CELL MEMBRANE LIPID COMPOSITION IN RELATION TO PHYSICAL PROPERTIES AND FUNCTION: A METHODOLOGICAL STUDY.

by

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Abstract.

Abnormalities on membrane function are widespread in many diseased states. It has been for some time postulated that these abnormalities are not due to the membrane proteins themselves but the matrix surrounding them; the phospholipid bilayer. The composition of the membrane affects its fluidity, which in turn affects its function. This composition is complex and although methods have been published which examine some aspects, few have succeed in providing a comprehensive analysis. This was the principle aim of the current study

A method has been developed to separate the lipid groups in the membrane and to analyse their fatty acid content individually. Cell membrane lipids were extracted then separated using a combination of silica Sep-pak and thin layer chromatography. Fatty acid methyl esters were then produced by acid catalysed transesterification and analysed by gas chromatography-mass spectrometry (GC-MS). This allows both for efficient separation and for positive identification of the esters. From these analyses, it was possible to determine indices of fatty acid desaturation and the saturated/unsaturated fatty acid ratio. The concentration of phospholipids present in the membrane could also be determined. Cell membrane cholesterol was also analysed by GC-MS and membrane microviscosity was assessed by fluorescence anisotropy using the fluorescent probe trimethylammonium diphenylhexatriene (TMA-DPH). In order to evaluate the individual contribution of the various lipid groups to the physical properties of the membrane, membrane lipid from erythrocytes and from hepatocytes from three experimental situations were analysed.

When hypertension was induced in Wistar-kyoto (WKY) rats, using the nitric oxide synthase inhibitor N^G -nitro-L-arginine-methyl ester (L-NAME), erythrocyte membrane microviscosity was reduced. This coincided with changes in membrane composition which may explain this change in fluidity. There was an increase in the amount of fatty acid present in the L-NAME-treated membrane and an increase in desaturase activity as measured by fatty acid ratios. Also, the levels of membrane sphingomyelin were reduced and the levels of phosphatidylcholine increased. These changes should have increased membrane fluidity. In opposition to this, the level of membrane cholesterol was increased in the L-NAME-treated group, which would be expected to cause a reduction in membrane fluidity. This suggests that the changes in phospholipid composition more than compensate for the increased cholesterol. The liver cell membrane was also studied. The fatty acid composition was altered by L-NAME treatment although the magnitude of the changes was

not as great as for erythrocytes. Moreover, the changes in composition were confined to the smaller membrane constituents, suggesting a possible membrane homeostatic mechanism in the liver, possible since this tissue has a high capacity to vary its own lipid metabolism.

Stroke-prone spontaneously hypertensive rats (SHRSP) have increased erythrocyte membrane microviscosity. Two strains were studied, the $SHRSP_{(Glasgow)}$ and $SHRSP_{(Hiedleberg)}$, both were compared to their WKY control. The saturated fatty acid content of some of the major lipids increased as was amount of sphingomyelin present in the membrane. Both of these would make the membrane more rigid. Interestingly, there was also an increase in the amount of polyunsaturated fatty acid present in the membrane. In particular, arachidonic acid was increased in the total lipid, free fatty acid and phosphatidylinositol fraction. Arachidonic acid is a key substrate for the production of a number of active substances such as prostaglandins and thromboxanes. Thus, in addition to throwing light on the membrane physical nature, comprehensive analysis may also reveal changes of functional importance.

The $SHRSP_{(Hiedleberg)}$ cross was used to assess whether the gene(s) which control membrane composition cosegregates with the gene(s) which control with blood pressure. Although differences were observed between the parental strains, none was found to correlate with blood pressure. Therefore, this study provided no evidence that inherited membrane abnormalities are causally related to the increase in blood pressure.

In the final study, erythrocyte membranes from diabetic patients were analysed. Although the literature suggests that the membrane microviscosity may be altered by diabetes, no alterations were observed here. However, changes were observed in the membrane composition, with the most marked of these occurring in the insulin-dependant diabetic patients. This study highlighted the usefulness of this method at detecting differences in the smaller lipid fractions which may be physiologically relevant but undetectable in the total lipid analysis.

DEDICATION

This thesis is dedicated to my mother and father. Their endless support and encouragement will never be forgotten.

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DECLARATION

I declare that, unless specified otherwise in the text the work presented in this thesis is my own.

Anne M^{c}Laren Dorrance.

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Abbreviations.

14:0	Myristic Acid
16:0	Palmitic Acid
16:1	Palmitoleic Acid
18:0	Stearic Acid
18:1	Oleic Acid
18:2	Linoleic Acid
18:3	Linolenic Acid
20:0	Arachidic Acid
20:3	Dihomo-γ-linolenic Acid
20:4	Arachidonic Acid
22:0	Behenic Acid
22:4/DHA	Docosahexaenoic Acid
24:0	Lignoceric Acid
24:4	Nervonic Acid
AII	Angiotensin II
ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
ATP	Adenosine 5'-triphosphate
BB	Bio-breeding
BHT	Butylated hydroxytoluene
C18	Octadecyl silica
cAMP	Adenosine 3',5'-cyclic monophosphate
CE	Cholesterol esters
C/PL	Cholesterol / phospholipid ratio
CTP	Choline phosphotransferase
dH_2O	Distilled water
DAG	Diacylglycerol
DEAE	Diethyl amino ethyl
DOCA	Deoxycorticosterone acetate
DPA	Docosapentaenoic acid
DPG	Diphosphatidylglycerol
DPH	Diphenylhexatriene
DTA	Docosatetraenoic acid
EE	Ethyl ester
EPA	Eicosapentaenoic acid
ER	Endoplasmic reticulum
FFA	Free fatty acid
FID	Flame ionisation detection
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
GL	Glycolipids
HDL	High density lipoproteins
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography.
IDDM	Insulin-dependant diabetes mellitus
IP_3	Inositol-1,4,5-triphosphate
IRD	Insulin requiring non insulin dependant diabetic
LAT	Lysophosphatidylcholine acyl transferase
LDL	Low density lipoprotein
L-NAME	N^G -nitro-L-arginine-methyl ester

L-NMMA	N^G -monomethyl-L-arginine
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidyethanolamine
ME	Methyl ester
MS	Mass spectrometry
MTBE	Methyl-*tert*-butyl ether
MUFA	Mono unsaturated fatty acid
NIDDM	Non-insulin-dependant diabetes mellitus
NO	Nitric oxide
NOS	Nitric oxide synthase
PA	Phosphatidic Acid
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
PEMT	PE methyl transferase
PG	Phosphatidylglycerol
PGS	Partial glycerides
PI	Phosphatidylinositol
PKA	Protein kinase A
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PML	Polymorphonuclear lycocytes
PS	Phosphatdiylserine
PUFA	Polyunsaturated fatty acid
RFHLPC	Reverse phase high performance liquid chromatography.
SBH	Sabra hypertension prone
SBR	Sabra hypertension resistant
SFA	Saturated fatty acid
STZ	Streptozotocin
SHR	Spontaneously hypertensive rat
SHRSP	Stroke-prone spontaneously hypertensive rat
SHRSR	Stroke-resistant spontaneously hypertensive rat
SPG	Sphingomyelin
TEAE	Triethyl amino ethyl
TIC	Total ion current
TLC	Thin layer chromatography
TMA-DPH	Trimethylammonium diphenylhexatriene
TMAH	Tertramethyl ammonium hydroxide
TMS	Trimethyl sililyl ether
TMSH	Trimethyl sulphonium hydroxide
TRIG	Triglycerides
UV	Ultraviolet
VSMC	Vascular smooth muscle cells
WKY	Wistar-kyoto

Chapter 1 Introduction and Literature Review

1.1 Historical Overview of Membrane Structure.

Initial reports that the cell membrane has a bilayer structure came from Gorter and Grendel in 1925. In their experiments, the lipids from erythrocyte membranes were extracted and floated as a mono-layer on water. From the surface area which these lipids covered, they concluded that there was sufficient to cover the cell surface twice. It is now apparent that the surface area of the cell was underestimated and all the lipid was not extracted. However, these inaccuracies cancelled each other out and the theory of the phospholipid bilayer was born. In the same year, Fricke measured the electrical capacitance of erythrocytes in suspension and hypothesised that the cells were surrounded by a hydrocarbon layer 5nm thick (Fricke 1925). Danielle and Davson(1935) then suggested that the membrane has a lipid core with polar groups facing outwards. The surface of the membrane, they surmised, would be covered by a protein mono-layer. However, this would sequester water away from the hydrophilic polar head groups, making it an unlikely model.

With the invention of the electron microscope cell membranes could be visualised as 'railway tracks' around the outside of the cell. Robertson (1959) proposed that the cell membrane is a three layered structure 5ηm wide, as had been proposed 30 years earlier by Fricke. Low angle x-ray defraction analysis of the myelin sheath around nerve cells confirmed the presence of a bilayer structure and showed that the membrane contained dense areas, the polar lipid head groups, and less dense areas, the fatty acyl core (Lodish *et al* 1995).

Although all the evidence points toward the cell membranes having a bilayer structure, they were thought to be solid structures until the emergence of magnetic resonance data and experiments using fused heterakaryone cells. These showed that membrane proteins have lateral motion, suggesting that the membrane exists in a dynamic fluid state (Frye & Edidin 1970). Then in 1972, Singer and Nicolson published the fluid mosaic model of the cell membrane, which is now widely accepted.

1.2 Basic Structure and General Composition of Membranes.

The fluid mosaic model of the cell membrane is shown in fig 1.1a. Lipids are its major structural component. The membrane proteins either span the bilayer or are attached to the outside of it. Most of the lipid is amphipathic phospholipid and it is this property

which results in the formation of the bilayer. They have a polar head group and non-polar the fatty acyl chains and form a sheet-like structure spontaneously with the polar head groups facing the aqueous solutions on either side of the bilayer. The internal core of the membrane contains the fatty acyl chains which are repelled by the aqueous phase and form a continuous hydrophobic layer, stabilised by van der Waal's forces between the fatty acyl chains. Ionic and hydrogen bonds between the polar head groups stabilise the outer regions of the membrane. Most lipids in the membrane are phosphoglycerides, although sphingolipids and sterols are also present along with small amounts of triglycerides, free fatty acids and cholesterol esters (Lodish *et al* 1995).

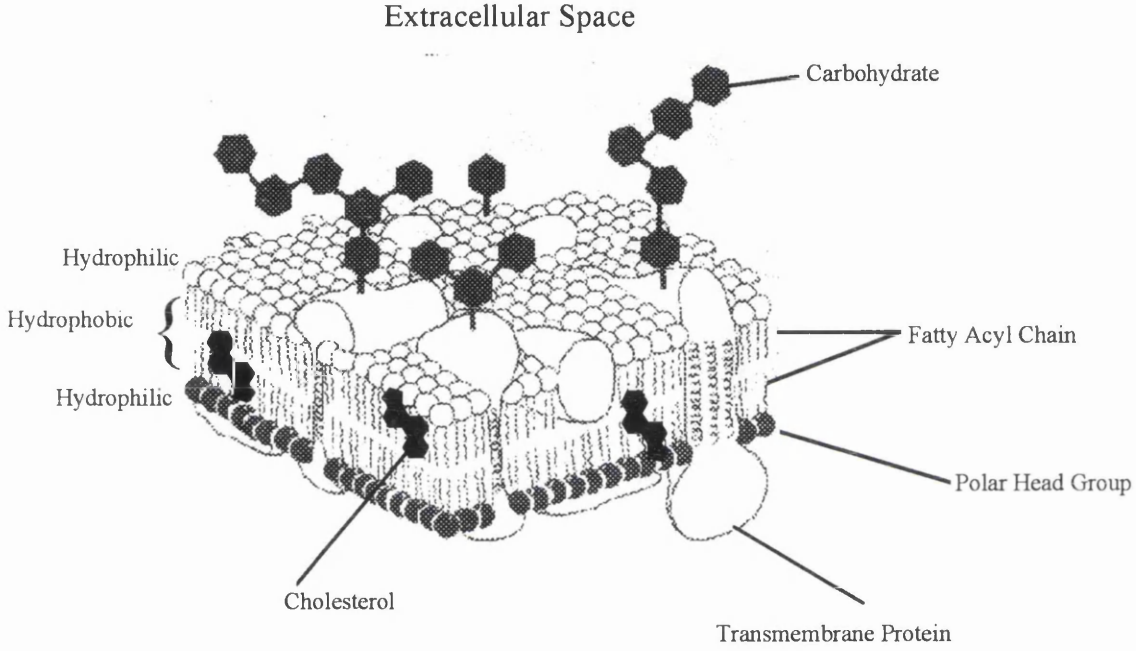


Fig 1.2 Diagram of a phospholipid bilayer showing the position of phospholipids, cholesterol, cell membrane proteins and glycolipids. Modified from Gurr and Harwood (1991).

Membrane proteins are responsible for the specialised functions of each cell type. The membrane-associated proteins of the cytoskeleton give the cell its shape and rigidity and are responsible for fusing membranes together at tight junctions and desmosomes to facilitate communication between cells. Other membrane proteins include receptors and ion channels which are involved in cell signalling. The ratio of protein to lipid in the cell membrane varies from cell to cell (table 1.2a).

Membrane	% of Protein	% of Lipid	% of Carbohydrate
Myelin	18	79	3
Plasma Membrane			
Human Erythrocyte	49	43	8
Mouse Liver	44	52	4
Rat Liver	58	42	5-10
Retinal rods (bovine)	51	49	4

Table 1.2a Relative proportions of lipid, protein and carbohydrate in cell membranes from a variety of cells.

There are two categories of membrane proteins, intrinsic and extrinsic. Intrinsic proteins insert into the lipid bilayer and span it at least once but often many times; seven transmembrane-spanning proteins are common and include the adrenoceptors, muscarinic and angiotensin II (AII) receptors. The amino acids in the membrane-spanning region are hydrophobic while those which lie outwith the membrane are hydrophilic. In glycophorin, an intrinsic erythrocyte membrane protein, 34 amino acids are involved in membrane binding, 23 of which are hydrophobic (Petty 1993). The bilayer-spanning region of most integral proteins has an alpha helical structure. The hydrophobic amino acids orientate their side chains into the outside of the helix where they form hydrogen bonds with each other to stabilise the helix. The hydrophobic amino acids orientate their side chains away from the helix to interact with the fatty acids in the membrane core. Some membrane proteins which only pass through one of the membrane leaflets are covalently linked to fatty acids which anchor them in the membrane.

Extrinsic proteins bind to the surface of the membrane through direct interactions with the phospholipid head groups or by indirect binding to intrinsic membrane proteins. Spectrin and ankyrin are extrinsic erythrocyte cytosketeton membrane proteins. Such proteins can usually be extracted with high ionic strength buffers which disrupt the ionic interactions between the membrane and the protein. In contrast, intrinsic proteins require detergents to extract them (Petty 1993). Extrinsic proteins can be attached to the membrane via a hydrocarbon chain. Glycosylphosphatidylinositol-anchored proteins (e.g. Thy 1 and alkaline phosphatase) attach to the outside of the membrane. Alternatively, proteins can attach to the inside of the membrane through myristate, a fourteen carbon fatty acid which forms an amide bond with a glycine residue at the N-terminus of the protein. Lastly, farnesyl or geranylgeranyl molecules form thiolester bonds with the C-terminus of proteins

for insertion into the membrane. Examples of these are the p60 protein and Ras, both of which are involved in controlling cell growth and division (Lodish *et al* 1995).

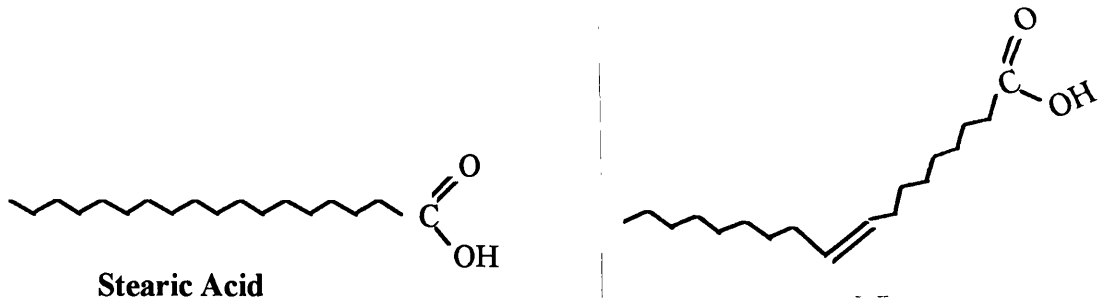
Also present in the membrane are glycoproteins and glycolipids. In glycoproteins, the sugar moieties, α-D-N-acetylglucosamine, α-D-N-acetylgalactosamine, N-acetylneuraminic acid or α-L-fructose, can be N-linked or O-linked to protein. In N-linkage, the sugar is attached to the amide group of an asparagine residue. O-linked sugars are attached to the oxygen of serine or threonine residues. Glycoproteins are primarily present in the outer leaflet of the membrane. The carbohydrate residues are important for the correct folding of the extracellular protein as the hydroxyl groups present form hydrogen bonds with the extracellular fluid. Glycolipids are formed from sphingosine. The most simple is cerebroside which contains one glucose or galactose residue while gangliosides can contain up to 7 sugar residues (Stryer 1988). The carbohydrate on the outside of the membrane is important for cell recognition.

1.3 Membrane Lipid Composition

A variety of different lipids are present in the cell membrane but fatty acids are common to them all. The membrane contains fatty acids of between 14 and 26 carbon atoms although those with 18 and 20 carbon atoms are most abundant. Their degree of unsaturation varies from 0 to 6 double bonds. *Cis* bonds are more common than *trans* and are responsible for a kink in the fatty acid chain (fig 1.3a & b). Table 1.3a shows the short hand notation, trivial and systematic names for some common fatty acids. This gives an indication of the number of carbon atoms, the number of double bonds and where the first double bond occurs, i.e. 16:0 is palmitic acid with 16 carbons and no double bonds, 18:1 n-9 is oleic acid with 18 carbons and one double bond at the 9 position (Petty 1993).

Cis double bond *Trans* double bond

Fig 1.3a Carbon to carbon double bonds in the *cis* and *trans* positions.



Stearic Acid

Saturated Fatty Acid

Monounsaturated Fatty Acid

Fig 1.3b Representative 18 carbon saturated and unsaturated fatty acids.

Notation	Trivial Name	Systematic Name
14:0	Myristic acid	*n* - tetradecanoic
16:0	Palmitic acid	*n*-hexadecanoic
16:1(n-9)	Palmitoleic acid	*cis* - 9 hexadecenoic
18:0	Stearic acid	*n* - octadecanoic
18:1(n-9)	Oleic acid	*cis* - 9 - octadecenoic
18:2(n-6)	Linoleic acid	*cic* - *cis* - 9, 12 - octadecadienoic
18:3(n-6)	Linolenic acid	all - *cis* - 6, 9, 12 - octadecatrienoic
20:0	Arachidic acid	*n* - eicosanoic
20:4(n-6)	Arachidonic acid	all - *cis* -5,8,11,14 - eicosatetraenoic
22:0	Behenic acid	*n* - docosanoic
24:0	Lignoceric acid	*n* - tetracosanoic

Table 1.3a Trivial and systematic names for some common fatty acids.

Phospholipids are the most abundant membrane lipid. Most are phosphoglycerides (fig 1.3c). The glycerol back-bone has three possible sites for hydrolysis known as *sn*- 1-3. The *sn*-3 position is esterified to a phosphoric acid residue. This in turn is attached to a base moiety. In phosphatidylethanolamine (PE) and phosphatidylserine (PS), this is an amino alcohol. For phosphatidylcholine (PC) and lysophosphatidylcholine (LPC), the base is choline. The glycerol *sn*-1 and -2 positions have fatty acids esterified to them, usually one of which is saturated and the other unsaturated. At neutral pH, most lipids have no net charge but cardiolipin and PS are negatively charged. The sphingolipids are also choline-containing lipids (fig 1.3d).

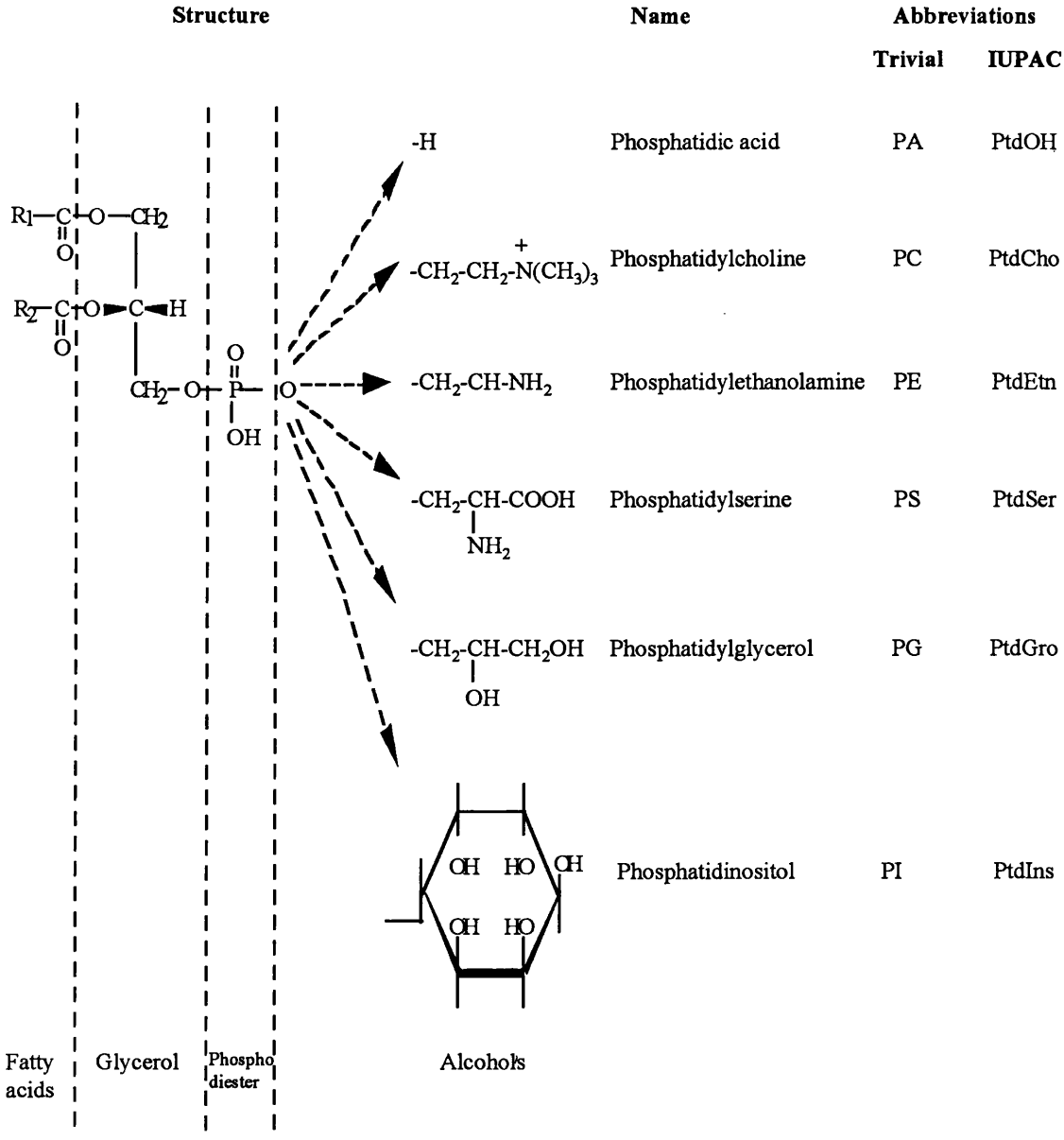


Fig 1.3c Chemical structures of common membrane phospholipids.

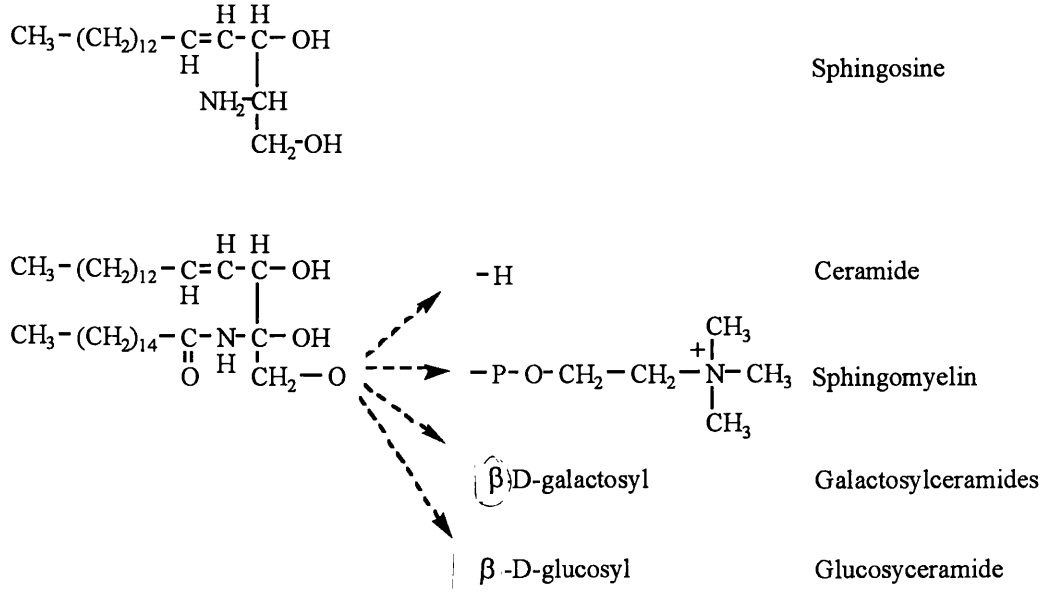
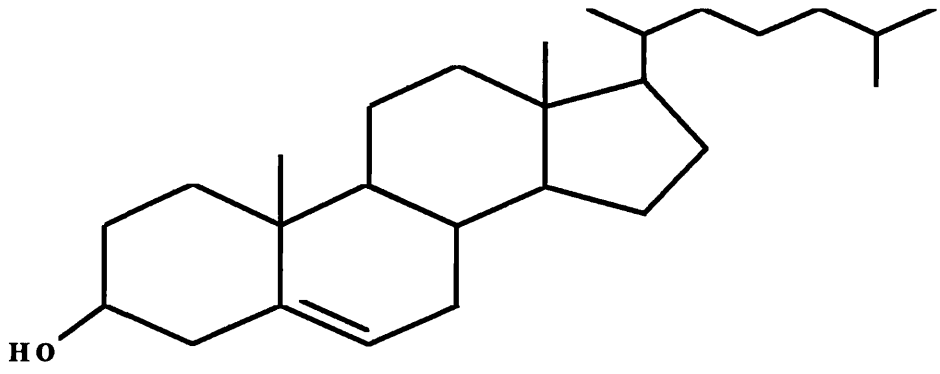


Fig 1.3d Chemical structures of common sphingosine lipids.

Cholesterol does not form a bilayer but its 3β-hydroxyl group interacts with the polar head groups of other lipids and the sterol ring structure is incorporated into the bilayer beside the hydrophobic fatty acid chains. Fatty acids can be esterified to the hydroxyl group to yield cholesterol esters (fig 1.3e) (Petty 1993).



Cholesterol

Fig 1.3e. Chemical structure of cholesterol.

The composition of the membrane varies from cell to cell (table 1.3b). Most is known about the erythrocyte membrane because it is simple to prepare free from contamination. Much is also known about hepatocyte membranes. The membranes of different organelles also have different compositions and therefore different properties.

Source	C	PC	SPG	PE	PI	PS	PG	DPG	PA	GL
Rat Liver										
Plasma membrane	30	18	14	11	4	9	-	-	1	-
ER (rough)	6	55	3	16	8	3	-	-	-	-
ER (smooth)	10	55	12	21	6.7	-	-	1.9	-	-
Mitichondria (inner)	3	45	2.5	24	6	1	2	18	0.7	-
Mitichondria (outer)	5	50	5	23	13	2	2.5	3.5	1.3	-
Nuclear membrane	10	55	3	20	7	3	-	-	1	-
Golgi	7.5	40	10	15	6	3.5	-	-	-	-
Lysososmes	14	25	24	13	7	-	-	5	-	-
Myelin	22	11	6	14	-	7	-	-	0	12
Rat erythrocyte	24	31	8.5	15	2.2	7	-	-	0.1	3

Table 1.3b Lipid composition of major cell organelles and erythrocytes. Abbreviations - C, cholesterol, DPG, diphosphatidyl glycerol (cardiolipin), PA, phospahtidic acid, SPG sphingomyelin GL, glycolipids ER endoplasmic reticulum (adapted from Jain & Wagner 1980).

1.4 Membrane Asymmetry.

The lipids in the membrane are asymmetrically distributed (fig 1.2a). This was first reported by Bretscher (1972) who used a non-cell-penetrating probe to label aminophospholipids (PS & PE). Whole erythrocytes were slow to be labelled whereas leaky cell ghosts were labelled rapidly. This suggested that the majority of the PE and PS is present in the inner-leaflet of the membrane and, by inference, most of the PC and sphingomyelin (SPG) must be in the outer-leaflet. The presence of most choline-containing lipids in the outer-leaflet was confirmed using phospholipases specific to certain lipids, combined with freeze-etch electron microscopy (Verkleij *et al* 1973). The phospholipases could not enter the cell and therefore could only digest lipids in the outer leaflet. Asymmetry has since been confirmed in erythrocytes and other cells (Zwall *et al* 1975, Tilley *et al* 1986, Gordesky & Marinette 1973, Fontaine *et al* 1980, Schick *et al* 1981).

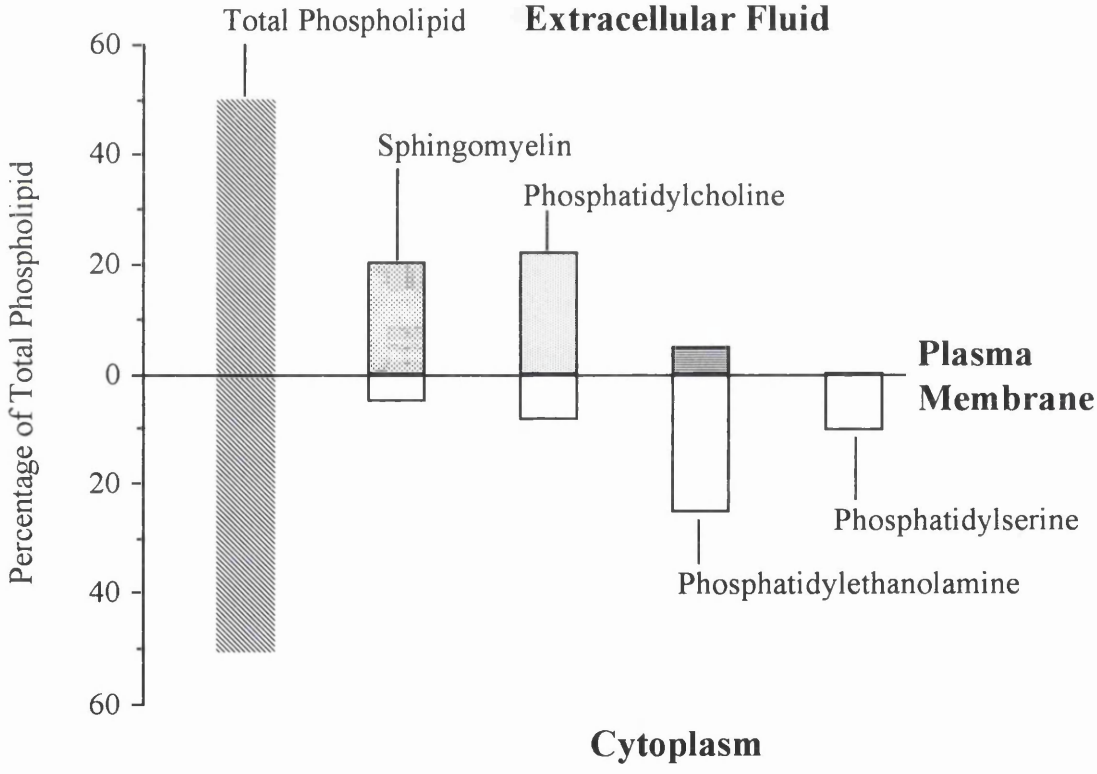


Fig 1.4a Asymmetric distribution of membrane phospholipids.

Not only are the lipids asymmetrically distributed in the membrane but there is also some molecular species asymmetry within some lipid groups. In erythrocytes, mono-unsaturated PE is located preferentially in the outer-leaflet and polyunsaturated PE, in particular arachidonic acid-containing PE, in the inner leaflet (Hullin *et al* 1991). Interestingly, no molecular species asymmetry for PE has been observed in platelets

(Schick *et al* 1981). Asymmetry has also been observed for SPG which contains relatively more long chain fatty acids in the outer- than inner-leaflet (Boegheim *et al* 1983). The same is not true for PC. Labelled fatty acids are mainly incorporated into inner leaflet PC but the distribution of the fatty acids across the two leaflets is similar although there is a greater proportion of PC in the outer leaflet (Renooij *et al* 1974). Experiments have shown that without the polar head group, lipids will diffuse through the membrane quickly (Ganong & Bell 1984). Therefore, the polar head groups may be partially responsible for maintaining this differential distribution about the membrane. This may explain why choline- and amino-containing lipids have different membrane distributions and why the maintenance of asymmetry is different for the two groups.

The maintenance of membrane asymmetry requires ATP. Cells incubated with labelled PS incorporated it into the inner membrane; none was present in the outer-leaflet (Middelkoop *et al* 1988). Similarly, spin-labelled PC remained in the outer leaflet while PE and PS were transported into the inner leaflet (Seigneuret & Devaux 1984). ATP may also be required for maintenance of molecular species asymmetry (Hullin *et al* 1991). Cell morphology studies have shown that the transport of PS and PE across the membrane requires ATP and Mg^{2+} (Daleke & Huestis 1985). The stoichiometry of lipid transfer and ATP utilisation is 1.13 for PS and 1.11 for PE (Beleznay *et al* 1993). The necessity for ATP and the knowledge that when asymmetry changes, cell shape also changes, may explain why erythrocytes require ATP to maintain their shape (Seigneuret & Devaux 1984). Although ATP is generally thought to be important for the maintenance of asymmetry, some believe that when ATP is absent, asymmetry is not lost immediately but further transport of new PE and PS is inhibited (Tilley *et al* 1986). Most movement of phospholipids occurs in an ATP-dependant manner but in the endoplasmic reticulum (ER), it is independent of ATP (Devaux & Zachowski 1994).

The need for ATP in the maintenance of asymmetry suggests the involvement of a protein in the process. Translocation of aminophospholipids into the inner leaflet is mediated by a specific enzyme; PE and PS compete for the active site for which PS has a higher affinity, making its transport faster. A 31 kDa protein, identified using iodinated thiolation reagents, is responsible for the translocation of PS across the bilayer. These thiolation reagents are based on pyridyldithioethylamine, a compound which is known to inhibit the transfer of lipids across the bilayer. The enzyme is the same size as integral membrane protein band 7 (Connor & Schroit 1988) and is inhibited by cytosolic concentrations of

Ca^{2+} greater than 1μm (Zachowski *et al* 1986), possibly because ATP is being consumed by increased Ca^{2+} pump activity (Bitbol *et al* 1987). The aminophospholipid translocase, as the protein has become known, has some stereospecificity for the polar head group. It will translocate L-α-phosphatidyl-D-serine but not D-α-phosphatidylserine (Hall & Huestis 1994, Smeets *et al* 1994).

The aminophospholipid translocase, a P-type ATPase (see later), has pronounced substrate specificity. In erythrocytes, PE methylation, a step in its conversion to PC, reduced activity while replacing the glycerol backbone with a ceramide abolishes it completely. The *sn*-2 position must be esterified but the acyl chain length is not important (Morrot *et al* 1989). It can also translocate PE plasmalogens (Fellmann *et al* 1993). The translocase has been identified in a wide variety of cells (Pomorski *et al* 1996a, 1996b, Cribier *et al* 1993) and tends to be most active in neonatal cell (Pomorski *et al* 1996b, Cribier *et al* 1993). Its importance has been shown by incorporating it into PC bi-layer where it is capable of PS translocation itself (Auland *et al* 1994). The bovine aminophospholipid translocase has been identified as a member of the P-type ATPase family (Tang *et al* 1996). A PC translocase, isolated from murine cell, has been identified as a p-glycoprotein which is coded for by the mdr2 gene (Ruetz & Cross 1994). It is ATP- and Mg^{2+}-dependant (Bitol & Devaux 1988, Ruetz & Cross1994). PC asymmetry is retained in isolated membranes but PC to equilibration is much slower (Kramer & Branton 1979).

Other cellular constituents and proteins are important in the maintenance of asymmetry (Haest & Deuticke 1976). In particular, spectrin, a cytoskeletal protein, is thought to prevent the movement of aminophospholipids into the outer leaflet of the membrane. Incubation of cells with SH-oxidising agents, which disrupt the cytoskeleton by forming disulphide bridges, increases the amount of PE and PS present in the outer leaflet (Haest *et al* 1978). Studies using merocyamine, a fluorescent dye which only binds to fluid state membranes, have shown that disruption of spectrin causes a loss of the gel-like state of the outer leaflet. Vesicles shed from aged erythrocytes and erythrocytes from mice with spherocytic anaemia were used for these experiments. In both these cases, the amount of membrane-bound spectrin was less than normal (Williamson *et al* 1982). Spectrin binds to mono-layers of anionic but not neutral lipids, suggesting that it may interact directly with aminophospholipids (Mombers *et al* 1980). In erythrocyte ghosts from patients with hereditary spherocytosis, a disease where erythrocytes have defective cytoskeltons, asymmetry is disrupted and the activity of Mg^{2+}-ATPase is increased (Vereulen *et al*

1995). This may be the aminophospholipid translocase attempting to maintain asymmetry. Others have shown that in cells where the membrane is disturbed chemically and asymmetry is lost, the activity of this enzyme is greater than control (Bartosz *et al* 1994). Studies of PS asymmetry in erythrocytes suggest that its maintenance requires more than the presence of aminophospholipid translocase and spectrin (De Jong *et al* 1996) as transport can be inhibited without inhibiting the aminophospholipid translocase (Connor & Schroit 1990). The authors suggest that the second protein may be endofacial, perhaps part of the cytoskeleton. Both proteins must be active for translocation to occur.

Other possible candidates for this role are the Rh blood group polypeptides. When these proteins are precipitated with specific antibodies, aminophospholipids coprecipitate (Schroit *et al* 1990). Rh polypeptides also co-localise with the aminophospholipid translocase in erythrocyte vesicles, suggesting a multicomponent complex for aminophospholipid translocation (Bruckheimer *et al* 1995). These findings have been disputed on the grounds that asymmetry is ubiquitous to all cells but the Rh blood group system is not.

Divalent cations have also been implicated in the control of asymmetry. The presence of excess Ca^{2+} will disrupt asymmetry (Connor *et al* 1990). High concentrations of Ca^{2+}, Sr^{2+} and the calcium ionophore A23187 cause increased movement of LPC into the inner leaflet of the membrane. Possibly these ions activate a proteinase which cleaves ankarin and disrupts the cytoskeleton (Henseleit *et al* 1990). Increasing cytoplasmic calcium levels activates transbilayer bi-directional lipid redistribution in human erythrocytes. The rates of movement of PC, PE and PS are all the same and continue as long as the concentration of Ca^{2+} is high. This redistribution was not caused by the aminophospholipid translocase which would not transport PC (Williamson *et al* 1992). However, the concentration of Ca^{2+} in these experiments was very high, in the non-physiological range. If the aminophospholipid transferase is involved in Ca^{2+}-induced lipid redistribution, it looses its stereochemical preference. Ca^{2+}-induced movement of lipids is bi-directional and applies to all lipid groups, with SPG being slightly slower to disperse (Smeets *et al* 1994). The redistribution observed here is probably not dependent on the aminophospholipid translocase.

Others have suggested that aminophospholipid translocase is involved in the Ca^{2+}-induced breakdown of membrane asymmetry. Redistribution of PE begins to change as soon as

aminophospholipid translocase activity is altered; PS is slower to change, possibly because it has a higher affinity for the enzyme. Other cations are also important. Sub-physiological concentrations of Mg^{2+} inhibit lipid transport, probably through inhibition of the Mg^{2+}-ATPase. Replacement of Mg^{2+} with Mn^{2+} does not affect transport but Co^{2+} inhibits it. High extracellular Ni^{2+} and VO^{2+} (vanadyl) caused inhibition of aminophosphlipid translocase at an extracellular site. Vanadate (VO_4^{3-}) is also an inhibitor but it appears to act at an intracellular site (Bitol *et al* 1987); an intracellular site of action has also been established for vanadate inhibition of (Na^+-K^+)-ATPase (Cantley *et al* 1978). The presence of Mg^{2+} in the lysis buffer for preparing erythrocyte ghosts is necessary for the maintenance of asymmetry (Connor *et al* 1990).

Asymmetry is lost from erythrocyte and platelet membranes during calcium influx-induced vesicle formation and shedding. The cell sheds vesicles which have a random lipid composition and, as more vesicles are lost, the asymmetry of the remaining cell membrane is lost too. Removing Ca^{2+} from the cells reactivates the ATP-dependant aminophospholipid translocase but asymmetry is not restored to the vesicles (Comfurius *et al* 1990). This may be because the vesicles do not contain adequate aminophospholipid translocase proteins or because the removal of Ca^{2+} from them is not sufficient to prevent this continued redistribution.

The aminophospholipid translocase in bovine aortic cells may be modulated by basic fibroblast growth factor in culture (Julien *et al* 1995).

It is interesting to note that the metabolism of lipids in the membrane is also asymmetric. Turnover of PE on the outer surface is four times faster than the inner surface. This effect is greatest for palmitic acid (Marinetti & Cattieu 1982).

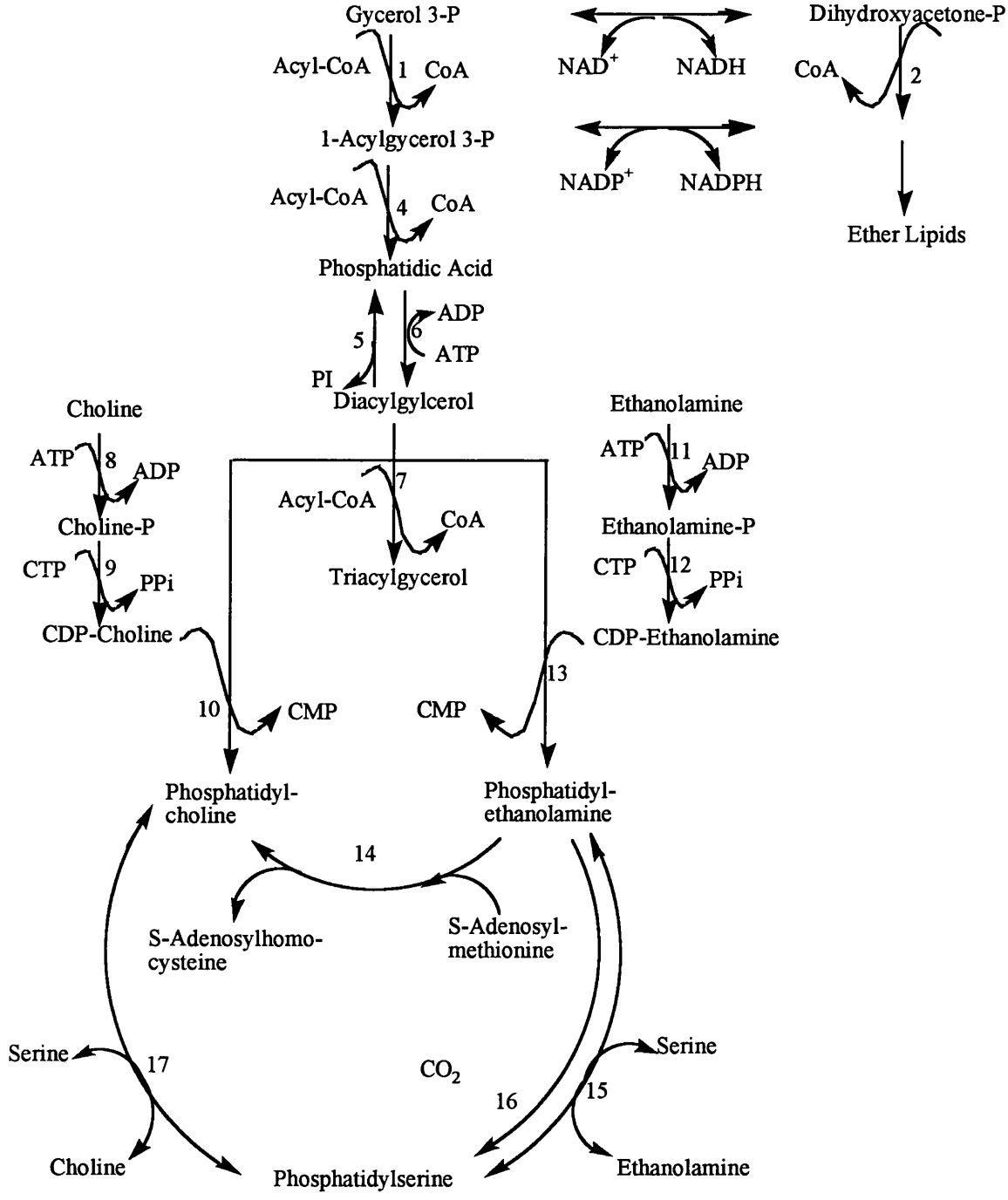
A breakdown in asymmetry, in particular PS asymmetry, has a variety of physiological consequences. The movement of PS from the inner to the outer leaflet occurs as a prelude to apoptosis, irrespective of the stimulus for cell death (Martin *et al* 1995). Movement also occurs during platelet activation and has a procoagulant effect which is not related to breakdown of the cytoskeleton or inhibition of the aminophospholipid translocase (Gaffet *et al* 1995a). There is no concomitant movement of PC into the inner leaflet during PS movement, suggesting that the movement of PS into the outer leaflet is not due purely to lipid scrambling but may be due to a reverse aminophospholipid translocase (Gaffet *et al*

1995b). The presence of PS in the outer leaflet during platelet activation has been confirmed using annexin V, a protein which specifically binds to PS (Stuart *et al* 1995).

1.5 Locus of Membrane Lipid Synthesis

Synthesis of phospholipids occurs in the ER via the pathways outlined in figs 1.5a and 1.5b. The composition of the phospholipids can be changed by *de novo* synthesis or by replacement of the fatty acids moieties by reacylation of lysophospholipids by acyltransferases and deacylation by esterases.

Synthesis of phospholipids begins with the esterification of glycerol by two fatty acids. Two different acyl-CoA:glycerol phosphate-O-acyl transferase enzymes are required which are specific for the *sn*-1 or *sn*-2 positions. The first step is the formation of phosphatidic acid (PA) from glycerol-3 phosphate, via 1-acylglycerol 3-phosphate synthesis. PA can also be formed from dihydroxyacetone phosphate and by turnover of pre-existing lipids. Most PA is hydrolysed by PA phosphatase to diacylglycerol (DAG) which is used for PC, PE, PS and triglyceride synthesis. PA phosphatase activity is found in both the cytosolic and microsomal fractions of the cell and is stimulated by glucocorticoids, growth hormone and cAMP but inhibited by insulin (Pittner *et al* 1986). Activity is regulated by varying the proportion of the enzyme bound to the membrane. Increasing fatty acid and fatty acyl CoA levels increase binding and thus increase activity, allowing the enzyme to respond to fatty acid loads (Bishop & Bell 1988).



Enymes of phospholipid biosynthesis,
1,*sn*-glycerol-3-phosphate acyltransferase, 2, dihydroxyacetonephosphate acyltransferase, 3, acyl (alkyl) dihyrodroxyacetonephosate acyltransferase, 4, lysophosphatidic acid acyltransferase, 5, phosphatidic acid phosphatase, 6, diacylglycerol kinase, 7, diacylglycerol acyltranferase, 8, choline kinase, 9, choline phosphat-cytidylyltransferase, 10, diacylglycerol:choline phosphotransferase, 11, ethanolamine kinase, 12, ethanol-amine phosphate cytidylytransferase, 13, diacylglycerol:ethanolamine phosphotransferase, 14, phosphatidyl-ethanolamine N-metyltransferase, 15, PE:serine O-phosphatidyltransferase, 16, phosphtidylserine decarboxylase17, PC:serine O-phosphatidyltransferase.

Fig 1.5a Pathway for the synthesis of the major membrane phospholipids, PS, PC and PE from glycerol.

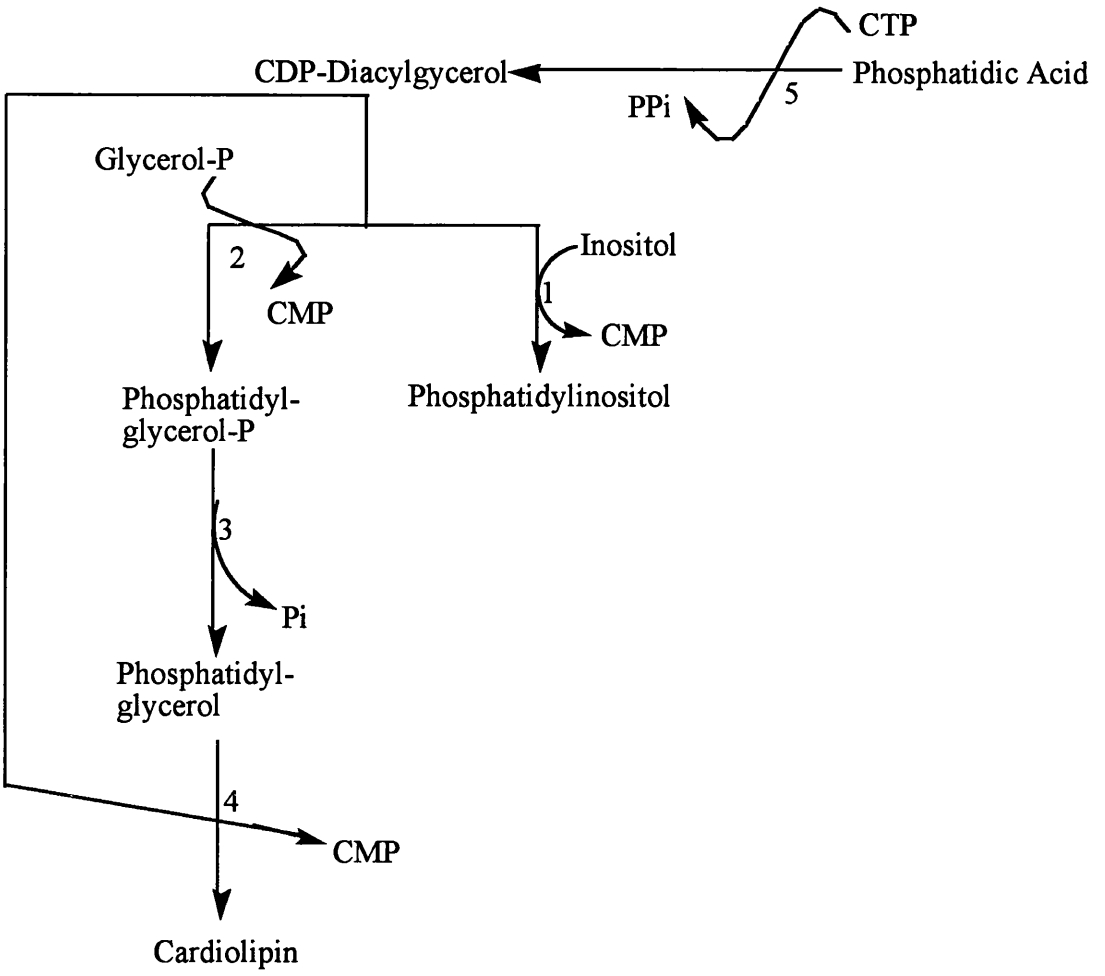


Fig 1.5b Pathway of PI and phosphatidylgycerol biosynthesis.

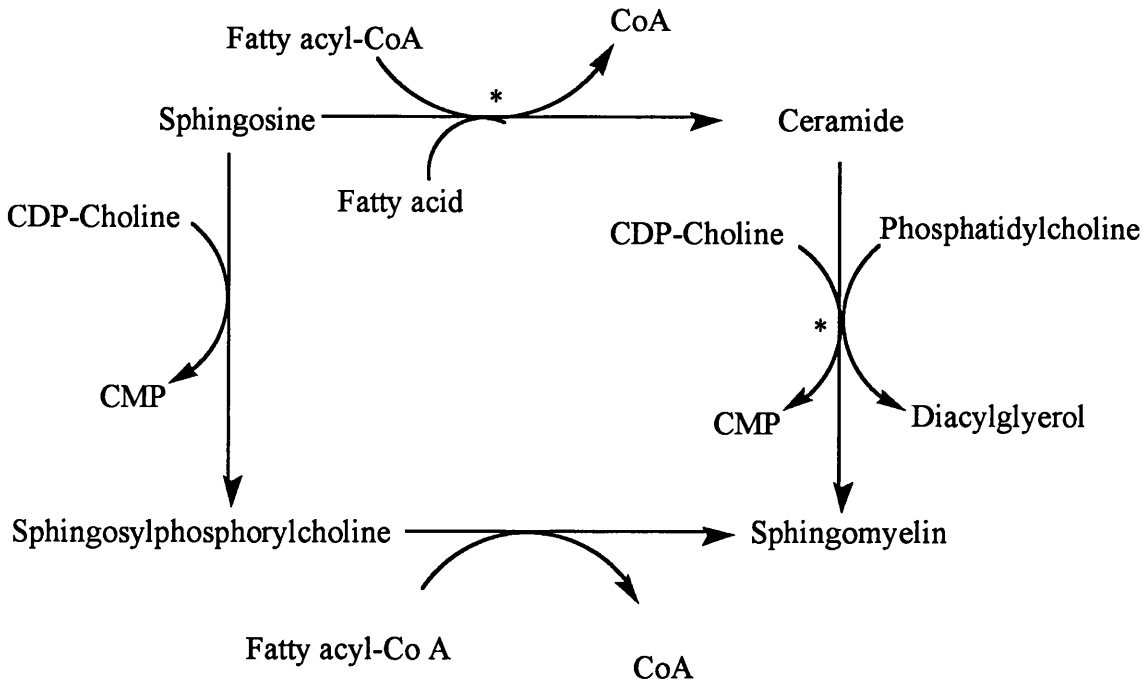
PC biosynthesis occurs primarily through the Kennedy pathway which involves the production of CDP-choline, which requires CTP, and is catalysed by the enzyme CDP-phosphocholine cytidyltransferase. This is the rate limiting step. The cytidyltransferase, like PA phosphatase, exists in both a membrane-bound and cytosolic form and again, the activity of the enzyme increases as more becomes membrane bound. PC biosynthesis increases with intracellular fatty acid levels (Pelech & Vance 1984) and when protein kinase C (PKC) is activated by phorbol esters (Pelech *et al* 1984). As cell growth is activated by PKC, this will produce more PC for new cell membranes. Protein kinase A (PKA)-mediated phosphorylation reduces the activity of the enzyme. This may be involved in the β adrenoreceptor response to stress as PKA is then activated, causing less cellular energy to be consumed in PC production (Petty 1993). Choline kinase may also contribute to the regulation of PC biosynthesis as growth factors increase its activity (Warden & Friedkin 1985). The final step in PC biosynthesis is catalysed by choline phosphotransferase which releases CMP. PC can also be produced by Ca^{2+}-dependant base exchange with PE or PS or by acylation of monoacyl PC. This process is thought to be particularly important in the formation of arachidonic acid-rich lipid. PC can result

from transacylation of monoacyl PC, important in the production of disaturated PC for lung surfactant (Gurr & Harwood 1991). Alternatively, PC can be synthesised by methylation of PE. Two methyl transferases exist in the erythrocyte membrane of Sprague-Dawley rats. The first is present on the inside of the membrane and produces phosphatidyl-N-monomethanolamine from PE using S-adenosyl-L-methionine as the methyl donor. The second enzyme is situated in the outer leaflet and converts the phosphatidyl-N-monomethanolamine into PC. The PC is therefore effectively being translocated across the cell membrane as it is produced (Hirata & Axelrod 1978).

PE is formed by a similar pathway to PC with phosphatidylethanolamine cytidyltransferase being a cytosolic enzyme and rate-limiting. This pathway is used for the formation of PE when ethanolamine is present in the cells. When ethanolamine concentration is limiting, the cell produces PE by the decarboxylation of PS. Like PC, PE can also be formed by Ca^{2+} -dependant base exchange and by acylation of monoacyl PE (Bishop & Bell 1988). PS cannot be synthesised independently of PC or PE. It is synthesised from these two lipids.

Production of anionic phospholipids, PI, PG and cardiolipin, occurs via CDP-diacylglycerol, the formation of which is catalysed by PA:CTP cytidyltransferase, thought to be present in both the ER and the mitochondria (Bishop & Bell 1988).

Sphingosine is formed from palmitoyl-CoA and serine in the presence of pyridoxal phosphate (fig 1.5c). The 3-ketodihydrosphingosine produced is then reduced to sphingosine using a flavoprotein enzyme (Gurr & Harwood 1991). Unlike the other lipids, SPG synthesis is predominately in the Golgi apparatus with only a small amount in the plasma membrane and the rough ER (Futerman *et al* 1990). Transport of SPG to the membrane is not dependant upon protein. Inhibition of cellular energy production with potassium cyanide slowed down the production of SPG but did not alter the percentage being transported to the cell surface. Brefeldin A, which inhibits protein transport through the Golgi apparatus, did not prevent the movement of SPG to the cell membrane. Both these facts point to SPG transport being vesicle- and not protein-mediated (Shiao & Vance 1993).



* = main route

Fig 1.5c Proposed pathway for sphingomyelin biosynthesis. Adapted from Gurr and Harwood (1991).

1.6 Cell Membrane Functions

The plasma membrane is the only structure ubiquitous to all cells. However, it is highly specialised and performs a wide variety of functions. Maintaining the composition and fluidity of the cell membrane is therefore important as membrane proteins will only function optimally in a precisely controlled physical environment.

The plasma membrane defines the boundaries of the cell and thus separates the cell contents from the extracellular milieu. Intracellular membranes compartmentalise cellular functions, allowing organelles to have internal conditions ideal for specialised groups of reactions. For example, lysosomes have an acidic pH to facilitate the breakdown of proteins. The cell membrane acts as a permeability barrier, preventing free movement of solutes and other molecules into and out of the cell. Flux of compounds across the membrane occurs by a variety of transport systems, the simplest being diffusion (Bangham *et al* 1965). Molecules with a molecular weight of less than 200 will diffuse across the membrane down the concentration gradient. Net flux stops when equilibrium is reached. This form of transport is non-saturable and does not require energy. Protein-mediated membrane transport is faster, can be saturated and has a degree of chemical specificity.

Facilitated transport is energy-independent but cannot move molecules against the concentration gradient. Its mechanism is unclear. It appears that the proteins involved span the membrane, perhaps forming a shield to protect the transported substance from the hydrophobic core of the membrane. Glucose transport into the erythrocyte occurs in this way. The concentration gradient is maintained by the immediate phosphorylation of glucose as it enters the cell (Petty 1993).

Primary and secondary active transport are energy requiring and allow the transfer of solutes against the concentration gradient. The transport protein must be phosphorylated by ATP. For example, Na^+K^+ATPase transports 3 Na^+ molecules out of the cell and 2 K^+ molecules into the cell for every high energy phosphate group consumed. This is a P-type ATPase, so called because of the formation of a phosphorylated intermediate. It is important for the regulation of cell volume and creates an electrochemical potential across the membrane which is used by secondary active transport processes to facilitate movement of substances against the concentration gradient. Secondary active transport often involves the movement of Na^+ down the concentration gradient, releasing energy which is harnessed to the movement of a second compound such as amino acids against its concentration gradient (Berne & Levy 1993). Apart from the P-type ATPases, two other types of ion transporting ATPase exist. The F-type ATPase which uses the H^+ gradient across the inner mitochondrial membrane to produce ATP and the V-type ATPase which generates an H^+ electrochemical gradient across the membranes of lysosomes and endosomes, making the inside of the organelle acidic (Berne & Levy 1993). Membrane ion exchange proteins are important for many cell functions. The Na^+/H^+ exchanger maintains intracellular pH balance. The Na^+-K^+-Cl^- transporter simultaneously moves 1 Na^+, 1 K^+ and 2 Cl^- into the cell. It is stimulated by cell shrinkage to increase the ion concentration inside the cell, leading to the movement of water into the cell by osmosis.

Endocytosis and exocytosis are quite different transport procedures. Endocytosis occurs in two ways, phagocytosis, the uptake of particulate material and pinocytosis, the uptake of soluble material. These are sometime referred to as cell 'eating' and 'drinking'. In both processes, the cell engulfs the extracellular material by extending pseudopodia around it. Some forms of pinocytosis require the deposition of clathrin under the cell membrane, forming structures known as clathrin-coated pits. Macromolecules bind to the outside of the membrane above these pits before internalisation. For example, low density lipoprotein (LDL) is taken up into the cell by this mechanism; LDL receptors are

contained within clathrin-coated pits in the membrane (Petty 1993). Phagocytosis is the internalisation of large particles. Macrophage-mediated, antibody-dependant phagocytosis is a component of the immune response. Exocytosis is important for the export of substances. Compounds may stored in secretory granules near the cell membrane surface and fuse with the membrane prior to release. Examples of exocytosis are mast cells releasing histamine or neural cell release of neurotransmitters (Petty 1993).

Most regulatory substances act by binding to a specific receptor in the cell membrane. These receptors may be ligand-gated ion channels which cause the cell membrane to depolarise when a ligand is bound or linked to a signal transduction pathway. Many processes involve the activation of G-protein-linked signal transduction pathways. G-proteins are heteromeric proteins attached to the inside of the cell membrane. For signal transduction to occur the α, β and γ subunits of the G-protein complex must link together. Efficiency of G-protein-linked receptors may therefore regulated by the fluidity of the membrane. G-proteins also affect the activities of K^+ channels, phospholipase C (PLC) and phospholipase A_2 (PLA_2)

Other effects of agonists binding to a receptor include the activation of protein kinases and phosphatases. Some second messenger-mediated pathways involve alterations to the membrane itself. PLC, activated by a G-protein-linked receptor, cleaves phosphatidylinositol-bis-phosphate to produce inositol-1,4,5-triphosphate (IP_3) and DAG. IP_3 binds to ion channels in the ER and increases intracellular free Ca^+ concentration. DAG activates PKC. PLA_2 is also activated by a G-protein-linked receptor and cleaves the fatty acid at the *sn*-2 position of the cell membrane phospholipids, leading to the release of arachidonic acid for prostaglandin and leukotriene synthesis.

The carbohydrates on the surface of the cell are responsible for immunogeneity and cell adhesion. Glycoproteins which terminate with a sialic acid residue have a negative charge which allows cells to adhere to positively charged surfaces. Cell surface antigens are glycoproteins and glycolipids which carry the blood group antigens or major histocompatability complex antigens.

Some cells, in particular fibroblasts and tumour cells, are mobile. They form lamellipoda which allow them to crawl along a surface. This is possible because of the rapid depolymerisation and repolymerisation of the cytoskeleton and the fluid nature of the

membrane. Membranes of other cells can deform and return to their original shape. For example the erythrocyte is 7.7μm in diameter and 1.9μm thick, but it must pass though capillaries much smaller than these dimensions.

1.7 Functions of individual fatty acids and lipids.

Cell membrane fluidity, the inverse of microviscosity, is affected by fatty acid content. In the most simple terms, unsaturated and short chain fatty acids make the membrane more fluid and saturated and long chain fatty acids make it more rigid. An unsaturated fatty acid has a kink in the acyl chain which reduces the interactions between it and its neighbours. This reduces the melting point of the membrane and the ordering of its fatty acyl chains, making it more fluid. Short chain fatty acids which have less surface area to interact with other fatty acids have the same effect (Lodish *et al* 1995). However, this is a gross oversimplification. Insertion of one double bond into a fatty acyl chain decreases the order of the membrane. The insertion of a second double bond will decrease it more but not twice as much. Insertion of four double bonds into the acyl chain has little more effect on the order than two double bonds (Stubbs *et al* 1981). Also, double bonds inserted into the middle of the acyl chain have more effect on fluidity than those inserted into the end of the chain (Stubbs & Smith 1984). Moreover, these effects are complicated by the presence of protein, by specialised regions of the membrane and by cholesterol. Although the membrane is referred to as a liquid-crystalline structure, most is in a highly ordered state with only a small region at the centre in a fluid state (Yeagle 1989).

An increase in the PC/SPG ratio in the membrane increases fluidity (Borochov *et al* 1977). SPG is a rigidifying lipid because it contains a large proportion of long chain fatty acids and the hydrophobic region contains a trans double bond and an amide bond, both of which decrease fluidity (Shinitzky & Barenholz 1974). Cholesterol is one of the best known membrane rigidifiers; it inserts into the phospholipid bi-layer preventing the free movement of the fatty acyl chains (Shinitzky 1984). Similarly, proteins are relativity incompressible and reduce membrane fluidity (Shinitzky & Inbar 1976, Schinitzky & Rivnay 1977).

Some lipids, in particular poly unsaturated fatty acids (PUFA), have specific effects on the cell. These occur at two levels, on gene transcription and on membrane function.

1.7.1 Nuclear Effects.

The list of proposed modulatory actions of PUFA is endless. Although the precise mechanism is unclear, evidence suggests that they regulate transcription of a variety of genes via a PUFA response element in the nucleus. This appears to be different form the fatty acid-activated nuclear factor with is also activated by saturated and monounsaturated fatty acids neither of which are know to affect gene transcription (Clarke & Jump 1994, & 1996). By preventing endogenous fatty acid biosynthesis, PUFA ensure that they will be required and inserted into the membrane (Clarke & Jump 1994). PUFA in particular arachidonic acid, inhibit the transcription of the stearoyl Co-A desaturase (Tebbey and Buttke 1993, Landschulz *et al* 1994). An additional metabolic effect of PUFA's, again demonstrated for arachidonic acid, is to inhibit transcription of the GLUT-4 glucose transported gene and increase the rate of its mRNA turnover in adipocytes but it increases the GLUT-1 gene transcription (Tebbey *et al* 1994). PUFAs affect cell growth. In addition to activating PKC (McPhail *et al* 1984), arachidonic acid induces *c-fos* transcription (Tebbey and Buttke 1993) but γ-linolenic acid (but not linoleic acid) inhibits the growth of human heptoma cells in culture (Booyens *et al* 1984). They also potentiate the TGF-β inhibitory action of carcinoma cells (Newman 1990). These last two actions may be of therapeutic value. Similarly, fatty acids may be antiviral, antibacterial, antimutagenic and modulate T and B cell response (Das 1991).

1.7.2 Effects on Protein Function.

Most effects of lipid on protein function appear to be mediated by free fatty acids. For example, extracellular docosahexaenoic acid (DHA) blocks the delayed rectifier K^+ current in cells from the pineal gland. Other fatty acids containing at least four double bonds have the same effect (Polling *et al* 1995). The effect varies from tissue to tissue. Thus arachidonic acid inhibits the delayed rectifier K^+ channel function in cardiac cells (Barhanin *et al* 1994) but activates it in rat neurocortical neurones (Palma *et al* 1993). Toad stomach smooth muscle cell K^+ channels were activated by fatty acids and negatively charged monoacyl lipids. Neutral lipids had no effect and positively charged lipids inhibited the channels. The site of action is cytosolic and there may be a fatty acid binding site on the protein itself or on an associated protein (Petrou *et al* 1995). Arachidonic acid and DHA potentiate the NMDA receptor response (Miller *et al* 1992, Nishikawa *et al* 1994) and a putative fatty acid binding site, homologous to known fatty acid binding proteins, has been identified on the receptor (Petrou *et al* 1993). Arachidonic acid inhibits the uptake of glutamate *in vitro* in brain liposome systems containing reconstituted

glutamate transporters. The fatty acid must be in the unesterified form for inhibition to occur (Trotti *et al* 1995).

Studies have been carried out on synthetic membrane systems to investigate the effects of phospholipids on reconstituted Ca^{+}-activated K^{+} channels. Changing the membrane lipid from mono-methylated PE to PC increases the size of the phospholipid head group by 10A and increases the channel opening time Changes in the ordering of the fatty acid chains by changing the fatty acids esterified to the phospholipids had no effect on the channels (Chang *et al* 1995a).

Diets rich in n-3 fatty acids reduced the plasma triglyceride levels and increased *in vivo* insulin sensitivity in non-insulin dependant diabetic (NIDDM) patients without affecting the microviscosity of either platelets or erythrocytes (Rabini *et al* 1993), suggesting a direct action of the fatty acids on the insulin receptor. The membrane environment of the insulin receptor affects its binding and kinase activity. When purified insulin receptors are inserted into vesicles containing PE and PC, they lose their receptor kinase sensitivity and the affinity for insulin is increased. It is only when PS is included in the vesicles that the insulin sensitivity to the receptor kinase is restored (Lewis & Czech 1987). Phospholipid mixtures containing phosphatidyl inositol (PI) affect the autophosphorylation of the β-receptor subunit of the insulin receptor. This is biphasic, PI stimulates when present at low concentrations and inhibits at higher concentrations (Sweet *et al* 1987). Changes in membrane fluidity in cultured heptomas cells accomplished by incubation of cells with linoleic acid or 25-hydroxycholesterol caused insulin resistance. Basal amino acid uptake fell in the 25-hydroxycholesterol-treated cells but increased in the linoleic acid-treated cells. However, insulin-stimulated amino-acid uptake and glycogen synthesis were reduced in both cell types compared to nonsupplemented cells (Bruneau *et al* 1987). Previously, it has been suggested that diet might affect insulin sensitivity in subjects genetically predisposed to NIDDM. An increase in the prevalence of obesity, NIDDM and hypertension occurs in those who consume a high saturated fat diet (Simopoulos 1994). In light of the effects of fatty acids on insulin sensitivity, this may be a mechanism for this response.

Fatty acids, particularly C16-C20 PUFAs, inhibit the binding of AII to its receptor in cultured bovine adrenal cells. Fatty acids also non-specifically inhibit aldosterone production (Goodfriend & Ball 1986). When fatty acids are removed from the surface of

adrenal glomerulosa cells, the aldosterone response to AII is increased (Goodfriend *et al* 1991a). Salt loading increases plasma fatty acid levels in inverse correlation to the suppression of plasma aldosterone levels (Goodfriend *et al* 1991b). PUFAs also inhibit the binding of glucocorticoids to their receptors in liver cells. The fatty acids bind to the receptor at a site different from the hormone binding site. The inhibition appears to be of the mixed non-competitive-type and is a function of fatty acid chain length, degree of unsaturation and concentration (Vallette *et al* 1991). In studies where the protein was partially digested with trypsin, the fatty acid and hormone binding sites were located on the same area of the protein. DHA acid could not inhibit the binding of the glucocorticoid antagonist, RU486, as effectively as it inhibited the binding of dexamethasone, a synthetic glucocorticoid. This suggests that the antagonist induces conformational changes in the protein, preventing fatty acid binding (Sumida *et al* 1993). Fatty acids have also been implicated in the binding of estradiol to rat uterine receptors (Vallette *et al* 1988). At low estradiol concentrations, fatty acids inhibit binding but at high concentrations, the opposite is true.

Nuclear binding of triiodothyronine (T_3) is competitively inhibited by fatty acids such as oleic acid in rat liver (Van der Klis *et al* 1989). Using purified proteins from a bacterial expression system, fatty acids were shown to interact directly with the T_3 receptor, although the exact mechanism of action remains unclear (Van der Klis *et al* 1991).

1.7.3 Effects on Membrane Function.

Fatty acids interfere with platelet aggregation. In human subjects *cis*- but not *trans*-unsaturated fatty acids have this affect (MacIntyre *et al* 1984). The authors of this report claim that inhibition is due to free fatty acids. However, unsaturated fatty acids increase membrane fluidity and the potency of inhibition is inversely related to the fatty acid melting points (MacIntyre *et al* 1984). Thus inhibition of aggregation may be due solely to elevated membrane fluidity. Diets high in n-3 PUFAs inhibit platelet aggregation and thromboxane A_2 production and reduce plasma triglyceride levels. Post-treatment platelet aggregation remained impaired for 14 weeks; the membrane concentration of n-3 fatty acids remained elevated for the same time, suggesting that composition determines aggregation (Tremoli *et al* 1995). Conversely, platelets from rats fed a high saturated fatty acid diet were less responsive to aggregatory stimuli than those fed diets high in n-3 and n-6 fatty acids. The high saturated fat diet caused an increase in the amount of arachidonic acid in the membrane and reduced the cholesterol/phospholipid (C/PL) ratio

compared to the other groups. *In vitro* modification of the C/PL ratio showed that platelet reactivity increases with increasing ratio (Heemskerk *et al* 1995).

Membrane fluidity can modulate the binding of opioids to their receptors in the brain. Incorporation of C18:1 (n-6, 9 and 11) into synaptosomes decreases membrane microviscosity and inhibits the binding of *mu, delta* and *kappa* opioids. (Lazar & Medzihradsky 1992). Also in the brain, lipid peroxidation, which will alter the PUFA content of the membrane, alters the kinetics of muscarinic receptor binding by decreasing Bmax and increasing Kd for the receptor. Under these conditions, the cell membrane microviscosity was increased (Ghosh *et al* 1993). The effects of membrane phospholipid on adrenergic receptor function have been reviewed by Williams *et al* (1995). Both α and β adrenoceptors are regulated by the fluidity of their membrane environment. All of the reported alterations in α receptor binding related to variations in the receptor Kd whereas for the β receptor, both Kd and Vmax were affected.

High concentrations of PS and PE cause cells to crenate and then to revert to discoid shape and finally to become stomatocytic. The extent of these shape changes is dose-dependant and increases with increasing hydrophobicity of the lipid. ATP and Mg^{2+} are also required (Daleke & Huestis 1985). The membranes of human erythrocytes will only tolerate small changes in the fatty acid composition of their phospholipids. Using a PC-specific transfer protein, the erythrocyte membrane PC can be replaced with disaturated PC. Replacing 40% of the cellular PC in this way causes the cells to lyse. Replacing 30% of the PC with di-unsaturated PC causes the cells to become leaky to potassium and to lyse. The cell functions best when the PC contains palmitic acid and either linoleic or linolenic acid. In general, the stability of the cell is lost when the degree is unsaturation is increased; permeability of the membrane increases with increasing unsaturated fatty acid (Kuypers *et al* 1984).

The activity of the erythrocyte Na^+ pump is reduced by incorporation of short chain fatty acid PC (Dwight & Hendry 1995). The authors believe this to be related to the reduction in the width of the bilayer but the residual long chain fatty acids should maintain the bilayer width. They also show that increasing the amount of C10:0 PC reduces the amount of arachidonic acid present in the membrane, possibly due to increased phospholipase activity caused by cell membrane deformation (Dwight & Hendry 1995).

1.7.4 Effects Of Increased Membrane Cholesterol

Cholesterol makes the membrane more rigid by preventing the fatty acids from moving freely. Increased cholesterol in vascular smooth muscle cell (VSMC) membranes increases intracellular Ca^{2+} concentration by increasing unidirectional Ca^{2+} uptake. Removal of the cholesterol restores intracellular Ca^{2+} concentration to normal (Bialecki *et al* 1991). Rabbit VSMC enriched with cholesterol have an increased C/PL ratio and increased unstimulated Ca^{2+} influx and efflux and cystolic Ca^{2+} concentration. A reduction in membrane fluidity was observed in microsomes prepared from these cells. The authors of this report suggest that increased cholesterol content opens a new or normally silent L-type Ca^{2+} -channel (Gleason *et al* 1991).

Increasing erythrocyte membrane cholesterol increases microviscosity and affects many ion transport systems. The ouabain-sensitive Na^+ influx is inhibited and the activity of the Na^+-K^+-ATPase, Ca^{2+}-ATPase and Mg^{2+}-ATPase are reduced, as are the activities of the Na^+-K^+-cotransporters and Na^+-Li^+-countertransporters and basal leakage of Na^+-K^+ (Lijnen & Petrov 1995). In rabbits fed a high cholesterol diet, erythrocytes have increased Na^+-K^+-pump activity compared to those fed a normal diet. A positive correlation between plasma cholesterol levels and pump activity was observed. Cell Na^+ content correlated with both pump activity and with membrane cholesterol levels. Unexpectedly, the authors report that the membrane composition was not altered and they suggest that changes in fluidity were not responsible although this was not measured (Makarov & Kuznetsov 1995). Other studies using rabbits fed high cholesterol diets showed that the increase in plasma cholesterol caused an increase in membrane cholesterol and an inhibition of the Na^+-K^+ pump activity (Uysal 1986).

Incubation of human lymphocytes with cholesterol-rich serum reduces β adrenergic receptor density but does not affect membrane microviscosity. Incubating the cells with PE and PC to remove the cholesterol restored the receptor density to normal. The receptor density is not therefore related to the cell membrane microviscosity although it is related to membrane cholesterol content (Pieri *et al* 1992). Incubation of human neural cells with cholesterol hemisuccinate increases membrane microviscosity and the binding of opioid μ-selective agonists to the high affinity state of the receptor. This binding can normally be inhibited by pertusis toxin but, in the rigidified membrane, it has no effect (Emmerson & Medzihradsky 1994). Increasing the cholesterol content in lipid bilayers from rat brain reduced the Ca^{2+}-activated K^+ channel open time and its probability of

opening. The channel conductance was also reduced. Increasing the cholesterol in the membrane encourages this channel to move from the open to the closed state (Chang *et al* 1995b).

1.18 Determinants of Membrane Lipid Composition

Membrane lipid composition is controlled by a variety of factors, including desaturase enzymes and diet. Desaturase enzymes control the insertion of carbon-carbon double bonds into fatty acids. They are in turn affected by diet, age and hormones. Diet also affects membrane composition directly as cells can incorporate lipids and fatty acids from the circulation into their membranes (see earlier).

1.8.1 Desaturase enzymes and their activity.

The activity of desaturases is a crucial determinant of the physical properties of the cell membranes. Their nature and the factors controlling their action must therefore be examined in detail.

The desaturase enzymes are ER-bound. Only the stearoyl-CoA desaturase (Δ9 desaturase) has been completely purified (Strittmatter *et al* 1974) and the gene identified (Thiede *et al* 1986). Two other desaturase enzymes are known to exist, the Δ5 and Δ6 desaturases. A fourth, a Δ4 desaturase, has been postulated and will be discussed further later. The nomenclature of the enzymes indicates where the double bond is inserted in the fatty acid chain. For example, the Δ5 desaturase inserts a double bond between carbon five and six. The Δ5 and Δ6 desaturase enzymes act on both n-3 and n-6 fatty acids, although the enzyme affinity is greater for n-3 fatty acids (Geiger *et al* 1993, Hrelia *et al* 1995). The activity of the Δ5 desaturase increases with increasing chain length of the fatty acids from C16-C20. The activity of the Δ6 desaturase is not similarly affected (Pollard *et al* 1980). The pathway of desaturation was first demonstrated in yeast where it was observed that double bonds can be inserted into stearic acid provided that oxygen and a reduced cofactor were present. Fatty acids must be activated prior to desaturation by conversion to acid CoA:thiolesters. The availability of fatty acids for desaturation appears to be controlled by fatty acid binding proteins (Gurr & Harwood 1991).

The Δ9 desaturase, which is sometimes referred to as cyanide-sensitive factor, is a 53kDa mixed function oxygenase. Two other proteins are required for desaturation, the flavoprotein, NADH-cytochrome b_5 reductase, and the haem-containing protein,

cytochrome b_5. These electron transport proteins are membrane-bound, although most of their structure is in the cytoplasm (Gurr & Harwood 1991). The desaturase is more firmly attached to the membrane. Hydropathy analysis measures the hydrophobic or hydrophilic nature of the constituent amino acids of the protein, making it possible to elucidate which sections of the protein, if any, span the bi-layer. This has shown that the enzyme contains 3 hydrophobic areas in all, long enough to pass through the membrane twice (Shanklin *et al* 1994).

A cytoplasmic fraction maybe necessary for Δ6 desaturation to occur. This fraction has lipoprotein characteristics and contains PC and free fatty acids. Its function may be to remove the γ-linolenic acid produced by the Δ6 desaturase, preventing product inhibition (Leikin & Brenner 1986). It is not clear whether similar fractions exist for the other desaturases or if this fraction can remove other desaturation products.

The Δ9 desaturase has been inserted into PC vesicles to study its substrate specificity. Providing that NADH and O_2 are available, the enzyme will bind the acyl-CoA derivatives of 9 to 20 carbon fatty acids but will only desaturate 12 to 19 carbon fatty acid derivatives (Enoch *et al* 1976). The length of the fatty acid chain which can be desaturated is limited possibly due to the size of the fatty acid binding site (Brett *et al* 1971). The methylene chain of the stearoyl-CoA is in the eclipsed (gauche) conformation at carbons 9-10 in the enzyme substrate complex, suggesting that the hydrogen atoms can only be removed in the *cis* conformation leading to the formation of *cis* double bonds. Dehydrogenation is the rate limiting step in desaturation (Enoch *et al* 1976).

The Δ9 desaturase is a single polypeptide which contains one non-haem iron moiety (Strittmatter *et al* 1974). The actual mechanism of desaturation remains unclear. It is possible that the electron transport proteins transfer electrons from NADH to the non-haem iron which may then bind oxygen to facilitate desaturation. During desaturation, both hydrogen atoms are removed from the fatty acid together, suggesting that desaturation does not occur through a hydroxylated intermediate followed by dehydration (Gurr & Harwood 1991). The amino acid sequence of desaturases from various sources has been compared and an area of amino acid homology containing eight histidine residues has been detected. This conserved area may act as a binding site for the non-haem iron on the cytoplasmic face of the ER membrane (Shanklin *et al* 1994). It now appears possible that there are different homologues of Δ9 desaturase in different tissues; in particular

oligodendrocyte and myelin-specific genes have been identified from a cDNA library (Schaeren-Wiemers *et al* 1995).

Fig 1.8 shows the pathway for desaturation of essential fatty acids. It has long been accepted that long chain PUFA are formed by sequential desaturation by the Δ6, Δ5 and Δ4 desaturases with elongation occurring between each desaturation. However, the existence of a Δ4 desaturase has been difficult to prove. Rat liver microsomes will not desaturate 22:5 (n-3) to 22:6. However, when the substrates for chain elongation, malonyl-CoA and NADPH, are included in the suspension, 22:6 is produced. This suggests that 22:5 fatty acid is elongated to 24:5 which is then desaturated by a Δ6 desaturase. The 24:6 produced could then undergo β oxidation to produce the 22:6 n-3 fatty acid (Voss *et al* 1991). The same is true for production of n-6 fatty acids in rat liver microsomes (Selma Mohammed *et al* 1995). This pathway requires shuttling of the fatty acid around the cell as it must move from the ER to peroxisomes for oxidation and then return to the ER for incorporation into phospholipids (Sprecher 1996, Sprecher *et al* 1995). The same pathway has now been identified for n-3 fatty acid production in human retinoblastoma cell lines. This work suggests the presence of two chain length-specific Δ6 desaturases, one capable of conversion of 18:3 n-3 to 18:4 n-3 and the other to convert 24:5 n-3 to 24:6n-3 (Marzo *et al* 1996). However, work in liver microsomes does not suggest the presence of two enzymes (Geiger *et al* 1993). Fatty acids esterified to PC can be desaturated by a phospholipid-specific desaturase while esterified to glycerol (Pugh & Kates 1977). This avoids the need for fatty acid conversion to thiolesters. It also raises questions about the active site of the desaturase enzymes and why there is no steric hindrance to desaturation. The authors suggest that this system may only become effective when essential fatty acids are deficient. As yet no evidence has been presented to suggest that the same is true for other lipids.

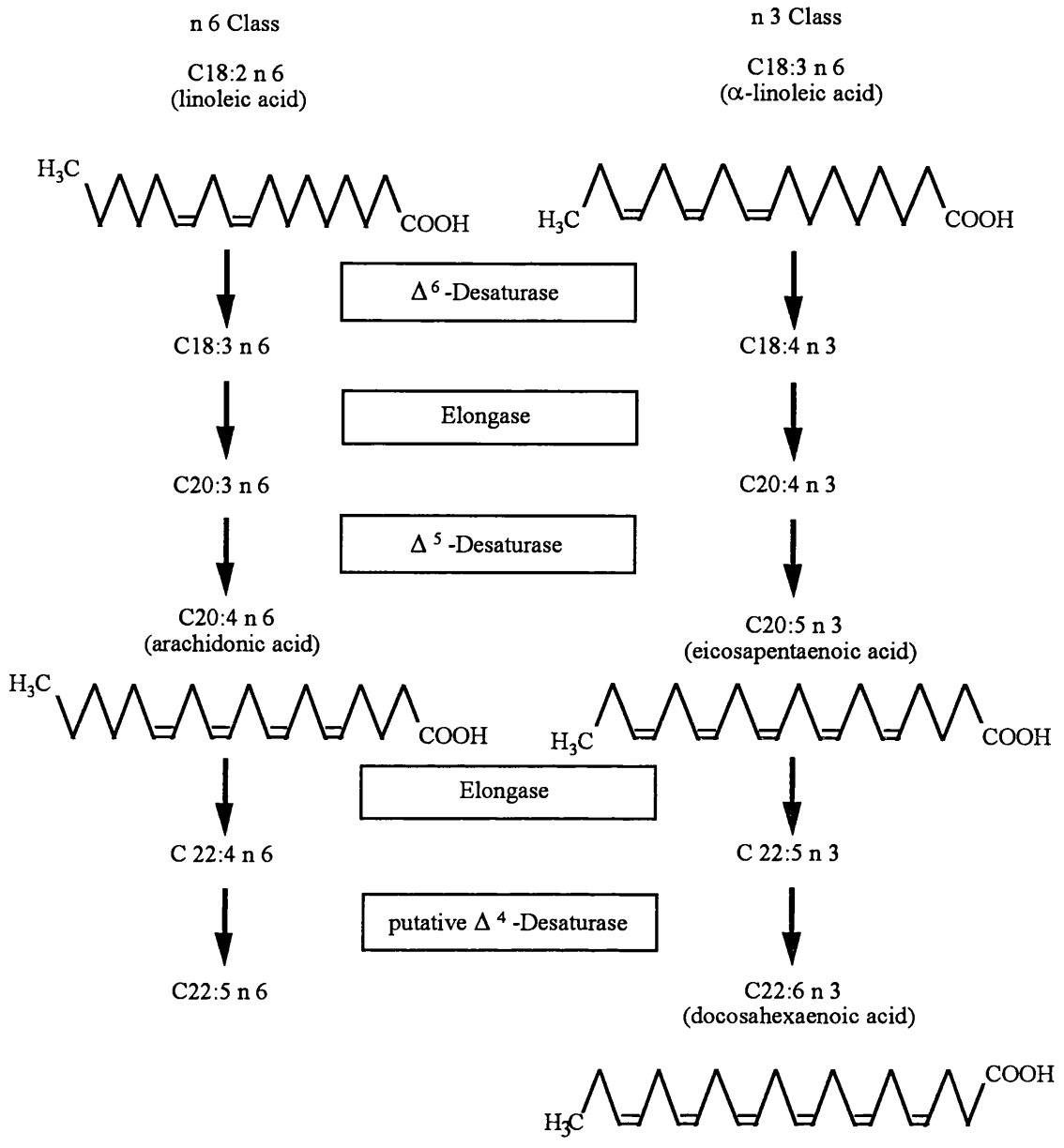


Fig 1.8 Pathway for essential fatty acid desaturation by the Δ6, Δ5 and putative Δ4 desaturase enzymes in the liver.

Although most structural information is available for the Δ9 desaturase, many studies of the physiological control of desaturation have centred around the Δ5 and Δ6 desaturases because they are required for the production of prostaglandins and leukotrienes from essential fatty acids. Although the Δ6 desaturase has not been cloned, the complex has been isolated from liver microsomes and the membrane surrounding it analysed. It contained mostly PC and cholesterol in a 4:1 ratio. This area of the membrane is in a gel-phase state at physiological temperature (Leikin & Schinitzky 1995). Increasing enzyme activity will increase the amount of PUFA in the membrane and therefore increase the fluidity of the membrane. This movement away from the gel-state environment in which the enzyme resides may control enzyme function in that the enzyme may become inhibited when the membrane becomes too fluid. When extracting the desaturase from the liver

microsomes, NADH-cytochrome b_5 reductase and cytochrome b_5 are co-extracted (Leikin & Shinitzky 1994).

Desaturases are not ubiquitous. Rat and human placenta (Ravel *et al* 1985, Chambaz *et al* 1985) and rat and human epidermis (Chapkin & Ziboh 1984, Chapkin *et al* 1986) have no activity while human platelets have very little (de Bravo *et al* 1985). Human foetal liver has active Δ5 and Δ6 desaturase enzymes (Chambaz *et al* 1985) but only the Δ6 desaturase is active in human endothelial cells from umbilical vein (Rosenthal & Whitehurst 1983). Liver microsomes from human neonates have active Δ5 and Δ6 desaturase enzymes although the activity of these enzymes is lower than in adults (Poisson *et al* 1993a). Cells from the rat small intestine, in particular jejunum and ileum cells, possess Δ9 and Δ6 desaturase activity (Garg *et al* 1988a). Cultured rat cardiac myocytes and human enterocyte cell lines can also desaturate essential fatty acid (Hrelia *et al* 1995, Dias & Parsons 1995). Desaturases were thought to be limited to the ER but rat liver nuclei have now been reported to have Δ5 desaturase activity (Ves Losada & Brenner 1995). This may be important for the regulatory effects of arachidonic acid on gene transcription (see earlier).

Before a discussion of the factors which affect desaturases, it should be noted that their activities in rats are strain-dependant. Studies of Δ5 and Δ6 desaturases in normotensive rats strains have shown that Sprague-Dawley rats have lower activities than Wistar rats. There are even differences between rats of the same strain from a different source. Wistar rats from Charles River have a lower activities that those from Bio-breeding (de Antueno *et al* 1994). Some of these changes may be due to diet or housing conditions.

The activities of these enzymes are generally measured using two techniques. The first is a direct enzyme assay; whole cells or microsomes are incubated with radioactive substrates for desaturation. The rate of conversion of substrate to product can then be measured. The second way is to study the fatty acid composition of the membrane and calculate indices of desaturation. These are the ratios of product to substrate. This method is useful when the sample size is small as measurements for all the desaturases can be made from one sample.

1.8.2 Dietary Induced Changes In Desaturation.

Trace elements affect desaturation in Sprague-Dawley rat liver. Iron, copper and manganese deficiency reduce the activity of the Δ9 desaturase. This is an iron-containing enzyme and iron deficiency therefore reduces its activity. The mechanism of action of the other elements is less clear, although the authors suggest that copper is a component of the desaturase. Δ5 and Δ6 desaturase activities are also reduced by iron and copper deficiency. Zinc deficiency does not directly affect the enzyme activity but alters the fatty acid composition of the liver cells because zinc deficient rats have a reduced food intake (Eder *et al* 1996). The lack of effect of zinc deficiency has been reported previously in Long-Evans rats (Kramer *et al* 1984), although others have reported Δ9 desaturase activity to be decreased and Δ6 desaturase activity to be increased under the same conditions (Fogerty *et al* 1985). Neither of these studies directly measured enzyme activity; the fatty acid composition of the membrane lipids was analysed. The later study attributes most of these changes to neutral lipids. Since Kramer *et al* (1984) only studied phospholipids, these alterations may have been overlooked. Direct enzyme activity studies have shown Δ9 desaturase activity to be reduced in rats fed a zinc deficient diet with a high fat content, preventing the possible effects of essential fatty acid deficiency (Eder & Kirchgessner 1995). As the control rats were also fed a high fat diet and all rats were force fed, these changes cannot be attributed to diet. The effects of Mg^{2+} deficiency has been studied in LLC-PK cells derived from porcine kidney. Mg^{2+} deficiency impairs PUFA biosynthesis, possibly by reducing Δ5 and/or Δ6 desaturase activity which would reduce the amount of PUFA incorporated into cell membrane phospholipids (Mahfouz *et al* 1989). Others have observed a reduction in the arachidonic and stearic acid content and a reduction in oleic and linoleic acid content in erythrocyte membranes from Mg^{2+} deficient rats (Rayssiguier 1986).

Vitamin A-deficiency increases rat Δ9 desaturase activity which is reversed when vitamin A is restored to the diet (Alam & Alam 1985). Vitamin E is important for membrane homeostasis because it is a potent antioxidant, preventing the breakdown of PUFAs (Burton & Traber 1990, Buttriss & Diplock 1988). However, vitamin E may also affect desaturase activity. Increased levels of vitamin E intake have opposite effects; the activity of the Δ6 desaturase increases in brain tissue and decreases in liver tissue (Despret *et al* 1992). The fact that the action is different in the two tissues suggests that this is not a general effect on the cell membrane. However, vitamin E decreases membrane fluidity in rat erythrocytes (Kameda *et al* 1985) and there is a negative correlation between Δ6

desaturase activity and membrane microviscosity (Brenner 1984). This explains the results in the brain but not the liver. If there are two forms of the Δ6 desaturase as there may be for Δ9 desaturase this may explain the two different effects.

N-3 fatty acids and cholesterol inhibit Δ6 desaturase in rat liver microsomes although their effects are not additive (Garg *et al* 1988b). The ability of rats to recover from this inhibition has been studied. Diets high in n-3 fatty acids were fed to young and old rats to inhibit the enzymes. After 3 days of feeding an n-3 deficient diet, the activity of the enzymes had returned to normal in the young rats; the activity in the older rats never completely recovered (Dinh *et al* 1995). Although n-3 fatty acids reduce desaturase activities, this inhibition is only effective as long as large quantities of n-6 fatty acids are not present. High concentrations of n-6 fatty acids overcome the inhibition (Garg *et al* 1990). Some fatty acids inhibit desaturases. In particular, C20:5 n-3 reduces Δ5 desaturase activity in erythrocyte, plasma, liver and kidney but not heart tissue (Nassar *et al* 1986). In the liver of Zucker rats, Δ9 desaturase activity is inversely related to the proportion of linoleic acid in the liver lipid (Weekes *et al* 1986). Linoleic acid also regulates the expression of Δ9 desaturase mRNA in neonatal mice. The brain isoform of the gene has increased expression and the liver isoform decreased expression in rats nursed by mothers fed a high linoleic acid diet (De Willie & Farmer 1993). Unsaturated fatty acids in general reduce Δ9 desaturase activity in mice. After returning the mice to a normal diet, the activity increased but did not reach control levels within the time course of the study (De Antueno *et al* 1992). Highly purified 20:5 (n-3) and 22:6 (n-3) fatty acid supplemention reduced liver Δ5 and Δ6 desaturase activity. Very high doses of the fatty acids caused this inhibitory effect to disappear. None of the doses of fatty acids affected the putative Δ4 desaturase and all doses increased the β oxidation of fatty acids (Gronn *et al* 1992). The activity of the Δ6 desaturase in rat liver microsomes is also increased by diets high in 18:3 (n-3) and 18:2 (n-6). The same was found to be true for the Δ5 desaturase while the Δ9 desaturase was inhibited by the above diets and a diet high in 20:5 (n-3) and 22:6 (n-3) (Christiansen *et al* 1991).

PE or ethanolamine supplementation in rats reduces the activity of the Δ6 desaturase. Ethanolamine also inhibited the desaturation of linoleic acid by hepatocytes in culture (Imaizumi *et al* 1989), suggesting that this may be an action of the base group and not the fatty acid. Feeding rats a choline-free diet reduces the amount of PC in the membrane and increases the amount of PS. It also causes a reduction in the activity of the Δ5 desaturase

in rat liver microsomes (Leikin & Brenner 1992). The reduction in PC increases the C/PL ratio making the membrane more rigid. The reduction in Δ5 desaturase activity will further exacerbate this decrease in membrane fluidity. PC (18:2) incorporation reduced Δ6 desaturase activity and increased the fluidity of the membrane (Koba *et al* 1995).

Trans fatty acids can regulate desaturation. The n-9 *trans* fatty acid, elaidate, is a potent inhibitor of Δ5 desaturase in human skin fibroblasts (Rosenthal & Doloresco 1984). Geometrical isomers of 18:3 (n-3) fatty acid do not inhibit Δ6 desaturase in rat liver microsomes but they do increase the activity of Δ5 desaturase (Blond *et al* 1990). These two results suggest that the position of the *trans* double bond determines the effect of the fatty acid.

Low fat diets in human subjects increase Δ6 desaturase activity in lymphocytes while Δ5 desaturase activity is unaffected (Hagve *et al* 1986). Desaturation in rat liver also increases during a fat-free diet (Voss *et al* 1991). This dietary control of desaturation may stabilise membrane lipid composition, keeping the concentration of arachidonic acid constant by increasing the conversion of linoleic acid. The increased activity may be due to lack of inhibition by the low fatty acid level in the diet. N-3 fatty acid deficiency prevented the age-related reduction in Δ6 desaturase activity in Wistar rat liver microsomes (Dinh *et al* 1993).

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Increasing the cholesterol content of liver microsomal membranes makes them less fluid and increases their activity of Δ5, Δ6 and Δ9 desaturases. Increased dietary cholesterol induced similar changes in rat adrenal microsomes (Igal & Dumm 1994). Increased Δ9 desaturase activity in spontaneously hypertensive rats (SHR) fed a high cholesterol diet (Ueno *et al* 1985) may be a device to increase fluidity to override the effects of increased cholesterol (Garda & Brenner 1985). Others, however, have observed that cholesterol feeding reduces the Δ6 desaturase activity (Garg *et al* 1986).

As desaturases are membrane-bound, they are ideally situated to maintain membrane composition and therefore cellular homeostasis. When fatty acids in the diet are plentiful, their activity is reduced but when this is limiting, activities increase to maintain the membrane levels of PUFA. Activity may be modulated by product or substrate inhibition. The enzymes also respond to membrane fluidity which is a complex result of cholesterol and phospholipid content.

Δ5 and Δ6 desaturases are also affected by dietary protein content. Low protein diets in rats initially reduced activity but this reduction was not consistent and fluctuated with time. Returning to a normal diet for 2 days, increased the activity of the enzyme which was maximal by 13 days (Narce *et al* 1988). The authors postulate that these changes may relate to changes in insulin, thromboxane, thyroid hormones or corticosterone. Decreased Δ9 desaturase activity has been shown in growing rats fed a low protein diet; subsequent feeding with a normal diet returned activity to a level greater than it was before the low protein diet began (Narce *et al* 1992a). Casein increases serum and liver cholesterol levels as well as increasing the desaturation of linoleic acid to arachidonic acid more than soybean protein. Here, casein protein increased Δ6 desaturase activity to a greater extent than soybean protein. Dietary protein appears to affect desaturation by changing the membrane fluidity which was correlated with Δ6 desaturase activity (Koba *et al* 1993). *In vitro,* an increase in membrane fluidity reduces activity and a decrease in fluidity has the opposite effect (Garda & Brenner 1984, 1985). Casein diets increase the desaturation of linoleic acid although at least 35% of the dietary protein must be casein for this effect to occur. This protein-rich diet increases Vmax but does not alter Km (Peluffo & Brenner 1974).

The effect of ethanol, which decreases liver and brain Δ6, Δ9 and Δ5 desaturase activity, also may be excerted by changing membrane fluidity (Wang & Reitz 1983).

1.8.3 Hormonal Control of Desaturation

Thyroid hormone excess increases lipogenesis, microsomal Δ9 desaturase activity (Towle & Mariash 1986) and the activity of PLA_2 (Szymanska *et al* 1991). Direct assay of Δ5 and Δ6 desaturases showed an acute suppression of activity but this was not reflected in changes in membrane composition (Ves-Losada & Peluffo 1993). In these experiments, the expected fall in membrane fatty acid composition did not occur, possibly because the PLA_2 is also less active. Conversely, on evidence of fatty acid composition, Δ6 desaturase activity is subnormal in hypothyroid patients (Van Doormal *et al* 1986), again possibly secondary to higher PLA_2 activity.

In vivo and *in vitro* ACTH administration inhibits PUFA biosynthesis in rat liver and adrenocortical cells by inhibiting Δ5 desaturase. Isolated liver and adrenocortical cells from ACTH-treated animals have a reduced incorporation and rate of metabolism of

dihomo-γ-linolenic acid (Mandon *et al* 1987). ACTH has a similar effect on Sertoli and Leydig cells (De Catalfo *et al* 1992), suggesting that this is not solely due to corticosterone. ACTH acts through cAMP. In rat adrenocortical cells, dibutyryl cAMP mimics the effect of ACTH (Mandon & De Gomez Dumm 1989). Other lipid-metabolising enzymes are inhibited by ACTH, in particular the acyl-CoA synthase in adipose tissue and the adrenal gland (Hall & Saggerson 1985, Mandon *et al* 1988). *In vitro* corticosteroid administration also inhibited arachidonic acid production by these cells (De Catalfo *et al* 1992). Since glucocorticoid receptors are widespread, this may be an important effect. Dexamethasone, a glucocorticoid analogue, also reduces the activity of the Δ5 desaturase (Marra *et al* 1986, Mandon *et al* 1987).

Rat liver microsome Δ5 desaturase activity is also inhibited by the mineralocorticoids, 11-deoxycorticosterone and aldosterone. Both reduced the conversion of dihomo-γ-linolenic acid to arachidonic acid. The inhibition caused by 11-deoxycorticosterone requires the presence of a soluble cytoplasmic 25 kDa protein fraction but aldosterone-induced inhibition does not, possibly suggesting a direct action of aldosterone on the membrane (De Alaniz & Marra 1992, Marra & De Alaniz 1991). Because the administration of these steroids does not change the C/PL ratio, the reduction in Δ6 desaturase activity caused by mineralocorticoids appears to be solely responsible for their effects on membrane fluidity in liver microsomes (Marra & De Alaniz 1990). 11-Deoxycorticosterone can also activate Δ9 desaturase in cultured hepatoma cells (Marra *et al* 1988).

In normal male rats, Δ9 desaturase activity decreases with age; oestrogen administration to castrated males prevents this. As the reduction is slower in female rats than males, oestrogens may have a role in maintenance of enzyme activity (Thorling & Hansen 1995). However, others have not confirmed that oestrogen affects desaturation in human subjects (Medeiros *et al* 1995). In this study, activity was compared with endogenous oestrogen levels. Other studies have shown the activities of Δ5 and Δ6 desaturases to be higher in postmenopausal than premenopausal women, suggesting that oestrogen may reduce the desaturase activity (Liu & Medeiros 1995). However, the age difference between these two groups would also account for differences in desaturation. It is difficult to compare these experiments as the first was carried out on male rats and the other two on female patients. A better experiment would have been to treat post-menopausal rats with oestrogen and to compare them with postmenopausal untreated rats. The activity of Δ9

desaturase is increased by testoserone whereas the activities of Δ5 and Δ6 desaturase is reduced. These effects are greater in females than males (Marra & De Alaniz 1989).

Transgenic mice have been used to study the effect of growth hormone on Δ6 desaturase activity. Animals which expressed the growth hormone transgene had a significantly greater Δ6 desaturase activity leading to an increased arachidonic acid and DHA content in the liver phospholipids but not those of adipose tissue (Nakamura *et al* 1996). Others have previously reported that growth hormone administration to hypophysectomized rats caused the ratio of arachidonic to linoleic acid to increase rapidly (Maddaiah & Clejan 1986), suggesting an increase in enzyme activity.

Adrenaline reduced the conversion of linoleic acid to γ-linolenic acid, i.e. Δ6 desaturation. This effect could be blocked by propanolol, the β receptor antagonist (de Gomez Dumm & Brenner 1978). The authors therefore state that the reduction in desaturation by catecholamines is mediated through the β receptor by an increase in cAMP. Thus this may be a general response to cAMP (see above).

1.8.4 Hypertension and Fatty Acid Desaturation.

Higher Δ5 and Δ6 desaturase activity have been reported in SHR compared to Wistar-kyoto (WKY) rats at one month and 6 months (Δ6 only) of age. However, compositional analysis of the membranes showed an unexpected reduction in arachidonic acid content; increasing enzyme activity should have increased the arachidonic acid in the membrane. The authors postulated that this might be due to inefficient esterification of arachidonic acid into phospholipids or increased utilisation of arachidonic acid in eicosanoid production (Narce *et al* 1994). A year later, the same group published results showing reduced Δ5 and Δ6 desaturase activity in SHR microsomes. Enzyme activity was inversely related to blood pressure. Analysis of membrane lipids showed an increase in linoleic acid and a reduction in arachidonic acid content as expected (Narce & Poisson 1995). The authors suggest that these alterations may be due to increased insulin sensitivity in SHR or increased prostaglandin production. It is difficult to reconcile the conflicting results of these two similar experiments. No attempt was made in the 1995 paper to explain these anomalies.

The two previous studies (Narce *et al* 1994, Narce & Poisson 1995) only investigated the rate of desaturation of n-6 fatty acids; investigations of the desaturation of n-3 fatty acids

further adds to the confusion. Hepatocyte Δ6 desaturase activity for n-3 fatty acids was higher in the young SHR rat than in the normotensive controls, but there was no difference when 12 month old rats were compared. Fatty acid analysis of the hepatocytes showed that the enzyme activity and n-3 fatty acid content of the membrane were not related. The authors postulate that the change in n-3 fatty acid desaturation is important in the pathogenesis of hypertension as it alters the eicosanoid balance (Narce *et al* 1995). There is, as yet, no evidence to indicate whether there are two different desaturase enzymes specific for n-3 and n-6 fatty acids. Therefore, it is difficult to explain why the activity of the enzyme can be increased for one fatty acid type but not the other. When the enzyme assay was carried out in whole hepatocytes, the enzyme activity was increased compared to control but when the assay was carried out using microsomes, a reduction activity was observed. A methodological artefact cannot therefore be excluded.

Stress, induced by housing animals one to a cage, reduced Δ5 and Δ6 desaturase activity in WKY rats. In SHR rats, only Δ5 desaturase activity was affected but this inhibition was greater than in WKY rats. Adrenaline administration reduced essential fatty acid desaturation in Sprague-Dawley rats (Mills *et al* 1994). This suggests that the effects of stress-induced hypertension may be due to release of adrenaline, which is known to be increased in this situation (Mills & Ward 1989). Blood pressure was increased in the stressed rats (Mills & Ward 1986a) but the levels of ACTH and corticosterone were not measured.

Both hypertension and salt loading reduced Δ5 and Δ6 desaturase activity while salt loading also reduced the incorporation of fatty acids into phospholipids. Again this effect was more marked in SHR than WKY rats (Poisson *et al* 1993b). Earlier studies had however suggested that Δ5 and Δ6 desaturase activities were increased in liver microsomes by salt loading (Narce *et al* 1992b). The concentration of salt in the drinking water used by Poisson *et al* (1993b) was 10% for four weeks whereas Narce *et al* (1992b) used 3% for 60 days. The difference in duration and magnitude of salt loading makes it difficult to compare these studies. Salt loading will suppress the renin-angiotensin-aldosterone system and increase the blood pressure which may also have had independent effects on desaturation. In patients, salt-loading caused the levels of unesterified plasma fatty acids to increase and plasma aldosterone levels to fall (Goodfriend *et al* 1991b). This is also a complex situation and it is unlikely that the aldosterone level is an important determinant of the lipid status here.

1.8.5 Diabetes Mellitus and Fatty Acid Desaturation.

Three different animal models have been used to study the effects of diabetes on the desaturase enzymes; streptozotocin (STZ)-induced diabetic rat, alloxan diabetic rats and spontaneously diabetic bio-breeding (BB) rats. STZ induces extrapancreatic toxicity which causes diabetes by an unknown action, possibly related to free radical formation. Δ5 desaturase activity is impaired in heart, liver, kidney, aorta, serum (Holman *et al* 1983), the renal cortex (Ramsammy *et al* 1993), testes (Wilder & Coniglio 1984) and in cells from the mesenteric vasculature (Fujii *et al* 1987). Interestingly, the levels of fatty acids from the n-3 series were less effected than those from the n-6 series (Holman *et al* 1983). Further studies of the renal cortex and isolated glomeruli showed that there is a reduced activity of the Δ5 and Δ6 desaturase, increased activity of the long-chain acyl-CoA synthetase and 1-acyl-*sn*-glycero-3-phosphocholine acyltransferase. Elongase activity was not altered by STZ-induced diabetes. Lipid peroxidation was also lower in diabetic rats, as measured by monoalondialdehyde, the end product of lipid peroxidation. The authors claim that the changes were not due to increased β oxidation (Ramsammy *et al* 1993). The adrenal glands of STZ-diabetic rats make less arachidonic acid, due to impaired Δ5 and Δ6 desaturase activity and this is partially reversed by insulin treatment. Insulin also increased arachidonic acid production in cultured diabetic rat adrenocortical cells (Igal *et al* 1991). Others have shown that insulin increases the activity of the Δ6 desaturase in diabetic rats (Shin *et al* 1995). Vitamin E, possibly acting as an antioxidant, improved desaturase activity and reversed several other STZ-induced changes (Douillet & Ciavatti 1995). However, unlike normal rats, STZ-diabetic rats fed either a diet high in either n-6 or n-3 fatty acids did not have altered desaturase activity by direct enzyme assay although the membrane contained more PUFA (Igal & de Gomez Dumm 1995).

Alloxan-diabetic rats have raised Δ6 desaturase activity in the kidney compared to controls but Δ9 desaturase activity is unchanged. In liver cells, Δ6 desaturase activity was unchanged but Δ9 desaturase was depressed (showing different tissue effects). The authors suggest that the rate limiting step in Δ6 desaturation is the formation of the fatty acid thiolester as the activity of the acyl-CoA synthetase is increased in the renal cortex (Clarke & Queener 1985). Neither Δ5 desaturase nor membrane fatty acids were analysed.

In spontaneously diabetic (BB) rats, the activities of the putative Δ4 desaturase, Δ9, Δ5 and Δ6 desaturases are all lower than control and normalised at least partially by insulin

(Mimouni *et al* 1992, Mimouni & Poisson 1990, 1992). However, the authors did not find any changes the cell membrane composition consistent with reduced activity but the experimental groups were small (n=3) (Mimouni *et al* 1994). Reported insignificant changes in arachidonic/linoleic acid ratio and membrane palmitoleic acid content consistent with enzyme changes might be significant in a larger experiment.

Although platelets have little desaturase activity, they have been used to study enzyme activity in human insulin-dependant (IDDM) and NIDDM patients by the direct enzyme assay. Δ5 and Δ6 desaturase activities were impaired (Jones *et al* 1986). Short term ketoacidosis, a feature of poorly controlled patients, reduced the arachidonic acid content of the plasma. There was an inverse correlation between the plasma glucose levels and arachidonic acid content. These results suggest that metabolism of essential fatty acids by the Δ5 and Δ6 desaturases is impaired. It can be corrected by insulin (Bassi *et al* 1996).

1.8.6 Effects of Age

In rat liver microsomes, Δ6 desaturase activity decreases with age while that of the Δ9 desaturase increases (Peluffo & Brenner 1974). This reduction in Δ6 desaturase can be prevented by the administration of γ-linolenic acid (Hrelia *et al* 1989) which increases the desaturation of both n-6 and n-3 fatty acids (Biagi *et al* 1991). Previous studies, however, have suggested that diets high in unsaturated fatty acids actually reduce desaturation (Nassar 1986, Weekes 1986, Gronn 1992). For Δ6 desaturase Km for linoleic acid increases with age but Vmax is not changed. For n-3 fatty acids, Km was unaffected by age (Hrelia *et al* 1990) possibly because the enzyme has a higher affinity for n-3 fatty acids. Others have reported that desaturation fluctuates with time. For example, desaturation rate of α-linolenic acid increases in WKY rats from 1.5-3 months and then decreases until 24 months when it returns to the same levels as at 1.5 months. Linoleic acid desaturation rate increases until 6 months and remains constant thereafter. The activity of the Δ5 desaturase decreases from 1.5 to 3 months and then increases again to reach the 1.5 month level at 24 months (Maniongui *et al* 1993). The decrease in membrane fluidity which occurs with age and which may be related to desaturase action, can be reduced by restricting the food intake of animals. Feeding rats on an 'every other day' regime reduces microviscosity of splenic lymphocytes compared to control fed rats (Pieri 1991).

1.8.7. Exogenous Inhibitors and Activators of Desaturation.

A number of inhibitors of microsomal desaturase have been identified but their use in the study of membrane lipid composition has been limited. Various inhibitors of microsomal desaturase enzymes have been identified. Curcumin (1,7-bis(4-hydroxy-3methoxyphenyl)-1,6-heptadiene-3,5-dione) inhibits Δ5 and Δ6 desaturases; its 4-hydroxyl group is essential for this effect (Kawashima *et al* 1996a). The same enzymes are inhibited by the structurally related gallic acid esters (Kawashima *et al* 1996b) and by benzyl viologen which induces free radical formation (Muriana *et al* 1991). Finally, sesamin, an aromatic constituent of sesame seeds, specifically inhibitor Δ5 desaturase (Shimizu *et al* 1991). Fibrates are used in the treatment of hyperlipidaemia syndromes. Their effects on lipid metabolism are complex and changes in Δ5, Δ6 and Δ9 desaturase activities inconsistent (Vazquez *et al* 1995, Alegret *et al* 1995, Sanchez *et al* 1993). It seems likely that these changes are secondary to altered lipid metabolism or possibly to peroxisomal proliferation (Alegret *et al* 1995). The activity of the Δ6 desaturase is inhibited by foetal bovine serum in enterocytes *in vitro* (Dias & Parsons 1995), possibly because it binds fatty acids, reducing their availability for desaturation.

1.9 Activities Of Enzymes Other Than The Desaturases.

From the foregoing discussion, it is clear that the desaturases play a major role in determining membrane composition. However, it is not an exclusive role; several other enzymes are involved.

The concentration of arachidonic acid in the membranes of cultured cardiac myocytes from young SHR (3 days old) is lower than in WKY but there is more of this acid in their growth medium. Thus, more arachidonic acid is lost from SHR membranes. This is due to relatively higher deacylation rate in SHR cell membranes by a more active Ca^{2+}- PLA_2. The Ca^{2+}-independent PLA_2 was not different between the two strains. Membrane lysophospholipid content was also not different so that increased reacylation must compensate for increased PLA_2 (Millanvoye-Van Bussel & Devynck 1995). The increase in PLA_2 activity could be due to raised intracellular [Ca^{2+}] which occurs in SHR or to a higher enzyme concentration. PKC, which regulates PLA_2, activity (Exton 1994) is increased in SHR aortic cells (Murakawa *et al* 1988). Lipid peroxidation is also more marked in their myocardial cells (Janero & Burchardt 1989) and PLA_2 has a higher affinity for peroxilipids than their unoxidised precursors (McLean *et al* 1993). The increase in

PLA_2 activity may therefore be a device to remove these peroxilipids. Neonatal SHR are normotensive Millanvoye-Van Bussel & Devynck (1996) therefore suggests that these changes have a genetic basis. However, these rats had hypertensive mothers and consequent changes in the uterine environment may at least have been partly responsible. Moreover, neonatal SHR rats have cardiac hypertrophy (Clubb *et* al 1987).

Renal PLA_2 activity is increased in stroke-prone SHR (SHRSP) rats (Kawaguchi *et al* 1986, Okamoto *et al* 1989). Their levels of PE and PC decrease with age, as does the arachidonic acid content of the cell membrane. In contrast, the levels of these lipids remain constant in WKY rats over time. Antihypertensive drugs, Nicardipine, Hydralazine and Enalapril, prevent the decrease in SHRSP and increased membrane arachidonic acid. The PLA_2 activity was also corrected by antihypertensive treatment (Okamoto *et al* 1989). Thus the increase in PLA_2 activity in SHRSP is probably due to increased blood pressure. PLA_2 acts preferentially on PC and PE (Kawaguchi *et al* 1986) which may explain why only these were affected in renal membranes. Interestingly, in the Okamoto *et al* (1989), study the levels of PE and PC in the membrane begin to fall before a detectable increase in PLA_2 activity, suggesting that something else is controlling their levels. PLA_2 activity is also increased in SHR aortic rings compared to WKY. Esterification of arachidonic acid into phospholipids is increased in SHR rats but esterification into neutral lipids was not different between groups. The increase in the amount of arachidonic acid in phospholipids does not appear to be due to reacylation of lysophospholipids but to *de novo* synthesis of lipids (Limas *et al* 1981).

PLC activities are increased in the renal membranes of SHRSP compared to WKY (Kawaguchi *et al* 1987). In SHR platelets, the activity of PLC is also enhanced. This leads to increased platelet reactivity as PLC breakdown of phosphatidylinosoitol 4,5-bisphosphate is an early step in the activation by thrombin (Koutouzov *et al* 1987).

It was suggested that turnover of PC is increased in SHR, SHRSP and Dahl salt-sensitive rats platelets compared to their control strains (Remmal *et al* 1988, Marche *et al* 1990) but not in those from deoxycorticosterone acetate (DOCA) -salt hypertensive rats (Marche *et al* 1990). However, when these studies were repeated with VSMC, conflicting results were obtained. When incubated with ^{32}P, the total lipid-associated radioactivity was greater in the WKY cells. The labelling of PE was also greater in the WKY but there was a small reduction in the amount of labelled PS compared to SHRSP. The actual

composition of the lipids was not different between the hypertensive and control rats (Dominiczak *et al* 1991a). Membrane fluidity is dependant on its lipid composition at any given time. That an increased turnover rate does not necessary mean that composition changes is emphasised by these observations of Dominiczak *et al* (1991a).

Diabetic subjects synthesise less lipid and there is an increased breakdown of the body's fat stores. In STZ-diabetic rat liver, lysophosphatidylcholine acyl-CoA transferase (LAT) activity is increased and PLA_2 activity is decreased (Dang *et al* 1984). This contradicts previous reports that reacylation of PC in diabetic rat erythrocytes is less efficient than in control rats (Le Petit-Thevenin *et al* 1988). Others have observed that, in STZ-diabetic rats, membranes incorporate less palmitic, oleic and linoleic acid into erythrocyte membranes than controls (Arduini *et al* 1995), again suggesting that reacylation and the activity of LAT may be impaired in STZ-diabetics. Propionyl-L-carnitine treatment reduces the vascular damage observed in diabetes rats (Corsico *et al* 1993), promoting investigation of its effect on erythrocyte membrane composition. This treatment corrects the deficit in reacylation of PC with linoleic acid; reacylation with the other fatty acids was partially restored (Arduini *et al* 1995). As Le Petit-Thevenin *et al* (1988) and Arduini *et al* (1995) both studied reacylation in erythrocytes, this impairment may be restricted to these cells. The incorporation of radioactive fatty acids into erythrocyte membrane phospholipids is reduced in diabetic rats and cannot be corrected by insulin treatment (Dang *et al* 1991). This is strong evidence that reacylation is impaired.

The arachidonic acid concentration in platelet membranes from STZ-diabetic rats is normal 3 weeks post-induction of diabetes but is increased compared to controls 6 weeks post-induction. Arachidonyl-CoA synthetase, acyl-CoA:lysophophatidylcholine acyltransferase and PLA_2 activities in platelets were not different at 3 and 6 weeks post induction of diabetes (Dang *et al* 1992), strongly suggesting that the alterations observed in platelet membranes are not caused by impaired activity of the deacylaction-reacylation cycle.

Diabetes is associated with an increase in enterocyte microsomal membrane total phospholipids, including PE and SPG. The phospholipid composition of the cells did not correspond with the activities of choline phosphotransferase (CTP), PE methyltransferase (PEMT) and 1,2-diacylglycerol:CDPcholine esterase which were similar for both diabetic and control cells. As there was no change in the activity of the enzymes responsible for converting PE to PC, the observed difference in membrane composition may be due to

alterations in *de novo* PE biosynthesis (Keelan *et al* 1994). Insulin increases sphingomyelinase activity (Petkova *et al* 1990) and, as insulin levels were low in these animals, activity should be lower than normal. This may have increased the amount of SPG in the cell observed by Kellan *et al* (1994) which would lead to a reduction in membrane fluidity.

It is worth noting that the liver microsomal acyltransferase system (1-acylglycerol 3-phosphocholine (1-acyl-GPC) acyltransferase) does not appear to differentiate between n-3 and n-6 fatty acids when esterifying PC. The system does, however, have a preference for unsaturated fatty acids and a degree of chain length specificity (Lands *et al* 1982).

1.10 Uptake of Fatty Acids and Lipids into the Cell Membrane.

Most information about lipid uptake into the membrane has been derived from studies of erythrocytes. These unique cells are incapable of lipid production and therefore any alteration in composition must be due to uptake. The composition of erythrocyte membranes changes throughout their life span and often reflects changes in the lipid composition of the plasma.

Rabbits fed diets containing large quantities of linoleic acid show an increase in the proportion of this fatty acid in the membrane. Linoleic acid is preferentially incorporated into the *sn*-2 position in PC and PE at the expense of oleic acid. Uptake is dependant on the presence of an acyltransferase which may be *sn*-2 specific (Mulder *et al* 1963). The *in vitro* uptake of fatty acids into phosphoglycerides is ATP- and coenzyme A-dependant. With these present, rabbit erythrocytes take up more linoleic, oleic and palmitic acid than stearic, myristic or lauric acid. The amount of fatty acid incorporated into the membrane parallels its plasma concentration (Mulder & Van Deenen 1965a). Fatty acids have been shown to be incorporated into erythrocyte ghosts in the presence of ATP, MgCl and coenzyme A, although C16:0 was incorporated from C16:0-CoA without cofactors. Incorporation of linoleic acid was the highest. Again, most of this was incorporated into the *sn* 2 position of PC (Oliveira & Vaughan 1964).

Human erythrocytes will take up fatty acids *in vitro*. Each fatty acid is taken up into the cell at a different rate and the rates of membrane association and esterification of the fatty acid into lipids are different. Palmitic and stearic acid become membrane-associated to a greater extent than oleic and linoleic acid but the actual incorporation rate into membrane

lipids is greater for oleic and linoleic (Donabedian & Karmen 1967). The presence of different pools of fatty acids in the membrane has been suggested. Palmitic acid is taken up rapidly, independent of cell metabolism, into a pool from where it can be removed using defatted albumin. This suggests that the fatty acid is loosely associated with the outside of the membrane. From this pool, fatty acids pass into a deeper pool from which they cannot be removed. This may not actually be physically deeper in the membrane but the fatty acids may be more tightly associated. Incorporation into this pool is energy-dependent, as is the subsequent incorporation of fatty acids into phospholipids (Schohet *et al* 1968). Fatty acids are preferentially incorporated into the lipids in the inner leaflet; this may be the deeper fatty acid pool suggested by Schohet (Renooij *et al* 1974).

Phospholipids exchange between plasma and the erythrocyte membrane in both man and the dog. Approximately 60% and 30% of the total PC and SPG respectively is exchangeable with a turnover time of 5 days. PE and PS are thought to be stable, in that they do not exchange with plasma lipids (Reed *et al* 1968). This may be due to their absence from plasma or because they are inner-membrane lipids. LPC, PC and SPG also exchange. The rate is greater for LPC than PC with SPG exchange being slowest (Sakagami *et al* 1965). Little exogenous free ^{32}P is taken up by the cell (Reed *et al* 1968) but what is taken up is incorporated into phosphatidic acid (Sakagami *et al* 1965). Intravenous infusion of PUFA-PC for 3 days decreased the cholesterol content and increased the PUFA content of the membranes. The increase in PUFA was not confined to PC; the arachidonic acid content in PS and PE also increased (Catafora *et al* 1992). Cholesterol can also be transfered between plasma and the erythrocyte membrane (Lange *et al* 1977). Increasing dietary cholesterol also increases its erythrocyte membrane content. Incubating cells with cholesterol will increase membrane cholesterol by as much as 47% (Lijnen & Petrov 1995).

Lysed rabbit erythrocytes can convert LPC to PC. This transacylation requires ATP and a fatty acid-CoA derivative. When no ATP is present, the cell will convert LPC to PC and glycerophosphorylcholine (Mulder *et al* 1965). The same authors suggest that erythrocytes accept lysophospholipids from the plasma as they cannot deacylate lipids. The erythrocyte could then reacylate the lysopospholipids and insert them into the membrane. Because there is more LPC than lysophosphatidylethanolamine (LPE) in the plasma, the composition of PC changes faster than that of PE (Mulder & Van Deeden 1965b).

Changes in membrane composition with diet are not restricted to erythrocytes. Diets high in n-3 and n-6 fatty acids changed the composition of mouse liver hepatocytes within 7 days. These changes were reversed by a normal diet with the correction of 20:5 n-3 taking longest. The unsaturation index was not altered, suggesting a concomitant increase in saturated fatty acid. The C/PL ratio was also unchanged by these manipulations and so the changes in fatty acid composition observed were not due to increased incorporation of phospholipids (De Antueno *et al* 1992).

While lipids can be taken up by the erythrocyte, they can also be released. If the lipid is taken into the cell as a total lipid molecule, it is also released that form. The uptake and release rates are similar. However, if the lipid was taken into the cell as free fatty acid and then esterified to a lipid, then it is released from the cell as free fatty acid. Fatty acid is often transferred from PC to PE and neutral lipids before being released in its free form (Schohet 1970).

The knowledge that erythrocyte lipid composition changes with diet has been used to check compliance in studies involving feeding of lipids. Treatment with γ-linolenic acid increased the erythrocyte concentration of n-6 fatty acids, in particular dihomo-γ-linolenic acid (Biagi *et al* 1994). Increased n-3 fatty acids in the diet for 4 months (3g/day) leads to increased n-3 fatty acids in erythrocyte membranes (Russo *et al* 1995).

The concentrations of oleic acid and arachidonic acid in rat liver microsomal membranes were increased by diets high in olive oil (high in oleic acid) or evening primrose oil (high in linoleic acid) respectively. Addition of cholesterol to the diet increased membrane cholesterol, membrane PC, C/PL and PC/PE ratio. There was a increase in the mono-unsaturated fatty acid (MUFA) and a slight reduction of saturated fatty acid (SFA). Arachidonic acid content was reduced and that of linoleic acid was increased; the ratio of these fatty acids was reduced by high cholesterol diet. This suggests that cholesterol increases the activity of the Δ9 desaturase and decreases the activity of the Δ5 and Δ6 desaturases. Cell membrane microviscosity measured by fluorescence anisotropy (see later) was increased and membrane fluidity correlated with the unsaturation index (Murina *et al* 1992).

Although erythrocytes cannot synthesise fatty acids, they can produce acyl:CoA esters and have one acyl:CoA synthetase enzyme. Its role in the cell is uncertain but it may participate in the transport of unsaturated fatty acids from the circulation (Davidson & Cantrill 1985). Others have shown that n-3 fatty acids are specifically incorporated into plasmalogens and phospholipids in the inter leaflet (Knapp *et al* 1994).

1.11 Physiological Conditions Affecting the Cell Membrane

1.11.1 Metabolic disease

During combined hyperlipidemia, the levels of plasma triglycerides and cholesterol are increased. Therefore, erythrocyte membranes should be altered. However, the expected correlation between plasma and membrane cholesterol does not exist. The membrane levels were normal but the membranes from the combined hyperlipidemic group were more fluid than those of the control group and basal Ca^{2+} pump activity are decreased (Muzulu *et al* 1995). These results suggest that the membrane possesses an a adaptive process to prevent excessive amounts of cholesterol entering the membrane. Platelet membranes from hyperlipidemic patients have an increased C/PL ratio; the opposite is true for membranes from hypercholesterolemic patients and the PC/SPG ratio is also decreased in these patients (Levy *et al* 1994). SPG is a membrane rigidifier while PC is a membrane fluidiser. A fall in the ratio should increase membrane fluidity (Borochov *et al* 1977). Others have observed that hypercholesterolemia increases platelet cholesterol content and does not alter the trimethylammonium diphenylhexatriene (TMA-DPH) or diphenylhexatriene (DPH) anisotropy, hypertriglycerideaemia decreases the C/PL ratio and decreases the DPH anisotropy (Le Quan Sang *et al* 1993a).

1.11.2 Hormones

Oestrogens have antioxidant actions. For example, they inhibit metal ion-dependant lipid peroxidation in liposomal and microsomal membrane preparations (Wiseman & Halliwell 1993). Synthetic oestrogens reduce membrane fluidity in phospholipid microsomes produced from ox brain and this correlates with the antioxidant action. It is not clear whether the mechanism of the antioxidant action is the reduced fluidity (Wiseman & Quinn 1994). Tamoxifen, another oestrogen-like compound, inhibits lipid peroxidation in this way (Wiseman *et al* 1993). Combined oestrogen and progesterone therapy decreases membrane PUFA and increases SFA and MUFA. The concentration of arachidonic acid is also decreased (Ranganath *et al* 1996). This decrease in PUFA may explain reduced membrane fluidity. It is difficult to reconcile the reported cardio-protective effects of

oestrogen with a decrease in membrane fluidity especially when the evidence of the adverse effect of reduced membrane fluidity in hypertension is considered.

1.11.3 Diabetes

1.11.3a Animal Models of Diabetes.

Changes in cell membrane structure and function have been reported in animal models of diabetes. Some of these have been discussed previously (see sections 1.8.5) with reference to lipid metabolising enzymes. Many reports of changes in the physical properties and composition of the membrane are conflicting (see also chapter 6).

In STZ-diabetic rats ileal enterocyte (Brasitus & Dudeja 1985) polymorphonuclear lycocytes (PML) (Masuda *et al* 1990) and renal cell membrane fluidity (Ramsammy *et al* 1993) has been reported to be reduced but that of synaptosomal membranes to be increased (Medow *et al* 1994). Others have found membrane fluidity to be unchanged in cerebral microvessels (Mooradian & Smith 1992) and intestinal brush border cells (Gourley *et al* 1983)

Microviscosity can be affected by a variety of factors, some of which change in diabetes. For instance, erythrocyte membrane C/PL ratio is elevated in diabetic rats suggesting reduced membrane fluidity. This can be corrected by Brazilin, a compound used to reduce blood glucose and blood viscosity (Moon *et al* 1993). A similar increase in the C/PL ratio and membrane cholesterol, prevented by insulin, has been observed in ileal enterocytes from STZ-diabetic rats (Brasitus & Dudeja 1985). Again these changes should increase membrane microviscosity. Synaptosomal and microvessel membrane cholesterol and C/PL ratio were not altered by STZ (Medow *et al* 1994 Mooradian & Smith 1992). Ramsammy *et al* (1993) suggest that cholesterol is not responsible for the changes in membrane fluidity in diabetes. Removing proteins and cholesterol from renal cells increased membrane fluidity but those from diabetic rats were still more rigid than control. This suggests that fatty acid composition may be important in determining fluidity. There is much evidence for this.

STZ reduces membrane palmitic, palmitoleic, arachidonic, docosatetraenoic (DTA) and docosapentaenoic acids (DPA) but increases linoleic, stearic acid and DHA (Dang *et al* 1991, Shin *et al* 1995, Moon *et al* 1993, Lin *et al* 1985). Many of these changes may be

caused by alterations in desaturase activity or to impaired reacylation of the membrane lipids in some cell types (see earlier). There is considerable variation in the effect of experimental diabetes between tissues (Lin *et al* 1985, Ramsammy *et al* 1993, Black *et al* 1993, Medow *et al* 1994, Mooridan & Smith 1992) which may be real or due to differences in analytical technique.

Because membrane fluidity may be reduced in diabetics, diets rich in n-3 fatty acids may be therapeutic as they should increase membrane fluidity. In cardiac cell membranes from diabetic animals fed diets high in n-3 fatty acids, membrane EPA and DHA increased and DTA in PE fell compared to non-diabetic controls. The fatty acid composition of PC was unaltered. The authors postulate that the beneficial effects of n-3 fatty acids are through mechanisms other than changes in the membrane phospholipid composition even though the diet led to increases in the amount of unsaturated fatty acid in the membrane (Black *et al* 1993). However, the size of the experimental groups for some fatty acids (n=2) reduced the usefulness of these results. Increased fatty acid peroxidation, by decreasing membrane unsaturation, should reduce membrane fluidity in STZ rats (Mooradian & Smith 1992)

Insulin treatment, particularly from the onset of diabetes, prevents membrane abnormalities occurring (Lin *et al* 1985, Medow *et al* 1994, Dang *et al* 1991, Shin *et al* 1995). However, the efficiency of metabolic control is important. PML membrane fluidity correlated negatively with serum glucose levels measured the previous week. In animals with a low serum glucose level (below 10mM), the membrane fluidity returned to normal over time. Interestingly, when glycosylated protein from diabetic rat serum was incubated with normal cells, cell membrane fluidity was also reduced (Masuda *et al* 1990), suggesting that protein glycation may affect membrane fluidity.

1.11.3b Human Diabetics.

Changes in erythrocyte structure and function are involved in the pathogenesis of diabetic vascular disease. (Isogia *et al* 1981 Schmid-Schonbein & Volger 1976). However the state of the literature on membrane biochemistry is confused and reflects the difficulty in carrying out rigidly controlled studies.

The erythrocyte membrane fluidity has been variously reported to be less than (Caimi *et al* 1992, Mazzanti *et al* 1989, Bryszewska *et al* 1986, Paolisso *et al* 1993, Kamada & Otsuji

1983, Dowd *et al* 1994) greater than (Bryszewska & Leyko 1983, Testa *et al* 1988, Mazzanti *et al* 1992) or the same as normal (Freyburger *et al* 1988, Hill & Court 1983, Candiloros *et al* 1995). If the literature on PML is reviewed (Caimi *et al* 1995, Kantar *et al* 1991), the outcome is similar. Similarity, there is little agreement on the effect of human diabetes on membrane composition. While, most studies indicate reduced fluidity (Watala 1993) affecting cell function, the reported fall in NIDDM membrane cholesterol and phospholipids and C/PL ratio should have the opposite effect (Baldini *et al* 1989). Both erythrocyte membrane C/PL ratio and plasma cholesterol correlate with fluidity in diabetic subjects (Bryszewska *et al* 1986). Others have failed to show a difference in erythrocyte or platelet membrane phospholipid or cholesterol content or the ratio of cholesterol/phospholipid (Hill & Court 1983, Urano *et al* 1991, Caimi *et al* 1995).

Erythrocytes and platelets from diabetic patients have an increased SPG content and/or a reduced PE concentration compared to control (Kamada & Otsuji 1983, Caimi *et al* 1995) tending to reduce fluidity because SPG contains most long chain saturated fatty acids and PE contain primarily unsaturated fatty acids (Rouser *et al* 1968). Membrane fluidity correlated negatively with membrane PS and PC concentrations in controls and negatively with the C/PL ratio and the membrane SPG content in the diabetics (Caimi *et al* 1995). The PC content was greater in young IDDM than controls (Baldini *et al* 1989).

Membrane fatty acid composition is altered in a complex fashion in diabetes and this is determined to a large extent by the altered desaturase activities (see earlier). In NIDDM erythrocyte membranes, the unsaturation index is higher than normal according to Pelikanova *et al* (1991) with higher levels of n-3,n-6 arachidonic acid and DHA (Urano *et al* 1991, Pelikanova *et al* 1991). In complete contradiction, others found reduced levels of PUFA, DHA and arachidonic acid while saturated fatty acid levels were increased (Parthiban *et al* 1995, Ruiz Gutierrez *et al* 1993). Platelet membrane analysis showed higher stearic acid and dihomo-γ-linolenic acid levels in NIDDM patients but arachidonic acid, an important factor in platelet aggregation, was normal (Rodier 1995). In a more detailed study, Baldini *et all* (1989) analysed the fatty acid content of erythrocyte membrane PS, SPG and PC and found relatively more saturated fatty acid in those from NIDDM patients. Similar results for PS and PC have been reported by Perssom *et al* (1996) who also found more C22:5 (n-3) in these fractions. It has been suggested that insulin resistance is associated with reduced skeletal muscle membrane PUFA (Borkman *et al* 1993).

Treatment of erythrocytes and erythrocyte membranes with *in vitro* physiological concentrations of insulin increases membrane fluidity (Dutta-Roy *et al* 1985, Bryszewska & Leyko 1983, Juhan Vague *et al* 1986). At supraphysiological insulin doses, this effect is reversed. These effects are independent of plasma glucose (Dutta-Roy *et al* 1985).

As with the rat models, the degree of metabolic control affects membrane fluidity in diabetics. Erythrocytes membrane from IDDM patients with poor metabolic control were less fluid; this was most marked in ketoacidosis, suggesting that hyperglycaemia and ketone bodies are key factors (Candiloros *et al* 1995). Anisotropy values correlate positively with the amount of membrane protein glycation, lipid peroxidation and membrane cholesterol content (Watala & Winocour 1992, Winocour *et al* 1990 1992a 1992b). Incubation of platelets with glucose increases protein glycation and reduces membrane fluidity. Acetylation of proteins with aspirin or acetyl chloride has a similar effect. Therefore, it is not the glucose moieties which change the membrane fluidity but the attachment of a molecule to a glycation site (Winocour *et al* 1992c). Incubation of erythrocyte with glucose reduced membrane fluidity according to Bryzewska & Szosland (1988) but Birlouez-Aragon *et al* (1990) found normal values even though glycation was increased in the diabetics patients.

Membrane fluidity also affects insulin binding and receptor number. Supplementation of Y79 retinoblastoma cells with DHA increases membrane fluidity, insulin binding and insulin receptor number (Yorel *et al* 1989). The opposite is also true; increasing membrane SFA of 3T3-L1 adipose cells caused a reduction in insulin binding by decreasing both receptor number and binding affinity (Grunfeld *et al* 198). Diets high in n-3 fatty acids fed to NIDDM patients improved *in vivo* insulin sensitivity (Popp Snijders *et al* 1987).

1.11.4 Blood pressure

1.11.4a Animal Models Of Hypertension

A variety of rat stains have been used to study the effects of hypertension on cell membrane structure and function. These include the genetically hypertensive rats, the SHR strain and its stroke-prone sub-strain, the SHRSP rat. Other stains which have been used are the Dahl and Sabra hypertensive (SBH) rats, both of which develop salt-

dependant hypertension, and the Lyon hypertensive strain. The results have varied between investigator, model and tissue.

Using the fluorescent probe DPH, Montenay-Garestier *et al* (1981) was the first to observe that the erythrocyte membranes of young SHR were less fluid than those of control WKY rats. These results have since been confirmed for erythrocytes (Orlov *et al* 1982) hepatocytes, synaptosomes and cardiomyocytes (Devynck *et al* 1982). Others have only been able to show erythrocyte and platelet membrane fluidity to be reduced in male SHR rats compared to WKY. Salt loading increased membrane microviscosity in platelets but had no effect on erythrocytes. No difference was observed between the microviscosities of vesicles produced from lipids extracted from erythrocyte ghosts from SHR and WKY rats (Aragon-Birlouez *et al* 1984, 1982).

Electron spin resonance techniques using 5'-nitroxide stearate as a probe have been used to investigate the membrane microviscosity of erythrocytes and cultured VSMC from SHR compared to WKY rats. Fluidity was reduced in both cell types from SHR. This reduction was enhanced when the Ca^{2+} ionophore A23187 was used to load the cell with calcium (Tsuda *et al* 1992a). Similar alterations in membrane microviscosity have been observed in VSMC anderythrocytes from SHRSP rats compared to WKY (Dominiczak *et al* 1991, McLaren *et al* 1993). However, when VSMC cells were incubated with calcium, the cell membranes from the SHRSP rats became more fluid, becoming more like the control cells (Dominiczak *et al* 1991). McLaren *et al* (1993) showed that membrane microviscosity did not correlate with blood pressure either in the F_2 population of an SHRSP * WKY cross or in two subgroups with either a WKY or an SHRSP male progenitor. The same was found to be true for membrane microviscosity of an F_2 population of SHR * WKY (Norman & Achall 1993).

Conversely, others have observed membrane fluidity to be increased in small intestine and jejunal brush border cells (Lau *et al* 1986 Vazquez *et al* 1996), microsomes from VSMC and endothelial cells (Jaiswal *et al* l 1983) and erythrocytes and synaptosomes (Yamori *et al* 1982). It is difficult to explain these disagreements except perhaps on technical grounds. Jaiswal *et al* (1983) used 1μM DPH with a microsomal suspension containing 100μg protein/ml which may itself have affected microviscosity. Dominiczak *et al* (1991) reported that a probe to phospholipid ratio of 1:200 did not perturb the membrane microviscosity but higher probe concentrations did. This ratio was achieved by incubating

5nmol/l of probe with membrane at a protein concentration of 1 mg/ml. The same may also be true for the report by Yamori *et al* (1982), where DPH was used as a probe at a concentration of 20μmol/l.

Platelet and erythrocyte membrane microviscosity was increased in SBH rats compared to their specific control. Salt loading increased platelet microviscosity but not erythrocyte membrane microviscosity, suggesting that the composition of these two membranes may be controlled by different mechanisms (Aragon-Birlouez *et al* 1984 1982). In contradiction to this, Kunes *et al* (1994) showed SBH rats to have lower membrane microviscosity than control but only after salt loading when hypertension was present. Erythrocyte membranes from Dahl salt-sensitive rats are more fluid than control prior to salt loading (Kunes *et al* 1994). The reduction in membrane microviscosity in SBH rats may be in response to increased blood pressure whereas, in Dahl rats, the membrane abnormality appears to be blood pressure-independent. In all the rat strains studied here, membrane microviscosity was negatively correlated with blood pressure (Kunes *et al* 1994). Others have observed that the membrane microviscosity is reduced when platelets from SBH rats are suspended in Na^+ free medium, a difference independent of dietary salt intake. The authors postulate that this may be an inherited effect which may contribute to the susceptibility of these animals to hypertension caused by salt loading (Le Quan Sang *et al* 1994a). An earlier study failed to show any difference in the membrane microviscosity between the platelets and erythrocytes from SBH and Sabra hypertension resistant SBR rats (Le Quan Sang *et al* 1991).

Lyon hypertensive rats have lower core membrane microviscosity than their control strain which correlated negatively with blood pressure and cystolic free Ca^{2+}. The microviscosity measurements obtained with TMA-DPH, a measure of fluidity at the outer regions of the membrane, was correlated positively with cytosolic free Ca^{2+} (Le Quan *et al* 1994b), suggesting that the core and outer regions of the membrane are affected in opposite ways by Ca^{2+} concentrations.

Differences in membrane microviscosity appear to be limited to genetically hypertensive rat strains. Membrane microviscosity is unaffected by DOCA-salt-induced hypertension or two-kidney, one clip Gold-Blatt renal hypertension (Orlov *et al* 1982). Cardiac sarcolemmal plasma membrane cholesterol and phospholipid composition were not altered in portal hypertension compared to control (Ma *et al* 1995).

A variety of alterations have been observed in the membrane composition of hypertensive and control animals. Studies in SHR and WKY rats have shown that linoleic acid is reduced in the total lipid extract from SHR mesenteric arterial beds. The SHR phospholipid fraction contained more palmitic, oleic and linoleic acid (Mtabaji *et al* 1988). The ratio of arachidonic acid to other fatty acids in VSMC membranes was higher in SHRSP than WKY. The membranes of the SHRSP cells were significantly less fluid (Dominiczak *et al* 1993). WKY renal membrane lipid has increased levels of PE compared to SHRSP . The levels of PC are also lower and the PC/SPG ratio is decreased in SHRSP compared to WKY. There was a decrease in the amount of arachidonic acid in the renal medulla of older SHRSP (Okamoto *et al* 1989). When SHRSP were compared with their stroke-resistant controls, SHRSR, VSMC cholesterol levels and cholesterol biosynthesis were lower in SHRSP and SHRSR compared to WKY. Membrane phospholipid content and phospholipid biosynthesis were increased in SHRSP compared to WKY but the cholesterol/phospholipid ratio was reduced in SHRSP. The levels of arachidonic, linoleic EPA were decreased in SHRSP compared to WKY. Incorporation of linoleic acid into the membrane was reduced in both SHRSP and stroke-resistant (SHR) SHRSR compared to WKY (Nara *et al* 1986).

In young SHR (4 weeks), the amount of DAG produced by stimulated aorta was significantly greater than in the WKY rats. By twenty weeks of age there was no longer a difference. The fatty acid composition of the DAG was similar for both groups as was the PE, PC and triglycerides (TRIG) composition of the membranes. This may be important in regulation of blood flow. The cholesterol content of SHR aortas was higher than controls (Okumura *et al* 1990).

The activity of many ion transport systems is altered in hypertension. Many of these investigations have been carried out in VSMC as changes in ion-transport are thought to be involved in the vascular hypertrophy and hyperplasia seen in hypertension. Early reports showed the VSMC from SHR to be more permeable to Na^+ and K^+ than cells from WKY rats (Jones 1973, Friedman & Friedman 1976, Friedman 1979). Friedman (1979) only found the active transport of ions to be increased in SHR rats over sixteen weeks of age, suggesting that this may be an effect of blood pressure in this strain. A generalised effect of blood pressure was excluded by inducing hypertension in WKY rats by renal artery constriction. No changes in ion transport were observed. Similar changes in ion

transport have been observed in erythrocytes from SHR compared to WKY (Van de Ven & Bohr 1983). The novel sodium-sensitive fluorescent dye, sodium-binding benzofluran-isophthalate, has been used to study intracellular cytosolic sodium ($[Na^+]_i$). Resting intracellular sodium was lower in SHR VSMC than those from WKY rats (Tepel *et al* 1995). As Na^+ influx is greater in SHR, $[Na^+]_i$ should be increased. This is not the case suggesting that Na^+ efflux must also be increased. This has also been found to be true in DOCA-salt induced hypertension where permeability of cells to Na^+ and Na^+ extrusion rate are both increased in both tail artery and aortic VSMC. Neither of there cell types showed an increase in the $[Na^+]_i$ (Friedman *et al* 1975, Jones & Hart 1975). Changes in the Na^+ transport in rat tail arteries have been observed ten days after the administration of DOCA-salt prior to the blood pressure rise (Friedman *et al* 1986), suggesting a possible genetic effect.

The Na^+-H^+ exchanger in SHR VSMC is more active under tissue culture conditions than in control cells. This suggests that this is a primary change and is not due to increased blood pressure, hypertrophy or hyperplasia. The increase in exchanger activity is concurrent with the increased growth rate in the VSMC from SHR compared to WKY. In later passages, when the two growth rates become similar, the activity of the exchanger also becomes similar. Intracellular pH is also greater and the recovery from acidification faster in SHR- than WKY-derived cells (Berk *et al* 1989). The exchanger is involved in the growth of vascular smooth muscle cells. It controls intracellular pH, which is elevated by growth factor stimulation. It is active in the early stages of the mitotic cell cycle and is required for the cells to enter the S phase of the cycle (Bobik *et al* 1991). Also as the exchanger is responsible for maintaining cell volume, its activity is increased when cells are actively dividing and during hyperplasia; in both these situations, the cell size is increased. This goes some way to explaining why the exchanger activity is increased in VSMC. It is more difficult to explain why the activity of the exchanger is also increased in skeletal muscle (Syme *et al* 1990), neutrophils (Feig *et al* 1986), lymphocytes (Feig *et al* 1987) and erythrocytes (Orlov *et al* 1989). There is probably a genetic basis to this abnormality but whether this is in the exchanger gene or in a factor which controls the exchangers activity, such as the cell membrane, is not clear. The increase in activity is not due to increased gene transcription as the messenger RNA levels in the cells are similar to control (La Pointe *et al* 1995). It is possible that the increased activity is caused by the lipid environment surrounding the cell.

The blood lymphocyte count was lower and the intralymphocyte Na^+ content was increased in SHRSP, DOCA-salt induced hypertensive rats and in the two kidney one clip hypertensive rat compared to their controls but in none was the K^+ content altered. Net sodium and potassium fluxes were increased in the SHRSP rats but only net sodium flux was increased in the DOCA-salt induced hypertensive rats (Furspan & Bohr 1985). As these studies were carried out a 4°C, these were passive ion fluxes and not active transport and the increased fluxes may be caused by an alteration in the cell membrane. However, as the membrane structure changes with temperature, it is difficult to estimate what would occur at physiological temperature. Later studies showed that the abnormality in K^+ flux correlated positively with blood pressure in an F_2 cross of SHR and WKY rats (Furspan *et al* 1987).

VSMC intracellular Mg^{2+} concentration is reduced in cells from SHR compared to WKY rats (Kisters *et al* 1996). This may be due to a disturbance in the membrane magnesium ion transport. Erythrocyte Mg^{2+} levels were significantly lower in hypertensive subjects, the membrane microviscosity was also increased in these patients (Paolisso *et al* 1987).

Intracellular Ca^{2+} content is a controlling factor in the VSMC contractile process. An increase leads to vasoconstriction. Total calcium concentration is higher in SHR VSMC compared to control and also in DOCA-salt induced hypertension. Using patch clamp technology, it has been observed that the long-lasting Ca^{2+} current is greater in cells from SHR (Rush & Hermsmeyer 1988). The authors suggest that this is an abnormality of the Ca^{2+} channel but it could just as easily be caused by abnormalities in the cell membrane. In erythrocytes, increasing intracellular Ca^{2+} concentration leads to a reduction in membrane fluidity (Weed *et al* 1969). The work of Tsuda *et al* (1992b) confirms this. This theory is further advanced by the knowledge that hepatocytes membranes have an increased microviscosity during endotoxic shock, but that this effect is prevented by calcium channel blockers (Salgia *et al* 1993). Thus cellular calcium homeostasis is important in the maintenance of membrane fluidity and membrane transport functions.

The activity of the erythrocyte Ca^{2+}-Mg^{2+}-ATPase has been investigated in an F_2 cross of SHR and WKY rats. Blood pressure was found not to correlate with either enzyme activity or cell membrane microviscosity, suggesting that neither are causally related to blood pressure. The authors assume that similar results would have been observed in

VSMC and therefore that cell membrane abnormalities do not play a role in hypertension (Norman & Achall 1993).

During salt-induced hypertension, there is an increase in intracellular free calcium but no change in calcium influx rates and a reduction in intracellular sodium concentration compared to the salt resistant rats. High salt intake decreases membrane microviscosity in SBH rats and this is inversely correlated with blood pressure. Intracellular free calcium concentration correlated positively with diastolic blood pressure and negatively with intracellular sodium concentrations (Pernollet *et al* 1994). When there is no external sodium present, rat and human platelet membranes become less fluid; this as not the case in erythrocyte ghost membranes (Le Quan Sang *et al* 1991).

Salt-loaded hypertensive rats suffer from renal failure leading to proteinururia. Administration of PUFA prevented renal damage while reducing the increase in blood pressure. Other oils containing shorter chain fatty acids did not have a similar effect, suggesting that this is a selective effect of the long chain unsaturated fatty acids found in fish oil (Hobbs *et al* 1996). The authors suggest that this is an effect of incorporation of long chain fatty acid into renal cell membranes. However, no attempt was made to quantify this.

Borderline hypertensive rats, (cross of WKY * SHR) stressed by isolation did not develop prolonged hypertension when fed γ-linolenic acid while those not fed the fatty acid developed non-neurogenic hypertension. The response to noradenaline in the γ-linolenic acid-fed rats was not affected whereas the pressor response to angiotensin II was more prolonged but less severe. During stress, Δ6 desaturase activity was reduced by the presence of increased adrenaline and corticosterone. Thus γ-linolenic acid may reduce the peripheral or the sympathetic response to stress (Mills *et al* 1985). It would have been interesting to see the effect of removing the γ-linolenic acid diet. Would the stress response still be as great? A low dose of γ-linolenic acid also reduced the increment in blood pressure sustained during stress isolation of Sprague Dawley rats but did not prevent it. However, a higher dose did prevent the increase in blood pressure (Mills & Ward 1984). There were no changes in heart rate, suggesting that the fatty acid did not act through the central nervous system. In WKY rats, Mills and Ward (1986a) showed that γ-linolenic acid administration reduced blood pressure in the unstressed state. The administration of arachidonic acid attenuated the increase in blood pressure only in the

first two weeks of stress-induced hypertension. Blood pressure was not affected by the administration of linoleic acid or α-linolenic acid, the n-3 fatty acid. The pressor response to noradrenaline was unaffected. Olive oil affected the response to AII stimulation in a similar way to γ-linolenic acid. Therefore, the blood pressure lowering and AII effects of γ-linolenic acid are probably by different mechanisms (Mills & Ward 1986a). Δ6 Desaturase is known to be inhibited by stress hormones. In these animals, therefore, the administration of the γ-linolenic acid may compensate for this. Mills and Ward (1986b) continued their study of stress-induced hypertension in WKY rats by testing the effectiveness of EPA which is also desaturated and should compensate for desaturase deficiency. However, EPA attenuated but did not inhibit the increase in blood pressure induced by stress. Since the amount of EPA used here was equimolar to the amount of γ-linolenic acid able to inhibit the increase in blood pressure completely, n-3 fatty acids do not have the same therapeutic potential as n-6. EPA also appears to have some tachicardic action, increasing the heart rate both during and before stress induction; the response to noradrenaline was increased in the EPA-treated rats (Mills & Ward 1986b).

The attenuation of experimental hypertension by linoleic acid administration appears to be strain-dependant. Dahl salt sensitive rats and 2 kidney 1 clip rats on high salt diets are both renin-independent forms of hypertension while the 2 kidney, 1 clip, low salt diet rat shows a renin-dependant form of hypertension. Linoleic acid administered from 4 weeks of age is only effective in attenuating hypertension in renin-dependant hypertension (Reddy & Kotchen 1996). This may be due to linoleic acid inhibiting renin and the acute *in vivo* pressor response to renin (Kotchen *et al* 1975). High linoleic acid diet is thought to inhibit the secretion of renin (Codde *et al* 1984). Inhibition of cyclooxygenase reduced the antihypertensive effect of linoleic acid (Ten Hoor & Vandengraft 1978). Thus, prostaglandin may be involved in the hypotensive effect of this fatty acid.

1.11.4b Human

Platelet membranes are less fluid in patients with untreated essential hypertension than in normotensive subjects. The cholesterol content of their platelet membranes is similar to controls but they contain less linoleic acid (Naftilan *et al* 1986). Others have suggested that platelets from hypertensive patients have a more fluid outer region than those from control patients. The anisotropy values for TMA-DPH correlated negatively with cystolic free Ca^{2+} and blood pressure. DPH anisotropy did not correlated with blood pressure (Le Quan Sang 1991).

Electron spin resonance studies have shown erythrocytes from essential hypertensive patients to have a reduced membrane fluidity and this reduction was more marked when the calcium ionophore A23187 was used to load the cell with calcium (Tsuda *et al* 1992a). In the same study, patients with secondary hypertension, (primary hyperaldosteronism, pheochromocytoma and renovascular hypertension) showed no abnormality in cell membrane fluidity or composition. The platelet membranes of patients with primary (essential) hypertension have an increased C/PL ratio which would make the membrane more rigid. This was not the case in the erythrocyte membranes (Benjamin *et al* 1990).

Platelet intracellular pH is thought to be increased in essential hypertension. This and membrane microviscosity were measured in human subjects with essential hypertension. Cytosolic pH was inversely related to the membrane microviscosity as measured using the fluorescent probe TMA-DPH. Here the cytosolic pH was not affected by specific inhibitors of Na^+-H^+-exchangers (Astarie *et al* 1992). This would suggest that membrane fluidity is increased in hypertension. The sialic acid content of glycolipids is higher in essential hypertensive patients than renal hypertensive patients or normotensive controls. This could affect Ca^{2+} binding which is also increased in hypertension (Reznikova *et al* 1984).

Although Carr *et al* (1995) could not confirm abnormalities in erythrocyte in patients with essential hypertension using either TMA-DPH or DPH, they claim to have identified a subgroup with a family history for hypertension where TMA-DPH anisotropy was increased. Na^+-Li^+-countertransport activity was increased and its Km for sodium was lower in patients with a family history of hypertension than those without. The authors suggest that the increase membrane microviscosity in these patients may affect Na^+-Li^+-countertransport (Carr *et al* 1995). Although the studies show variable effects of hypertension on membrane microviscosity the consensus of opinion from rat and human studies would suggest that membrane fluidity is reduced and microviscosity increased in genetic hypertension. A variety of serum fatty acid concentrations correlate with blood pressure. Cholesterol ester (CE) -bound palmitoleic acid increases with increasing systolic blood pressure while stearic acid increases with decreasing diastolic blood pressure. CE-bound dihomo-γ-linolenic acid increases with increased diastolic blood pressure as does phospholipid-bound eicosatrienoic acid (Simon *et al* 1996). The inverse association of stearic acid concentration with blood pressure may explain why increased stearic acid is

not thought to be an increased cardiovascular risk factor. Studies of fatty acid composition of erythrocyte membranes in control and untreated hypertensive patients revealed that the linoleic acid content was lower in the hypertensive patients and the arachidonic acid and oleic acid content was higher (Ollerenshaw *et al* 1987). Platelet membrane PC was higher in hypertensive patients compared to control although membrane fluidity was not different between the two groups (Caimi *et al* 1996).

There is considerable evidence that cell membrane function is altered in essential hypertension. Platelet Ca^{2+} concentration increases with increasing blood pressure and increasing plasma triglycerides (Le Quan *et al* 1995) but only in patients with a family history of hypertension. This report suggested a link between intracellular calcium concentration and blood pressure but the activity of the platelet Ca^{2+} pump was not investigated. Sodium transport is affected by hypertension. Platelet Na^+-H^+-exchange is increased in hypertensive patients compared to controls (Livne *et al* 1987). Whether this is due to an increase in the number to exchangers or to alterations in the kinetics of exchange is not clear. Erythrocyte Na^+-K^+-countertransport activity and Na^+K^+-cotransport activity is negatively related to membrane cholesterol, phospholipid and free fatty acid content in normal subjects (Lijnen *et al* 1994). Na^+-K^+-ATPase activity is also inversely related to membrane cholesterol and phospholipid content. Thus elevated erythrocyte membrane lipids in normal men are associated with lower cation transport rate (Lijnen *et al* 1994). Digitalis-like factor, an endogenous Na-K-ATPase inhibitor, is increased in essential hypertension and its plasma concentration also correlates with reduced membrane fluidity in these patients (Tsuda *et al* 1992a). Na^+-H^+-countertransport activity is greater in erythrocytes from essential hypertensive patients than from normotensive controls (Canessa *et al* 1980). Na^+-Li^+-exchange activity has also been studied in a group of white hypertensive patients using atomic absorption spectrophotometry. It is higher in the patients than in controls. There was also more PS and less PE in the hypertensive patient's membranes than the control. The authors hypothesise that the changes in lipid to protein interaction in the membrane are responsible for weaker Na^+ binding and faster rates of ion exchange in the membrane (Chi *et al* 1996).

Studies to evaluate how erythrocytes respond to the increased shear flow observed in hypertension have shown that the fluidity of the outer region of the membrane is negatively correlated with shear rate, shear flow and blood velocity. The inner regions of the membrane were not affected by this. This suggests that either shear flow affects

membrane fluidity or that the membrane changes to cope with the increased flow (Le Quan Sang *et al* 1993a & b).

N-3 fatty acids reduce cardiovascular risk factors and atheroschlerotic plaque formation. Diets high in n-3 fatty acids reduced blood pressure (5 mmHg SBP, 4 mmHg DBP) which was further improved by dietary salt restriction. This is thought to be due to a change from the production of vasoconstricting to vasodilating eicosanoids produced from n-3 fatty acids (Semplicini& Valle 1994). In contradiction to this, a 4 month diet high in EPA and DHA (n-3 fatty acids) had no effect on the blood pressure or heart rate in mild essential hypertension (DBP 105 mmHg) although the amount of erythrocyte membrane n-3 fatty acids was increased (Russo *et al* 1995).

Diets high in essential fatty acids decrease plasma TRIGs, increase high density lipoprotein (HDL) cholesterol, decrease blood pressure, increase vasodilation, decrease fibrinogen, decrease platelet aggregation and decrease cardiac arrhythmias (Horrobin 1995). On the other hand, high membrane C20 and C22 fatty acid levels may predispose to coronary and peripheral vascular disease; their levels are increased in patients with diabetes and hypertension (Horrobin 1995). There is also an opinion that an alteration in the composition of arterial cell membranes precedes the onset of atherosclerosis. At the branch points in human and swine arteries, there is an increase in the membrane content of SPG and a decrease in the other phospholipids compared to the non-branching areas of the arteries. The branch points are the areas most prone to the formation of atherscherotic plaque formation (Kummerow *et al* 1994). As SPG has a higher transition temperature, it is thought that this will effect an increase the rigidity of cell membranes. It is also thought that these alterations will change the surface charge on the cell which may lead to calcium binding to the cell.

It is clear that, while there is much evidence that changes in the physical properties and chemical composition of the membranes of many cell types is altered in a variety of diseases, the precise changes and their direct causes remain obscure. While much of the confusion has been due to the difficulty in comparing very different experimental models and designs or tissues, much has been contributed by the differences in analytical techniques which are continuously improving.

1.12 Techniques of Lipid Analysis.

Most lipid analysis techniques follow the same protocol; the lipids are extracted, purified or separated and then derivatised for analysis. Before discussion of these techniques, it is necessary to consider one of the major problems of lipid analysis, that of lipid oxidation.

1.13 Prevention of Oxidation

Lipids, in particular unsaturated fatty acids, are prone to oxidation. The oxidation reaction has a low activation energy and is therefore difficult to prevent. It can be minimised by keeping all samples in a dark, metal-free environment at -20°C and by degassing all reagents and flushing all sample containers with nitrogen (Christie 1989).

Initiation of oxidation occurs when lipids are attacked by any species which is reactive enough to remove a hydrogen atom (equation 1). The carbon radical, formed by the removal of hydrogen, is stabilised to form a conjugated diene, which reacts with oxygen to form a hydroperoxide radical (equation 2). Hydroperoxide radicals can remove hydrogen from other lipid molecules leading to a chain reaction (equation 3). Reduced iron complexes react with hydroperoxides to produce alkoxy radicals (equations 4a & b) (Halliwell & Gutteridge 1984).

$$\text{Lipid-H} + \text{OH} \rightarrow \text{Lipid}\bullet + H_2O \quad (1)$$

$$\text{Lipid}\bullet + O_2 \rightarrow \text{Lipid-}O_2\bullet \quad (2)$$

$$\text{Lipid-}O_2\bullet + \text{Lipid-H} \rightarrow \text{Lipid-}O_2\text{H} + \text{Lipid}\bullet \quad (3)$$

$$\text{Lipids-}\ O_2\text{H} + Fe^{2+}\text{complex} \rightarrow \text{Lipid-O}\bullet + Fe^{3+}\text{complex} + \text{OH} \quad (4a)$$

$$\text{Lipids-}\ O_2\text{H} + Fe^{3+}\text{complex} \rightarrow \text{Lipid-}O_2\bullet + Fe^{2+}\text{complex} + \text{H} \quad (4b)$$

Although it is necessary to keep lipid samples in a metal free environment (Christie 1989), transition metals do not appear to initiate oxidation but they do cause the breakdown of hydroperoxides which are present in most samples. How the hydroperoxides are formed is not clear but the mechanism is thought to involve oxygen in the activated singlet state (Halliwell & Gutteridge 1984). As transition metals catalyse oxidation, so do compounds which contain transition metals such as haem or haem products, haematin and photophorin (Tappel 1961, Dodge & Phillips 1966). These compounds are non-specific catalysts of peroxide decomposition. The catalytic activities of myoglobin, haemoglobin, haemin and cytochromes are all equal (Tappel 1955). Also, haemoglobin is capable of oxidising lipids (Yoshida *et al* 1994).

Chain breaking antioxidants prevent initiation and propagation of oxidation. They are aromatic and phenol amino compounds which form loose molecular complexes with free radicals. Butylated hydroxytoluene (BHT) acts as an antioxidant because the phenol group is able to donate a proton to neutralise free radicals (Pryor 1994). In this form, the radicals are too unreactive to initiate or maintain a chain reaction. BHT is the most commonly used chain breaking antioxidant but its use is not without its difficulties. At high temperatures, BHT reacts with oxygen, leading to the formation of free radicals (Frenkel 1980). It thus becomes a pro-oxidant (Christie 1989). Although BHT is volatile and can be removed from samples during evaporation of solvents under nitrogen (Christie 1989), some analysts have observed contaminating peaks arising from BHT during gas chromatography (Moffat *et al* 1991, Eder *et al* 1992b, Zang *et al* 1996). Others report that BHT is itself esterified during methylation reactions with boron trifluoride methanol (BF_3MeOH) (Heckers *et al* 1987) and methanolic (*m*-trifluoromethylphenyl) trimethyl ammonium hydroxide (Zang *et al* 1996), leading to contamination of gas chromatographs. The esterification of BHT also causes it to loses its antioxidant ability as methylation of the hydroxyl group in BHT prevents the removal of a proton (Pryor 1994). The compounds into which BHT is converted during BF_3MeOH methylation have been identified by mass spectrometry as 2,6-di-*tert*-butyl-4-methoxyphenol, 2,6-di-*tert*-butyl methylphenol and 2,6-di-*tert*-butyl-4-methoxy-5-hydroxyphenol (Moffat *et al* 1991).

As transition metals, in particular iron, have been implicated in the generation of free radicals (Halliwell & Gutteridge 1984, Schaich 1992), the use of chelating agents such as citric, phosphoric and ascorbic acids in lipid analysis solutions has been recommended. It is also possible to use synthetic chelating agents such as diethylenetriamine-pentaacetic acid (Zang *et al* 1996). Chelating agents are preventative antioxidants and a synergistic effect of adding these together with chain breaking antioxidants to samples has been claimed (Frenkel 1980).

1.14 Extraction of Lipids

Extraction of lipids from tissues is the first stage in lipid analysis and some basic considerations apply. The tissue must be removed immediately post mortem and either processed or frozen in liquid nitrogen. This prevents enzymes such as lipases, which remain active after death, from altering the composition of the membrane. They catalyse the conversion of diacylphospholipids to lysophospholipids and free fatty acids and the conversion of diacylglycerols to phosphatidic acid. Therefore, the presence of large

quantities of lysophospholipids and phosphatidic acid are indicative of membrane degradation by these enzymes (Hamilton *et al* 1992). The use of hot acetic acid may prevent enzymatic degradation of lipids (Phillips & Privett 1979). This method was developed for plant tissue but was reported as being useful for mammalian systems too. However, its use is rarely reported, probably due to the hazards of working with hot acid and the risk of lipid hydrolysis.

For lipid extraction, organic solvents are used to disrupt the interactions between membrane lipids and proteins. In particular, methanol is required to break the hydrogen bonds between protein and lipid. There are three commonly used lipid extraction techniques, the methods developed by Folch *et al* (1957) and Bligh and Dyer (1959) and that developed by Ways and Hanahan (1964) which is a modification of the Folch *et al* (1957) method. Folch *et al* (1951) extracted lipids by homogenising the tissues in chloroform/methanol (2:1 v/v) followed by a water wash. This led to a loss of 1% of the lipid. In a modified procedure, salts were added ($CaCl_2$, $MgCl_2$, NaCl or KCl) to the wash and its volume reduced. Some of these salts are extracted from the tissue along with the lipid. The authors state that acidic lipids are extracted as salts of Na, K, Ca or Mg, and are present in the organic phase in their undissociated form and in the aqueous phase in the dissociated form. The addition of extra salt to the system will cause more of the lipid to move into the organic phase, thus increasing lipid recovery. The losses incurred using this technique were small (0.3% and 0.6% for brain white and grey matter) (Folch *et al* 1957).

In Bligh and Dyer's (1959) modification of this method, the composition and volume of the chloroform and methanol mixture were adjusted according to the water content of the tissues. For efficient extraction, the solvents and the sample water must form a monophasic solution after homogenisation. Extra chloroform was then added to produce a biphasic solution allowing for removal of non-lipids. This method led to a loss of 1% of the total lipid, which according to the authors, compares favourably with the Floch *et al* (1957) method (Bligh & Dyer 1959).

The third commonly used lipid extraction procedure was developed by Ways and Hanahan (1964) specifically for use with erythrocytes. Membrane lipids were extracted with methanol by mixing the cells with 5 times their volume of methanol at room temperature. An equal volume of chloroform was then added to the mixture and the mixture filtered. Multiple extractions of the residue increased recovery. Potassium chloride was then added

to the combined extract which was allowed separate into an organic and aqueous phase in a separating funnel (Ways & Hanahan 1964). Although others report this method as being useful, it is cumbersome. Moreover, many of the steps in the procedure require the samples to stand at room temperature for prolonged periods which will lead to oxidative loss of lipids.

Most lipid analysts choose one of the above techniques, probably because the solvents can be purchased relatively inexpensively in pure form, but frequently modify them. For example, when using the Floch *et al* (1957) extraction procedure, the recovery of lipid may be improved if chloroform/methanol/water (3:48:47 v/v/v) is used to wash the extract (Hamilton *et al* 1992). Similarly, keeping the pH of the solvents in the Bligh and Dyer procedure low (pH < 2.5) makes it possible to extract the intermediates of glycerol synthesis, acyl and alkyl dihydroxyacetone phosphate (Hajra 1974). In another modification to improve recovery of glycosphingolipids, the membrane lipids were extracted and the residue peracetylated with formamide, pyridine and acetic anhydride. The acylated products were then extracted with chloroform (Millar-Podraza *et al* 1993). The complete extraction of polyphosphoinositides has necessitated many modifications including the addition of calcium chloride (Hausser & Eichberg 1973) and extraction with chloroform and methanol followed by chloroform/methanol/hydrochloric acid (Wells & Dittmer 1965).

Although chloroform and methanol are most frequently used, there are alternative solvents such as isopropanol. Rose and Oklander (1965) observed that, on extraction of lipids from whole erythrocytes with chloroform and methanol, the cholesterol content was lower than expected and that extracts were heavily contaminated with haem. The amount of cholesterol extracted was inversely proportional to the concentration of chloroform in the solvent and the haem contamination increased with increasing polarity of the alcohol used. The authors report effective extraction of all lipids with little haem contamination using chloroform/isopropanol (7:11 v/v) (Rose & Oklander 1965). However, the technique requires the extract to stand for long periods of time at room temperature so that extra care should be taken to prevent lipid oxidation. Others have used isopropanol in conjunction with hexane for lipid extraction but this should not be used when quantitative extraction of gangliosides is required (Hara & Radin 1978). Isopropanol has been employed by other groups for the extraction of lipids from very small samples (Wang *et al* 1994). Samples were sonicated in isopropanol/hexane/water (3:1:3 v/v/v); the water

present is that derived from the sample. After sonication, 0.9% NaCl in hexane was added to make the ratio of solvents isopropanol/hexane/water (3:3:6 v/v/v) (Wang *et al* 1994). The use of isopropanol alone for lipid extraction has also been recommended for whole erythrocytes and was as effective as the other systems mentioned above (Peuchant *et al* 1989). This latter method will be discussed further later with reference to esterification of lipids. The risk of oxidation caused by the long standing times at room temperature required by this technique can be overcome by the addition of BHT (Eder *et al* 1992b). Butanol can extract most lipids at neutral pH (Hajra 1974). Lipids from brain white matter can also be extracted with tetrahydrofluran. The resulting extract contains only trace amounts of protein (Diaz *et al* 1992).

Most lipid extracts produced using these methods do not require further purification but some, in particular extracts of bone and brain, do. Cellulose columns have been used for the removal of non-lipid contaminants but losses of PI are high (Garcia *et al* 1956). Sephadex resin has also been used (Wells & Dittmer 1963) but reproducibility is poor as a very slow flow rate through the column is required for the complete removal of non-lipids. Sephadex beads (G-25) may be used instead of the resin. The amount of water in the stationary phase can be increased to allow the flow rate to be increased (Wurthier 1966). More complex clean up procedures have used C18 reverse phase columns to remove non-lipid contaminants from PC, cerebrosides, sulphatides and gangliosides. This system was developed to study the synthesis of lipids and its use for purifying membrane lipid extracts is probably limited (Figlewicz *et al* 1985).

1.15 Separation of Lipids Groups

Before discussion of the methods used for the separation of lipid groups, it is first necessary to explain the principles behind the chromatographic procedures used. There are three main types of liquid chromatography commonly used, adsorption, partition and ion exchange chromatography.

1.15.1 Absorption Chromatography.

Adsorption chromatography utilises an adsorbent which has an affinity for the lipids. Lipids with polar functional groups bind to the adsorbent by dipole-dipole interactions and are retained on the adsorbent. Non-polar lipids do not bind and will be eluted. Silica is the most commonly used adsorbent for lipid separation. Various factors affect its ability to do so. Silanol groups (Si-OH) on the surface have a strong affinity for water. Thus,

the water content of solvents must be carefully controlled. The pore size, volume and the surface area of the silica column or plate affect separation. Metal ions also affect the chromatography as do basic solvents which dissolve silica (Hamilton *et al* 1992). In general, the polarity of the mobile phase should be opposite to that of the adsorbent.

1.15.2 Partition Chromatography.

There are two forms of partition chromatography, normal phase and reverse phase. In normal phase chromatography systems, the stationary phase is more polar than the mobile phase. The opposite is true for reverse phase chromatography which is often used to separate the components of a single lipid class as a function of their fatty acid chain length. Octadecylsilyl silica (C18), which is synthesised by reaction of the silanol groups with organochlorosilane, is most commonly used.

(Surface Si) - O - Si - C18

This is often referred to as bonded phase chromatography because the stationary phase has been chemically bonded to the surface of the support material, in this case silica. Partition chromatography relies on the relative solubility of the solute in the mobile or stationary phase; a compound which is more soluble in the mobile phase will be eluted more rapidly. Paper chromatography is also partition chromatography.

1.15.3 Ion-Exchange Chromatography

Commonly used ion-exchange packings are diethylaminoethyl (DEAE)- and triethylaminoethyl (TEAE)- cellulose. Ion change chromatography involves the competitive substitution of ions on sites of opposite charge on the stationary phase. Thus only ionic forms of molecules can be separated. The retention times of lipids can be controlled by altering the solvent pH or by addition of counter ions.

1.15.4 Other Types of Chromatography

In argenation chromatography, silica gel is impregnated with silver nitrate. Polar complexes are formed between the double bonds in the lipids and the silver ions. *Cis* double bonds interact more strongly than *trans* and lipids can be separated as a function of the acyl chain length and number of double bonds.

In ion-pair chromatography, the stationary phase is non-polar and the mobile phase contains a large counter-ion such as tetrabutyl ammonium. This forms an ion- pair with ionic lipids, which will undergo normal chromatography. Exclusion chromatography uses

the pore structure of the stationary phase to control the elution of compounds. Small molecules can permeate the solid phase and are retained in the column while large molecules elute. A difference of 10% in molecular weight between the compounds is required for compound separation, making its use for lipid analysis limited (Sewell 1992).

A variety of chromatography methods is used to study lipids and fatty acids. These include thin layer chromatography (TLC), solid phase chromatography, high performance liquid chromatography (HPLC) and gas chromatography (GC). Some methods utilise a combination of these.

1.16 Thin Layer Chromatography.

TLC is adsorption chromatography. The adsorbent, usually silica or alumina, is coated on a thin layer on a support, usually glass, polyester or aluminium. Lipid samples are applied to a plate and the plate developed in ascending mode using the solvent of choice. Lipids have characteristic mobilities on the plates which are quantified as their Rf values.

There are many solvents systems for separating different lipid groups by TLC (see also chapter 3). In general, using one dimensional chromatography, it is only possible to separate the constituents of neutral or polar lipids. To separate both completely, it is normally necessary to use 2 dimensional TLC. For instance, phospholipids, free fatty acids, mono-, di- and triglycerides, cholesterol and cholesterol esters were separated using long silica plates and a double development system (Freeman & West 1966). Silica gel H has been used to separate PE, PC, PI, PS, sphingomyelin and neutral lipids (Vitiello & Zanetta 1978). Two dimensional TLC has been used to separate non-polar lipids, cerebrosides and sulphatides and PC, PI, PS, PE, LPC. PA, PGS and sphingomyelin (Rouser *et al* 1970). Most separations use silica plates but silicic acid TLC has also been used (Mangold & Malins 1960). Separate TLC systems have been developed for specific uses. For example, in argenation TLC, lipids are separated as a function of the number of double bonds present; lipids with 1 to 6 double bonds can be separated using this technique (Kennerly 1986). In high performance TLC (HPTLC), silica particle sizes are smaller (4.5-5 μm) and the layer is thinner than standard TLC. Less solvent and sample is required, resolution is better and nanogram quantities of neutral lipids can be separated. However, three different solvent systems are necessary, drying the plate between each development with hot air introducing a clear oxidative risk (Conte & Bishop 1988). One recent report showed that 8 major phospholipids can be separated on one dimensional

TLC without any pre-treatment of the plates. The fatty acid composition of the separated lipids was then analysed by HPLC (Miwa *et al* 1996).

Lipids can be identified on the plates in a variety of ways. Once identified, the lipids can either be quantitated on the plate or removed from the plate prior to quantitation. Lipid spots can be identified by charring the plates but this is destructive. Iodine vapours can be used but are toxic. Moreover, losses of polyunsaturated fatty acids may be caused by iodination of the double bonds (Nichaman *et al* 1963). The most commonly used detection systems are fluorescent dyes such as Rhodamine B or 6G which bind to the lipid groups. It is also possible to convert lipids into fluorophores, which allows their quantification on TLC or HPTLC plates using a spectrofluorimeter (Segura & Gotto 1974, Segura & Navarro 1980, Harrington *et al* 1980). Densitometery can also be useful (Bitman *et al* 1983, Macala *et al* 1983). Finally, they can be removed from the plate and quantitated by their fatty acid composition (Bitman *et al* 1984) but a major problem is to remove of the lipid spots quantitatively. This can be overcome by using polyester-backed plates which can be cut easily.

One of the major advances has been the development of TLC with flame ionisation detection (FID). Known as Iatroscan TLC/FID, it uses re-usable silica or silicic acid-coated quartz columns called chromarods. The rods are developed like a TLC plate and then passed through a stationary flame in an FID system. However, the results may be variable, depending on the sample size and the speed at which the rod is passed through the flame (Crane *et al* 1983). Reproducibility for neutral lipid separation was poor, due to variability between the rods, although this could be improved using an internal standard (Farnworth *et al* 1982). TLC/FID compares favourably with phosphorus assay as a means of analysing phospholipids but the results obtained by Kramer *et al* (1985) were higher than those from GC. Others found it compares well with GC (Rao *et al* 1985). Like TLC, the rods can be chemically treated. For example, silver nitrate impregnated rods have been used to study the degree of unsaturation of TRIG; this is argentation chromatography (Sebdio *et al* 1985). Copper (II) sulphate (Kaimal & Shantha 1984) and oxalic acid impregnation improved both resolution and reproducibility. Oxalic acid is a strong organic acid which alters the chromatographic properties of the silica (Banerjee *et al* 1985). Okumura *et al* (1988, 1990) used TLC/FID to study diacylgycerols and Peuchant *et al* (1989) to separate phospholipids. Lipids cannot be recovered from

TLC/FID. It is therefore possible to quantitate the individual lipids but not to determine their specific fatty acid composition.

1.17 Solid Phase Separation

Solid phase separation procedures use small, often disposable, columns. The most common is silica in the form of Sep-pak columns. These have been used to separate the neutral lipids from polar lipids (Bitman *et al* 1983, Juaneda & Rocquelin 1985). Neutral lipids are eluted with either hexane or chloroform and the polar lipids with methanol. Judicious manipulation of the composition of the eluting solvents allows for more demanding separations. Hamilton & Comia (1984) could separate neutral lipids and then separate PE and PC and their later report claimed separation of CE, TRIG, free fatty acids (FFA), partial glycerides (PGS) and cholesterol. PI + PE are eluted together followed by PC + LPC + sphingomyelin (Hamilton & Comia 1988). This latter study has, however, been difficult to reproduce (Christie 1992) (see chapter 3). Another modification allows the separation of cholesterol from polar lipids (Wang *et al* 1994). The separation of free fatty acids, PE and PC is also possible. Here this technique was used to study the oxidation products of FFA by converting them to ammonium salts which gave them a much longer retention time (Ansari & Shoeman 1988). Janero & Burghardt (1990) used silica Sep-pak cartridges to remove neutral and non-choline-containing lipids from choline-containing lipids and platelet activating factor.

Better resolution can be obtained using larger columns. Silica gel column chromatography has been used to extract FFA and TRIGs from human plasma. The individual fatty acids present in these groups were then analysed by GC-MS (Ingalls *et al* 1995). The same group earlier reported a method of separation of FFA, CE, TRIGs cholesterol, di- and monoglycerides, ethanolamine-containing phospholipids and choline-containing phospholipids, also using silica column chromatography. The lipids were separated in seven elution steps with only cholesterol and diglycerides being eluted together. The recovery of added heptadecanoic acid after the extraction and separation steps was 81% and the recovery of synthetic lipid analogues was between 80 and 91% (Ingalls *et al* 1993).

Using aminopropyl-bonded phase columns, it was possible to recover CE, TRIG, di- and mono-glycerides, cholesterol, FFA and phospholipids separately and quantitatively (Kaluzny *et al* 1985). The author refers to this as 'chromatographic mode sequencing'.

The compounds were eluted by changing either the solvent, the solid phase or both. This method is complicated, involving the use of many disposable columns linked together in sequence. Kim & Salem (1990) failed to reproduce it precisely but this modification separated cholesterol, FFA and TRIG followed by the elution of PC + PE + SPG together and PI + PS + PA together. The final solvent, which eluted PI, PS and PA, contained formic acid and 5% phosphoric acid, which introduces the risk of lipid breakdown and selective loss of PUFA. However, the authors state that these losses are no greater then those incurred during TLC. Aminopropyl silica columns have been used to purify fatty acid ethyl esters. Fatty acid ethyl ester and cholesterol esters can be eluted with hexane. Cholesterol esters can then be removed from the sample using C18 columns (Bernhart *et al* 1996).

The antibiotic neomycin is an aminoglycoside with an affinity for phosphoinositides. Neomycin was first used, coated on to glass beads, to remove polyphosphoinositides from phospholipids (Schacht 1978). It has since been used to separate non-acidic lipids from phospholipids which elute sequentially: PS, PA, PI, phosphatidyl glycerol and the polyphosphoinositides (Palmer 1981). The use of this technique has been limited by its relatively inadequate separation of the lipids groups and the difficulty of producing reproducible batches of neomycin-coated beads in the laboratory.

Silicic acid column chromatography is ion exchange chromatography. It has been widely used for lipids. A variety of factors affect separation, such as the water content of the silicic acid, the amount of silicate present, the particle size of the silicic acid and the amount of lipid loaded. PC binds to the column more tightly than PE and LPC binds the tightest because of its ability to form hydrogen bonds. This technique was used to prepare phospholipids from erythrocytes, but it is a complex procedure requiring large volumes of blood (Williams *et al* 1966). DEAE cellulose has also been used. Here the zwitterionic lipids, PC and sphingomyelin have longer retention times that the non-acidic lipids. (Rouser *et al* 1961). Neutral lipids, glycolipids and polar lipids can be separated using a series of solvents (Wing *et al* 1986). Others have used DEAE cellulose to separate neutral lipids from choline-containing lipids and PE (Christie *et al* 1987). DEAE and TEAE cellulose chromatography are expensive, require large amounts of solvents and TEAE in particular, shrinks in the column, leading to packing irregularities. To overcome some of these difficulties, quaternary ammonium glycophase, covalently bound to controlled pore size glass, will bind ionic and polar lipids which allows sequential elution of neutral lipids,

PC + sphingomyelin followed by PE and PS and finally PI + sulphatides (Bandi *et al* 1982).

1.18 High-Performance Liquid Chromatography.

HPLC is a form of solid phase separation, carried out on a large column which is reused. It can be both preparative and quantitative. The stationary phase can be a liquid, an ion-exchange resin or a porous polymer in a metal column through which the mobile phase passes at high pressure. The sample is injected into the mobile phase and the components are detected as they are eluted. In this way, a much higher degree of resolution and reproducibility can be obtained. The mobile phases used in HPLC, like other chromatography systems, usually contain organic solvents. Both isochratic and gradient elution programmes have been used. The type of detection used often determines which solvents are used (see below).

No detection device is ideal. Ultraviolet (UV) spectrophotometric detectors are the most commonly used but the compounds must absorb UV light or be derivatised to do so. Moreover, many solvents absorb in the same range (200-210 nm) as the lipids as well as impurities which increase background readings. These detectors are most sensitive to compounds with conjugated double bonds or aromatic rings. Other lipids need to be derivatised. Fatty acids are converted to aromatic esters, glycolipids are benzolated and phospholipids are converted to diacylglycerols before esterification with aromatic acid derivatives. Fluorescence detectors are more sensitive than UV detectors provided that the conditions of use are well-controlled. Fatty acids must be converted to fluorescent derivatives. Refractive index detectors respond to molecules which have a different refractive index from the mobile phase. This detector is less sensitive than the UV detector and is sensitive to changes in the ambient temperature. It cannot be used with gradient elution systems. Infra-red spectrophotomeric detectors are as sensitive as refractive index detectors but, since all organic compounds absorb infra red light, including the solvents, high background levels are a problem. Mass detectors or light scattering detectors rely on the solvent being rapidly evaporated as it elutes from the column. The solute is not evaporated but nebulised and the small droplets pass through a light beam and are refracted or reflected. The amount of scattered light is proportional to the amount of compound present. This system will cope well with gradient elution systems but all solvents must be volatile and a supply of dry filtered compressed air is required (Robinson & Macrae 1984). For use with preparative HPLC, a stream-splitting system is inserted

before the detection system. Flame ionisation detectors are perhaps the most widely used detection and quantitation system but only volatile solvents can be used. The use of mass spectrometry with HPLC is now becoming more common (Sewell 1992).

Visible and UV light detection systems for HPLC have been compared. Visible light systems could detect 400 fmol-1 pmol and UV systems between 100 and 200 fmol. Recoveries were quantitative and the inter- and intra-assay variability low (Miwia & Yamamoto 1987). Possibly the best identification results are obtained combining both UV and mass detector together (Sothiros *et al* 1985). Mass spectrometry will be discussed further later in connection with gas chromatography.

As with TLC, many HPLC systems have been described in the literature; a few examples will be discussed here. Molecular species of diacylglycerols have been studied using HPLC to separate them from other lipids and reverse-phase HPLC (RFHPLC) to separate the individual molecular species after benzolation. This led to a 99% recovery of the lipids (Lee & Hajra 1991). Hamilton & Comia (1984) report the separation of CE, FFA and TRIG by HPLC with the additional separation of some of the major molecular species within these groups. Preparative separation of lipids by HPLC followed by GC of their fatty acids has been used to quantitate lipids as well as to study their fatty acid composition (Eder *et al* 1992b). A similar combination has been reported by Seewald & Eichinger (1989) although the authors report a difficulty with drifting GC baseline. Patton *et al* (1982) also reported a similar type of separation using RPHPLC using a C18 column. A similar column was used by Dominiczak *et al* (1993) to separate chromophore esters of fatty acids and by Sotirhos *et al* (1985) to separate individual molecular species in phospholipids. Separation of plasma CE by fatty acid chain length has been achieved (Smith *et al* 1980). Neutral lipids separate on a silicic acid HPLC column but the recoveries of TRIG and CE were unreliable (Greenspan & Schroeder 1982). Others have reported the separation of major phospholipids by HPLC using a silica gel column and a diol column (Soudant *et al* 1995).

1.19 Gas Chromatography

Gas chromatography is partition chromatography; the mobile phase is a gas and the stationary phase is liquid. It is possible to separate the molecular species of simple lipids by GC but separation of more complex lipids requires them to be broken down into their component parts by either enzymatic or chemical hydrolysis. The original stainless steel

columns used in gas chromatography techniques caused the selective loss of PUFA but this problem has been overcome by the use of silica or glass columns. The most commonly used columns are capillary, wall-coated open tubular columns where the liquid phase is attached to silica capillary columns with an internal diameter of 0.1-0.3 mm. Gas chromatography columns are situated in a temperature-controlled oven to allow for high column temperatures. The more volatile lipids will elute at lower temperatures; higher temperatures are needed to elute the less volatile compounds. The stationary phases in GC are often poysiloxanes bonded to the column by cross linking between polymer chains. These bonded phase columns are more thermally stable than columns with non-bonded stationary phases. Polarity of the stationary phase can be changed. For instance, it can be increased by adding phenyl groups. It is also possible to separate lipids in thermally stable, polarisable stationary phases such as phenyl methyl polysiloxane polymer which becomes more polar with increasing temperature

Samples are introduced into a heated injection port above the capillary column in a small volume of solvent. The solvent and the sample are then volatilized into the carrier gas which permanently flows though the column. During 'split' injection, there is a high flow of carrier gas through the injection zone and the sample is volatilized into the carrier gas. Much of the sample is lost because of the high flow rate of gas through the injection zone. This prevents column overloading. It is not suitable for thermally labile compounds but is adequate for fatty acid methyl esters. For 'splitless' injection, the carrier gas flow rate is low. The initial column temperature is also low, allowing for the samples to condense at the top of the column. After a time, a flow of gas is introduced to the injector area to clear any remaining sample. This technique cannot easily differentiate between high and low molecular weight compounds.

Eluting lipids can be detected and identified by flame ionisation or mass spectrometry. It is possible to identify lipids by their retention time compared to standard mixtures, although ideally these should be checked on more than one column. There is a wealth of information in the literature on the GC properties of lipids using different columns, temperature programs and lipids derivatives. TRIGs can be separated by carbon number and to some extent by the number of double bonds (Evershed 1992). It is also possible to perform total lipid profiling by GC. Phospholipase is used to convert the phospholipids into diacyl glycerols which are then analysed as trimethyl silyl ethers. Glass columns are suitable for long, repetitive use (Lanza 1980). Fatty acid methyl esters (FAME) have been

separated using a capillary column with a free fatty acid solid phase, the poly (ethylene glycol) ester of nitroterphthalic acid (Beamelle & Vial 1986). GC analysis has shown that there are odd numbered FAME in the membrane along with methylated isomers, double bond position isomers dimethyl acetals and branched chain fatty acids in erythrocyte ghost lipids (Alexander *et al* 1985).

Multiple dimensional GC has been employed to analyse furan fatty acids. For this, two columns are connected in series, a precolumn and an analytical column. The precolumn partially fractionates the samples and the fraction of interest can be diverted to the analytical column for compete analysis (Wahl *et al* 1995). This twin column technique may be useful for samples containing isomers of fatty acids.

1.20 Mass Spectrometry

Mass spectrometry (MS) is the most sensitive detection and identification technique available for lipids analysis. It can be used in conjunction with GC or with HPLC. MS relies on the fragmentation of the compound and quantitation of the ions formed by chemical or electrical ionisation. The mass spectrum (i.e. the pattern of ions formed) is characteristic of the structure of the compound. For example, fragmentation sites frequently occur at double bonds. The mass spectrometer consists of a sample inlet, an ion source, a mass analyser and ion detector. The ion source, mass analyser and ion detector are all maintained in a vacuum by a turbomolecular pump. For electron ionisation, electrons are produced from a hot filament as an electron beam which ionises compounds in its path. The molecular ion is a radical cation formed by the removal of an electron from the compound. If this species is stable, it will be seen as the highest mass ion in the spectrum. Usually, it is unstable and breaks down into smaller ions. The pattern is reproducible and the total ion current can be used for quantitation.

Although MS can be used to identify lipids, my interest was to use it in conjunction with a separation technique to analyse fatty acid mixtures released from lipids, that is, to use it as a quantitative method with positive identification of individual fatty acids. For GC-MS, the fatty acids eluting sequentially from the capillary column pass into the mass spectrometer.

1.21 Esterification of Fatty Acids

Fatty acids must be volatile under GC conditions. Converting them to their methyl esters enhances volatility. When this process is carried out with fatty acid attached to lipids, it is called transesterification. Esterification of fatty acid residues in lipids and free fatty acids can be acid-catalysed. Free fatty acids will not undergo esterification by base catalysis although fatty acid attached to lipid will (reactions 1 & 2).

$$\text{R`-CO-OR``} + \text{R-OH} \underset{}{\overset{H^+ \text{ or } {}^-OCH_3}{\rightleftharpoons}} \text{R`-CO-OR} + \text{R``-OH} \qquad [1]$$

$$\text{R`-CO-OH} + \text{R-OH} \overset{H^+}{\rightleftharpoons} \text{R-CO-OR} + \text{HOH} \qquad [2]$$

Esterification is a reversible reaction which under certain conditions will reach equilibrium. To drive the reaction to the right, it is necessary either to remove the products or to have a large excess of alcohol present. For acid-catalysed esterification, reagents and samples must be completely dry to avoid ester hydrolysis (which is reversible). Ester hydrolysis which occurs during base-catalysed esterification is irreversible. The carboxylate anion formed does not react with alcohol and will react with sodium or potassium to form a soap (Lui 1994). An advantage of acid-catalysed transesterification is that it can take place in the presence of silica so that there is no need to extract lipids after TLC (Christie 1972). Here, the methods of acid-catalysed esterification will be discussed, followed by those of base-catalysed esterification.

Fatty acid methyl ester synthesis by heating lipids with BF_3MeOH (produced by bubbling BF_3 though cold methanol) has been reported as being useful (Liu 1994). Inclusion of benzene improved the esterification of fatty acids present in neutral lipids (Morrison & Smith 1964). As BF_3MeOH has a greater ability to esterify free fatty acids than to transesterify lipids, it has been suggested that alkaline hydrolysis should be carried out prior to esterification with BF_3MeOH (Metcalfe *et al* 1966). Although this technique was recommended by the American Oil Chemists Society in 1969, it is not without difficulties. In 1964, Lough reported that it led to the formation of methoxy-substituted fatty acid esters, probably by the addition of methanol to the double bond of oleic acid. They were therefore thought to be a mixture of the 9- and 10- methoxy derivatives of stearic acid methyl ester. The formation of artefacts increased with the time the sample was heated

and age of the reagents (Lough 1964, Klopfenstien 1971). A 30% reduction in the recovery of oleic acid was observed (Fulk & Shorb 1970). As in the study by Lough (1964), this appears to have been caused by the addition of methanol to oleic acid. Klopfenstein (1971) confirmed that artefacts were formed at concentrations of boron trifluoride greater than 50%. BF_3MeOH from different sources was also contaminated to a variable level which may have caused the artefacts. The authors of this study advocate the use of boron trichloride methanol. In a study of methylation of fish oils containing high quantities of PUFA, 7 and 14% BF_3MeOH reduced the yield of oleic acid compared with sulphuric or hydrochloric acid in methanol, sodium methoxide in methanol and tetramethylguanidine in methanol and produced an artefact which eluted between the 20:5 and 24:1 fatty acid methyl esters (Medina *et al* 1992). In this case, the BF_3MeOH used was one month old and the reaction was only allowed to continue for 2-3 mins so that these problems are unlikely to have been due to old reagents or long reaction times (see above).

Esterification of lipids by heating with hydrochloric acid and methanol is popular. The reagent can be prepared either by bubbling anhydrous hydrogen chloride through methanol, by adding liquid acetyl chloride to methanol (Kishimoto & Randin 1959) or by adding hydrochloric acid to methanol (Jham *et al* 1982). Sulphuric acid in methanol is also easy to prepare and was adopted as their standard method by the Association of Official Analytical Chemists in 1965. It has been claimed that a reaction times as short as 10 min at 60 °C quantitatively produced esters from mouse erythrocytes and synaptosomal membrane phospholipids (Wing *et al* 1986). However, because the fatty acid in sphingomyelin is attached via an amide bond, transesterification requires high concentrations of acid and longer reaction times which may cause fatty acid losses (Eder *et al* 1992a). As mentioned previously, Peuchant *et al* (1989) favoured the use of ispropanol as an extracting solvent for lipids. If 1% sulphuric acid was then added and heated, isopropyl esters were formed. These are less polar then methyl esters but recoveries of unsaturated fatty acid and the fatty acids present in sphingomyelin were quantitative. Only one test-tube was used and losses caused by transferring of samples between tubes were avoided.

Transesterification with a mixture of chloroform, methanol and acetylchloride is useful for smaller samples. Heating releases hydrogen chloride which is neutralised with silver carbonate. The sample is centrifuged and the supernatant analysed for fatty acid methyl

esters without further treatment (Stransky & Jursik 1996). This method appears to be more suitable for esterification of short chain fatty acids, although caution should be exercised in heating mixtures containing chloroform.

Base-catalysed transesterification is not suitable for use with free fatty acids or sphingomyelin or for samples containing silica (Christie 1989). Treatment with methanolic sodium methoxide at ambient temperature is the only way to get quantitative formation of methyl esters (Eder *et al* 1992a). However, the experimental conditions used in this report were inconsistent and no fair comparison with other studies can be made. Bannon *et al* (1982) suggest that this is the best method for esterification of triglycerides but the mixture needs to be refluxed briefly.

Another base-catalysed procedure for transesterification uses methanolic tetramethyl ammonium hydroxide (TMAH). In this process, the fatty acids in lipids are converted into fatty acid methyl esters. The free fatty acids are converted into TMAH salts which can then be pyrolytically alkylated to produce fatty acid methyl esters (Metcalfe & Wang 1981). Some artefacts are produced and trimethylsulphonium hydroxide (TMSH) has been suggested as a suitable alternative. It is less expensive and does not form artefacts (Butte 1983). TMSH compared favourably with the sodium methoxide-catalysed procedure. The method does cause loss of some polyunsaturated fatty acids when the TMSH is injected straight into the injection port of the GC-MS as has been recommended (Elhamdy & Christie 1993). Interestingly, this reaction can be accomplished *in situ* in the GC injection port. The process is referred to as thermally-assisted hydrolysis and methylation pyrolysis derivatisation. Free fatty acids and methanolic TMAH are injected together into the injector port of a GC. The TMAH salts which are formed then undergo thermal decomposition to produce fatty acid methyl esters. One problem is that the PUFAs undergo isomerisation but this can be prevented by reducing the concentration of TMAH or by using trimethyl(trifluoro-m-tolyl) ammonium hydroxide. Submicrogram quantities of lipid can be handled and no separation and reaction steps are required prior to injection (Challinor 1996).

Shantha *et al* (1993) compared four methods; 1). sodium methoxide in methanol, 2). methanolic sodium hydroxide and boron trifluoride in methanol, 3). tetramethylguanidine in methanol and 4). methanol- benzene- acetyl chloride to synthesise methyl esters of linoleic acid isomers. Methods 1 and 4 destroyed some fatty acid isomers and produced

an artefact which co-eluted with fatty acids esters. Methods 2 and 3 were deemed suitable for low concentrations of free fatty acids (Shantha *et al* 1993).

Enzymatic methods of esterification of fatty acids have been described. Haas & Scott (1996) reported a method combining normal transesterification and lipase-mediated esterification. Lipid extracts were treated with alcohol and potassium hydroxide, leading to the formation of esters from glycerides and phosphoglycerides. The reaction could be carried out at room temperature and was compete within 1 hour. The pH of the reaction mix was then reduced and the free fatty acid present was esterified by means of a lipase-dependant esterification reaction. The yield of fatty acid esters was low. The efficiency of the alkaline transesterification was not greater than 90% and only 63% of the free fatty acid present in the reaction mixture was esterified in the lipase-mediated reaction (Haas & Scott 1996). This method was only suitable for use with primary alcohols. It was ineffective with isopropanol.

Diazomethane can be used to catalyse the esterification reaction. The esterification of free fatty acids is rapid but it is not possible to transesterify phospholipids. It is toxic, potentially explosive and has a short self life (Liu 1994).

It is normally accepted that lipids must be extracted into organic solvents prior to derivatisation. This may not be the case. Abel *et al* (1963) found that it was possible to esterify the lipids in bacterial cells using BF_3MeOH without prior extraction and Dugan *et al* (1966) reported that it was possible to transmethylate lipids directly in animal tissue with sulphuric acid and methanol. There have also been reports of *in situ* esterification by basic catalysts such as methanolic sodium methoxide and potassium hydroxide in methanol (Hougen & Bodo 1973, Long *et al* 1988). The fatty acid profiles obtained by direct and extraction methods were comparable in tissue containing high quantities of PUFA (Guillou *et al* 1996). However, this may not be suitable for tissues containing haemoglobin which will catalyse oxidation. In his review of methylation techniques, Liu (1994) reports that *in situ* methylation is simple, highly sensitive and is useful for analysis of total membrane fatty acids.

Methods of esterification described as on-line techniques have also been reported. Fatty acids in oils were methylated with acetyl chloride in methanol. The lipid was dissolved in n-hexane and pumped along a thin tube together with the derivatisation mixture. When

the reaction tube was full, the flow was stopped and the tube was heated to 80°C. A stream of water was then introduced to the reaction tube distal to the heated area to remove residual acetyl chloride. The hexane solution of derivatives could then be injected directly into a gas chromatograph. This method is reported to compare well with methanolic sodium hydroxide and boron trifluoride methanol methods (Ballesteros *et al* 1993).

From the preceding reviews, evidence suggests that cell membrane microviscosity varies physiologically in some diseases. What changes in their composition account for these changes? The literature on the subject is inconclusive. Moreover, changes in the fatty acid/lipid composition of the membrane may alter its function without significantly changing viscosity. By establishing and evaluating a method of comprehensive analysis of membrane lipids, it was intended to address these questions.

Chapter 2 Materials and Methods

2.1 Materials

All reagents employed were of the highest quality available and were obtained from the following suppliers.

2.1.1 General Reagents.

The following reagents were purchased from Sigma Chemical Company, Poole Dorset: ammonium acetate, bovine serum albumin, butylated hydroxytoluene, Folin & Ciocalteu's phenol reagent, Krebs ringer bicarbonate buffer mix, sodium potassium tartate, Tris base, Rhodamine B spray reagent, pyridine, hexamethyldisilazane, trimethyl chlorsilane, N,N-dimethylformamide.

The following reagents were purchased from BDH Chemicals Ltd., Dorset, England. Hydrochloric acid, calcium chloride, dipotassium orthophosphate, potassium dihydrogen orthophosphate, sodium chloride, sodium carbonate, sodium hydroxide pellets, cupric sulphate, pH buffer tablets pH 4, 7 and 9, ethanol.

The following reagents were purchased from Rathburn Chemicals, Walkerburn, Scotland: hexane, methanol. Prolabo, Manchester supplied the chloroform and concentrated sulphuric acid.

2.1.2 Fatty Acid and Lipid Standards

All free fatty acids, fatty acid methyl and ethyl esters and all lipid and cholesterol standards were purchased from Sigma Chemical Company Ltd. Poole, Dorset, U.K.

2.1.3. Chromatography Apparatus

The following chromatography products were purchased from or donated by Whatman Ltd, Maidstone, Kent, England; polyester-backed PE SIL G thin layer chromatography plates, glass-backed 150A and 60A LK5DF fluorescent plates and 3mm chromatography paper and thin layer chromatography tanks.

Waters Corporation, 34 Mapel Street, Milford Massachusetts supplied the silica Sep-pak cartridges.

2.1.4 GC-MS equipment.

Finnigan MAT Ltd (Paradise, Hemel Hempstead, Herts) supplied the Finnigan MAT ITS40 GC-MS, the auto-sampler, dedicated computer and the Magnum software. J&W Scientific (91 Blue Ravine Road, Folsom, California) supplied the 30m DB-5 capillary chromatography column.

Sigma Chemical Company (Poole Dorset) supplied the Supelco pre-treated septa. Burke Analytical (Glasgow, Strathclyde) supplied the injection port liners, Hamilton syringes, auto-sampler vials and lids and sample vials with Teflon lined screw caps. BOC (Guildford Surrey) supplied the CP grade helium.

2.1.5. Fluorescence Anisotropy Equipment.

The fluorescent probe, trimethylammonium diphenylhexatriene (TMA-DPH) was obtained from Molecular Probes Inc. (Eugene, Oregon, USA). Perkin-Elmer LS-50 luminescence spectrophotometer was obtained from Perkin-Elmer Ltd (Beaconsfield, Buckinghamshire, UK).

2.2. Blood Samples.

Human blood samples were taken using Vacutainer needles and bottles containing lithium heparin (Becton Dickinson, Vacutainer Systems Europe, Meylan, Cedex-France). Rat blood samples were obtained by cardiac puncture into 10ml syringe which were pre-washed with lithium heparin. The blood was then transferred into Vacutainer bottles as above.

2.3 Animals

All experimental animals were obtained from the breeding colonies of SHRSP and WKY rats housed in the Department of Medicine and Therapeutics, Gardiner Institute, University of Glasgow. Rats were housed two to a cage with free access to food and water. The animals were maintained on a 12 hour light dark cycle. Blood pressure was measured by the tail cuff method; rats were prewarmed to 30°C. The blood pressure measurements for each rat were an average of 8 consecutive readings. Rats were anaesthetised with halothane prior to blood sampling.

2.4 General Procedures.

2.4.1 Glassware

All test-tubes were treated in a sonicating water bath for 30 minutes in a solution of Decon 75 (Decon Laboratories Ltd., Hove, United Kingdom), rinsed thoroughly with tap water and then distilled water (dH_2O) and dried in an oven at 37^0 C.

2.5 Membrane Preparation.

2.5.1 Preparation of Erythrocyte Cell Membranes.

Erythrocyte membranes were prepared following the method of Saito *et* al (1986). Blood was drawn into lithium heparin Vacutainers, mixed and placed on ice. Erythrocytes were separated from the plasma by centrifugation at 1000g (200 r.p.m.) for 10 minutes at 4°C. The plasma and buffy coat were removed and the erythrocytes resuspended in 5 volumes of cold erythrocyte wash buffer. The cells were pelleted again by centrifugation, the supernatant was removed and the cells resuspended in erythrocyte wash buffer. This washing procedure was repeated until the supernatant was clear after centrifugation. The erythrocytes were then lysed with 40 volumes of cold erythrocyte lysis buffer. The cell lysate was then centrifuged at 15,000g (20,000 r.p.m.) for 20 min at 4°C. The supernatant was removed and the pellet resuspended in erythrocyte lysis buffer and centrifuged again. This washing procedure was continued until there was no haemoglobin present in the membrane pellet and the supernatant was clear. The white membrane pellet was resuspended in 1ml of buffer and frozen at -70°C under an atmosphere of nitrogen until use.

2.5.2 Preparation of Liver Cell Membranes.

Liver samples were obtained and kept on ice in Krebs ringer bicarbonate (Krebs) buffer until use. The tissue was minced into pieces not more then 2mm across using sterile scissors. The suspension of tissue was then washed in erythrocyte lysis buffer to eliminate erythrocytes from the preparation. The tissue suspension was centrifuged at 1000g (200 r.p.m.) for 10 minutes at 4°C. The supernatant was removed and the tissue transferred into a cold, hand-held homogeniser and homogenised for 0.5-1 min. The homogenate was then transferred to a centrifuge tube and washed with Krebs buffer. This was then centrifuged at 15,000g (20,000 r.p.m.) for 20 min at 4°C. The supernatant was removed and the pellet resuspended in Krebs buffer. The washing procedure was repeated until the supernatant was clear after washing. The liver cell membranes were then resuspended in Krebs buffer and stored at - 70°C under and atmosphere of nitrogen until use.

2.6 Lowry Protein Estimation.

Protein concentrations were measured by the method of Lowry *et al* (1951). A standard curve was constructed using concentrations of bovine serum albumin ranging from 0.1-1mg/ml.

2.7 Measurement of Cell Membrane Microviscosity

Erythrocyte membranes were resuspended in 50mmol/l TRIS-HCl (pH 7.4) at a protein concentration of 1mg/ml. This was incubated with TMA-DPH (solution in N,N-dimethylformamide) at a final concentration of 5nmol/l at 37 °C for 30 min. Fluorescence anisotropy was measured in quadruplicate with a Perkin Elmer LS-50 luminescence spectrophotometer with computer-controlled excitation and emission polarizers.

Fluorescence intensity (I) was measured at excitation and emission wavelengths of 340 and 450nm respectively. The slits were set at 20nm. Anisotropy (A) was calculated according to the following equations:

$$A = (I_{vv} - GI_{vh}) / (I_{vv} + 2GI_{vh})$$

$$G = I_{hv} / I_{hh}$$

where the first element of the subscript pair is the excitation orientation and the second element is the emission orientation (v, vertical; h, horizontal). G, the correction factor for the optical system, was evaluated prior to each experiment. The auto-fluorescence of the erythrocyte membranes was checked before each reading and subtracted from the experimental readings (McLaren *et al* 1993).

2.8 Lipid Analysis Techniques

The following techniques are a summary of the methods developed in chapter 3 for the analysis of membrane lipids.

2.8.1 Extraction of Membrane Lipids

Cell membranes were thawed and sonicated in a cold sonicating bath for 10mins. The volume of membrane corresponding to 2mg of protein was placed in a cold test tube on ice. The internal standards for CE, TRIG, FFA, PE, PC, and LPC were added to the test tube and mixed (see chapter 3). Methanol (2ml) was added to the test tube, mixed and then sonicated for 2 min. Chloroform (4ml) was added to the tube, mixed and sonicated as previously. Aqueous Na_2CO_3 (1.2 ml of a 0.73% solution) was then added to the tube

and mixed before centrifugation at 1000g (200 r.p.m) for 5 min. This led to the formation of a biphasic solution. The upper aqueous layer of this was removed with a Pasteur pipette and the interface between the two layers was also carefully removed. The remaining organic phase was evaporated under nitrogen at room temperature. The lipid residue was resuspended in hexane (1ml). The dead space in the test tube was flushed with nitrogen prior to storage at -20°C. All solvents contained BHT (10mg/100ml) and where possible, all procedures were carried out on ice.

2.8.2 Separation of Lipid Groups

The protocol for lipid separation is detailed in chapter 3. Briefly, hexane was removed from the lipid samples by evaporation under nitrogen and the residue was redissolved in methyl-*tert*-butyl-ether (MTBE) (2ml). This was applied to a washed Sep-pak cartridge and the neutral lipids eluted with MTBE (12ml) and MTBE/MeOH/ammonium acetate (25:4:1:v/v/v) (15ml). The solvent was removed from the neutral lipid fraction by evaporation under nitrogen and the lipid residue resuspended in hexane/MTBE (200:3 v/v) and stored under nitrogen at -20°C until further analysis. The polar lipids were eluted from the Sep-pak cartridge following the revised elution system outlined in chapter 3. The solvents were evaporated from the polar lipid fractions prior to the further separation of the polar lipids by TLC (see chapter 3). The lipid spots on the TLC plate were visualised using Rhodamine B spray diluted with ethanol (1:20 v/v). The neutral lipids were also applied to a washed Sep-pak and separated as described in chapter 3.

Fatty acid methyl esters were prepared and extracted from the various lipid fractions using the method outlined in chapter 3.

2.8.3 Analysis of Fatty Acid Methyl Esters

Fatty acid methyl ester solution dissolved in hexane (1μl) was applied to the GC-MS. The GC oven temperature programme was as follows:- 160°C for 1min, increase to 229°C at 2°C per min, to 275°C at 2.5°C per min and finally to 290°C at 5°C per min. Fatty acid methyl esters were then quantitated using the Magnum soft-ware.

The indices of desaturation were calculated as follows:- Δ5 desaturase: arachidonic acid/dihomo-γ-linolenic acid, Δ6 desaturase: linolenic acid/linoleic acid, total desaturase: arachidonic acid/linoleic acid, Δ9 desaturase: oleic acid/stearic acid. The

saturated/unsaturated fatty acid ratio was calculated by summing the quantities of the two fatty acid groups and calculating a ratio of the two amounts.

The following formulae were used to convert the measurements of fatty acid methyl esters into the amount of phospholipid present in the membrane (Seewald & Eichinger 1989).

1. Calculation of the total fatty acid (FA) molecular weight of the phospholipid (ng):

$$\chi \text{ ng}_{FA1} + \chi \text{ ng}_{FA2} + \chi \text{ ng}_{FA2} + ...+ \chi \text{ ng}_{Fai} = \chi \text{ ng}_{\Sigma FA}$$

2. Calculation of the relative amount of a single fatty acid (%)

$$(\text{ng}_{FA1} / \text{ng}_{\Sigma FA}) \bullet 100 = \% \text{ FA1}; \quad (\text{ng}_{FA\chi i} / \text{ng}_{\Sigma FA}) \bullet 100 = \% \text{ FA}\chi i$$

3. Calculation of average FA molecular weight(ΦMWFA) of the phospholipid

% FA1/100	•	MWFA1	=	.....
%FAi/100	•	MWFAi	=	.....
-------		----------		-------
Σ= 100%				Σ = ΦMWFA

4. Determination of the phospholipid molecular weight (MWPL)

$$(n \bullet \Phi\text{MWFA}) + \text{MWPL}_{CORE} = \text{MWPL}$$

Where n = number of fatty acid linked to the phospholipid, ΦMWFA = average fatty acid molecular weight, MWPL_{CORE} = molecular weight of the phospholipid core which has no fatty acids bonded to it.

5. Calculation of the relative amount of fatty acids in the whole phospholipid molecule (% FAPL).

$$(n \bullet \Phi\text{MWFA} \bullet 100) / \text{MWPL} = \%\text{FAPL}$$

6. Determination of the absolute amount of phospholipid;

$$(\text{ng}_{\Sigma FA} / \%\text{FAPL}) \bullet 100 = \text{absolute amount of PL in ng/ml total extract.}$$

2.8.4 Estimation of Membrane Cholesterol

The protocol for measuring of membrane cholesterol concentration is detailed in Chapter 3, part 2. Briefly, membrane lipids were extracted as described previously from a volume of sample which corresponds to 0.1mg/ml of protein. The solvent was removed from the extract and trimethyl silyl ethers of cholesterol were synthesised. These were then analysed on the GC-MS using the same GC oven temperature program as for the analysis of fatty acid methyl esters.

2.9 Buffers.

Erythrocyte lysis buffer contained 5 mM K_2HPO_4 dissolved in dH_2O. The pH of the solution was adjusted to pH 8 by addition of KH_2PO_4. Erythrocyte wash buffer was produced from erythrocyte lysis buffer by adding 0.15M NaCl.

Krebs buffer contained sachet of Krebs ringer bicarbonate buffer mixture dissolved in the appropriate amount of dH_2O. Calcium chloride (1.2mM) and sodium bicarbonate (25.8mM) were added and the pH adjusted to 7.4.

Chapter 3 The Development of a Method of Analysis of Cell Membrane Lipids

3.1 Introduction

The following chapter describes the development of a method for the complete analysis of the fatty acids and lipids present in the cell membrane. In part 1 the development of the method for the fatty acid analysis will be presented. The methods studied here include the production and extraction of fatty acid alcohol esters and the separation of the lipid groups in the cell membrane, followed by the quantitative recovery of the fatty acids esterified to these. Although several different techniques will be discussed here, some basic principles are the same for all. These will be detailed here with a brief discussion of each aspect. The production of standard curves for analysis by GC-MS is explained and the inter and intra assay variability for the method will also be discussed. In the second part of this chapter, the development of an assay for membrane cholesterol is described.

Part 1

3.1.1. Samples and Reference Standards

Neutral Lipids

For cholesterol esters, free fatty acids and triglycerides, two types of standards were used, one containing the internal standard, heptadecanoic acid, and one containing a fatty acid commonly found in the membrane. These naturally occurring lipids have been used as research standards to evaluate the method as they behave in a similar manner to membrane lipids. No internal standard-containing lipid was available for partial glycerides; in this case therefore, heptadecanoic acid was added directly to the test tube in which the neutral lipids were to be methanolysed. The composition and quantities of the lipids used to evaluate the methods are given in Table 3.1.1.a. The quantities of lipids have been calculated to yield fatty acids at the following concentrations; research standard fatty acids: 50 ng/μl and heptadecanoic acid: 20 ng/μl. In all cases, the concentrations stated assume one hundred percent esterification and recovery.

Lipid Group	Fatty Acid Composition Of Lipids Used	Concentration Of Fatty Acid In Final Sample	Volume Of 1 mg/ml Solution Used
CE	Heptadecanoic Acid	20 ng/µl	24 µl
CE	Oleic Acid	50 ng/µl	58 µl
TRIG	Heptadecanoic Acid	20 ng/µl	10 µl
TRIG	Linoleic Acid	50 ng/µl	27 µl
FFA	Heptadecanoic Acid	20 ng/µl	10 µl
FFA	Palmitic Acid	50 ng/µl	25 µl
FFA	Stearic Acid	50 ng/µl	25 µl
PGS	Stearic Acid	50 ng/µl	27.5 µl

Table 3.1.1a Concentration and fatty acid composition of neutral lipids used during method development.

Polar lipids

For PE, PC and LPC, two types of reference standards were used, one research standard lipid, as previously described, and one lipid containing the internal standard, heptadecanoic acid which was a 1,2-diheptadecanoyl derivative. For the research standards, the fatty acids at positions *sn*1 and *sn*2 differed from each other, as they would in the membrane. Although the recoveries are given for both fatty acids, it is only possible to calculate for one fatty acid to be present 50 ng/µl, (see table 3.1.1.b). No commercially available internal standards were available for the remaining lipid groups. In these cases, free heptadecanoic acid was used as internal standard as described for partial glycerides (see above). The composition and quantities of the lipids used are listed in table 3.1.1.b. These concentrations again assume 100% esterification and recovery.

Lipid Group	Fatty Acid Composition Of Lipids	Concentration of Fatty Acid In Final Sample	Volume of 1 mg/ml Solution Used
FFA	Heptadecanoic Acid	20 ng/µl	10 µl
PC	Palmitic Acid	50 ng/µl	74 µl
PE	Stearic Acid	50 ng/µl	33 µl
PE	Heptadecanoic Acid	20 ng/µl	13 µl
LPC	Palmitic Acid	50 ng/µl	48 µl
LPC	Heptadecanoic Acid	20 ng/µl	19 µl
PI	Stearic Acid	50 ng/µl	88 µl
SPG	Palmitic Acid	50 ng/µl	70 µl

Table 3.1.1b Concentration and fatty acid composition of the polar lipids used during method development.

3.1.2 Sample Analysis and Estimation of Lipid Recovery

The fatty acid methyl esters formed from each fraction or esterification reaction were analysed by GC-MS (see chapter 1). The amount of fatty acid present is proportional to the total ion current (TIC) at the appropriate retention time. This value was used to assess the recovery of the lipids after chromatography. The TIC values produced by fatty acids esterified to lipids which underwent chromatography (referred to as sample) were compared to those from unchromatographed standards. Efficiency of methanolysis was assumed to be equal for both the unchromatographed standards and the samples.

Where heptadecanoic acid-containing lipid was available, a direct comparison was made. The TIC value for the unchromatographed standard was taken as maximum and the TIC value for the sample expressed as a percentage of that. The percentage recoveries of the internal standard and research standard were also compared to each other, as a measure of whether both groups behaved in a similar manner.

Where no internal standard-containing lipid was available, the analysis was carried out as above and the results were further analysed as follows:- A ratio of the TIC produced by the fatty acids from the lipids and the heptadecanoic acid was calculated. As there was 2.5 times more of the research standard fatty acid present than there was internal standard, the expected ratio was greater than 1. The value obtained for ratio derived from unchromatographed standards was taken as maximum and the value obtained for the chromatographed sample was compared to this and expressed as a percentage. This method of evaluating the results was chosen because the quantitation computer software package for the GC-MS uses this ratio to calculate the amount of fatty acid present in the sample.

For the evaluation of the esterification method, the TIC values for fatty acid methyl esters produced by methanolysis were compared to those for commercially available standard fatty acid methyl esters at the same concentration.

Where the samples and internal standards were shown to be recovered in the same proportion, there was no need to correct further for losses during analysis. Results are expressed as mean percentage of maximum with standard deviations in brackets. Unless otherwise stated, n=6. The results were analysed using Students t-test: the probability

level required for significance was taken to be $p<0.05$. Where appropriate, 2 way analysis of variance was used.

3.2.1 Esterification Study

To allow their analysis by GC-MS, it is necessary to volatilise fatty acids. This is usually facilitated by their conversion to alcohol esters and can be achieved using a variety of methods (see chapter 1). The aim of this study was to evaluate the use of acid-catalysed transesterification for the production of esters and to evaluate their subsequent extraction using diethyl ether.

3.2.2 Method.

The basic method (Christie 1989) for acid catalysed transesterification and extraction is described below. Modifications will be explained later.

3.2.2a Esterification.

1. The lipids were extracted from the appropriate tissue using chloroform/methanol (2:1 v/v) containing BHT (10mg/100ml) (During the development of the method, standard solutions of lipids in hexane were used).

2. The solvent was evaporated under nitrogen at room temperature and the lipid residue was resuspended in 1% concentrated sulphuric acid in methanol (2ml). The tubes were stoppered and the contents mixed well before incubating at 50°C overnight in a dry incubator. The dead space in the tube was flushed with nitrogen before incubation.

3.2.2b Extraction of Fatty Acid Esters.

1. After incubation, the tubes were removed from the incubator and allowed to cool to room temperature.

2. Diethyl ether (5ml) was added to each tube and the contents mixed. Distilled water (5ml) was then added and mixed. The tubes were placed in a freezing ethanol bath to freeze the lower aqueous layer. The diethyl ether layer was then transferred into a clean test tube and the frozen aqueous layer discarded.

3. The diethyl ether was washed with 0.05% $NaHCO_3$ in distilled water (5ml). The diethyl ether phase was again separated in a freezing bath and the washing procedure repeated with distilled water (5ml).

4. The diethyl ether was dried with anhydrous sodium sulphate and evaporated to dryness under a stream of nitrogen at room temperature. The fatty acid ester residue was

resuspended in hexane (500µl) containing BHT (10mg/100ml), the dead space in the vial was flushed with nitrogen and the vial stored at -20°C until required for analysis.

3.2.3 Experimental 1.

3.2.3.a Alcohol Esters Study.

Although methyl esters are most commonly used in the analysis of fatty acids, it seemed possible that the production of other alcohol esters might have some advantages, for example, by simplifying GC separation of the C18 fatty acids which is notoriously difficult. The properties of both saturated and unsaturated fatty acid esters were studied. The fatty acids ranged from C16-C22 and these were esterified using methanol, ethanol, butanol and isopropanol using the protocol described in section 3.2.2a.

3.2.3.b Efficiency of Esterification

To allow for valid quantitation, it is necessary to ensure that all fatty acids, internal and research standards, are being esterified and recovered uniformly. A number of factors in the procedure described above could affect this. The following were tested using the esterification and extraction procedure detailed in section 3.2.2:-

1. The effect of the absence or presence of BHT on esterification and extraction was studied by carrying out two parallel experiments, one with BHT (10mg/100ml) present in all reagents and a second with no BHT present.
2. The effect of removing the water from the alcohols was tested by drying the alcohols with anhydrous sodium sulphate (300g/1litre w/v). The mixture was allowed to settle for at least 4 hours prior to use.
3. The effect of using different reaction tubes was tested by replacing glass stoppered test tubes with test tubes with Teflon-lined screw caps.

3.2.4 Results 1

3.2.4.a Alcohol Esters Study

The retention times of the fatty acid esters analysed by gas chromatography are summarised in table 3.2.4.a1. Table 3.2.4.a2. details the retention times of saturated fatty acids and the difference in retention time between each saturated fatty acid ester and the one most closely adjacent to it. This gives an index of separation efficiency. Table 3.2.4.a3. summarises the same information for the unsaturated fatty acid esters.

	Fatty Acid Ester Type			
Fatty Acid	Methyl	Ethyl	Butyl	Isopropyl
Palmitic C16:0	23:54	24:45	25:35	27:10
Palmitoleic C16:1	22:28	24:15	27:35	26:22
Heptadecanoic C17:0	25:24	27:13	28:01	28:27
Stearic C18:0	27:48	29:26	30:06	30:34
Oleic C18:1	27:11	28:48	29:45	29:54
Linoleic C18:2	26:59	28:43	29:23	29:31
Linolenic C18:3	27:39	28:49	27:33	27:59
Arachidonic C20:4	30:14	32:13	33:02	34:30

Table 3.2.4.a1. Retention times (min:sec) for a variety of fatty acid esters on the gas chromatograph.

Fatty Acid + Ester Type	Retention Time	Adjacent fatty acid and difference in retention time
Methyl Esters		
Palmitic	23:54	
Heptadecanoic	25:24	17:0 - 16:0 = 1:30
Stearic	27:48	18:0 - 17:0 = 2:24
Ethyl Esters		
Palmitic	24:45	
Heptadecanoic	27:13	17:0 - 16:0 = 2:28
Stearic	29:26	18:0 - 17:0 = 2:13
Butyl Esters		
Palmitic	25:35	
Heptadecanoic	28:01	17:0 - 16:0 = 2:26
Stearic	30:06	18:0 - 17:0 = 2:02
Isopropyl Esters		
Palmitic	27:10	
Heptadecanoic	28:27	17:0 - 16:0 = 1:17
Stearic	30:34	18:0 - 17:0 = 2:07

Table 3.2.4.a2 Retention times (min:sec) of saturated fatty acids and the difference between retention times of closely adjacent saturated fatty acid esters.

Fatty Acid + Ester Type	Retention Time	Adjacent fatty acid and difference in retention time
Methyl Esters		
Palmitoleic	22:28	
Oleic	27:11	18:1 - 18:2 = 0:12
Linoleic	26:59	18:2 - 16:1 = 4:31
Linolenic	27:39	18:3 - 18:1 = 0.28
Arachidonic	30:14	20:4 - 18:3 = 0:12
Ethyl Esters		
Palmitoleic	24:15	
Oleic	28:48	18:1 - 18:2 = 0:05
Linoleic	28:43	18:2 - 16:1 = 4:31
Linolenic	28:49	18:3 - 18:1 = 0.01
Arachidonic	32:13	20:4 - 18:3 = 3:24
Butyl Esters		
Palmitoleic	27:35	
Oleic	29:45	18:1 - 18:2 = 0:22
Linoleic	29:23	18:2 - 16:1 = 1:48
Linolenic	27:33	18:3 - 16:1= 0:02
Arachidonic	30:02	20:4 - 18:1 = 0:17
Isopropyl Esters		
Palmitoleic	26:22	
Oleic	29:54	18:1 - 18:2 = 0:23
Linoleic	29:31	18:2 - 18:3 = 1:32
Linolenic	27:59	18:3 - 16:0 = 1:34
Arachidonic	34:30	20:4 - 18:1 = 4:36

Table 3.2.4.a3 Retention times (min:sec) of unsaturated fatty acids and the difference in retention times between closely adjacent unsaturated fatty acid esters.

1. All fatty acid esters were adequately separated including the esters of the C18:0, C18:1, C18:2 and C18:3 fatty acids. The retention times for all the fatty acid esters were at least 5 seconds away from the nearest fatty acid ester, allowing for individual quantitation. For any given fatty acid, the retention time for each ester increased with increasing molecular weight of the alcohol used in the esterification.

2. The different fatty acid alcohol esters provided different degrees of separation of fatty acid esters. However, there was no constant pattern to these differences. The difference between C18:0 and C17:0 and between C18:2 and C18:3 appears to decrease with increasing molecular weight of the alcohol. The results for other fatty acids were more variable.

3.2.4.b Efficiency of Esterification.

The coefficients of variation for the following experiments are detailed in tables 3.2.5.b1-b2.

Fatty Acid	ME	ME +BHT	ME Dry	ME Teflon
Palmitic	20.69	11.51	6.99	8.24
Palmitoleic	16.78	15.17	23.34	14.06
Heptadecanoic	8.33	6.94	20.19	7.48
Stearic	15.09	12.33	4.67	11.74
Oleic	9.94	17.46	23.92	13.58
Linoleic	6.12	7.14	7.98	6.86
Linolenic	13.31	11.39	9.03	14.74
Arachidic	14.75	26.51	22.59	9.51
Arachidonic	14.63	7.34	23.62	12.81
Behenic	15.17	13.59	6.20	11.67
DHA	9.06	26.70	23.44	13.95
Lignoceric	11.14	20.22	9.10	10.72
Nervonic	11.79	24.21	6.16	7.13
Hexacosanoic	28.86	27.17	36.01	

Table 3.2.4.b1. Coefficients of variation for the experiments to produce fatty acid methyl esters under a variety of conditions as described in section 3.2.3b.
Abbreviations, ME: methyl esters, ME+BHT: methyl esters with BHT present, ME Dry: Methyl esters produced using dried methanol, ME Teflon: methyl ester produced in Teflon lined screw capped vials.

Fatty Acid	EE	EE +BHT	EE Dry	EE Teflon
Palmitic	12.32	4.26	45.51	28.75
Palmitoleic	17.30	19.47	14.01	18.91
Heptadecanoic	8.74	7.76	10.56	7.85
Stearic	6.50	5.08	13.59	24.09
Oleic	14.51	26.32	11.76	20.13
Linoleic	.56	8.53	6.44	17.22
Linolenic	16.85	16.42	6.62	12.78
Arachidic	8.17	10.75	8.20	16.40
Arachidonic	4.62	6.26		
Behenic	13.16	7.25	5.63	16.99
DHA	6.73	10.62	13.41	18.55
Hexacosanoic			46.43	62.98

Table 3.2.4.b2 Coefficients of variation of the experiments to produce fatty acid ethyl esters as described in section 3.2.3b.
Abbreviations, EE: ethyl esters, EE+BHT: ethyl esters with BHT present, EE Dry: ethyl esters produced using dried ethanol, EE Teflon: ethyl ester produced in Teflon lined screw capped vials.

3.2.4.b1 Effects of the presence or absence of BHT.

The results for esterification in the absence and presence of BHT (tables 3.2.4.b3-b4) can be summarised as follows:-

1. With no BHT present, the recoveries varied from 19.91% (5.41) to 155.32% (19.16) for methyl esters and 62.93% (5.37) to 214.03% (13.39) for ethyl esters. The coefficients

of variation ranged from 6.12-28.86% for methyl esters and 4.62-17.30% for ethyl esters. Analysis of variance showed there was no statistical difference between the TIC values of the laboratory-produced methyl esters and the standards ($p=0.78$). There was a significant difference between the ethyl ester standards and the laboratory produced esters ($p=0.02$). The values which were statistically significantly different are indicated in table 3.2.4.b3.

2. With BHT present, the recoveries varied from 44.15% (4.92) to 168.56% (25.44) for methyl esters and 29.76% (5.15) to 101.18% (8.27) for ethyl esters. The coefficients of variation ranged from 6.96-27.17% for methyl esters and 4.26 to 26.32% for ethyl esters. Analysis of variance showed there was no statistical difference between the TIC values of the laboratory-produced methyl esters and the standards ($p=0.55$). There was a significant difference between the ethyl ester standards and the laboratory produced esters ($p<0.001$). The values which were statistically significantly different are indicated in table 3.2.4.b4.

3. No definite trend was observed between saturated and unsaturated fatty acids in either group. The same fatty acids gave high recoveries for methyl esters both with and without BHT. These were C16:0, C18:0, C22:0 and C22:6. The coefficients of variation were in general lower for the ethyl esters than methyl esters. BHT had no effect on this. The variability of the results for each fatty acid was not consistent for all four treatments. For stearic acid, the coefficient of variability ranged from 5.08-15.09% over the four treatments. Comparison by analysis of variance of the values obtained in the presence and absence of BHT showed no statistical difference between the groups ($p=0.51$).

Fatty Acid.	ME	ME STD	% of Max.	EE	EE STD	% of Max.
Palmitic	28.60	24.34	117.49	21.74	27.01	80.49
C16:0	(5.92)	(0.71)	(24.31)	(2.68)	(0.67)	(9.92)
Palmitoleic	20.21	36.36	53.32	19.27 *	64.74	29.76
C16:1	(3.87)	(1.69)	(8.95)	(3.33)	(3.21)	(5.15)
Heptadecanoic	20.95	27.81	75.34	22.14 *	38.11	58.09
C17:0	(1.74)	(1.10)	(6.28)	(1.93)	(5.08)	(5.08)
Stearic	38.26	22.70	168.56	29.36	29.50	99.53
C18:0	(5.78)	(0.74)	(25.44)	(1.91)	(3.51)	(6.47)
Oleic	31.23	50.65	61.66	32.90 *	49.72	66.16
C18:1	(3.20)	(3.74)	(6.13)	(4.77)	(3.74)	(9.60)
Linoleic	20.96	30.50	68.74	20.84 *	40.40	51.59
C18:2	(1.28)	(1.32)	(4.21)	(1.16)	(5.44)	(2.87)
Linolenic	14.58	22.83	63.86	20.13 *	27.67	72.74
C18:3	(1.94)	(1.75)	(8.50)	(3.39)	(1.99)	(12.26)
Arachidic	16.71	12.30	135.42	21.38	21.13	101.18
C20:0	(2.46)	(0.67)	(19.98)	(1.83)	(1.27)	(8.27)
Arachidonic	20.04	40.51	49.48	20.30 *	11.21	181.10
C20:4	(2.93)	(1.64)	(7.24)	(0.94)	(1.33)	(8.36)
Behenic	27.49	26.47	103.85	20.58 *	27.93	75.52
C22:0	(4.17)	(1.17)	(15.75)	(2.66)	(1.33)	(9.94)
DHA	22.92	22.26	102.86	26.98	27.61	97.14
C22:6	(2.07)	(1.18)	(9.32)	(1.82)	(1.24)	(6.54)
Lignoceric	8.18	18.52	44.15	5.28	NA	NA
C24:0	(0.91)	(1.14)	(4.92)	(0.71)		
Nervonic	17.20	23.07	76.77	18.34	NA	NA
C24:1	(2.03)	(2.45)	(9.05)	(1.46)		
Hexacosanoic	5.33	15.57	34.23	2.37	NA	NA
C26:0	(1.54)	(1.60)	(9.88)	(0.65)		

Table 3.2.4.b3 Total ion currents (10^6) and percentage of maximums produced by fatty acid esters synthesised in the absence of BHT, results are expressed as mean (SD), n=6..
Abbreviations NA: not available all others as described in table 3.2.4b1 & b2.
Statistics ANOVA for methyl esters p=0.78, for ethyl esters p=0.02 * = statistically different from maximum using unpaired Students t-test.

Fatty Acid.	ME + BHT	ME STD	% of Max.	EE + BHT	EE STD	% of Max.
Palmitic	28.37	24.34	116.57	25.21	27.01	93.33
C16:0	(3.27)	(0.71)	(13.45)	(1.08)	(0.67)	(3.98)
Palmitoleic	26.73	36.36	73.51	26.92 *	64.74	41.55
C16:1	(4.05)	(1.69)	(11.15)	(5.23)	(3.21)	(8.09)
Heptadecanoic	23.19	27.81	88.22	25.74 *	38.11	67.53
C17:0	(1.71)	(1.10)	(6.13)	(2.00)	(5.08)	(5.24)
Stearic	35.26	22.70	155.32	29.64	29.50	100.47
C18:0	(4.35)	(0.74)	(19.16)	(1.50)	(3.51)	(5.10)
Oleic	39.98	50.65	78.93	38.28 *	49.72	76.98
C18:1	(6.98)	(3.74)	(13.78)	(10.07)	(3.74)	(20.26)
Linoleic	22.65	30.50	74.26	25.42 *	40.40	62.93
C18:2	(1.61)	(1.32)	(5.30)	(2.17)	(5.44)	(5.37)
Linolenic	17.55	22.83	74.91	21.22 *	27.67	76.69
C18:3	(2.06)	(1.75)	(8.53)	(3.48)	(1.99)	(12.59)
Arachidic	16.17	12.30	131.06	18.83 *	21.13	89.12
C20:0	(4.28)	(0.67)	(34.74)	(2.03)	(1.27)	(9.58)
Arachidonic	21.84	40.51	53.92	23.99 *	11.21	214.03
C20:4	(1.60)	(1.64)	(3.96)	(1.50)	(1.33)	(13.39)
Behenic	21.36	26.47	80.71	17.73 *	27.93	63.47
C22:0	(2.80)	(1.17)	(10.91)	(1.28)	(1.33)	(4.60)
DHA	21.44	22.26	97.89	24.34	27.61	87.62
C22:6	(5.47)	(1.18)	(26.14)	(2.59)	(1.24)	(9.31)
Lignoceric	4.80	18.52	25.92	4.05	NA	NA
C24:0	(0.97)	(1.14)	(5.24)	(0.53)		
Nervonic	13.61	23.07	60.75	15.09	NA	NA
C24:1	(3.30)	(2.45)	(14.71)	(2.32)		
Hexacosanoic	3.10	15.57	19.91	1.14	NA	NA
C26:0	(0.84)	(1.60)	(5.41)	(0.42)		

Table 3.2.4.b4 Total ion current (10^6) produced by fatty acid esters synthesised in the absence of BHT and the percentage of maximum, results are expressed as mean (SD) n=6. Statistics ANOVA for methyl esters p=0.55, for ethyl esters p>0.001 * = statistically different from maximum using unpaired Students t-test.

3.2.4.b2 The Effect of Removing Water from the Alcohol.

The recoveries of the fatty acid ester produced with alcohol dried with Na_2SO_4 are shown in table 3.2.4.b5 and can be summarised as follows:-

1. The recoveries of methyl esters ranged from 34.95% (3.18) to 133.26% (31.24). The recoveries of ethyl esters ranged from 36.68% (5.14) to 290.86% (30.71). Coefficients of variance ranged from 4.67-36.01% for methyl esters and 5.63-45.51% for ethyl esters. Analysis of variance showed a statistical difference between the TIC values of the laboratory produced methyl esters and the standards ($p<0.001$). The same was true of ethyl esters ($p<0.001$). The values which were statistically difference by t-test are indicated in table 3.2.4.b5.

2. Under these conditions, there was no trend between the yields obtained for saturated and unsaturated fatty acids. The two fatty acid methyl esters which produced the highest

recoveries were C18:2 and C22:6 (these were not statistically different from maximum). For ethyl esters, the highest yields were for PUFA in particular C18:2 and C18:3. Comparing these recoveries with the recoveries obtained in the previous experiment (no BHT), this experiment gave fewer yields of greater than 100%. Analysis of variance showed the that two experiments gave different results ($p<0.001$ for both methyl and ethyl esters). For methyl esters, only the recovery of linoleic acid was increased using dried alcohol. All other fatty acids were either less well recovered or not affected. For ethyl esters, the recoveries of oleic, linolenic and arachidic acid were greater using dried alcohol.

Fatty Acid	ME	ME STD	% of Max.	EE	EE STD	% of Max.
Palmitic	12.75 *	25.24	50.51	15.78 *	28.08	55.37
C16:0	(0.89)	(1.12)	(3.53)	(7.19)	(1.13)	(25.20)
Palmitoleic	15.77 *	30.86	51.11	24.31 *	66.28	36.68
C16:1	(3.70)	(1.77)	(11.93)	(3.40)	(14.87)	(5.14)
Heptadecanoic	15.70 *	27.48	57.13	36.45 *	12.53	290.86
C17:0	(0.78)	(1.38)	(2.83)	(3.85)	(0.88)	(30.71)
Stearic	18.70 *	27.82	67.18	22.79 *	34.17	66.65
C18:0	(0.87)	(1.14)	(3.14)	(3.07)	(1.51)	(9.06)
Oleic	19.10 *	40.57	47.08	28.82	31.73	90.83
C18:1	(4.57)	(2.99)	(11.26)	(3.30)	(3.26)	(10.68)
Linoleic	25.32	24.18	104.73	30.23 *	11.20	269.67
C18:2	(2.02)	(1.34)	(8.36)	(1.95)	(0.79)	(17.36)
Linolenic	13.73 *	28.61	47.96	17.87 *	10.85	164.67
C18:3	(1.24)	(1.46)	(4.33)	(1.18)	(0.82)	(10.91)
Arachidic	23.53	27.73	87.26	31.90 *	27.67	115.29
C20:0	(5.35)	(2.31)	(19.71)	(2.62)	(1.85)	(9.45)
Arachidonic	16.66 *	29.55	56.40	23.07	NA	NA
C20:4	(3.88)	(2.70)	(13.12)	(2.23)		
Behenic	14.93 *	38.71	38.56	20.19 *	28.71	72.80
C22:0	(0.92)	(3.99)	(2.39)	(1.18)	(1.63)	(4.10)
DHA	16.10	12.70	133.26	21.67	20.67	108.08
C22:6	(3.77)	(1.08)	(31.24)	(2.11)	(1.34)	(14.55)
Lignoceric	10.37 *	29.68	34.95	10.47	NA	NA
C24:0	(0.94)	(3.63)	(3.18)	(0.72)		
Nervonic	36.09 *	40.01	90.20	43.10	NA	NA
C24:1	(2.22)	(1.27)	(5.56)	(2.76)		
Hexacosanoic	40.05	43.45	92.06	19.39	10.82	179.20
C26:0	(14.37)	(2.41)	(33.15)	(9.00)	(0.86)	(83.20)

Table 3.2.4.b5 Total ion current (10^6) produced by fatty acid esters synthesised using dried alcohol, and the % of maximum obtained as in table 3.2.5.b1 (n=6)
Statistics ANOVA for methyl esters $p > 0.001$, for ethyl esters $p > 0.001$

3.2.4.b3 The Effect of Different Reaction Vessels

The results for the experiments carried out in Teflon-lined screw capped test tubes (table 3.2.4 b6) can be summarised as follows:-

1. The yields of methyl esters ranged from 28.52% (4.01) to 132.74% (14.23). The yields of ethyl esters ranged from 23.48% (4.44) to 201.61% (126.61). The coefficients of variation for methyl ester ranged from 6.86-27.36% and for ethyl esters from 7.85-62.98%. Analysis of variance showed a statistical difference between the TIC values of the laboratory produced methyl and ethyl esters and the standards ($p<0.001$), the values which were statistically significantly different are indicated in table 3.2.4.b6.

2. Most of the fatty acids which gave high yields were saturated fatty acids, in particular C16:0, C18:0 and C24:0. The yields of ethyl esters, although different to those of the methyl esters, were not consistently higher or lower. The highest yield fatty acid ethyl esters were a mixture of saturated and unsaturated fatty acids (C17:0, C22:0, C18:2 and C18:3). The variability of the recoveries here is in general lower for the methyl esters compared to the original method. The same is not true for ethyl esters. Comparing these results with those obtained using the original protocol, the results are statistically different for both methyl and ethyl esters ($p<0.001$ for both). The only methyl ester which gave a significantly higher recovery using the Teflon-lined screw capped vials was lignoceric acid methyl ester; all other esters gave yields either the same or lower than previously. For ethyl esters, palmitic, heptadecanoic, linoleic, linolenic and behenic acid ethyl esters gave higher recoveries than previously.

Fatty Acid	ME	ME STD	% of Max.	EE	EE STD	% of Max.
Palmitic	33.20 *	25.24	116.06	19.32 *	28.08	68.80
C16:0	(10.90)	(1.12)	(9.56)	(5.55)	(1.13)	(19.78)
Palmitoleic	8.80 *	30.86	28.52	15.56 *	66.28	23.48
C16:1	(1.24)	(1.77)	(4.01)	(2.94)	(14.87)	(4.44)
Heptadecanoic	17.79 *	27.48	64.74	23.63 *	12.53	188.60
C17:0	(7.41)	(1.38)	(4.84)	(1.86)	(0.88)	(14.81)
Stearic	34.02 *	27.82	125.61	31.24	34.17	91.43
C18:0	(6.05)	(1.14)	(14.75)	(7.53)	(1.51)	(22.03)
Oleic	10.81 *	40.57	26.65	18.14 *	31.73	57.18
C18:1	(1.47)	(2.99)	(3.62)	(3.65)	(3.26)	(11.51)
Linoleic	15.48 *	24.18	64.28	19.79 *	11.20	176.55
C18:2	(1.11)	(1.34)	(4.41)	(3.38)	(0.79)	(30.14)
Linolenic	8.78 *	28.61	30.67	12.56 *	10.85	115.79
C18:3	(1.31)	(1.46)	(4.52)	(1.54)	(0.82)	(14.18)
Arachidic	8.51 *	27.73	30.70	13.11 *	27.67	47.37
C20:1	(0.80)	(2.31)	(2.92))	(2.15)	(1.85)	(7.77)
Arachidonic	10.45 *	29.55	35.36	15.46	NA	NA
C20:4	(1.34)	(2.70)	(4.53)	(2.98)		
Behenic	29.38	38.71	94.89	32.76	28.71	114.12
C22:0	(5.55)	(3.99)	(11.07)	(5.57)	(1.63)	(19.39)
DHA	8.67 *	12.70	68.25	15.45 *	20.67	74.76
C22:6	(1.21)	(1.08)	(9.52)	(2.87)	(1.34)	(13.87)
Lignoceric	29.03	29.68	132.74	35.05	NA	NA
C24:0	(6.03)	(3.63)	(14.23)	(7.39)		
Nervonic	23.19 *	40.01	57.95	15.64	NA	NA
C24:1	(1.65)	(1.27)	(4.13)	(1.22)		
Hexacosanoic	18.88 *	43.45	43.45	21.81	10.82	201.61
C26:0	(5.17)	(2.41)	(11.89)	(13.74)	(0.86)	(126.97)

Table 3.2.4.b6 Total ion currents (10^6) for the esters produced using Teflon-lined screw capped tubes and % of maximum data (n=6). Abbreviations are as above.
Statistics ANOVA for methyl esters $p > 0.001$, for ethyl esters $p > 0.01$

Comment.

In the light of the results obtained from these experiments the extraction procedure which follows was evaluated and then used for all subsequent experiments.

3.2.3 Experimental 2

3.2.3c Alteration to The Extraction Procedure.

Methyl esters were produced as described previously using reagents containing no BHT. Dried alcohol and Teflon-lined screw capped tubes were used. The extraction procedure was as follows:-

1. The test-tubes were removed from the incubator and allowed to cool to room temperature. Diethyl ether (3ml) was added to each tube and mixed well. Water (5ml) was then added mixed and allowed to settle.

2. The diethyl ether phase was then transferred by means of a Pasteur pipette into a clean test tube. The pipette was washed with 1ml of the salt solution (0.05% $NaHCO_3$) by pipetting it down the inside of the pipette allowing the salt solution to run into the test tube containing the diethyl ether. The residual water was back-extracted with diethyl ether (3ml); this was added to the first extract with a further 4ml of salt solution which was also washed down the inside of the pipette.

3. The diethyl ether layer was separated from the salt solution into a clean test tube. The pipette was washed with distilled water (2.5ml) also into the clean test tube.

4. The residual salt solution was back-extracted with diethyl ether (1ml) which was added to the previous extract. The pipette was washed with distilled water (2.5ml) into the test tube. The diethyl ether phase was separated from the water by freezing (-20^{0}C).

5. Finally, the diethyl ether layer was decanted into a clean test tube and evaporated to dryness under a stream of nitrogen at room temperature. The residue was resuspended in 500ml of hexane containing BHT (10mg/100ml). The vial was flushed with nitrogen and stored at -20^{0}C.

3.2.3.d Improving the Efficiency of Esterification

The following experiments were carried out using the new extraction procedure to improve the efficiency of esterification.

1. Effect of acid concentration on the efficiency of esterification was tested by using 1% and 2% H_2SO_4 in methanol for the esterification reaction. Heat was applied using an aluminium heat block.

2. The effect of incubation temperature was tested by increasing the temperature to 60°C and reducing the time of incubation to 2 hours.

3. Effects of these procedures on the stability of standard fatty acid methyl esters were tested. Standard esters were dissolved in 2% H_2SO_4 in methanol and immediately extracted using the method in 3.2.3.c to evaluate losses of fatty acid methyl esters during

the extraction procedure. To evaluate the losses of fatty acid during the incubation, the standard esters were incubated at 60^0C for two hours in the incubator or heat block and at 50^0C in the incubator overnight before extraction.

3.2.4 Results 2

3.2.4c Alteration To The Extraction Procedure.

The results for the recovery of fatty acids extracted using the modified extraction procedure are shown in table 3.2.5.c1 and can be summarised as follows:-

1. The yield of fatty acid esters ranged from 39.65% (1.18) to 73.76% (3.45). The coefficients of variation ranged from 2.98-10.75%.

2. None of the fatty acid esters produced a yield of greater than 100% and the variability of the results was reduced. Comparing this with the previous experiment, showed that the two methods provided different recoveries of fatty acid methyl esters ($p<0.001$) (see table 3.2.5.c2). The overall yield of the fatty acid esters was more uniform (i.e. low coefficients of variation) using the new procedure with most of the fatty acid methyl esters being recovered at between 50 and 70% of maximum.

Fatty Acid	ME	ME STD	% of Max.	CV
Palmitic	13.74 (1.17)	25.24 (1.12)	54.44 (4.65)	8.54
Palmitoleic	13.89 (1.49)	30.86 (1.77)	45.01 (4.84)	10.75
Heptadecanoic	19.98 (1.59)	27.48 (1.38)	72.71 (5.78)	7.95
Stearic	18.32 (1.35)	27.82 (1.14)	65.84 (4.84)	7.35
Arachidic	20.45 (0.95)	27.73 (2.31)	73.76 (3.45)	4.68
Behenic	15.35 (0.46)	38.71 (3.99)	39.65 (1.18)	2.98
Nervonic	20.01 (1.32)	40.04 (1.27)	50.02 (3.31)	6.62
Lignoceric	18.05 (0.82)	29.68 (3.63)	60.81 (2.77)	4.55

Table 3.2.4.c1 Total ion currents (10^6) from the fatty acid methyl esters. The esters where produced using the original procedure (incubating at 50°C) and then extracted using the washing procedure described in section 3.e. The % of maximum for the synthesised esters compared to the standard esters is also shown as is the coefficient of variation (CV).

Fatty Acid	% of Max.Original Protocol	CV Original Protocol	% of Max. New Protocol	CV New Protocol
Palmitic	117.49 (24.31)*	20.69	54.44 (4.65)	8.54
Palmitoleic	53.32 (8.95)	16.78	45.01 (4.84)	10.75
Heptadecanoic	75.34 (6.28)	8.33	72.71 (5.78)	7.95
Stearic	168.56 (25.44)*	15.09	65.84 (4.84)	7.35
Arachidic	135.42 (19.98)*	14.75	73.76 (3.45)	4.68
Behenic	103.85 (15.75)*	15.17	39.65 (1.18)	2.98
Nervonic	76.77 (9.05)*	11.79	50.02 (3.31)	6.62
Lignoceric	44.15 (4.92)	11.14	60.81(2.77)	4.55

Table 3.2.4.c2. Comparison of the percentage of maximum TIC for the fatty acid methyl esters extracted using the original protocol and the modified protocol.

3.2.4d Optimisation of the Esterification Procedure.

3.2.4d1 Effect of acid concentration.

The results produced by increasing concentration of the acid present in the methanol are shown in table 3.2.4.c3 and can be summarised as follows:-

1. The recoveries of the fatty acid methyl esters produced with 1% acid present in methanol ranged from 46.27% (10.60) to 116.42% (21.82). The recoveries of the esters produced with 2% acid in methanol ranged from 52.39% (6.35) to 112.16% (7.27).

2. The recoveries of the fatty acid methyl esters produced using 2% acid were in general slightly greater, although not significantly, than those produced using 1% acid, the only exception to this being arachidic acid. The variability in the yield of fatty acid methyl esters was reduced when esters were produced using 2% acid (CV: 6.48-12.12%) compared to 1% (CV: 18.74-23.66%). Comparison of the recoveries obtained using 1 and 2% acid by analysis of variance showed no significant difference (p=0.38).

Fatty Acid	ME STD	1% TIC	1% % max.	1% CV	2% TIC	2% % max.	2% CV
Palmitic	25.24 (1.12)	14.46 (3.15)	57.33 (12.24)	21.35	16.16 (1.49)	64.04 (5.89)	9.20
Palmitoleic	30.86 (1.77)	14.29 (3.26)	46.27 (10.60)	22.91	16.17 (1.95)	52.39 (6.35)	12.12
Heptadecanoic	27.48 (1.38)	16.85 (3.44)	61.33 (12.53)	20.43	18.60 (1.45)	67.69 (5.26)	7.77
Oleic	40.57 (2.99)	20.92 (4.95)	51.57 (12.20)	23.66	21.92 (2.30)	54.03 (5.66)	10.47
Linoleic	24.18 (1.34)	14.48 (3.49)	59.87 (14.41)	24.07	14.97 (1.58)	61.91 (6.53)	10.55
Arachidic	27.73 (2.31)	32.29 (6.05)	116.42 (21.82)	18.74	31.10 (2.02)	112.16 (7.27)	6.48

Table 3.2.4.c3 The total ion currents (10^6) for fatty acid methyl esters when passed through the gas chromatograph and fatty acid methyl esters produced in either 1% or 2% sulphuric acid in methanol and the % of maximum data and coefficients of variation.

3.2.4.d2 Effect of Incubation Temperature.

The results produced when the incubation temperature was increased to 60^{0}C (table 3.2.4.c4) can be summarised as follows (here the concentration of the acid was 2%).

1. The yield of fatty acid methyl esters produced ranged from 86.41% (7.83) to 103.13% (10.44) (CV: 4.69-10.47%).

2. The results were less variable than previously and none of the TIC values produced by the laboratory-esterified fatty acids were significantly different from those produced by the standard fatty acids.

Fatty Acid	ME STD	2% H_2SO_4 in MeOH 2h 60^o HB	% Of Maximum	CV
Heptadecanoic	15.35	13.48 (0.98) *1	87.79 (6.43)	7.32
Stearic	16.87 (0.39)	15.78 (1.65) *2	93.52 (9.79)	10.47
Linolenic	10.61 (0.26)	10.94 (1.11) *3	103.13 (10.44)	10.12
Arachidic	15.74 (0.42)	15.22 (0.71) *4	96.67 (4.53)	4.69
Arachidonic	20.98 (0.53)	18.13 (1.65) *5	86.41 (7.83)	9.06

Table 3.2.4.c4 Total ion currents (10^6) from the fatty acid methyl esters where the samples were incubated for 2hours at 60^{0}C, the percentage of maximum is also shown. The abbreviations are as described previously. *1 P=0.136, *2 P=0.252, *3 P=0.997, *4 P=0.607 *5 P=0.373.

3.2.4.d3 Effect of the extraction and incubation procedures on standard fatty acid methyl esters.

The results (table 3.2.4.c5-7) can be summarised as follows.

1. The yields of fatty acid esters produced by dissolving fatty acid methyl esters in the acid methanol mix and immediately re-extracting ranged from 92.95% (6.22) to 140.16% (7.03). The yield of fatty acid methyl esters produced after incubation for 2 hours at 60^{0}C in the heat block ranged from 80.98% (5.43) to 120.43% (4.85), a similar incubation in the incubator produced yields ranging from 72.05% (2.87) to 105.16% (7.99). Incubation in the incubator overnight at 50^{0}C produced yields ranging from 61.46% (5.36) to 194.59% (15.22)

2. The yields of fatty acid esters obtained after incubation in the heat block were greater than those obtained for either incubation in the dry incubator. The results obtained after incubating overnight in the incubator were the most variable. Linolenic acid methyl ester gave yields of consistently greater than 100% irrespective of the treatment, suggesting the standard mixture may not be 100% pure and may contain some unesterified fatty acid which has been esterified by these procedures.

Fatty Acid	STD	RE EXT	2h 60° C HB	2h 60°C INC	24h 50°C INC
Heptadecanoic	16.69	15.51	13.98	12.52	10.69
	(0.36)	(1.04)	(0.63)	(0.36)	(0.95)
Stearic	17.60	18.10	15.44	14.09	11.99
	(0.35)	(1.10)	(0.95)	(0.66)	(1.01)
Linolenic	8.27	11.59	9.96	8.70	16.09
	(0.24)	(0.58)	(0.40)	(0.66)	(1.26)
Arachidic	17.40	20.18	16.45	14.55	12.84
	(0.38)	(1.54)	(1.23)	(0.82)	(1.44)
Arachidonic	20.51	19.51	16.61	14.78	12.60
	(0.43)	(0.75)	(1.11)	(0.59)	(1.10)

Table 3.2.4.c5. TIC values of for the standard study described in section 3.2.3d.

Abbreviations RE EXT; immediate retraction, 2h 60° C HB; two hour incubation in heat block, 2h 60°C INC; two hour incubation in incubator, 24h 50°C INC; overnight incubation in incubator.

Fatty Acid	RE EXT	2h 60° C HB	2h 60°C INC	24h 50°C INC
Heptadecanoic	92.95	83.79	75.01	64.06
	(6.22)	(3.76)	(2.18)	(5.68)
Stearic	102.94	88.72	80.04	105.67
	(6.33)	(5.41)	(3.94)	(8.92)
Linolenic	140.16	120.43	105.16	194.59
	(7.03)	(4.85)	(7.99)	(15.22)
Arachidic	115.97	94.53	84.15	73.80
	(8.83)	(7.04)	(4.87)	(8.29)
Arachidonic	95.13	80.98	72.05	61.46
	(3.68)	(5.43)	(2.87)	(5.36)

Table 3.2.4.c6. Percentage of maximum recoveries for standard study described in section 3.2.3d.

Fatty Acid	RE EXT	2h 60° C HB	2h 60°C INC	24h 50°C INC
Heptadecanoic	6.69	4.49	2.91	8.87
Stearic	6.15	6.10	4.92	8.44
Linolenic	5.01	4.03	7.60	7.82
Arachidic	7.61	7.45	5.79	11.23
Arachidonic	3.89	6.70	3.98	8.72

Table 3.2.4.c7 Coefficients of variation for the standard study described in section 3.2.3d.

3.2.5. Discussion.

Because the molecular weights of the fatty acids studied here do not differ greatly, they can be difficult to separate by gas chromatography. In particular, the 4 fatty acids containing 18 carbon atoms only vary from each other by 2 atomic mass units each. However, the use of the graduated temperature program for the gas chromatograph (see section 2.8.3) allows adequate separation of the fatty acid esters. Although all the alcohols used produced esters which were adequately separated by gas chromatography

and the different esters were separated by different amounts, only the study of methyl and ethyl ester production was continued for the following reasons. Methanol and ethanol are available at low cost and high purity. Pure methyl esters of all naturally occurring fatty acid are available commercially for use as standards; pure ethyl esters are available for most fatty acids. While butyl and propyl ester standards could have been synthesised, the separation of these was no more efficient than methyl or ethyl esters so that the expenditure of time and money was not justified. It became apparent after the initial experiments that there was no advantage in the production of ethyl esters and this too was abandoned. Some groups do routinely use other alcohol esters. For example isopropyl ester production was favoured by Peuchant *et al* (1989). This method allows for the lipids to be extracted with isopropanol.

Using the original extraction procedure, the experiments to test the esterification reaction efficiency provided results which were inadequate. In particular the recoveries were variable, ranging from less then 50% to greater than 150%. The low recoveries may have been due to incomplete esterification or extraction or losses incurred during the extraction procedure possibly, due to oxidation. These will be discussed further later. Greater than theoretical recoveries must have been due to differences in recovery between the standard and the synthesised esters. Identical recovery of the internal standard and sample esters is essential for valid quantitation using the computer quantitation package. It is not a necessity for esters to be recovered 100% but all fatty acid esters must be recovered in similar proportions. This was not the case here.

As mentioned previously, the low recoveries observed may have been due to incomplete esterification of the fatty acids. The esterification reaction is impaired when water is present in the reaction mix (Christie 1989). Any residual traces of water were removed from the alcohol with anhydrous sodium sulphate. Although the water content of the alcohol was not tested, the drying procedure used is widely accepted as being adequate. Also, the addition of the sulphuric acid, itself a dehydrating agent, should have eliminated any residual water from the mixture. Other groups (Fixter, personal communication) report that for esterification to proceed, the methanol must bubble when the acid is added to it; this was routinely observed. Drying of alcohol did not increase the efficiency of esterification. In fact, in many cases the yields produced with dry alcohol were lower than before. Many of the previous results showed yields of greater than maximum; drying the alcohol prevented this. The reason is not clear. As it is not an expensive or time

consuming, the drying procedure was adopted as an extra precaution. Other sources of error were investigated. It is interesting, however, that there are reports of esterification continuing in the presence of up to 10% water in the sample (Eder *et al* 1992a).

Fatty acid esters may have been lost during the extraction procedure or have not been completely extracted. One of the major problems with the extraction procedure was the loss of solvent from the test-tubes during mixing. Ground glass stoppers formed an inadequate seal and allowed the diethyl ether to escape, reducing recoveries. Other groups recommend the routine use of test tubes with Teflon-lined screw caps (Eder *et al* 1992a). Therefore, these were evaluated. Teflon liners are necessary since the solvents leaching plasticizers from the plastic which may interfere with the subsequent gas chromatography of the fatty acid esters. This is discussed further elsewhere (see section). Great care was taken to avoid residual water in the cap after washing (see above).

The screw cap allowed for better mixing of the solutions and prevented the escape of the diethyl ether during the extraction procedure. The tight seal also reduced the risk of evaporation of methanol during incubation. Although the use of these tubes did not improve the recoveries or the reproducibility of the results, they were more convenient and were therefore used throughout subsequent experiments. Again, using Teflon lined screw capped vials reduced the number of yields which were greater the maximum.

As mentioned previously, it was possible that oxidation was causing loss of fatty acid esters during the extraction. Contrary to previous reports that the presence of the antioxidant BHT is beneficial in all lipid extractions (Hamilton *et al* 1992) and esterification reactions (Eder *et al* 1992a), no benefit was observed here. Moreover, the presence of large quantities of BHT produced GC-MS peaks which interfered with the gas chromatography of the esters, particularly those of the C18 fatty acids. BHT is also reported to form free radicals at high temperatures (Frenkel 1980) and at high concentration is a pro-oxidant (Christie 1989), leading to the oxidation of fatty acids (see chapter 1). With BHT present in all the extracting solvents, its final concentration was difficult to control as the repeated evaporation of solvents leads to a build up of high concentrations. With BHT present in only the storage solvents, the final concentration is much lower and does not affect gas chromatography. BHT was therefore omitted from the esterification and extraction procedure but included in the hexane used to redissolve the samples for storage. All samples were stored under an atmosphere of nitrogen at -

20°C, which should minimise sample oxidation. The autosample facility on the GC-MS was routinely used throughout this work. BHT was included in the hexane used here to prevent oxidation while the samples awaited injection.

Oxidation could occur during the extraction procedure. For example, while in the freezing bath, the diethyl ether layer containing the fatty acids was not submerged in the ethanol, because the freezing bath was not deep enough, and was exposed to light, (both heat and light catalyse oxidation of fatty acids) (Frenkel 1980). The time the tubes spent in the freezing bath varied from sample to sample and day to day as did the temperature of the laboratory. To avoid these problems as far as possible, the time taken for extraction was reduced from up to six hours to 45 minutes and was carefully standardised. Phase separation by freezing was discontinued in favour of separation using a Pasteur pipette and the aqueous washings were back extracted.

A variety of steps were taken to optimise the esterification reaction. Heating during the esterification was changed from using a dry incubator to using an aluminium heat block which may improve the transfer of the heat as aluminium is a better conductor of heat than air. Recoveries of esters were also improved by increasing the concentration of the catalyst, H_2SO_4, to 2% from 1%. This may have had two effects, improved efficiency of catalysis or possibly better dehydration of the alcohol.

The final modification made was to increase the temperature of incubation from 50°C to 60°C. The maximum temperature is limited by the boiling point of methanol (64.7°C). At this higher temperature, the incubation time could be reduced, reducing the risk of oxidation. Although none of the small modifications made significantly improved the results, cumulatively they increased the recoveries of fatty acid methyl esters to quantitative and reduced the variability.

Using standard methyl esters, it was shown that the losses during the revised esterification and extraction procedure were negligible. In the second part of this study, where standard esters were incubated in the incubator, some losses were incurred possibly due to oxidation. The products of oxidative breakdown of fatty acids are aldehydes and ketones which are not detected on the gas chromatograph using the conditions employed here.

There are several alternative methods of fatty acid ester synthesis and choice may depend on the aims of the experiment and the local conditions. Here it was desired that the esterification procedure should be suitable for the study of all the lipid groups. For example, free fatty acids and sphingomyelin cannot be esterified using base-catalysed esterification (Christie 1989) whereas acid-catalysed esterification is universally effective.

In 1968, the American Oils Chemists Society recommended the use of boron trifluoride methanol as an approved method for ester formation (Firstone 1969). A variety of authors had previously reported the successful use of this reagent (Morrison & Smith 1964). Boron trifluoride methanol is toxic and unstable and the use of old regent has been reported to cause the decomposition of PUFA and production of methoxy-substituted derivatives and other artefacts (Lough 1964, Fulk & Shorb1970, Shepard & Inverson 1975). It is possible to use other trihalides. For example, boron trichloride methanol has been reported to produce fatty acid esters without destruction of PUFA (Klofenstein 1971). The same group suggest that boron trifluoride in methanol only produces artefacts when the conditions employed are too harsh and that, when esterification is carried out in an inert environment, no artefacts occur (Klofenstein 1971). Because of the toxicity of boron trifluoride methanol and the risk of polyunsaturated fatty acid degradation, this reagent was not investigated here.

The use of acid-catalysed esterification is simple, rapid and, providing the conditions are carefully controlled, the results are highly reproducible. The use of methanolic hydrochloric acid has been preferred by some groups (Christie 1989) while others recommend the use of sulphuric acid (Hitchcock & Hammond 1980). The reagents are inexpensive and can be prepared fresh daily, avoiding the problems of degradation and concentration variation with time.

Eder *et al* (1992a) has evaluated the use of a variety of esterification procedures, paying particular attention to the esterification of small amounts of phospholipids. The methods tested were based on boron-trifluoride methanol, methanolic sodium methoxide and methanolic sulphuric acid. This paper suggests that the only suitable method for methylation of phospholipid-bound fatty acid is methanolic sodium methoxide. However, as this is a base-catalysed esterification, it was not suitable for use here. The use of methanolic sulphuric acid is not recommended by the author but the conditions they employed were harsh (6% sulphuric acid and 90°C). The authors state that under these

conditions, there was no loss of polyunsaturated fatty acids (Eder *et al* 1992a). However, in the same paper they also suggest that high concentrations of acid and high temperatures may be responsible for the loss of fatty acids esterified to sphingomyelin, which may explain why the authors observed poor recoveries of fatty acids from phospholipids. It is clear from this study and others that there is a great variation in the performance of methods.

Although some of the evidence would dissuade from the use of acid-catalysed esterification, many people report it as their method of choice because the reagents are stable, relatively safe and inexpensive. The results here show that the method can be both accurate and reproducible.

3.3.1 Separation of Neutral Lipids Using Silica Sep-Pak Cartridges

Complete analysis of the cell membrane requires that the neutral lipid fraction is separated into its constituent groups. The method of Hamilton and Comia (1988) reports the complete and quantitative separation of cholesterol ester, triglycerides, partial glycerides and free fatty acids. It utilises radioactively labelled standards to estimate the recoveries. Here an attempt has been made to recover fatty acids from the individual lipid groups both quantitatively and without mutual contamination. This method evaluated here is based on the use of silica Sep-pak cartridges as recommended by Hamilton and Comia (1988).

3.3.2 Methods

The separation method (Hamilton & Comia 1988) is as follows:-

1. Silica Sep-pak cartridges were activated with hexane/MTBE (96:4 v/v) (4ml) followed by hexane (12ml). The eluate was discarded.

2. The lipid sample was dissolved in hexane/MTBE (200:3 v/v) (2ml) which was applied to the cartridge. The test tube was washed with a further 2ml of solvent which was also applied to the cartridge followed by a further 10ml of the solvent. The total eluate was saved as **fraction 1** containing cholesterol esters.

3. Hexane/MTBE (96:4 v/v) (12ml) was then applied to the cartridge and the eluate saved as **fraction 2** containing triglycerides.

4. Hexane/acetic acid (100:0.2 v/v) (12ml) was added to the cartridge and the eluate saved as **fraction 3**.

5. Hexane/MTBE/acetic acid (100:2:0.2 v/v/v) (12ml) was then applied to the cartridge and the eluate saved as **fraction 4** containing free fatty acids.

6. MTBE/acetic acid (100:0.2 v/v) (12ml) was applied to the cartridge and the eluate saved as **fraction 5** containing partial glycerides.

Fractions were collected in Pyrex tubes and evaporated to dryness under nitrogen at room temperature. Fatty acid methyl esters were produced and extracted as described previously (section 3.2.3c). The volume of eluting solvent was on occasion increased to improve the recovery of lipids; the volume used will be given in the results section. In general, where the recovery was not adequate, the volume of eluting solvent was increased to 15 ml.

3.3.3 Experimental procedures.

Each lipid group was studied in turn using standard lipids (section 3.1.1). The samples were added to a test tube and the solvents removed by evaporation under nitrogen. The samples were then applied to the Sep-pak cartridge and eluted following the protocol above.

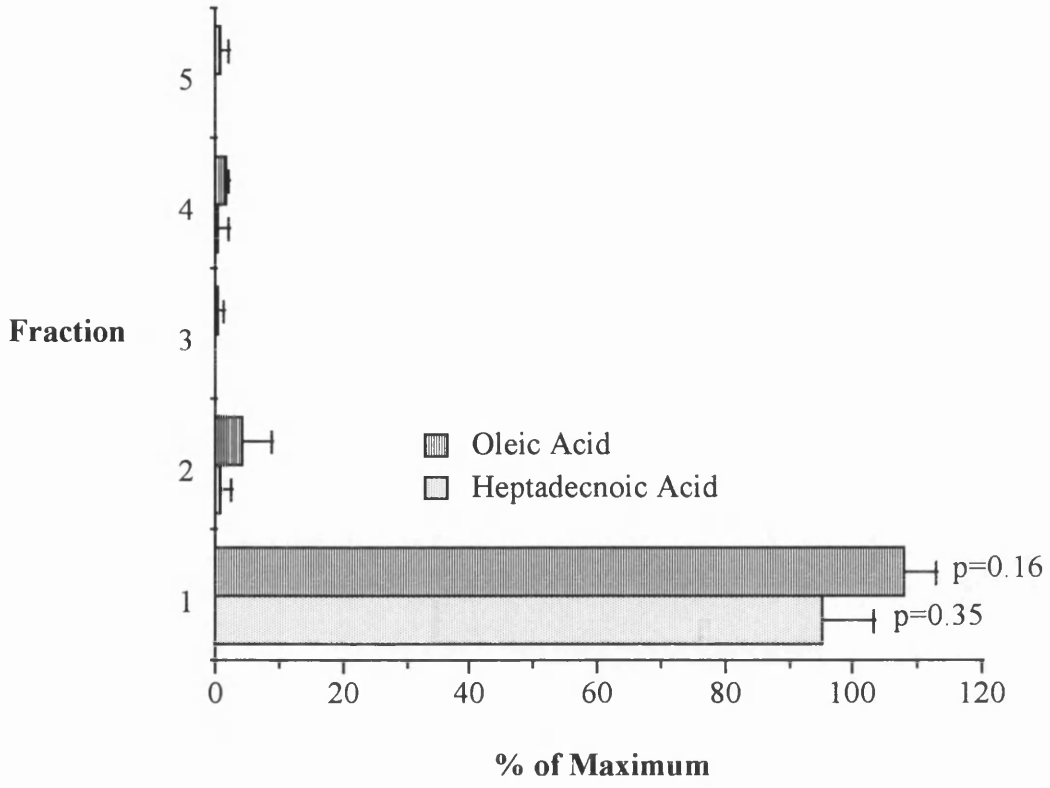
3.3.4 Results

Early gas chromatograms contained contaminating peaks. These were avoided if the elution rate from the Sep-pak cartridge was maintained with a constant negative pressure from a water pump which shortens the elution time and if all solvents were stored in glass bottles with glass lids. The probable origin of these peaks will be discussed later. Initially, BHT was present in all the eluting solvents to prevent oxidation of the lipids during the elution procedure. However, BHT was later eliminated from all solvents except those used for sample storage for the reasons described above. Recoveries for the individual lipid groups were as follows:-

3.3.4.a Cholesterol Esters.

The results (graph 3.3.4.a) can be summarised as follows:-

Cholesterol esters were recovered quantitatively in fraction 1, which contained 95.15% (8.10) and 107.92% (4.95) of maximum heptadecanoic and oleic acid methyl esters from cholesterol esters respectively. This did not differ significantly from maximum ($p=0.35$ and 0.16). There was no difference between the percentage recovery of research and internal standards ($p=0.06$).



Graph 3.4.4a Elution pattern of cholesterol esters from silica Sep-paks showing the percentage of maximum recoveries of the fatty acid methyl esters produced and standard deviation for each fraction and the p values for an unpaired T-test (n=6).

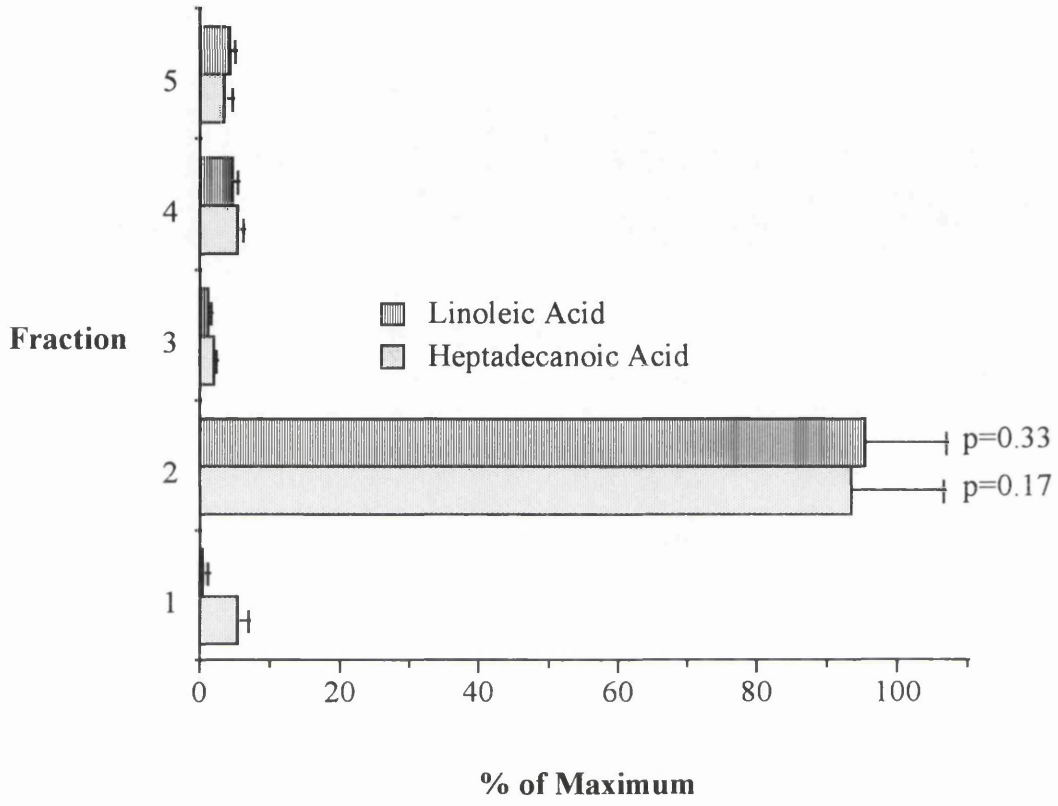
3.3.4.b Triglycerides.

The results (table 3.3.4.b and graph 3.3.4b) can be summarised as follows:-

Triglyceride recovery was not quantitative. Fraction 2 contained 68.89% (4.72) and 72.16% (7.48) of the maximum for linoleic acid and heptadecanoic acid respectively. The elution volume for fraction 2 was therefore increased to 15ml. The recoveries of trigylcerides were then quantitative. The percentage of maximum recoveries of heptadecanoic and linoleic acid methyl ester liberated from triglycerides were 93.38% (13.29) and 95.35% (11.39) respectively (p=0.17 and 0.33). There was no difference between the percentage recovery of research and internal standards (p=0.64).

Fraction	% of Maximum of Heptadecanoic Acid Methyl Ester	% of Maximum of Linoleic Acid Methyl Ester
1	6.81 (4.51)	1.74 (1.12)
2	72.16 (7.48)	68.89 (4.72)
3	0.74 (0.84)	2.14 (1.17)
4	0 (0)	2.91 (2.39)
5	0.37 (0.90)	3.48 (1.03)

Table 3.3.4.b Percentage of maximum recoveries and standard deviations for each fraction from the Sep-pak cartridges for fatty acid methyl esters liberated from triglycerides (n=6).



Graph 3.3.4b. Elution pattern of triglycerides from silica Sep-pak cartridges showing the percentage of maximum recoveries of the fatty acid methyl esters produced and the standard deviation and p values for an unpaired T-test (n=6)..

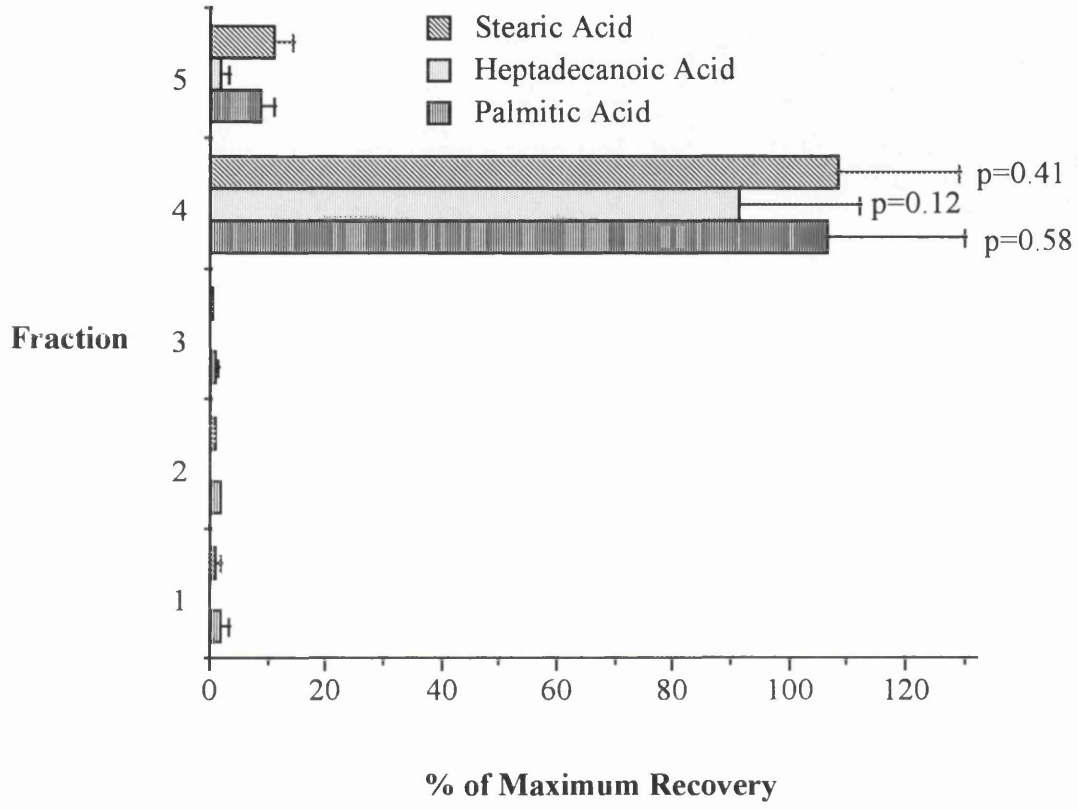
3.3.4.c Free Fatty Acids.

The results (table 3.3.4.c and graph 3.3.4c) can be summarised as follows:-

Initially the recovery of free fatty acids was not quantitative. Fraction 4 contained 51.61% (3.62), 47.24% (2.85) and 50.92% (5.01) of palmitic acid, heptadecanoic acid and stearic acid methyl esters respectively. The volume of eluate for fraction 4 was increased to 15ml. This made recovery of free fatty acids quantitative; the percentage of fatty acid recovered was 106.63% (23.21), 91.61% (20.51) and 108.65% (20.44) for palmitic, heptadecanoic and stearic acid respectively. These recoveries did not differ significantly from maximum (p=0.58, 0.12 and 0.41). There was no difference between the percentage recovery of research and internal standards (p=0.09 and 0.1 for palmitic and stearic acid).

Fraction	% of Maximum Palmitic Acid Methyl Ester	% of Maximum Heptadecanoic Acid Methyl Ester	% of Maximum Stearic Acid Methyl Ester
1	0.63 (0.33)	0.03 (0.08)	0.22 (0.12)
2	0.97 (0.30)	0.02 (0.06)	0.34 (0.10)
3	0.32 (0.27)	0 (0)	0.14 (0.10)
4	51.61 (3.62)	47.24 (2.85)	50.92 (5.01)
5	7.00 (6.44)	0.20 (0.34)	5.62 (3.16)

Table 3.3.4.c Percentage of maximum recoveries for free fatty acids eluted from a Sep-pak cartridge. Volume of fraction 2 equals 15 ml (n=6).

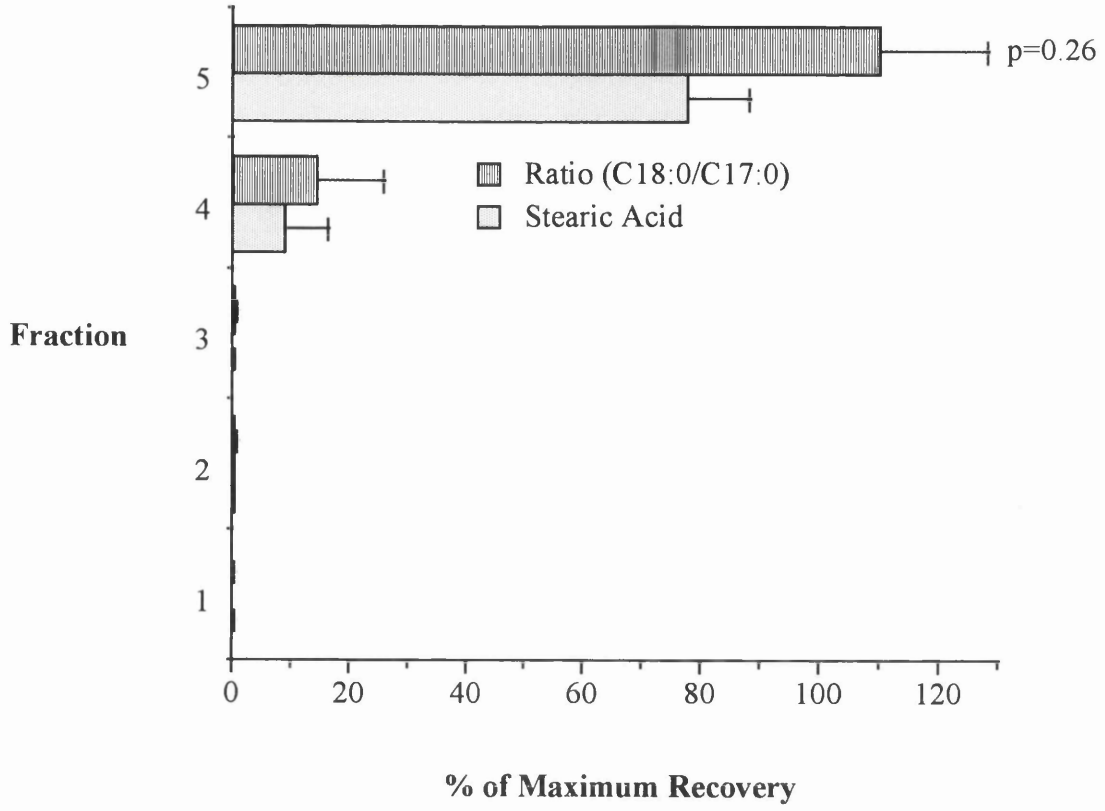


Graph 3.3.4c. Elution pattern of free fatty acids from Sep-paks showing the percentage of maximum recoveries of the fatty acid methyl esters produced and the standard deviation and p value for and unpaired T-test (n=6).

3.3.4.d Partial Glycerides

The results (graph 3.3.4.d) can be summarised as follows:-

As the volumes of the previous fractions were increased to 15ml, the volume of fraction 5 was also increased. The recoveries of partial glycerides were not quantitative. The percentages of maximum stearic acid and heptadecanoic acid recovered from fraction 5 were 77.94% (10.53) and 73.55 (5.79) respectively. The ratio of C18:0/C17:0 eluted with fraction 5 was not significantly different from the maximum (p=0.26) and the research and internal standard behaved in the same way (p=0.22).



Graph 3.4.4d Elution pattern of partial glycerides from silica Sep-pak cartridges showing the percentage of maximum recoveries of the fatty acid methyl esters produced and the standard deviations and p values for and unpaired T-test (n=6).

3.3.5. Discussion

In the initial experiments, contaminating peaks were observed on the GC-MS which interfered with the chromatography of the C18 and C20 fatty acids. These appear to have come from the plastic surfaces with which the solvents came into contact (the Sep-pak cartridge and the lids of the solvent bottles) as the peaks disappeared when the solvents were stored in all-glass bottles and the flow rate of the solvents through the Sep-pak cartridge was increased. In general, the greater the flow rate of solvent through a chromatography column, the poorer the resolution of the compounds to be separated. However, here a balance had to be found between adequate resolution of the lipid groups, good recoveries and avoidance of contaminating peaks. The occurrence of contaminating peaks during gas chromatography after the use of Sep-pak cartridges has been reported elsewhere. In his discussion of solid phase extraction, Christie (1992) suggests that these are plasticisers or more precisely phthalates. He states that these should be removed from the cartridge during the washing procedure. It would be possible to overcome this by purchasing glass silica-columns or producing silica columns in the laboratory. The cost of the first option is prohibitive, the second option would lead to variability in the results as the packing density and water content of the silica could not be easily controlled. Most of

the contaminating peaks observed here were probably derived from the plastic lids of the storage bottles.

After the initial experiments BHT, was omitted from the eluting solvents. All the solvents used for chromatography were degassed with nitrogen or by sonication to prevent oxidation. The reasons for omitting BHT have been discussed previously.

In the initial experiments involving the separation of free fatty acid and triglyceride fractions, the recoveries were poor and variable. These were improved to approximately 100% by increasing the elution volume. The use of larger volumes of solvent than necessary is, however, to be avoided, again due to the risk of oxidative losses during evaporation of eluates to dryness. This is discussed further in the next section. The fatty acid liberated from the partial glycerides could not recovered quantitatively. However, since both the internal standard and the research standard had similar recoveries, valid quantitation by gas chromatography was possible using the Magnum software package without further correction for losses. Fraction three contained no lipid and functions to acidify the column. Altering the elution volumes of the other fractions did not alter this situation. Increasing the volume of earlier fractions did not lead to premature elution of free fatty acids or partial glycerides in this fraction.

The modified method is fast and efficient and does not require large amounts of specialised equipment or large volumes of solvent. The recoveries of all the lipid groups either did not differ significantly from maximum or from the recovery of the internal standard. It has also been established that the recoveries of neutral lipids containing the internal standard heptadecanoic acid were identical to those containing naturally occurring fatty acids. Therefore these are valid recovery standards for lipids from biological samples.

The most commonly used techniques for the separation of neutral lipids are thin layer chromatography (TLC) and high performance liquid chromatography (HPLC). TLC is effective for the separation of neutral lipids, but many of the solvent systems reported to separate lipids contain acetic acid (Mangold & Malins 1960, Henderson & Tocher 1992) which introduces the risk of hydrolysis. As the recovery estimate for the lipids depended on subsequent assessment of fatty acid content, the use of acetic acid in solvent systems should be avoided. This will be discussed in more depth in the next section. Other TLC techniques involve a double development of the plate with drying between (Conte &

Bishop 1988), which again introduces the additional risk of oxidation. Thin layer chromatography has a limited sample capacity and overloading causes poor resolution of the lipid groups. There are also difficulties in recovering samples quantitatively because they must be scraped off the plate which is inefficient and time consuming. It also exposes the lipids to oxidation because recovery from the plate cannot be performed in an inert atmosphere. A modified TLC method has been reported for the isolation of partial glycerides which involves TLC followed by flame ionisation detection (Okumura *et al* 1988). This requires specialised equipment but it has been used successfully to separate cholesterol and phospholipids (Rosasromero *et al* 1996). A full discussion of this method is contained within the introduction (section 1.16).

HPLC, although efficient at separating lipid groups, also requires expensive equipment and, depending on the detection system used, identification of the lipid groups can be difficult. Retention times on HPLC vary with changes in atmospheric conditions, making identification by retention time alone unreliable. Also, because the lipids have a range of fatty acids esterified to them, the peaks they produce are diffuse. It would be possible to separate the lipids by preparative HPLC and then reapply the individual lipid groups under different conditions to separate the lipids with reflect their fatty acid composition.

Many groups use silica cartridges to separate the neutral lipids from polar lipids (Bitman *et al* 1983, Ansari & Shoeman 1988, Hamilton & Comia 1984, Janero & Burghardt 1990). Also, the use of aminopropyl-bonded phase disposable columns has been shown to be effective for the separation of the lipids studied here, providing quantitative recoveries of lipids (Kalunzy *et al* 1985). This method was not tested here because the recoveries of the polar lipids have proved unreliable and it was desirable to use the same method to separate the neutral and polar lipids. This will be discussed further later.

The use of disposable Sep-pak cartridges allows for a high capacity, fast, efficient and relatively inexpensive separation of lipid groups. The lipids are separated as a function of their own polarity and that of the solvent with the least polar lipids being eluted first. The principle of this and other solid phase extraction procedures are similar to those of HPLC involving adsorption and partition chromatography (see chapter 1).

3.4.1 Separation of Polar Lipid Groups Using Silica Sep-Pak Cartridges.

For the complete analysis of the cell membrane, it was necessary to isolate and separate the major phospholipid groups. The method chosen, as for the neutral lipids, was that of Hamilton and Comia (1988). In this method, polar lipids were separated into two groups, with PI and PE in one group and PC, LPC and sphingomyelin in the other. Recoveries were measured using standard phospholipids in which the base group was radioactively labelled. In the following modifications to this method, particular attention has been paid to obtaining quantitative recoveries of non-radioactive lipids. Here, this has been monitored by measuring the recovery of fatty acid methyl esters produced from recovered lipids as previously described.

3.4.2. Method

The use of a gradient solvent systems to elute neutral lipids from a silica Sep-pak cartridge was as described on section 3.3.1. The initial method for the separation of the polar lipids is a continuation of this method and was as follows.

1. Neutral lipids were eluted from the cartridge as described previously (see section 3.3.1), ending with the elution of partial glycerides with MTBE/Acetic acid (100:0.2 v/v) (15ml) **fraction 5**.

2. MTBE/methanol/aqueous ammonium acetate (25:4:1 v/v/v) (pH 8.6) (8ml) was applied to the cartridge and the eluate saved as **fraction 6** containing PI and PE.

3. MTBE/methanol/aqueous ammonium acetate (10:4:1 v/v/v) (pH 8.6) (12ml) was applied to the cartridge and the eluate saved as **fraction 7** also containing PI and PE. Fraction 6 and 7 are pooled at this stage.

4. MTBE/methanol/aqueous ammonium acetate (5:8:2 v/v/v) (pH 8.6) (12ml) was applied to the cartridge and the eluate saved as **fraction 8** containing LPC, PC and sphingomyelin.

5. MTBE/methanol/aqueous ammonium acetate (5:4:1 v/v/v) (pH 8.6) (12ml) was applied to the cartridge and the eluate saved as **fraction 9** also containing LPC, PC and sphingomyelin. This was repeated and the eluate saved as **fraction 10** also containing LPC, PC and sphingomyelin. Fractions 8, 9 and 10 are pooled at this stage.

The concentration of aqueous ammonium acetate used in the preparation of the solvents was 0.3mM dissolved in distilled water.

To evaluate recovery and specificity, each lipid group was studied separately using standards (the fractions were not pooled but analysed separately). The preparation of fatty acid methyl esters and their subsequent analysis was as described in 3.1.1.

3.4.3 Experimental

As a preliminary evaluation of this method, the following questions were addressed:-

1. Was the elution pattern of the lipids as published? To test this, the method outlined in 3.4.2 was used to evaluate the recovery and fractional distribution of PI.

2. Was the acetic acid present in the solvents for the elution of neutral lipids affecting the elution and recovery polar lipids? This was investigated by replacing solvents 3-5 (neutral lipid separation) with solvents containing a range of acid concentrations. The volume of solvent 3 was also manipulated. Table 3.4.3.b shows the composition and volume of the solvents used. The solvents will be referred to by the numbers in the table. Here the results are expressed as % recovery of the total fatty acid.

Solvent	Volume of Acid (per 100ml solvent)	Volume of Fraction 3 Applied to the Cartridge
Solvent 1	0	12 ml
Solvent 2a	0.1	2 ml
Solvent 2b	0.1	4 ml
Solvent 2c	0.1	12 ml
Solvent 3a	0.2	2 ml
Solvent 3b	0.2	4 ml
Solvent 3c	0.2	12 ml
Solvent 4a	0.4	2 ml
Solvent 4b	0.4	4 ml
Solvent 4c	0.4	12 ml

Table 3.4.4.b3 Volume of acid present in all solvents and volume of fraction 3 applied to the cartridge in experiment 2.

3. Is it possible to remove the neutral lipids from the Sep-pak cartridge with one solvent, allowing for subsequent separation of polar lipids? This was tested using the protocol described below. The final solvent in the elution system for the neutral lipids was MTBE/acetic acid (100:0.2 v/v), which should remove all the neutral lipids from the Sep-pak cartridge. As the omission of acid from the solvent system was necessary, the ability of MTBE alone to elute the neutral lipids was tested using the protocol described below.

A. Palmitic acid, heptadecanoic acid, trilinolein, cholesterol oleate and PI (containing mostly stearic and arachidonic acid) were dissolved in MTBE (2ml) and applied to a prewashed Sep-pak cartridge. A further 2ml of MTBE was added to the test tube and

mixed before being added to the cartridge. MTBE (10ml) was then added to the cartridge. The combined eluate was collected in Pyrex culture tubes. This formed **fraction 1**.

B. The polar lipid separation procedure described in 3.4.2 was carried out beginning with the addition of MTBE/MeOH/aqueous ammonium acetate (25:4:1 v/v/v) (pH 8.6) (12ml) to the cartridge. This formed **fraction 2.**

C. MTBE/MeOH/aqueous ammonium acetate (10:4:1 v/v/v) (pH 8.6) (12ml) was then added to the cartridge forming **fraction 3**.

D. MTBE/MeOH/aqueous ammonium acetate (5:4:1 v/v/v) (pH 8.6) (12ml) was then added to the cartridge forming **fraction 4.**

E. MTBE/MeOH/aqueous ammonium acetate (5:8:2 v/v/v) (pH 8.6) (12ml) was then added to the column forming **fraction 5**. This was repeated forming **fraction 6**.

The above procedure was repeated using distearin to assess whether partial glycerides were quantitatively removed by MTBE. The samples were analysed as described previously (see section 3.1.1 and 3.1.2.).

4. Was the recovery of the polar lipid groups improved using the above method? To test this, each of the polar lipid groups were studied in turn; the sample analysis was as described previously. Where the volume of the solvent used was altered to increase the recovery of the lipids, the volume used is given in the results. In general, where the recovery of the lipid was inadequate, the volume of eluting solvent was increased to 15ml.

5. Were the lipids being applied to the cartridge in a quantitative manner? To test this, the lipid was dissolved in MTBE as usual and then transferred to another test tube before the removal of the solvent and methylation. This simulated the addition of the sample to the Sep-Pak cartridges. Samples were also dissolved in MTBE (2ml) and sonicated in a water bath for 2 min prior to treatment as above. The effect of removal of solvents by evaporation under nitrogen on the recovery of the fatty acids was evaluated by adding the lipid to of appropriate solvent (15 ml) and to dry under nitrogen prior to methylation (PE and LPC were used for this study).

3.4.4 Results

N.B. Where ratio of research standard/internal standard was expressed as a percentage of maximum. The value of the ratio for the unchromatographed standards was taken as

maximum and the value of ratio obtained for the chromatographed samples was compared to this and expressed as a percentage.

3.4.4a Elution of PI using the original method.

The method of Hamilton and Comia (1988) did not separate the polar lipid groups adequately (table 3.4.4.a). Fraction 7 contained 84.53% (12.17) of the maximum fatty acid liberated from PI. However, fractions 4, 5 and 6 contained 20.44% (10.73), 25.78% (23.39) and 17.33% (10.73) of the fatty acids respectively. The variability of the results obtained were unacceptably high.

Fraction	% of Maximum Fatty Acid
4	20.4 (10.73)
5	25.78 (23.39)
6	17.33 (11.36)
7	84.53 (12.17)
8	12.67 (3.29)
9	4.5 (0.61)

Table 3.4.4. a Elution pattern of PI showing the percentage of maximum fatty aids for the fatty acids liberated from PI following the original method of Hamilton and Comia (1988).

3.4.4b The effect of acetic acid on the elution pattern of the lipids.

The results (tables 3.4.4 b1-b4) can be summarised as follows:-

The elution pattern which most closely matched the published pattern was obtained with no acid present in the solvent. No lipid was found in fraction 4. With the other eluting solvents, the volume of fraction 3 was also varied. In general, the greater the volume of fraction 3, the greater the quantity of lipid observed in fractions previous to fraction 6 (i.e. the fractions where neutral lipids elute). Solvent 4, which contained 0.4% acetic acid caused the elution of lipid earliest in the protocol.

In a separate experiment, PI was added to a test tube containing 0.2% acetic acid in methanol, mixed and left to stand in the bench for 10 min. Subsequent extraction of showed a small amount of fatty acid methyl esters has been released from PI.

Fraction	% of Total fatty acid eluted with solvent 1
3	0
4	0
5	14.17
6	7.18
7	63.26
8	13.02
9	2.36

Table 3.4.4.b1. Percentage of maximum recoveries of the fatty acid methyl esters produced from PI after elution from the Sep-pak cartridgesusing solvent 1, containing no acid.

Fraction	% of total fatty acid eluted with each solvent		
	Solvent 2a	Solvents 2b	Solvent 2c
3	0	0	0
4	0	0	12.63
5	46.27	42.63	53.86
6	10.68	7.93	13.94
7	26.08	32.46	13.66
8	11.70	13.51	5.90
9	5.27	3.467	0

Table 3.4.4.b2 Percentage of maximum recoveries of the fatty acid methyl esters from PI after elution from the Sep-pak cartridge using solvent 2 a, b and c containing 0.1 ml acetic acid

Fraction	% of total fatty acid eluted with each solvent		
	Solvent 3a	Solvent 3b	Solvent 3c
3	0	0	0
4	0	10.47	18.8
5	6.24	37.80	19.6
6	7.45	7.24	5.8
7	20.92	25.90	39.29
8	7.63	13.49	13.68
9	2.75	5.09	2.72

Table 3.4.4.b3 Percentage of maximum recoveries for the fatty acids produced from PI after elution from the Sep-pak cartridges using solvent 3 a, b and c containing 0.2 ml acetic acid.

Fraction	% of total fatty acid eluted with each solvent		
	Solvent 4a	Solvent 4b	Solvent 4c
3	0	1.09	0.96
4	18.26	18.87	21.49
5	32.45	24.68	15.07
6	9.22	7.49	6.09
7	23.72	37.22	39.45
8	12.83	7.98	13.79
9	3.52	1.62	3.14

Table 3.4.4.b4 Percentage of maximum recoveries for the fatty acids produced from PI after elution from the Sep-pak cartridges using solvent 4 a, b and c containing 0.4 ml acetic acid.

3.4.4c Removal of Neutral Lipids from the Sep-pak cartridge using MTBE.

The results (table 3.4.4.c) can be summarised as follows:-

The neutral lipids were eluted with both fractions 1 and 2 of the solvent system described in 3.4.3. The amount lipid of recovered did not vary significantly from maximum. Fraction 1 contained 95.76% (10.27) of linoleic acid from trilinolein, 99.76% (15.30) of oleic acid from cholesterol oleate and 93.66% (16.72) of stearic acid from partial glycerides. Fraction 2 contained 97.33% (10.38) of the free fatty acid, palmitic acid ($p=0.2645$ for palmitic acid $p=0.2899$ for the linoleic acid, $p=0.9729$ for oleic acid, $p=0.47$ for stearic acid). The 62.10% (11.43) of PI was eluted from the column with fraction 3 none was eluted in fractions 1 and 2. Therefore, there was no residual neutral lipid to interfere with the polar lipid analysis.

Fraction	Palmitic	Linoleic	Oleic	Stearic	Stearic From PGS
1	6.96 (4.44)	95.13(10.27)*1	99.76(15.30)*2	6.29 (1.16)	93.66(16.74)*4
2	97.33(10.38)*3	0	2.05 (0.53)	9.04 (2.53)	4.88 (1.96)
3	3.47 (3.84)	1.40 (2.18)	9.25 (1.98)	62.10 (11.43)	2.59 (0.71)
4	7.41 (0.66)	0.22 (0.53)	3.08 (0.45)	10.15 (2.30)	1.38(0.1)
5	2.36 (1.19)	0	0.55 (0.47)	2.54 (0.50)	0
6	2.72 (0.29)	0	0.69 (0.36)	1.86 (0.22)	0

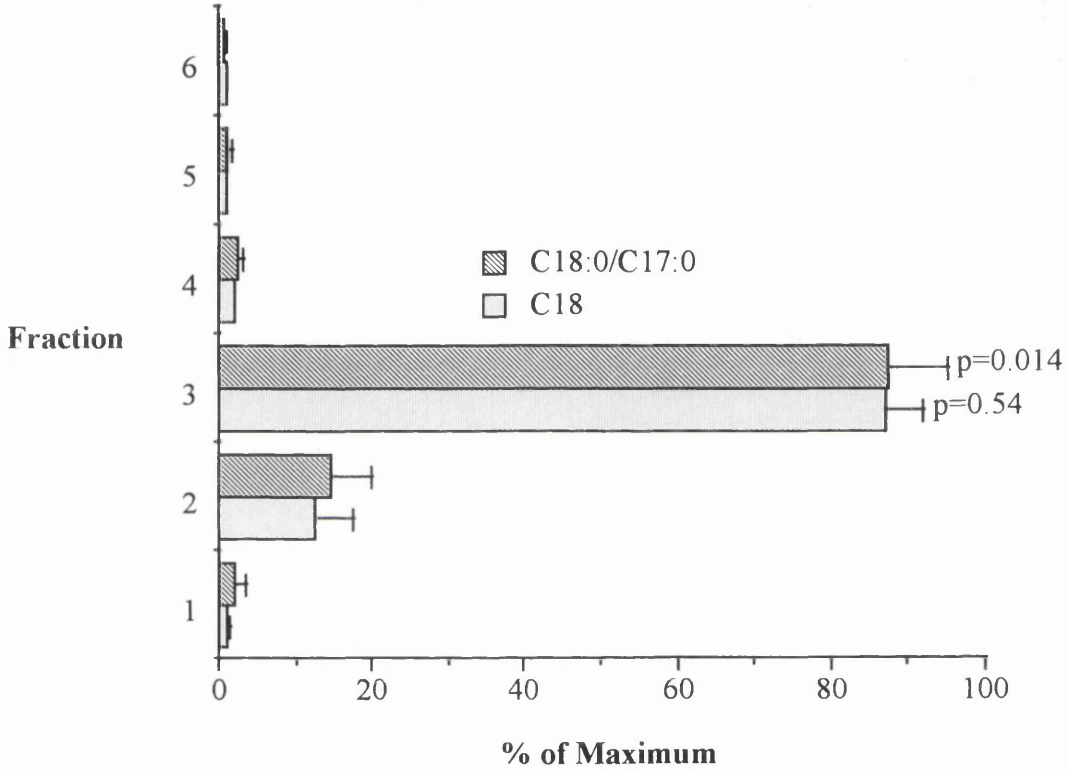
Table 3.4.4.c. Percentage of maximum fatty acids for the lipids stripped from the Sep Pak cartridge to allow for the elution of polar lipids. Palmitic acid was present as a free fatty acid, linoleic acid was liberated from trilinolein and oleic acid was liberated from cholesterol oleate. Stearic acid was liberated from PI. (n=6). *1 $p=0.290$ *2 $p=0.265$ *3 $p=0.973$ *4 $p=0.48$.

3.4.4d Efficiency of Separation and recovery of the Polar lipids after removal of the neutral lipids.

The results (table 3.4.4.d1) can be summarised as follows:-

3.4.4d1 Phosphatidylinositol

As has been shown previously, PI was present in fraction 3 of the modified elution system. The volume of the eluting solvent for fraction 3 was increased to 15 ml, which recovered then all the PI. The percentage of C18:0 liberated from PI present in fraction 3 was 87.10% (4.90), which did not differ significantly from maximum (p=0.54). The value obtained for C18:0/C17:0 in fraction 3 was 87.43% (7.685) (p=0.014). There was no difference between the recovery of research and internal standards (p=0.59).



Graph 3.4.4d1 Elution pattern of PI from silica Sep-pak cartridges showing the percentage of maximum recoveries of the fatty acids from PI, the standard deviations and p values for an unpaired T-test (n=6).

3.4.4d2 Phosphatidylcholine

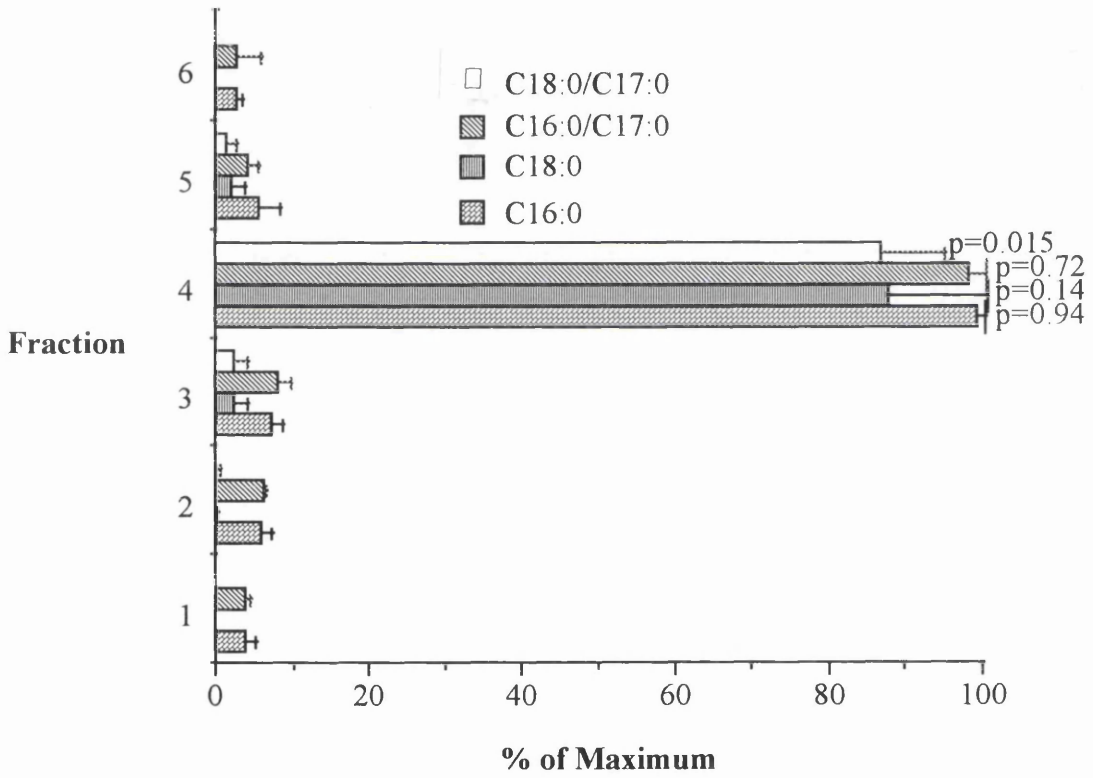
These results (table 3.4.4.d2 and graph 3.4.4.d2) can be summarised as follows:-

Most of the PC was eluted with fraction 4. The percentage C16:0/C17:0 in fraction 4 was 79.68 (3.598) and for C18:2/C17:0, the percentage present in fraction 4 was 69.23 (7.72). The volume of fraction 4 was increased to 15 ml, which completely recovered the PC. The percentage of maximum recoveries for palmitic and linoleic acid liberated from PC in fraction 4 were then 99.41 (12.95) and 87.85 (12.69) respectively (p=0.96 and 0.14). The percentage of C16:0/C17:0 present in fraction 4 was 98.218 (10.06), which was not

significantly different from maximum (p=0.72). The percentage of C18:2 / C17:0 present in fraction 4 was 86.60 (8.50) (p=0.015). There was no difference between the recovery of research and internal standards (p=0.74 and 0.19 for palmitic and linoleic acid).

Fraction	% of Maximum C16:0	% of Maximum C18:0	% of Maximum C16:0/C17:0	% of Maximum C18:2/C17:0
1	7.06 (1.45)	0.61 (0.79)	7.22 (1.298)	0.742(0.976)
2	5.66 (0.36)	0.24 (0.59)	5.705 (0.808)	0.248(0.608)
3	18.38 (2.56)	11.71 (2.49)	16.13 (1.635)	10.752 (3.668)
4	91.43 (12.56) * 1	79.37 (12.10) * 2	79.68 (3.598)	69.23 (7.718)
5	17.98 (6.63)	15.01 (5.94)	16.485 (5.509)	13.728 (5.019)
6	2.07 (0.64)	0	2.048 (0.403)	0

Table 3.4.4.d2. Percentage of maximum C16:0/C17:0 and C18:2/C17:0 for Phosphatidyl choline elution for Sep=pak cartridges standard deviations shown in parenthesis. Volume of fraction 3 = 15 ml. (n=6) * 1 p= 0.37 * 2 p=0.018



Graph 4.3.3d2 Elution pattern of PC from silica Sep-pak cartridges showing the percentage of maximum recoveries and ratios for the fatty acids from PC, the standard deviations and p values for an unpaired T-test (n=6).

3.4.4d3 Phosphatidylethanolamine

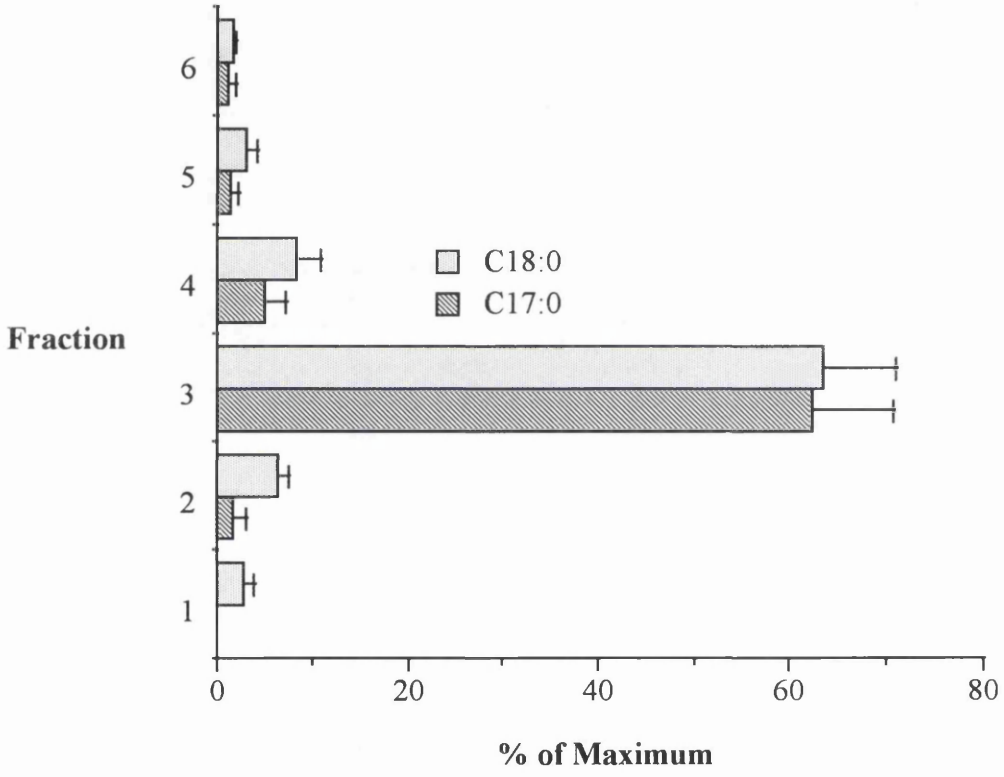
These results (tables 3.4.4.d3 3.4.4.d3) can be summarised as follows:-

The majority of PE was recovered in fraction 3, which contained 54.36% (23.38) of C17:0 and 56.93% (24.63) of C18:0 liberated from PE (p=0.0029 and 0.0052). The volumes of fractions 3-6 were increased to 15ml and the samples were sonicated for 2 min before their

application to the Sep-pak cartridge (see section xxxx). PE was adequately separated from the other lipid groups, but the recoveries were significantly less than 100%. Fraction 3 contained 62.27% (8.38) of C17:0 and 63.41% (7.76) of C18:0 liberated from PE. There was, however, no difference between the recovery of research and internal standards (p=0.81).

Fraction	% of Maximum C17:0	% of Maximum C18:0
1	0.29 (0.47)	1.24 (0.88)
2	0.76 (0.46)	3.79 (0.78)
3	54.36 (23.38)	56.93 (24.63)
4	3.50 (1.38)	4.52 (0.94)
5	0.51 (0.56)	1.71 (0.92)
6	0.61 (0.46)	1.68 (0.25)

Table 3.4.4.d3 % of maximum recoveries of the fatty acids liberated from PE were both fraction 3 and 4 were 15 ml in volume.



Graph 3.4.4.d3. Elution pattern of PE from silica Sep-pak cartridges showing the percentage of maximum recoveries for the fatty acid from PE, the standard deviation and p values of an unpaired T-test (n=6).

3.4.4d4 Lysophosphatidylcholine.

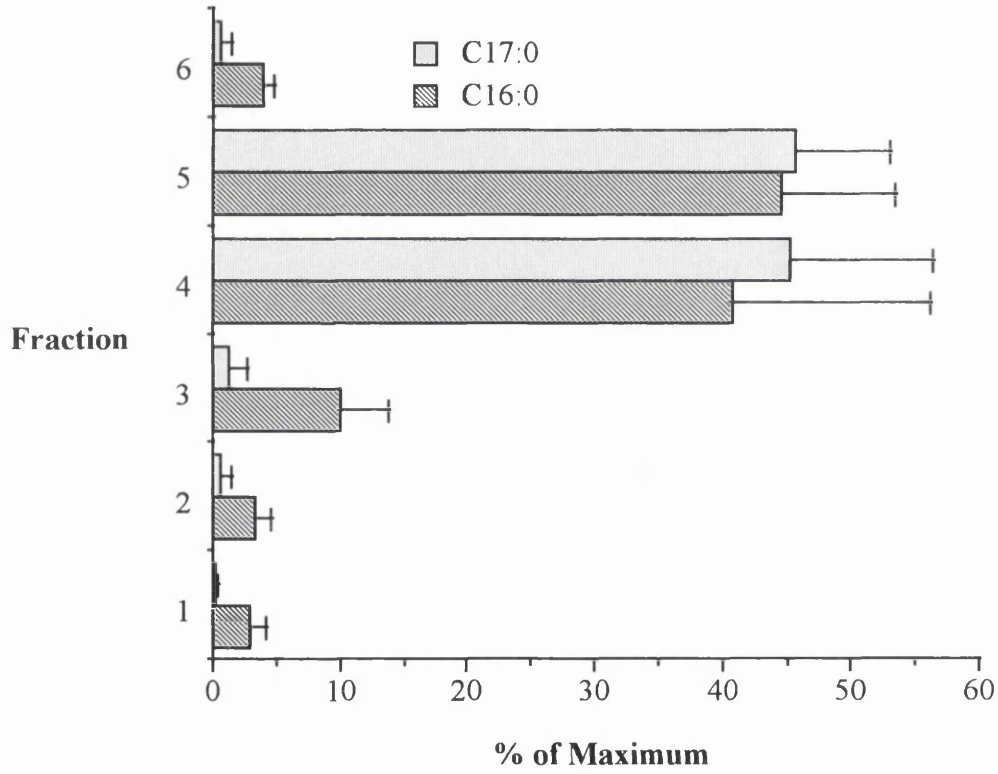
These results (table 3.4.4.d4 & graph 3.4.4.d4) can be summarised as follows:-

The recovery of LPC was significantly less than maximum. The percentage of C16:0 and C17:0 recovered from the lipid was 14.67% (10.76) and 13.24% (12.27) in fraction 4 and 14.67% (7.50) and 26.43 % (19.12) in fraction 5 respectively. Sonication of the sample prior to its application to the Sep-pak cartridge increased the recoveries of LPC. 40.94 %

(15.25) of C16:0 from LPC was eluted with fraction 4 and 44.62% (8.84) with fraction 5. Fraction 4 contained 45.28% (11.19) of C17:0 liberated from LPC and fraction 5 contained 45.79% (7.21). These were still significantly less than maximum. Adding to two fractions together gave a recovery of 92.20% (13.18) for C16:0 and 92.51% (12.62) for C17:0 ($p=0.25$ for both lipids). LPC was quantitatively recovered in fractions 4 and 5 combined. There was no difference between the recovery of research and internal standards ($p=0.97$).

Fraction	% of Maximum C16:0	% of Maximum C17:0
1	4.17 (1.55)	1.47 (1.59)
2	3.99 (0.75)	0.86 (0.67)
3	9.31 (1.97)	1.95 (1.60)
4	14.67 (10.75)	13.24 (12.27)
5	14.67 (7.50)	26.43 (19.12)
6	3.81 (1.25)	2.32 (1.31)

Table 3.4.4.d4. Percentage of maximums for fatty acids liberated from LPC fractions 3-6 were 15 ml.



Graph 3.4.4.d4 Elution pattern of LPC from silica Sep-pak cartridges showing the percentage of maximum recoveries for the fatty acids from LPC, the standard deviations and p values for an unpaired T-test (n=6).

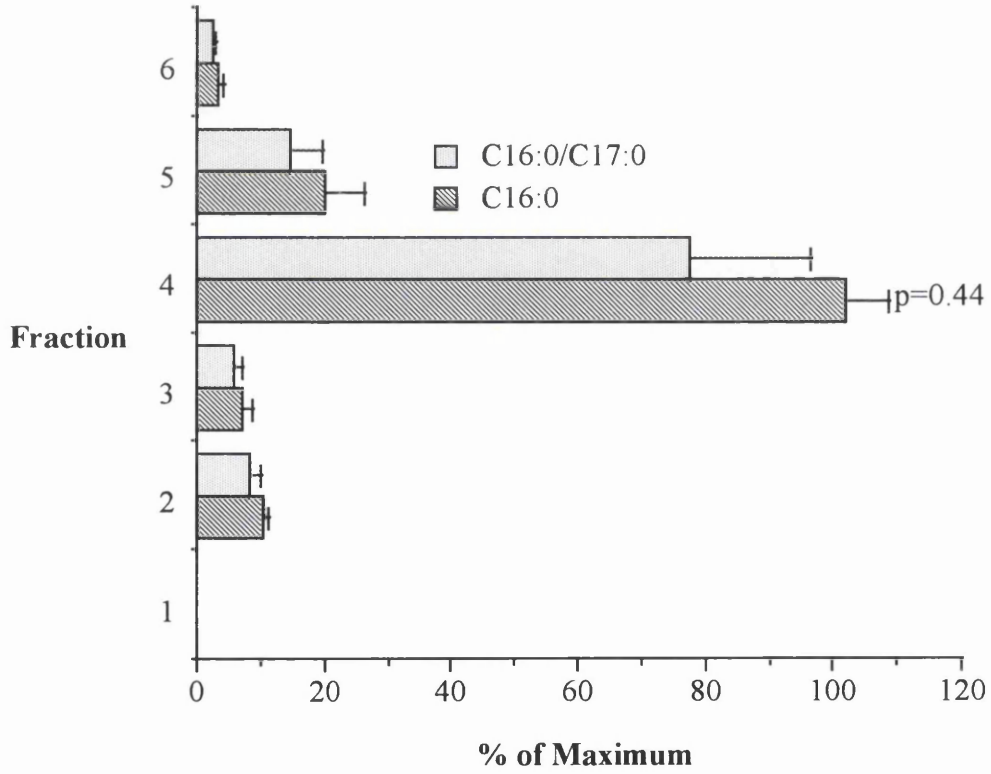
3.4.4d5 Sphingomyelin

As has been the case for all the lipids, fatty acid methyl esters were produced by acid catalysed transesterification. As there is no internal standard for sphingomyelin, recoveries were calculated using the ratio C16:0/C17:0. Using 2% sulphuric acid in methanol, the ratio obtained of the unchromatographed standards was lower than expected {0.61

(0.03)}. Using 5% acid in methanol for the esterification the ratio was still less then expected {0.68 (0.015)}. Overnight incubation at 50°C with hydrochloric acid : methanol (5:1 v/v) provided a ratio of 1.33 (0.03). This was closer to the expected ratio. The amount of heptadecanoic acid esterified and recovered using this method and the method using 1% sulphuric acids were not significantly different (p=0.54). This esterification protocol was therefore used routinely for sphingomyelin analysis.

The results for the separation of sphingomyelin using this esterification method (graph 3.4.4.d5) can be summarised as follows:-

Sphingomyelin was recovered quantitatively with fraction 4; 101.91% (6.71) of the palmitic acid liberated from sphingomyelin was recovered there. This was not significantly different from maximum (p=0.44). This fraction contained 77.42% (18.93) of the ratio C16:0/C17:0 maximum fatty acid (p=0.04). There was no difference between the recovery of research and internal standards (p=0.05).



Graph 3.4.4.d5 Elution pattern of SPG from silica Sep-pak cartridges showing the percentage of maximum recoveries of the fatty acids and the ratio of sample/internal standard for the fatty acid from SPG, the standard deviation and p values for an unpaired T-test (n=6).

3.3.5e Are the lipids being applied to the Sep-pak cartridge quantitatively ?

The results of experiments to simulate the application of the lipid to the cartridge can be summarised as follows:-.

In the absence of sonication, the recovery of PE and LPC was not quantitative, the percentages of C17:0 and C18:0 liberated from PE recovered being 78.66% (6.65) and 75.93% (8.19) respectively. For LPC, the percentages of C16:0 and C17:0 recovered were 3.94% (3.23) and 1.89% (3.34) respectively. Sonication of the sample led to recoveries of C17:0 and C18:0 from PE of 73.26% (31.04) and 75.34% (22.89) respectively which were not significantly different from maximum (p=0.15 and 0.08). For LPC recoveries for C16:0 and C17:0 were 96.39% (11.38) and 94.06 % respectively which were not significantly different from maximum (p=0.55 and 0.24).

The results of the experiment to test the effects of evaporation of solvent on the recoveries of PE can be summarised as follows:-

For PE, where the lipid was dissolved in the solvent, dried and esterified, the percentages of C17:0 and C18:0 recovered were 99.87% (3.97) and 99.38% (1.93) respectively which were not different from maximum (p=0.98 and 0.86).

3.4.1 Discussion

In the original report of this method, the polar lipids were eluted after the sequential elution of neutral lipids (Hamilton & Comia 1988). The polar lipids were not completely separated but were eluted in two distinct fractions, one containing PI and PE and one containing PC, LPC and sphingomyelin. Here this was not the case. Table 3.4.5 compares the expected elution pattern with the one obtained. PI was identified in fractions both before and after it was expected to elute.

Fraction Original	Reported Results	Obtained Results	Fraction Modified	Separation of Polar Lipids
1	CE	CE		
2	TRIG	TRIG		
3	FFA	FFA + PI		
4	PGS	PGS + PI	1	PGS + TRIG +CE
5	PI	PI	2	FFA
6	PI + PE	PI	3	PI + PE
7	PC+LPC+SPHING	PI	4	PC+SPHING+LPC
8	PC+LPC+SPHING	PI	5	LPC
9	LPC	PI	6	

Table 3.4.5 Difference in the elution pattern from reported using the original method and the modified method.

The method of estimating recovery may partly explain these differences. One method of estimating phospholipid recovery is to measure the phosphate content of samples (Bartlett 1959). This technique cannot differentiate between different phospholipids and is now rarely used. Hamilton and Comia (1988) used phospholipids with radioactively labelled base groups to differentiate between different phospholipid groups. Neither of these methods gives a measure of whether the lipids are being recovered intact. The assessment of recovery used here was to measure the recovery of fatty acids after methanolysis. That this could be done quantitatively had been ascertained using standards. The quantity of phospholipid present can be calculated from concentrations of fatty acids (Seewald & Eichinger 1989, Wang *et al* 1994). The results using this technique, in contrast to other methods, will take account of the chain lengths and molecular weights of the fatty acids present. The results obtained for the fatty acids can also be used, as discussed previously, to calculate indices of enzyme activity and the proportion of saturated and unsaturated fatty acids present. As the separation properties of the lipid groups have been assessed individually, it is not possible that the fatty acids could have come from any source other than the lipid being studied. When studying polar lipids, contamination of the free fatty acid fraction may be indicative of lipid breakdown.

Several other factors could influence the elution pattern. Lipid breakdown, caused by either acid hydrolysis or lipid oxidation, would obviously allow for fatty acid elution and elution of other fragments in the wrong fraction. This cannot have been the sole reason for the differences observed because increasing the eluting solvent volume considerably improved recoveries in some cases. The lipids may adsorb to either the column or the test tube, delaying or preventing recovery. These possibilities will be discussed in turn.

Sulphuric or hydrochloric acid is used during the production of fatty acid esters to catalyse the hydrolysis of the fatty acid to glycerol ester bond. Most reports suggest that strong acids, high incubation temperatures and long incubation times are required for the complete esterification of fatty acids (Eder *et al* 1992a). The purpose of the acetic acid in the solvent system is to acidify the column during the neutral lipid separation for the subsequent elution of free fatty acids and partial glycerides. Acetic acid is weak but its presence in the chromatography solvents may cause some hydrolysis of the phospholipid, thereby liberating the fatty acids to be eluted as free fatty acids. This hypothesis was tested by manipulating the concentration of acid in the eluting solvents to range from 0-0.4% (Hamilton and Comia used 0.2%). Whether acid catalysed hydrolysis of fatty acids

could occur under these conditions was tested by adding PI to a test tube containing methanol and 0.2% acetic acid. After a short incubation at room temperature, fatty acid methyl esters were found to be present, confirming that acetic acid is capable of catalysing the hydrolysis of fatty acids from lipid. The amount of fatty acid hydrolysed from the lipid on the column appears to increase with both acid concentration and volume of solvent used. The amount hydrolysed is small in comparison to the total amount present. Therefore, this loss may not significantly effect the results obtained for the lipids present in the membrane at high concentrations. However, the liberated fatty acid appears to be contaminating the free fatty acid fraction. In biological samples, the concentration of free fatty acid is low and even a small contamination will grossly distort its concentration.

As mentioned previously, Hamilton and Comia (1988) used radioactively labelled standard lipids to measure recovery and the loss of fatty acids from the lipids would not have been evident. That is, their recoveries were probably spuriously good. The amount of phosphate or base recovered was not measured here. However, the finding that the phospholipids were quantitatively eluted in a fraction distant from the free fatty acid fraction and that the recoveries of each constituent fatty acid was equal is good evidence of the accuracy of this recovery index.

The omission of acid from the solvents was the most obvious way to solve the problem of phospholipid breakdown. However, for the separation of neutral lipids it was retained in the solvents since quantitative recoveries were obtained. Therefore, the possibility of analysing neutral and polar lipids separately was investigated. Because the size of the available sample is often small, both fractions must be derived from the same sample. A neutral lipid fraction which contained no phospholipid was first eluted from the Sep-pak cartridge. The polar lipids could then be sequentially eluted in the absence of acid. Results show that there was no significant overlap of the neutral and polar fractions, nor were there significant loses of neutral lipids using this modified separation. An interesting observation is that under these conditions, the free fatty acids elute after the partial glycerides. In the separation of neutral lipids described in section 3.1, free fatty acids elute before partial glycerides. It is difficult to explain why the elution of these two lipids was inverted here but one possibility is that the absence of the acid is affecting the pH of the solvents and therefore the ionisation state of the free fatty acids and their binding to the silica. The use of the final solvent from the neutral lipid separation protocol to elute lipids

from the Sep-pak cartridge has been employed by other groups to recover cholesterol and phospholipids (Wang *et al* 1994).

Using a different procedure from that for the neutral lipids, the recoveries of most of the phospholipids were quantitative. However, PE and LPC presented some difficulties. Lipids, in particular unsaturated fatty acids, are prone to oxidation (Frenkel 1980). This may explain the loss of lipid. Oxidation can be overcome by the addition of antioxidants to all the reagents but for the reasons discussed previously, BHT was not included in the solvents used here. However, other steps were taken to minimise oxidation which have also been discussed previously. Most of the phospholipids studied here possess two fatty acid moieties. Usually one of these is unsaturated, making all lipids equally prone to oxidation. The risk of oxidation increases with the time spent on the cartridge which was longest for PC, LPC and sphingomyelin but these were recovered quantitatively. PE elutes before these lipids, suggesting that oxidation on the cartridge is not the cause of the low recoveries observed. Oxidation could also be occurring during the removal of the solvents by evaporation. However, direct testing suggests that this was not the case. Loss of PE has been observed in lipid extracts from erythrocyte membranes separated on Silica gel H; oxidised lipids may bind more tightly to this. However, in this study, the lipids were stored in a dry form prior to analysis with no BHT present. This is not normal practice and may allow some lipid breakdown. The authors could provide no reason why PE should be more prone to oxidation (Dodge & Phillips 1966).

The third possible reason for the low recoveries of some lipids is that the elution volume used was not sufficient. In most cases, increasing the volume of eluting solvent to 15 ml maximised recoveries. However, this was not effective for PE or LPC. The absence of either lipid in subsequent fractions suggests the low recoveries were not due to insufficient solvent; had the elution volume been too small, the lipids should have eluted with the next fraction. Increasing the volume above 15ml unnecessarily was avoided. Increasing the volume of solvent, which was wet, will increase the time required for evaporation and therefore increase the risk of oxidation. Increasing the temperature of evaporation was avoided for the same reason. It might have been possible to speed up this process by drying the extract before evaporation with anhydrous sodium sulphate or by freezing. This was not tested here. Had internal standards been available for all the lipid groups, the use of these methods may have been easier to apply since any losses of lipid caused by the

drying technique would be corrected for by the presence of the appropriate internal standard.

It is possible that LPC was not adequately dissolved in the solvent used for application to the cartridge although recoveries were increased by sonicating the samples in a cold water bath prior to their application. Why LPC was more difficult to dissolve than other polar lipids is not clear. It differs from PC by the absence of one fatty acid moiety and, as fatty acids are non-polar, this may increase the polarity of the lipid, making it difficult to dissolve in organic solvent.

PE may be more prone to adsorption to the column or glass surfaces. It was the only lipid studied with a terminal nitrogen group. Sonication of the sample should have ensured complete solution but, although the recovery of PE after sonication was on average maximum, the results were variable. Three subsequent and more polar aliquots of solvent were applied to the cartridge after the fraction where PE elutes and very little PE was recovered from these fractions. The lipid may be adsorbed to the glass. A possible way of preventing this is to siliconise the test tubes. However, with GC-MS, it leads to the appearance of peaks which interfere with the gas chromatography. Oxidised lipids bind more tightly to silica gel H (Dodge & Phillips 1966) but it is not clear in the current study whether the lipids were oxidised and if so, whether they would bind more tightly to the silica used here. In the case of PE, internal standard and research standard were recovered in the same proportion to each other. This makes it possible to accept the low recoveries and still obtain valid quantitation. PE is eluted cleanly in one fraction which means any residual PE will not interfere with other lipids.

The expected value for the maximum ratio C16:0/C17:0 produced by sphingomyelin was estimated from the values obtained for other C16:0-containing lipids and was lower than anticipated, suggesting that the C16:0 was not being completely hydrolysed from the sphingomyelin. The fatty acid is attached via an amide bond which is more stable than the hydroxyl (ester) bonds and therefore its hydrolysis requires methanol and hydrochloric acid (Eder *et al* 1992a). Quantitative sphingomyelin recoveries were obtained by incubating the lipid with 5% hydrochloric acid in methanol overnight at 50 °C.

The method of Hamilton and Comia (1988), as they reported it, provided perhaps the most efficient means of separating the various lipid groups available, although Christie (1992)

observes that it has been difficult to reproduce in other laboratories. Other groups (Bitman *et al* 1983, Ansari & Shoeman 1988, Hamilton & Comia 1984, Janero & Burghardt 1990) used silica Sep-pak cartridges for the simple separation of neutral and polar lipids and for the separation of PE from PC (Hamilton & Comia 1984). Realistic alternatives to silica Sep-pak chromatography for the separation of lipids are few. Successful use of one dimensional TLC has been reported (Vitiello & Zanetta 1978, Rouser 1970). Also, using a chloroform/methanol/water solvent system, Lepage (1964) reported the separation of the lipids studied here. Our experience using this solvent system could not confirm this (see section 3.5). Two dimensional TLC is not economical and is logistically less convenient. Silver nitrate TLC has also been applied (Kennerly 1986). Again, it is more expensive than Sep-pak cartridges and the plates must be handled in the dark (Henderson & Tocher 1992). The problems of recovering samples quantitatively from TLC plates is discussed elsewhere.

HPLC efficiently separates polar lipids although the detection of the lipid classes remains difficult. This has been discussed previously (see Chapter 1 part 2). Success has been reported using a UV detector (Yeo & Horrlock 1988, Hundrieser & Clarke 1988) and a mass detector (Stolyhwo *et al* 1987). Neither method gives information about the fatty acid composition of the polar lipids which was the key aim of this study. An alternative is to use reverse phase HPLC (Sothiros *et al* 1985) to study the fatty acid composition of lipid groups or to use HPLC as a preparative step, as with the Sep-pak chromatography, followed by the separate analysis of fatty acids (Seewald & Eichinger 1989).

Neomycin binds phospholipids and has proved valuable in the isolation of polyphosphoinositides (Schacht 1978). Neomycin-coated beads have since been employed in systems for the separation of phospholipids (Palmer 1981) but this technique did not separate as extensive a range of lipid groups as Sep-pak cartridges or HPLC. Moreover, preparation of the antibiotic-coated beads in the laboratory may be difficult to standardise between batches.

A method using disposable aminopropyl-bonded phase columns has been reported by Kaluzny *et al* (1985) to separate phospholipids from neutral lipids. Not only is this method complex with many columns being used and columns being 'piggy backed' together to provide separation but it has also been proved unreliable by other groups who

observed that PI and phosphatidic acid were retained on the column (Christie 1992, Egberts & Buiskool 1988, Kim & Salem 1990).

It is apparent from the literature that there is no ideal method for the separation of all lipid groups. The altered method used here provides quantitative recovery of the lipids studied. Where the recovery differs from maximum, the use of an internal standard-containing lipid still allows for valid quantitation of the fatty acids present. The method yields two distinct groups of lipid which can then be separated easily using thin layer chromatography. This will be the topic of the next section.

3.5.1. Thin Layer Chromatographic Separation Of Lipids

Membrane lipids consist of a complex mixture of compounds with relatively similar physical properties (see chapter 1). For this project, it was necessary to separate each lipid type completely. As discussed previously, silica Sep-pak cartridges separate neutral lipids but the polar lipids are eluted in two groups, one containing PI and PE and a second containing LPC, PC and sphingomyelin. These individual lipids must then be separated and recovered quantitatively, or at least with high reproducibility. The lipids are present at low concentration and, to avoid non-specific contamination, the analytical system has to have a high level of cleanliness. With these requirements in mind, the ability of three types of chromatographic support-silica gel thin layer chromatography (TLC) on glass plates and TLC on polyester backed plates and paper chromatography using heavy duty (Whatman 3mm) paper, were tested. The solvent system composition was also manipulated. An initial decision was taken to use one dimensional chromatography. Two dimensional chromatography requires one plate or paper for each sample which is expensive for large scale studies; moreover, it is not possible to run reference standards alongside the biological samples.

3.5.2. Methods

The source and specification of the TLC plates and chromatography paper are given in Materials (Chapter 2). Samples of lipid standards corresponding to the groups obtained for the Sep-pak cartridge separation of the polar lipid groups (see table 3.1.1.b) were evaporated to dryness under nitrogen and redissolved in chloroform/methanol (2:1 v/v) (20 µl). The resuspended lipids were applied quantitatively to the TLC supports as spots under a stream of nitrogen to minimise oxidation. The test tubes were washed with a further volume of solvent and that too was applied to the plate. The chromatograms were

developed at 4°C using a variety of solvent systems (see section 3.5.3.a). All solvents, including those used for spotting, contained BHT (10mg/100 ml). For all chromatography, the glass tanks were lined with 3mm Whatman chromatography paper, sealed using a glycerol and starch sealant and equilibrated at 4°C for at least 4 hours. Where possible, the tanks were allowed to equilibrate overnight. Chromatography tanks were washed and fresh solvent added after every second chromatogram. The developed chromatograms were dried under nitrogen and stained with Rhodamine B spray reagent (Rhodamine B/ethanol 1:20 v/v). The appropriate spots were located under UV light and removed, either by scraping the silica gel off the support (TLC-glass plates) or by cutting out the appropriate areas (TLC-polyester plates; paper), followed by elution of the lipids using chloroform : methanol (2:1 v/v). These samples were transesterified, extracted and analysed by GC-MS as described previously. Recoveries were measured only when the separation of the lipid groups was complete as it is impossible to determine the lipid group from which the fatty acids have been liberated when separation is incomplete.

3.5.3. Results

3.5.3a Paper Chromatography.

The solvents used for the separation of the lipids and the results obtained are listed in table 3.5.3a. As can be seen, none of the solvents used were capable of separating the lipids completely.

Solvent	Support	Results
chloroform : acetone; methanol: acetic acid: water (6:8:2:2:1 v/v/v/v/v)	paper	PI and PE were not viable PC, LPC and sphingomyelin were not resolved (Rf = 0.94)
chloroform: methanol: 28% aqueous ammonia (65:35:5 v/v/v)	paper	All the lipid groups migrated the same distance (Rf = 0.70)
Chloroform : methanol: 28% aqueous ammonia (65:25:5 v/v/v)	paper	PI and PE were unresolved (Rf = 0.7). LPC and PC migrated with the solvent, sphingomyelin was not visible.
Chloroform : methanol : 28% aqueous ammonia (65:15:5 v/v/v)	paper	LPC migrated with the origin, sphingomyelin (RF =0.81) PC (Rf = 0.78)
chloroform : methanol: 28% aqueous ammonia (75:15:5 v/v/v)	paper	PC and LPC were not resolved (Rf = 0.51)

Table 3.5.3.a. Solvent systems used and the results obtained for paper chromatography of lipids.

3.4.3b TLC-glass backed plates.

The solvent systems used and the results obtained using them are listed in table 3.5.3.b. Where solvents A and B are listed, the plates were developed to half of their total distance with solvent B, dried and then developed fully with solvent A.

As can be seen from the table, the only solvent capable of separating all the lipid groups was chloroform: methanol: water (65:25:4 v/v/v). The Rf values for the lipids in this solvent system are listed in table 3.5.3 b. The other solvents used were capable of moving sphingomyelin and PC from the origin but could not resolve them.

Solvent	Support	Results
Solvent A : Hexane : diethyl ether: glacial acetic acid (80:20:2 v/v/v) Solvent B : ethyl acetate : propan-2-ol: chloroform : methanol: 0.25 aqueous KCl (25:25:25:10:9 v/v/v/v/v)	Silica on glass	PC and Sphingomyelin were removed from the origin together, LPC remained at the origin. PI and PE were not visible.
Solvent A: hexane : diethyl ether : glacial acetic acid (70:30:2 v/v/v) Solvent B was as above	Silica on glass	PC and sphingomyelin were removed from the origin but not resolved, LPC was not removed. PI was moved from the origin .
Solvent A: hexane : diethyl ether : glacial acetic acid (60:40:2 v/v/v) Solvent B was as above	Silica on glass	Sphingomyelin and PC were removed from the origin but not resolved, LPC was not removed from the origin. PI and PE were not visible.
Redevelopment of the above plate in solvent A	silica on glass	PC and LPC and sphingomyelin were removed form the origin but not removed. PI was removed from the origin.
Chloroform : methanol : acetic acid (65:25:4 v/v/v)	silica on glass	All the lipid groups were separated and removed form the origin. (RF values in table 3.5.3.b2)

Table 3.5.3.b1. Solvent systems used and the results obtained using glass backed TLC plates. Where two solvents are listed the plates were developed half way in solvent B and then fully in solvent A.

Lipid	RF Value
Phosphatidylcholine	0.235
Lysophosphatidylcholine	0.075
Sphingomyelin	0.115
Phosphatidylethanolamine	0.345
Phosphatidylinositol	0.175

Table 3.5.3.b2. The RF values for the lipids separated on the Glass Backed TLC plates.

3.5.3c Recoveries of Fatty Acid Methyl Esters Liberated From the Separated Lipids.

The recoveries of the lipids were again assessed by recovery of the fatty acids esterified to the lipids. The recoveries are listed in table 3.5.3.c. The solvent system used was chloroform/methanol/water (65:25:4 v/v/v) (see above). The recoveries of both the research standard fatty acid (C18:0) and the internal standard fatty acid liberated from PE did not differ significantly from maximum, ($p=0.58$ and 0.447 respectively). Comparison of the recovery of the research and internal standard showed no significant difference between the groups ($p=0.34$).

The recovery of LPC was not quantitative but the recovery of research standard fatty acid did not differ significantly from that of the internal standard fatty acid ($p=0.812$) showing that both lipid groups are behaving in a similar manner. Although the fatty acids from sphingomyelin were not recovered quantitatively, there was no significant difference between the ratio of C16:0/C17:0 for the chromatographed and unchromatographed samples ($p=0.083$). Comparison of the recovery of the research and internal standard showed no significant difference between the groups ($p=0.44$).

The recoveries of fatty acids liberated from PI and PC were significantly different from maximum.

Lipid	% of Maximum Naturally Occurring Fatty Acid	% of Maximum Internal Standard	Ratio Where Applicable
Lysophosphatidylcoline	42.65 (25.13)	42.16 (24.27)	
Phosphatidylcholine	55.68 (30.88)	61.87 (36.28)	
Phosphatidylethanolamine	104.14 (16.57)	94.42 (17.38)	
Sphingomyelin	44.39 (21.04)	53.56 (17.40)	80.87 (21.39)
Phosphatidylinositol	63.15 (12.62)		

Table 3.5.3.c1 The recoveries of the fatty acid methyl esters liberated from the lipids separated on the glass backed TLC plates (n=6).

3.5.3d TLC-Polyester backed plates.

The recoveries of the fatty acids liberated from the lipids separated on polyester backed plates (table 3.5.3.d) can be summarised as follows:-

The recovery of LPC was not quantitative but comparison of the percentage recovery of research standard and internal standards showed both groups to be behaving in the same

way (p=0.21). The same was true for PC (p=0.864) and therefore no further correction was required for valid quantitation.

The recoveries of PE was not significantly different from maximum (p=0.05 and 0.41 for the internal standard and research standard respectively). Comparing the maximum recoveries for the two lipid groups showed no significant difference (p=0.469). The recovery of sphingomyelin was assessed using the ratio of C16:0/C17:0, which did not differ significantly from maximum (p=0.23). Comparison of the recovery of the research and internal standards showed no significant difference between the two (p=0.14). PI was analysed in the same way by comparing the ratio of C18:0/C17:0. There was no significant difference between the unchromatographed and unchromatographed samples (p=0.14). Comparing the percentage of maximum recoveries for the internal standards and research standard showed no significant difference from maximum (p=0.34).

Lipid	% of Maximum Naturally Occurring Fatty Acid	% of Maximum Internal Standard	Ratio Where Applicable
Lysophosphatidylcoline	87.30 (9.15)	79.02 (12.02)	
Phosphatidylcoline	87.49 (6.04)	88.01 (2.94)	
Phosphatidylehanolamine	105.65 (12.75)	110.42 (9.05)	
Sphingomyelin	87.90 (6.63)	95.34 (9.12)	92.78 (12.02)
Phosphatidyliositol	55.23 (15.57)	47.60 (9.58)	115.85 (22.32)

Table 3.5.3.c2 The recoveries of the fatty acid methyl esters liberated from the lipids separated on the polyester backed TLC plates (n=6)

3.5.4. Discussion

The physical properties which determine the separation of mixtures of compounds by paper chromatography or TLC are solubility and adsorption. In paper chromatography, the paper fibres are coated with a liquid stationary phase, usually aqueous alcohol, during equilibration. During development of the paper, a less polar mobile phase which is saturated with stationary phase moves over the layer of stationary phase on the paper. The movement of the compounds depends on their partition coefficient between the two phases, hence the term partition chromatography. Good separation of compounds can be obtained with only very small difference in the partition coefficient and solvent systems are chosen to provide maximum differences. TLC is adsorption chromatography. The compounds have an affinity for the solid support, in this case silica, the molecules in the solvent compete with those in the solute for the adsorption sites. Non-polar molecules

have only weak affinity for the silica and are easily displaced and therefore have large Rf values. Polar lipids form dipole-dipole interactions with the silica and are retained.

Paper chromatography is inexpensive and the recovery of the samples by elution is simple. It is also easy to prepare in a high state of purity. Unfortunately, paper chromatography was not able to separate the mixture of membrane lipids using the solvent systems evaluated here. The Rf values obtained were not reproducible. These inconsistencies in the Rf values may have been due to differences in the saturation of the atmosphere in the tank, causing alterations in the coating of the paper with stationary phase which in turn will lead to alterations in the movement of the mobile phase over the paper. However, these variations should have been minimised by the carefully standardised conditions of temperature and equilibration time. In retrospect, the use of a descending chromatography systems may have given better resolution because longer development is possible. However, longer development times in an air atmosphere might increase oxidative losses. Another serious problem was that a high background fluorescence was present when the lipids were visualised with Rhodamine B under UV light which made locating the lipid spots difficult. There was also evidence of significant losses due to irreversible lipid adsorption at the origin. Finally, the loading capacity of paper is less than that of TLC. On a practical note, sheets of 3mm paper were less easy to handle than plates. There are reports of the use of paper chromatography to separate phospholipids, but the experimental details are vague (Wuthier 1966). In his review of the subject, Rouser *et al* (1961) suggests that paper chromatography should only be used in method development and it is not appropriate for quantitative recovery of lipids.

The use of thin layer chromatography for the separation and recovery of lipids is commonplace and its use has been discussed in both the previous sections. As can be seen from the table 3.5.3.b, several solvent systems were evaluated for their ability to separate the membrane lipids but only chloroform/methanol/water (65:25:4 v/v/v) performed adequately. The differences in the Rf values were small but separation was reproducible and reliable using both glass and polyester backed plates. It is difficult to say why some of the solvents recommended elsewhere failed to separate the membrane lipids here. All the solvents investigated here were used at 4°C, which should not only standardises the vapour pressure and development time, but also help to minimise lipid oxidation. There will be small difference in the pore and particle size of the silica purchased from different manufacturers but this should make only small differences in the

retention of lipids on the plates. The use of a fluorimeter to measure the florescence produced by the Rhodamine B may have enabled the detection of small differences in the retention times between the lipids. The use of radioactive lipids would also have allowed for mechanical detection of the lipid spots but it was felt preferable to avoid their use if possible.

The quantitative removal of lipid spots from the glass-backed plates was difficult, leading to poor recovery. The silica gel had to be scraped off the plate and transferred to a test tube for the transesterification reaction during which time there is risk of oxidation. Moreover, the silica plates had been sprayed with Rhodamine B and creation of aerosols of silica containing this toxic compound is difficult to avoid. These factors made the routine use of the medium unattractive. It is possible to stain the TLC plates with iodine but this causes loss of polyunsaturated fatty acids which are proportional to the number of double bonds in the fatty acids. These losses are due to iodination of double bonds (Nichaman *et al* 1963). Also, iodine vapour is toxic.

The use of the polyester-backed plates from which the appropriate areas can be cut improved the recovery of all lipid groups both in terms of efficiency and reproducibility. Only PC and PI were not recovered quantitatively but the recovery of the internal standard closely mimicked the recovery of the research standard, allowing for correction for losses.

Other systems have been suggested for the separation of polar lipids by TLC. Wing *et al* 1986) reports a technique using plates coated with a mixture of silica gel HF and silica gel G. As the plates have to be produced in the laboratory, their use was not assessed here. Other modifications include over-developing the plate (Bitman *et al* 1984). High performance TLC (HPTLC) has been reported to separate twenty lipids classes using four different solvent systems (Yoa & Rastetter 1985). The need for multiple drying of the plates and the difficulty in recovering lipids from HPTLC plates makes this system unacceptable.

3.6.1 Fatty Acid Methyl Ester Standard Curve Production and Main Ion Identification.

During the development of the method analysis, of fatty acid methyl ester recovery has been achieved using the total ion current produced on the GC-MS. It is possible, however, to use the Magnum Soft-ware (Version 2.40) on the GC-MS to identify and

quantitate the fatty acid methyl esters. For this to be possible, standard curves must be applied to the GC-MS and stored in the memory of the soft-ware. This allows identification of the esters by comparison with the spectrum of standards and quantitation of the esters present. Quantitation requires the internal standard to be at a fixed concentration in all samples (20ng/µl). To produce the standard curve, the soft-ware calculates the ratio of the peak area of the sample/peak area of the internal standard. This ratio is plotted on the Y axis; the concentration of the sample fatty acid is plotted on the X axis. The amount of fatty acid present in the sample is calculated using the same ratio.

3.6.2 Methods.

3.6.2a Selection of Principle Ions for Compound Identification

Standard fatty acid methyl esters were applied to the GC-MS and a spectrum obtained. Three or four ions were then identified from the spectrum on the basis of both their concentration and uniqueness to each fatty acid. The retention time was also noted.

3.6.2b Standard Curve Production and Evaluation.

Standard fatty acid methyl esters were dissolved in hexane at a final concentration of 1 mg/ml. These stock solutions were then used to produce a standard curve with a final concentration ranging from 5ng/µl to 400ng/µl. The final concentration of heptadecanoic acid methyl ester was 20ng/µl. Each concentration was applied to the GC-MS in replicate (n=6). The total ion currents produced were assessed for variability. The linearity of the standard curves was also assessed. Results are expressed as mean ± (SEM).

3.6.3 Results.

3.6.3a Principle Ion Identification.

Table 3.6.3a shows the formula, retention time, molecular weight and the principle ions for all the fatty acid methyl esters to be studied. All have distinct spectra making their identification in a biological sample simple.

3.6.3b Standard Curve Evaluation.

Table 3.6.3b shows the peak areas of a variety of fatty acid methyl esters. Table 3.6.3c shows the correlation coefficient for the standards curves and the linear equation.

Common Name Of Methyl Ester	Formula	Molecular Weight (AMU)	Retention Time (Secs)	Main Ions
Palmitic	$C_{17}H_{34}O_2$	270.5	793	143, 171, 227, 271
Palmitoleic	$C_{17}H_{32}O_2$	268.4	762	110, 123, 237, 269
Stearic	$C_{19}H_{38}O_2$	298.5	1111	143, 255
Oleic	$C_{19}H_{36}O_2$	296.5	1071	110, 123, 264, 297
Linoleic	$C_{10}H_{34}O_2$	294.5	1063	109, 121. 263, 294
Linolenic	$C_{19}H_{33}O_2$	292.6	1075	107, 121, 261, 292
Arachidic	$C_{21}H_{42}O_2$	326.4	1439	143, 227, 284
Arachidonic	$C_{21}H_{34}O_2$	318.5	1336	105, 119, 147, 319
Dihomo γ linolenic	$C_{21}H_{36}O_2$	320.5	1375	107, 121, 135, 320
Behenic	$C_{23}H_{46}O_2$	354.6	1665	143, 255, 311, 355
DHA	$C_{23}H_{34}O_2$	342.5	2093	105, 117, 313, 388
Lignoceric	$C_{25}H_{50}O_2$	382.7	2134	143, 241, 340, 382
Nervonic	$C_{25}H_{48}O_2$	380.7	2134	111, 135. 348, 381
Hexacosanoic	$C_{27}H_{54}O_2$	410.7	2500	143, 255, 368, 411
Heptadecanoic	$C_{18}H_{36}O_2$	284.5	949	149, 185, 241, 285

Table 3.6.3a Formula and molecular weights of the methyl esters of the fatty acids to be studies and the retention time in seconds of the fatty acids on the gas chromatograph.

Fatty Acid Methyl Esters	Concentration of Fatty Acid Methyl Esters							
	400	300	200	100	50	25	10	5
Palmitic	12.41	10.48	8.01	3.46	2.24	1.27	0.66	0.26
	(0.28)	(0.18)	(0.15)	(0.03)	(0.07)	(0.02)	(0.04)	(0.02)
Palmitoleic	21.47	17.20	13.30	6.81	2.93	1.17	0.98	0.235
	(0.39)	(0.80)	(0.36)	(0.15)	(0.07)	(0.04)	(0.01)	(0.02)
Stearic	17.98	14.28	12.20	7.62	4.42	2.56	0.68	0.39
	(0.75)	(0.6)	(5.95)	(0.55)	(0.31)	(0.31)	(0.13)	(0.1)
Oleic	15.55	14.05	11.87	5.33	3.36	1.76	0.76	0.24
	(0.27)	(0.21)	(0.18)	(0.09)	(0.14)	(0.05)	(0.04)	(0.03)
Linoleic	37.50	33.81	28.48	17.05	7.68	3.01	2.10	0.38
	(1.42)	(0.19)	(0.47)	(0.3)	(0.19)	(0.07)	(0.16)	(0.03)
Arachidonic	25.52	22.35	19.52	10.88	4.17	1.00	0.55	0.14
	(0.59)	(1.24)	(0.49)	(0.59)	(0.28)	(0.05)	(0.04)	(.007)
Behenic	10.69	8.99	7.86	4.70	2.61	1.20	0.35	0.16
	(0.49)	(0.25)	(0.15)	(0.36)	(0.18)	(0.12)	(0.08)	(0.05)
Lignoceric	7.27	5.69	4.84	2.31	1.05	0.42	0.13	0.06
	(0.41)	(0.24)	(0.18)	(0.28)	(0.09)	(0.05)	(0.03)	(0.02)
Hexacosanoic	2.44	1.40	1.05	0.54	0.30	0.12	0.06	NA
	(0.11)	(0.1)	(0.02)	(.006)	(.008)	(.002)	(.002)	

Table 3.6.3b Peak areas produced by a selection of fatty acid methyl esters on the gas chromatograph for the concentrations used in the standard curve.

Fatty Acid Methyl Esters	Correlation Coefficient	Linear Equation
Palmitic	0.991	y = 0.391 + 0.04x
Palmitoleic	0.967	y = 0.128 + 0.03 x
Stearic	0.989	y = 0.579 + 0.04x
Oleic	0.995	y = 0.283 + 0.05x
Linoleic	0.994	y = 0.479 + 0.06x
Arachidonic	0.986	y = 0.347 + 0.04x
Behenic	0.982	y = 0.476 + 0.02x
Lignoceric	0.984	y = 0.308 + 0.02x
Hexacosanoic	0.997	y = -0.08 + 0.01x

Table 3.6.3c Correlation coefficient and linear equations for the fatty acid methyl ester standard curves using the ratio peak area of sample / peak area of internal standard.

3.6.4 Discussion

The main ions obtained will allow the positive identification of specific fatty acids. This a major benefit of the GC-MS over other systems. The spectra for the saturated fatty acids have a characteristic 143 ion. The fragmentation pattern of palmitic acid methyl ester is shown below with the molecular weights which relate to each fragment (Alexander *et al* 1985). The spectrum obtained here is in good agreement with this (see fig 3.6.4a).

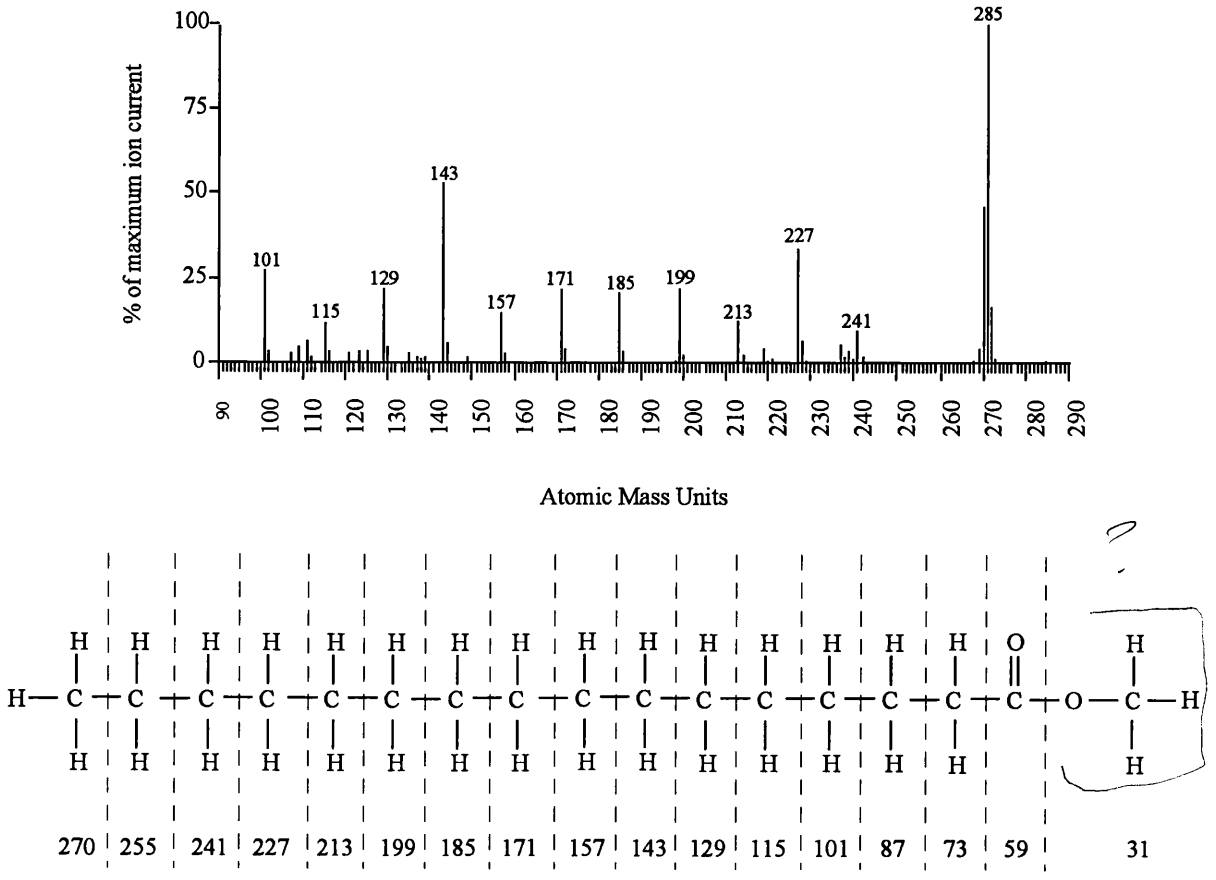


Fig 3.6.4a Mass spectrum and fatty acid structure of palmitic acid, showing break sites in the fatty acids chain and the atomic weight of the fractions produced.

The more highly unsaturated fatty acids also have characteristic ions. Linolenic and dihomo-γ-linolenic acid methyl esters both have three double bonds and a main ion of 107. The spectra for all the unsaturated fatty acids follow similar step wise pattern; the most abundant ion has the smallest molecular weight and the abundance decreases as the molecular weight increases. Fragmentation occurs at double bonds (see fig 3.6.4b).

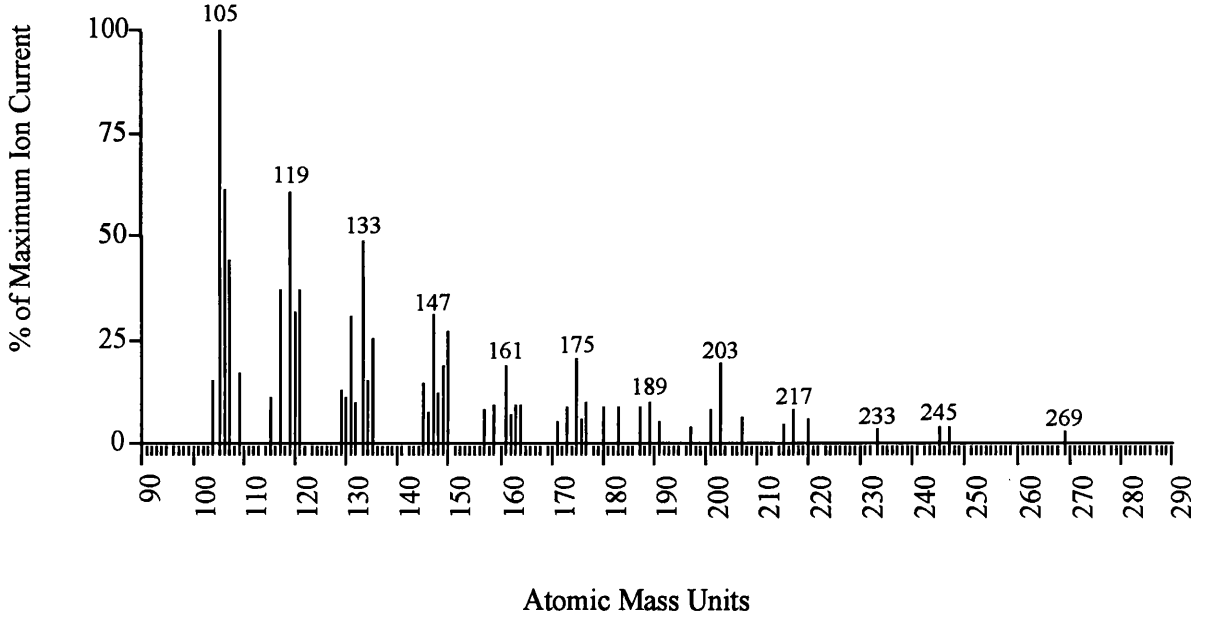


Fig 3.6.4b Mass spectrum of arachidonic acid showing the typical step wise fragmentation pattern caused by multiple double bonds.

The reproducibility of the spectrum and retention times were good and all the standard curve studied were linear. Quantitation using these standard curves should therefore be accurate.

3.7.1 Inter- and Intra-Assay Variability for the Lipid Separation.

A sufficiently large blood sample was obtained to allow for repeated analysis of erythrocyte membrane lipid fatty acid composition. The preparation was stored at a protein concentration of 2 mg/ml of protein. Samples were assayed in replicate (n=6) on six separate occasions. Coefficients of variation were used to assess intra and inter assay variability. To asses variation between batches (inter-assay variability), the scatter of the batch means was measured.

3.7.2 Results

The intra-assay variabilities are shown in table 3.7.2a, the inter-assay variabilities are shown in table 3.7.2b. The intra- and inter-assay variabilities for the indices of desaturation and the saturation/unsaturated fatty acid ratio and the phospholipid composition are shown in tables 3.7.2c, 3.7.2d and 3.7.2.e.

Lipid	C16:1	C16:0	C18:2	C18:1	C18:0	C18:3	C20:4	C20:3	C20:0	C22:6	C22:0	C24:4	C24:0
CE	53.57	23.27	52.34	11.14	41.17	51.22	54.22	23.52	85.71	50.72	49.78	21.12	30.43
TRIG	47.89	10.80	27.26	20.00	43.70	22.47	107.4	35.9	36.27	44.75	30.22	26.47	21.54
FFA	32.72	10.94	74.85	16.91	5.33	44.21	37.59	24.83	61.90	69.00	66.87	18.66	27.83
PGS	33.78	9.69	51.93	15.69	6.16	38.51	41.18	34.88	36.11	76.76	59.95	44.19	39.14
PI	36.56	21.13	19.72	14.99	39.43	34.13	35.25	41.63	22.24	202.9	44.89	43.55	46.39
PE	38.46	15.48	39.07	7.86	17.07	36.03	17.21	27.71	64.52	86.70	166.6	62.5	71.89
PC	40.53	29.22	1.43	17.61	22.08	20.90	39.32	13.93	90.77	18.25	171.3	101.1	79.92
LPC	45.16	24.71	41.63	62.68	28.74	42.71	42.86	54.22	31.16	45.11	105.3	24.75	39.99
SPG	130.1	17.59	55.84	27.72	19.60	44.40	40.81	9.65	155.9	38.85	200.9	78.38	22.20
TOTAL	40.21	15.72	43.55	10.66	12.61	16.67	33.37	27.53	72.27	74.58	45.97	46.21	51.72

Table 3.7.2a Inter assay variability (coefficients of variation) for the fatty acid methyl esters produced by the lipid separation technique.

Lipid	C16:1	C16:0	C18:2	C18:1	C18:0	C18:3	C20:4	C20:3	C20:0	C22:6	C22:0	C24:4	C24:0
CE	27.43	20.50	47.41	17.29	42.66	30.63	48.19	38.83	65.19	55.79	77.68	47.51	54.59
TRIG	25.58	14.00	36.14	13.17	25.62	21.89	61.01	30.23	73.40	86.94	105.17	36.76	43.00
FFA	39.44	8.70	104.8	16.45	7.60	26.96	60.16	30.97	38.09	39.14	78.31	53.23	55.99
PGS	52.43	12.62	45.12	24.39	11.78	30.99	58.82	24.51	55.55	74.20	105.20	75.51	63.11
PI	38.50	8.24	10.96	10.67	9.14	44.80	10.36	15.85	67.48	54.80	65.17	64.15	61.56
PE	35.90	14.77	35.35	11.28	26.11	32.25	18.05	24.81	64.52	48.05	38.09	45.42	26.96
PC	19.77	4.57	7.92	15.35	5.37	28.68	10.94	22.39	40.61	59.40	52.78	29.97	43.56
LPC	64.88	13.25	56.27	25.77	19.16	33.13	51.95	15.12	60.34	43.16	46.74	29.52	27.34
SPG	23.77	5.23	55.03	34.92	6.69	24.05	43.14	29.94	67.49	50.46	30.74	25.13	47.75
TOTAL	29.27	16.44	22.48	6.32	18.66	20.44	30.84	25.27	74.56	38.05	51.72	30.57	27.79

Table 3.7.2b Intra assay variability (coefficients of variation) for the fatty acid methyl esters produced by lipid separation technique.

Lipid	Δ5 desaturase	Δ6 desaturase	total desaturase	Δ9 desaturase	sat/unsat
CE	28.57	108.34	65.62	43.13	34.21
TRIG	103.63	50.03	69.17	21.21	25.62
FFA	46.87	48.10	95.72	18.05	28.89
PGS	62.50	30.98	46.67	30.08	38.56
PI	12.39	24.24	59.60	11.19	36.32
PE	32.50	48.54	34.17	26.30	22.30
PC	34.59	21.53	41.17	18.96	59.07
LPC	85.71	67.83	69.13	19.33	26.46
SPG	57.76	85.06	116.50	34.67	59.52
TOTAL	40.00	44.38	28.40	14.57	18.44

Table 3.7.2c. Inter assay variability (coefficient of variation) for the indices of desaturation and the saturated/unsaturated fatty acid ratios derived from the results obtained for the lipid separation technique.

Lipid	Δ5 desaturase	Δ6 desaturase	total desaturase	Δ9 desaturase	sat/unsat
CE	57.14	64.33	62.50	29.11	28.95
TRIG	50.53	31.91	58.26	40.16	26.54
FFA	62.50	58.57	99.80	11.89	38.92
PGS	50.00	55.29	60.55	20.70	26.23
PI	20.23	50.26	16.77	35.07	7.88
PE	26.85	43.43	30.34	18.03	21.30
PC	17.79	31.22	26.78	32.67	17.54
LPC	50.00	76.05	61.11	22.68	15.54
SPG	48.39	50.63	24.26	27.95	25.46
TOTAL	26.31	18.65	20.93	13.58	23.23

Table 3.7.2d Intra assay variability (coefficient of variation) for the indices of desaturation and the saturated/unsaturated fatty acid ratios derived from the results obtained for the lipid separation technique.

Lipid	Inter assay variability	Intra assay variability
PI	15.86	20.85
PE	20.16	56.57
PC	17.18	12.07
LPC	18.70	36.37
SPG	22.70	27.91

Table 3.7.2e Inter and intra assay variabilities for the derived phospholipid concentrations obtained from the results of the fatty acid analysis after separation of the lipid groups. .

3.7.3 Discussion

The separation efficiency of the lipid groups was good (see section 3, 4 & 5) but the variability of some of the measurements made was high. Interpretation of the analyses on the three subsequent studies, particularly of measurements of those fatty acids present in low concentration, must take this into account.

With any multistep analysis, large variabilities are inevitable as each step in the process introduces more variability. Every effort was made to keep the variability of the method to a minimum by the addition of internal standards to the sample at the time of lipid extraction. All the samples were also processed in an identical manner. However, it is difficult to control laboratory conditions such as temperature and water pressure (used to operate Sep-pak chromatography). Both may have affected the results. High variability was not limited to a particular lipid group, suggesting it is not a function of the lipids themselves. Nor was the high variability limited to unsaturated fatty acids, suggesting that oxidation was not solely responsible.

Samples were injected into the GC-MS automatically. They were in the autosampler for up to 10 hours. During this time, the samples were contained in dark glass tubes containing BHT but it is still possible that a small amount of sample degradation occurred. Manual injection of the very large number of samples required too much time to be a realistic option.

Part 2.

3.8.1 Measurement of Cholesterol By GC-MS

The current in-house colorimetric technique for the measurement of cholesterol is a modification of the method of Gamble *et al* (1978). This method requires larger sample sizes than were available here. Moreover, the precision is poor variable. The measurement of cholesterol esters is described in section 3.3. There are several well-characterised, sensitive methods for the measurement of cholesterol using GC-MS. The method evaluated here involves the conversion of extracted cholesterol to its trimethyl silyl ether (TMS) (Christie 1989).

3.8.2 Methods.

3.8.2a Production of cholesterol TMS ether.

Lipid was extracted from the sample as previously described. The solvent was evaporated under nitrogen and the residue resuspended in pyridine (500µl). Hexamethyldisilazane (150µl) was added to this and mixed. Trimethylchlorosilane (50µl) was then added and mixture was shaken for 30 seconds and left at room temperature for 5 min.

The solvents were removed by evaporation and the residue resuspended in hexane (2ml). The solution was washed with water (2ml) which was removed by freezing. The

remaining hexane was transferred to a clean test tube and the solvent evaporated to dryness under nitrogen. The residue was resuspended in hexane containing BHT (500µl) in preparation for analysis by GC-MS.

3.8.2b Main Ion Identification

The mass spectrum was obtained for cholesterol TMS ethers and the internal standard, 5β cholestan-3-α-ol. The spectra were compared and 3 unique ions identified for each compound.

3.8.2c Standard Curve Production.

Standard curves were produced by derivatising a range of cholesterol concentrations with the internal standard. The internal standard concentration was 20 ng/µl. Cholesterol concentration ranged from 10-400ng/µl.

3.8.2d Linearity of measurements with increasing sample size.

An extract of erythrocyte membranes was prepared as previously described. Aliquots with protein content ranging from 0.05-0.5 mg/ml were analysed for cholesterol.

3.8.2e Inter- and Intra-Assay Variability.

A large sample of erythrocyte membrane was prepared at a protein concentration of 0.1 mg/ml. Cholesterol was determined in replicate (n=6) on six different occasions. The inter and intra-assay variability was calculated as described in section 3.7.1.

3.8.3 Results.

3.8.3a Main Ion Identification.

The ions selected for identification of cholesterol TMS ether were 368, 329 and 129 atomic mass units. For 5β-cholestan-3α-ol ether they were 370, 355, and 215 atomic mass units.

3.8.3b Standard Curve Production

The standard curve was linear ($y = 0.046x - 0.08$) with a correlation coefficient of 0.995.

3.8.3c Linearity of the Cholesterol Concentration in Biological Samples

Table 2.13.1 shows the concentration of cholesterol obtained at the various protein concentrations used. The results are expressed as mean ± (SEM) (n=3). The correlation coefficient R = 0.897. Linearity was tested using linear regression analysis (p = 0.011).

Protein Concentration (mg/ml)	Concentration of cholesterol present (ng/μl)
0.05	34.704 (0.55)
0.1	59.167 (2.89)
0.2	95.452 (2.23)
0.3	186.000 (3.68)
0.4	446.917 (42.87)
0.5	872.271 (74.79)

Table 3.8.3 Concentration of cholesterol present in samples with a range of protein concentrations.

3.8.3d Inter and Intra Assay Variability.

Intra	Mean	50.569	**Inter**	Mean	50.569
	SD	2.745		SD	2.906
	CV	5.43%		CV	5.74%

3.8.4 Discussion.

The advantages of this method over the colorimetric methods are two fold. It requires very little sample and it is very precise. The method is rapid, effective and the intra- and inter-assay variabilities are low. The GC-MS analysis can be automated, reducing further the time spent in the analysis.

The concentration of the cholesterol over the various protein concentrations shows a linear trend but the correlation coefficient was less than 1. Additional errors are contributed by the extraction and the estimation of protein concentration. The method had adequate precision for the sample sizes available in subsequent studies.

3.9 Summary.

A method of extracting, separating and measuring the concentrations of individual lipid groups from cell membranes has been devised and assessed. In order to test its performance further, it was used to study membrane composition in hypertension and diabetes mellitus.

Chapter 4 L-NAME Induced Hypertension.

4.1 Introduction.

The lipid and fatty acid composition of the cell membrane determines its physical properties. To date, changes in membrane fluidity or microviscosity have only been observed in patients with essential hypertension and in rat models of genetic hypertension. Studies of secondary hypertension in the DOCA-salt induced hypertension and two-kidney, one-clip Goldblatt hypertension rat models have failed to show any alteration in membrane microviscosity (Orlov *et al* 1982). The same is true for human renal hypertension (Orlov & Prostnov 1982). However, alterations in membrane function, such as an increase in net sodium fluxes across the lymphocyte membrane (Furspan & Bohr 1985) and increased intracellular calcium (Pernoller *et al* 1994), have been observed in secondary hypertension, suggesting that some abnormality of the membrane may be present.

In recent years, interest in the potent vasodilator, nitric oxide (NO), has been increasing exponentially. NO is produced during the conversion of L-arginine to L-citrulline by NO synthase (NOS). NO, produced in the endothelial cells, activates guanylate cyclase in VSMC. Guanylate cylcase then catalyses the conversion of GTP to cGMP which activates a cGMP-dependant protein kinase and this in turn causes vasorelaxation. Inhibition of NOS induces hypertension in Brattleboro, Long-Evans and Wistar rats (Gardiner *et al* 1990a, Gardiner *et al* 1990b, Morton *et al* 1993). This increase in blood pressure is accompanied by an increase in plasma renin (Morton *et al* 1993, Johnson & Freeman 1992, Ribeiro *et al* 1992, Baylis *et al* 1992, Gardiner *et al* 1992), suggesting that it is a renin-dependant form of hypertension. Several analogues of L-arginine have been shown to act as competitive inhibitors of NOS. Two of the most commonly used are N^G-nitro-L-arginine methyl ester (L-NAME) and N^G-monomethyl-L-arginine (L-NMMA). Three types of NOS have been identified, type I (brain NOS), type II (inducable NOS) and type III (endothelial NOS).

Therefore, L-NAME appears to cause malignant hypertension by a mechanism which resembles that in the two-kidney, one-clip Goldblat hypertension rat model. A small increase in blood pressure activates renin secretion which further increases blood pressure, leading to a vicious circle of increasing blood pressure and increased plasma renin. Morton *et al* (1993) showed that L-NAME-induced hypertension could be inhibited by administration of Captopril, an inhibitor of angiotensin converting enzyme, suggesting a

role for the renin-angiotensin system. This might be a logical conclusion if antagonism of NOS has a purely vasoconstrictor effect. However, Morton *et al* (1993) also noted considerable vascular and cardiac hypertrophy which itself may have exerted a positive feedback effect on blood pressure.

Here the effects of L-NAME-induced hypertension on membrane lipid composition and membrane microviscosity in the rat have been studied.

4.2 Materials and Methods.

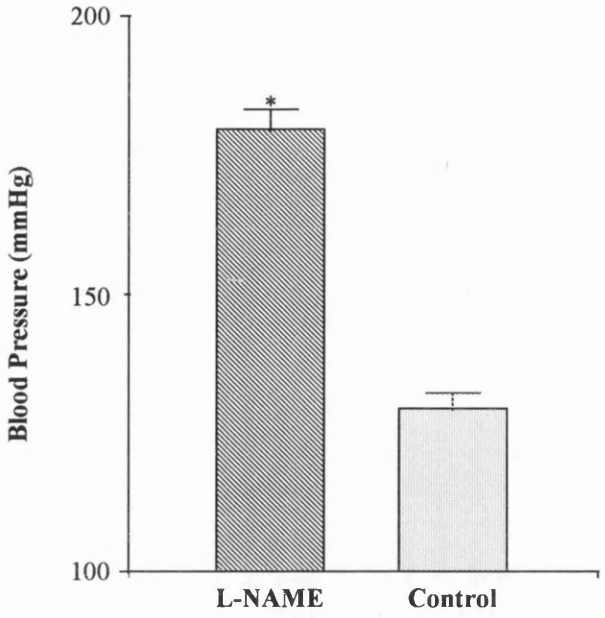
32 WKY rats (male/female 50:50) were split equally into treated and control groups. Treatment began at 8 weeks of age and continued until 11 weeks. The treated group received L-NAME (10mg/kg/day) in their drinking water while the controls received water only. Both groups were maintained in a twelve hour light-dark cycle and food and water were available *ad libitum*. At the end of the treatment period, blood and liver samples were obtained and analysed as previously described. Lipid analysis was carried out in 8 animals from each group and the microviscosity was measured in all 16 animals from each group.

Data were analysed by ANOVA. Only when p was less then 0.05 were unpaired Students t-tests carried out to compare the concentrations of the individual fatty acids. Only statistically significant results (i.e. $p<0.05$) are shown $p<0.01$ is indicated by an *. All graphs show the means and SEMs; where appropriate p values for ANOVA are shown beneath the X axis. All graphs show the variable measured on the Y axis and the fatty acid or lipid group on the X axis. Where data for multiple lipid groups or enzymes are shown on one graph the group or enzyme is detailed below the graph.

4.3 Results.

4.3.1 Blood Pressure.

Graph 4.3.1 shows the mean systolic blood pressure from the L-NAME-treated and control groups at the end of the treatment. The blood pressure of the L-NAME-treated group was significantly higher than that of the control group.



Graph 4.3.1 Systolic blood pressure (mmHg) for 16 L-NAME treated and 16 control rats measured using the tail cuff method.

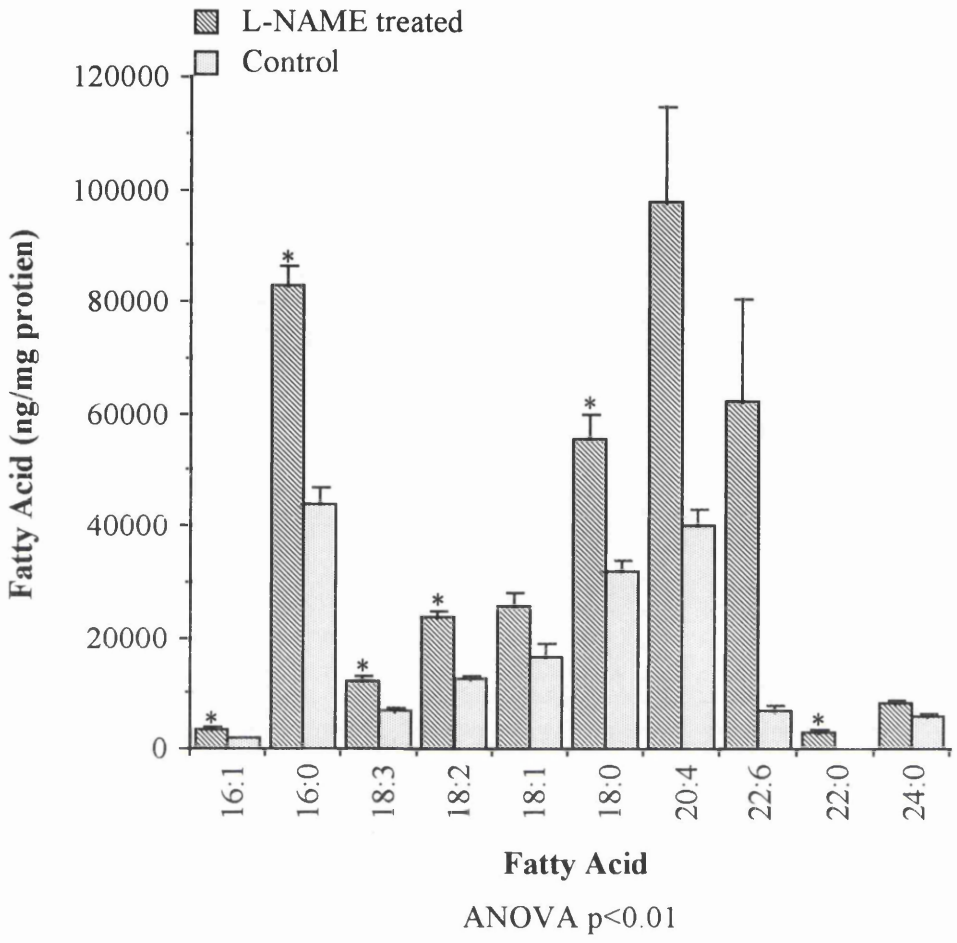
4.3.2. Total Fatty Acids.

Erythrocytes

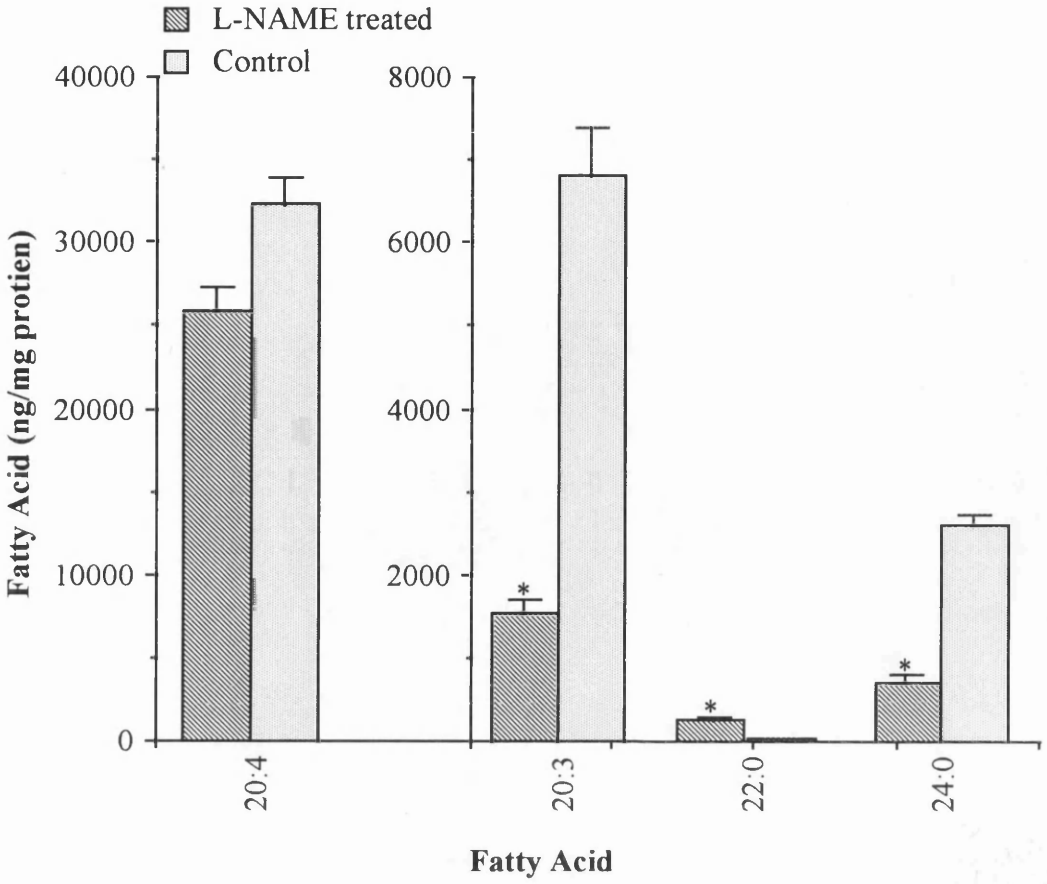
Graph 4.3.2a illustrates the differences in the total fatty acids composition between the erythrocyte membranes from the L-NAME-treated and control animals. The levels of the following fatty acids were significantly higher in the L-NAME-treated group compared to control; palmitic, palmitoleic, linolenic, linoleic, oleic, stearic, arachidonic, docosahexaenoic, behenic and lignoceric acid.

Liver Cell Membranes

In graph 4.3.2b, the composition of the liver cell membranes from L-NAME-treated and control rats are compared. The concentration of the following fatty acids were significantly lower in the L-NAME-treated group compared to the control group; arachidonic, dihomo-γ-linolenic, behenic and lignoceric acid.



Graph 4.3.2a Total fatty acid composition (ng/mg protein) of erythrocyte membranes from L-NAME treated (n=8) and control (n=8) rats. Results shown as mean and SEM, all results shown are statistically significantly different ($p<0.05$) * indicates $p<0.01$.



Graph 4.3.2b Total fatty acid composition (ng/mg protein) of liver cell membranes from L-NAME treated (n=8) and control rats (n=8), statistics as described in graph 4.3.2a. Only results found to be statistically significantly different by t-test are shown in the graph.

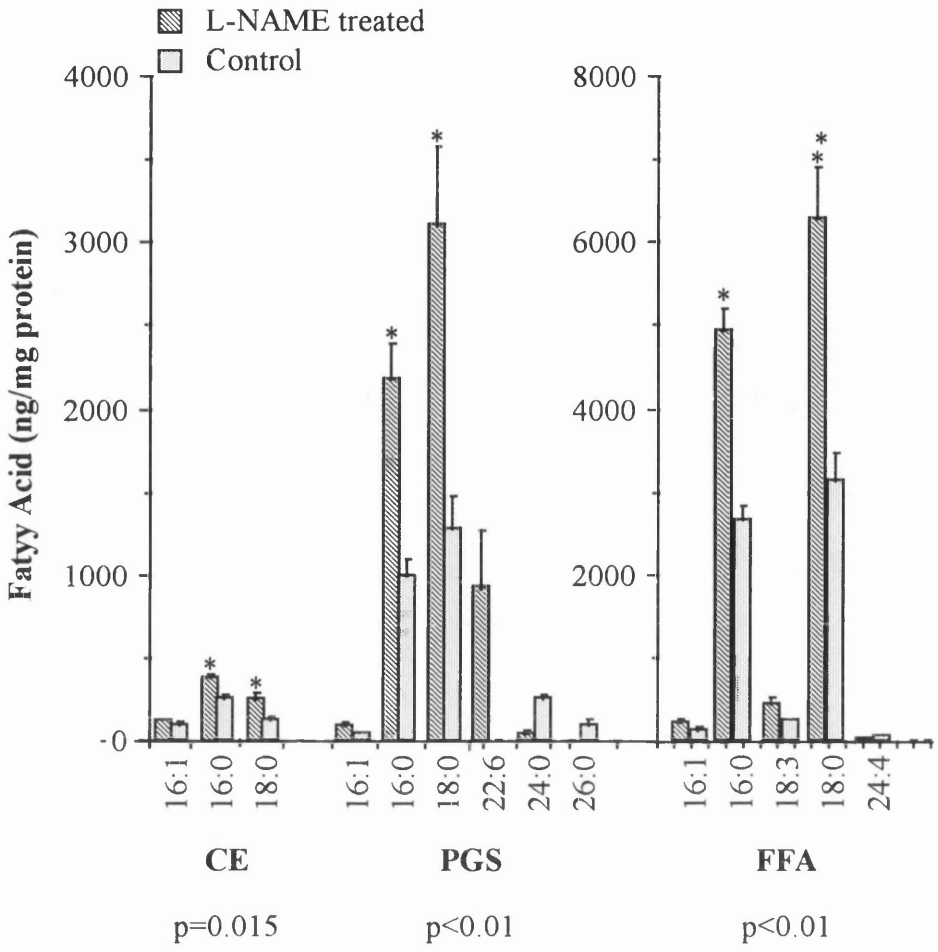
4.3.3 Neutral Lipids.

Erythrocytes

Graph 4.3.3a illustrates the differences observed in the fatty acids esterified to neutral lipids from erythrocyte membranes from the L-NAME-treated animals compared to the untreated controls. Of the fatty acids altered in the total lipid fraction (4.3.2), the following were significantly altered in the neutral lipids; in CE, there was a higher concentration of palmitoleic, palmitic, and stearic acid in the L-NAME-treated group compared to control. In the FFA fraction, palmitoleic, palmitic, linolenic and stearic acids were present at a higher concentration in the L-NAME-treated group compared to control. In the L-NAME-treated group, the PGS fraction contained more palmitoleic, palmitic, stearic, docosahexaenoic and lignoceric acid.

Although not significantly altered in the total lipid fraction by L-NAME-treatment, a reduction in the amount of nervonic acid present in the FFA fraction of the L-NAME-

treated group compared to the control was observed. Also the level of hexacosanoic acid in the PGS fraction reduced in the L-NAME-treated group compared to control.

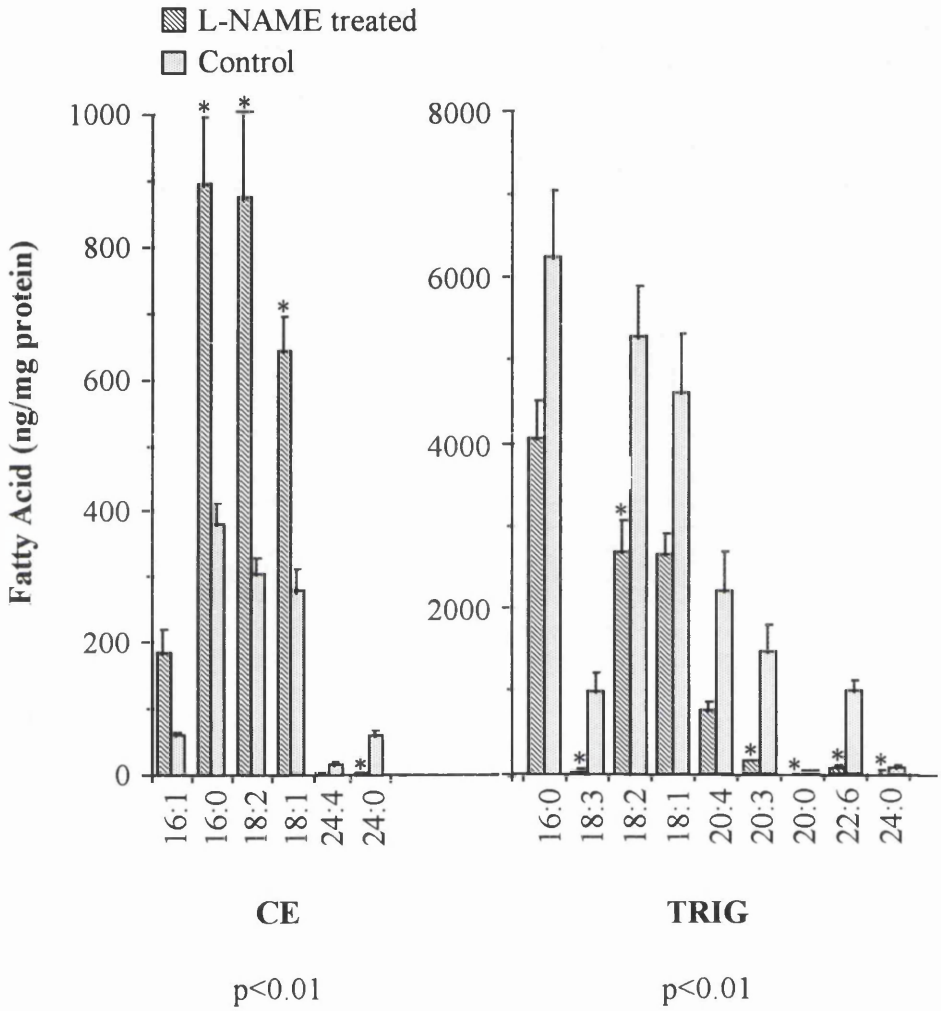


Graph 4.3.3a Fatty acid composition of erythrocyte neutral lipids (ng/mg protein) from L-NAME treated and control rats. Statistics as described previously, p values obtained for ANOVA are shown below the graph.

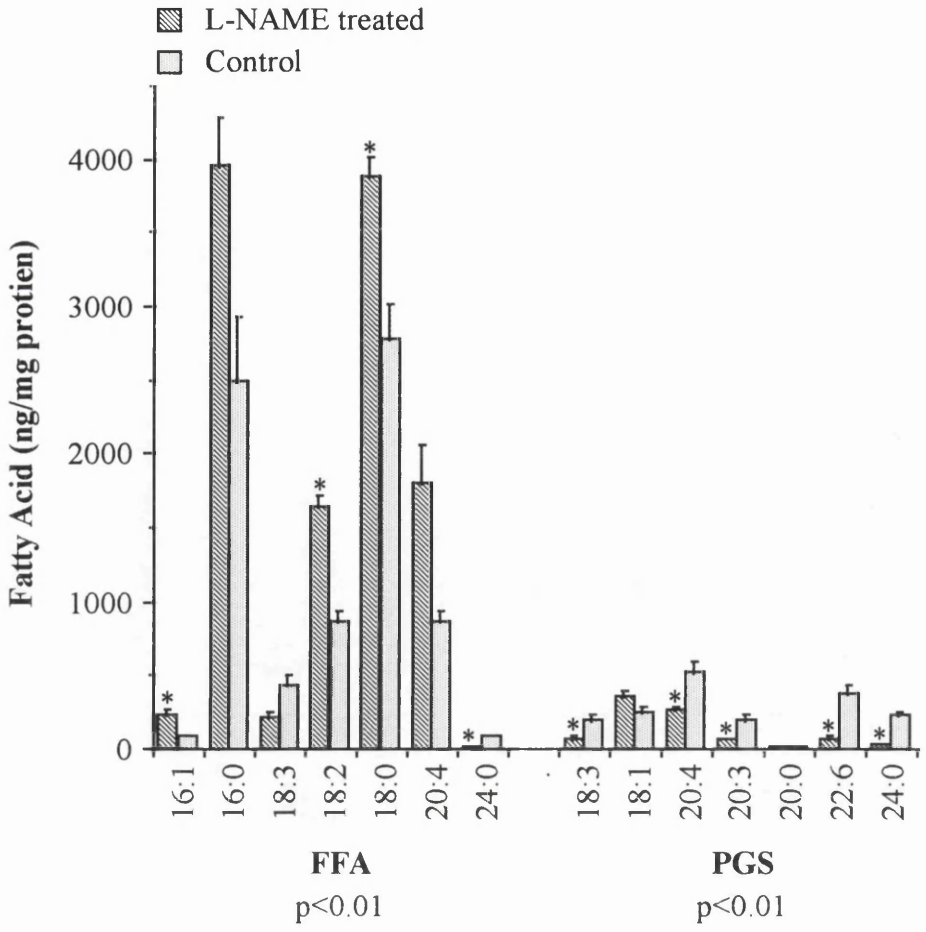
Liver Cell Membranes.

Graphs 4.3.3b and 4.3.3c summarise the differences in fatty acids composition of the liver membranes between L-NAME-treated and control rats. Arachidonic acid was present at a lower concentration in the CE and TRIG fractions but increased in the FFA fraction of the L-NAME-treated group compared to control. Dihomo-γ-linolenic and behenic acid were reduced in the TRIG and PGS fractions of the L-NAME-treated animals compared to control. L-NAME-treatment reduced the lignoceric acid content of all the neutral lipids studied.

Although not significantly altered in the total lipid fraction, the levels of the following fatty acids were altered by L-NAME-treatment. Palmitoleic acid was increased in the CE and FFA fractions. Palmitic and linoleic acid were increased in CE and FFA and reduced in the TRIG fractions. Linolenic was reduced in the TRIG, FFA and PGS fractions. Oleic acid was increased in CE and PGS and reduced in the TRIG fraction. Stearic acid was increased in the FFA fraction and arachidic acid was reduced in the CE, TRIG and PGS fractions.



Graph 4.3.3b Fatty acid composition (ng/mg protein) of CE and TRIG from liver cell membranes form L-NAME treated (n=8) and control (n=8) rats. Statistics as described previously.



Graph 4.3.3c Fatty acid composition (ng/mg protein) of FFA and PGS from liver cell membranes from L-NAME treated (n=8) and control (n=8) rats.

4.3.4 Polar Lipids.

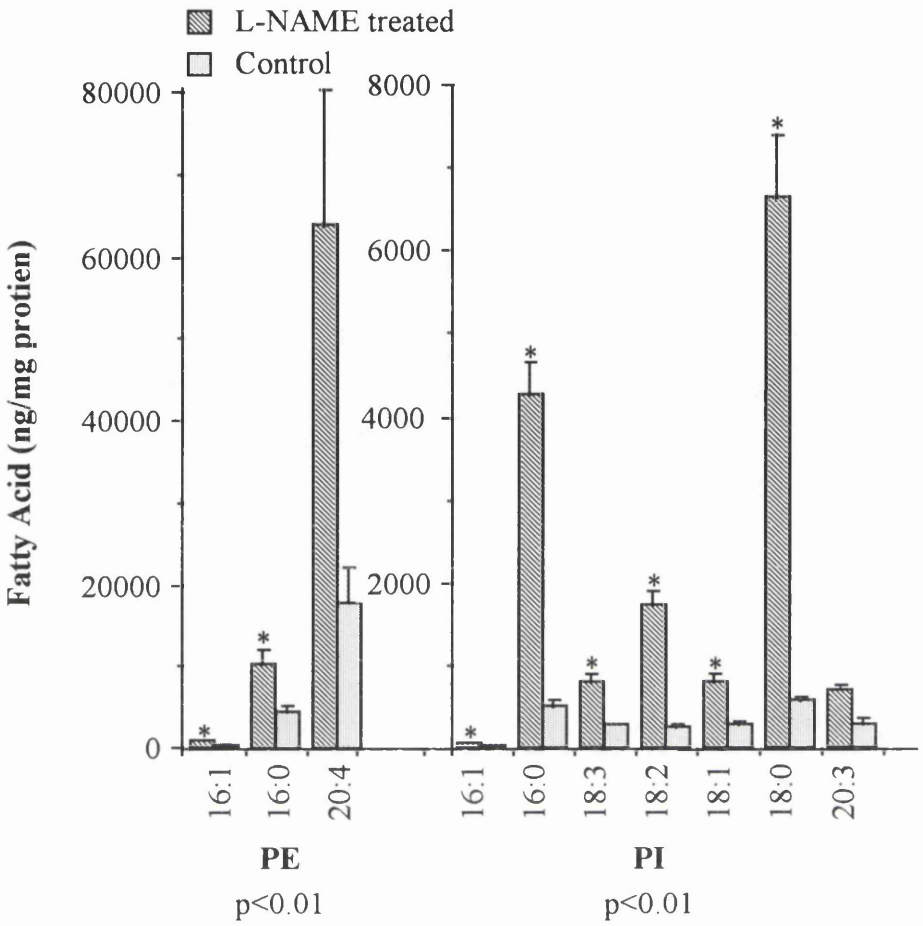
Erythrocytes

L-NAME-treatment caused alteration in the levels of fatty acids in all the polar lipid groups studied. The results are summarised in graphs 4.3.4a and 4.3.4b.

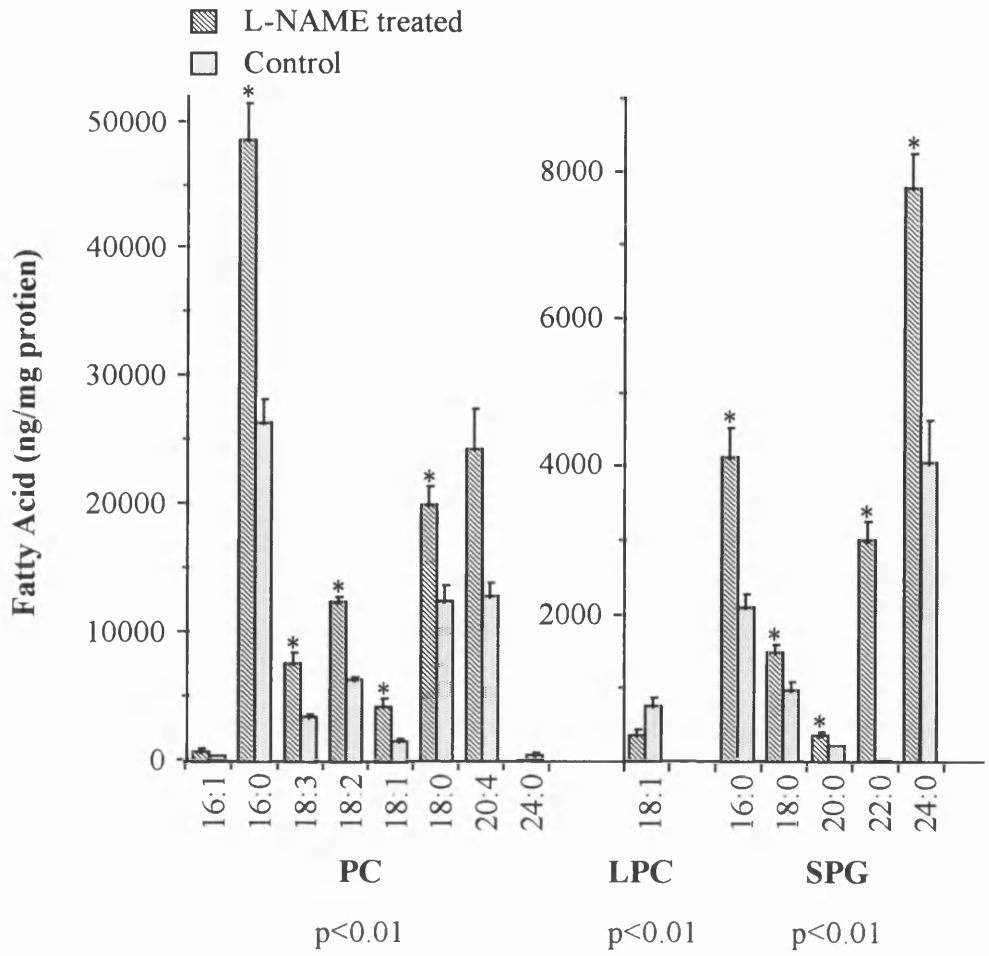
In the PI fraction, the following fatty acids were increased in the L-NAME-treated group compared to control; palmitoleic, palmitic, linolenic, linoleic, oleic and stearic acid. In the PE fraction, the levels of palmitoleic, palmitic and arachidonic acid were increased in the L-NAME-treated group compared to control.

In the PC fraction from the L-NAME-treated group, the concentrations of the following fatty acids were increased compared to control; palmitoleic, palmitic, linolenic, linoleic, oleic, stearic and arachidonic acid. In the same fraction, the levels of lignoceric acid were reduced in the L-NAME-treated group. The same was true for oleic acid in the LPC fraction. In the SPG fraction, the concentrations of the following fatty acids were

increased compared to control; palmitic stearic, behenic and lignoceric acid. L-NAME-treatment increased the amount of arachidic and dihomo-γ-linolenic acid in the polar lipid fraction although these were not significantly altered in the total lipid fraction.



Graph 4.3.4a Fatty acid composition (ng/mg protein) of PE and PI from erythrocyte membranes from L-NAME treated (n=8) and control rats (n=8).

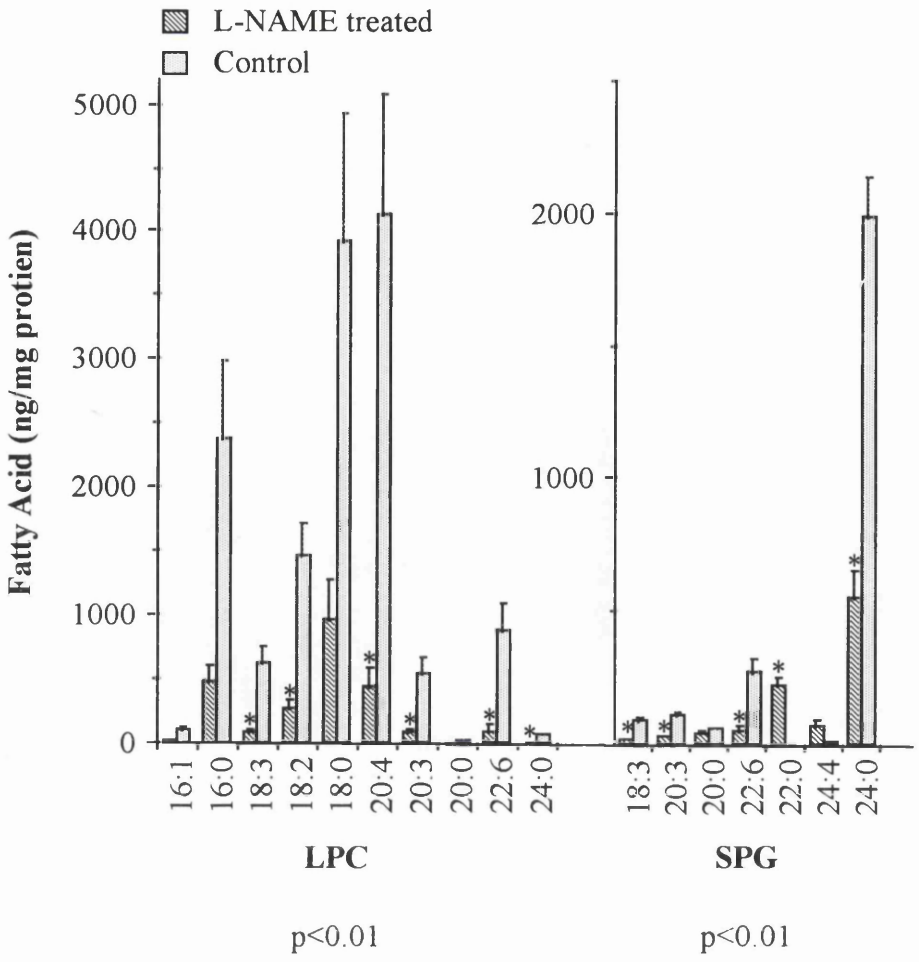


Graph 4.3.4b Fatty acid composition (ng/mg protein) of PC, LPC and SPG from erythrocyte membrane from L-NAME treated (n=8) and control rats (n=8).

Liver Cell Membranes.

In the liver cell membranes, alterations in fatty acid composition were limited to LPC and SPG. In the LPC fraction, the levels of arachidonic, dihomo-γ-linolenic and lignoceric acid were reduced in the L-NAME-treated group compared to control. In the SPG fraction, the levels of dihomo-γ-linolenic and lignoceric acid were reduced compared to control. L-NAME-treatment increased the levels of behenic acid in the SPG fraction compared to controls.

Although not altered in the total lipid fraction, the concentrations of the following fatty acids were altered by L-NAME-treatment. Palmitic, palmitoleic, linoleic and stearic acid were reduced in the LPC fraction. Linolenic and arachidic acid were reduced in both fractions and nervonic acid was increased and hexacosanoic acid reduced in the SPG fraction.



Graph 4.3.4c Fatty acid composition (ng/mg protein) of LPC and SPG from liver cell membranes from L-NAME treated (n=8) and control (n=8) rats.

4.3.5 Desaturase Activities.

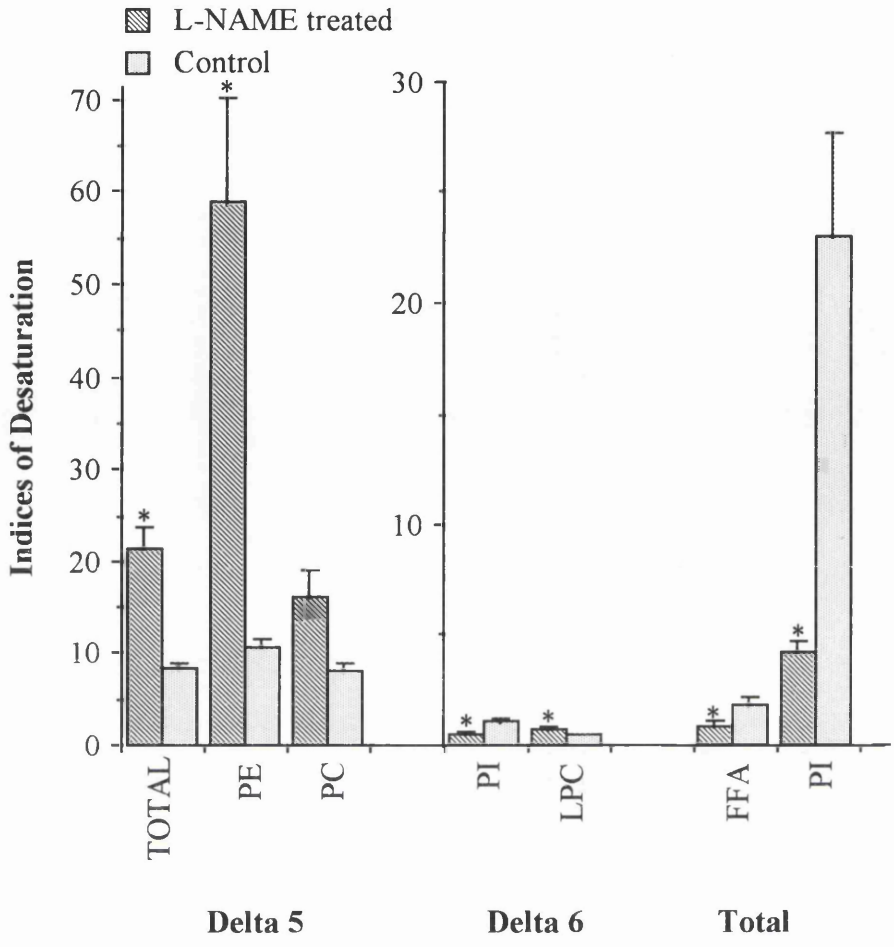
The indices of desaturation were calculated as follows;

Δ5 desaturase	=>	arachidonic / dihomo-γ-linolenic acid
Δ6 desaturase	=>	linolenic / linoleic acid
Total desaturase	=>	arachidonic / linoleic acid
Δ9 desaturase	=>	oleic / stearic acid

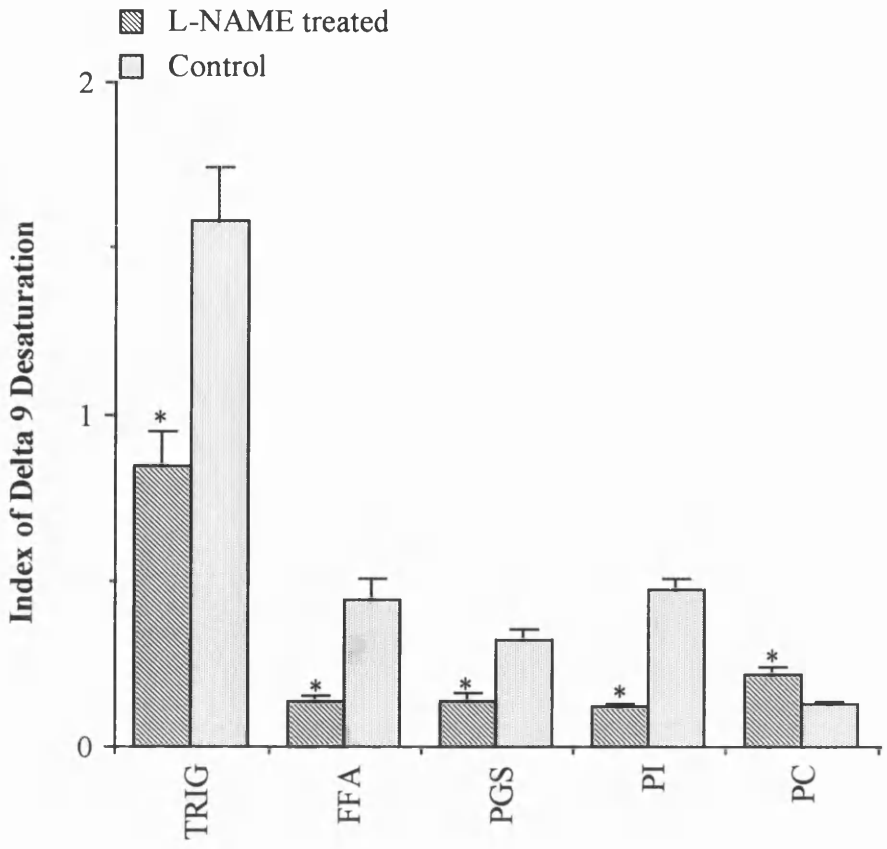
Erythrocytes

Graphs 4.3.5a and 4.3.5b show all the desaturase activities as assessed by erythrocyte membrane composition. Δ5 Desaturase was increased by L-NAME-treatment. This change is particularly marked in the PE and PC fraction. Δ6 Desaturase activity was not altered as assessed by the total membrane lipids. However, in the PI fraction of the L-NAME-treated group Δ6 desaturase was reduced compared to control; the results obtained for LPC was the opposite. Total desaturase activity was reduced in the L-

NAME-treated group when FFA and PI were considered but was unaltered in the total fatty acid fraction. Δ9 Desaturase activity was not altered in the total fatty acid fraction by L-NAME-treatment. However, a reduction was observed in the TRIG, FFA, PGS, PI and PC fractions.



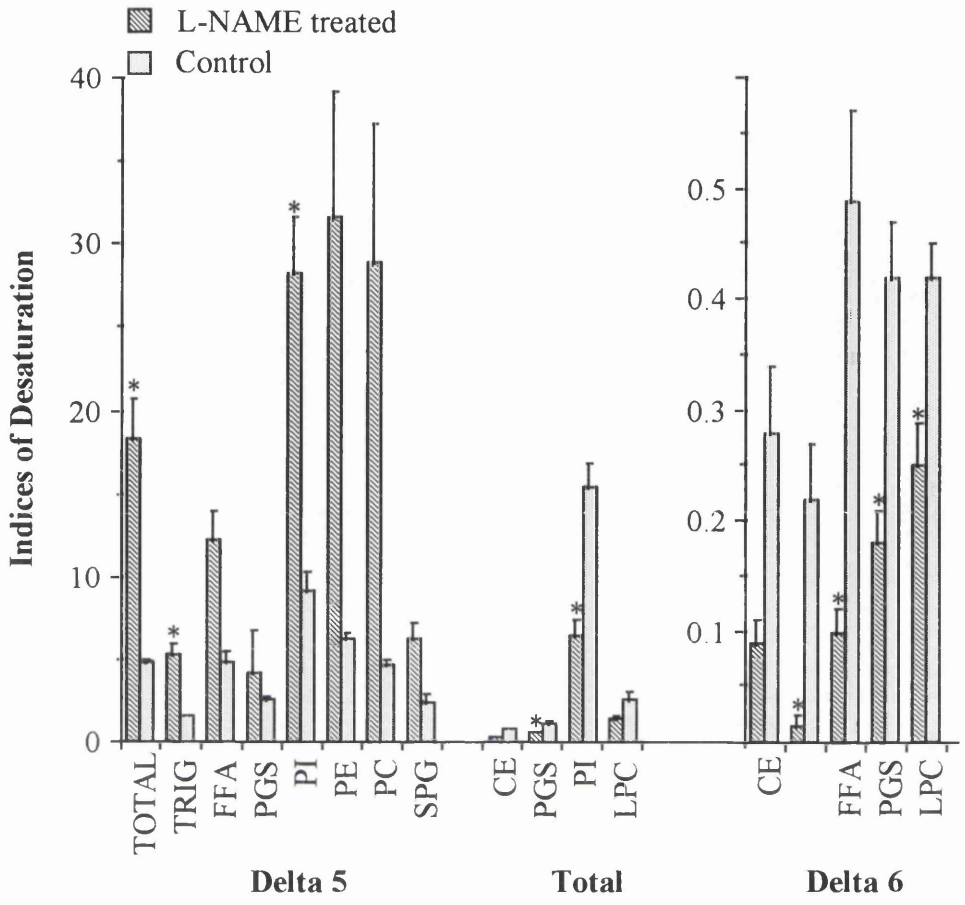
Graph 4.3.5a Indices of essential fatty acid desaturation for erythrocytes from L-NAME treated and control rats (n=8).



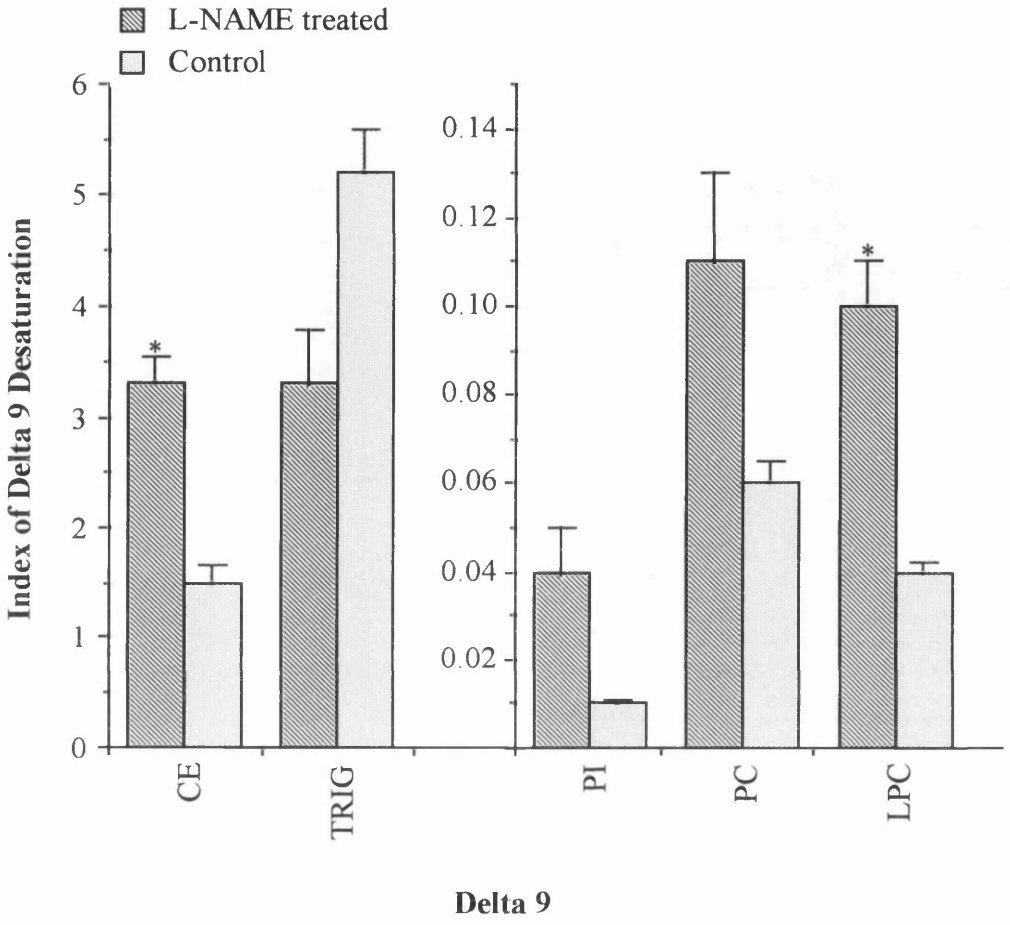
Graph 4.3.5b Indices of Δ9 desaturation for erythrocyte membranes from L-NAME treated and control rats (n=8 for both groups).

Liver Cell Membranes.

Graphs 4.3.5c and 4.3.5d shows the indices of desaturation for the lipids from liver cell membranes. Δ5 Desaturase activity was increased in the total lipid fraction of the L-NAME-treated group compared to control. This was apparent in the TRIG, FFA, PGS, PI, PC PE and SPG fractions. No change was observed in the Δ6 desaturase in the total lipid fraction from the two groups. However, a reduction in Δ6 desaturase activity was observed for the CE, TRIG, FFA, PGS and LPC fraction. The same was true for the total desaturation of the essential fatty acids; reductions in desaturase activity were observed in the CE, PGS, PI and LPC fractions. There was no significant change in the activity of the Δ9 desaturase when total fatty acids were considered. However, there was an increase in the index of Δ9 desaturase activity in the CE, PI, PC and LPC fractions in the L-NAME treated group. The activity was reduced for the TRIG fraction from the L-NAME-treated group compared to control.



Graph 4.3.5c Indices of essential fatty acid desaturation for liver cell membranes from L-NAME treated and control rats (n=8 for both groups).

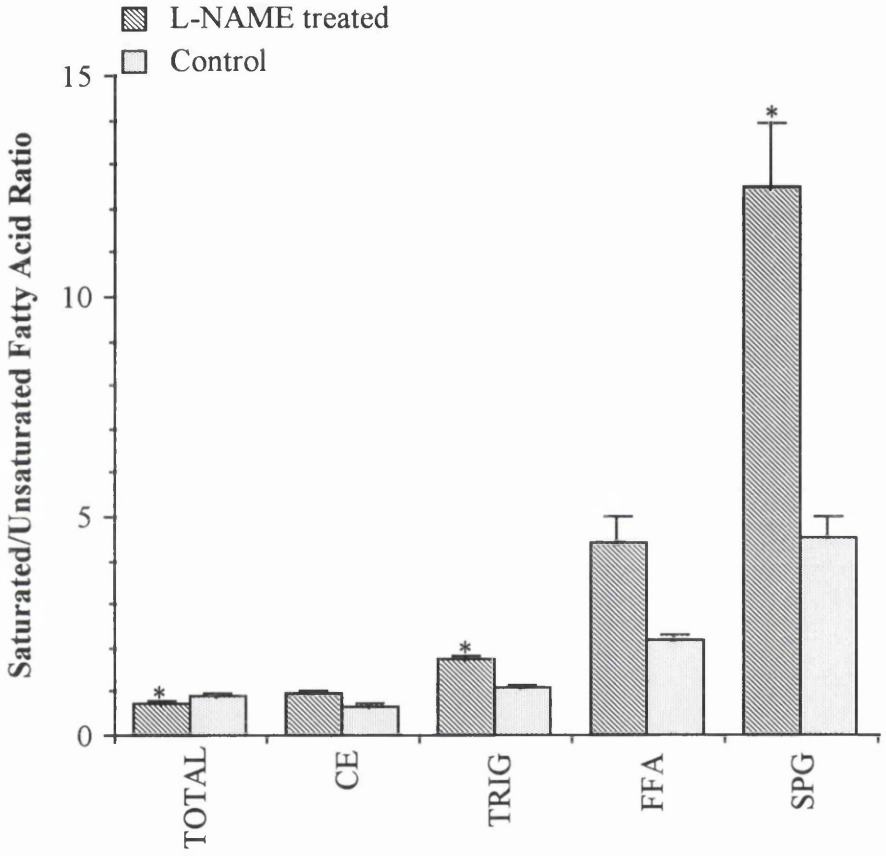


Graph 4.3.5d Indices of Δ9 desaturation for liver cell membranes from L-NAME treated and control rats (n=8 for both groups).

4.3.6 Saturated/Unsaturated Fatty acid Ratio.

Erythrocytes

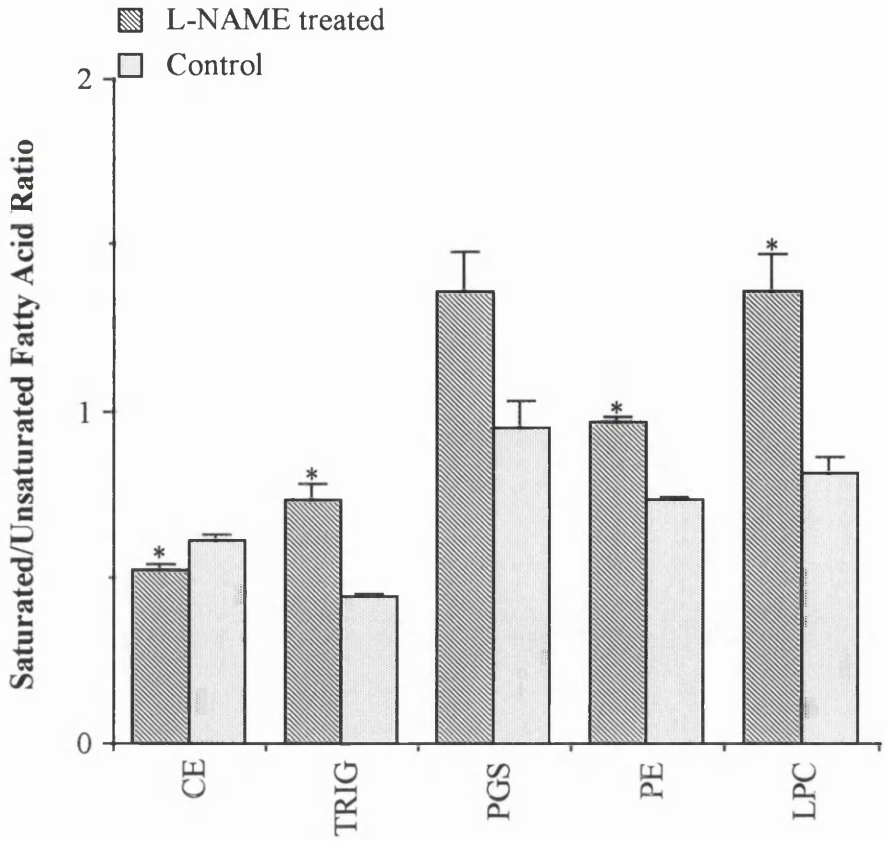
Graph 4.3.6a compares the saturated/unsaturated fatty acid ratios for the fatty acids in the erythrocyte membrane from the L-NAME-treated and control rats. Considering the total membrane fatty acids, there was a reduction in the saturated/unsaturated fatty acid ratio in the L-NAME-treated group compared to control. In the individual lipid groups, the opposite was observed for the CE, TRIG, FFA and SPG fractions. No significant change was seen in other fractions.



Graph 4.3.6a Saturated/unsaturated fatty acid ratio for lipids from erythrocyte membranes from L-NAME treated and control rats (n=8).

Liver Cell Membranes.

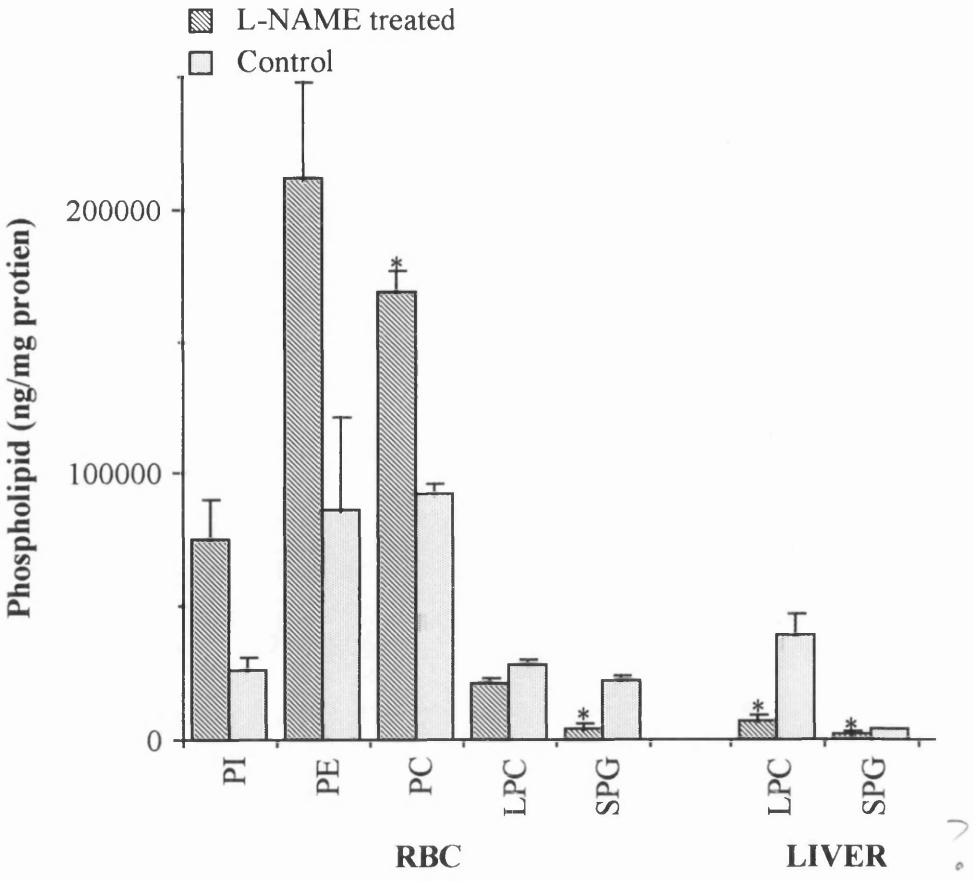
Graph 4.3.6b shows a similar comparison of this ratio in the liver cell membranes form L-NAME-treated and control rats. There was no change in the ratio in the total fatty acid fraction but it was increased in the TRIG, PGS, PE and LPC fractions and reduced in the CE fraction in the L-NAME-treated group compared to control.



Graph 4.3.6b Saturated/unsaturated fatty acid ratio for lipids from liver cell membranes from L-NAME treated and control rats (n=8 for both groups).

4.3.7 Phospholipids.

Graph 4.3.7 shows the phospholipid composition (derived for the average fatty acid and core phospholipid molecular weight) of the liver and erythrocyte membranes from the L-NAME-treated group compared to control animals. In the erythrocyte, the L-NAME-treated animals had significantly more PI, PE and PC and less LPC and SPG than the controls. In the liver cell membranes, the levels of LPC and SPG were reduced in the L-NAME-treated group compared to control.



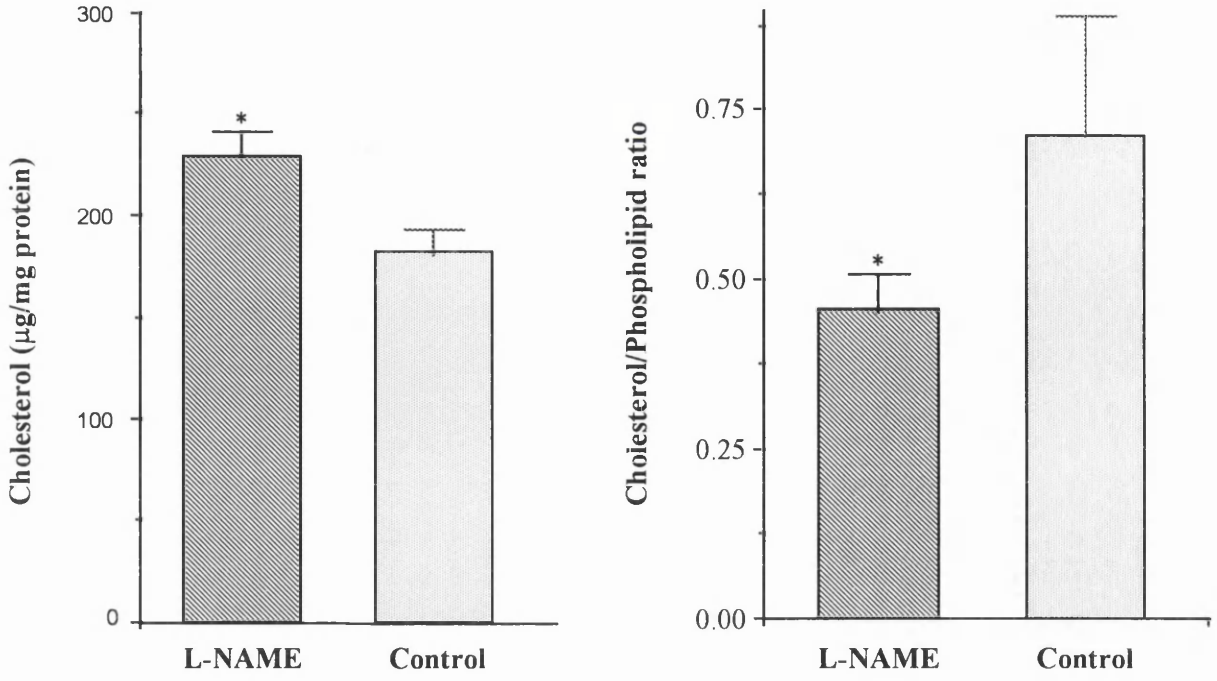
Graph 4.3.7 Derived phospholipid composition (ng/mg protein) for erythrocytes and liver cell membranes from L-NAME treated and control rats (n=8 for both groups).

4.3.8 Membrane Cholesterol and Cholesterol/Phospholipid Ratio.

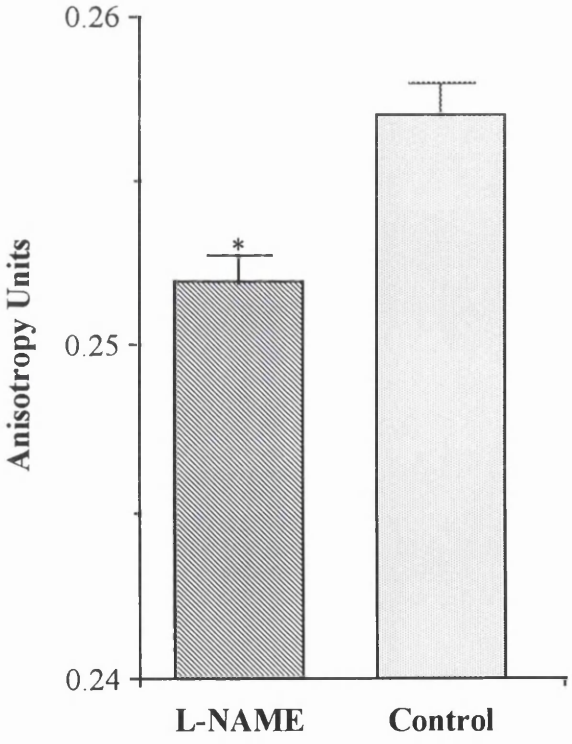
Graph 4.3.8a shows the erythrocyte membrane cholesterol measurements for the L-NAME-treated and control animals. L-NAME-treatment increased the membrane cholesterol content. The cholesterol/phospholipid ratio (graph 4.3.8b) was reduced by L-NAME-treatment.

4.3.9 Membrane Microviscosity.

Erythrocyte membrane microviscosity was lower in the L-NAME-treated group than in the control group (graph 4.3.9).



Graph 4.3.8a & b Erythrocyte membrane cholesterol content (µg/mg protein) for L-NAME and control rats and the cholesterol/phospholipid ratio for L-NAME treated and control rats (n=8 for both groups)..



Graph 4.3.9 Erythrocyte membrane microviscosity for L-NAME treated and control rats measured using fluorescent probe TMA-DPH (n=8 for both groups).

4.4 Discussion.

Inhibition of NO synthesis by L-NAME caused a rapid increase in systolic blood pressure to a level similar to that observed in mature SHRSP. L-NAME treatment also caused erythrocyte membrane microviscosity to decrease compared to control. Parallel analysis of membrane lipid composition showed a complex pattern of changes consequent, directly or indirectly, on L-NAME treatment. To what extent can these changes explain the physical alteration in the erythrocyte membrane? In which lipid component are these changes most significant? Moreover, alteration in the levels of some fatty acids, while possibly having little effect in microviscosity, may never-the-less have important physiological implications. Finally, it is interesting to compare changes in erythrocyte membrane with those of the liver cell membranes.

The fatty acid composition of the membrane affects fluidity. At the most simple level of analysis, total fatty acid composition. L-NAME treatment increased the levels of endogenously synthesised fatty acids and essential fatty acids and their metabolites. There are two possible reasons for these changes. Firstly, there may indeed be more lipid present in the membrane from the L-NAME-treated group, or alternatively, because the fatty acid concentrations were calculated relative to membrane protein concentration, there may be less protein. However, in the later case, changes in fatty acid composition would have been the same for all the lipid groups and in the same proportion. This was not the case. A combination of saturated (rigidifying) and unsaturated (fluidising) fatty acid were altered by L-NAME treatment. Since these have opposite effect, the overall change is difficult to estimate. However, as more unsaturated fatty acids were affected than saturated fatty acids and the increases in the amounts of arachidonic and docosahexaenoic acid were very large, it is possible to suggest that fluidity would be increased.

The differences observed in erythrocyte neutral lipid fatty acid composition closely mimicked those of the total lipid fraction, with the exception of nervonic acid which was higher in the FFA fraction but not in the total lipids. The neutral lipids are present in small quantities in the membrane and changes in their composition will probably not have a significant effect on membrane physical properties (The combined fatty acids from the neutral lipids make up less than 10% of the total fatty acid).

The changes in the fatty acid composition of erythrocyte membrane polar lipids were also similar to those observed in the total fatty acid composition. Some of these changes may

have contributed the increase in membrane fluidity. The arachidonic acid content of the PE fraction was increased by L-NAME treatment. PE is the major inner leaflet lipid and this increase in an unsaturated fatty acid suggests that this region would have become more fluid. In contrast, the changes in the fatty acid composition of the choline-containing lipids should predispose to lower fluidity. Thus, increased amounts of long chain fatty acids, behenic and lignoceric acid, in the PC and SPG fraction should have increased the membrane microviscosity (Lodish *et al* 1995). PC and SPG are located primarily in the outer leaflet, suggesting that this area of the membrane may be less fluid. It would be interesting to check these differential effects by using fluorescent probes for the inner and outer leaflet of the membrane. Unfortunately, haemoglobin interferes with the fluorescence making this impossible. An alternative cell, the platelet, is difficult to obtain from rats in large enough quantities to carry out both lipid analysis and membrane microviscosity measurement. It is interesting to note that the changes in the LPC fraction did not mimic those of the total lipid fraction as well as the other choline-containing lipids; only stearic acid was increased by L-NAME treatment. LPC is a minor membrane component, (there is roughly 10 times more PE in the membrane than LPC in the treated group and 3 times more in the untreated group) and some of these changes may have been undetectable.

Another determinant of membrane fluidity is the activity of the desaturases. Increased activity leads to the production of more unsaturated fatty acid, thus decreasing microviscosity. Erythrocytes have no desaturase activity and their fatty acid composition reflects the fatty acid composition of the plasma. This in turn is controlled, in part, by the liver which is a major site of fatty acid desaturation. Desaturase activity in the liver contributes to the fatty acid composition of erythrocyte membrane. The ratio of fatty acid product and substrate for the enzyme in the erythrocyte thus provides an index of desaturase activity. Δ5 Desaturase activity was increased in the total fatty acid fraction by L-NAME treatment. This increase was also apparent in the fatty acids esterified to PE and PC, the two major membrane lipids, although the amount of arachidonic acid relative to dihomo-γ-linolenic acid was much greater for the PE fraction than for the PC fraction. This increase in desaturation may have contributed to the increased membrane fluidity observed here. Δ6 and Δ9 Desaturase activity in the total membrane lipid fraction was not altered by L-NAME treatment. Δ6 Desaturase activity was increased in the LPC fraction and reduced in the PI fraction. However, the significance of this is questionable as there are no reports of desaturases acting on fatty acids esterified to LPC and PI. Neither LPC

or PI are major membrane lipids and these changes probably do not significantly affect membrane fluidity. Changes in the plasma lipid composition were not studied. Whether L-NAME prevented the erythrocyte membrane from equilibrating with the plasma or whether the plasma composition was altered cannot therefore be determined.

The ratio of saturated/unsaturated fatty acid in the membrane can also determine membrane fluidity. This ratio was reduced in the total lipids fraction, suggesting more unsaturated fatty acid in the membrane. This should cause the membrane to become more fluid (as was the case). In contrast, in CE, TRIG, FFA and SPG, there was an increase in the saturated/unsaturated fatty acid. However, apart from SPG, these are minor membrane components. SPG contains primarily saturated fatty acid and it is possible that a further increase, as seen here, would not further affect membrane fluidity.

Lipid composition, in addition to fatty acid composition affects membrane fluidity. L-NAME treatment increased the derived concentrations of PI, PE and PC and reduced the concentration of LPC and SPG. SPG is a membrane rigidifying agent and PC is a membrane fluidising agent (Shinitzky & Barenholz 1974, Borochov *et al* 1977). Thus the changes in phospholipid composition agree with the direct physical measurements of membrane fluidity. Another determinant of fluidity is cholesterol. L-NAME treatment increased the levels of erythrocyte membrane cholesterol which should make the membrane more rigid, in contradiction to the results of direct measurement. However, the membrane cholesterol/phospholipid ratio was lower in the L-NAME treated group, in agreement with direct measurement. Thus, increased membrane fatty acid and phospholipid content, which reduced the cholesterol/phospholipid ratio in the L-NAME-treated group, appears to have more than counteracted the influence of membrane cholesterol. This highlights the necessity of analysing both lipid composition and membrane microviscosity when considering membrane dynamics.

L-NAME treatment induced changes in liver cell membranes which were different from those observed in erythrocytes. These differences may have been due to different effects of L-NAME or blood pressure on the two membrane types. Liver cells actively synthesise fatty acids and are a major site of essential fatty acid desaturation. Conversely, erythrocytes have no desaturase activity and their fatty acid composition is controlled in part by the fatty acid composition of the plasma (Donabedian & Karmen 1967). The liver, therefore, may be more capable of maintaining cell membrane homeostasis than the

erythrocyte. Also, the liver cell has a nucleus which allows it to control more precisely which proteins are expressed to respond to changes in the environment. It is therefore probable that the lipid environment to which the erythrocyte is exposed is more variable and subject to short term alteration than that of the liver cell. Thus, it is not unexpected that there are differences in the effects of L-NAME in the two membranes, although it has been for some time a common misconception that the erythrocyte membrane is an index of the fatty acid composition of all other membranes. Obviously, for a study of the changes caused by L-NAME and blood pressure, the VSMC would be more relevant. Unfortunately, it was not possible to study these cells in the current experiment. However, the erythrocyte is probably more similar to VSMC than the hepatocyte as there is no evidence to suggest that VSMC have desaturase activity and they are not major sites of fatty acid biosynthesis. Thus the levels of at least the PUFA present in this membrane are probably determined in a similar fashion.

→ ? effects on chain elongation.

The alterations in the fatty acid composition of the total lipid fraction were limited to the levels of four long chain fatty acids, three (arachidonic, dihomo-γ-linolenic and lignoceric acid) of which were reduced and the other (behenic acid) increased by L-NAME treatment. Again, these were a combination of saturated and unsaturated fatty acids, making their contribution to membrane fluidity difficult to compute. However, as two unsaturated fatty acids were reduced (arachidonic, dihomo-γ-linolenic) and one saturated fatty acid greatly increased (behenic acid) by L-NAME treatment a reduction in membrane fluidity could be postulated. The reduction in lignoceric acid would increased membrane fluidity, but the relative concentrations of the fatty acids would suggest that the other three fatty acid would override this effect leading to reduced fluidity. There were no changes in the fatty acid composition of the major lipids PE and PC, suggesting that the liver's homeostatic mechanism maintains lipid and fatty acid composition. Changes in fatty acid composition were limited to minor lipid groups with the exception of SPG.

The changes observed in the liver cell membrane neutral lipid fraction were more widespread and different from those observed for the total lipid fatty acids. Neutral lipids, as discussed previously, play a minor role in membrane structure and functioning and the fatty acid composition of these lipids will probably not affect membrane fluidity. Interestingly, there was a general reduction in the amount of fatty acids present in the TRIG and PGS fractions, implying a reduction in the amount of these lipids present in the membrane. TRIG and PGS have a similar origins and are precursors of phospholipid

molecules. TRIGs, although present in the membrane, are primarily fuel storage molecules. A reduction in their membrane levels may suggest that L-NAME affects storage, perhaps reducing the amount of TRIG produced by the cell. Alternatively, the reduction in TRIG may be due to an increased phospholipid synthesis.

The levels of lignoceric acid were reduced in the L-NAME-treated SPG fraction, the converse of what occurs in the erythrocyte. Although all the fatty acids which were altered in the total lipid fraction were also altered in the SPG fraction, other fatty acid were also affected which were unchanged in the total lipids. Many of the fatty acids present in the LPC fraction were reduced by L-NAME treatment; calculation of the LPC concentration confirmed that there was less LPC present. This could result from an increase in acyltransferase activity in the L-NAME-treated animals or a reduction in the activity of phospholipase. Further studies are necessary to distinguish between these possibilities. The SPG concentration in the membrane was reduced in the L-NAME treated group suggesting that, like the erythrocyte, the liver cell membranes will be more fluid. Unfortunately, it was not possible to measure membrane microviscosity in these cells.

The indices of desaturation measured in the liver cell membranes are more directly relevant to enzyme activity. As in the erythrocyte membrane, Δ5 desaturase activity was increased in the total lipid fraction of the L-NAME-treated animals. This effect was observed in all membrane lipid groups except CE and LPC. The results of the Δ6, Δ9 and total desaturase were similar to those in the erythrocyte, in that changes were observed in some individual lipid groups but not in the total lipid fraction. The increase in the activity of the Δ5 desaturase in both in the liver and the erythrocyte suggests that the erythrocyte may in fact provide a good measure of this enzyme.

The mechanisms by which L-NAME treatment alters the membrane remain unclear. It is possible that the increase in blood pressure may have affected the cell membrane directly. However, the change in membrane fluidity observed here is opposite to that observed in genetic hypertension (Montenay-Garesterier *et al* 1981, Dominiczak *et al* 1991, McLaren *et al* 1993). The absence of NO production may also have affected cell membrane composition. It is interesting to note that SHRSP have less tissue NO than WKY controls despite higher NOS activity (McIntyre *et al* 1996). SHRSP have higher membrane microviscosity. Also, in the L-NAME-treated rats, there is effectively no NO production

and membrane microviscosity was reduced. Thus, changes in microviscosity found in the current experiment may relate to the severely reduced amount of NO produced. Others have reported that intravenous infusion of the NO donor, nitroglycerine, into dogs causes an increase in platelet membrane microviscosity (Bassenge & Fink 1996). It is also possible that L-NAME may itself have had a direct effect on the membrane.

Some previous studies have considered the effect of lipids on NOS activity and the ability of NO to permeate the membrane. In bovine aortic endothelial cells, PC, LPC and PE increased the activity of NOS in a dose-dependant manner by increasing Vmax (Hirata *et al* 1995). In the current study, the levels of PE and PC were increased and may have had the same effect. It is possible that this represents an attempt by the cell to increase the amount of NO produced and overcome the inhibition caused by L-NAME. The levels of LPC in the membrane were reduced in this study; this would not increase NO production. Conversely, arachidonic acid may inhibit NOS in aortic endothelial cells (Hirata *et al* 1995). The same is true for oleic acid which is thought to act through a protein-kinase C-independent mechanism (Davda *et al* 1995). In the current study, the levels of erythrocyte arachidonic and oleic acid were increased by L-NAME treatment which would further exacerbate the inhibition of NOS by L-NAME.

Interestingly, NO has been reported to increase arachidonic acid release from membrane lipids by stimulating the activity of a Ca^{2+}-independant phospholipase A in a macrophage-like cell line (RAW-264.7) (Gross *et al* 1995). If the same is true for erythrocytes, then L-NAME may reduce the activity of this phospholipase which may explain the high levels of arachidonic acid remaining in the membrane. Such a mechanism would not involve changes in desaturase activity. A reduction in the activity of the phospholipase would explain the reduction in the amount of LPC present in both liver and erythrocyte membranes. Direct measurement of the enzyme activity would be required to confirm the mechanism.

The rise in erythrocyte membrane cholesterol may be a response to the reduction in the available NO. It has previously been shown that, by increasing the membrane cholesterol artificially, the amount of NO transported into the inside of the bilayer increases (Subczynski *et al* 1996). However, these experiments were carried out in artificial PC membranes and their physiological relevance is not clear. If the changes which occur in the cholesterol composition of the erythrocyte membrane also occur in VSMC, then this

might increase the proportion of vasodilator entering from adjacent endothelial cells, thus increasing vasodilation and reducing blood pressure. Others have observed that cholesterol is capable of modulating the activity of NOS. In fact, cholesterol appears to have a biphasic effect on NOS activity. Liposomes containing high and low concentrations of cholesterol were incubated with endothelial cells; high cholesterol concentration reduced the activity of NOS and low concentrations increased the activity (Deliconstantinos *et al* 1995). However, because the actual membrane cholesterol content was not measured, it is impossible to say how these results relate to those obtained here.

Inhibition of NOS by L-NAME has been shown to produce a renin-dependent form of hypertension (Morton *et al* 1993), which has the advantage over the two-kidney one-clip Goldblatt model that it is non-invasive. To the best of my knowledge, this is the first study which shows changes in membrane physical properties in hypertension of this type.

Thus biochemical analysis and physical measurement of the changes in membrane properties are in broad agreement. More detailed information is produced by biochemical analysis, some of which has functional as opposed to structural significance.

dependent
dependant.

Chapter 5 Genetic Hypertension in the Stroke-Prone-Spontaneously Hypertensive Rat.

5.1 Introduction.

Cell membrane microviscosity is probably increased in genetically hypertensive rats (Montenay-Garestier *et al* 1981, Orlov *et al* 1982a, Devynck *et al* 1982, McLaren *et al* 1993) and in patients with essential hypertension (Orlov & Prostnov 1982, Naftilan *et al* 1986, Tsuda *et al* 1987). Various abnormalities have also been observed in membrane transport systems, such as sodium and potassium channels (Jones 1973, Furspan & Bohr 1985, Furspan *et al* 1986) and sodium-calcium exchangers (David-Dufilho *et al* 1986) in hypertension. Because so many membrane functions are altered in hypertension, it has been suggested that this is due not to changes in the protein composition but to the matrix in which this protein is embedded. Altering the composition of the cell membrane alters the activity of some proteins. For example, opioid receptor function can be modulated by membrane fatty acid composition (Remmers *et al* 1990). Others studies have shown that decreasing membrane microviscosity increases the β-adrenergic receptor affinity for adenylate cyclase (Rimon *et al* 1978) and increases the activity of Na^+-K^+-ATPase (Yeagle 1989). Moreover, by altering the composition of the fatty acids present in PC and PE, it is possible to alter the activity of Na^+-Li^+-counter transport (Engelmann *et al* 1993). The increased membrane microviscosity observed in genetic hypertension could be caused in several ways, including increased membrane protein concentration, a reduction in the unsaturated fatty acid content of the membrane or an increase in the amount of long chain saturated fatty acids present.

A variety of differences have been reported in the composition of the cell membrane in hypertension. Naftilan *et al* (1986) observed that the platelet membranes from patients with essential hypertension contain less linoleic acid than those from normotensive controls. In the same study, there was a small increase in the amount of arachidonic acid present in the membranes from patients with essential hypertension. However, because of the small sample number, this difference was not significant. Similar changes have been observed in erythrocyte membranes from patients with essential hypertension (Ollerenshaw *et al* 1987). Dominiczak *et al* (1993) reported an increase in the proportion of arachidonic acid in cultured vascular smooth muscle cells from SHRSP as compared to WKY. The opposite has been observed in the mesenteric arterial bed, although the results did not reach statistical significance (Mtabaji *et al* 1988). In this case, the lipids were extracted

from the whole mesenteric arterial bed which is surrounded with fat. It is not clear whether the fat was completely removed from the mesenteric bed prior to lipid extraction. This may have affected the accuracy of the results.

Most previous studies have concentrated on the fatty acid composition of the total membrane lipids. No attempt has so far been made to ascertain whether these abnormalities occur in particular lipid groups. The aim of the first part of this study was to compare the membrane fatty acid composition of both liver cell and erythrocyte membranes from young $SHRSP_{(Glasgow)}$ and WKY. The aim of the second part of this study was to compare the fatty acid composition of the erythrocyte membrane from $SHRSP_{(Heidelberg)}$ and WKY. An F_2 segregating population, produced by brother-sister back-crossing the F_1 population, was then used to study whether any of the traits observed in the parental strains segregated with blood pressure.

5.2 Materials and Methods.

5.2.1 Study 1.

$SHRSP_{(Glasgow)}$ and WKY rats were fed normal rat chow and water *ad libitum.* At nine weeks of age, the systolic blood pressure was measured by the tail cuff method as described previously (Chapter 2). Liver and blood samples were obtained at 11 weeks of age and analysed as previously described (n=8). Data were analysed as described in chapter 4 and results are presented in a similar fashion.

5.2.2 Study 2

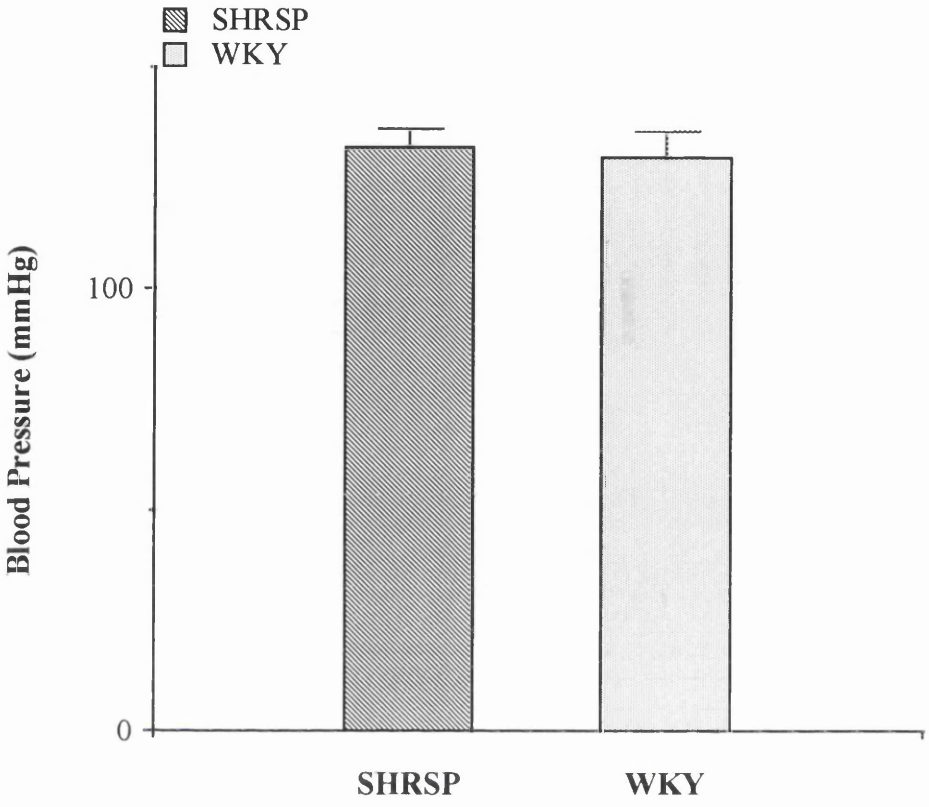
Blood samples from the parental strains of $SHRSP_{(Heidelberg)}$ and WKY and the F_2 population of this cross were obtained from the laboratory of Prof. D. Ganten and Dr K. Lindpaintner (Berlin, Germany). Samples were obtained when the rats were 20 weeks of age. The animals were housed under normal laboratory conditions, fed normal rat chow and received sodium chloride in their drinking water. The samples were transported from Berlin to Glasgow on ice. Erythrocyte membranes were prepared on arrival.

The samples from the parental strain were analysed as for study 1 (n=14). Where statistically significant differences were obtained, these traits were then studied in the F_2 population (n=18). Regression analysis was used to assess whether the traits correlated with blood pressure.

5.3. Results.

5.3.1 Blood Pressure.

There was no significant difference between the systolic blood pressures from the $SHRSP_{(Glasgow)}$ and the WKY rats (graph 5.3.1) at 9 weeks of age.

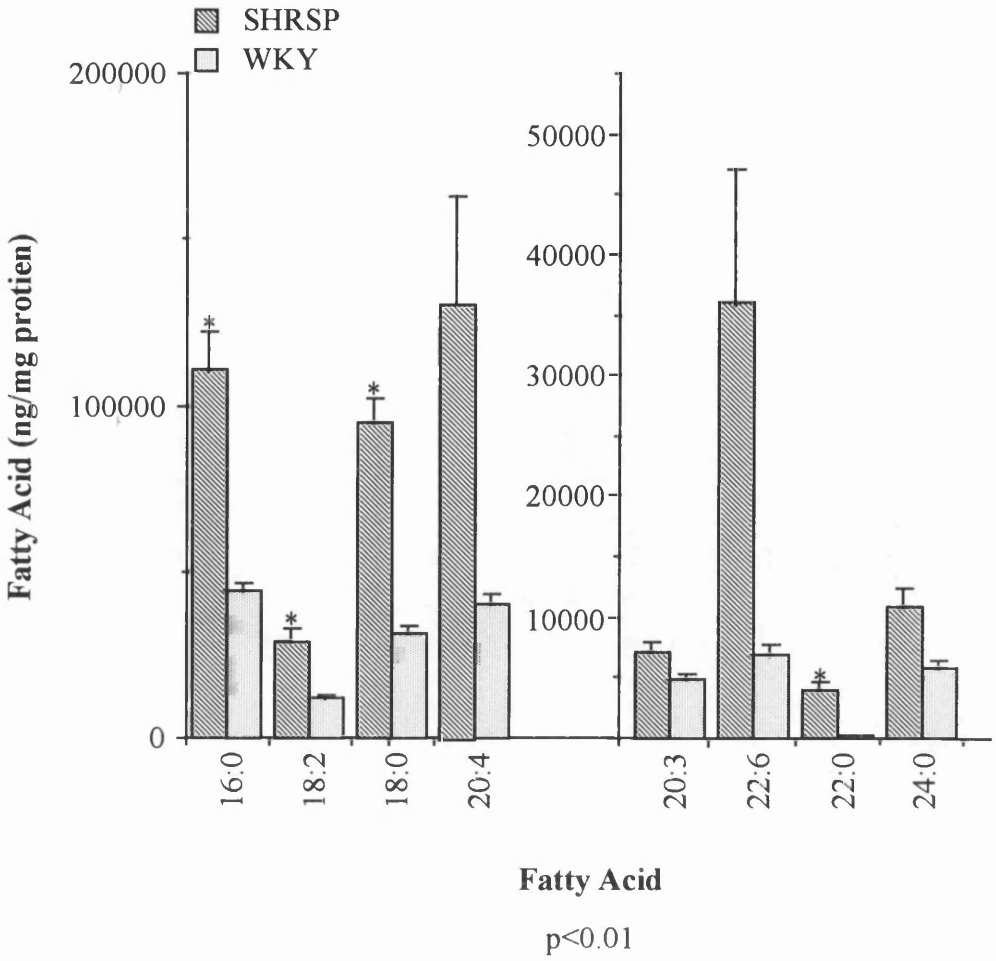


Graph 5.3.1 Tail cuff systolic blood pressure measurements (mmHg) for $SHRSP_{(Glasgow)}$ and WKY rats at 9 weeks of age (n=8 for both groups),

5.3.2 Total Fatty Acid Composition.

Erythrocytes

Graph 5.3.2a shows the differences observed in the total fatty acid composition of the erythrocyte membranes from $SHRSP_{(Glasgow)}$ and WKY. Palmitic, linoleic, stearic, arachidonic, dihomo-γ-linolenic, docosahexaenoic, behenic, lignoceric and hexacosanoic acid were all higher in the erythrocyte membranes from $SHRSP_{(Glasgow)}$ compared to the WKY.



Graph 5.3.2a Fatty acid composition (ng/mg protein) of total erythrocyte lipids from $SHRSP_{(Glasgow)}$ and WKY rats. P value for ANOVA shown beneath the graph, all results ate statistically significantly different by T-test ($p<0.05$), * indicates $p<0.01$.

Liver Cell Membranes.

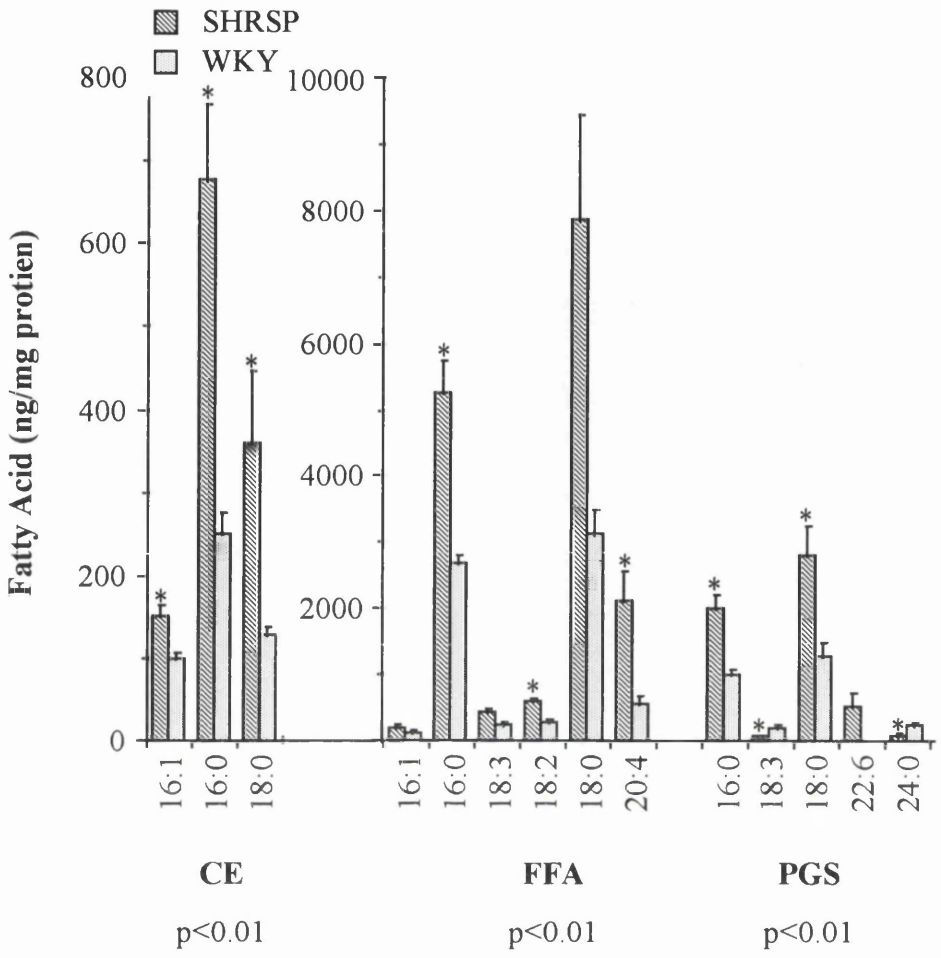
There was no difference in the total fatty acid profiles of liver cell membranes between $SHRSP_{(Glasgow)}$ and WKY.

5.3.3 Neutral Lipids

Erythrocytes

Graph 5.3.3a details the differences observed in the fatty acid composition of the neutral lipids from $SHRSP_{(Glasgow)}$ and WKY. Palmitic and stearic acid were higher in the CE, FFA and PGS fractions of $SHRSP_{(Glasgow)}$ compared to WKY. In the FFA fraction, linoleic and arachidonic acid were also higher in $SHRSP_{(Glasgow)}$. There was more docosahexaenoic acid and less lignoceric acid present in the PGS fraction from $SHRSP_{(Glasgow)}$ compared to WKY

Although not different in the total lipid fraction, palmitoleic acid was higher in the CE and FFA fractions from $SHRSP_{(Glasgow)}$ compared to WKY. Linolenic acid was higher in the FFA fraction and lower in the PGS fraction of the $SHRSP_{(Glasgow)}$.

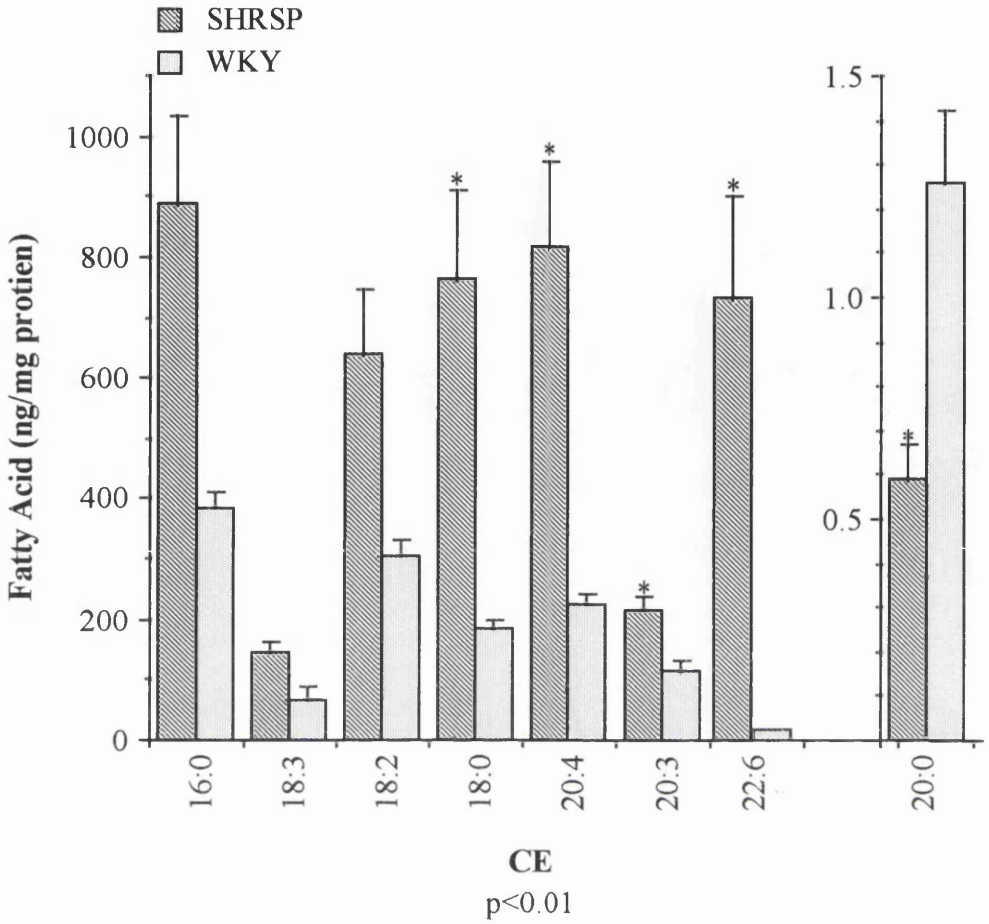


Graph 5.3.3a Fatty acid composition of neutral lipids (ng/mg protein) for erythrocytes from $SHRSP_{(Glasgow)}$ and WKY rats. Statistics as described previously (n=8 for both groups).

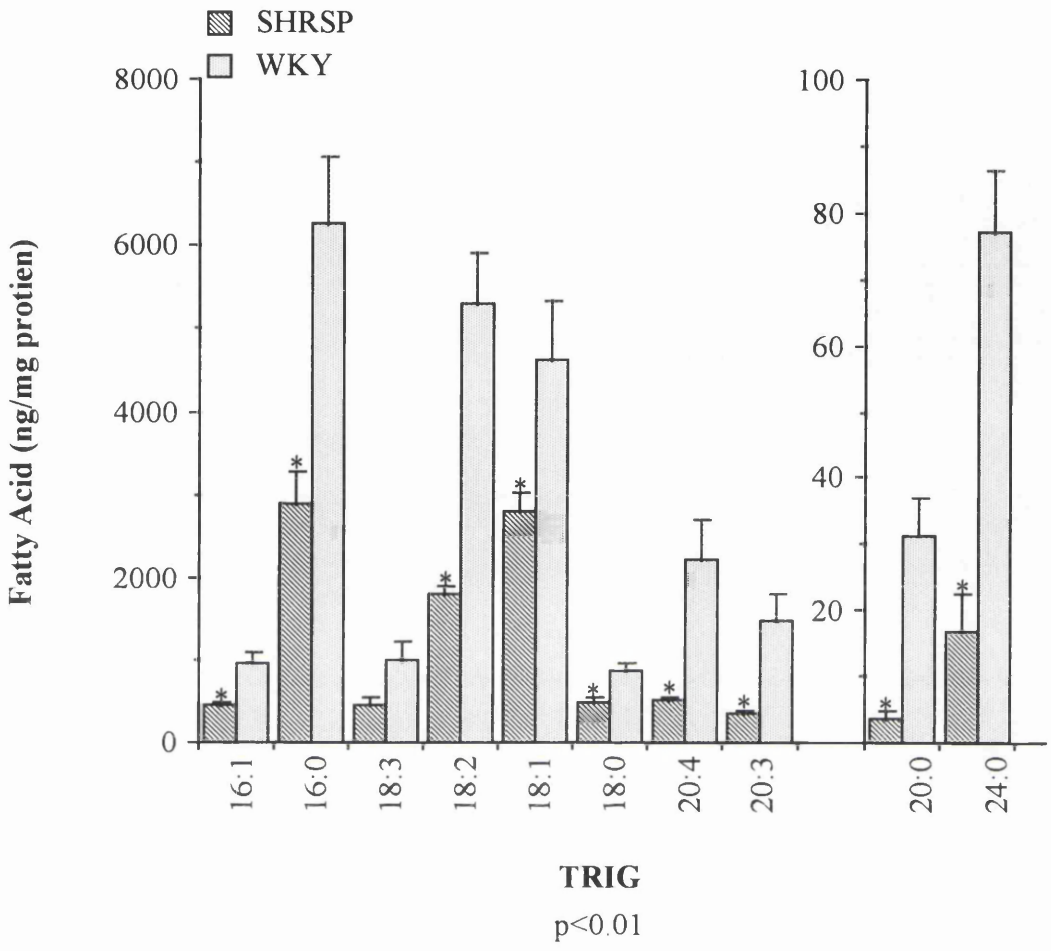
Liver Cell Membranes

Although there were no significant differences in the fatty acid composition of the total lipid fraction between $SHRSP_{(Glasgow)}$ and WKY, there were differences in the composition of the neutral lipids. In the CE fraction, palmitic, linolenic, linoleic, stearic, arachidonic, dihomo-γ-linolenic and docosahexaenoic acids were higher in the membranes from $SHRSP_{(Glasgow)}$ compared to WKY (Graph 5.3.3b). In the same fraction, behenic acid was lower in $SHRSP_{(Glasgow)}$. In the TRIG fraction, the levels of the following fatty acids were lower in $SHRSP_{(Glasgow)}$ compared to WKY; palmitoleic, palmitic, linolenic, linoleic, oleic, stearic, arachidonic, dihomo-γ-linolenic, behenic and lignoceric (Graph 5.3.3c).

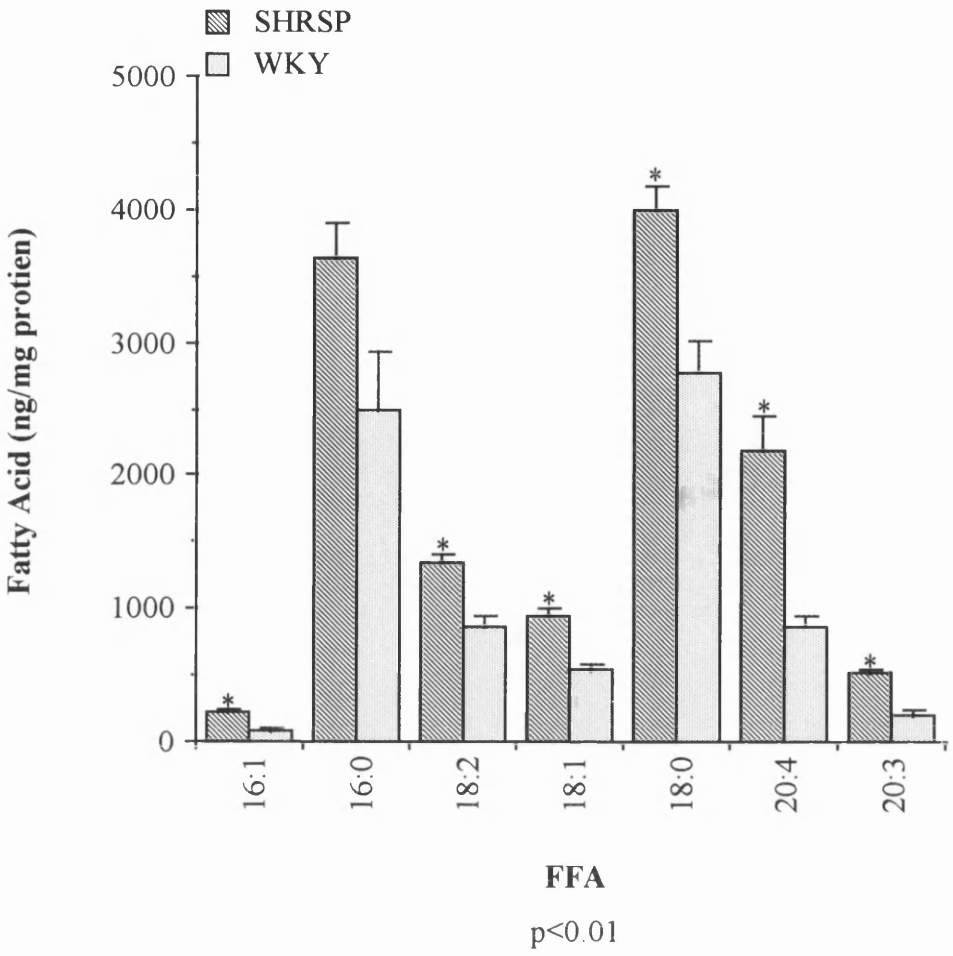
In the FFA fraction, the following fatty acid concentrations were higher in the $SHRSP_{(Glasgow)}$ compared to WKY; palmitoleic, palmitic, linoleic, oleic, stearic, arachidonic, and dihomo-γ-linolenic acid (Graph 5.3.3d).



Graph 5.3.3b Fatty acid composition of liver cholesterol esters (ng/mg protein) from $SHRSP_{(Glasgow)}$ and WKY. Statistics as described previously (n=8) for both groups.



Graph 5.3.3c Fatty acid composition of liver cell membrane TRIG (ng/mg protein) from $SHRSP_{(Glasgow)}$ and WKY. Statistics as described previously (n=8) for both groups.



Graph 5.3.3d Fatty acid composition of liver cell membrane FFA (ng/mg protein) from $SHRSP_{(Glasgow)}$ and WKY. Statistics as described previously (n=8) for both groups.

5.3.4 Polar Lipids.

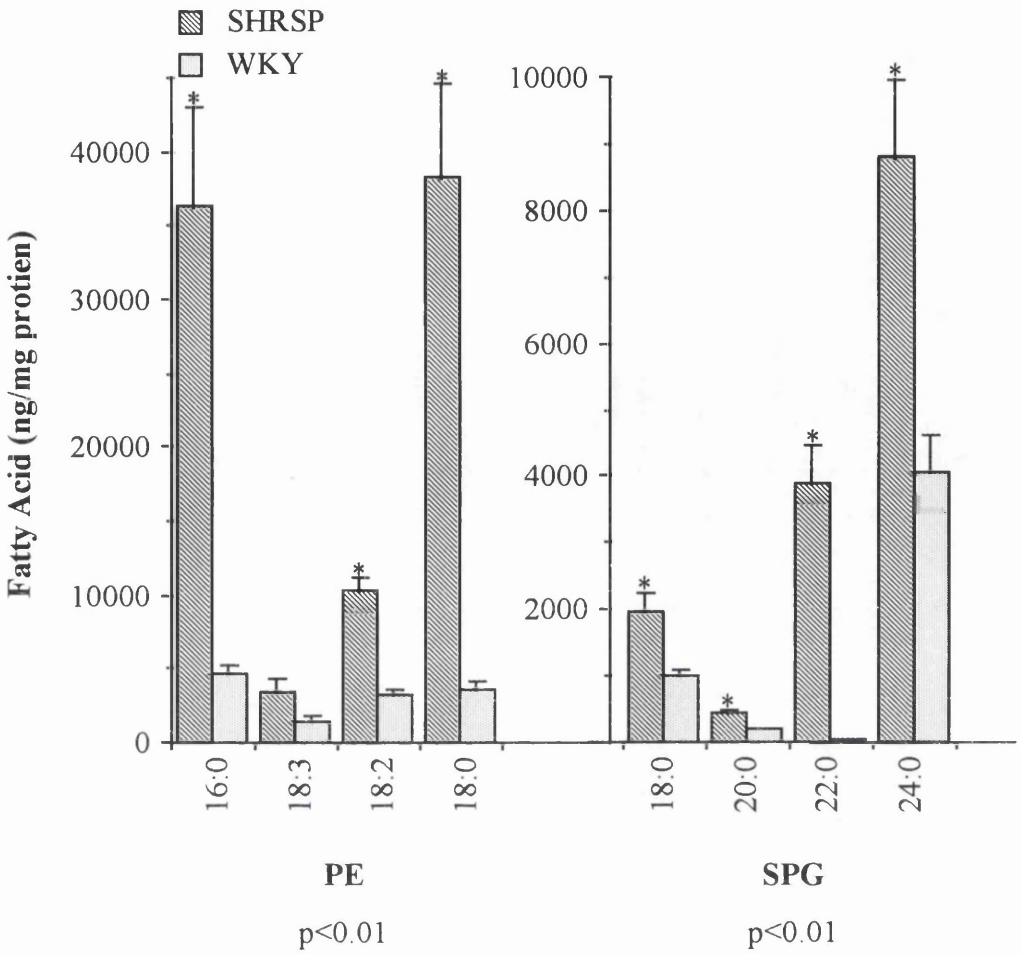
Erythrocytes.

The following fatty acids were increased in the PE fraction from $SHRSP_{(Glasgow)}$ compared to WKY; palmitic, linoleic and stearic acid. In the SPG fraction the levels of stearic, behenic and lignoceric acid were increased in the $SHRSP_{(Glasgow)}$ compared to the WKY. These results are summarised in graph 5.3.4a.

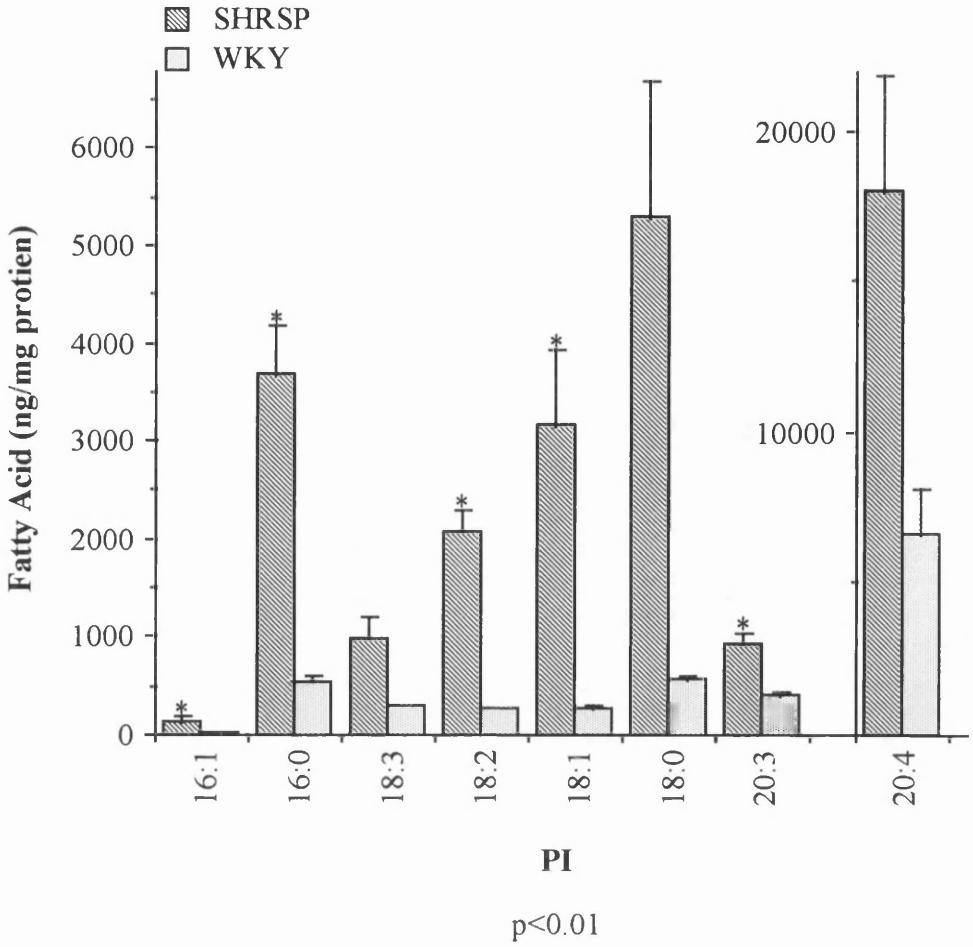
Graph 5.3.4b details the differences in fatty acid composition if the PI fraction. Here the levels of palmitic, linoleic, stearic, arachidonic, and dihomo-γ-linolenic acid were increased in the $SHRSP_{(Glasgow)}$ compared to the WKY.

Although not altered in the total lipid fraction, there was an increase in the amount of linolenic acid in the PE fraction and an increase in the amount of arachidic acid present in the SPG fraction from the $SHRSP_{(Glasgow)}$ compared to WKY. In the PI fraction,

palmitoleic, linolenic, and oleic acid were increased in the $SHRSP_{(Glasgow)}$ compared to the WKY. These results are also summarised in graphs 5.3.4a and 5.3.4b.



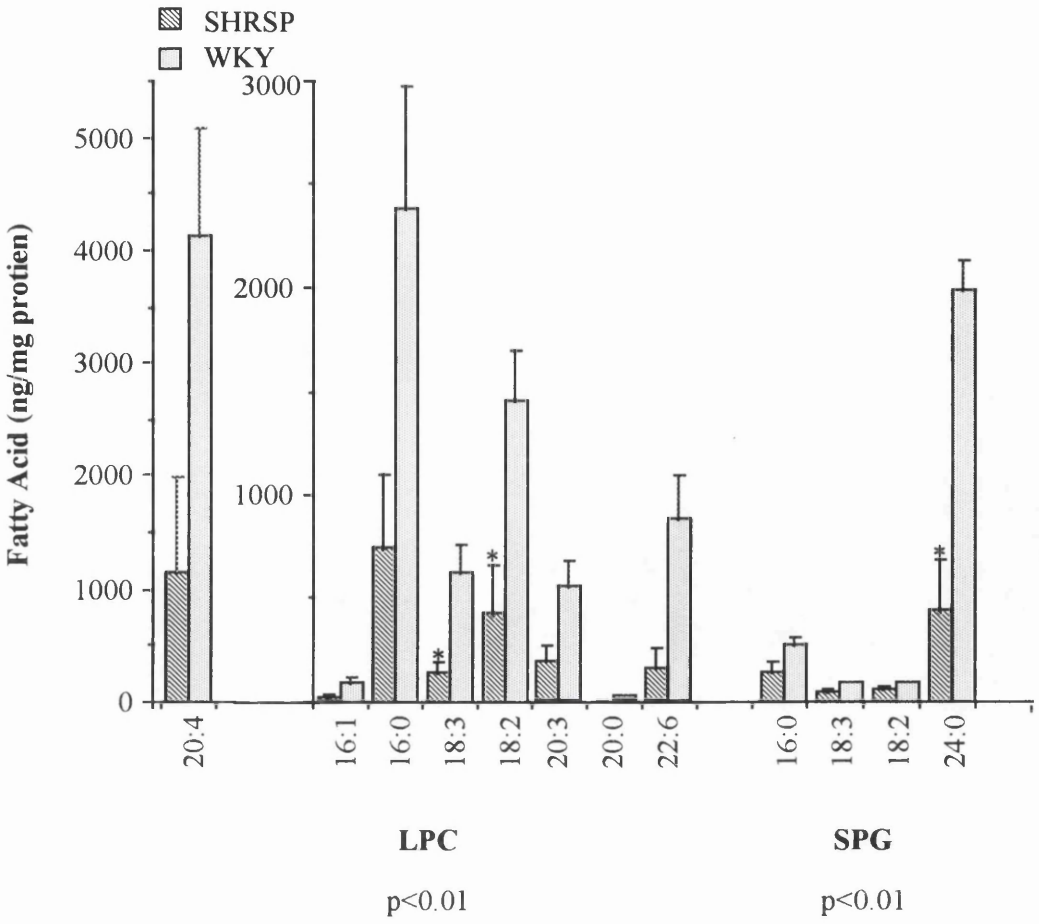
Graph 5.3.4a Fatty acid composition of erythrocyte membrane PE and SPG (ng/mg protein) from $SHRSP_{(Glasgow)}$ and WKY. Statistics as described previously (n=8) for both groups.



Graph 5.3.4b Fatty acid composition of erythrocyte membrane PI (ng/mg protein) from $SHRSP_{(Glasgow)}$ and WKY. Statistics as described previously (n=8) for both groups.

Liver Cell Membranes.

In the LPC fraction, the following fatty acids were decreases in $SHRSP_{(Glasgow)}$ compared to WKY; palmitoleic, palmitic, linolenic, linoleic, arachidonic, dihomo-γ-linolenic, behenic and docosahexaenoic acid. In the SPG fraction the levels of palmitic, linolenic, linoleic and arachidonic acid were decreases in the $SHRSP_{(Glasgow)}$ compared to WKY. These results are detailed in graph 5.3.4c.



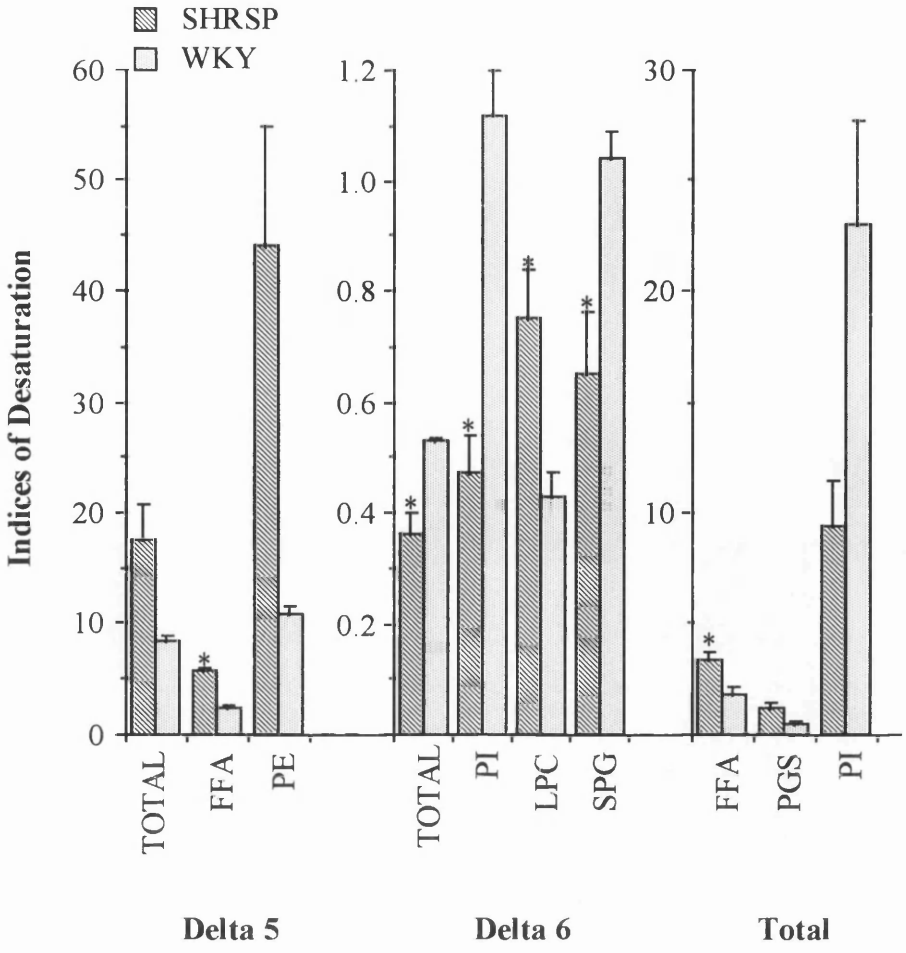
Graph 5.3.4c Fatty acid composition of liver cell membrane LPC and SPG (ng/mg protein) from $SHRSP_{(Glasgow)}$ and WKY. Statistics as described previously (n=8) for both groups.

5.3.5 Indices of Fatty Acid Desaturation.

Erythrocytes

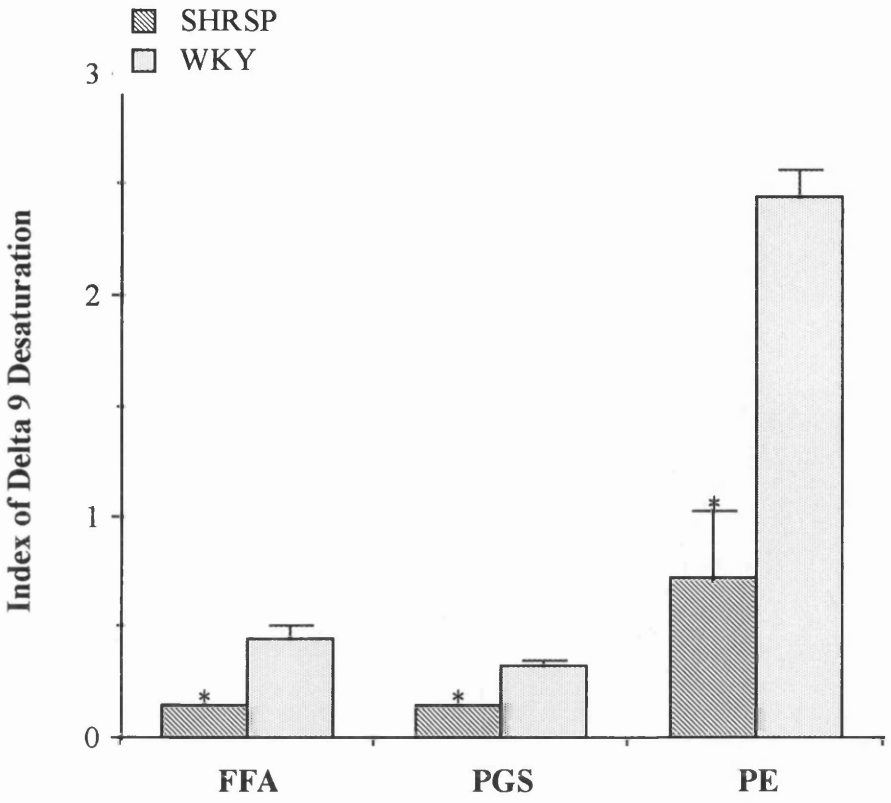
Graph 5.3.5a summarises the differences in essential fatty acid desaturation between $SHRSP_{(Glasgow)}$ and WKY. The activity of the Δ5 desaturase was increased in the total lipid fraction of $SHRSP_{(Glasgow)}$ compared to WKY. This increase was also observed in the FFA and PE fractions. The index of Δ6 desaturation was reduced in the total lipid fraction of the $SHRSP_{(Glasgow)}$ compared to WKY. This was observed in the PI and SPG fractions but in the LPC fraction, the index of Δ6 desaturation was increased.

There was no difference in the index of total desaturation for total fatty acids between $SHRSP_{(Glasgow)}$ and WKY. This index was increased in the FFA fraction and PGS and reduced in the PI fraction of $SHRSP_{(Glasgow)}$ compared to WKY.



Graph 5.3.5a Indices of essential fatty acid desaturation for erythrocytes from $SHRSP_{(Glasgow)}$ and WKY.

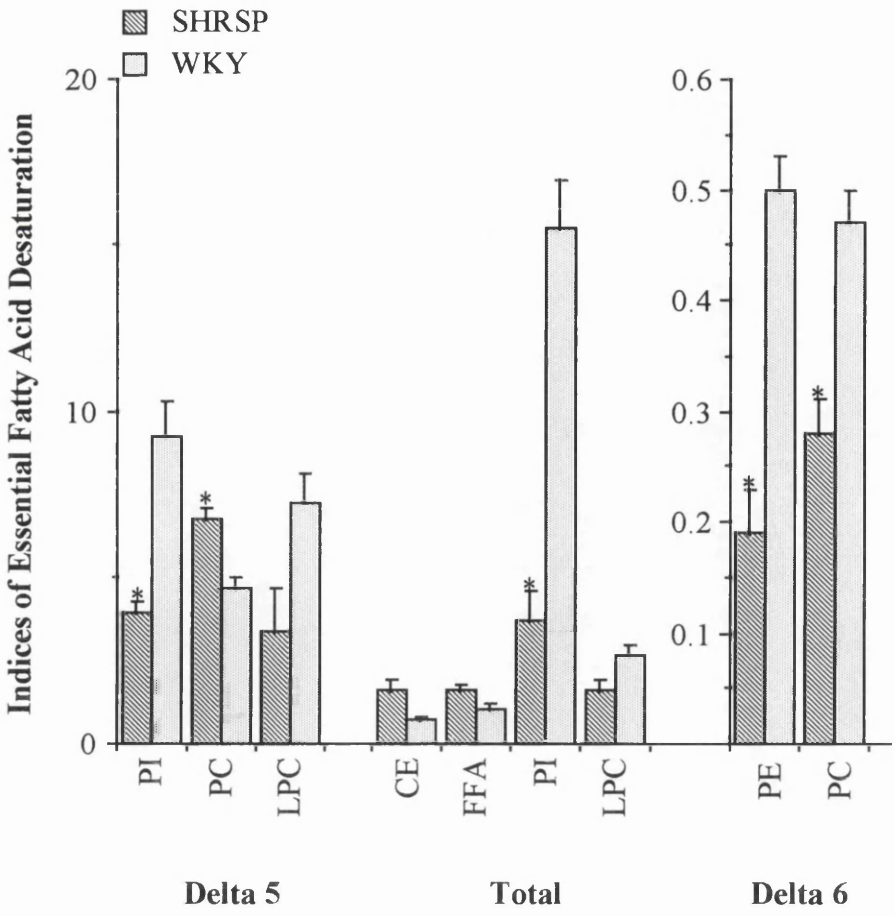
The index of Δ9 desaturation was not altered in the total fatty acid fraction but was reduced in the FFA, PGS and PE fractions of erythrocytes from $SHRSP_{(Glasgow)}$ compared to WKY. These results are summarised in graph 5.3.5b.



Graph 5.3.5b Indices of Δ9 desaturation for erythrocytes form $SHRSP_{(Glasgow)}$ and WKY.

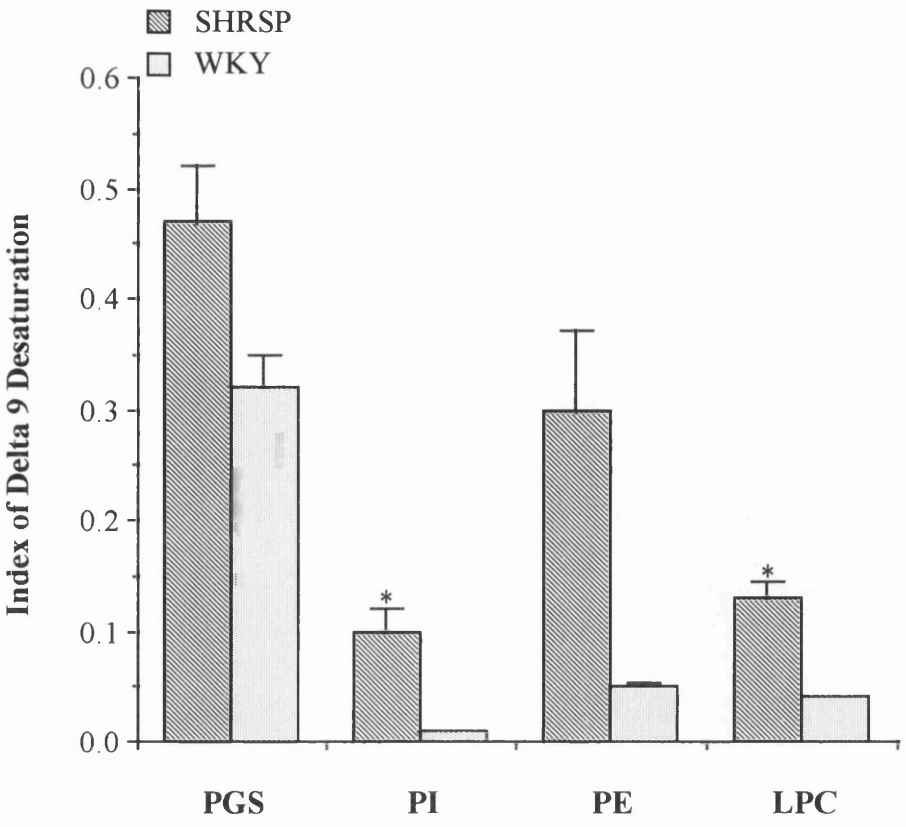
Liver Cell Membranes.

In the total lipid fraction, there were no differences in the indices of desaturation for the total lipid fraction. The index of Δ5 desaturation was decreased in the PI and LPC fractions and increased in the PC fraction of $SHRSP_{(Glasgow)}$ compared to WKY. The index of Δ6 desaturation was reduced in both the PE and PC fractions from $SHRSP_{(Glasgow)}$ compared to WKY. The index of total desaturation was increased in the CE and FFA and reduced in the PI and LPC fractions from $SHRSP_{(Glasgow)}$ compared to WKY (Graph 5.3.5c).



Graph 5.3.5c Indices of essential fatty acid desaturation for liver cell membranes from $SHRSP_{(Glasgow)}$ and WKY.

The index of Δ9 desaturation was increased in the PE, PC and LPC fractions and reduced in CE and SPG fractions of liver cell membranes from $SHRSP_{(Glasgow)}$ compared to WKY. These results are summarised in 5.3.5d.



Graph 5.3.5d Indices of Δ9 desaturation of liver cell membranes from $SHRSP_{(Glasgow)}$ and WKY.

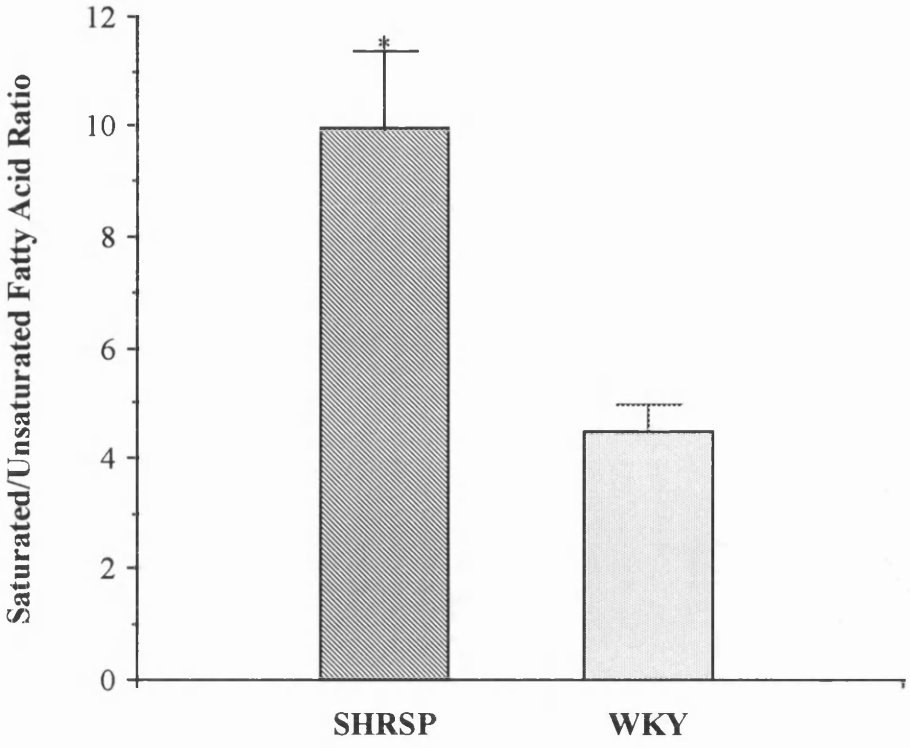
5.3.6 Saturated/Unsaturated Fatty Acid Ratio.

Erythrocytes

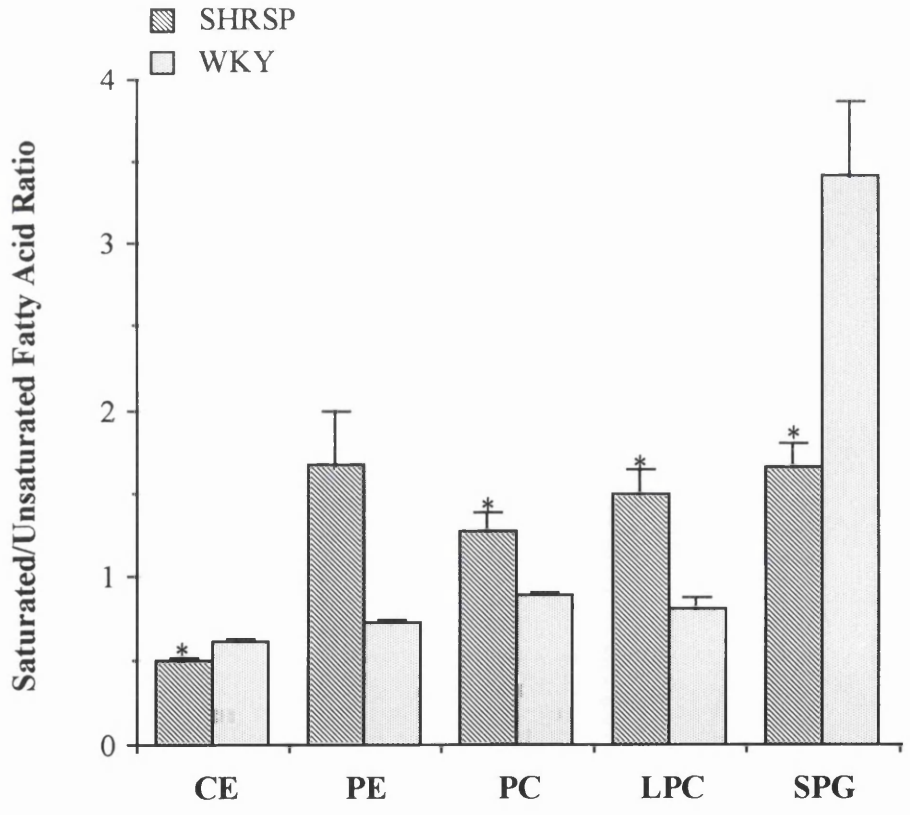
The saturated/unsaturated fatty acid ratio was increased in the SPG fraction of erythrocyte membrane from $SHRSP_{(Glasgow)}$ compared to WKY. These results are summarised in graph 5.3.6a.

Liver Cell Membranes

There was an increase in the saturated/unsaturated fatty acid ratio in the PE, PC and LPC fraction and a reduction in the CE and SPG membrane fractions from $SHRSP_{(Glasgow)}$ compared to WKY (graph 5.3.6b).



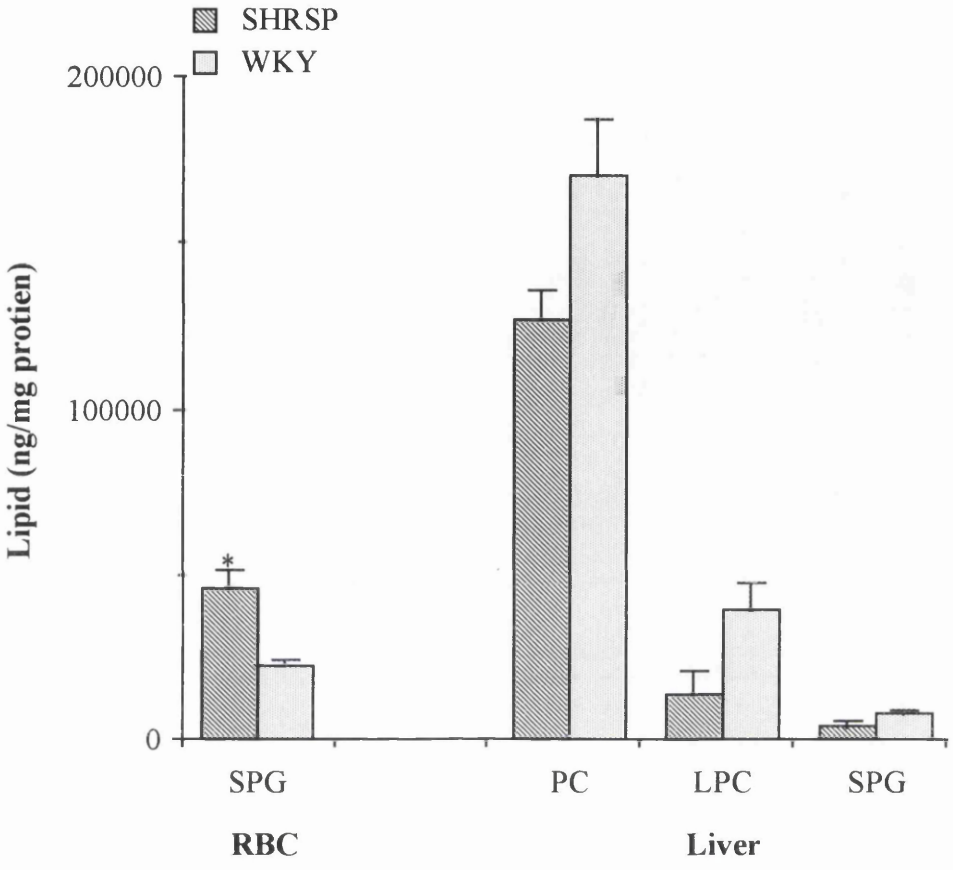
Graph 5.3.6a Saturated/unsaturated fatty acid ratio for erythrocytes from $SHRSP_{(Glasgow)}$ and WKY.



Graph 5.3.6b Saturated/unsaturated fatty acid ratio for liver cell membranes from $SHRSP_{(Glasgow)}$ and WKY.

5.3.7 Derived Phospholipid Composition

The differences in phospholipid composition are shown in graph 5.3.7. In the erythrocyte membranes, there was an increase in the amount of SPG present in the $SHRSP_{(Glasgow)}$ compared to WKY. In the liver cell membrane the concentrations of PC, LPC and SPG were reduced in $SHRSP_{(Glasgow)}$ compared to WKY.

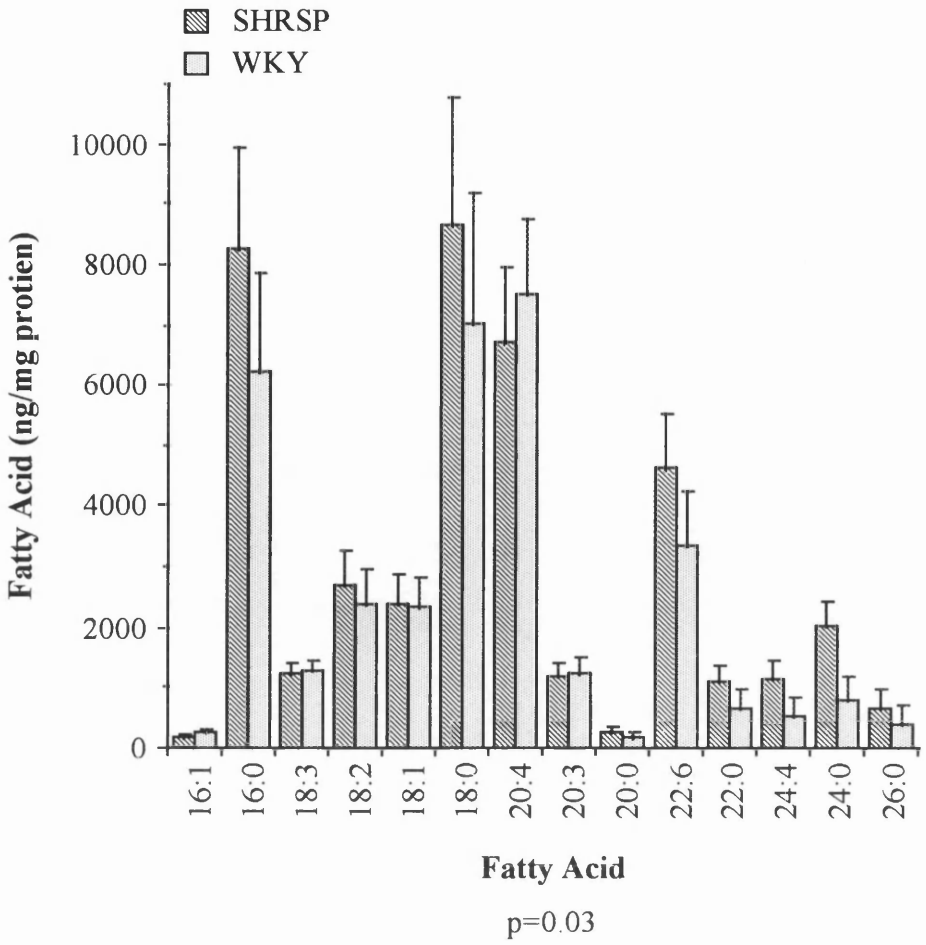


Graph 5.3.7 Derived phospholipid composition of erythrocytes and liver cell membranes from $SHRSP_{(Glasgow)}$ and WKY.

Part 2

5.3.8 Total Fatty Acids

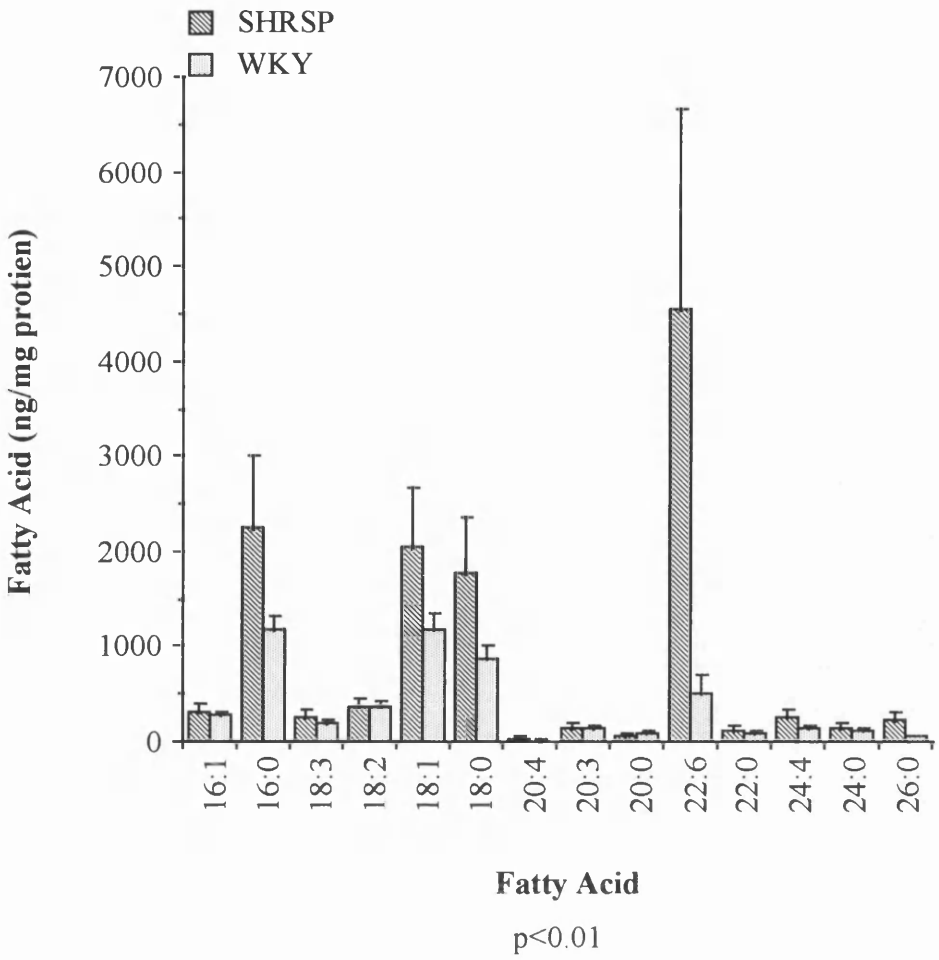
Although ANOVA of the total fatty acid profile for the erythrocyte membrane from $SHRSP_{(Heidelberg)}$ compared to WKY suggested the presence of significant differences, no single fatty acid could be identified to account for these (Graph 5.3.8).



Graph 5.3.8 Fatty acid composition of total erythrocyte lipids (ng/mg protein) from $SHRSP_{(Heidelberg)}$ and WKY rats. Statistics as described previously (n=14) for both groups.

5.3.9 Neutral Lipids

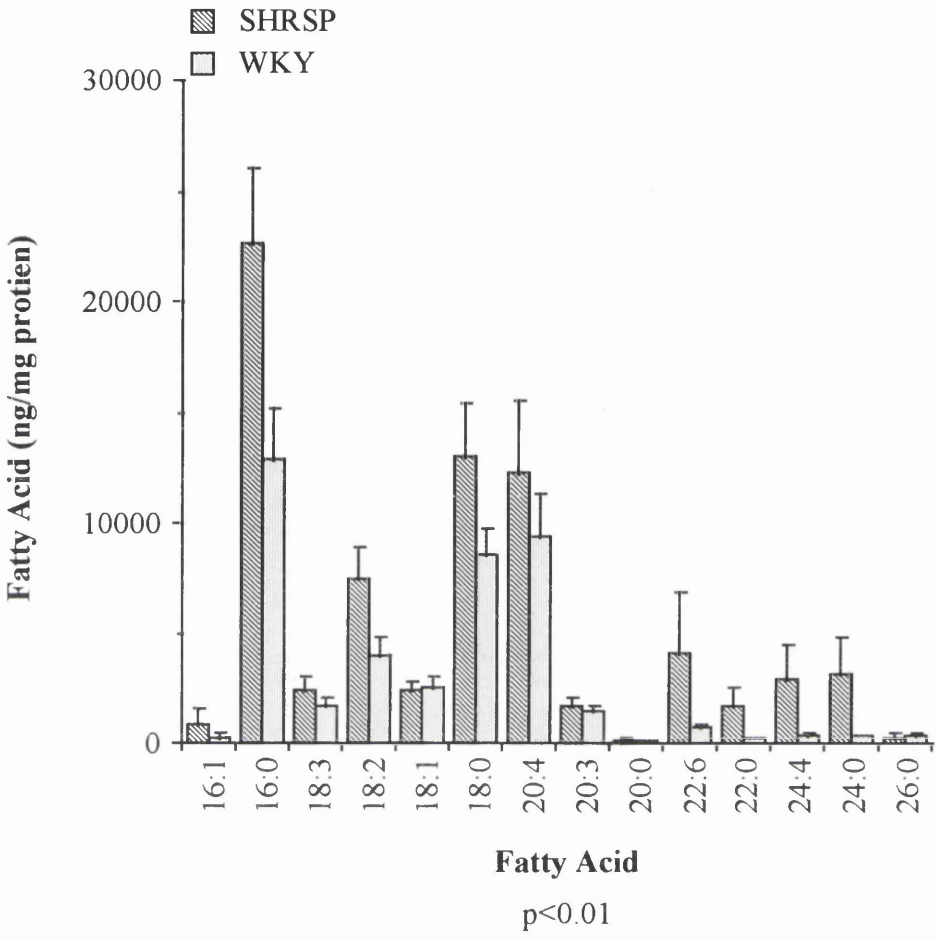
The fatty acid composition of TRIG fraction was found to be different between $SHRSP_{(Heidelberg)}$ and WKY. However, comparison of the individual fatty acids did not provide any statistically significant differences (graph 5.3.9).



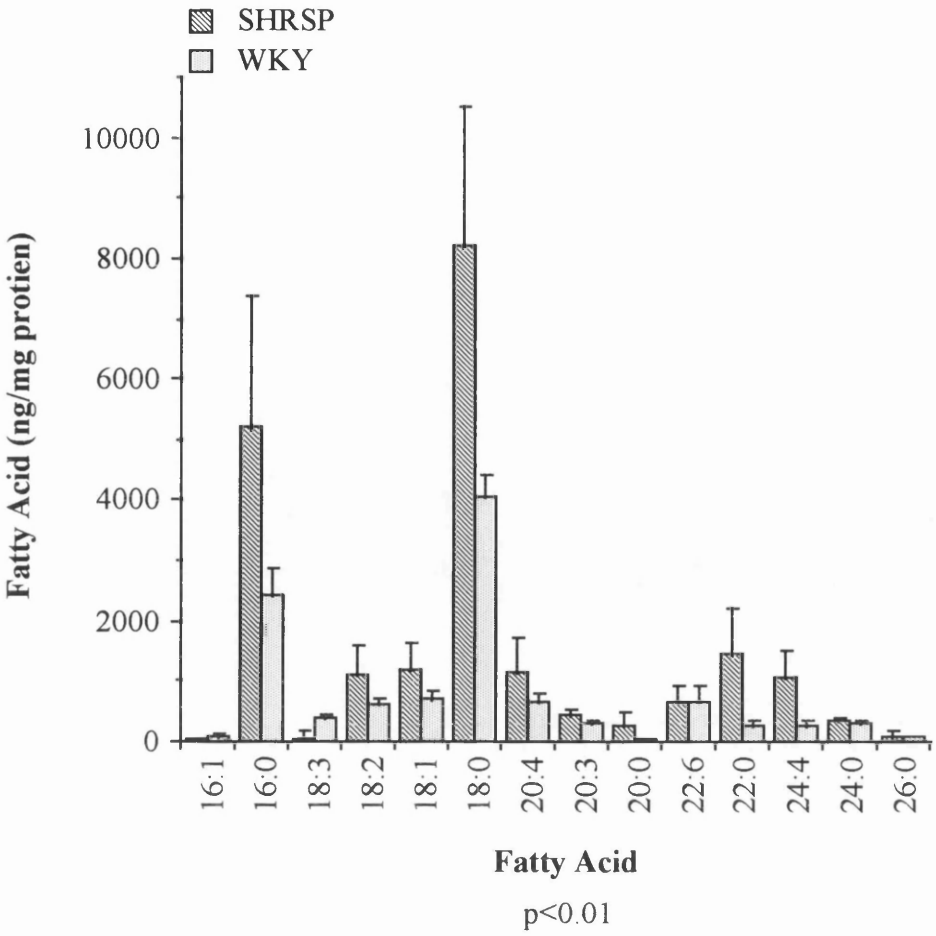
Graph 5.3.9 Fatty acid composition of erythrocyte TRIG (ng/mg protein) from $SHRSP_{(Heidelberg)}$ and WKY. Statistics as described previously (n=14) for both groups.

5.3.10 Polar Lipids

Statistically significant differences were obtained for the fatty acids composition of PC and LPC. In both cases there was a general increase in the amount of fatty acid present in the $SHRSP_{(Heidelberg)}$ compared to WKY although none of these fatty acids returned a statistically significant t-test. See graphs 5.3.10a and 5.3.10b.



Graph 5.3.10a Fatty acid composition of erythrocyte PC (ng/mg protein) from $SHRSP_{(Heidelberg)}$ and WKY. Statistics as described previously (n=14) for both groups.

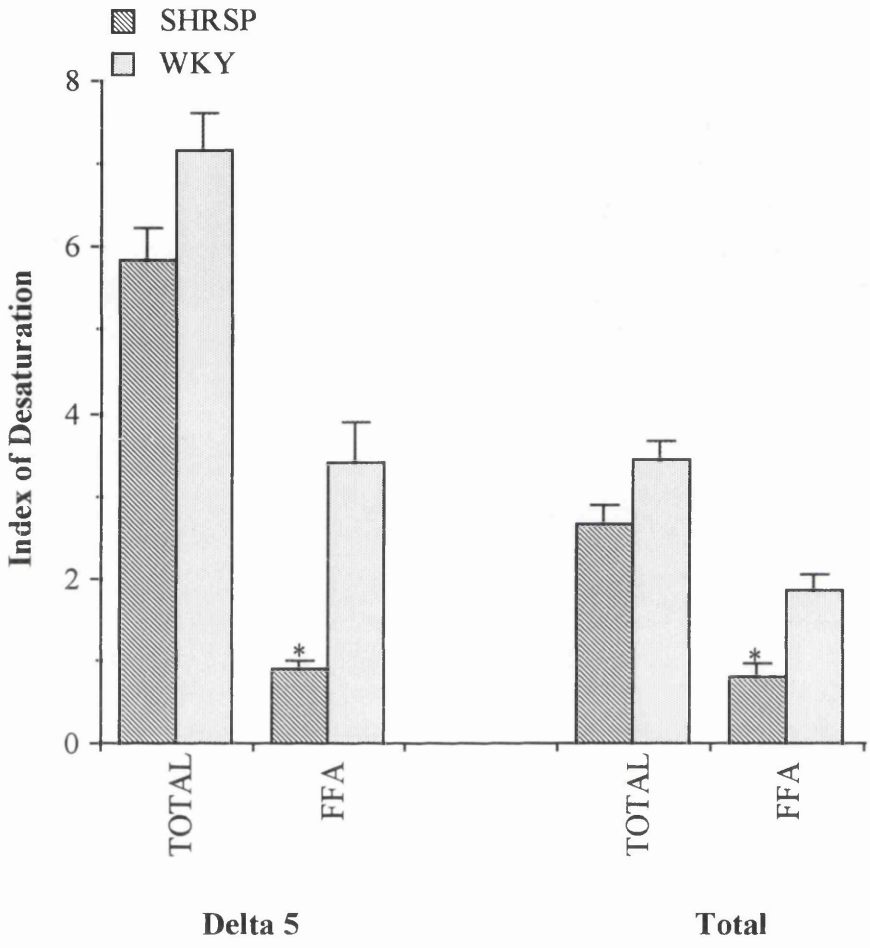


Graph 5.3.10b Fatty acid composition of erythrocyte LPC (ng/mg protein) from $SHRSP_{(Heidelberg)}$ and WKY. Statistics as described previously (n=14) for both groups.

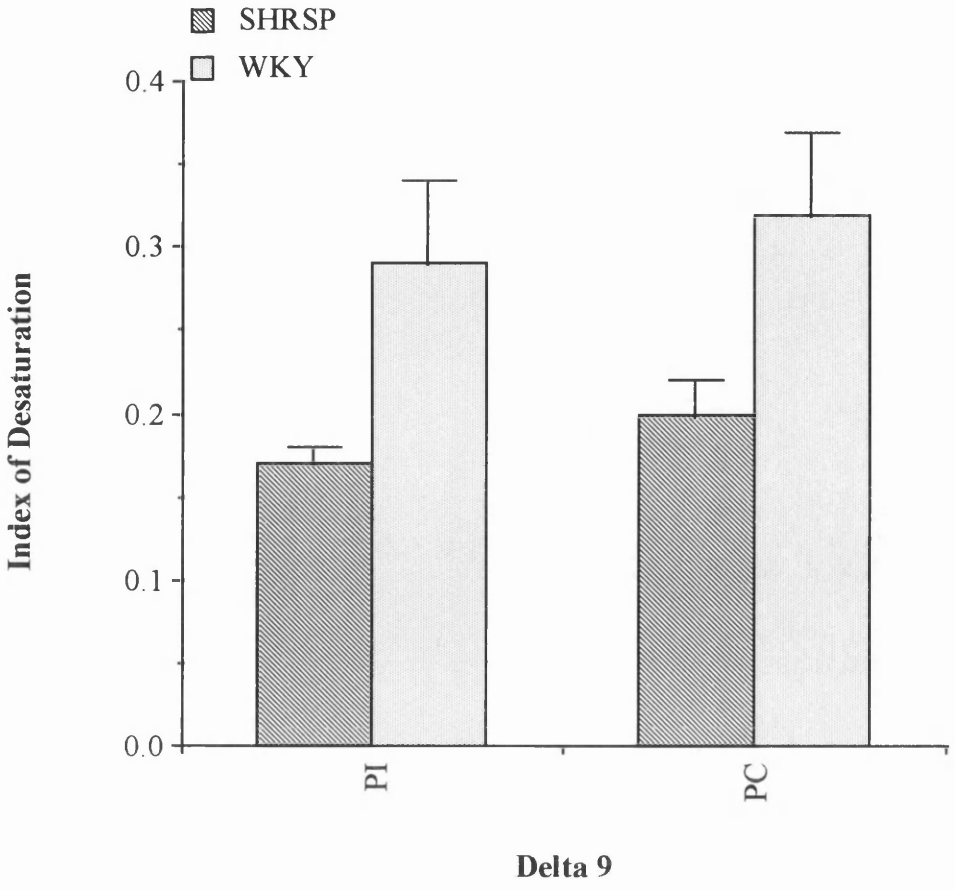
5.3.11 Indices of Fatty Acid Desaturation.

The results for the indices of fatty acid desaturation are shown in graphs 5.3.11a. For the total fatty acid fraction the index of Δ5 desaturation was reduced in $SHRSP_{(Heidelberg)}$ compared to WKY. This difference was also apparent in the FFA fraction. The index of Δ6 desaturation was not altered for any of the lipid fractions. The index of total desaturation was also reduced in the total fatty acids fraction and in the FFA fraction of the $SHRSP_{(Heidelberg)}$ compared to WKY.

These results are summarised in graph 5.3.11b. The index of Δ9 desaturation was not altered in the total lipid fraction but was reduced in the PI and PE fractions of $SHRSP_{(Heidelberg)}$ compared to WKY.



Graph 5.3.11a Indices for essential fatty acid desaturation for erythrocyte membranes for $SHRSP_{(Heidelberg)}$ and WKY.



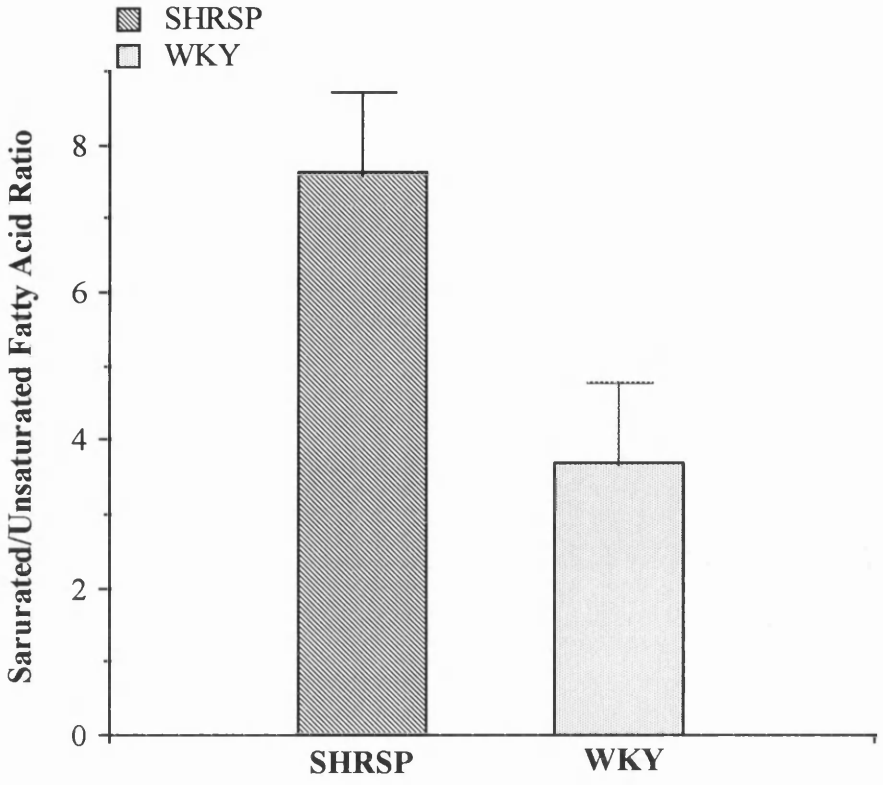
Graph 5.3.11b Indices of Δ9 desaturation for erythrocyte membranes from $SHRSP_{(Heidelberg)}$ and WKY.

5.3.12 Saturated/Unsaturated Fatty Acid Ratio

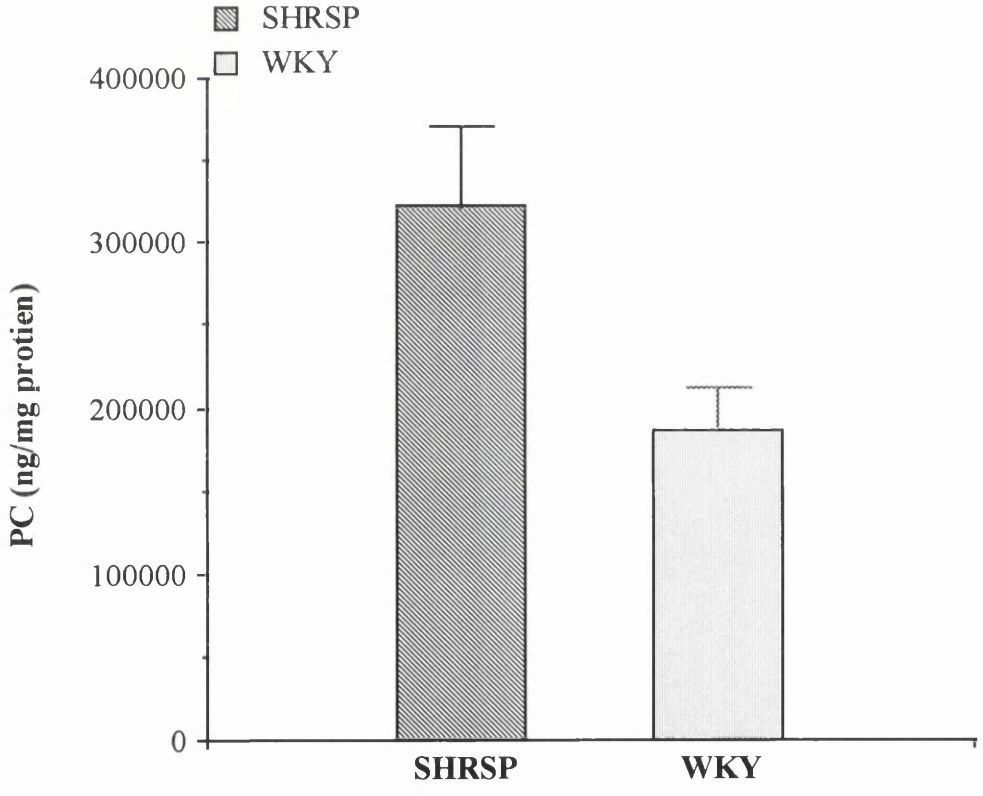
These results are summarised in graph 5.3.12. The saturated/unsaturated fatty acid ratio was reduced in the SPG fraction of $SHRSP_{(Heidelberg)}$ compared to WKY.

5.3.13 Derived Phospholipid Composition.

Graph 5.3.13 shows the changes in the phospholipid composition between the two strains. The levels of PC were increased in the erythrocyte membranes from $SHRSP_{(Heidelberg)}$ compared to WKY.



Graph 5.3.12 Saturated/unsaturated fatty acid ratio of SPG from erythrocyte membranes from $SHRSP_{(Heidelberg)}$ and WKY.



Graph 5.3.13 Derived phospholipid concentration of erythrocyte membrane PC from $SHRSP_{(Heidelberg)}$ and WKY.

5.3.14 Analysis of the F_2 Population.

Table 5.3.1 details the R and R^2 values and the p value obtained for regression analysis for the systolic blood pressure and the traits observed to be different between the SHRSP (Heidelberg) and WKY. None of the traits studied were found to correlate with systolic blood pressure.

Trait	p	R value	R^2 Value
Δ5 in total fatty acid	0.189	0.324	0.105
Δ5 in FFA	0.27	0.274	0.075
total desaturase in total fatty acids	0.671	0.109	0.012
total desaturase in FFA	0.248	0.288	0.083
Δ9 in PI & PE	0.068	0.440	0.194
Sat/Unsat in SPG	0.86	0.044	0.002
Derived PC	0.435	0.197	0.039

Table 5.3.14 Values for regression analysis of traits in the F_2 population of an $SHRSP_{(Heidelberg)}$ and WKY cross.

5.4 Discussion.

Analysis of the cell membrane composition of genetically hypertensive rats showed that there were differences in the fatty acid composition of liver and erythrocyte membranes between the $SHRSP_{(Glasgow)}$ and WKY but these were not the same in both tissues. Differences were also observed between the erythrocyte membranes from $SHRSP_{(Heidelberg)}$ and WKY. Also, different changes from control were observed in the erythrocyte lipid composition of the two SHRSP strains.

Why the $SHRSP_{(Glasgow)}$ and $SHRSP_{(Heidelberg)}$ membranes behaved differently is difficult to explain. The $SHRSP_{(Heidelberg)}$ were nine weeks older than the $SHRSP_{(Glasgow)}$ and had marked hypertension probably of some duration whereas the blood pressures of the $SHRSP_{(Glasgow)}$ and age-matched WKY were not significantly different at nine weeks although a difference might be predicted at 11 weeks (Davidson *et al* 1995). Even so the duration of hypertension still remains different for the two groups. Hypertension may itself affect membrane lipid composition. Also it is worth noting the $SHRSP_{(Glasgow)}$ were maintained on a normal diet while the $SHRSP_{(Heidelberg)}$ were given sodium chloride in their drinking water; this will tend to elevate blood pressure. Changes in lipid metabolism occur

with increasing age. Peluffo & Brenner (1974) reported that the activity of the desaturases decreases due to a reduction in affinity of the enzyme for its substrate (Hrelia *et al* 1990) but others have observed that activity fluctuates with time (Maniongui *et al* 1993). Either way, the differences may relate to the differences in ages of the two samples. It has also been shown that the desaturases activities can be different between rats of the same strain from different suppliers (de Antueno *et al* 1994); this was the case here. These differences suggest that the fatty acid composition of the membrane is not controlled by the same genetic factors which control blood pressure. Although both strains become hypertensive, some membrane changes were opposite. For instance, the index of Δ5 desaturase activity is increased in $SHRSP_{(Glasgow)}$ compared to WKY but reduced in $SHRSP_{(Heidelberg)}$. To ascertain whether this difference is due to high blood pressure, it would be necessary to analyse lipid composition sequentially as hypertension develops. However, it is impossible to carry out this type of experiment without activating the renin-angiotensin system The changes in the two hypertensive strains will be discussed separately. The two strains were separated over 20 years and it is possible that genetic differences in the two have become fixed in subsequent generations.

5.4.1 $SHRSP_{(Glasgow)}$ Compared to WKY.

There was no statistical difference between the systolic blood pressure of the $SHRSP_{(Glasgow)}$ and WKY rats as measured at 9 weeks of age. Unfortunately, the blood pressure was not measured again at 11 weeks when the tissue was sampled. Hypertension in the SHRSP rat is fully developed by 12 weeks (Davidson *et al* 1995). It is therefore likely that blood pressure was higher in the SHRSP rats at 11 weeks (Dominiczak *et al* (1991) found a difference as early as 44 days of age). If so the changes in fatty acid composition may be secondary to this increase in blood pressure. If not, the differences observed between these two groups will not have been secondary to hypertension and may have played a causative role in raising the blood pressure. However, it also is possible that the maternal blood pressure, high in SHRSP, may exert some effect. It has been observed that 3 week old SHR rats have elevated erythrocyte membrane microviscosity in comparison to age-matched WKY. This would be prior to the onset of hypertension (Montenay-Garestier *et al* 1981).

The total lipid composition of the erythrocyte membrane was different in the $SHRSP_{(Glasgow)}$ compared to WKY. This applied to both endogenously-produced fatty acids and the essential fatty acids and their metabolites. Notably, the amount of long chain PUFA, in particular arachidonic and docosahexaenoic acid, present in the membrane was increased.

This increase in arachidonic acid is in good agreement with previous studies (Naftilan *et al* 1986, Ollerenshaw *et al* 1987 Dominiczak *et al* 1993) but is in contradiction with Horrobins' (1995) theory that inadequate amounts of arachidonic acid may be a causal factor in coronary artery disease. PUFA are believed to increase membrane fluidity. Thus, the increase here is the opposite of the previously observed membrane microviscosity data. Arachidonic and docosahexaenoic acid are, however, only two of many unsaturated fatty acid present in the cell membrane. Although these are polyunsaturated, their effect on membrane fluidity is not directly proportional to the number of double bonds present. Studies of synthetic membranes have shown that changing a fatty acid from saturated to monounsaturated has a far greater effect on membrane fluidity than changing a monounsaturated fatty acid to a polyunsaturated fatty acid. By separating the lipids fractions and analysing their individual fatty acid composition, it should be possible to determine whether these were general changes or specific to one or more fractions.

In terms of exerting a physical effect on the membrane, alterations in the fatty acid composition of the lipids which are present in the largest quantities in the membrane will have most effect. PC, PE and SPG these constitute 31, 15 and 8.5 percent of the membrane lipid respectively. However, some other lipid fractions may be of particular physiological importance, for example for cell signalling.

The changes in neutral lipid fatty acid composition were similar to those observed in total lipid composition. Difference were observed in the fatty acids present in CE, FFA and PGS. Although there was a general increase in the amount of fatty acid present in these fraction in the $SHRSP_{(Glasgow)}$ compared to WKY, these fractions are quantitatively small and therefore may not have any measurable effect on the membrane's physical properties. However, an increase in the amount of fatty acid present in the CE fraction of $SHRSP_{(Glasgow)}$ suggests that there may be more CE present in the membrane, which would be in accordance with an increased membrane microviscosity in these rats. In the FFA fraction, the level of arachidonic acid was increased in $SHRSP_{(Glasgow)}$ compared to WKY. The possible relevance of this will be discussed further later.

The fatty acid composition of the polar lipids was also different between the $SHRSP_{(Glasgow)}$ and WKY. These changes were similar to those observed in the total lipids fraction. The lipid groups which were affected were PE, SPG and PI. In terms of altering the physical properties of the membrane, the lipids present in the largest quantities were PE and SPG. In

the PE fraction, there was a large (7 fold) increase in the levels of saturated fatty acids (18:0 and 16:0) in the $SHRSP_{(Glasgow)}$ and WKY. This increase should be reflected in an increase in membrane microviscosity. Although there was also an increase in some of the polyunsaturated fatty acids present, the magnitude of the increase was much smaller than that for the saturated fatty acids. The changes in the fatty acid present in the SPG fraction were limited to saturated fatty acids. The saturated fatty acids ranging form C18 to C24 were all increased in the membranes of $SHRSP_{(Glasgow)}$ compared to WKY. This again will have tended to increase membrane microviscosity. The derived membrane content of SPG was also increased in $SHRSP_{(Glasgow)}$ compared to WKY. SPG is known to increase membrane microviscosity. The same is true for long chain fatty acids. It is not clear from the literature whether these two effects are additive but either one or both may be responsible for the increase in microviscosity observed by McLaren *et al* (1993) in the erythrocyte in the SHRSP rat compared to WKY. The saturated/unsaturated fatty acid ratio in SPG was increased in the $SHRSP_{(Glasgow)}$ as compared to WKY, this is in agreement with the changes in fatty acid composition.

Of particular physiological interest is the increase in the arachidonic acid content in the PI and FFA fraction from $SHRSP_{(Glasgow)}$. Arachidonic acid is increased in the membranes from subjects with genetic hypertension (see 5.1). These current results further show that this increase appears to be limited to lipid groups, PI and FFA.

This may be important for cellular signalling, particularly if similar changes occur in other cell types such as vascular smooth muscle. Phosphatidylinositol 4,5-bisphosphate (PIP_2) is produced by phosphorylation of PI. In response to an external stimuli, cells hydrolyse PIP_2 to produce inositol 1,4,5-triphosphate (IP_3), diacylglycerol and arachidonic acid, all of which are important cell signalling agents (Sperelakis 1995). Arachidonic acid can be converted into eicosanoids, a group of vasoactive compounds, consisting of prostaglandins, thromboxanes and leukotrienes (Rang & Dale 1991). Without a full scale analysis of the cycloxygenase pathway, it is impossible to ascertain whether the compounds produced will promote vasoconstriction or relaxation. It is possible that increased vasoconstrictor compounds could lead to elevated blood pressure in the $SHRSP_{(Glasgow)}$. However, an increase in the amount of vasodilator present could be an 'effort' to compensate for elevated blood pressure.

Moreover, arachidonic acid and other polyunsaturated fatty acids have been shown to inhibit gene transcription. PUFA, but not saturated or monounsaturated fatty acids, inhibit

hepatic lipogenesis (Armstrong *et al* 1991, Blake & Clarke 1990), possibly at the level of enzyme synthesis (Clarke & Jump 1994), since high PUFA diets reduce the transcription of the fatty acid synthase gene (Armstrong *et al* 1991, Blake & Clarke 1990, Jump *et al* 1993, Limatta *et al* 1994). Acetylcarboxylase (Katsurada *et al* 1990), malic enzyme and glucose 6-phosphate dehydrogenase (Schwartz & Abraham 1983, Tomlinson *et al* 1988) are similarly affected. The suppression of transcription has a rapid onset, suggesting that the fatty acids exert a direct effect (Jump & Clarke 1994). Although other PUFA are capable of inhibiting gene transcription, it appears that they must undergo desaturation to be effective. This has been confirmed using the arachidonic acid analogue, eicosa-5,8,11,14-tetraynoic acid (Abraham *et al* 1977, Clarke & Clarke 1982, Tomlinson *et al* 1988). Conversely, PUFA have been found to induce the expression of peroxisomal enzymes, in particular acyl CoA oxidase (Flatmark *et al* 1988). It is now thought that fatty acids regulate gene transcription by affecting the activity of transcription factors which target *cis*-linked elements associated with specific genes (Clarke & Jump 1996). Free PUFA have also been shown to have other effects, including the activation of potassium channels in smooth muscle cells (Ordway *et al* 1989). As red cells have no nucleus, this affect is only important if the same changes occur in other cell types. What is important is that the composition of the erythrocyte membrane is thought to reflect that of the plasma. Therefore if arachidonic acid is increased in the plasma or in lipoproteins, then this may have implications for many cell types.

The absence of differences in the saturated/unsaturated fatty acid ratio in any lipid group other than SPG is in contradiction to the evidence that the membrane microviscosity is increased in the SHRSP compared to WKY. Many factors affect cell membrane microviscosity including the membrane protein and cholesterol composition. In biological membranes, the control of membrane fluidity is complex and a simple comparison of saturated/unsaturated fatty acids will only provide the crudest of estimates.

The above changes in the membrane composition may reflect differences in the fatty acid desaturases. Direct measurements of the activity of the hepatic Δ5 and Δ6 desaturases have shown them to be increased in the SHR compared to WKY (Narce *et al* 1994). This analysis was accompanied by an compositional analysis of the cell membrane which showed a surprising reduction in the amount of arachidonic acid present. However, a later study by the same group suggested the enzyme activities to be reduced (Narce & Poisson 1995).

Others have shown that physiological stress, which induces hypertension, reduced the activity of the desaturases (Mills & Ward 1989).

It was of interest here to evaluate whether the results obtained using the fatty acid data were in agreement with published data obtained using the direct enzyme assay. The Δ5 desaturase activity was greater in the $SHRSP_{(Glasgow)}$ compared to WKY, agreeing with their relative membrane arachidonic acid content. The difficulty in comparing these measurements with previously obtained data is that Narce *et al* used much older animals than those used here.. Δ6 desaturation was reduced in the total lipid fraction of $SHRSP_{(Glasgow)}$ compared with WKY. The reduction in Δ6 activity and the increase in Δ5 activity resulted in there being no net change in the total desaturase activity.

It was also of interest to evaluate whether the desaturation index for the individual lipid groups agreed with that of the total lipid fraction. Changes were not observed in every lipid group but only in PE and FFA. The change was most marked in PE. The index of Δ6 desaturation was reduced in the PI and SPG fractions and increased in the LPC fraction . Alteration in the total desaturase could only be observed in the relatively minor lipid fractions. The Δ6 desaturase is thought to be the rate limiting enzyme in the desaturation, elongation pathway (Bernert & Sprecher 1975). Here the activity of this enzyme was reduced, yet the activity of the Δ5 was increased. It is possible that in the SHRSP, rats the Δ6 desaturase is not rate limiting or that the levels of dihomo-γ-linolenic acid present in the membrane or the diet were such that the activity of the Δ6 desaturase was not rate limiting.

The index of Δ9 desaturation was reduced in the FFA, PGS and PE fractions but this affect was not seen in the total fatty acid analysis. The index of Δ9 desaturase activity is a ratio of oleic acid/stearic acid. As mentioned previously, changing a saturated fatty acid to a monounsaturated fatty acid has a major effect on membrane fluidity. As this occurs in the PE fraction, a quantitatively important component, this is in agreement with the increases membrane microviscosity in hypertensive rats.

Many of the differences from normal occurred in the PE fraction. This is an inner membrane lipid and it would be of interest to examine whither the changes in membrane microviscosity only occur in the inner leaflet. However, it is difficult to use fluorescent probes specific for different areas of the membrane with erythrocytes because of the

interference from haemoglobin. Most studies of membrane microviscosity have been carried out in membrane preparations or erythrocyte ghosts.

It has previously been noted (chapter 4) that changes in response to L-NAME occurring in the fatty acid composition of the erythrocyte membrane appeared to be greater and more generalised than those seen in liver cell membranes. Reasons for this were discussed. A similar situation existed in the current experiment. One of the major differences from erythrocytes is that, in the liver cell membranes, the total fatty acid composition was not different between the two strains.

Like the erythrocytes, there was a general increase in the amount of fatty acid present in the CE fraction of the membrane in $SHRSP_{(Glasgow)}$ to WKY. Devynck *et al* (1982) showed that hepatocyte membranes from SHR are less fluid than those from WKY. If the same is true for SHRSP, then this may be the mechanism responsible. As in the erythrocyte membranes, the concentration of arachidonic acid was increased in the FFA fraction from the $SHRSP_{(Glasgow)}$ compared to the WKY. Unlike the erythrocytes, the fatty acid present in TRIG appear to be reduced in $SHRSP_{(Glasgow)}$ compared to WKY. How membrane TRIG levels relate to cellular fuel storage or phospholipid synthesis is not clear but this difference in TRIG content may be important.

The derived LPC concentration in the liver cell membrane was reduced in the $SHRSP_{(Glasgow)}$ compared to WKY. This could be interpreted in two ways; a) as a reduction in the activity of the phospholipase enzymes or, b) as an increase in the activity of the acyl transferase enzymes. Previously, the activity of phospholipase A_2 has been shown to be increased in cardiac myocytes from SHR (Millanvoye-Van Bussel & Devynck 1995) and in renal cells in SHRSP (Okamoto *et al* 1989). The increased activity of these enzymes in renal membranes is corrected by antihypertensive treatments, suggesting that the increase in blood pressure caused the increase in enzyme activity. Although the activity of this enzyme was not studied directly in the two rat strains, from the results obtained it is possible to suggest that the activity of the phospholipase is reduced as less LPC is produced. The absence of an increased phospholipase A_2 activity may be due to the absence of an increase in blood pressure. Care should be taken not to over interpret the results obtained for LPC. In the $SHRSP_{(Glasgow)}$, the derived levels of PC and SPG were also reduced compared to WKY. Therefore, there is an overall reduction in the amount of choline-containing lipids present in the membrane. This alteration was not observed in erythrocyte membranes although there

was a reduction in the amount of some of the fatty acids present. As the composition of the erythrocyte is probably more similar to the vascular smooth muscle cells than liver cell membranes, this difference may not be relevant in terms of vascular physiology.

The changes in the fatty acids composition of SPG were different in the liver from the erythrocytes. As mentioned previously, the levels of SPG lignoceric acid were increased in the erythrocyte membrane of $SHRSP_{(Glasgow)}$ compared to WKY but not in liver cell membranes. Shorter unsaturated fatty acids were increased in the $SHRSP_{(Glasgow)}$ which would tend to promote membrane fluidity. This again may suggest that the membrane has an ability to maintain homeostasis.

Although the liver is the major site of desaturation, the indices of fatty acid desaturation assessed in the total fatty acid were not altered. Direct measurement of hepatic desaturase activities SHR and WKY rats gave conflicting results as discussed previously (Narce *et al* 1994, 1995, Narce & Poisson 1995). Thus in the current study, changes were only observable in the erythrocyte membranes, the composition of which is dependant on the plasma lipid composition.

It was again of interest to study whether the results obtained for the total lipids were mimicked by all the lipid groups. The changes observed for the individual lipid groups were not uniform. For Δ5 desaturase, the index was reduced in PI and LPC and increased in PC. As LPC is derived from PC and vice versa, it is difficult to explain why the index of desaturation is increased in one lipid and reduced in the other. PC is a major membrane lipid and the increase observed here agrees the situation in erythrocytes. The index of Δ6 desaturase activity was reduced in both PE and PC. Again these are major lipids and the situation agrees with that observed with the erythrocytes.

There was no change in the index of Δ9 desaturation between the two strains but there was an increase in the index in PE and PC. This would tend to make the membrane more fluid, possibly an attempt by the liver cell to maintain membrane fluidity, although fluidity has been reported to be lower in hepatocytes form the SHR rats compared to the WKY (Devynck *et al* 1982). There have been no previous studies of the Δ9 desaturase in genetically hypertensive rats. This is surprising since it is the best characterised enzyme and the only one yet cloned.

Although the saturated/unsaturated fatty acid ratio was not different for the total fatty acids it was different in PE, PC and LPC. Here there was an increase in the ratio, suggesting that there is relatively more saturated fatty acid present in the membrane in the $SHRSP_{(Glasgow)}$ compared to WKY and that these changes occur in both the inner and outer leaflet. This would be in agreement with the Devynck *et al* study (1982) which showed that hepatocyte membrane in SHR rats is less fluid than in control animals. The saturated/unsaturated ratio for SPG fraction from the liver cell membranes also provided interesting results. SPG, as mentioned previously, contains primarily saturated fatty acids. However, in $SHRSP_{(Glasgow)}$, the levels of unsaturated fatty acid in this lipid group were increased. This is opposite to what was observed in the erythrocyte membrane.

5.4.2 Cosegregation Study.

The aim of the second part of this study was to assess whether the gene(s) which control blood pressure cosegregate with putative gene(s) which control membrane composition. The membrane compositional traits in the F_2 population produced by crossing $SHRSP_{(Heidelberg)}$ and WKY rats were studied. In this F_2 progeny the genes for membrane traits and blood pressure traits will have had a chance to recombine. Therefore, if the genetic loci which control membrane composition are the same or closely related to the loci which control blood pressure a correlation between the two traits will be observed. If the phenotypic indices of membrane composition and blood pressure are correlated then the genes for the two traits can be said to cosegregate and may be causally related or the changes in the membrane may be secondary to hypertension. If they are found not cosegregate then it is possible to carry out a total genome search to identify the gene(s) responsible for controlling membrane composition.

Difference between the two parental strains were first identified. The index of $\Delta 5$ and total desaturation in both the total lipid fraction and the free fatty acid fraction, the index of $\Delta 9$ desaturation in the PI and PE fractions and the saturated /unsaturated ratio in the SPG fraction were all reduced in the $SHRSP_{(Heidelberg)}$ compared to WKY. The derived concentration of PC in the membrane was increased in the $SHRSP_{(Heidelberg)}$.

None of these membrane traits correlated with blood pressure in the F_2 population. Therefore, this study provided no evidence that the genes which control blood pressure and those which control the fatty acid composition of the membrane cosegregate. This suggests that membrane compositional abnormalities are not causally related to hypertension in the

genetically hypertensive rat. The differences between the two strains of rats may therefore be due to chance fixation of the genes during the inbreeding of the SHRSP and the unrelated to the occurrence of increase blood pressure. However the sample size used here was small and this may have compromised the results.

Most of the enzymes involved in lipid biochemistry in the cell are membrane-bound, either all the time or at certain times during the production of lipids. Thus the enzymes may themselves be affected by the membrane fluidity and it may be this effect which leads to differences in the membrane composition between the two strains. For this to be true, membrane fluidity must be altered by something other than the fatty acids and lipids present, possibly protein concentration or cholesterol concentration, neither of which were measured here. McLaren *et al* (1993) failed to show a correlation between membrane microviscosity and blood pressure in an F_2 population also produced by crossing $SHRSP_{(Heidelberg)}$ and WKY rats.

Other phenotypes which relate to ion transport and vascular function have undergone similar analysis in F_2 populations produced from genetically hypertensive and normotensive rats. Lymphocyte potassium efflux rate has been shown to correlate with blood pressure but lymphocyte sodium influx did not (Furspan *et al* 1987). The contractile activity to noradrenaline has also been studied; the osccillartyory contractile activity in tail arteries (Bruner *et al* 1986) and mesenteric arteries (Mulvaney 1988) correlated with blood pressure. It has also been observed that the aortic smooth muscle contractile response to cobalt correlates with blood pressure (Rapp 1982). Other phenotypic variables which have failed to show a correlation with blood pressure include whole body exchangeable sodium and sodium intake (Harrap 1986).

It is of interest that the traits which do correlate with blood pressure appear to be primarily dependant on one protein (and therefore one gene) such as the noradenaline receptor. The process of maintaining the membrane composition requires many proteins, and therefore many genes. Their relationships with the development of hypertension will be correspondingly complex.

The analysis of the F_2 population may have been compromised by the small numbers of samples available. However the membrane samples did cover the full range of blood pressures obtained for the F_2 generation (i.e. blood pressures ranged from 120 mmHg to

160 mmHg). A large amount of variability may have resulted form the way the samples were handled prior to lipid analysis. During transit from Germany to Glasgow, some haemolyisis occurred which made preparation of milky white membrane, as have been used in the previous studies, difficult. In my experience, when samples of whole blood are left on ice for prolonged periods before membrane preparation, it becomes very difficult to remove all the haemoglobin. However, prolonged washing of the membranes reduces the yield of membrane obtained. Therefore, a compromise had to be made. The presence of some residual haemoglobin may have affected the lipid results or the protein analysis. Finally, the membranes were collected and prepared over many months. Thus the samples were stored for different lengths of time possibly contributing to the variability in the results.

Chapter 6 Cell Membrane Composition in Diabetes.

6.1 Introduction.

It has been suggested that changes in erythrocyte structure and function are implicated in the pathogenesis of diabetic vascular disease (Isogia *et al* 1991, Schmid-Schonbein & Volger 1976). Cell membrane microviscosity is inversely related to cell deformability and has been extensively studied in diabetic patients. Unfortunately the results of these studies have frequently been conflicting (See section 1.13).

Thus, erythrocyte membranes diabetic patients have variously been found to be more fluid, (Bryszewska & Leyko 1983) unchanged (Freyburger *et al* 1988, Hill & Court 1983) or less fluid (Caimi *et al* 1992) compared to control patients. As discussed earlier, comparison of these results is compromised by the variation in the success of metabolic control between the study groups (Candiloros *et al* 1995, Watala & Winocour 1992, Winocour *et al* 1990). Few reliable studies of membrane lipid composition have been published. Kamada & Otsuji (1983) found more SPG and less PE in the membranes of diabetic subjects compared to control. This would mediate toward a less fluid membrane. Indeed membrane fluidity was negatively correlated with membrane SPG content (Caimi *et al* 1995). Clearly, many other membrane lipids contribute to its physical characteristics and a comprehensive analysis may help to establish the nature of these changes.

6.2 Materials and Methods.

Whole blood was obtained from diabetic patients (8 NIDDM, 8 IDDM and 8 IRD {IRD are NIDDM patients requiring treatment with insulin}) attending the diabetic clinic at Gartnavel General Hospital, Glasgow. Blood was transported the laboratory on ice and erythrocyte membranes prepared. Age matched controls were obtained from within the laboratory and the staff of the Western Infirmary, Glasgow.

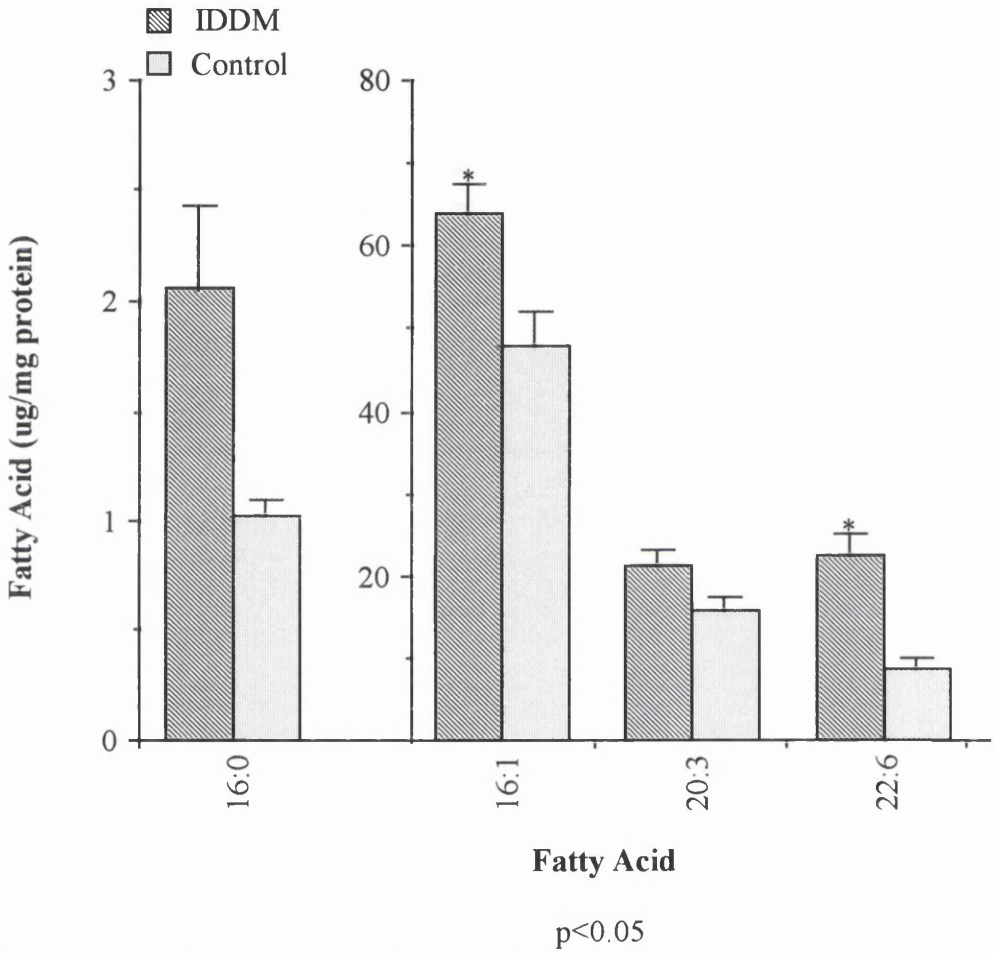
Membrane lipid composition and membrane microviscosity were analysed and presented as previously described (n=8 for all study groups and controls). All results are significant ($p<0.05$), * indicates $p<0.01$.

6.3 Results.

All patients were treated either with insulin or metformin ± glibenclamide, see appendix 1.

6.3.1 Total Fatty Acid

When the fatty acid composition of the total lipid fraction was analysed IDDM patients membranes contained significantly more palmitic, palmitoleic, dihomo-γ-linolenic acid and DHA than normal (Graph 6.3.1), but those from NIDDM and IRD patients did not differ from normal.



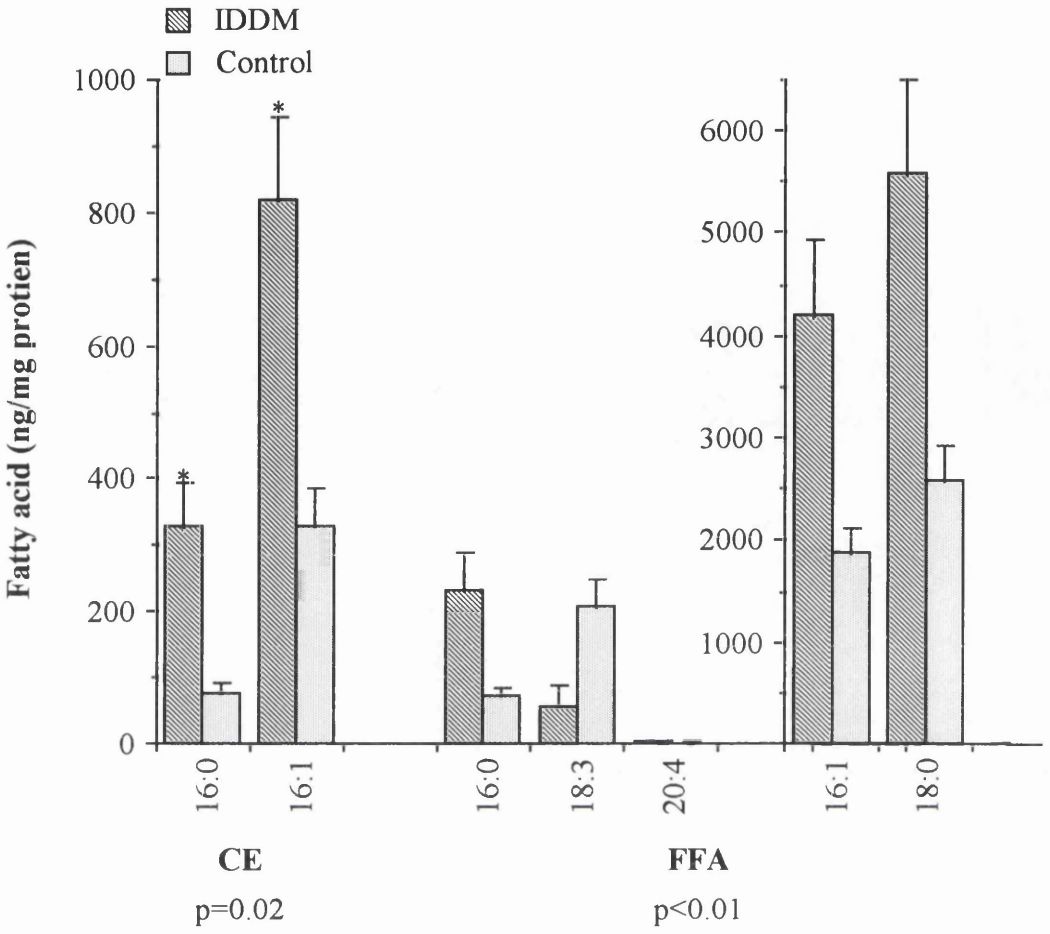
$p<0.05$

Graph 6.3.1 Fatty acid composition of erythrocyte membrane lipids (ng/mg protein) from IDDM patients and controls. All results are statistically significantly different ($p<0.05$) * indicates $p<0.01$ ($n=8$ for both groups).

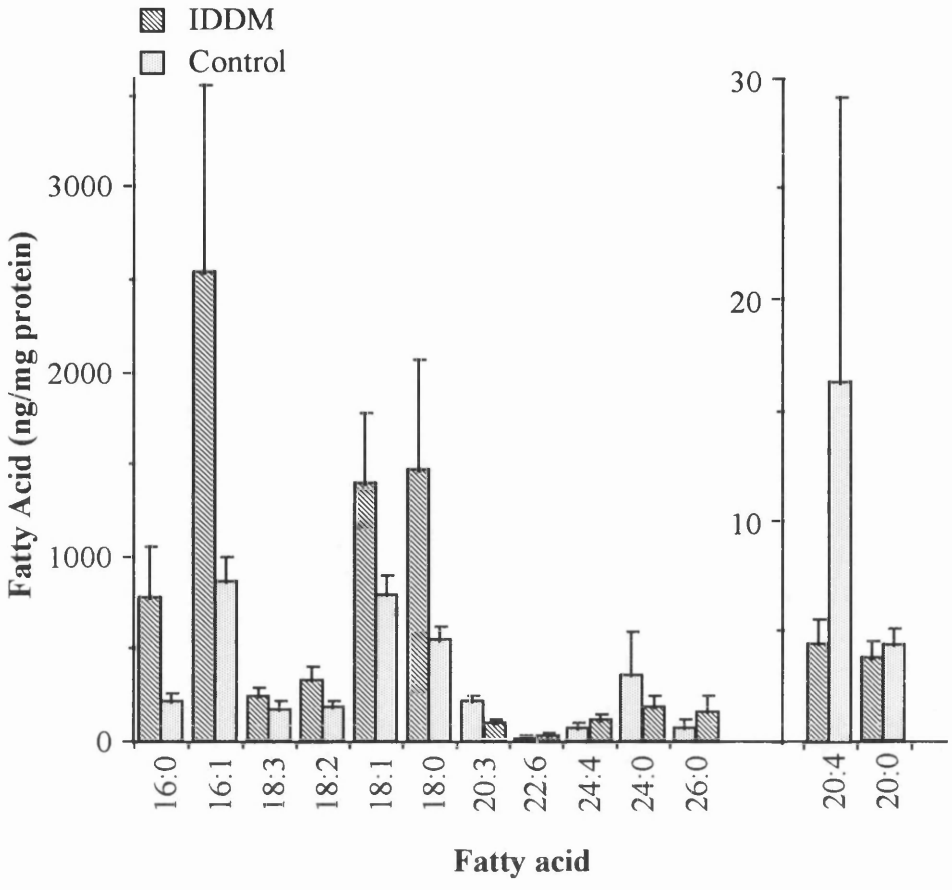
6.3.2 Neutral Lipids.

Palmitic and palmitoleic acid were also increased in the CE and FFA fractions from IDDM patients. In addition, linolenic acid was reduced and stearic and arachidonic acid were increased in IDDM patients compared to control (Graph 6.3.2). These differences were not detectable in the total lipid analysis. Although ANOVA indicated differences in the fatty acid composition of the TRIG fraction from IDDM patients no individual differences

could be identified (Graph 6.3.2b). Again NIDDM and IRD membrane did not differ significantly from normal.



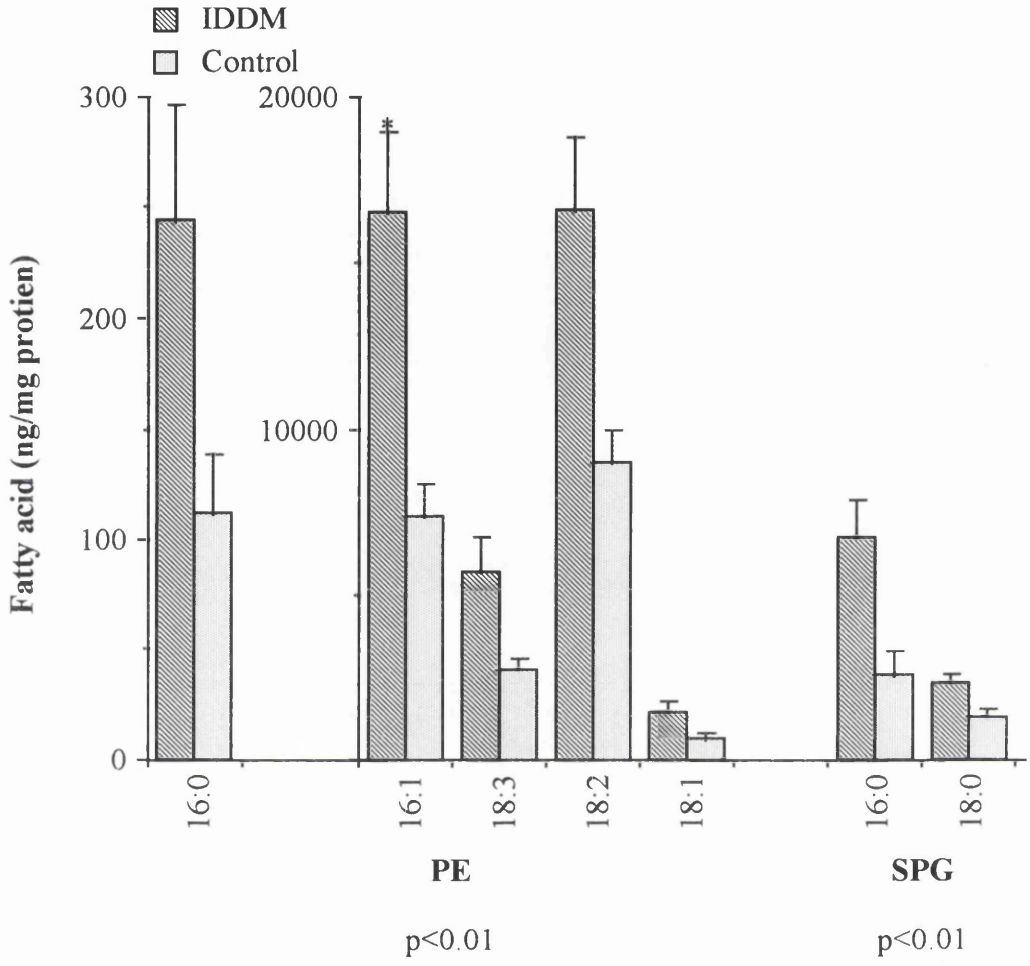
Graph 6.3.2a Fatty acid composition of erythrocyte membrane CE and FFA (ng/mg protein) from IDDM and control patients. All results are statistically significantly different ($p<0.05$) * indicates $p<0.01$ (n=8 for both groups).



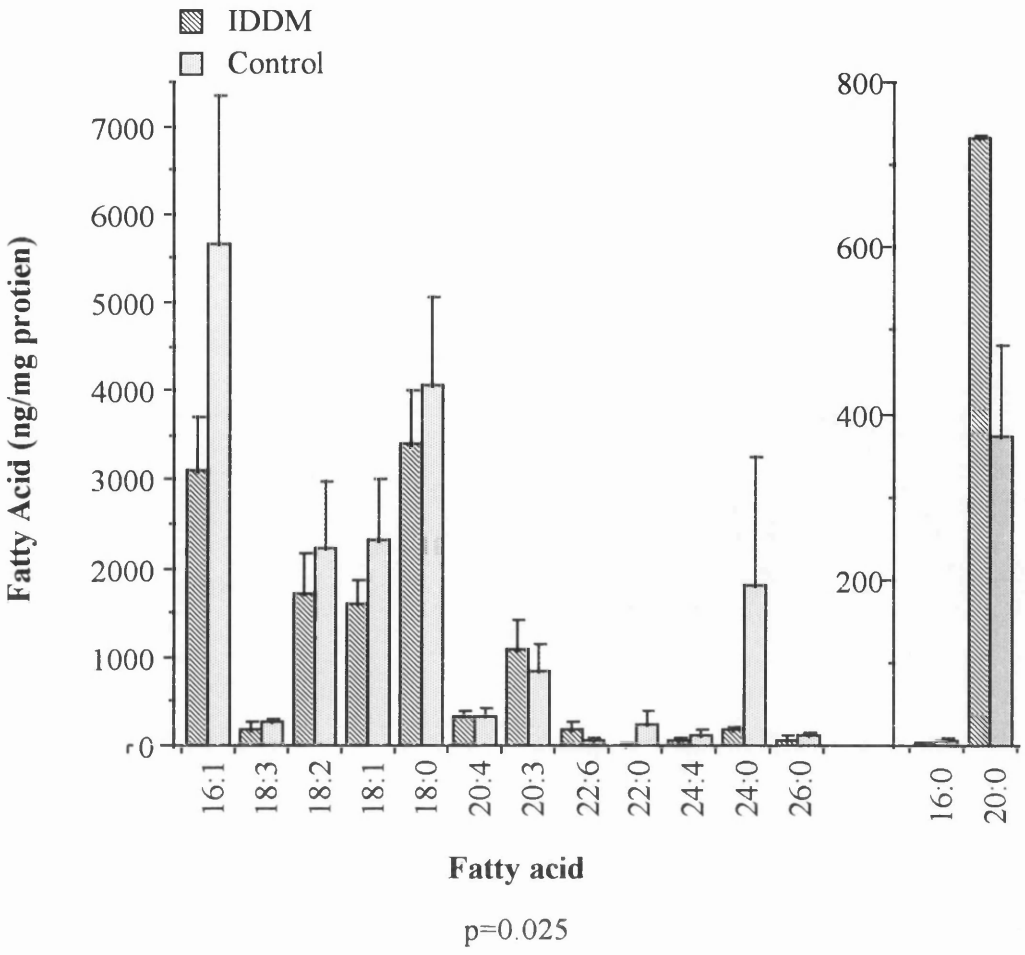
Graph 6.3.2b Fatty acid composition of erythrocyte TRIG (ng/mg protein) from IDDM patients and control. All results are statistically significantly different ($p<0.05$) * indicates $p<0.01$ ($n=8$ for both groups).

6.3.3 Polar Lipids

In the IDDM PE fraction, palmitic and palmitoleic acid were increased and in the SPG fraction, palmitic acid was increased. The linolenic, linoleic and oleic acid content of the PE fraction were also increased as was stearic acid in the SPG fraction (Graph 6.3.3a). These differences were not identifiable in the total lipid analysis. Although ANOVA indicated possible differences between normal and IDDM in the LPC fraction, individual differences could not be identified (Graph 6.3.3b).

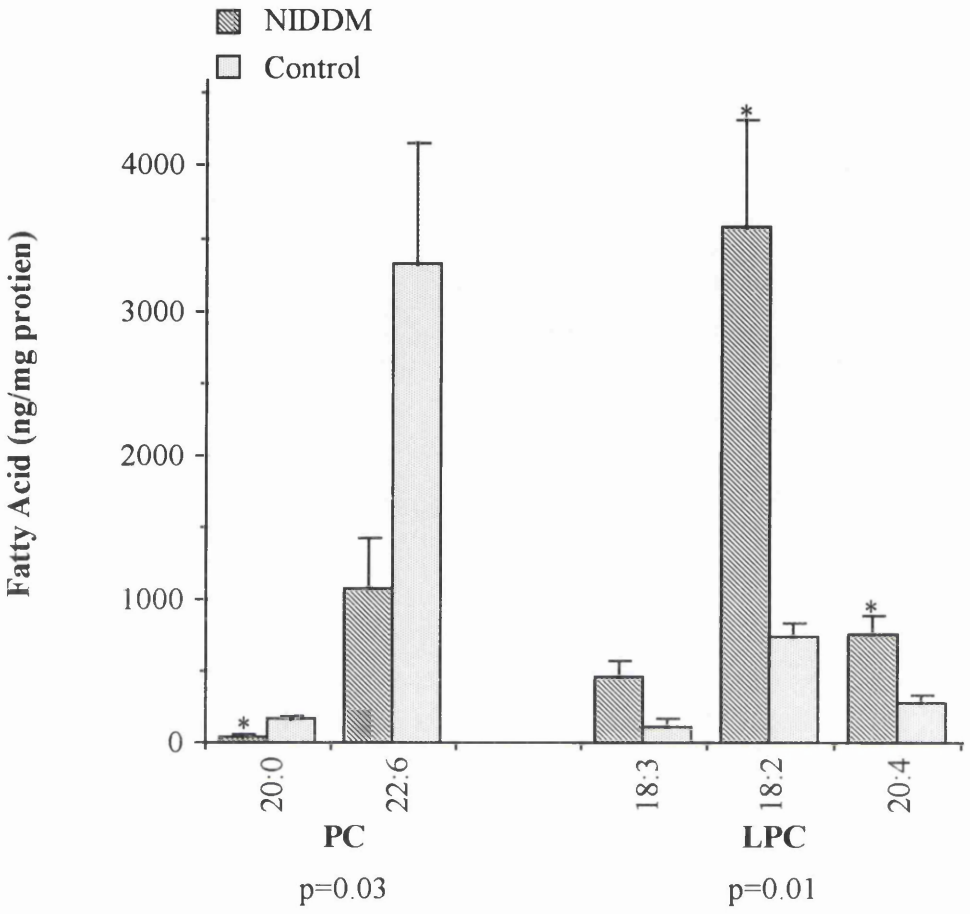


Graph 6.3.3a Fatty acid composition of erythrocyte PE and SPG (ng/mg protein) from IDDM patients and controls. All results are statistically significantly different ($p<0.05$) * indicates $p<0.01$ (n=8 for both groups).

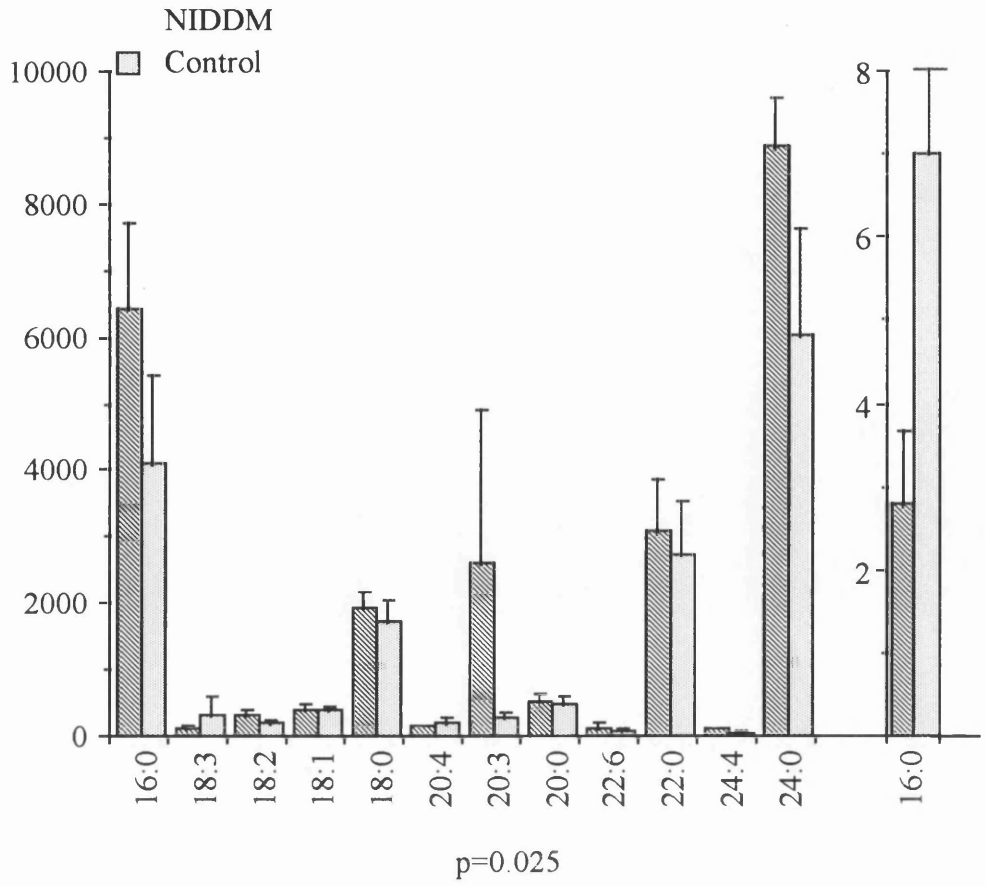


Graph 6.3.3b Fatty acid composition of erythrocyte LPC (ng/mg protein) from IDDM patients and controls. All results are statistically significantly different ($p<0.05$) * indicates $p<0.01$ (n=8 for both groups).

Some changes were also identified in the fatty acid content of the NIDDM PC and LPC fractions which were not apparent in the total lipid analysis. In the PC fraction, arachidic acid and DHA were reduced in NIDDM patients while in the LPC fraction, linolenic, linoleic and arachidonic acid were higher than normal (Graph 6.3.3c). No specific changes in the SPG fraction could be identified despite ANOVA indicating the contrary (Graph 6.3.3d).

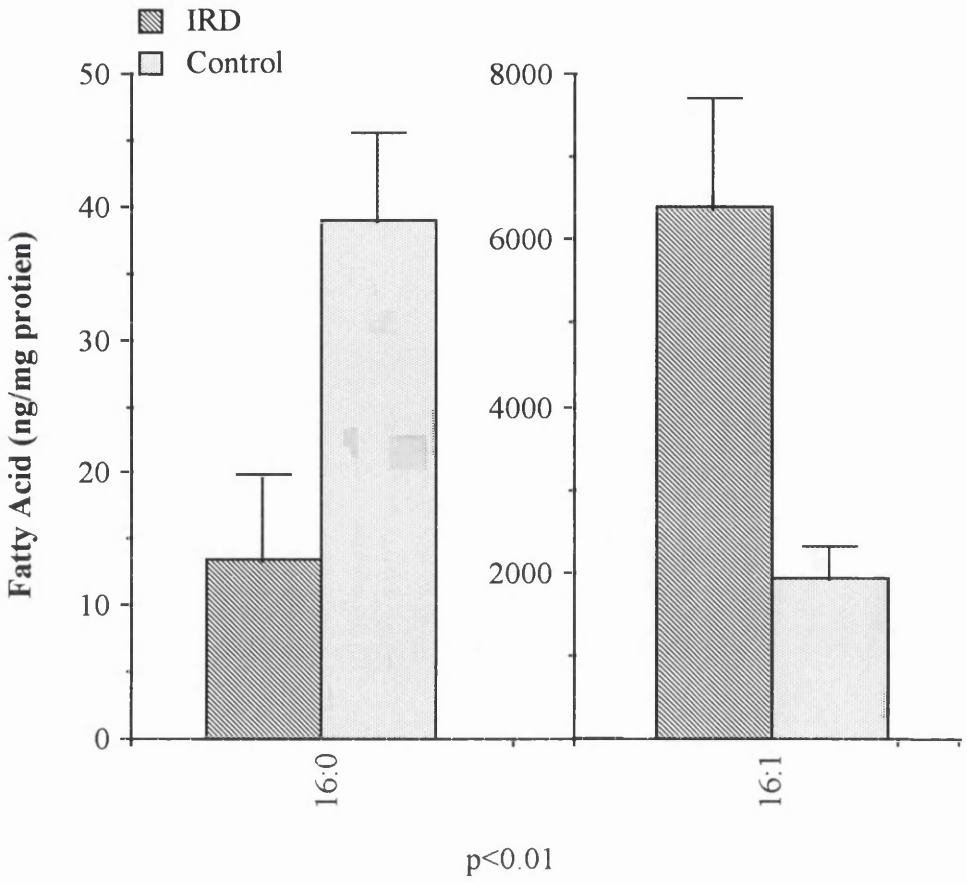


Graph 6.3.3c Fatty acid composition of erythrocyte PC and LPC (ng/mg protein) from NIDDM patients and control. All results are statistically significantly different ($p<0.05$) * indicates $p<0.01$ (n=8 for both groups).



Graph 6.3.3d Fatty acid composition of erythrocyte SPG (ng/mg protein) from NIDDM patients and controls. All results are statistically significantly different ($p<0.05$) * indicates $p<0.01$ (n=8 for both groups).

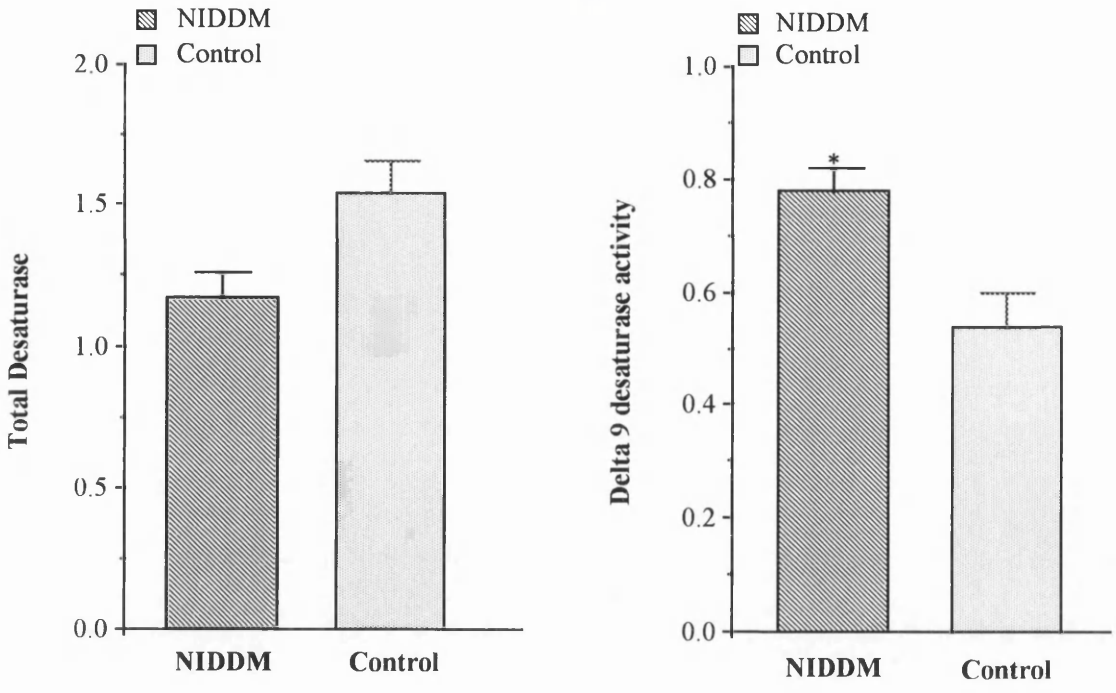
Finally, a significant increase in the palmitic and palmitoleic acid content of LPC was observed in IRD compared to control. This was not obvious in the total lipid analysis (Graph 6.3.3e).



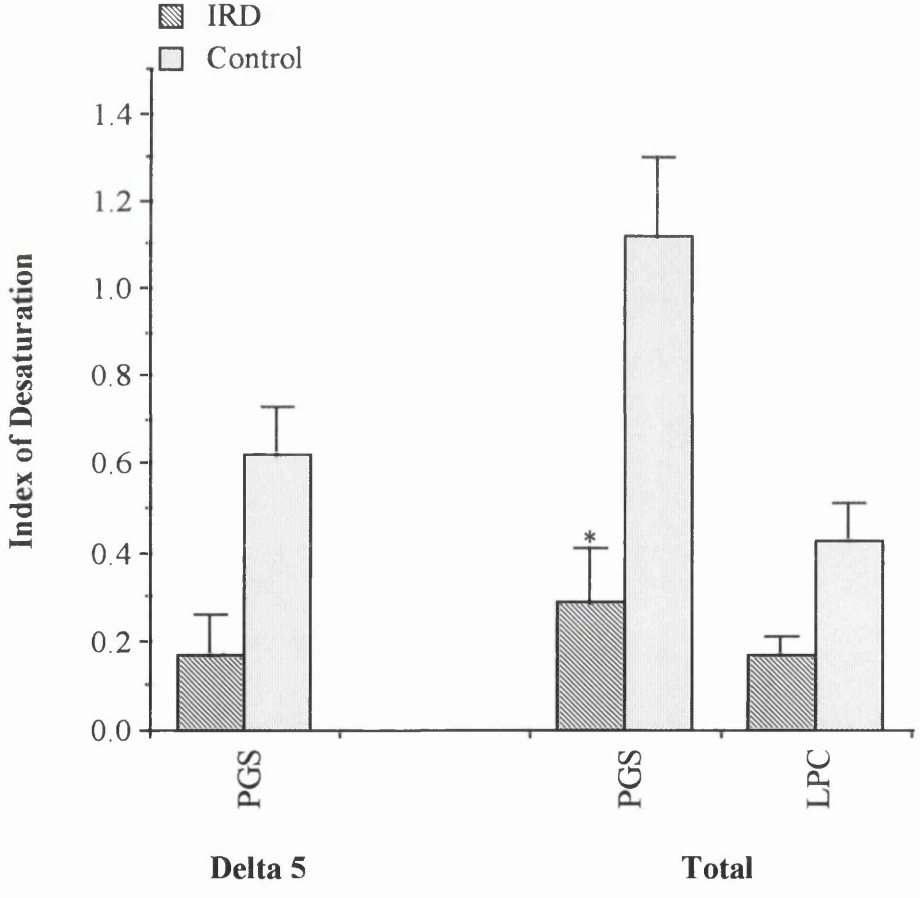
Graph 6.3.3e Fatty acid composition of erythrocyte LPC (ng/mg protein) from IRD patients and controls. All results are statistically significantly different ($p<0.05$) * indicates $p<0.01$ (n=8 for both groups).

6.3.4 Indices of desaturation

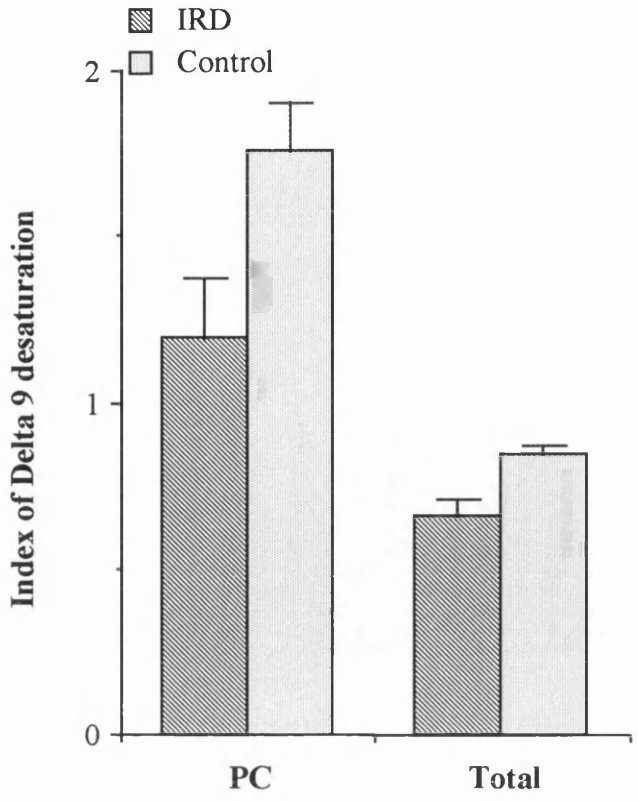
Desaturase activity was normal in the IDDM group but there were identifiable changes in the other groups. There was evidence of less total desaturase activity (total lipid fraction) but increased Δ9 desaturase activity (LPC fraction) in the NIDDM patients compared to control (Graphs 6.3.4a & b). Conversely, in IRD patients both Δ5 desaturation (PGS fraction), total desaturation (PGS and LPC fractions) and Δ9 desaturation (total lipid and PC fractions) were reduced compared to control (Graph 6.3.4c &d).



Graph 6.3.4a & b. Indices of total and Δ9 desaturase activity for erythrocytes from NIDDM patients and controls (n=8 for both groups).



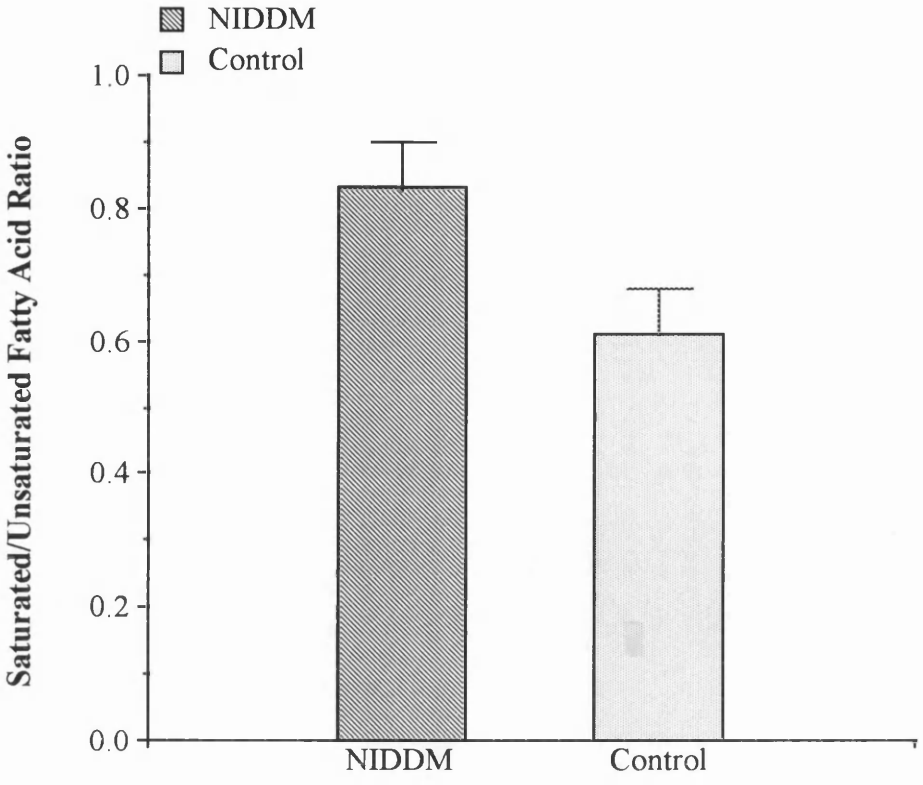
Graph 6.3.4c Indices of essential fatty acid desaturation for erythrocytes from IRD and control patients (n=8 for both groups).



Graph 6.3.4d Indices of Δ9 desaturase activity for erythrocytes from IRD patients and controls (n=8 for both groups)..

6.3.5 Saturated/unsaturated fatty acid ratios.

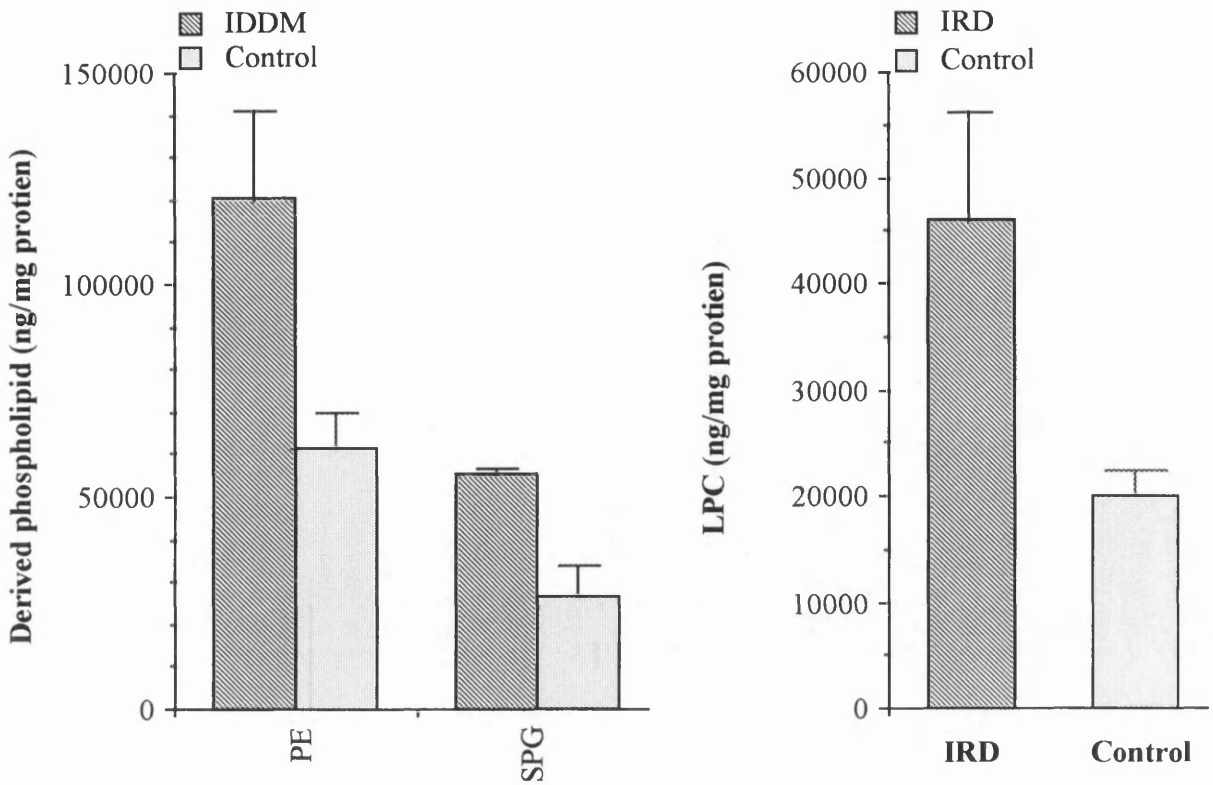
There were no significant differences between the saturated/unsaturated fatty acid ratios of IDDM or IRD patients and control subjects. However, in the CE fraction from NIDDM patients, this ratio was higher than normal (graph 6.3.5).



Graph 6.3.5 Saturated/unsaturated fatty acid ratio for erythrocyte cholesterol esters form NIDDM patients and control (n=8 for both groups).

6.3.6 Derived phospholipid composition.

The levels of both PE and SPG were increased in IDDM patients compared to control (Graph 6.3.6a). In membranes from IRD patients, there was significantly more LPC than normal (Graph 6.3.6b). No differences could be detected between NIDDM patients and controls.



Graph 6.3.6a & b Derived phospholipid composition of erythrocyte membranes from IDDM patients and control and IRD patients and controls (n-8 for both groups).

6.3.7 Membrane cholesterol and cholesterol/phospholipid ratios

There was no significant difference in membrane cholesterol from IDDM patients (237.35±36.17 μg/mg protein) and controls (158.81±19.68 μg/mg protein) (p=0.080). There was also no significant difference between the C/PL ratio from the IDDM (0.41±0.10) and control patients (0.29±0.02) (p=0.29). There were no significant differences in the membrane cholesterol between NIDDM patients (259.55±41.21μg/mg protein) and controls (206.67±23.41μg/mg protein) (p=0.28) or in the C/PL ratio from NIDDM patients (0.46±0.06) and control (0.35±0.03) (p=0.14). There was no significant differences in the membrane cholesterol between IRD patients (193.48±19.8μg/mg protein) and controls (207.69±23.40μg/mg protein) (p=0.66) or in the C/PL ratio from NIDDM patients (0.52±0.11) and control (0.49±0.06) (p=0.80).

6.3.8 Membrane microviscosity.

There were no significant differences in erythrocyte membrane microviscosity as measured using TMA-DPH between any of the groups and control values. IDDM patients: (0.258±0.002) compared to control (0.256±0.001) (p=0.43), NIDDM patients:

(0.258±0.003) and controls (0.257±0.002) (p=0.96), IRD patients: (0.259±0.001) and control (0.255±0.002) (p=0.15).

6.4 Discussion.

The groups of diabetic subjects matched the control subjects well for age. However, it is important to emphasise that all the patients were undergoing treatment and that the degree of metabolic control varied considerably, tending to be less good in the IRD group (see appendix 1) None of the patients were seriously hypertensive. As previously discussed, this affects the cell membrane properties (Candiloros *et al* 1995, Watala & Winoccour 1992, Winocour *et al* 1990). In agreement with some previous studies (Hill & Court 1983, Freyburger *et al* 1988), no evidence of altered membrane microviscosity was obtained. However, interesting abnormalities in lipid composition were identified which might have had conflicting and therefore compensating effects on membrane microviscosity. Many of these differences were not obvious when the total fatty acid analysis was carried out and were revealed only by more detailed analysis of the lipid groups. The changes were most marked in the IDDM group.

The changes in the fatty acid composition of the IDDM the total lipids included an increase in two endogenously produced fatty acids, palmitic and palmitoleic acid. This suggests that endogenous fatty acid production is not impaired in IDDM patients. As one fatty acid is saturated and the other unsaturated, their increase will have opposing effects on membrane fluidity.

The increase observed in the levels of dihomo-γ-linoleic acid in IDDM patients suggests that there is possibly a reduction in the activity of the Δ5 desaturase, thereby causing a build-up of the enzyme substrate. Bassi *et al* (1996) suggested that essential fatty acid metabolism was impaired in IDDM patients during ketoacidosis and that this could be corrected by insulin. The activity of the Δ5 and Δ6 desaturases has also been shown to be impaired in platelets from IDDM and NIDDM patients (Jones *et al* 1986). Although the levels of dihomo-γ-linoleic acid were increased in the IDDM patients, there were no alterations in the actual index of desaturase activity. This may have been because the IDDM patients were insulin-treated. DHA is a long chain PUFA; an increase in this would have been thought to increase membrane fluidity. This was not the case.

Unlike the IDDM patients, the fatty acid composition of the membrane was not altered by diabetes in the IRD or NIDDM patients. These results are in keeping with the direct measurements of membrane fluidity which were also not different in the two groups of diabetics and their controls.

Analysis of the neutral lipids from IDDM membranes showed that some of the changes observed in the total lipid analysis were conserved here. In particular, the levels of palmitic and palmitoleic acid were increased in the FFA and CE fraction. Also in keeping with the total lipid analysis, no differences were observed in the fatty acid composition of the neutral lipids from NIDDM and IRD patients compared to control. The changes which were observed in the IDDM fraction were in comparatively minor lipid groups and thus will not have had a major effect on the membrane physical properties.

Analysis of the membrane polar lipids revealed more differences in the fatty acid composition of the membrane from diabetics and controls. Both the fatty acid composition and the derived phospholipid composition of PE and SPG were different in IDDM patients compared to control. The levels of both were increased in the erythrocyte from the diabetic patients. As SPG causes a reduction in membrane fluidity and PE an increase, the effects of these two lipids appear to have cancelled each other out as there was no difference in the observed membrane microviscosity between patients and control. The fatty acid composition of these lipids reflects their effect on membrane fluidity. In PE the only fatty acids which were altered were unsaturated with the exception of palmitic acid. This would be effective at increasing membrane fluidity. The fatty acids which were increased in the SPG fraction of the IDDM patients were saturated; these would have been effect at reducing membrane fluidity. The results for the derived PE concentration were in contradiction to previous reports suggesting that the levels of PE in the membrane are reduced in diabetic patients (Caimi *et al* 1995). Others have observed in increase in SPG in the membrane and a reduction in PE (Kamada & Otsuji 1983).

Although no differences were observed in the total fatty acid composition of NIDDM and IDDM patients, there were differences in the composition of the polar lipid groups. In the PC fraction, there was a reduction in the amount of arachidic acid and DHA in the NIDDM patients. In the LPC fraction, there was an increase in the amount of linolenic, linoleic and arachidonic acid present in the NIDDM subjects. It is interesting that the fatty acids which are altered in PC are not the same as those which are altered in LPC. This

may mean that in the diabetic subjects, there is more *de novo* synthesis of PC than reacylation of LPC. This would be in agreement with others who have suggested that the reacylation of lipids is impaired in diabetics (Le Petit-Thevenin *et al* 1988, Arduni *et al* 1995). Others have analysed the fatty acid composition of PC in NIDDM patients and found there to be an increase in palmitic, oleic and arachidonic acid and a reduction in palmitic and oleic acid (Perrsom *et al* 1996). This does not agree with the results obtained here.

In the IRD patients, there was an increase in the amount of LPC present in the erythrocyte membrane compared to control. This increase may suggest an increase in the activity of the phospholipase enzymes or a reduction in the reacylation of lipids. A reduction in lipid reacylation has previously been suggested (Le Petit-Thevenin *et al* 1988, Arduni *et al* 1995). The only lipid group where an alteration was observed between IRD and control was in the LPC fraction where the levels of palmitic and palmitoleic acid were increased. These fatty acid have also been seen to be increased in other lipid groups in IDDM patients. Also, a similar trend toward more LPC was observed in IDDM patients and in NIDDM patients. The fatty acid composition of PC and LPC suggests that reacylation of LPC may be impaired. Thus, this may be a generalised defect in diabetes although further experiments would be required to confirm this.

The saturated/unsaturated fatty acid ratio for the total lipids was not different between any of the diabetic and control groups. This is in agreement with the direct physical measurements which showed no difference in membrane microviscosity. Further analysis of the membrane lipid composition revealed that in the NIDDM patients, there was an increase in the saturated/unsaturated fatty acid ratio in the CE fraction. This is such a minor group that it may not be of physiological relevance.

Although there were few observable changes in the degree of fatty acid unsaturation, there were some changes in the indices of fatty acid desaturation. The index of total essential fatty acid desaturation was reduced in NIDDM patients compared to controls although this change in desaturation could not be observed in any of the individual lipid groups. Others have observed that essential fatty acid desaturation is reduced and humans (Jones *et al* 1986, Bassi *et al* 1996). Although the indices of essential fatty acid desaturation were not different in the total lipid fraction between the IRD patients and control there were alterations in some of the lipid groups. The index of $\Delta 5$ desaturation was seen to be

reduced in the PGS fraction of the IRD patients. The PGS fraction also had a reduced index of total desaturase activity as had the LPC fraction. Both of these are minor membrane fractions which may not affect the physical properties of the membrane.

The index of Δ9 desaturation was seen to be increased in the LPC fraction from NIDDM patients compared to control. This, as mentioned previously, is a relatively small membrane component and may not be physiologically relevant. In the IRD patients the index of Δ9 desaturation in the total lipid fraction was reduced compared to control This was also observed in the PC fraction, a major outer membrane lipid. To the best of my knowledge, desaturase enzymes have never been studied in a group of IRD patients. This reduction in the production of oleic acid would lead to the membrane being less fluid. However, the direct membrane microviscosity measurements made here did not reflect this.

None of the diabetic groups studied showed a difference in membrane microviscosity compared to control. This may relate to the membrane lipid composition as discussed previously, but it is also possible that the treatment the patients were receiving affected the membrane fluidity. Insulin is known to increase membrane fluidity directly (Dutta-Roy *et al* 1985, Bryzewska & Leyko 1983, Juhan Vague *et al* 1986). Therefore, if there were changes in fluidity caused by diabetes, these may have been corrected by insulin treatment. Fluidity is also known to be influenced by the metabolic control of the patients, with poor control reducing membrane fluidity (Candiloros *et al* 1995, Watala & Winocour 1992, Winocour *et al* 1990). The only group with consistently poor metabolic control were the IRD group, but these were also insulin-treated and the two effects may have compensated for each other. The absence of a difference in the C/PL and membrane cholesterol content for any of the groups is in agreement with other previous studies in erythrocytes (Hill & Court 1983) and platelets (Caimi *et al* 1995).

The published evidence for changes in membrane structure and function in diabetes is varied and often conflicting. Although in this study some of the results obtained were in agreement with the previously published observations many, like membrane microviscosity, were not. In the case of the IRD and IDDM patients, insulin treatment may have affected the results obtained. Another confounding factor is that in human studies of this nature, it is impossible to control the dietary lipid intake as with laboratory

animals. Also, the duration of diabetes can be controlled in rat models. The groups studied here had been suffering from diabetes for various lengths of time.

The results obtained here are in agreement with some of the studies carried out in rat models. For instance, the increase in the concentration of membrane DHA observed in IDDM patients is in agreement with studies carried out in STZ-diabetic rats (Dang *et al* 1991). Moreover it has previously been suggested that the activity of the Δ5 desaturase is reduced in heart, liver, kidney, aorta (Holman *et al* 1983) and adrenal gland (Igal *et al* 1991) as well as other tissues in STZ-diabetic rats. This is in agreement with some of the results observed here. The same is true of the results obtained for the Δ9 desaturase which has also been shown to have reduced activity in alloxan diabetic rats (Clarke & Queener 1985) and BB spontaneously diabetic rats (Minouini *et al* 1992). Some of the results obtained here are, however, in contradiction to the results seen in diabetic rats. Erythrocytes from STZ-diabetics rats incorporate less palmitic acid and that this could not be fully corrected for with insulin treatment (Arduini *et al* 1995).

Chapter 7 General Discussion.

Cell membrane abnormalities occur in a variety of diseases including hypertension and diabetes. As discussed in chapter 1, the membrane controls many cell function but whether these abnormalities initiate or contribute to the disease process or are merely responses to it remains unclear. To study this problem, good analytical methods are obviously necessary. The fluidity (microviscosity) of the membrane can be measured using fluorescent probes which are able to distinguish physical changes in the inner and outer leaflets. The biochemical basis of the changes is much less easily accessible; published data on membrane composition in these diseases are sparse and often conflicting (see 1.11.3 & 1.11.4). Never-the-less, it is desirable for several reasons. Without complete analysis of the membrane lipids it is impossible to distinguish whether changes in microviscosity are due to a generalised defect in the fatty acid metabolism or to an effect on a specific membrane lipid. Thorough analysis of fatty acid composition may be a more informative index of microviscosity, especially where it is not possible to use specific probes for the inner and out leaflet. Furthermore, it might reveal real changes in composition which have no net effect an fluidity. Some of these, such as alterations in arachidonic acid content (see chapter 5), may have obvious implications for cell function. The current study has sought to devise such a method and to test its performance in three situations in which membrane changes are recognised.

The method has been based on that of Hamilton and Comia (1988). Careful evaluation, however, revealed a serious flaw which required major reorganisation. Firstly, the recovery of lipids was less efficient than reported. It was also found that acetic acid, recommended by Hamilton and Comia (1988) as a component of solvents for eluting lipids from Sep-pak cartridges, caused significant hydrolysis of lipid ester bonds. This caused serious distortion of the assessment of FFA content (see chapter 3 sections 3 & 4). These problems were solved by adjusting the volumes of eluting solvents, by removing the acid from the solvents and by separating the components of neutral lipids and polar lipids on different Sep-pak cartridges. The performance criteria were determined (see 3.7.1). The complexity of the protocol, while it ensured a high level of specificity, meant that precision was variable. This has been taken into account when discussing the results of the three studies. It cannot be claimed that the method is a simple routine but it provides a comprehensive analysis of membrane lipids with their fatty acid content. From this, it has also been possible to assess desaturase activity. It is possible that, if major reproducible changes are identified in specific situations, the method could be simplified to focus on these.

Cell membrane microviscosity in SHRSP and SHR is higher than in WKY rats (see 1.11.4a). In diabetic rats and human patients, the evidence is more conflicting with both increased and reduced membrane fluidity reported (see 1.11.3). In rats treated with L-NAME, from which they developed hypertension, membrane fluidity was found to be increased (Chapter 4). A primary aim of the current study was to ascertain whether these changes could be explained by specific changes in lipid composition.

In general terms, the cell membrane composition by-and-large reflected the changes in membrane microviscosity. When membrane microviscosity was increased, the amount of unsaturated fatty acid present was decreased and vice versa. Also, the index of $\Delta 5$ desaturation appeared to follow a similar pattern with increased desaturation leading to increased membrane fluidity. The concentrations of the phospholipids also appeared to agree in most cases with the direct physical measurements. In some cases, however, the direct measurement and the compositional measurement disagree, as was the case for cholesterol in the L-NAME-treated rats. Membrane cholesterol increased and membrane microviscosity decreased, the opposite to what was expected. This highlights the great value of carrying out a full compositional analysis of the membrane. As the changes in phospholipid composition appear to have compensated for the increased cholesterol, it would be interesting to manipulate membrane composition to study the relative effects of various lipids on fluidity. Some such studies have been carried out on artificial membranes but their physiological relevance is questionable.

Analysis, as carried out here, can reveal changes which, while not altering the physical properties of the membrane, might change the function. In the IDDM patients, there was an increase in the amount of PE and SPG present in the membrane. As these have opposite effects on fluidity, no net change was observed. The increase may reflect a change in the activity of enzymes involved in the production or degradation of these lipids and may be a contributor or a consequence of the altered intermediary metabolism characteristic of this disease.

The membrane exists in a constantly changing sate with the lipids perpetually being shuttled into and out of the membrane. The analyses made here are at best a "snap-shot" of this process and gives no indication of over what time scale these alterations have occurred. To evaluate this, it would be necessary to carry out a time course experiment, sampling at

various intervals. With the present method sensitivity, however, the amount of blood required would certainly activate the renin-angiotensin system, compromising the results. Thus ways of markedly increasing the sensitivity must be sought. Many of the results obtained here suggest that enzyme activity is altered and it would now be of interest to assess these activities directly to confirm the observation. The method could readily be adapted for use with radioactive lipids to measure the rate at which they are taken up into the membrane. Radioactive fatty acid could be administered to rats to follow the time course of their fate *in vivo*. Finally, although the method was developed specifically for cell membranes it is applicable to all other lipid containing tissues, for example atheromatous plaques.

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Appendix 1

Patient data for diabetic study, see chapter 6.

DOB	Duration of diabetes (years).	Treatment	Blood Pressure (mmHg)	Weight (Kg)	HSA_{1c}
11.7.65	8	ins	120/80	82	4.5
9.2.44	5	ins	110/60	66	6.1
12.2.62	5	ins	110/70	76	5.7
6.2.47	5	ins	110/60	63	6.6
17.5.54	2	ins	116/78	76	NA
29.2.64	14	ins	110/70	59	9.0
26.3.65	18	ins	120/80	81	7.7
3.6.72	6	ins	120/70	73	5.2

Table 1. Patient information for the IDDM patients studied in chapter 6. Abbreviations: DOB-date of birth, ins-insulin, HSA_{1c},-Haemoglobin A_{1c}

DOB	Duration of diabetes (years).	Treatment	Blood Pressure (mmHg)	Weight	HSA_{1c}
18.4.63	1	met	130/90	81	4.2
7.11.26	3	glic/met	170/110	89	5.4
15.2.42	8	glic/met	110/70	77	9.1
15.6.41	4	glic/met	160/80	102	6.2
8.3.43	7	glic/met	135/85	94	8.6
17.1.34	7	glic/met	150/74	71	5.5
8.6.31	4	glic	110.50	62	6.2
27.1.56	1	glic	130/82	83	8.2

Table 2 Patient information for the NIDDM patients studied in chapter 6. Abbreviations as above , met-metformin, glic-glibenclamide

DOB	Duration of diabetes (years).	Treatment	Blood Pressure (mmHg)	Weight (Kg)	HSA_{1c}
3.7.34	1/9	ins	160/70	72	6.3
12.10.29	20/25	ins	120/90	54	8.9
4/8/22	4/10	ins	170/70	56	
5.5.37	10/23	ins	112/70	71	7.3
1.6.37	13/27	ins	135/80	81	10.5
2.3.30	10/22	ins	135/70	88	10.3
31.8.46	3/4	ins	138/90	68	9.1
31.10.20	2/8	ins	150/80	81	5.9

Table 3 Patient information for the IRD patients studied in chapter 6. Abbreviations as before. Duration of diabetes shows the number for years that the patient has been requiring insulin for and the total number of years since diagnosed as NIDDM.

Publications.

A.M. Dorrance, K. Lindpainter A.F. Dominiczak, R. Fraser.

Evidence of a reduced delta 5 desaturase activity in stroke prone spontaneously hypertensive rats.

Journal of Hypertension, 14, Supplement 1, P1277, 1995.

A.M. Dorrance, D. Graham, W.K. Lee, A.F. Dominiczak, R. Fraser.

Nitric oxide synthase inhibition alters cell membrane composition.

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Nitric oxide synthase inhibition increases erythrocyte membrane fluidity and alters membrane lipid composition.

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