanley, 1990). Integral proteins may span both leaflets of the phospholipid bilayer or be partially embedded. The surfaces of these proteins in the region of the bilayer are apolar. Peripheral proteins interact electrostatically with the polar surfaces of other proteins and lipids in the bilayer. The protein content of different membranes varies. For example, the protein content of myelin is approximately 25% by weight compared to 75% in inner mitochondrial membranes (Houslay and Stanley, 1990). This difference reflects the varied functions carried out by membranes. The main function of myelin is its role as an insulator of neuronal axons and it has little catalytic activity. In contrast the inner mitochondrial membrane is the site of many metabolic reactions such as those involved in oxidative phosphorylation.

The lipid composition of biological membranes is important in determining the physical properties of the bilayer. The physical behaviour of membranes is believed to exert an influence over membrane function, for example by the modulation of membrane protein activity. The most widely studied physical property of biological membranes is membrane fluidity and this will now be considered.

9.1.2 Membrane Fluidity

Fluidity is the property of a liquid that describes its ease of movement and is the inverse of viscosity. Membrane fluidity is a term that is often used in a general sense to describe the overall degree of mobility of membrane components. However it must be appreciated that phospholipids, proteins and other membrane components can undergo several modes of movement. Biological membranes at physiological temperatures are in a liquid crystalline or "fluid" state. Phospholipid molecules can rotate about their long axis and carbon-carbon bonds of the acyl chains can undergo transient trans - gauche isomerisations. The formation of gauche isomers results in kinks in the acyl chains and weakens Van der Waal interactions between neighbouring acyl chains. The mobility of
acyl chains increases towards the core of the bilayer with a greater frequency of trans-gauche isomerisation (Houslay and Stanley, 1990). If phospholipids bilayers are cooled below a certain temperature (the transition temperature), trans-gauche isomerisations cease and an all-trans configuration is assumed by the acyl chains. This allows regular packing of the fully extended, parallel acyl chains in a quasi-hexagonal array. The bilayer then becomes gel-like or solid in nature. The temperature at which this transition occurs depends upon the length and unsaturation status of the phospholipid acyl chains, and the nature of the head groups. In addition to intramolecular movements, phospholipids can rapidly diffuse laterally in the plane of the bilayer. Phospholipids can also exchange, or “flip-flop”, between the two leaflets of the bilayer. In synthetic bilayers this is a very slow process with a half-life of days (Houslay and Stanley, 1990). This is because it is thermodynamically unfavourable to move the polar head group of phospholipids through the hydrophobic core. In membranes there is evidence that this process may be mediated by proteins in an energy dependent manner (Tocanne et al., 1994). This is believed to contribute to an asymmetric distribution of different phospholipid species between the two leaflets of biological membranes (Tocanne et al., 1994).

Integral membrane proteins can also undergo fast axial rotation and are able to diffuse laterally within the plane of the bilayer. However, unlike phospholipids, integral membrane proteins are unable to “flip-flop” between leaflets.

The original concept of the free lateral diffusion of membrane lipids and proteins with respect to each other as proposed by the fluid mosaic model is not now generally accepted. Although proteins and lipids are able to diffuse laterally across a leaflet, there is evidence that these movements are restricted. Membrane proteins are believed to form a network through associations of integral membrane proteins with the cytoskeleton and other integral and peripheral proteins (Houslay and Stanley, 1990; Tocanne et al., 1994). This network can act as a barrier to the lateral diffusion of membrane proteins and lipids resulting in a lateral compartmentalisation of membrane components. An example of this is the barrier to lateral diffusion of components of the outer leaflet of epithelial
plasma membranes between apical and basal domains imposed by epithelial tight junctions (Tocanne et al., 1994).

9.1.3 The Relationship Between Membrane Structure and Function

Biological membranes define the boundaries of living cells, serving as hydrophobic barriers to the free diffusion of substances into and out of the intracellular compartment. They are also responsible for communication, transport and enzymatic activities that are mediated by membrane proteins. Depending upon their position, membrane proteins interact hydrophobically in the core of the bilayer or electrostatically at its surface with the adjacent lipids. It is therefore reasonable to assume that the activities of integral membrane proteins are sensitive to the nature of their lipid environment in terms of both its specific chemical nature, such as polar head group species, and its physical properties, such as the degree of fluidity. Therefore, in order to understand the relationship between membrane structure and function it is important to consider the effect of membrane lipids on membrane proteins.

Membrane lipids may modulate the function of membrane proteins such as receptors, transporters and enzymes by specific interactions that affect their conformation. Integral proteins are surrounded by a ring or annulus of lipid with which they directly interact. There is a requirement that annular lipids adequately seal proteins into the bilayer if the membrane is to retain its barrier function. Interactions at the protein-lipid interface necessary for such a seal may exert a conformational constraint upon the protein and so affect its ability to undergo conformational changes that accompany its biological activity. Thus the conformation of binding and active sites of membrane proteins may well be affected by such interactions. Conformational distortions of integral proteins and their neighbouring lipids may also occur due to differences in the thickness of their hydrophobic sections (Houslay and Stanley, 1990; Tocanne, 1994). Thermodynamic forces will favour changes in their conformation that eliminate the mismatch. Thus the nature of the annular lipid accommodating integral proteins in a bilayer may influence the expression of the activity of that protein. Annular lipids can interchange with neighbouring lipid, a process that is influenced by electrostatic and van der Waal interactions between protein and lipid. However, a preference for specific lipid species by some proteins may be shown. For instance rhodopsin and Na⁺/K⁺-ATPase
preferentially accumulate acidic phospholipids within their annular domain (Houslay and Stanley, 1990). There have also been reports of co-purification of specific lipids with membrane proteins (Kinnunen, 1991) and of specificity in the interactions of integral proteins not only with phospholipid head groups, but also with acyl chains (Kinnunen, 1991). Peripheral proteins also demonstrate preferences for certain phospholipid head group species (Tocanne, 1994).

In addition to specific interactions between membrane proteins with lipids, there is considerable evidence to suggest that the bulk physical properties of a membrane, such as its fluidity and permeability, can affect membrane function. For example the insulating property of the myelin membrane can be attributed to its high proportion of phospholipids with long saturated acyl chains (Viret et al., 1990). Spector and Yorek (1985) proposed that membrane fluidity may exert an influence on membrane protein activity. Investigations on the dependence of membrane protein activity on the fluidity of the bilayer have included studies on the effect of temperature changes as a means of altering the fluidity. These experiments are analysed using Arrhenius plots, where the logarithm of reaction rate is plotted against the reciprocal of temperature. Abrupt changes in the gradient of these linear plots at a particular temperature reflect changes in the activation energy of the protein mediated activity. In some cases such break points occur at the phase transition temperature of the lipid, and hence indicate that membrane fluidity is affecting membrane protein activity. For example, Amatruda and Finch (1979) demonstrated a correlation between the phase transition of adipocyte ghosts with a breakpoint in glucose uptake. However not all break points occur at the transition temperature and care needs to be taken in the interpretation of these plots (Carruthers and Melchior, 1988; Houslay and Stanley, 1990).

Other studies have investigated the effect of altering the lipid composition of membranes on membrane physical parameters and the activity of membrane proteins (reviewed in Spector and Yorek, 1985; Viret et al., 1990). Correlations between the physical state of membranes and the activity of membrane enzymes, receptors, and transport processes have been shown. The effect of membrane fluidity on the function of membrane proteins is varied with both positive and negative correlations and biphasic
effects having been observed (Viret et al., 1990). In a number of situations the influence of membrane fluidity on the activity of membrane proteins may be less important than other membrane parameters. For instance, reconstitution studies have shown that the nature of the phospholipid head group and backbone and the length and degree of unsaturation of the phospholipid acyl chains exert a greater influence on the activity of the erythrocyte red cell sugar transporter than the fluidity of the bilayer (Carruthers and Melchior, 1988). However, as discussed above the term “fluidity” can encompass many different types of movement and the techniques used to measure this property will determine which movements are being probed and may affect the interpretation and comparison of results. Again, the mechanism through which the mobility of membrane lipids influence membrane protein activity could be due to conformational effects, or for example by the modulation of the lateral movement of the protein in the plane of the membrane (Spector and Yorek, 1985).

The influence of the lipid composition of the rod outer segment membrane sacs on the function of rhodopsin is discussed by Brown (1994) and represents one example where there is strong evidence for the modulation of a protein function by a physical property of a membrane. When a photon of light is absorbed by the 11-cis retinal prosthetic group of rhodopsin it isomerises to the all-trans conformation and activates a sequence of conformational changes in rhodopsin. The transition of metarhodopsin-I to metarhodopsin-II is believed to be the triggering event for the visual process (a conformational change exposing the recognition sites for transducin) and has been shown to be affected by the nature of the lipid environment. The rod outer segment membranes have an unusual fatty acid composition being enriched with PUFAs, especially DHA (see section 1.7.3.). These membranes favour the transition of metarhodopsin-I to metarhodopsin-II which are in an acid-base equilibrium. The effect of altering the lipid environment on this transition was investigated. It was found that the transition was sensitive to the bilayer thickness, the polar head group species and the acyl chain composition. Brown concluded from his studies that it was the overall physical properties of the bilayer and not a chemical specificity that influenced the transition. Non-lamellar forming lipids with a small head group size relative to the cross-sectional area of the acyl chain favoured the transition. Native photochemical
behaviour was achieved when rhodopsin was recombined with phospholipids where DHA had been replaced with phytanic acid which, like DHA has a large cross-sectional area compared to saturated fatty acids. The presence of such lipids exerts a curvature elastic stress on a bilayer resulting in a relatively unstable membrane. In the case of rhodopsin, such a structure appears to provide an energetically favourable environment for the conformational changes required for its function. These in vitro rhodopsin reconstitution studies suggested that phytanic acid incorporation into photoreceptor outer membrane discs was unlikely to perturb the function of rhodopsin. However, as discussed in section 1.7.7, it has not been shown in vivo whether phytanic acid can be stably incorporated into the phospholipids of these membranes. In addition, the retina appeared to have a specific requirement for DHA in vivo as a compensatory increase in docosapentaenoic acid (22:5n6), which is structurally similar to DHA, during a dietary deficiency of DHA, did not prevent visual dysfunction (see section 1.7.6).

The relationship between bilayer lipid composition and membrane protein activity is complex and involves general physical properties such as bilayer fluidity and specific biochemical interactions. In order to investigate the molecular distortion hypothesis, the effect of phytanic acid on membrane fluidity (Part I) and on the membrane transport of $[^3H]$choline (Part II) which is a membrane associated function, were investigated.
9.2 The Effect of Phytanic Acid Supplementation on the Fluidity of Rat RPE and Y79 Cell Membranes

9.2.1 Measurement of Membrane Fluidity

There are a number of techniques available for the measurement of specific movements of molecules in membranes that contribute towards its overall fluidity. For example, fluorescence recovery after photobleaching is a technique used to measure the lateral diffusion coefficients of fluorometrically labelled membrane components (Houslay and Stanley, 1990) and electron spin resonance (ESR) has been used to determine the rate of flip-flop of nitrooxide labelled phospholipids in liposomes and intact erythrocytes (Houslay and Stanley, 1990). There are also a range of techniques that provide information on the overall degree of mobility or fluidity of membrane bilayers. For example, the melting or transition of bilayers from the crystalline phase to the liquid crystalline phase with change in temperature can be followed using differential scanning calorimetry. The temperature at which this transition occurs is indicative of the degree of fluidity of a membrane. Freeze fracture electron microscopy is a means of visualising the interior of membranes and can reveal the presence of solid and fluid domains. The mobility of molecules at various depths in membrane bilayers may be measured using ESR, nuclear magnetic resonance (NMR) and fluorescence polarisation techniques that utilise labelled phospholipids or probes incorporated into a bilayer. The mobility of these molecules will depend on the local viscosity. More specifically, it is the average orientation of bilayer molecules relative to the membrane surface and the rates of reorientation that are generally considered to determine the measured mobility of these incorporated components. In this study, fluorescence polarisation using diphenylhexatriene (DPH) as the probe was used as a measure of membrane fluidity.

9.2.2 Fluorescence Polarisation

Fluorescence polarisation or anisotropy (the quality of having unequal properties in different directions) is a measure of the degree of movement (rotation) of an extrinsic fluorophore incorporated into a membrane. When a fluorophore is excited with
monochromatic light which is polarised, the emitted light will also be polarised. Rotation of the fluorophore within the lifetime of the excited state will result in a depolarisation of the emitted polarised light. Thus the degree of polarisation of emitted light can be used as a measure of the degree of rotation of the probe which in turn is related to its ease of movement in the membrane. Figure 9-3 is a representation of the excitation and emission of a rod shaped fluorophore such as DPH (see below) which has an absorption and emission dipole moment parallel to the long axis of the molecule. Excitation of the fluorophore with light polarised parallel to the Z-axis will result in emission of light polarised in the same direction (I_{VV}) unless the fluorophore rotates. In the measurement of fluorescence anisotropy, polarised emission is detected at right angles parallel (I_{VV}) and perpendicular (I_{VH}) to the direction of polarisation of the excitation light (see figure 9-3). Steady state fluorescence anisotropy (r_s) is the ratio of intensities of the polarised component of emitted light to the total emitted light following continuous illumination.

\[ r_s = \frac{I_{VV} - I_{VH}}{I_{VV} + 2I_{VH}} \]  

(Eq. 9-1)

where,

I_{VV} = \text{Intensity of light when both the excitation and emission polarisers are vertically positioned.}

I_{VH} = \text{Intensity of light when the excitation polariser is in the vertical position and the emission polariser is horizontal.}

In the above equation, the total intensity is equal to I_{VV} + 2I_{VH} (Lakowic, 1983). This arises from the assumption that a fluorophore such as that depicted in figure 9-3 is symmetrically orientated about the Z axis. Using this model, the emitted light can therefore also be expected to be symmetrically orientated about the Z axis. Thus equal intensities of light would be emitted along the Z, X and Y axes (i.e. in one vertical and two horizontal planes).
Figure 9-3: Measurement of steady state fluorescence anisotropy using the fluorescence probe diphenylhexatriene (DPH). The probe is excited using plane polarised light and polarised emission is detected in the same plane and at right angles to the excitation light. Rotation of the probe within the lifetime of the excited state will result in a depolarisation of fluorescence.
**Diphenylhexatriene**

Diphenylhexatriene is a probe molecule commonly used in fluorescence depolarisation studies (see figure 9-4). It is a polyene hydrocarbon with a stable all-trans configuration of an elongated rectangular shape (Shinitzky and Barenholz, 1978). DPH will readily partition into the hydrophobic domains of lipid bilayers. It partitions equally well into solid and fluid lipid domains and hence can be considered to be equally distributed in membranes with heterogeneous viscosity (Shinitzky and Barenholz, 1978). Within a bilayer it is oriented parallel to the long axis of the phospholipid molecules, although a small population orientates perpendicular to this position in the bilayer centre (van Ginkel, 1989) (see figure 9-5). The absorption dipole moment of DPH is orientated parallel to the long axis of the molecule and the emission dipole is nearly colinear with the absorption dipole. Rotation of the long axis can therefore be measured as a depolarisation of the fluorescence. The absorption and emission maxima are 355 and 425 nm respectively (Shinitzky and Barenholz, 1978). In an aqueous environment the quantum yield is negligible (Shinitzky and Barenholtz, 1974).

**Interpretation of Steady State Fluorescence Anisotropy**

To understand the interpretation of $r_s$ it is necessary to briefly consider its mathematical derivation. The orientation of a molecule in a membrane can be described in terms of the angular displacement ($\beta$) between its axis of symmetry and the bilayer normal (see figure 9-5). An orientational distribution function $f(\beta)$ characterises the average orientation of a molecule in a membrane. The distribution function may be expressed as an infinite series of goniometric (concerning the measurement of angles) functions of the angle $\beta$, the coefficients of which are called order parameters (van Ginkel et al., 1989). It is only possible to determine the lowest order parameters, denoted $\langle P_2 \rangle$ and $\langle P_4 \rangle$, experimentally.

$$\langle P_2 \rangle = \frac{\langle (3 \cos^2 \beta - 1) \rangle}{2}$$  \hspace{1cm} (Eq. 9-2)

$$\langle P_4 \rangle = \frac{\langle (35 \cos 4\beta - 30 \cos 2\beta + 3) \rangle}{8}$$  \hspace{1cm} (Eq. 9-3)
Figure 9-4:

a) Structure of the fluorescent probe diphenylhexatriene (DPH) which localises to the hydrophobic interior of membranes.

b) Structure of 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH) which localises closer to the polar surface of membranes.
Figure 9-5: Positioning of DPH in membranes can be described by its angular displacement ($\beta$) between its axis of symmetry and the bilayer normal (see text).
Steady state fluorescence anisotropy of DPH is a function of the order parameter $\langle P_2 \rangle$ and the rotational diffusion coefficient $D_\perp$ of the long axis, (the tumbling motion).

$$r_s = f(\langle P_2 \rangle, D_\perp) \quad \text{(Eq. 9-4)}$$

It has been shown that when $r_s > 0.2$, the contribution of $\langle P_2 \rangle$ dominates, but for $r_s < 0.2$, $D_\perp$ is the major influence (van Ginkel, 1989).

### 9.2.3 Experimental Aim

Branched chain fatty acids are not normally found in the membranes of eukaryotes. Thus the incorporation of phytanic acid into membrane lipids in the place of straight chain acyl groups may well be expected to alter the interactions between neighbouring acyl chains and hence the degree of mobility of these components. It was the intention of the following studies to investigate whether an alteration in membrane fluidity could be detected in cultured retinal cells (RPE and Y79) exposed to phytanic acid.

### 9.2.4 Materials

Diphenylhexatriene, phosphate buffered saline (PBS) (Sigma-Aldrich Company Ltd., Poole, Dorset, UK); tetrahydrofuran (Rathburn Chemicals Ltd., Walkerburn, Scotland).

### 9.2.5 Steady State Fluorescence Anisotropy Measurements Using DPH as the Probe

A 2 mmol/l stock solution of diphenylhexatriene in tetrahydrofuran was stored at $-20^\circ\text{C}$ in the dark. A fresh 5 μM working solution was prepared daily by dilution of the stock solution with PBS, stirring in the dark at $4^\circ\text{C}$ for 2 to 3 hours before use.

Sonicated cell suspensions in PBS from a 75 cm$^2$ confluent flask were centrifuged at 100000 g for 1 hour. The membrane pellet was then rinsed 3x with PBS and homogenised in approximately 150 μl PBS, using a hand held 0.1 ml glass homogeniser. Appropriate aliquots (typically 5 and 10 μl) were taken for protein determination using the bicinchoninic acid protein assay (see section 6.3.4).
Membrane suspensions equivalent to approximately 100 μg of protein were incubated with 2 ml 5 μmol/l DPH in PBS at 37°C for 1 hour in the dark. It had been determined that this quantity of membrane suspension resulted in a measurable fluorescence emission when incubated with 5 μmol/l DPH (see below). Fluorescence anisotropy was determined using a Perkin Elmer LS3 fluorescence spectrophotometer with excitation and emission wavelengths of 360 nm and 430 nm respectively and band widths of 10 nm. Fluorimeter cells were maintained at 37°C using a circulating water bath. Excitation and emission wavelengths were polarised vertically (0°) and horizontally (90°).

Fluorescence was measured at the following polariser settings for each sample.

\[
\begin{array}{cccc}
\text{Excitation} & 0^\circ & 90^\circ & 0^\circ & 90^\circ \\
\text{Emission} & 0^\circ & 90^\circ & 0^\circ & 90^\circ \\
\end{array}
\]

Fluorescence readings of DPH in PBS alone were subtracted from the readings given by DPH and membranes for each polariser setting. RPE membrane suspensions in PBS alone produced negligible fluorescence (see below).

Steady state anisotropy \( r_s \) was calculated using the following equation:

\[
r_s = \frac{\left( \begin{array}{cc} 0 & 90 \\ 0 & 90 \end{array} \right) - \left( \begin{array}{cc} 90 & 0 \\ 0 & 90 \end{array} \right)}{\left( \begin{array}{cc} 0 & 90 \\ 0 & 90 \end{array} \right) + 2 \left( \begin{array}{cc} 90 & 0 \\ 0 & 90 \end{array} \right)} \quad \text{(Eq. 9-5)}
\]

9.2.6 The Effect of the Quantity of Membrane Suspension on Fluorescence Emission and Anisotropy Measurements

To establish the protocol for the determination of \( r_s \), the effect of incubating different amounts of membrane suspension with 5 μmol/l DPH on the individual fluorescence
readings at different polariser settings and the overall steady state fluorescence anisotropy ($r_s$) measurement was investigated. Volumes of between 10 to 100 µl of two separate membrane suspensions of known protein concentration, prepared from parent RPE cells grown in normal medium, were added to 2 ml 5 µmol/l DPH solution. Membranes were incubated and fluorescence readings were taken as described in 9.2.5. Blank readings of DPH in PBS without membranes (DPH blank) and of membranes incubated in PBS without DPH (membrane blank) were taken. Due to a limited amount of sample, it was not possible to take blank membrane readings for all the volumes of membrane suspension used. However, it was found that the fluorescence readings of membranes alone was very low. For example 154 µg of a membrane suspension in 2 ml of PBS gave fluorescence readings ranging from 0.1 to 0.4 depending on the polariser setting. The results are shown in table 9-1. The intensity of the individual fluorescence readings at different polariser settings increased with increasing amount of membrane added to the incubation mixture. For example when 20 µg of membrane suspension 1 was used, the reading at $0^\circ$ setting was 4.9 compared to 45.3 when 192 µg was used. However the overall steady state anisotropy values ($r_s$) remained relatively unchanged. The variation in $r_s$ values obtained did not follow a trend and most likely reflected the reproducibility of the method (see below). The corrected values, obtained by subtracting the individual fluorescence readings of the DPH blank and membrane blank from the sample reading at the same setting before calculation of $r_s$, are also shown for those samples where membrane blanks were taken. From these results it was determined that incubation of DPH with an amount of membrane suspension equivalent to 100 µg protein would be suitable for the measurement of steady state fluorescence anisotropy.

**9.2.7 Reproducibility of Method**

The reproducibility of the above method was assessed by determining the coefficient of variation for repeated measurements of a single pool of cells (intra-flask variability) and for single measurements of equivalent cells grown in parallel (inter-flask variability). This study was performed on confluent parent RPE cells, seeded from the same source. The results are shown in figure 9-6 and in table 9-2. The coefficient of variation for the intra-flask experiment was 1.5 % and for two inter-flask studies were 4.3 % and 1.8 %. These results demonstrated that the method was reproducible when comparing the $r_s$.
Table 9-1: The effect of quantity of membrane (µg protein) on individual fluorescence readings at different polariser settings and the calculated steady state fluorescence anisotropy ($r_s$) of RPE membrane suspensions incubated with 2 ml 5 µmol/l DPH.

Corrected $r_s$ values were calculated after subtracting DPH and membrane blanks from the uncorrected fluorescence readings at each polariser setting.

IS = insufficient sample for correction to be made.
Steady State Fluorescence Anisotropy Measurements of RPE Cell Membranes

Figure 9-6: Reproducibility of steady state fluorescence anisotropy ($r_s$) measurements of rat RPE membranes. Reproducibility within a cell population (intra-flask) and between cell populations (inter-flask).

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Mean $r_s$</th>
<th>SEM</th>
<th>c.v. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intra-flask</td>
<td>10</td>
<td>0.159</td>
<td>0.0007</td>
<td>1.5</td>
</tr>
<tr>
<td>Inter-flask 1</td>
<td>9</td>
<td>0.162</td>
<td>0.0023</td>
<td>4.3</td>
</tr>
<tr>
<td>Inter-flask 2</td>
<td>10</td>
<td>0.157</td>
<td>0.0009</td>
<td>1.8</td>
</tr>
</tbody>
</table>

Table 9-2: Reproducibility of steady state fluorescence anisotropy ($r_s$) measurements in RPE cell membranes.

SEM = standard error of the mean

c.v. = coefficient of variation
values of cells from both within the same population and between equivalent populations.

9.2.8 Effect of Phytanic Acid Supplementation on the Membrane Fluidity of RPE Cell Membranes

Confluent parent RPE cells were supplemented with ±150 μmol/l phytanic acid (8 x 75 cm² flasks per group) for seven days, and $r_s$ values of membrane suspensions from each flask were determined and compared. The results are shown in figure 9-7. The mean ± SEM $r_s$ value for the phytanic acid supplemented group was 0.148 ± 0.011 compared with 0.183 ± 0.010 for the control group ($p < 0.001$). The lower steady state fluorescence anisotropy value of the phytanic acid supplemented group compared to the control group corresponded to a lower degree of order and hence a higher degree of fluidity of the membrane lipids in this group.

9.2.9 Membrane Fluidity of Y79 cells Supplemented with Phytanic Acid

Y79 cells were seeded at a density of 4.5 x 10⁵ cells/ml in 20 ml of medium with or without 100 μmol/l phytanic acid for 7 days. This supplementation concentration of phytanic acid was chosen for this study as it had previously been shown to result in its uptake by Y79 cells that was approaching a maximum (see section 4.2.3). The results of the membrane fluidity measurements are shown in figure 9-8. The mean (± SEM) $r_s$ value was 0.070 ± 0.008 ($n = 4$) for the control group and 0.009 ± 0.002 ($n = 4$) for the phytanic acid supplemented group ($p < 0.01$). Again this result can be interpreted as an increase in the membrane fluidity of Y79 cells supplemented with 100 μmol/l phytanic acid compared to controls.

9.2.10 Discussion

Supplementation of RPE and Y79 cells with pathological concentrations of phytanic acid (150 and 100 μmol/l respectively) decreased the steady state fluorescence anisotropy of DPH in suspensions of crude membrane preparations. This can be interpreted as an increase in the fluidity of the membranes from these supplemented cells. The values of $r_s$ obtained in these studies were < 0.2 which indicated that $r_s$ was primarily determined by the rotational motion of the long axis of DPH (see 9.2.2). It can therefore be reasoned that there is a decrease in the restriction to this motion in
Figure 9-7: Steady state fluorescence anisotropy ($r_s$) of RPE membranes from cells supplemented with ± 150 μmol/l phytanic acid for 7 days.

$n = 8$ and bar represents the mean.
Figure 9-8: Steady state fluorescence anisotropy ($r_s$) of Y79 membranes from cells supplemented with ± 100 μmol/l phytanic acid for 7 days.

$n = 4$ and the bar represents the mean.
membranes from phytanic acid supplemented cells. The analysis of the fatty acid composition in phytanic acid supplemented cells showed that a significant proportion of phytanic acid (~20%) taken up by cells was incorporated into the phospholipid fraction (see section 5.3). Thus the effect of phytanic acid on \(r_s\) was likely to be due to the presence of phospholipids containing one or two phytanyl side chains. The presence of four methyl groups along the phytanyl chain would be expected to decrease hydrophobic interactions and increase the distance between neighbouring fatty acyl groups, thus reducing the packing order of phospholipids.

A number of studies on the effect of phytanic acid on the properties of membranes have been described. The effect of phytanic acid supplementation on the biochemical composition and electrophysiological properties of membranes of a strain of the fungus Neurospora crassa, which is unable to synthesise fatty acids, was described by Friedman and Glick (1982). This group showed that an incorporation of phytanic acid into phospholipids of this fungus was associated with a lowering of the phase transition temperature \(T_g\), an increased dependence of membrane potential on temperature, and an alteration in the species of sterols present in the lipid fraction. Another study reported the effect of free phytanic acid on liposomes composed of dipalmitoylphosphatidylcholine:1-palmitoyl, 2-stearoylphosphatidylcholine (3:1) and free phytanic acid (Yue et al., 1988). \(T_g\) was lowered and there was a broadening of the range over which it occurred. The enthalpy of transition was also decreased. A lowering of \(T_s\) in liposomes containing free phytanic acid, phytol or \(\alpha\)-tocopherol was also reported by Fukuzawa et al (1980). This effect of phytanic acid, free and phospholipid bound, on \(T_s\) is strong evidence that phytanic acid disrupts the phospholipid packing order in the gel phase and increases the membrane fluidity.

The effect of free phytanic acid and phytol intercalated between bilayer phospholipids on the order of phospholipid acyl chains has been investigated (Fukuzawa et al., 1980; Yue et al., 1988). Using deuterium nuclear magnetic resonance, an order parameter for phospholipid vesicles containing free phytanic acid and phytol above \(T_s\) was determined. Free phytanic acid and phytol slightly increased the mean order parameter to an extent comparable to the non-branched fatty acid, palmitic acid (16:0).
ordering effect was much smaller than that observed for \( \alpha \)-tocopherol (Yue et al., 1988). Similarly free phytol and phytanic acid in liposomes above \( T_s \) had little or only a slight lowering effect on the fluorescence polarisation of DPH (indicating a slight increase in membrane fluidity), whereas \( \alpha \)-tocopherol increased the polarisation (Fukuzawa et al., 1980). These results show that whereas \( \alpha \)-tocopherol increases the order (hence decreasing the membrane fluidity) of phospholipid bilayers above \( T_s \), free phytanic acid and phytol do not share this property. The membrane ordering effect of \( \alpha \)-tocopherol has been attributed to its chromanol moiety and not to the phytyl side chain (Fukuzawa, et al., 1980; Yue et al., 1988). These results are however not directly relevant to the present study as the majority of phytanic acid in membranes is likely to exist as phospholipid acyl chains. The effect of phospholipid bound phytanic acid on membrane fluidity has not been reported.

Although the results of the studies in this chapter correlate well with the effect of phytanic acid on the thermotropic behaviour of membranes, the presence of triacylglycerols in these membrane preparations may have complicated the interpretation of the results. McVey et al. (1981) found a difference in fluorescent polarisation measurements when comparing whole cells and isolated plasma membranes and suggested that this resulted from the presence of lipid droplets in the whole cell. It is therefore possible that contaminating triacylglycerol in the membrane preparations may have interfered with the fluorescence polarisation measurements. In order to confirm the results of the present studies, it would be necessary to repeat them using purified membrane preparations shown to be free of triacylglycerol contamination.

The measurement of fluorescence anisotropy of DPH provided information about the mobility of this probe in the inner parts of the bilayer. A number of other probes are available for the measurement of membrane fluidity that can provide information on different parts of the membrane and also on different types of molecular motion. For example, TMA-DPH (see figure 9-4b) localises closer to the lipid/aqueous interface than DPH and hence can be used to measure the membrane fluidity in the region of the polar head group. Pyrene is another fluorescent probe that can be used to measure the lateral movement in a lipid bilayer. Deuterium nuclear magnetic resonance is a useful
tool for investigating molecular interactions and has the advantage that the deuterium labelled molecule, unlike the fluorescence probes, will not perturb its environment. As described above, this technique was used by Yue et al (1988) to investigate the effect of free phytanic on physical properties of liposomes. It would be interesting to repeat such a study using phospholipid bound phytanic acid.
Part II

9.3 The Effect of Phytanic Acid On The Uptake of [methyl-3H]Choline by Y79 Cells

Choline is required by cells for the synthesis of the membrane lipids, phosphatidylcholine and sphingomyelin. It is also the precursor of the neurotransmitter acetylcholine and of betaine, a source of methyl groups involved in the remethylation of homocysteine to methionine. Most choline is obtained from the diet, with only relatively small amounts being synthesised de novo by animals including man. Choline may be delivered to cells in the form of phospholipids transported and taken up by cells as a constituent of lipoproteins or as free choline. The mechanisms of uptake of free choline have been studied in a number of cell types and various modes of transport have been identified. For example, in presynaptic membranes of cholinergic neurons a Na⁺-choline high affinity cotransport mechanism has been identified (Lerner, 1989). Choline uptake by passive diffusion at high choline concentrations (100 - 500 μmol/l) and by carrier mediated transport (facilitated diffusion) at low choline concentrations (6 - 31 μmol/l) was demonstrated in Ehrlich-Lettré ascites (Haeffner, 1975). A carrier mediated transport system for choline has been identified in the brush border membrane of rat intestine (Saitoh et al., 1992) and a conductive mechanism, whereby transport was favoured by an inside-negative K⁺ diffusion potential, has been identified in human placental brush-border epithelial membranes (Grassl, 1994).

9.3.1 Experimental Aim

The transport of choline by Y79 retinoblastoma cells was used as a model with which to study the effect of pathological concentrations of phytanic acid on a membrane function. Hyman and Spector (1982) demonstrated that, like isolated photoreceptor cells (Masland and Mills, 1980), Y79 retinoblastoma cells possess mechanisms for both high (0.15 to 1.0 μmol/l) and low (5 - 100 μmol/l) affinity choline uptake. Hyman and Spector investigated the effect of increasing polyunsaturated fatty acid (PUFA) content of Y79 cell membranes on the kinetics of the high affinity system and found that the efficiency of transport by this system was increased in PUFA enriched cells with both
the maximum velocity and Michaelis constant of this system being reduced. Treen et al (1992) also investigated choline uptake in Y79 cells enriched with DHA (22:6n3). An increase in choline uptake in DHA supplemented cells compared to unsupplemented cells was observed when cells were exposed to a choline concentration in the range of the low affinity uptake system. Thus it appears that both modes of uptake are affected by the composition of membrane lipid acyl chains. The high affinity system was postulated to be mediated through a facilitated diffusion mechanism (Hyman and Spector, 1982). Although the mechanism of uptake by the low affinity system has not been characterised it is likely that it occurs through a passive diffusion process as it occurs at high choline concentrations. In the following studies, the effect of supplementing Y79 cells with phytanic acid on the low affinity choline uptake system was investigated.

9.3.2 Materials

[Methyl-$^3$H]choline chloride (Amersham International plc, Buckinghamshire, UK); choline chloride, Dulbecco’s phosphate buffered saline (DPBS), fatty acid free bovine serum albumin (fafa-BSA) (Sigma-Aldrich Company Ltd., Poole, Dorset, UK); scintillant Hi-Safe 3 (Wallac UK Ltd. Milton Keynes, UK).

9.3.3 Y79 Cell Culture and Supplementation Conditions

The uptake of choline was studied in Y79 cells supplemented with phytanic acid. In the following studies Y79 cells were seeded at $4.5 \times 10^5$ cells/ml in 10 or 20 ml of media supplemented with 0 to 100 µmol/l phytanic acid. Cells were fed a further 5 or 10 ml of the same medium after two days and harvested on the 5th day after seeding.

9.3.4 Determination of the Uptake of [Methyl-$^3$H]Choline by Y79 Cells

The determination of choline uptake was based on the method of Treen et al (1992). At the end of the supplementation period, Y79 cells were collected by centrifugation and washed twice in DPBS + 0.3 %[fafa-BSA. The cells were then suspended in DPBS and dilutions were made for cell counting using a haemocytometer (Neubauer). Cell concentrations were adjusted to $2.5 \times 10^6$ cells/ml with DPBS and 200 µl aliquots were incubated with 20 µl 2.5 mmol/l [methyl-$^3$H]choline (with a specific activity of 3.54 nCi/nmole) for 30 minutes at 37°C in a shaking water bath. The final concentration of
choline was 227.3 μmol/l. The uptake of choline was stopped by the addition of 1 ml ice-cold DPBS + 0.3 % jaf-BSA. Cells were washed with this buffer, initially with 3 x 0.5 ml, and in later studies with 4 x 1 ml washes. The cells were collected after each wash by centrifugation at 13 000 g for 1 minute. The cells were then suspended in 50 μl of DPBS and sonicated in a water bath at 20°C for 5 minutes. Sonicates were transferred to 5 ml scintillation vials containing 2 ml scintillant (Hi-Safe 3), with 2 x 50 μl DPBS washes. Dispersion of the sonicate in the scintillant was achieved by a succession of vortexing (40 seconds), bath sonication (5 minutes), followed by further vortexing (40 seconds). Disintegrations per minute were counted using a Wallac 1410 Liquid Scintillation Counter with external standardisation for quench correction. The mean DPM of two cycles of counting was obtained and the uptake of choline by the cells calculated.

9.3.5 Background Radiation
The amount of background radiation in 200 μl of cell suspension (n = 2) and 200 μl of cell suspension washed with 1 ml of DPBS + 0.3 %/jaf-BSA (n = 3) was determined. There was no difference in the amount of background radiation in these two groups. Therefore 200 μl untreated cell suspension was used for the determination of background radiation which was subtracted from the measured radioactivity in the following studies.

9.3.6 Efficiency of the Washing Procedure
The efficiency of the washing procedure in removing external radioactivity from the cell pellet was assessed by measuring the amount of radioactivity in the last wash. It was found that when the washes were increased to 4 x 1 ml, a mean of 0.03 % (i.e. 50.8 pCi or 113 dpm; n = 10) and 0.06 % (n = 6) of the starting radioactivity (177 nCi) remained in the last wash in two separate studies. This compared to a mean of 0.1 % (179.7 pCi; n = 8) when cells were washed with 3 x 500 μl DPBS.

9.3.7 Reproducibility of the Uptake of [Methyl-3H]Choline by Untreated Y79 Cells
The reproducibility of choline uptake was assessed in Y79 cells that had been grown in unsupplemented culture media. 8 x 200 μl aliquots from a single pool of cells were incubated with [methyl-3H]choline as described above. Post incubation stopping and
wash volumes were 500 μl. The mean uptake ± SEM was 546.1 ± 18.8 pmol/10^6 cells/hr, with c.v. of 9.7 %. When repeated, with increased washing volumes (4 x 1 ml), the mean uptake of choline by the cells was 337.2 ± 22.7 pmol/10^6 cells/hr and the c.v. value was 18.8 %. The results are shown in figure 9-9. The variation of this assay was therefore higher than desired. There could be several sources of error in this assay, such as inaccuracies of the cell count, loss of viability of cells during the procedure, or loss of cells during washing. The variability could possibly be improved, for instance, by using an automated cell counter or by performing the uptake in situ in the culture flask rather than using a cell suspension. The lack of reproducibility did cause problems and the protocol was altered as described below. However an initial study was carried out prior to these changes and these results are described in 9.3.8.

9.3.8 The Effect of Phytanic Acid on the Uptake of [Methyl-^3H]Choline by Y79 Cells

The uptake of [methyl-^3H]choline by Y79 cells supplemented with 100 μmol/l phytanic acid for 5 days was compared to the uptake by cells grown in unsupplemented medium for the same time. Cells from 5 x 75 cm^2 flasks were used for each group, counted and assayed separately. The results are shown in figure 9-10. The uptake by the phytanic acid supplemented group was significantly increased (p = 0.005) at 791.1 ± 80.7 pmol/10^6 cells/hr (mean ± SEM), whereas the control group had an uptake of 473.7 ± 21.5 pmol/10^6 cells/hr. The c.v. was 10.1 and 22.8 % respectively. Thus supplementation of Y79 cells with 100 μmol/l over 5 days appeared to result in an increased uptake of choline.

9.3.9 Alteration in Protocol

It was noted that the reproducibility of this method varied and a number of studies were conducted in which there was a large scatter which tended to obscure statistical differences between control and treated groups. To try and reduce the within group scatter, the protocol was altered. Cells from separate flasks of the same group were pooled and three cell counts per group were made to try and improve the accuracy of the cell counting. Replicates of each pool were then assayed for choline uptake. The effect of phytanic acid supplementation of Y79 cells on choline uptake was investigated using this altered protocol. The mean ± SEM uptake of [methyl-^3H]choline
The Uptake of [Methyl-\textsuperscript{3}H]Choline by Untreated Y79 Cells

Figure 9-9: The uptake of [methyl-\textsuperscript{3}H]choline by untreated Y79 cells, measured on two separate occasions.

<table>
<thead>
<tr>
<th>[Methyl-\textsuperscript{3}H]Choline Uptake (pmol X 10\textsuperscript{6} cells\textsuperscript{-1} x hr\textsuperscript{-1})</th>
<th>0.5 ml Washes</th>
<th>1.0 ml Washes</th>
</tr>
</thead>
<tbody>
<tr>
<td>800</td>
<td>■</td>
<td>▲</td>
</tr>
<tr>
<td>600</td>
<td>■</td>
<td>▲</td>
</tr>
<tr>
<td>400</td>
<td>■</td>
<td>▲</td>
</tr>
<tr>
<td>200</td>
<td>■</td>
<td>▲</td>
</tr>
<tr>
<td>0</td>
<td>■</td>
<td>▲</td>
</tr>
</tbody>
</table>

Cells were washed with 3 x 0.5 ml (■) or with 4 x 1.0 ml (▲) DPBS + 0.3 % faf-BSA.

\( n = 8 \) and bar shows the mean; replicates are repeated measurements from a pooled population of cells.
Figure 9-10: The uptake of [methyl-³H]choline by Y79 cells supplemented with 100 μmol/l (■) or 0 μmol/l (▲) phytanic acid over 5 days.

n = 5 and bar shows the mean; replicates are measurements from separate populations of cells.

p = 0.005
by phytanic acid supplemented cells (100 μmol/l) was 728.3 ± 30.3 pmol/10^6 cells/hr (n = 10), and was significantly increased (p = 0.0001) compared to the uptake by control cells (458.7 ± 11.5 pmol/10^6 cells/hr; n = 10). The c.v. was 13.2 and 7.9 % respectively. The results are presented in figure 9-11 and are comparable to the values obtained in the previous study.

This study was repeated using Y79 cells supplemented with 50 μmol/l phytanic acid. The results are presented in figure 9-12. The mean uptakes ± SEM were similar i.e. 372.8 ± 29.1 and 398.9 ± 21.8 pmol/10^6 cells/hr for the 50 and 0 μmol/l supplemented groups respectively (n = 10 for each group).

9.3.10 Discussion

In the above studies, a 58 and 67 % increase in choline uptake by Y79 cells was observed on two separate occasions when these cells were supplemented with 100 μmol/l phytanic acid, whereas 50 μmol/l supplementation had no measurable effect. Y79 cells were exposed to 227 μmol/l choline in these studies which is in the concentration range of the low affinity transport system identified by Hyman and Spector (1982), that probably operates through passive diffusion. The diffusion rate of a charged particle such as choline is determined by both the concentration and electrical potential gradients across a membrane. Whilst small uncharged water soluble particles are able to diffuse through the lipid matrix of membranes, charged particles such as choline are unable to do so (Byrne and Schultz, 1994). Diffusion of charged particles is thought to occur through aqueous channels or by a protein carrier mediated mechanism (facilitated transport) (Byrne and Schultz, 1994). Thus the uptake of choline by the low affinity transport system is likely to be a protein mediated event.

The supplementation of Y79 cells with 100 μmol/l phytanic acid over 7 days resulted in extensive modification of the lipid fatty acyl composition of total lipids and the phospholipid fraction whereby phytanic acid accounted for 46 and 31 % of total fatty acids respectively (see section 4.5.1). Supplementation of Y79 cells with 50 μmol/l phytanic acid over 7 days also resulted in a significant uptake of phytanic acid into crude membranes (32 % of total peak area of crude membrane extracts) (see section 4.2.3). Although the supplementation period was shortened to 5 days in the choline
The Effect of Phytanic Acid on [Methyl-\(^3\)H]Choline Uptake by Y79 Cells

Figure 9-11: The uptake of [methyl-\(^3\)H]choline by Y79 cells supplemented with 100 µmol/l (■) or 0 µmol/l (▲) phytanic acid over 5 days.

n = 10 and bar represents the mean; replicates of each group are measurements from a pooled population of cells.
The Effect of Phytanic Acid on [Methyl-^3H]Choline Uptake by Y79 Cells

Figure 9-12: The uptake of [methyl-3H]choline by Y79 cells supplemented with 50 µmol/l (■) or 0 µmol/l (▼) phytanic acid over 5 days.

n = 10 and bar represents the mean; replicates of each group are measurements from a pooled population of cells.
uptake studies, this is unlikely to result in major differences in phytanic acid uptake by the Y79 cells compared to the 7 day incubations as there was little difference in uptake at 5 days compared to 7 days in studies using rat RPE cells (see figure 4-4). The lack of an effect of 50 μmol/l supplementation on the uptake of choline suggested that the low affinity uptake system was sensitive only to an extensive uptake of phytanic acid into membrane phospholipid.

A number of other studies have been carried out on the effect of modulating the fatty acyl composition of membrane lipids of Y79 cells on the transport of choline and other molecules. Treen et al (1992) reported a 20 % increase in choline uptake by the low affinity system when Y79 cells were supplemented with 20 μmol/l DHA. Hyman and Spector (1982) reported an increased efficiency of choline uptake by the high affinity system of Y79 cells supplemented with 30 μmol/l linolenic acid (18:3n3), DHA, or arachidonic acid (20:4n6), whereas supplementation with the monounsaturated fatty acid, oleic acid (18:1n9) had no effect. Taurine and glycine uptake, but not leucine, serine, glutamate, and α-aminoisobutyrate uptake by Y79 cells have also been shown to be affected by polyunsaturated fatty acid enrichment (reviewed in Spector and Yorek, 1985). Taurine uptake like choline uptake was enhanced, whereas glycine uptake was reduced in these studies. Overall these studies demonstrated that not all membrane transport systems were affected by the same membrane lipid modifications.

In the literature there are numerous reports of investigations into the effect of modulating the composition of membrane lipids on membrane functions using in vitro cell culture systems, membrane proteins reconstituted in phospholipid vesicles and dietary studies in man and animals (for example, Sandra et al., 1984; Christon et al., 1988; Corrocher et al., 1992; Sohal et al., 1992; Coetzer et al., 1994; Mills et al., 1995; Murphy, 1995; Martin and Meckling-Gill, 1996; Vazquez et al., 1996; and see reviews by Spector and Yorek, 1985 and Carruthers and Melchior, 1988). Alterations in the phospholipid fatty acyl profiles of membranes have been correlated with changes in carrier-mediated transport and membrane enzyme activities, the binding properties and numbers of membrane receptors, phagocytotic and endocytotic processes and susceptibility to complement-mediated cytolysis (Spector and Yorek, 1985). Other
effects on membrane functions have also been associated with the modulation of phospholipid head groups and the cholesterol content of membranes (Spector and Yorek, 1985). Spector and Yorek (1985) have proposed that such modulations of membrane lipid composition may affect membrane protein functions by altering the physical properties of those membranes and/or through specific interactions between the protein and lipids (see section 9.1.3). In the current study it has been shown that supplementation of Y79 cells with 100 μmol/l phytic acid appeared to increase the membrane fluidity as measured by fluorescence anisotropy using DPH as the probe (see section 9.2.11). Thus it is possible that the low affinity choline transport by Y79 cells is affected through such a mechanism. In contrast however, Treen et al (1992) reported an increased uptake of choline by DHA supplemented Y79 cells, but did not observe a change in the DPH fluorescence anisotropy of intact cells. However they did observe an increase in the lateral fluidity of the membrane probe pyrene, demonstrating some change in the physical properties of the cellular membranes. The differences in results between the current study and that of Treen et al (1992) could be due to supplementation with different fatty acids and/or to the fact that this group measured DPH fluorescence anisotropy in intact cells.

The modulation of membrane protein activity by the fluidity of the bilayer, as mentioned earlier (section 9.1.3), may occur as result of altered abilities of proteins to undergo conformational changes that accompany their activity. In terms of the transport processes, this could conceivably apply to one model for facilitated diffusion whereby the protein carrier operates through gross movements of subunits and these movements may be rate limited by the membrane fluidity (Carruthers and Melchior 1990). However the simple diffusion of substrates through a fixed pore system is unlikely to involve conformational changes and it is unlikely that this process will be affected by membrane fluidity. The precise mechanism for choline uptake by the low affinity system has not been determined, but the effect of fatty acid supplementation on this system is consistent with facilitated diffusion involving a protein carrier.

The results of this study are by no means conclusive and further work is needed to verify the results. For instance the effect of supplementation of palmitate (16:0), the
unbranched analogue of phytanic acid could be measured as an additional control. The use of a choline concentration that falls within the range of the high affinity choline transport system would allow comparison with the results of Hyman and Spector (1982). Additionally other transport processes of Y79 cells such as those mentioned above could be investigated.

**9.4 Summary**

The results from the membrane fluidity and choline uptake studies have provided evidence that supplementation with pathological concentrations of phytanic acid increases the membrane fluidity of RPE and Y79 cells and affects a membrane function of Y79 cells. These results are therefore consistent with the molecular distortion hypothesis - that incorporation of phytanic acid into cellular membranes alters the physical properties and the function of those membranes. Reconstitution studies could be carried using phytanoyl-phospholipids to further study the effect of phytanic acid on membrane protein functions. Ultimately however, it would be necessary to demonstrate that membrane abnormalities were correlated with cellular dysfunction and degeneration in the affected tissues of ARD patients. The rarity of this disease would make this a difficult task.
10. GENERAL DISCUSSION

The aims of this study were to develop an in vitro model of ARD in the retina and to use this model to investigate the antimetabolite and molecular distortion hypotheses which have been suggested to explain the pathogenesis of this disease. The antimetabolite hypothesis proposes that phytanic acid accumulation in the tissues of patients with ARD may interfere with the function of vitamin E due to similarities in the structure of these two molecules. Interest in this hypothesis is relevant to the possible treatment of patients with phytanic acid accumulation as an induced vitamin E deficiency state could possibly be overcome by dietary supplementation. The molecular distortion hypothesis suggests that phytanic acid incorporation into membrane lipids may alter the function of those membranes through changes in membrane structure.

To investigate these hypotheses, methods were established for the in vitro culture of mammalian cell lines of retinal origin which included a primary bovine and an immortalised rat retinal pigment epithelial (RPE) cell line and a retinoblastoma cell line (Y79). Methods were also validated for the reproducible supplementation of lipids which included phytanic acid, α-tocopherol and linolenic acid. The antimetabolite hypothesis was investigated by studying the effect of exposure to pathological concentrations of phytanic acid on the concentration and uptake of α-tocopherol by the rat RPE cell line. The effect of phytanic acid on the susceptibility of α-tocopherol supplemented Y79 cells to in vitro peroxidative stress was also determined. The molecular distortion hypothesis was explored by assessing the effect of phytanic acid supplementation on the membrane fluidity of the rat RPE and Y79 cell lines and on the uptake of choline by Y79 cells.

In summary, no evidence was found in support of the antimetabolite hypothesis. Pathological concentrations of phytanic acid did not significantly influence the uptake or the concentration of α-tocopherol in rat RPE cells and did not appear to interfere with its antioxidant properties. However, evidence in favour of the molecular distortion hypothesis was provided by the membrane fluidity and choline uptake studies.
The results from these latter studies suggested that membranes which take up and incorporate significant concentrations of phytanic acid into their phospholipids are subject to altered physical properties which can be translated to changes in membrane function. In the retina this may be relevant to a number of cellular processes, such as those involved in the turnover and recycling of the photoreceptor outer segment membranes. This involves the phagocytosis, degradation and the return of material of the outer segment membranes by the RPE. Disruption of RPE membrane functions would in turn disrupt this recycling. It has been shown *in vitro* that phytanoyl phospholipids do not affect the function of rhodopsin. This indicates that *in vivo* the incorporation of phytanic acid into outer segment membranes is unlikely to disrupt the function of photoreceptors. However there appears to be a specific requirement for docosahexaenoic acid (DHA) (22:6n3) by photoreceptor outer segment membranes, as fatty acids of similar structure such as docosapentaenoic acid (22:5n6) are unable to compensate for a deficiency of DHA. DHA is conserved in the retina and this appears to involve recycling mechanisms specific for DHA (Bazan et al., 1992). It has been suggested that the recycling that occurs between the RPE and photoreceptors may involve transport proteins in the interphotoreceptor space that are specific for DHA (Anderson et al., 1992).

It has been reported that phospholipids with branched acyl chains in both the Sn1 and Sn2 positions are poor substrates for phospholipase A2 and human lecithin-cholesterol acyltransferase (DeBose et al., 1985; Pownall et al., 1987). Thus the presence of such phospholipids may disrupt the degradative processes in the RPE and contribute to the accumulation of phytanic acid in the retina. The presence of lipid inclusions in the nervous tissues of ARD patients has been reported and the results from the *in vitro* studies described in this current work suggested that an accumulation of lipid material in the cytoplasm of the RPE was likely to occur. The mere physical presence of large amounts of lipid in the cytoplasm of cells could cause a general disruption of cellular functions.

It would be appropriate to follow up the studies on the molecular distortion hypothesis by studying further the effect of the uptake of phytanic acid into membrane lipids on the
physical and functional properties of those membranes. The membrane fluidity studies described in chapter 9 could be repeated using a range of phytanic acid supplementation concentrations to determine if the effect followed a trend. In addition, the use of other fluorescent probes and techniques such as ESR to measure membrane fluidity would give information on the effect of phytanic acid on different movements in the phospholipid bilayer. It would also have been of interest to observe the effect of phytanic acid accumulation on a number of other membrane functions such as the high affinity choline uptake system of Y79 cells and the phagocytotic function of the RPE for which an in vitro assay has previously been developed (Chaitin and Hall, 1983).

In the studies described in this current work, a simple in vitro model was established using transformed cell lines. The use of such cell lines had the advantage that a sufficient number of cells could be easily cultured for the different studies involved in this investigation. However transformed cell lines are less differentiated than primary cell lines and the simplicity of such a model has its drawbacks in that it only vaguely approximates to the in vivo situation. Thus care needs to be taken when interpreting the results. No attempt was made to differentiate the RPE cells by growth on a permeable substratum and characterise the uptake of phytanic acid by the basal membranes. Such an approach would be more appropriate for modelling ARD in the retina as fatty acids that are utilised by the retina are first taken up by the basal membranes of the RPE which lie adjacent to the choriocapillaries. The fatty acids are then transported by proteins from the apical membranes to the neural cells. The fate of phytanic acid in the RPE is pertinent to the mechanism of retinal degeneration in ARD. If phytanic acid is transported from the RPE to the photoreceptors and other retinal neurons, the initiation of degeneration could occur through simultaneous disruption of the function(s) of a number of cell types. If alternatively the phytanic acid accumulates mainly in the RPE with little incorporation into the rest of the retina, then the dysfunction of this cell type will be the primary cause of the degeneration.

The creation of an appropriate animal model is possible now that the gene encoding an enzyme with phytanoyl-CoA \( \alpha \)-hydroxylase activity has been identified and shown to be mutated in patients with ARD (Jansen et al., 1997; Mihalik et al., 1997). It should
therefore be possible to create a knock-out animal model of the disease. This approach would overcome the problems that have hampered the creation of a successful animal model in the past (see section 1.6). An animal in which phytanoyl-CoA α-hydroxylase activity is deficient is likely to accumulate phytanic acid in its retinal tissues and thereby mimic the disease in humans. Thus the movement of radiolabelled phytanic acid in the retina could be followed. Such an approach, in addition to the studies on post-mortem tissues from patients, would provide the greatest insights on the mechanism of retinal degeneration in ARD.
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