

**Proton and carbon-13 NMR lipid profiles of cells, tissues and
body fluids**

by

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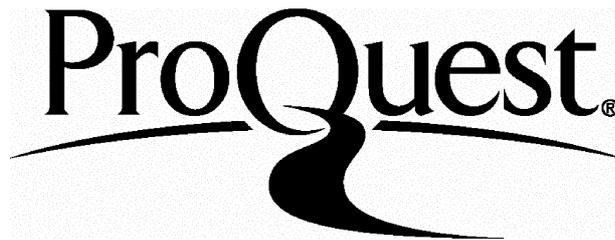
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To My Parents

ABSTRACT

This thesis describes the development of a novel, comprehensive and rapid biological lipid analysis via high-field NMR. Based upon the information obtained from various 2-dimensional (2D) NMR experiments, and by comparing NMR spectra of biological lipid extracts to standard lipid spectra, plus cross-references with existing data when available, the major lipids and fatty acids have been successfully identified and quantified. Significantly, information obtained from NMR experiments proved to be in agreement with previous published data, which was obtained using well-established but time-consuming conventional chromatographic techniques. The NMR lipid assay was then applied to the analysis of various biological tissues, cells and body fluids including rat liver, brain, heart, kidney, mast cell, human erythrocyte and plasma lipid extract. Assignments were mostly achieved using ^1H - and ^{13}C -NMR spectroscopy, although experiments involving other nuclei were also performed when necessary (e.g. ^{31}P). Applications to the detection of lipid and phospholipid changes in carbon tetrachloride treated animals were carried out.

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LIST OF ABBREVIATIONS

(In alphabetical orders)

1D and 2D	One- and two-dimensional
¹ H-NMR	Proton nuclear magnetic resonance spectroscopy
¹³ C-NMR	Carbon-13 nuclear magnetic resonance spectroscopy
³¹ P-NMR	Phosphorus nuclear magnetic resonance spectroscopy
C or CHOL	Cholesterol
CAR	Cardiolipin (Diphosphatidylglycerol)
CDCl ₃	Fully deuterated chloroform; D: deuterium
CD ₃ OD	Fully deuterated methanol; D: deuterium
CE	Cholesterol ester
COSY	Homonuclear correlation spectroscopy
COSYDQF	Double quantum filtered homonuclear correlation spectroscopy
COSYMQF	Multiple quantum filtered homonuclear correlation spectroscopy
DG	Diacylglycerol
FID	Free induction decay
g	gram
GAC	Galactocerebroside
GC	Gas chromatography
HETCOR	Heteronuclear correlation spectroscopy
HOHAHA	Homonuclear Hartmann-Hann spectroscopy
HPLC	High performance liquid chromatography
K _d	Dissociation constant (chemical kinetic value)
LPC	Lysophosphatidylcholine
LPE	Lysophosphatidylethanolamine
M	Molar (unit of concentration)
MG	Monoacylglycerol
MHz	Mega Hertz (unit of frequency)
mg	milligram

min.	Minute(s)
Mono.	Mono-saturated (fatty acid)
MS	Mass spectroscopy
NMR	Nuclear magnetic resonance spectroscopy
NOE	Nuclear overhauser enhancement
PA	Phosphatidic acid
PC	Phosphatidylcholine
PDE	Phosphatidyl- <i>N, N</i> -dimethylethanolamine
PE	Phosphatidylethanolamine
PG	Phosphatidic glycerol
PI	Phosphatidylinositol
PLA	Plasmalogen
PLA-C	Plasmalogen-choline
PLA-E	Plasmalogen-ethanolamine
PME	Phosphatidyl- <i>N, N</i> -mono-methylethanolamine
ppm	Part per million
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acid
Sat.	Saturated (fatty acid)
S.D.	Standard deviation
SPH	Sphingomyelin
TG	Triacylglycerol
TLC	Thin layer chromatography
TOCSY	Total correlation spectroscopy
Total P.	Total phospholipids
Unsat.	Unsaturated (fatty acid)
UFA	Unsaturated fatty acid
v/v	Volume per volume ratio

Chapter 1

Introduction

1.1 RESEARCH OBJECTIVE

1.1.1 Research background

The term "lipid" in molecular biochemistry, is commonly refers to a group of compounds including esterified or non esterified fatty acids, steroid and related compounds, phospholipids, glycerolipids, ether lipids, sphingolipids and glycolipids etc., and it is in this sense that the term is used herein. Lipids have been studied by scientists for about two centuries, and for many years lipids were considered to be molecules mainly used for structural and energy storage purposes. Being one of the essential building block in cells, phospholipids are the primary component in most cellular membranes, although cholesterol also acts as an important structural constituent of some eukaryotic membranes and its presence is essential to maintaining the specific membrane fluidity [Watala & Kordacka, 1987; Watala *et al*, 1987]. Cholesterol is also the precursor of bile acids, vital for lipid absorption in the digestive system and the precursor of steroid hormones [Vance & Jacobson, 1988a]. Yet myelin sheath in the nervous system contains *ca* 75% of lipid with a significant amount of sphingolipids. Furthermore, the triglycerides, stored primary in the adipose tissue, function as an energy reserve [Vance & Jacobson, 1988b]. More recently, over the past two decades, the existence of complex structures such as glycolipids and lipoproteins, plus the elucidation of important biological functions of lipids has received interest in the role of lipids in biology and medicine. The major biological functions of lipids are summarized as follows:

- 1) as structural elements for membranes
- 2) as efficient reserves for the storage of energy (e.g. triacylglycerols)
- 3) as detergent to help solubilize other lipid classes during digestion (e.g. bile acid)
- 4) precursors of many animal vitamins and hormones, and
- 5) as electro-or thermo-insulators.

In addition, there is one newly discovered role of lipids which revolutionized the modern lipid research over the past decade, that is, their potent biological activities in the generation of signals — both as intracellular second messengers acting on cytoplasmic targets, and intercellular messengers that act at specific receptors. Significant

developments in this area have included the discovery of signal transduction functions of inositol lipids [Exton, 1990], diglyceride lipids [May & Calder, 1993; Lameh *et al*, 1990; Nigam *et al*, 1990; Neer & Clapham, 1988], platelet activating factor (PAF) [Schulam *et al*, 1991] and the arachidonate cascade [Choi & Gao, 1991; DuBourdieu & Morgan, 1990; Rustenbeck & Sigurd, 1989]. Various theories suggesting body lipids play an active role in major biological events have also been proposed [Ordway *et al*, 1991; Exton, 1990; Martin & Michaelis, 1990; Pacini *et al*, 1990; Barbour *et al*, 1989; Williams *et al*, 1989; Bevan & Wood, 1987; Ragab-Thomas *et al*, 1987; Bell, 1986]. Such discoveries plus the association of lipids with certain protein functions and immuneresponse processes, have opened a new frontier for lipid biochemistry research.

Lipid chemists and biochemists have gradually focussed their attention away from pure structural studies to the study of the potent biological activities. This is probably one of the major driving forces which caused the rapid development of modern lipid analytical methodologies. Thin layer chromatography (TLC) [Pal & Davis, 1991; Jheem & Weigel, 1989; Jardetski & Roberts, 1981; Gurr & James, 1980; Touchstone, 1973] which was introduced in the 50's and has been used extensively for lipid separation since then. TLC is still widely used today because of its simplicity and comparatively inexpensive features. Later there was the introduction of gas chromatography (GC) [Kuksis *et al*, 1990; McGrath & Elliott, 1990; Beyer *et al*, 1989], particularly for fatty acid methyl ester analysis. The development and exploitation of this particular technique contributed greatly to the vast explosion of information on the chemistry and biochemistry of lipids over the last 20 years. More recently, the development of high performance liquid chromatography (HPLC) [Araki *et al*, 1990; Samet *et al*, 1989; Seta *et al*, 1990; Vercaemst *et al*, 1989; Christie, 1987; May *et al*, 1986; Yu *et al*, 1986]. It is rapidly gaining ground and becoming one of the most commonly used technique for lipid analysis since the last decade. Other developments included mass spectroscopy (MS) [Harvey, 1991; Toshiyuki *et al*, 1984] and radiolabelled lipids as the substrates for enzymatic studies. Enzymatic hydrolysis [Chilton, 1991; Gibbons *et al*, 1989] procedures have been developed for determining the positional distribution of esterified fatty acids

within complex lipids. Although most of these lipid analysis methodologies are well established and informative, there are drawbacks still waiting to be overcome. For instance, virtually all of the techniques mentioned above are not able to provide a comprehensive lipid analysis in one single step. More often the assay has to be repeated with different conditions or parameters, or a combination of multiple methods are used in order to yield a more satisfactory result. Such multi-step chromatographic approaches consequently leads to a tedious, time-consuming and labour intensive lipid assay. Furthermore, recovery of lipid samples can also be a problem — liberation of the esterified fatty acids via lipid hydrolysis are often required prior to GC analysis to allow the determination of fatty acid compositions. Other chromatographic technique such as TLC, which apparently is not designated for quantitative sample recovery; although lipids from TLC adsorbent can be re-extract or eluted to a certain extent with appropriate solvent systems [Christie, 1987].

Thus the scientific interest in lipids has been considerably widened as the importance of lipids in cellular signalling, cell metabolism, immunology etc. are steadily being discovered and recognized. This has leads to an ever-increasing demand for a comprehensive yet rapid lipid analysis as an alternative to the existing chromatographic approaches.

1.1.2 The introduction of NMR in lipid analysis

Nuclear magnetic resonance spectroscopy (NMR) is widely known as a powerful technique for molecular structural determination. Its capability has been thoroughly demonstrated in areas such as polypeptide and protein structural analysis. There have also been attempts to apply NMR to other biological macromolecules, including lipids. Since in the late 70's, NMR has been applied to the identification of lipid structures [Williams & Fleming, 1987; Kemp, 1986]. Nevertheless, due to the early technological limitations (such as applied magnetic field strength), information obtained via NMR was restricted only to a few distinctive functional groups and the precise characterization of the individual lipid or fatty acid classes within one natural lipid mixture was impossible.

Phosphorus (^{31}P) NMR has also been developed for lipid analysis [Radda *et al*, 1991; Sparling *et al*, 1989; Meneses & Glonek, 1988; Stoiros *et al*, 1986; Llida *et al*, 1977; Mahadevan *et al*, 1968], but their application has been limited to phospholipids. The other aspect of NMR compared to the majority of conventional analytical methods was its intrinsic insensitivity. Despite proton (^1H) NMR having the highest sensitivity among all nuclei, it was still significantly lower than its chromatographic rivals, besides the dipolar and J-coupling interactions of spectra were found to be too complicated for full interpretation. In principle broad band (proton) decoupled carbon-13 NMR can provide a tempting alternative since all carbon atoms give one distinctive signal and thus considerably reduce the spectral complexity. In practice however, the sensitivity of ^{13}C -NMR is even lower than its ^1H counterpart by *ca.* 6000 fold, owing to the low natural ^{13}C abundance (*ca.* 1 %). Thus large sample quantities were required which were not considered appropriate for most analytical purposes.

Fortunately, this situation has been changed dramatically after the introduction of Fourier transform (FT) technology, very high field super-conducting magnets (currently up to 17.6 tesla) and the improvement of computing technology. The combination of these advances significantly enhanced the sensitivity as well as resolution of NMR spectra, especially carbon-13 NMR. With the additional aid of modern multi-dimensional NMR technique the complex lipid profile assignments eventually became feasible.

Recently, a few laboratories [Sze & Jardetski *et al*, 1990; May *et al*, 1986; Yu *et al*, 1986; Toshiyuki *et al*, 1984; Canioni *et al*, 1983; Jardetski & Roberts, 1981; Drabkowski *et al*, 1980; Sawan *et al*, 1979; Llida *et al*, 1977; Mahadevan *et al*, 1968 etc.] including ours [Choi *et al*, 1993; Casu *et al*, 1991] have explored the potential of high-field NMR spectroscopy as a complementary technique for lipid profiling. In our studies, both ^1H and ^{13}C NMR were utilized and developed. Lipid extracts from rat liver, brain, heart and kidney, human erythrocytes and blood plasma were selected for our development work to illustrate the diversity of their lipid contents and the potential interest of these tissues in biochemical and medical research. The major advantages of this NMR assay over the other conventional technique can be summarized as follow:

- 1) Comprehensive lipid profile can be obtained by one single 1D experiment within a short period of time (< 30 min. for ^1H -NMR with *ca.* 5-10 mg of sample). In principle all lipids could be detected by proton and carbon-13 NMR.
- 2) Natural lipid mixtures can be measured simultaneously in both a quantitative and qualitative manner, and the relative concentration of each individual species can be determined via the integrated areas of the characteristic resonances.
- 3) It has been traditionally difficult to analyze esterified fatty acid compositions within the lipid mixture. In contrast, information of the fatty acid compositions and the degree of unsaturation are readily accessible from both 1D proton and carbon-13 NMR spectra.
- 4) The procedure for sample preparation is simple. Since NMR is a non-destructive experiment, samples are fully recoverable and can be retained for further analysis.

With the advantages of its rapidity, comprehensiveness and non-destructive nature, *in vitro* lipid profiling via NMR has been proved to be a promising alternative to the main stream chromatographic approaches as a rapid and comprehensive lipid assays.

1.1.3 Research purposes and objectives

The initial aim of this project was to develop a rapid, comprehensive lipid assay using high field NMR spectroscopy and to use this to monitor biological lipid metabolism *in vitro*. Ultimately such an assay should be applicable for routine analysis particularly in clinical medicine.

During the establishment of NMR lipid profile analysis, lipid extracts from different animal organs were used for development work, as mentioned in the previous section. Lipid extracts from cell culture and body fluid were also studied. After the primary verification, the technique was applied to various lipid metabolic studies, in order to evaluate its applicability. Assignments of proton and carbon-13 NMR were achieved by comparison with purified standard lipid and fatty acid spectra, plus structural information obtained by two dimensional NMR cross peaks. The quantitations of lipids were calculated and were in good agreement with the conventional TLC and HPLC assay

methods. Although the primary interest was to develop proton and carbon-13 NMR for lipid analysis, NMR of other nuclei, ^{31}P for example, were also used occasionally as a compliment to the project development.

In this introductory section, a brief description of the lipid structures will be included, since the characterization of lipids by the NMR approach is based principally upon their structural variety and specificity. Other topics that are going to be covered in this section include highlights of the functional importance of lipid as potent biological molecules, and a summary in regard to the basic principle of NMR.

1.2 STRUCTURES AND PROPERTIES OF LIPIDS

Since all distinguishable lipid NMR signals (and in fact any other kind of molecule) arise exclusively from their structural diversities, it is therefore relevant to include a brief overview of lipid structures. Some of their properties and occurrence will also be described. This summary however, is by no mean comprehensive. It will focus merely on those lipid species which are detected and assigned by ^1H or ^{13}C -NMR. Other relatively low abundance lipid molecules which lay beyond the detection capability of the NMR assay described here, such as some steroid hormones and lipid soluble vitamins, will not be discussed.

1.2.1 Fatty acids

Fatty acids are long chain hydrocarbon molecules with a terminal carboxyl group [Vance, 1988a; Christie, 1987]. Fatty acids can be saturated with no double bonds in the aliphatic chain, namely saturated fatty acids. The most common species among the saturated fatty acids are palmitic acid (16:0) and stearic acid (18:0) Fatty acids which consist of one and more double bonds are called monounsaturated and polyunsaturated fatty acids (PUFA) respectively. The double bond occurs between carbon atom 9 and 10 in most monounsaturated fatty acids of animals, thus the most common monounsaturated fatty acid find in animal cells is oleic acid (18:1). Polyunsaturated fatty acids are numerous and major examples included linoleic acid (18:2) and linolenic acid (18:3).

These two fatty acids contain olefinic group(s) between $\Delta-9$ and the terminal methyl group (ω -CH₃), and since animal cells are not able to desaturate fatty acid between $\Delta-9$ and the ω -CH₃ positions, these acids must be taken into the body through diet and hence are named essential fatty acids. Elongation of aliphatic chains and the desaturation of fatty acids between carboxyl group and $\Delta-9$ can take place in animal cells, and thus polyunsaturated fatty acids such as arachidonic acid (20:4), eicosapentaenoic acid (20:5) and docosahexaenoic acid (22:6) are producible in the body. Most types of PUFA found in animals are non-conjugated (i.e. double bonds are separated by one methylene group). Moreover, almost all naturally-occurring unsaturated fatty acids are in the cis-configuration; trans isomers are seldomly found. The length of most fatty acid chains found in animals is between 16 to 22 carbon chain, although longer (up to 24) and shorter variations (e.g. decanoic acid in milk) are also known (from 4 to 24) [Vance & Jacobson, 1988b]. With the exception of marine organisms, fatty acids with even number of carbons predominate in animal cells. Table 1.1 contains a list of common fatty acids found in animals together with their trivial and systematic names, as well as their shorthand designations. Although fatty acids occur in very large amounts as building block components of complex lipids, only trace amounts exist in non-esterified (free) form in cells and tissues. They are more often associated with alcohol as their "backbone" in order to form complex lipids. The principal lipid backbones found in animals are the trihydric alcohol — glycerol, and sphingosine (4-sphingenine), which yield glycerolipids and sphingolipids respectively.

Table 1.1

Some naturally-occurring fatty acids found in animals together with their trivial, systematic names and their shorthand designations

Systematic name	Trivial name	Shorthand designation	
<i>n</i> -Tetradecanoic acid	Myristic acid	14:0	
<i>n</i> -Hexadecanoic acid	Palmitic acid	16:0	
<i>n</i> -Octadecanoic acid	Stearic acid	18:0	
<i>n</i> -Eicosanoic acid	Arachidic acid	20:0	
<i>n</i> -Docosanoic acid	Behenic acid	22:0	
<i>n</i> -Tetracosanoic acid	Lignoceric acid	24:0	
<i>n</i> -Hexacosanoic acid	Cerotic acid	26:0	
<i>cis</i> -9-Hexadecenoic acid	Palmitoleic acid	16:1 Δ^9	(n-7)
<i>cis</i> -9-Octadecenoic acid	Oleic acid	18:1 Δ^9	(n-9)
<i>cis</i> -11-Octadecenoic acid	Vaccenic acid	18:1 Δ^{11}	(n-7)
All- <i>cis</i> -9,12-Octadecadienoic acid	Linoleic acid	18:2 $\Delta^{9,12}$	(n-6)
All- <i>cis</i> -9,12,15-Octadecatrienoic acid	α -Linolenic acid	18:3 $\Delta^{9,12,15}$	(n-3)
All- <i>cis</i> -5,8,11,14-Eicosatetraenoic acid	Arachidonic acid	20:4 $\Delta^{5,8,11,14}$	(n-6)
All- <i>cis</i> -5,8,11,14,17-Eicosapentaenoic acid	-----	20:5 $\Delta^{5,8,11,14,17}$	(n-3)
All- <i>cis</i> -4,7,10,13,16,19-Docosahexaenoic acid	-----	22:6 $\Delta^{4,7,10,13,16,19}$	(n-3)
<i>cis</i> -15-Tetracosenoic acid	Nervonic acid	24:1 Δ^9	(n-15)

1.2.2 Glycerolipids: Neutral glycerolipids

There are two major types of glycerolipids, namely neutral (uncharged) glycerolipids [Vance & Jacobson, 1988b; Christie, 1987; Myher, 1978; Hitchcock, 1975; Sprecher *et al*, 1965] and phosphoglycerolipids [Kanfer & Hakomori, 1983; Mangold & Paltauf, 1983; Hori & Nozawa, 1982; Ansell *et al*, 1973; Kates, 1972; Carter *et al*, 1969; Kittredge & Roberts, 1969]. The former have esterified fatty acids at all three glycerol moieties, the latter consist of two esterified fatty acids and one phosphodiester group at

the *sn*-3 position. Triacylglycerol is the principal uncharged glycerolipid found in animals. The monoacylglycerol and diacylglycerol are metabolites of triacylglycerol and phospholipids, thus are normally present in very small quantities. However, diacylglycerols have particular importance biosynthetically as precursors of triacylglycerols and complex lipids [Vance & Jacobson, 1988b]. Although triacylglycerols are found in the liver and intestine, they are primarily found in adipose tissue, which function as a storage depot for this lipid. The specialized cell in this tissue is called the adipocyte. The cytoplasm of this cell is full of lipid vacuoles that contain almost exclusively the triacylglycerols which serve as an energy reserve for mammals. At times when the diet or glycogen reserves are insufficient to supply the body's need for energy, the fuel stored as fatty acyl components (i.e. non-esterified fatty acids) of the triacylglycerols is mobilized and transported to other tissues in the body [Vance & Jacobson, 1988b]. A second important function of adipose tissue is insulation of the body from cold.

1.2.3 Glycerolipids: Glycerophospholipids

Glycerophospholipids, by definition, are glycerolipids that contain phosphate diester group [Vance & Jacobson, 1988c]. Phospholipids are amphipathic molecules since they have both polar and non-polar functional groups. The polar head groups prefer an aqueous environment (i.e. hydrophilic) whereas the non-polar acyl substituents are excluded from the aqueous environment (i.e. hydrophobic). It is such amphipathic property causes most phospholipids to arrange spontaneously into a bilayer when suspended in an aqueous environment. This phenomenon of phospholipid bilayers is the major structural feature of most biological membranes. Glycerophospholipids can be further classified according to their phosphate head group substituents. The common head groups found in cells include choline, ethanolamine, serine, inositol, hydrogen, hydroxyl group and phosphatidylglycerol; which respectively form phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidic acid (PA), phosphatidylglycerol (PG) and cardiolipin

(diphosphatidylglycerol) (CAR) [Christie, 1987; Hori & Nozawa, 1982; Ansell *et al*, 1973; Carter *et al*, 1969]. PC, commonly termed lecithin, is a non-acidic lipid and is often the most abundant glycerophospholipid in animal tissues. Saturated fatty acids are always found to occupy the *sn*-1 position while unsaturated fatty acids are mainly attached to the *sn*-2 position. PE is generally the second most abundant class of glycerophospholipid after PC. It is also a non-acidic lipid where fatty acid distributions are similar to PC, although the PUFA content may be higher than its PC counterpart. PS is weakly acidic and it occurs in considerable amounts in brain and erythrocytes but is low in other animal tissues. PI is strongly acidic and its importance in cellular signalling has recently been recognized [May & Calder, 1993; Exton, 1990; Lamah *et al*, 1990; Neer & Clapham, 1988]. PA is another strongly acidic lipid and exists in trace amounts in animals, yet it is extremely important biosynthetically as it is the precursor of all other glycerophospholipids and of triacylglycerols. PG is also an acidic lipid and exists in a limited amount of animal tissues. CAR is a major lipid component of mitochondria, especially in heart muscle [Ioannou & Golding, 1979] Cardiolipin from some animal species contain very high proportion of linoleic acid [Parson, 1988]. The headgroup variety of glycerophospholipids is summarized schematically in table 1.2.

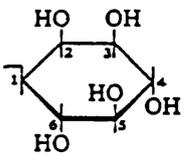
Another distinctive class of phosphoglycerolipid is the lysophospholipids, in which one of the acyl substituents (usually at the *sn*-2 position) is missing. The existence of lysophospholipids in cells is relatively low compared to their phospholipid counterparts; they account for only 1 or 2 % of the total phospholipids [Vance & Jacobson, 1988c].

1.2.4 Glycerophospholipids: Ether lipids

Glycerol ether lipids can be divided into two general types: alkyl-acyl ethers and alk-1-enyl-acyl ethers (or plasmalogens) [Mangold & Paltauf, 1983; Kates, 1972; Snyder, 1969]; the former has an alkyl ether linkage between the fatty acid and the *sn*-1 glycerol moiety, and the later an alkenyl linkage. The structure and diversity of the phospho head groups are identical to its glycerophospholipid counterparts. Plasmalogens contain mainly ethanolamine head group and they are the major ether lipid in animal cells.

Table 1.2

Summary of the structure and distribution of important glycerophospholipids.

Substituent (X)	Phospholipid	Remarks
	$ \begin{array}{c} \text{CH}_2\text{OCOR}^1 \\ \\ \text{R}^2\text{COOCH} \\ \quad \quad \quad \text{O} \\ \text{CH}_2\text{O} \quad \quad \text{P} \quad \quad \text{O} \\ \quad \quad \quad \quad \quad \\ \quad \quad \quad \quad \quad \text{O}^- \end{array} $	General formula
H	Phosphatidic acid (PA)	Negatively charged lipid. Important metabolic intermediate, only occurring in trace amounts.
$-\text{CH}_2\text{CH}(\text{NH}_3^+)\text{COO}^-$	Phosphatidylserine (PS)	Negatively charged lipid. Serine is the L-isomer. Widespread but minor lipid.
$-\text{CH}_2\text{CH}_2\text{NH}_3^+$	Phosphatidylethanolamine (PE)	Widespread and major lipid. N-Acyl derivative found in certain seeds.
$-\text{CH}_2\text{CH}_2\text{NMe}_3^+$	Phosphatidylcholine (PC)	Has a net neutral charge. The major animal phospholipid. Found in large amounts in plants and in small quantities in some bacteria.
$-\text{CH}_2\text{CH}(\text{OH})\text{CH}_2\text{OH}$	Phosphatidylglycerol (PG)	Negatively charged lipid. Head group glycerol has <i>sn</i> -1 configuration. The major phospholipid in photosynthetic tissues and many bacteria. Some bacteria contain O-aminoacyl groups (lysine, ornithine, arginine or alanine) attached to position 3 of the base glycerol. Bisphosphatidic acid, the fully acylated analogue of PG, has been found in some plant tissues.
	Phosphatidylinositol (PI)	Negatively charged lipid. Inositol is the <i>myo</i> -isomer. Widespread and usually minor lipid. The higher inositides, diphosphoinositide (1-(3- <i>sn</i> -phosphatidyl)-L-myoinositol 4-Phosphate) and triphosphoinositide (1-(3- <i>sn</i> -phosphatidyl)-L-myoinositol 4,5-bisphosphate) have only been detected in animals and yeast
Phosphatidylglycerol	Diphosphatidylglycerol (cardiolipin, DPG)	Negatively charged lipid. Common in bacteria. Localized in the inner mitochondrial membrane of eukaryotes.

¹R = long-chain alkyl groups.

²R = long-chain alkyl groups for glycerophospholipids, or hydroxyl groups for lysophospholipids

1.2.5 Sphingolipids

Sphingolipids contain a long-chain, hydroxylated secondary amine bases. Sphingosine being the most abundant long-chain base, and accounts for about 90 % or more of the total sphingolipid bases [Vance & Jacobson, 1988c; Kanfer & Hakomori, 1983; Kates, 1972]. Sphingosine is a toxic material to cells and is therefore present in trace quantities only. The sphingolipid base is acylated on the amine with a fatty acid to give ceramide (figure 1.1), and a head group connected to the primary hydroxyl group in order to form

complex lipids. The major head groups are either phosphocholine to give sphingomyelin or carbohydrate to give the class called glycosphingolipids [Kanfer & Hakomori, 1983; Gurr & James, 1980; Kates, 1972]. The carbohydrates most often associated with glycosphingolipids are galactose and glucose, which in turn form galactocerebroside and glucocerebroside. Brains and the myelin sheath of nerves apparently contains large amounts of galactocerebroside. There is a subdivision of the glycosphingolipids called gangliosides, which contain one or more molecules of sialic acid (*N*-acetylneuraminic acid) as the head group.

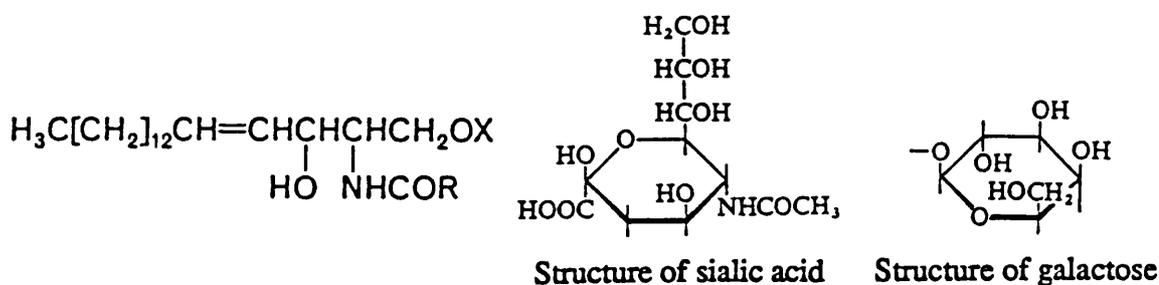


Figure 1.1 General structure of ceramide and cerebroside.

Cerebroside and neutral ceramides. X= H for simple ceramides. X= a monosaccharide (usually galactose) for cerebroside in animals. X= two to six sialic acids for gangliosides. The fatty acids in the R group are frequently 2-hydroxy compounds for the galactocerebrosides.

1.2.6 Steroid and related compounds

Cholesterol and other steroids are derivatives of the tetracyclic hydrocarbon perhydrocyclopentanophenanthrene. The cyclohexane rings of cholesterol and other steroids adopt either the chair or the boat conformation. The chair conformation is more stable and thus is the preferred conformation. Cholesterol is also the precursor for other important steroids in animals: bile acids and steroid hormones. Bile acids are the major degradation products of cholesterol, and act as detergents in the small intestine thereby assist the solubilization of lipids, which become more easily degraded by intestinal lipases. In addition, cholesterol is an important structural component of some eukaryotic membranes, and its presence is essential for maintaining membrane fluidity [Watala & Kordacka, 1987; Watala *et al*, 1987].

1.3 THE POTENT BIOLOGICAL ROLE OF LIPIDS

The importance of lipids in biological systems has been gradually recognized in the past decade. Nowadays, lipids are involved in many significant biochemical signal transduction pathways; the following is the summary of their major potent biological functions.

1.3.1 *Phosphoinositide turnover* [Nozawa *et al*, 1991]

The activation of Ca^{2+} mobilizing receptors on cell membrane surface via various agonists in turn activated phosphoinositide specific phospholipase C (PLC) [Rhee *et al*, 1989] to generate stimulatory second messengers [Firkin & Williams, 1967]. The diesteric degradation of PI was earlier thought to be a primary event [Imai *et al*, 1982a] and its hydrolytic product 1,2-diacylglycerol (DG) is subsequently phosphorylated to phosphatidic acid (PA) followed by resynthesis of phosphatidylinositol (PI). Recently, attention has been focused on the breakdown of polyphosphoinositides and the apparent disappearance of PI is considered to be due to its consumption for the replenishment of the phosphorylated derivatives, PIP and PIP_2 . Hydrolysis of PIP_2 subsequently yielded 1, 4, 5-triphosphate (IP_3) and DG, whereas IP_3 and DG are found to be the two receptor generates phosphoinositide-derived second messengers, which respectively act as Ca^{2+} mobilizer and protein kinase C activator [May & Calder, 1993; Lamah *et al*, 1990; Neer & Clapham, 1988]. Other lipid metabolites involved in the phosphoinositides turnover such as PA are also suggested to have a positive feedback effect to amplify the receptor-mediated PLC activation [Putney *et al*, 1980].

1.3.2 *Arachidonic cascade* [Choi, Gao & Gibbons, 1991; DuBourdieu & Morgan, 1990; Rustenbeck & Sigurd, 1989]

Arachidonic acid and its metabolites has become a major interest in lipid research with regard to their high biological potency, which modulate cellular functions such as platelet aggregation, smooth muscle contraction and neural excitation [Kurachi *et al*, 1989]. *In vivo*, arachidonic acid is preferentially esterified at the C-2 position of glycerophospholipids [Irvine, 1982], but it is not evenly distributed in phospholipids but

predominated in PI and PE [Marcus *et al*, 1969]. Liberation of arachidonic acid from membrane phospholipids can be induced by various agonists (e.g. hormones or growth factors). There are two major pathways proposed for the liberation of arachidonic acid; namely, PLA₂ pathway [Colard *et al*, 1987; Imai *et al*, 1982b; Jesse & Franson, 1979; Bill *et al*, 1977; Pickett *et al*, 1977] and PLC/ DG-lipase pathway [Prescott & Majerus, 1983; Bell *et al*, 1979]. Once released, arachidonic acid is rapidly converted into biologically active metabolites (eicosanoids) by the action of cyclooxygenase and lipoxygenase [Bevan & Wood, 1987; Williams *et al*, 1989; Barbour *et al*, 1989]. Such events have been extensively studied on blood platelets [Nozawa *et al*, 1991]. In human platelets, cyclooxygenase metabolizes arachidonic acid to prostaglandin endoperoxides, PGG₂ and PGH₂, which are further converted mainly to thromboxane A₂ (TXA₂), 12-hydroxy-5,8,10-hepadecatrienoic acid (HHT) and also PGE₂, PGD₂ and PGF₂α. Among them, TXA₂ and prostaglandin endoperoxides induce aggregation and secretion of granule constituents, whereas PGE₂, PGD₂ and PGF₂α inhibit platelet functions. The major products of 12-lipoxygenase are 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid (12-HPTET) and 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE). These particular lipoxygenase products are reported to stimulate neutrophil functions, while 5-lipoxygenase convert arachidonic acid into leukotrienes and lipoxins as the potential mediators of inflammation and hypersensitivity reactions [Ordway *et al*, 1991; Kurachi *et al*, 1989; Piomelli *et al*, 1987]. Arachidonic acid itself is known to activate protein kinase C and PLC, and also to stimulate Ca²⁺ release from intracellular stores [Nozawa *et al*, 1991; Rustenbeck & Lenzen, 1989].

1.3.3 Signalling through phosphatidylcholine (PC) breakdown [Dinh & Kennerly, 1991; Exton, 1990; Martin & Michaelis, 1990; Pacini *et al*, 1990; Pelech & Vance, 1989; Ragab-Thomas *et al*, 1987]

Receptor stimulation in a diversity of cells through a receptor-G-protein mechanism [Bell, 1986] is associated with the hydrolysis of PC by a variety of cellular phospholipases [Exton, 1990]. One aspect of the importance of PC hydrolysis lies in the ability of the formation of DG, which importantly contribute to the cell activation by

modulating the activity of protein kinase C [Kikkawa & Nishizuka, 1986]. In addition, increasing biophysical evidence points to a critical role for DG in the development of membranous intermediate that promote membrane fusion during exocytosis [Siegel *et al*, 1989; Das & Rand, 1984]. The DG can be generated either via the direct breakdown of PC by the action of phospholipase C [Gruchalla *et al*, 1990; Agwu *et al*, 1989; Billah, *et al*, 1989; Liscovitch & Amsterdam, 1989; Cabot *et al*, 1988a; Pai *et al*, 1988; Bocckino *et al*, 1987], or indirectly by the joint action of phospholipase D and phosphatidic acid phosphohydrolase, which formed phosphatidic acid by the former and then converted to DG by the latter enzyme [Cabot *et al*, 1988a; Slivka *et al*, 1988; Takuwa *et al*, 1987; Daniel *et al*, 1986; Mufson *et al*, 1981]. The PC generated DG was distinguished from its PIP₂ generated analogue by its distinctive fatty acid composition [Bocckino *et al*, 1985]. It was also observed that the time course of DG generation via PC breakdown was very different from the phosphoinositide cycle [Charest *et al*, 1985; Exton, 1988]. The prolonged formation of DG from PC may be important in cellular control mechanisms that require long term activation of protein kinase C [Agwu *et al*, 1989; Augert *et al*, 1989; Pessin & Raben, 1989; Uhing, *et al*, 1989; Cabot *et al*, 1988b; Wright *et al*, 1988; Bocckino *et al*, 1985].

Another major aspect of the degradation of PC, is the liberation of arachidonic acid by the action of phospholipase A₂. As described in the previous section, arachidonic acid and its metabolites are cellular second messengers which can trigger various biological events [Bevan & Wood].

1.3.4 Direct or indirect regulation of ion channels by fatty acids (arachidonic acid and others) [Ordway *et al*, 1991; Kim *et al*, 1989; Kurachi *et al*, 1989]

The action of fatty acids on ion channels can be classified as direct or indirect. Indirect effects arise principally from the metabolic conversion of one fatty acid, arachidonic acid, to active oxygenated metabolites, which then affect certain channels [Kim *et al*, 1989; Kurachi *et al*, 1989; Piomelli *et al*, 1987]. It has been demonstrated that agonist-stimulated liberation of arachidonic acid activate K⁺ channels in *Aplysia* neurons [Piomelli *et al*, 1989] and cardiac atrial cells [Kurachi *et al*, 1989].

In contrast, direct effects of fatty acids do not depend on such metabolic conversion, but instead elicit their effect by either interacting with the channel itself (or an accessory protein) directly, or some associated site within the plasma membrane, or by altering the lipid bilayer. Fatty acid-binding sites on a number of proteins have been well defined. The best-studied sites are those of albumin, a water soluble protein that binds structurally diverse fatty acids with K_D values that in many instances are in the micromolar range [Spector, 1975]. It is possible that analogous binding sites exist on ion channel proteins, either within the lipid bilayer or in the aqueous environment. Fatty acids might act by altering the interaction of channels with the lipid bilayer. It has been known that the composition of the lipid bilayer can dramatically affect protein behaviour. For example, negatively charged phospholipid head groups enhance the activity of purified glucose transporter [Carruthers & Melchior, 1988]. Furthermore, some membrane proteins appeared to be associated preferentially with certain lipids [Marsh & Watts, 1988]. Such findings suggest that specific chemical interactions with membrane lipids are important for protein function. Another possible mechanism of fatty acid action, in addition to the modification of specific protein-lipid interactions, is the alteration of bulk membrane fluidity. As it has been proposed that the fatty acid regulation of squid giant axon Na^+ channels [Takenaka *et al*, 1988; Takenaka *et al*, 1987] and aortic smooth muscle K^+ channels [Bregestovski *et al*, 1989] are both mediated by the effect of fatty acids on membrane fluidity.

Apart from their direct effects on purified proteins and ion channels, fatty acids have been found to regulate a variety of cell processes including synaptic transmission [Williams *et al*, 1989; Madden & van der Kloot, 1985], neurotransmitter uptake [Barbour *et al*, 1989] and suppression of Na^+ and Ca^{2+} currents [Linden & Routtenburg, 1989] via mechanisms that do not appear to involve any active oxygenated metabolites. Recently, fatty acids were found to block [Hwang *et al*, 1990] or inactivate [Anderson & Welsh, 1990] the Cl^- channels implicated in the pathogenesis of cystic fibrosis.

In addition, many of the vitamins and hormones found in animals are lipids or derivatives of lipids. Lipids might also be important modulators under pathological

conditions, whereas some diseases are related to lipid metabolism disorders, such as Niemann-Pick disease [Lehninger, 1981], high phosphatidylcholine hemolytic anemia (HPCHA) [Shohet *et al*, 1971], hepatocellular liver disease [Cooper *et al*, 1974] and cardiac diseases [Talmud & Humphries, 1991; Kim & Clapham, 1989; Naccache *et al*, 1985; Chin *et al*, 1984] etc. In diabetes [Watala *et al*, 1987; Frazee *et al*, 1985; Synder, 1985; Weisweiler *et al*, 1985; Connor, 1979] and tissue ischemia [Katz & Messineo, 1981; Bazan, 1970], which abnormal fatty acids level were observed in the circulation.

1.4 A BRIEF INTRODUCTION TO THE BASIC PRINCIPLES OF NMR SPECTROSCOPY

1.4.1 Basic theory of NMR

By the continuously spinning of nuclei with an angular momentum, certain isotopes which exhibit a paramagnetic spin property (non-even spin number) can give rise to an associated magnetic field. If one of such nucleus is subjected to a very powerful external magnetic field and being perturbed with a pulse of radio-frequency energy, the nucleus will resonate between different quantised energy levels at specific frequencies, absorbing some of the applied energy. The change of energy of the system as it relaxes back towards equilibrium after the radio-frequency pulse, although very small, can' be detected, amplified and displayed in a spectrum format. The trace obtained, of the variations in the intensity of the resonance signal with increasing applied magnetic field, is the NMR spectrum [Sander & Hunter, 1990].

Depending on the locations, resonance signals from the same nuclear species can be varied within one molecule, due to the slight differentiation of chemical-magnetic environment (shielding) that each chemical group is subjected to. In the other words, NMR resonance signals are structural dependent. Such phenomenon is defined as chemical shift. It is on this basis that NMR can be applied for molecular structural analysis. There are two established NMR spectroscopy — firstly is the continuous wave (CW) NMR and later the Fourier transform (FT) NMR. The former processes each experiment by sweeping the implied range of radio-frequency continuously in order to excite the nuclei individually, while in the latter experiment all nuclei are excited

simultaneously by the application of an intensive energy pulse. FT NMR surpasses its CW predecessor by enormously reducing the requirement of experimental time, which consequently leads to higher sensitivity and other technological breakthroughs such as multi-dimensional NMR experiments.

More specifically, in FT NMR, following the perturbation (radio-frequency) pulse, the subsequent time domain relaxation response, namely free induction decay (FID), is converted into digital form and stored as data points in a computer for analysis. In order to display the spectrum in a frequency manner, the time domain FID is converted to the frequency domain spectrum via a Fourier transformation. Like all the other spectroscopic techniques the resolution of NMR spectrum is affected by the signal-to-noise ratio. If the signal-to-noise ratio in the data is low, it can be improved by the accumulation of FIDs. The final signal-to-noise ratio is proportional to the square root of the number of FIDs which are co-added. There are limitations to the dynamic range of signals which can be successfully averaged.

The sensitivity of NMR can be reflected from the Boltzmann equation of energies distribution:

$$N_{\beta} / N_{\alpha} = \exp (-h\nu / kT)$$

where N_{α} is the population of the nuclear at lower energy state

N_{β} is the population of the upper energy state.

For NMR, with frequencies ν in the range of radiofrequency (RF), $h\nu \sim 10^{-2}$ cal (4.2×10^{-2} Joules) and therefore the excess population of spins in the lower state is only *ca.* 1 in 10^5 . This is the basic reason for the low sensitivity of NMR as compared to with infrared (IR) and especially ultraviolet (UV) spectroscopy. Nevertheless, there is a valuable compensation, as the coefficient of absorption is a constant for any nucleus, and thus the NMR signal obtained is directly proportional to the number of nuclei producing it. This particular feature allows the spectral quantitation to take place and it is also useful

in interpreting NMR spectra.

1.4.2 Two dimensional NMR

One of the most exciting advances in recent NMR technology is the introduction of two-dimensional (and multi-dimensional) experiment [Freeman & Morris, 1979]. A 2D spectrum is a map containing two frequency axes. One axis, F_2 , contain conventional chemical shifts. The other axis, F_1 , may contain shift or coupling information, or both. The position of a signal on the map reveal both the chemical shift of that signal, and another piece of information which depends on the nature of F_1 . Every 2D experiment can be described as a preparation-evolution-mixing-detection sequence. The magnetization that is experimentally detected by collecting FIDs appears along F_2 , while F_1 reports on the behaviour of the spins during the evolution period, labelled as τ . A picture of the evolution period is indirectly built up by acquiring a series of spectra with increasing evolution times. All 2D experiments have a 1D equivalent using a single value for the evolution time.

Below are descriptions of the 2D experiments used in this monograph:

1) Homonuclear correlation spectroscopy (COSY and COSY-45°)

The COSY-type experiments [Bax & Freeman, 1981] consisted of two identical ($SW_1 = SW_2$) chemical shift axes, F_1 and F_2 . F_2 was derived from the FIDs, which the total number was defined as NE by the computer. Each consecutive FID was different from the previous one by having D_0 increased by IN ($IN = 0.5 / SW_1$). After Fourier transformation, the first point of each F_2 spectrum, in the order of acquisition [$D_0 = 0.003$ ms (effectively zero) to $D_0 = (NE \times IN) + 0.003$ ms] became the first interferogram of F_1 . Thus the number of interferograms was the number of data points in F_2 ($TD_2 = SI_2$). Commonly the interferograms were zero-filled once ($SI_1 = 2TD_1$) to improve F_1 resolution without having to acquire too many increments. For maximum sensitivity, a 90° detection pulse was used. When peaks on the diagonal, which are normally the most intense, interfered with cross peaks closely (due to the

small difference in δ between connected spins), a 45° detection pulse was used, principally to reduce the intensity of the diagonal signal. The diagrammatic display of the pulse sequence is shown as follow:

D1(HG,S1) → D3,S2 → 90° → D0 → D0,D3 → 90° or 45° → AQ
 Relaxation presaturation Minimum decoupler presaturation pulse Evolution delay Decoupler gate off Detection pulse FID

2) Double and triple quantum filtered COSY (COSYDQF and COSYTQF)

This sequence is similar to that of COSY until after the evolution delay. All single quantum transitions (including solvent) were filtered out from the detection plane by a combination of multiple quantum coherence (MQC) selection pulses [Shaka & Freeman, 1983; Piantini *et al*, 1982] and phase cycling of the transmitter and detector. Two consecutive MQC pulses (separation by a short delay, D3 of 0.003 ms, for phase switching between the 2 pulses) selected for only those vectors with non-single (i.e. double, triple etc.) transitions. These vectors had modulated according to J (coupling constant) during the fixed delay D2 and therefore had remaining magnetization in the x-y plane. D2 was typically 0.05 - 0.35 s, for a range of J ($D2 = 0.25/J$). Selection for double or higher order quanta depended on the particular phase cycling of the 3 pulses and detection. The minimum phase cycle for DQF and MQF were 8 and 12 while the maximum are 16 and 48 respectively. These experiments required at least twice as many transients as COSY for the same S/N, all other parameters being the same. Longer D1 times were required for T1 relaxation. The diagrammatic display of the pulse sequence is shown as follow:

D1 → 90° → D0 → D2 → 90° → D3 → 90° → D2 → AQ
 Relaxation Preparation Evolution delay Fixed delay MQC pulse Phase switch Selection pulse Fixed delay FID

3) Homonuclear Hartmann-Hahn COSY (HOHAHA)

The HOHAHA employed a spin-lock field based on the MLEV17 [Bax & Davis,

1985] composite pulse decoupling cycle. MLEV17 was repeated a total of x times such that $66x$ (90° pulse length) = $0.1 / J$ for direct connectivities with coupling constant J or larger; x was preferably an even number. Indirect connectivities could also be obtained by prolonging the MLEV17 sequence, until total correlation was observed. The spin-lock required rapid consecutive pulsing which was beyond the capability of some earlier designed transmitter, but which was within the duty cycle of the decoupler channel of an instrument suitably equipped. Therefore this experiment was done in "reverse mode" with excitation and detection through a broadband decoupler. Two trim pulses of duration $n\pi \cong 2.5$ ms ($n = \text{integer}$) were applied before and after the MLEV17 sequences to defocus magnetization not parallel to the x - axis. Within MLEV17, the last pulse served to correct imperfections during MLEV16. All other parameters were as for COSY. 90° pulse widths were redetermined because the decoupler channel had different characteristic to the ^1H -transmitter channel. The diagrammatic display of the pulse sequence is shown as follow:

D1(D0,S1)	\rightarrow	90°	\rightarrow	D0	\rightarrow	$\pi.n$	\rightarrow	$(\pi/2-\pi-\pi/2)_{16}$	\rightarrow	π	\rightarrow	$n\pi$	\rightarrow	AQ
Relaxation decoupler set		Preparation pulse		Evolution		Trim pulse		Spin-lock cycle		Correcting pulse		Trim pulse		FID

4) Heteronuclear correlation spectroscopy (HETCOR)

In the heteronuclear shift correlation spectrum [Benn & Gunther, 1983], a low- γ heteronucleus is observed experimentally, and appears along F_2 . The other dimension contains the chemical shifts of the protons to which those spins are coupled. The polarization transfer that connects the spins depends on the size of J -coupling between them, so the experiments can be "tuned" to show connections between directly attached pairs, or to reveal longer range coupling. These spectra can be used to assign the X-spin spectrum, knowing the ^1H assignments, or vice versa, to spread out an otherwise overlapped ^1H spectrum, or to detect the ^1H spectrum indirectly. In this monograph, HETCOR was used to assigned and confirmed ^{13}C lipid resonances with the references of previously assigned ^1H spectra.

The abbreviations for NMR experiments used in this section are listed and described in table 1.3.

Table 1.3
Abbreviations for 2D NMR experiments

Abbreviation	Definition
δ	Unit of chemical shift
D0	Incremental delay
D1, D2, D3	Relaxation delay time, other preset delay time
F ₁	Vertical (second) axis in 2D spectrum
F ₂	Horizontal (first) axis in 2D spectrum
FW	Filter width in Hertz
HG	Homonuclear continuous wave decoupler
IN	0.5 / SW1
<i>J</i>	Spin-spin coupling constant
NS	Number of scan
NE	Number of experiment
S1, S2, S3	Preset decoupler power level
SI, SI1, SI2	Size of data file in 1D, F ₁ and F ₂ spectra
S/N	Signal-to-noise ratio
SW1	Spectral width of F ₁ in Hertz
SW2	Spectral width of F ₂ in Hertz
T ₁	Spin-lattice relaxation time
TD	Number of real data points

Chapter 2

Experimental

2.1 MATERIALS

2.1.1 Materials for $^1\text{H-NMR}$ analysis on rat liver, brain, heart and kidney, human erythrocytes and blood plasma

Phosphatidylcholine (PC), lysophosphatidylcholine (LPC), phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LPE), phosphatidyl-*N*-mono-methylethanolmine (PME), phosphatidyl-*N*-di-methylethanolmine (PDE), phosphatidylglycerol (PG), phosphatidic acid (PA), phosphatidylserine (PS), cardiolipin (CAR), sphingomyelin (SPH), 1-monoglycerol (MG), cholesterol (C), cholesterol ester (CE), palmitic acid, oleic acid, linoleic acid ethyl ester, linolenic acid ethyl ester, arachidonic acid ethyl ester, eicosapentaenoic acid ethyl ester, docosahexaenoic acid ethyl ester and 1,2 di-*O*-hexadecyl-3-palmitoylglycerol were purchased from Sigma Ltd. Chloroform (99.8%) and fully deuterated methanol (99.8%) were purchased from Aldrich Co. All the other solvents, fatty acids and standard components were of the highest grade available. Male Wister strain rats, weighing between 150 to 200 g were used in these studies. All animals were fed by standard diet. Human blood were obtained from healthy volunteers at the City Hospital of Cagliari, Sardegna, Italy.

2.1.2 Materials for Bond Elut (NH_2 aminopropyl) ion-exchange chromatography

Bond Elut (NH_2 aminopropyl) solid phase extraction columns were purchased from Analytichem International. Deuterated chloroform (99.8%) and fully deuterated methanol (99.8%) were purchased from Aldrich Co., HPLC grade hexane from May and Baker Co. and ammonium acetate from Pharmacos Ltd. All other solvents, including methanol, chloroform, diethyl ether and glacial acetic acid are reagent grade and were purchased from BDH Ltd.

2.1.3 Materials for $^{13}\text{C-NMR}$ analysis on rat liver, human erythrocytes and blood plasma

Deuterated chloroform (99.8%) and fully deuterated methanol (99.8%) were purchased from Aldrich Ltd. All lipid standards used for spectroscopic references were of highest purity available and were purchased from Sigma Ltd. Chloroform, methanol,

all other solvents for lipid extractions and standard components were purchased from BDH Ltd. and of the highest grade possible. Male Wister strain rats, weighing between 150 to 200 g were used in the study. Human blood were obtained from healthy volunteers between 50 and 60 years old at the City Hospital of Cagliari, Sardegna, Italy.

2.1.4 *Materials for the study of carbon tetrachloride induced hepatotoxicity in rat liver*

Deuterated chloroform (CDCl_3 , 99.80%) and fully deuterated methanol (99.80%) were purchased from Aldrich Ltd. Chloroform, methanol carbon tetrachloride, all other solvents and standard components were of the highest possible grade. Male Sprague-Dawley rats (Charles River) weighing 200-220 g were used.

2.2 TISSUES EXTRACTION AND SAMPLE PREPARATIONS

2.2.1 *Rat liver: Tissue extraction and sample preparation for $^1\text{H-NMR}$*

Rat liver was removed from rats following cervical dislocation and immediately frozen in liquid nitrogen. 0.5 g of frozen tissue was weighed while the temperature was maintained below 0°C . The lipids were extracted according to the method developed by Folch *et al* (1957). Liver tissue was homogenized by a Polytron[®] (type 2TA 10-35) 50 Hz homogenizer (manufactured by Kinematica, Switzerland) at sub-zero ($^\circ\text{C}$) temperature with 15.0 ml of chloroform-methanol mixture (2:1, v/v), then centrifuged at 800 rpm for 5 min., and the organic solvent phase was filtered through glass-wool. The lipids were re-extracted twice using, in turn, 10.0 and 5.0 ml of chloroform-methanol mixture (2:1, v/v) and all solvent extracts were combined together after filtration.

The extracted lipids were washed with 5.0 ml of 0.5 M potassium chloride in 50.0 % methanol, then the chloroform-methanol phase was separated from aqueous phase via centrifugation at 800 rpm for 5 min. The washing procedure was performed twice and finally the retained organic phase was evaporated under a stream of nitrogen. The residue was resuspended in 0.80 ml of $\text{CD}_3\text{OD-CDCl}_3$ (2:1) and transferred to 5 mm NMR tubes for $^1\text{H-NMR}$ experiments. Lipid extract of dried weight *ca.* 50.0 mg was used for

all experiments. Samples were bubbled with nitrogen prior to experiments in order to remove oxygen.

2.2.2 Rat brain: Tissue extraction and sample preparation for $^1\text{H-NMR}$

Rat brains were removed from Male Wister strain rats following cervical dislocation and immediately frozen in liquid nitrogen. A 0.50 g of frozen tissue was used for each lipid extraction. The samples were maintained at sub-zero temperature ($^{\circ}\text{C}$) throughout the homogenization and lipid extractions processes. The brain tissue was homogenized by a Polytron[®] (type 2TA 10-35) 50 Hz homogenizer (manufactured by Kinematica, Switzerland) in 15.0 ml of chloroform-methanol (2:1 v/v). After homogenization the brain extract was centrifuged at 800 rpm for 5.0 min. to separate the organic solvent phase from the tissue pellet, subsequently the solvent extract was collected and filtered through glass wool. Lipids were re-extracted twice from the remaining pellet using, in turn, 10.0 ml and 5.0 ml of chloroform-methanol (2:1 v/v) and all solvent extracts were combined together after filtration.

The extracted lipids were washed twice with 0.50 M potassium chloride in 50.0 % methanol and the lipid extract was evaporated under a stream of nitrogen. By this particular extraction procedure, 100.0 mg of brain tissue yield *ca.* 14.0 mg of dried lipids, as 70.0 mg of dried lipids was used for the 1D and 2D proton NMR experiments. The residue was resuspended in 0.80 ml of $\text{CD}_3\text{OD-CDCl}_3$ (2:1 v/v) and transferred to 5 mm NMR tubes for $^1\text{H-NMR}$ experiments. Samples were bubbled with nitrogen prior to experiments in order to remove oxygen.

2.2.3 Human erythrocytes and blood plasma: Tissue extraction and sample preparation for $^1\text{H-NMR}$

Blood from healthy adult volunteers was taken in the morning before breakfast and immediately anticoagulated with 0.50 % sodium citrate. Red blood cells were separated from plasma by centrifugation (800 g, 10 min., 20°C) and washed three times with 0.90 % sodium chloride. Isolated erythrocytes and plasma were subjected to lipid extraction according to the method by Folch *et al* (1957). For every 2.0 ml of erythrocytes or

plasma, 20.0 ml of chloroform-methanol (2:1 v/v) were added and shaken vigorously for 2 min. The lipid extract was then centrifuged (800 g, 10 min., 20°C) in order to separate the organic solvent extract from protein residues. The lipids were re-extracted twice from the residues using 10.0 ml of chloroform-methanol (2:1 v/v) and the organic solvent extract were filtered and combined together. The combined organic solvent extract was washed twice with 0.10 M potassium chloride and finally the organic solvent was evaporated under a stream of nitrogen. The dried lipid extract residues, at dried weight *ca.* 50.0 mg were resuspended in 0.80 ml of CD₃OD-CDCl₃ (2:1 v/v) and transferred to 5.0 mm NMR tubes. Samples were bubbled with nitrogen prior to spectroscopic experiments.

2.2.4 Tissue extraction and Bond Elut (NH₂ aminopropyl) ion-exchange chromatographic separation for rat liver

Procedures for liver lipid extraction were as described previously in section 2.2.1 except that 1.0 g of tissue was used instead of 0.5 g. The lipid extract was evaporated under a stream of nitrogen prior to Bond Elut fractionation.

Bond Elut separations were done in triplicate. All column elutions were achieved in five minutes under low speed centrifugation conditions (800 rpm) and the columns were washed with 8.0 ml of HPLC grade dry hexane prior to application of the lipid extracts. The extracted lipid residue was redissolved in 1.0 ml of chloroform. 0.20 ml of dissolved lipids was then applied to each Bond Elut column and another 0.20 ml was retained as control, for the determination of recovery after separations.

According to their different polarity, lipids were separated into four individual classes [Egberts & Buiskool, 1988; Kates *et al*, 1988] by passing different eluants through the columns in the following order: (1) chloroform [eluted non-polar lipids and cholesterol] (2) diethyl ether with 2.0 % acetic acid [eluted free fatty acids] (3) methanol [eluted nonacidic phospholipids] (4) 0.05M ammonium acetate in chloroform-methanol (4:1 v/v) plus 2.0 % (v/v) of 28.0 % aqueous ammonium solution [eluted acidic phospholipids]. A volume of 2 x 4.0 ml eluants was used for all elutions.

The 0.20 ml unseparated lipid extract and all eluates were evaporated under a stream of nitrogen. The residues were then resuspended in 0.6 ml of CD₃OD-CDCl₃ (2:1) and transferred to 5 mm NMR tubes. Samples were bubbled with nitrogen prior to recording the spectrum.

2.2.5 Tissue extraction and Bond Elut (NH₂ aminopropyl) ion-exchange chromatographic separation for rat brain, heart and kidney.

Rat brains, hearts and kidneys were removed from Male Wister strain rats following cervical dislocation and immediately frozen in liquid nitrogen. Procedures for rat brain lipid extraction was identical as in section 2.2.2, excepted 1.0 g of wet weight brain tissue was used instead of 0.5 g. The lipid extract was evaporated under a stream of nitrogen prior to Bond Elut fractionation.

The heart and kidney lipid extraction were performed according to the method developed by Folch *et al* (1957). 1.0 g of tissue was used for each lipid extraction. The samples were maintained at sub-zero temperature (in °C) throughout the homogenization and lipid extractions processes. Rat heart and kidney were both homogenized by a Polytron® (type 2TA 10-35) 50 Hz homogenizer (manufactured by Kinematica, Switzerland) in 15.0 ml of chloroform-methanol (2:1 v/v). After homogenization the lipid extract was centrifuged at 800 rpm for 5.0 min. to separated the organic solvent phase form the tissue pellet, subsequently the solvent extract was collected and filtered through glass wool. Lipids were re-extracted twice from the remaining pellet using, in turn, 10.0 ml and 5.0 ml of chloroform-methanol (2:1 v/v) and all solvent extracts were combined together after filtration. Finally the extracted lipids were washed twice with 0.50 M potassium chloride in 50.0 % methanol and the lipid extract was evaporated under a stream of nitrogen prior to Bond Elut fractionation.

All Bond Elut column separations were performed in triplicate; elutions were facilitated by low-speed centrifugation (500 rpm) for 5 minutes. Columns were washed with 4.0 ml of HPLC grade hexane prior to application of the lipid extracts. All lipid extracts were redissolved in 1.00 ml of chloroform, 0.20 ml of the dissolved lipids

applied to each Bond Elut column and another 0.20 ml retained as control for the determination of recovery after separations.

According to the polarity differences, lipids were separated into five individual fractions [Choi *et al*, 1993; Egberts & Buiskool, 1988; Kates *et al*, 1988] by passing different eluants through the columns in the following order: 1) chloroform [eluted non-polar lipids and cholesterol], 2) diethyl ether with 2.0 % acetic acid [eluted non-esterified fatty acids], 3) methanol [eluted non-acidic phospholipids], 4) glacial acetic acid [eluted phosphatidylserine] and 5) mixture of 0.05 M ammonium acetate in chloroform/methanol (4:1, v/v) and 2.0 % (v/v) of 28.0 % aqueous ammonium solution. After step 4 methanol was applied to the column for neutralizing any remained acetic acid, prior to proceeding to step 5. A volume of 2×4.0 ml of eluants was used for all elution. Due to the low abundance of phosphatidylserine, and for the convenience of lipid classification, step 4 (glacial acetic acid) was omitted in the heart and kidney lipid extract separations. Thus phosphatidylserine was eluted with other acidic-phospholipids in step 5.

The 0.20 ml unseparated lipid extract and all eluates were evaporated under a stream of nitrogen. The residues were then resuspended in 0.6 ml of $\text{CD}_3\text{OD}-\text{CDCl}_3$ (2:1, v/v) and transferred to 5 mm NMR tubes. Samples were bubbled with nitrogen prior experiment.

2.2.6 Rat liver: Tissue extraction and sample preparation for ^{13}C -NMR

The rat liver was removed following cervical dislocation and immediately frozen in liquid nitrogen. Lipid extraction were performed as section 2.2.1, according to the method developed by Folch *et al* (1957). After extraction, the lipids were lyophilised and 50.0 mg was used for each NMR experiment. Samples were dissolved in a 1.0 ml mixture of deuterated chloroform and methanol ($\text{CDCl}_3-\text{CD}_3\text{OD}$) with the volume ratio 1: 2 (v/v). NMR sample tubes with 5 mm diameter were used and samples were bubbled with nitrogen prior to running.

2.2.7 Human erythrocytes and blood plasma: Tissue extraction and sample preparation for ^{13}C -NMR

Human blood samples were collected in the morning before taking breakfast. Blood was being withdrawn via vein acupuncture and immediately anticoagulated with 0.5 % sodium citrate. Red blood cells were then isolated from plasma (800 rpm, 10 min., 20°C) and washed three times with 0.9 % sodium chloride. Separated erythrocytes and plasma were subjected to lipid extraction according to the method developed by Folch *et al* (1957). 20 ml of chloroform-methanol (2:1 v/v) were added to 2.0 ml of erythrocyte or 20.0 ml of plasma, shaken vigorously for 2 minutes, then centrifuged. The organic layers were collected and washed twice with 0.10 M potassium chloride. Finally the solvent was evaporated under a stream of nitrogen and the lipid extracts were then subjected to lyophilization. 40.0 mg of the lipid extracts were redissolved in 2.0 ml of deuterated chloroform-methanol ($\text{CDCl}_3\text{-CD}_3\text{OD}$) with the volume ratio of 1:1 (v/v). NMR sample tubes with 10 mm diameter were used and samples were bubbled with nitrogen prior to NMR experiments.

2.2.8 Lipid extraction, separations and sample preparation via ion-exchange column chromatography

Livers were removed from the rats following cervical dislocation and immediately frozen into liquid nitrogen. 0.5g of frozen liver was used for each lipid extraction. Lipid extraction were performed as described in section 2.2.1, according to the method developed by Folch *et al* (1957). Lipid extracts were separated into three classes, according to their polarities, by simple ion-exchange column chromatographic procedures as described in section 2.2.5.

Sample were dissolved in a 0.8 ml mixture of deuterated chloroform and methanol with volume ratio of 1:2. 5 mm NMR sample tubes were used and samples were bubbled with nitrogen prior to experiment.

2.3 ANIMAL TREATMENT FOR THE STUDY OF CARBON TETRACHLORIDE INDUCED HEPATOTOXICITY IN RAT LIVER

Male Sprague-Dawley rats (Charles River) weighing 200-220g were fed by standard diet (Ditta Piccioni, Brescia, Italy) with water ad libitum. Feeding was stopped 12 hours prior the administration of carbon tetrachloride (CCl₄). CCl₄ (26 mmol per kg body wt.) was dissolved in 2.0 ml of mineral oil then applied by stomach tube 24 hour before the animals were sacrificed. In control experiments animals received an equivalent amount of mineral oil.

2.4 ONE DIMENSIONAL NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

2.4.1 1D proton NMR spectroscopy for rat liver, brain, heart, kidney, human erythrocytes & blood plasma and Bond Elut lipid fractions

Spectra were recorded using Burker AM500 spectrometer equipped with an ASPECT 3000 array processor, and Varian Unity XR-300 spectrometer connected to a SUN workstation. The 1D proton spectra were recorded at 30°C in the Fourier transform (FT) mode with 32K data points, using a 45° detection pulse and 2.0 s acquisition time, with the relaxation delay (D1) of 6.0 s. The residual CD₃OD resonance at 3.30 ppm was used as the reference chemical shift.

2.4.2 1D Carbon-13 NMR spectroscopy for rat liver

The 1D spectra were recorded at 30.0°C at 150.869 MHz on a Varian Unity 600 spectrometer. In 1D experiments, a pulse of 90°, 2.0 seconds of acquisition time and 32 K data point were used. The CDCl₃ resonance signal at 77.0 ppm was used as reference for ¹³C spectra. The FIDs were processed via a SUN Sparc computer workstation, equipped with VnmrX software (version 4.1).

2.4.3 1D Carbon-13 NMR spectroscopy for human erythrocytes and blood plasma

All 1D experiments were performed by using Varian Unity XR-300 NMR spectrometer at 75.435 MHz. Spectra were recorded at 30°C with 32K data points, 90°

pulse and 6.0 s acquisition time. The CDCl_3 resonance signal at 77.0 ppm was used as reference for ^{13}C spectra. The FIDs were processed via a SUN Sparc computer workstation, equipped with VnmrX software (version 4.1).

2.4.4 1D proton NMR spectroscopy for the study of carbon tetrachloride induced hepatotoxicity in rat liver

Spectra were recorded using Bruker AM 500 NMR spectrometer. All spectra were recorded at 30°C in the Fourier Transform (FT) mode with 32K data points, using a 45° pulse and 2.0s of acquisition time. Chemical shifts were referred to the residual CD_3OD resonance at 3.30 ppm. All lipid and fatty acid quantitations were achieved by measuring the diagnostic integrated areas and normalized by the integrated area of the CDCl_3 resonance at 7.69 ppm.

TWO DIMENSIONAL NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

2.5.1 2D HOHAHA proton NMR spectroscopy for rat liver, brain, heart, kidney

Spectra were recorded using Bruker AM500 spectrometer, equipped with an ASPECT 3000 array processor. The 2D homonuclear Hartmann-Hann experiment (HOHAHA) was performed at 30°C, using the MLEV-17 spin-locking sequence [Bax & Davis, 1985]. The trim pulses were of 2.50 ms duration each, giving a total mixing time of 54 ms. The 2D spectra consisted of 2K data points, obtained from 512 FIDs of 64 scans each, with zero-filling in the F_1 dimension. The data were multiplied with a square sine-bell function in both dimensions prior to transformation. The residual CD_3OD resonance at 3.30 ppm was used as the reference chemical shift.

2.5.2 2D COSY proton NMR spectroscopy for human erythrocytes and blood plasma

Spectra were recorded using Bruker AM500 spectrometer equipped with an ASPECT 3000 array processor. The 2D COSY experiment was performed at 30°C by using standard pulse sequence, with a relaxation delay (D1) of 4.0 s and recorded in 2K data points, obtained from 400 FID's of 32 scans each, with zero-filling in the F_1 dimension.

The data were multiplied with a square sine-bell function in both dimensions prior to transformation. The residual CD₃OD resonance at 3.30 ppm was used as the reference chemical shift.

2.5.3 2D COSY proton NMR spectroscopy for rat liver lipid extract after Bond Elut chromatography

Spectra were recorded using a Bruker AM500 NMR spectrometer equipped with an ASPECT 3000 array processor. The 2-D homonuclear shift-correlated experiment (COSYHG) was performed at 30°C with the following pulse sequences: (1) high powered D1 45° pulse with relaxation delay of six seconds (2) low powered 90° pulse (3) evolution (4) 90° pulse (5) detection. The decoupler was on during D1 in order to presaturate H₂O signal. The 2D spectrum consisted of 2K data points, obtained from 512 FIDs of 64 scans each, with zero-filling in the F₁ dimension. The data were multiplied with a square sine-bell function in both dimensions prior to transformation. The residual CD₃OD resonance at 3.30 ppm was used as the reference chemical shift.

2.5.4 2D HETCOR NMR spectroscopy for rat liver

All 2D HETCOR spectra were recorded at 30.0°C at 150.869 MHz on a Varian Unity 600 spectrometer. The experiments were performed using pre-programmed sequences from Varian, with 2K data points, obtained from 512 FID's of 32 scan each. The CDCl₃ resonance signal at 77.0 ppm was used as reference for ¹³C spectra and for ¹H spectra the CD₃OD resonance at 3.30 ppm was used. All data were processed via a SUN Sparc computer workstation, equipped with VnmrX software (version 4.1).

2.5.5 2D HETCOR NMR spectroscopy for human erythrocytes and blood plasma

HETCOR experiments were performed on a Varian Unity XR-300 NMR spectrometer at 75.435 MHz. Spectra were recorded at 30°C using pre-programmed HETCOR sequences from Varian, with 2K data points, obtained from 512 FID's of 64 scan each. The CDCl₃ resonance signal at 77.0 ppm was used as reference for ¹³C spectra and for ¹H spectra the CD₃OD resonance at 3.30 ppm was used. The data were

processed via a SUN Sparc computer workstation, equipped with VnmrX software (version 4.1).

Chapter 3
1D and 2D proton NMR of lipids from rat liver

3.1 INTRODUCTION

Lipid assays are essential for the determination of lipid levels in membranes, cells, tissues and body fluids. This knowledge allows the comparison of lipids under normal and pathological conditions, detection of unusual lipids or metabolites and the study of control and regulation. Enzyme assays normally permit the study of the level of substrates or the products exclusively for one particular enzyme [Gibbons *et al*, 1989], but the use of the thin layer chromatography (TLC) [Touchstone, 1973], high performance liquid chromatography (HPLC) [Christie, 1985] or ion-exchange chromatography [Bandi & Ansara, 1985] has permitted the simultaneous elucidation of the relative concentrations of several lipids in a given sample [Gurr & James, 1989; Christie, 1987a; Christie, 1987b]. Despite the usually tedious and time-consuming assay procedures, the techniques mentioned above are the limited methods of choice for routine lipid assays.

One and two dimensional NMR, in principle, can achieve a comprehensive analysis in a rapid and non-destructive manner, and yield the levels of all lipids present in a given sample. For instance, one-dimensional phosphorus NMR spectra has been demonstrated to yield the relative levels of phosphorus-containing lipids [Meneses & Glonek, 1988; Sotiros *et al*, 1986]. Assignments of proton nuclear magnetic resonance spectra of lipids belonging to several classes have recently appeared [May *et al*, 1986; Yu *et al*, 1986; Toshiyuki *et al*, 1984; Jardetski & Roberts, 1981; Sawan *et al*, 1979; Drabkowski *et al*, 1980; Llida *et al*, 1977; Mahadevan *et al*, 1968]. Several groups have demonstrated the feasibility of proton NMR for the analysis of complex mixtures of synthetic lipids [Sparling, 1990; Sparling *et al*, 1989].

In this report, isolated lipids from rat liver obtained by the combination of classical freeze-clamp procedures and Folch extraction [Folch *et al*, 1957] were subjected to one- and two dimensional proton NMR spectroscopy to generate "NMR lipid profiles" of tissue. The relative concentrations of *ca.* 40 lipids were simultaneously determined by 1D and 2D NMR and compared with corresponding levels obtained by chromatographic methods. Significantly, the relative quantitations of cholesterol, glycerophospholipids,

ether phospholipids, sphingolipids and glycerides were shown to be in agreement with previous chromatographic determinations. In addition, considerable information on the nature of the fatty acid chains on intact lipids was also gleaned via the $^1\text{H-NMR}$ approach without resort to hydrolysis. Although this $^1\text{H-NMR}$ approach to lipid assays not reached its optimum and still has several reservations, its rapidity and the comprehensive profiles provide a tempting alternative for the studies of structures and the roles of lipids in biology and medicines [Gibbons *et al*, 1989; Gurr & James, 1989; Bax & Davies, 1985].

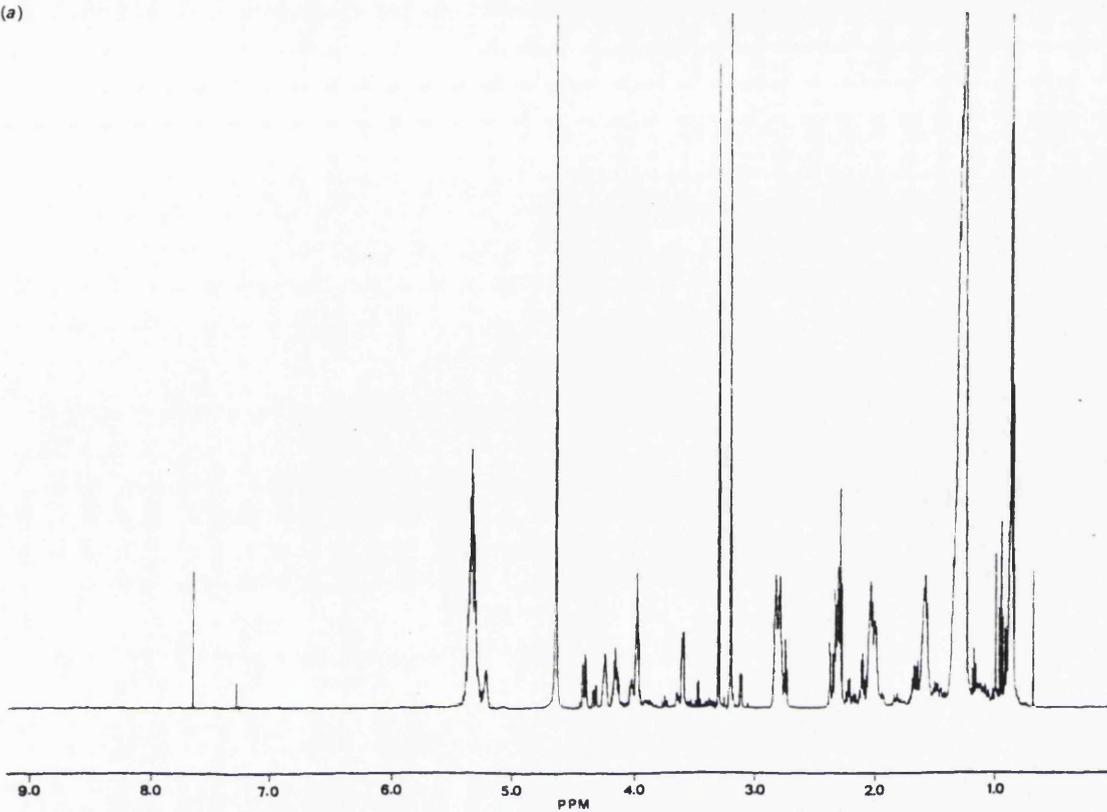
3.2 RESULTS

3.2.1 Lipid quantitation by proton NMR

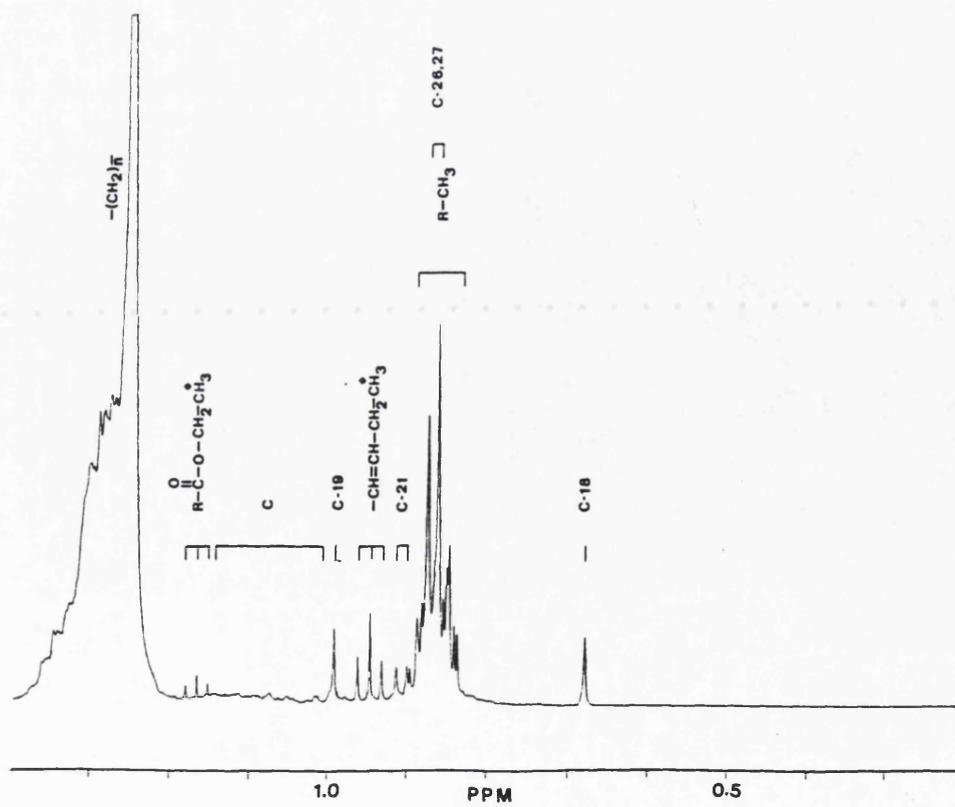
The quantities of individual lipids (in wt. %) in liver has been well established by many workers and the weight percentage of fatty acids of each class of lipid was also reported [Christie, 1987; Bandi & Ansara, 1985; Christie, 1985; Touchstone, 1973]. These data are compared with the values obtained by $^1\text{H-NMR}$ methods in table 3.1.

One dimensional spectra (Fig.3.1a - e) and 2D-HOHAHA (Fig. 3.2a - c) of the liver extract revealed the presence of many lipids. Generally, although there is a overlap of some resonances from different lipid components, each lipid has a structural-specific signal or set of resonances that permitted its identification and quantitation. The relative intensities of these structural-specific resonances (which are directly proportional to the lipid concentrations) from five different animals, measured in term of integrated areas and subsequently normalized by the integrated area of residual CD_3Cl , showed an agreement within 5.0 % for major lipids and between 5.0 to 50 % for low-abundance lipids. In the few cases (mainly among fatty acids) where no structural-specific information can be extracted due to excessive overlapping, indirect quantitation was still possible, by subtracting other resolved components from the overlapped signal-complex. With a few exceptions, relative lipid concentrations determined by the integral areas in the 1D spectra agreed adequately with the data obtained by conventional assay techniques (HPLC, TLC etc.) (Table 3.1). All the quantitation of lipids and esterified

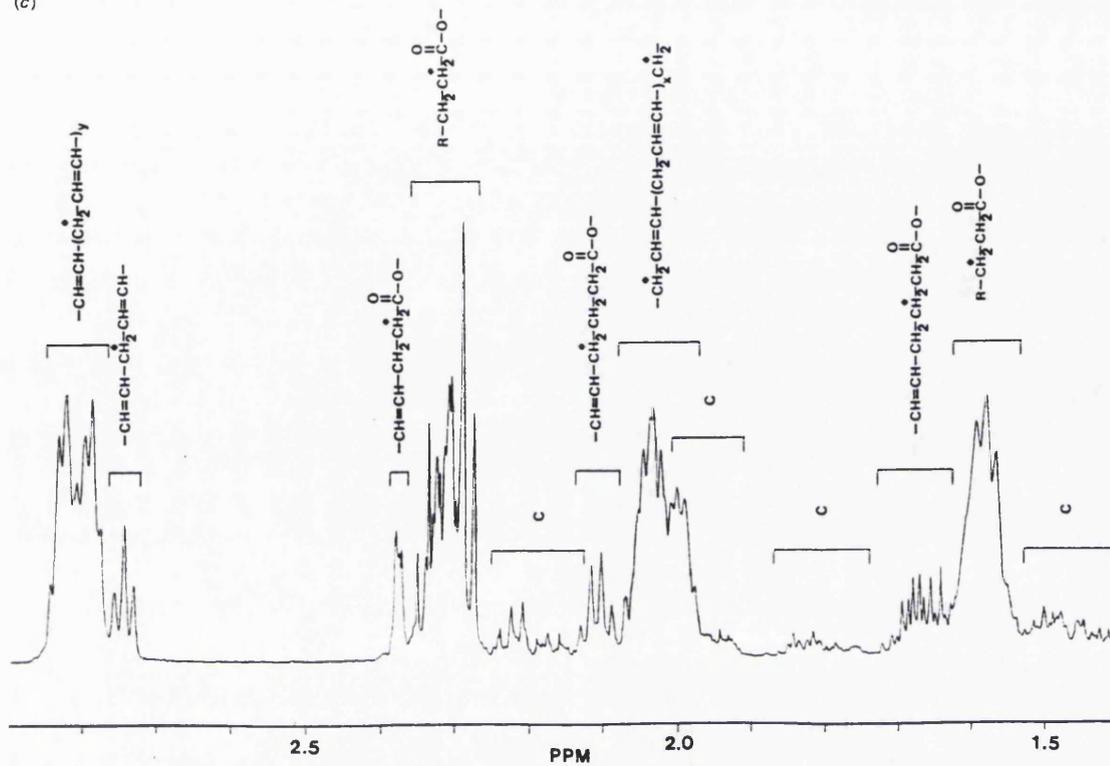
(a)



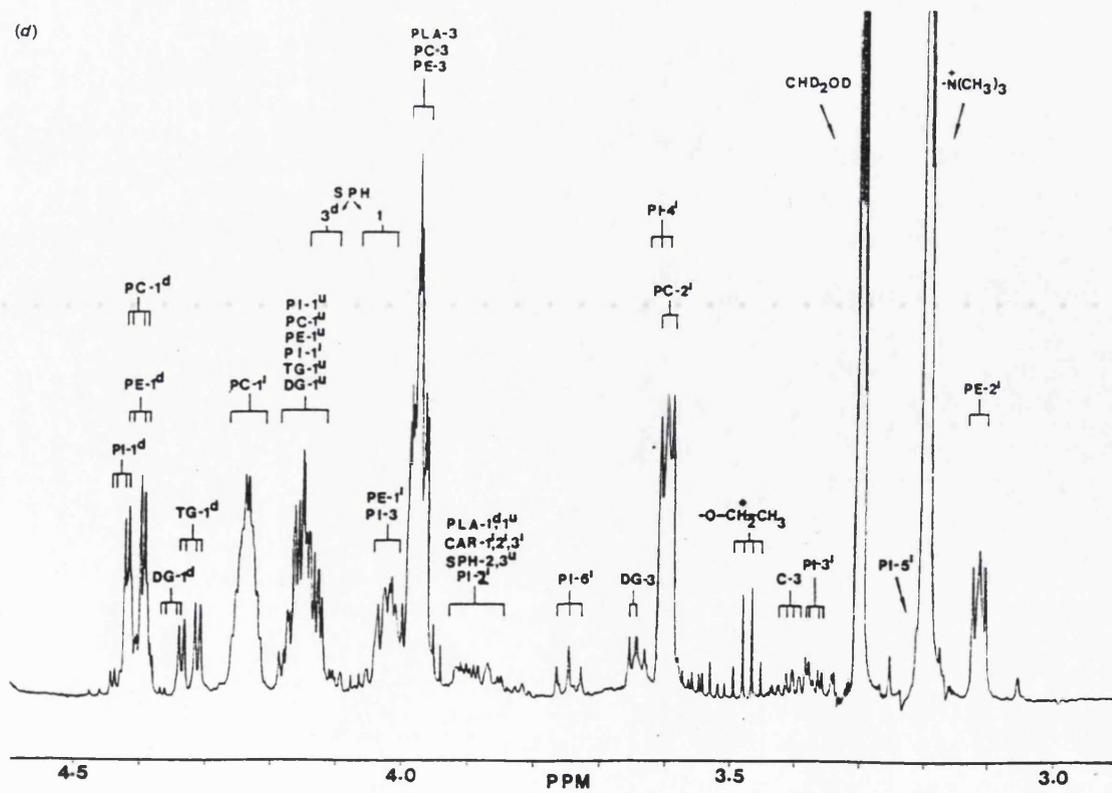
(b)



(c)



(d)



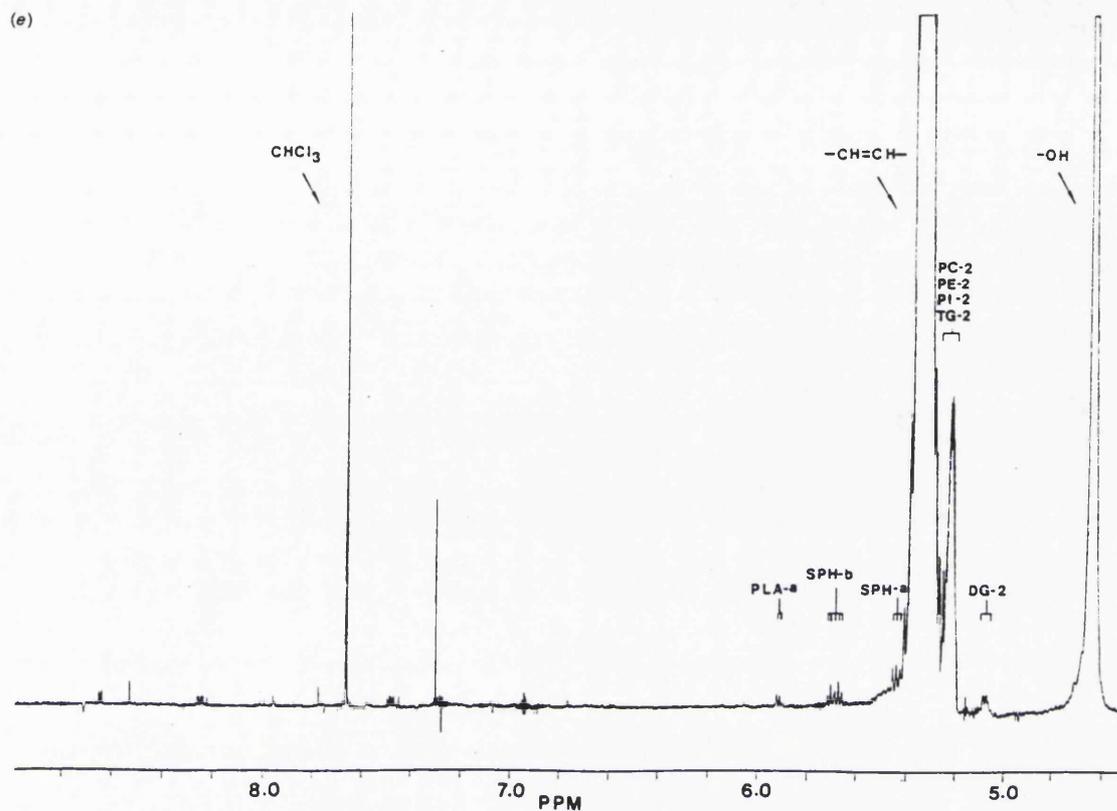
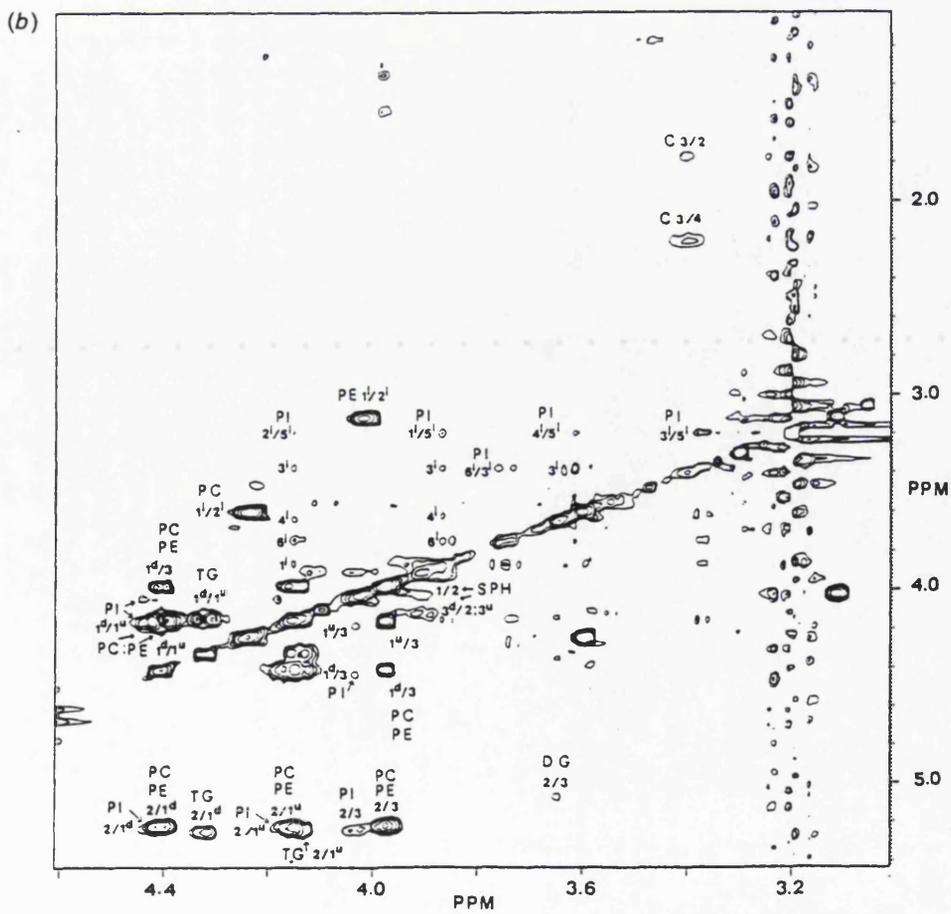
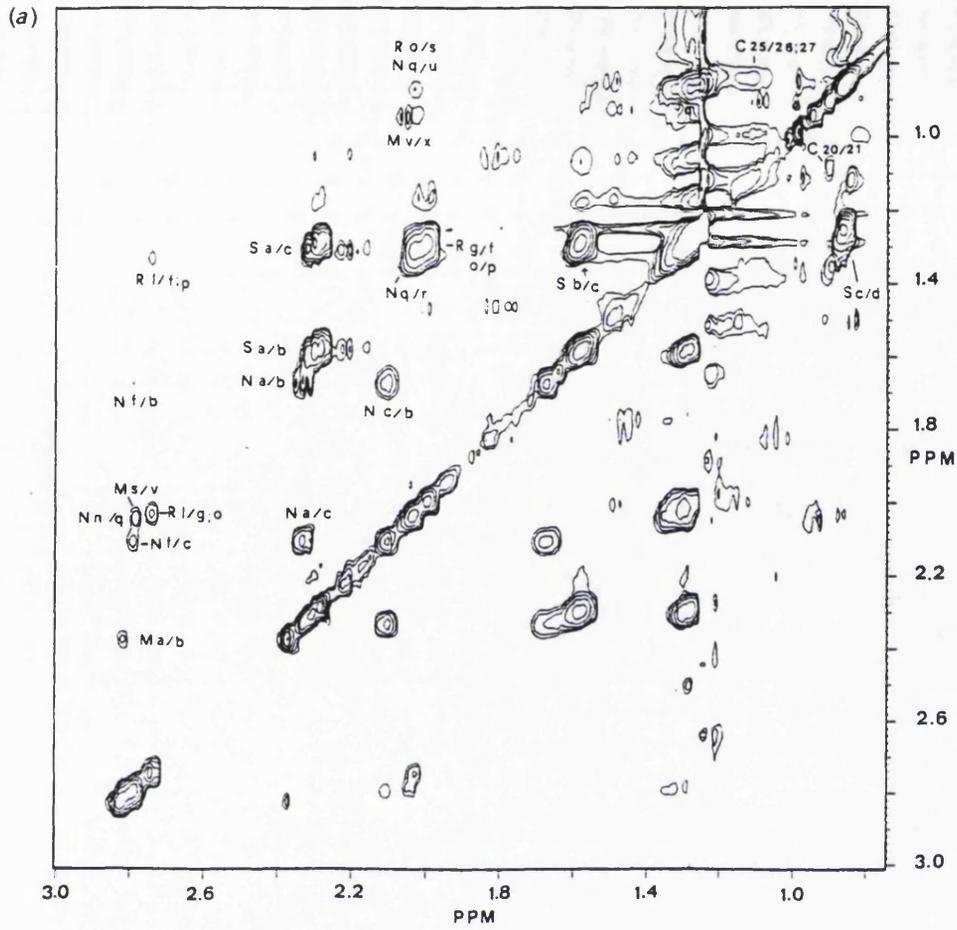


Figure 3.1 (a) One-dimensional proton NMR spectrum of lipids from rat liver [0.50 g of lipid extract in 0.80 ml of CDCl₃-CD₃OD (1:2)]. (b) to (e) Expansions from spectrum (a) showing resonance assignments. The designations \underline{u} or \underline{d} refer to the upfield and downfield protons of a CH₂ group; asterisk indicates the specific resonance in the fatty acid; \underline{x} and \underline{y} are used to indicate the number of repeated chemical group, where $\underline{y} \geq 2$ and $\underline{x} > 1$; only the CH₃ resonances of cholesterol were used for quantitation; nomenclature such as PI-1' and PC-2' are used to designate those protons attached to the specific phospholipid head groups; other designations are explained in the text.



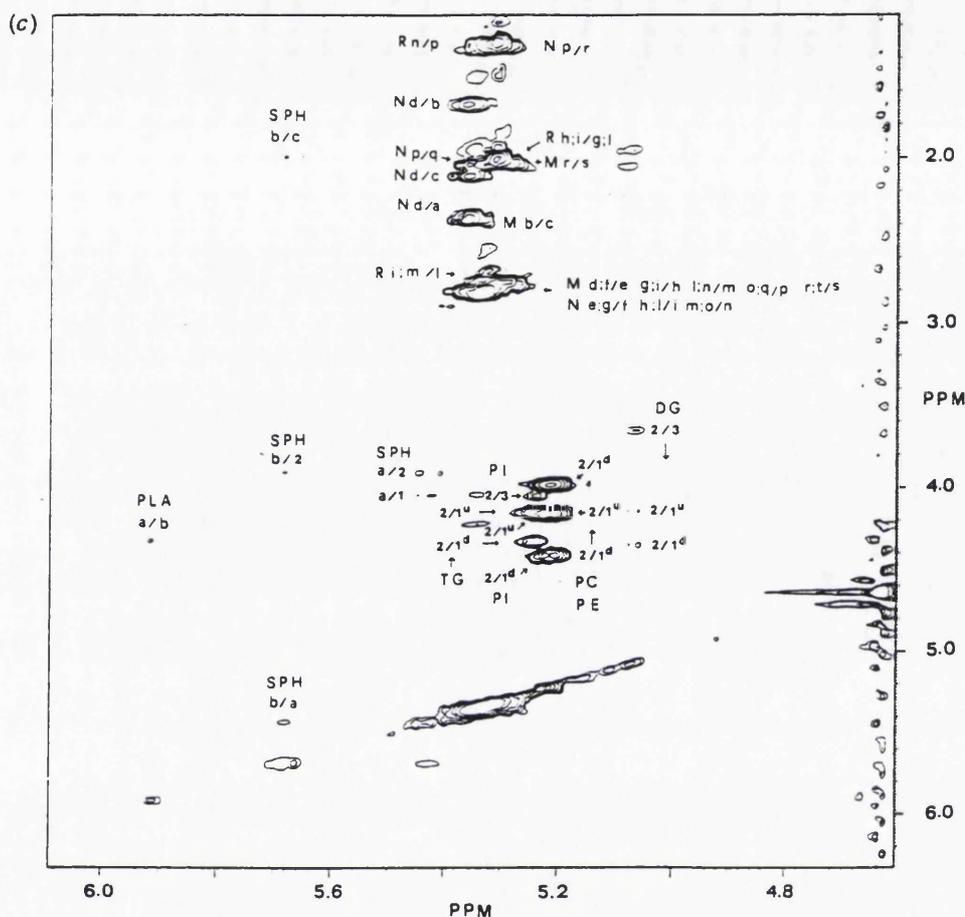
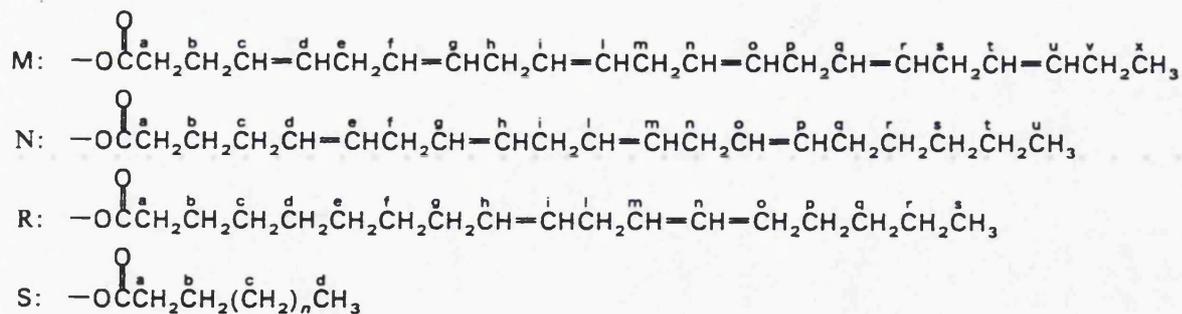


Figure 3.2 (a) Expansion of the 2D-HOHAHA spectrum of lipid extracts from rat liver showing the structural-specific resonances and cross-peaks from the fatty acids of intact lipids. Proton cross-peaks were labelled according to the formulae illustrated below:



For instance, Ma/b and Nc/b indicated respectively the cross-peaks between downfield proton **a** and upfield proton **b** in fatty acid chain N. (b) Expansion of the 2D-HOHAHA spectrum contains the major head group resonances of glycerol, serine, ethanolamine, choline, inositol, with the backbone resonances of glycerophospholipids, ether glycerophospholipids, sphingolipids and DG & TG glycerol moiety resonances. (c) Expansion of the 2D-HOHAHA spectrum of rat liver lipid extracts containing resonances from the olefinic groups of unsaturated fatty acids, the olefinic groups from unsaturated ether lipids plus the C-2 resonances of glycerol and sphingosine moieties.

fatty acids reported are in terms of weight percentage (wt. %). However, their absolute amount can be determined, by referring wt. % to the wet weight of tissue used for lipid extraction and the total weight of the dried lipid extract.

Table 3.1

Comparison of weight percentages (wt. %) of individual lipids determined by HPLC and NMR.

Lipid	NMR ^a	HPLC ^b
PC	46.4 ± 1.4	55.0
PE	20.2 ± 0.8	20.0
PI	4.9 ± 0.9	4.0
PS	-----	-----
CAR	1.8 ± 0.8	5.0
LPC	-----	-----
TG	11.6 ± 1.6	7.0
DG	1.4 ± 0.7	-----
Total unsat. ether lipids ^c	0.9 ± 0.6	-----
Total unsat. sphingolipids ^d	2.4 ± 0.6 ^e	2.0
Cholesterol	10.4 ± 0.5	7.0

^aErrors represented the ± S.D. from the analysis of five different livers.

^bData obtained from reference [Christie, 1985].

^cPlasmalogen being the principal component.

^dSphingomyelin being the principal component.

^eTotal sphingolipids, not measured here, can be estimated from the C-2 amide resonance if CD₃OH-CDCl₃ is used as solvent.

3.2.2 Proton NMR spectral assignment: Glycerophospholipids

This class of lipids possesses glycerol backbones with acyl chains at the *sn*-1 and *sn*-2 positions, with a phosphate diester at the *sn*-3 position joining the head groups to the acylglycerol moiety. The head groups include serine (PS), ethanolamine (PE), and its mono- and di-*N*-methylated precursors (PME & PDE), choline (PC), inositol (PI) and phosphorylated inositols (PIP_x), glycerol (PG) and proton atom (PA). Cardiolipin

(DAG-P_i-glycerol-P_i-DAG) is also a member of this class. It should be noted that these lipids, although they might possess identical head groups, can form subclasses according to the composition of the esterified fatty acids, which can be varied from saturated to various degrees of unsaturation. In addition, lysolipids, a minor species in healthy animal tissues, formed usually by the loss of the *sn*-2 chain, also belong to this class. The spectral assignments of all lipids in this class, as shown in figures 3.1 and 3.2, were achieved by comparison with lipid standard spectra, by spiking and by analysis of the cross-peaks in HOHAHA spectra or from cross-sections of these spectra.

Although the proton-proton 2D cross-peaks for the glycerol CH₂CHCH₂ moiety were slightly different from each member of this class, cross-peaks between head group protons were found to be more accurate and convenient to use for assignment purposes. Examples of this included the chemical shifts of head group methylene protons 1' and 2' from serine (4.28 & 3.93 ppm), ethanolamine (4.02 & 3.11 ppm), choline (4.22 & 3.59 ppm) and resonances from the inositol ring (C-1' at 3.90; C-2' at 4.20; C-3' at 3.40; C-4' at 3.60; C-5' at 3.20 & C-6' at 3.80 ppm). The weight percentage of these lipids in liver derived from the 1D spectra are shown in table 3.1. The characteristic spectral assignments in figures 3.1 and 3.2 are summarized schematically in table 3.2. All PC and PE signals were assigned unequivocally since they are the most abundant lipids in liver tissue and the weight percentage (wt. %) of PC was calculated to be 46.60%. Although PI was also assigned, the lack of detectable PIP and PIP₂ etc., could be attributed to their relative low abundances in liver tissue.

In principal, cardiolipin should have distinctive cross-peaks between the CH₂CHCH₂ protons of its diacylglycerol moieties. However, even under the high magnetic field of 500 MHz, these were still significantly overlapped (4.42, 4.17, 5.21 & 3.98 ppm) with the corresponding moiety cross-peaks of PS, PE, PC and PI. The CAR's central glycerol backbone moiety (CH₂CHCH₂) should provide two sets of degenerate protons (subjected to nonequivalent magnetic environments which resulted in upfield/ downfield splitting), namely, 1a, 1a', 3c and 3c' and hence a cross-peaks should be occurred at 3.84/ 3.98 ppm. Such cross-peak was indeed detected and was attributed to CAR (Fig.3.2b),

but it was very close to the diagonal and the insufficient spectral resolution restrict accurate quantitation. The glycerol moiety cross-peaks of PA and PG were found too weak to be distinguished unambiguously. As revealed by the standard, the glycerol head group (CH₂CHCH₂) of PG could provided a diagnostic set of signal at 3.88 (C-1'), 3.74 (C-3') and 3.58 (C-2') ppm. However, none of these signal were observed, again due to the low abundance of PG in liver. By referring to the standard 2D spectra (PA: 4.46/ 4.17 ppm & 5.27/ 3.97 ppm, PG: 4.41/ 4.17 ppm & 5.20/ 3.97 ppm), a low intensity of PA resonance was spotted at 4.46 ppm in the 1D liver extract spectrum, and both PA and PG were estimated to be less than in 0.1 wt. %. Furthermore, concentrations of PME and PDE were also known to be far below the detection limit of ¹H-NMR (<< 0.1 wt. %).

Table 3.2

Diagnostic proton chemical shifts for major lipids and fatty acids.

Chemical species or structures	Chemical shift (ppm)	General remarks
Total P.	4.40	Total phospholipids content
PC	3.20	-----
PE	3.08	-----
PI	3.75	-----
PS	3.75	-----
CHOL.	0.70	-----
SPH	5.70	-----
PLA	5.90	-----
TG	4.30	-----
DG	3.60	-----
-CH=CH-	5.61	Fatty acid olefinic groups
-CH=CH-(CH ₂ -CH=CH) _x -	2.80	[x>1]; PUFAs except linoleic acid
-CH=CH-CH ₂ -CH=CH-	2.75	Principally linoleic acid
-CH=CH-CH ₂ -CH ₂ -COO-	2.40	Δ ⁴ fatty acids, mainly docosahexaenoate
-CH ₂ -CH=CH-(CH ₂ -CH=CH) _v -CH ₂ -	2.00	[y≥0]; fatty acid allylic groups
-CH=CH-CH ₂ -CH ₂ -CH ₂ -COO-	1.70	Δ ⁵ fatty acids, principally arachidonate
CH ₃ -CH ₂ -CH=CH-	0.95	n-3 fatty acids
CH ₃ -CH ₂ -CH ₂ -R	0.85(total)	R = long-chain alkyl groups

3.2.3 Proton NMR spectral assignment: Mono-, Di- and Triglycerides

Triacylglycerol is the only major glyceride lipid that exists in liver, as has been demonstrated by HPLC chromatography [Christie, 1985] and was consistent with the data obtained by 1D and 2D proton NMR, which showed predominantly the backbone moieties signals of TG at 4.32, 4.15 and 5.25 ppm respectively. A limited amount of DG was also found, as indicated by its weak but observable C-2/ 3 glycerol moiety cross-peak at 5.10/ 3.65 ppm (Fig. 3.2b), and the weak C-3 resonance at 3.65 ppm in the 1D spectrum (Fig. 3.1d). The relative concentration of TG in wt. % was calculated to be 11.60 % and DG was estimated to have a wt. % of 1.40, which are both in good agreement with the corresponding HPLC values.

3.2.4 Proton NMR spectral assignment: Ether phospholipids

Ether phospholipids are glycerophospholipids in which the hydrocarbon chains at the *sn*-1 position is linked by a saturated or unsaturated ether linkage, as illustrated by the following structural formulae:



Those protons attached to carbon **a** and **b** in formula [i] provided the diagnostic resonances for saturated ether lipids. A cross-peak generated from protons **a**/ **b** occurred at 3.44/ 1.59 was found in the spectrum of standard saturated ether lipid, and the absent of this particular cross-peak in the 2D liver extract spectrum is consistent with the expected low abundance of this class of ether lipid. The resonance signals of the protons, located at **a**, **b** and **c** in formula [ii], provided the diagnostic resonances for unsaturated ether lipids, especially plasmalogen, the most abundant species in this class. These three pairs of protons generated the characteristic cross-peaks in the 2D HOHAHA spectrum

at 5.91/ 4.33 ppm (*a*/ *b* cross-peak), 5.91/ 2.00 ppm (*b*/ *c* cross-peak) and 4.33/ 2.00 ppm (*a*/ *c* cross-peak); all were clearly observed in the 2D liver lipid extract spectrum (fig.3.2). The glycerol moiety cross-peaks at 3.92/ 5.16 ppm and 3.87/ 5.16 ppm further confirmed the existence of this lipid. Quantitation by measuring the integrated area of 1D spectrum gave a concentration of *ca.* 1.70 wt. % for this class of lipid.

3.2.5 Proton NMR spectral assignment: Sphingolipids

Instead of the glycerol moiety, this class of lipids contains a sphingosine (4-sphingenine) or 4-sphinganine backbone moiety, as illustrated by the following structural formulae:



The principal compounds of this particular class are sphingomyelin (ceramidephosphocholine) and ceramidephosphoethanolamine, which contributed up to *ca.* 10.0 % to the total membrane-lipid content. However, proton resonance signals between sphingomyelin and ceramidephosphoethanolamine could not be distinguished since all the diagnostic cross-peaks between protons 1, *a*, *b* and *c* (see formulae [iii] & [iv]) are found to be overlapped (cross-peak of *b*/ *a* at 5.68/ 5.43 ppm, *a*/ 1 at 5.43/ 3.95 ppm and *b*/ *c* at 5.68/ 2.02 ppm). Nevertheless, these cross-peaks still allow the two lipids to be distinguished from other lipid classes (Fig.3.2c).

There are other relatively low-abundance sphingolipids (< 5.0 %) that exist in animal cells, namely cerebroside and ganglioside, which have the phospho-head group replaced by carbohydrate moieties. Since the concentration of these lipids in liver tissue is below the detection limit of ¹H-NMR, no corresponding signals were observed.

Nevertheless, proton resonance signals from cerebrosides were detected in rat brain tissue, and will be discussed in detail in following chapters.

3.2.6 Proton NMR spectral assignment: Steroidal lipids

The only member of this class detected in the liver lipid extract was cholesterol. The existence was confirmed by numerous cholesterol-specific resonances such as the chemical shifts at 1.76, 2.22 and 3.40 ppm (Fig. 3.1c & d). Figure 3.2 also revealed several cholesterol cross-peaks at 3.40/ 2.22 ppm, 3.40/ 1.76 ppm and 1.72/ 3.40 ppm. The weight percentage of cholesterol was easily calculated from the C-18 methyl resonance at 0.68 ppm (Fig. 3.1b).

3.2.7 Analysis of fatty acid composition by proton NMR.

Previous determination of individual free fatty acids via NMR [May *et al.*, 1986; Yu *et al.*, 1986] have been extended and refined in this study, and used for the determination of esterified fatty acid compositions in liver lipid extracts. Other publications which reported the composition of fatty acids in liver tissues used a combination of TLC and GC analysis [Christie, 1987; Christie, 1985; Touchstone, 1973], and the lipids were subjected to hydrolysis prior to the chromatographic assays.

The relative concentration of fatty acids was determined from the 1D spectrum by referring the area of the total CH_3 resonances (*ca.* 0.87 ppm) or the area of the CH_2COOR resonance at *ca.* 2.30 ppm as 100.0 %. This resulted in the value of the total unsaturated fatty acid chains obtained as 53.10 % by the integration of allylic CH_2 group resonance (formula [v]). These allylic resonances occurred between 1.98 and 2.10 ppm, as shown in figure 2.1c.





Significantly, the grand total of the *n*-3 polyunsaturated fatty acids including 22:5, 20:5, 22:6 and 18:3 were determined from their ω (omega)- CH_3 resonance at 0.95 ppm (formula [vi]), which yielded 10.40 wt. %. Arachidonic acid (20:4) and eicosapentaenoic acid (20:5) were collectively determined as 9.60 % from their characteristic C-3 methylene resonance at 1.65 ppm (formula [vii]). Quantity of di-unsaturated fatty acids were determined from their specific biallylic resonances at 2.74 ppm, which gave the wt. % value of 14.60 %. Linoleic acid (18:2) is the major fatty acid of this class. The corresponding biallylic resonances from other polyunsaturated fatty acids occurred at *ca.* 2.80 ppm. The amount of docosahexaenoic acid (22:6) was determined as 5.40 wt. % from its diagnostic C-2 methylene signal at 2.38 ppm (formula [viii]).

With the aid of simple algebraic calculations, the composition of esterified fatty acid in rat liver extract can be summarized as follow:

- (i) Since the specific amount of docosahexaenoic acid was calculated as 5.40 %, and the collective amount of (*n*-3) polyunsaturated fatty acids (i.e. 22:5, 20:5, 22:6 & 18:3) was found to be 10.40%, this implies that the combined value of 22:5, 20:5 and 18:3 fatty acids should be *ca.* 5.0 %.
- (ii) There is another combined polyunsaturated acid signal, which consisted of docosapentaenoic acid (22:5), docosahexaenoic acid (22:6) and linolenic acid (18:3) and constituted 10.0 wt. %. Thus by subtracting the known wt. % of docosahexaenoic acid from this collective value, showed that the combined wt. % of docosapentaenoic acid and linolenic acid [i.e. (22:5) + (18:3)] must be *ca.* 4.6 %.

(iii) By combining the conclusions of (i) and (ii) revealed that the wt. % of eicosapentaenoic acid (20:5) was *ca.* 0.4 % (i.e. 5.0 % - 4.6 %). Recall that the collective amount of arachidonic acid (20:4) and eicosapentaenoic acid (20:5) was 9.60 %, which meant that the specific wt. % of arachidonic acid was *ca.* 9.20 % (i.e. 9.6 % - 0.4 %).

The remaining undetected unsaturated fatty acids, principally monosaturated fatty acids because of their lack of diagnostic resonances, were quantified by subtracting all detectable fatty acids values from the of total unsaturated fatty acid (53.10 %), yielded 18.0 wt. %. Quantity of free fatty acids was found to be insignificant, as indicated by the absence of their resonances at 3.48 and 1.16 ppm. The analysis of fatty acid composition are summarized in table 3.3.

Table 3.3

Composition of esterified fatty acids from rat liver lipid extract obtained by proton NMR.

Systematic name	Trivial name	Shorthand designation	Content (wt. %)
9,12-Octadecadienoic	Linoleic	18:2 (<i>n</i> - 6)	14.60
5,8,11,14-Eicosatetraenoic	Arachidonic	20:4 (<i>n</i> - 6)	9.20 ^a
5,8,11,14,17-Eicosapentaenoic	-----	20:5 (<i>n</i> - 3)	0.40 ^a
9,12,15-Octadecatrienoic	Linolenic	18:3 (<i>n</i> - 3)	4.60 ^b
7,10,13,16,19-Docosapentaenoic	-----	22:5 (<i>n</i> - 3)	
4,7,10,13,16,19-Docosahexaenoic	-----	22:6 (<i>n</i> - 3)	5.40

^aDiagnostic signal was superimposed with signal from other fatty acid classes, thus quantitation was achieved indirectly by subtraction.

^bThe wt. % was obtained from a mixture of two fatty acids.

3.4 DISCUSSION

NMR spectroscopy has been exploited in many laboratories for the study of cell, tissue and body metabolism: both *in vitro* and *in vivo* approaches have been utilized. *In*

in vivo approaches to the study of lipids have been limited owing to the intrinsic broadening of the spectra of individual lipids in intact membranes due to lipid aggregation. Membranes of animal cells generally possess 50-100 different lipids belonging to separate classes which are related by common structural moieties. It is not surprising, therefore, that the individual spectra of the lipids have not been distinguished in these complex mixtures. Apart from specific examples in lipid analysis [Sparling, 1990; Sparling *et al*, 1989], lipid metabolism studies of tissues by proton NMR are few [May *et al*, 1986]. Progress has been limited by the lack of fully comprehensive metabolite "spectral maps" of membrane lipids of the various tissues. The *in vitro* lipid analysis of rat liver reported in this article, both quantitatively and qualitatively, was obtained by solely ^1H -NMR spectroscopy and the lipid extracts were prepared by classical freeze-clamp and lipid extraction procedures [Folch *et al*, 1957]. However, drawbacks to the freeze-clamp NMR lipid assay method included the incomplete profile of low-abundance lipids such as highly phosphorylated inositols or cholesterol esters, hydrophobic vitamins and cofactors, due to the intrinsic insensitivity of NMR, and the excessive overlapping in part of the spectra. Despite such reservations, even the simple ^1H -NMR procedures described here rapidly and quantitatively yielded information on an amount of 50 or more lipids in a period of 30 min. This NMR lipid profile should be contrasted with corresponding profiles obtained by TLC, HPLC, gas chromatography (GC) or the determination of lipid concentration in tissues by conventional enzyme assays. The agreement with the corresponding data obtained by HPLC indicates that the ^1H -NMR method is a powerful complement to other approaches, with the added advantages of rapidity and comprehensiveness, as well as using intact non-hydrolyzed lipids. Thus the application of this NMR procedure should assist in the study of many biological processes in the liver, including the livers in pathological situations such as diabetes and various lipidaemias.

Chapter 4
1D and 2D proton NMR of lipids from rat brain

4.1 INTRODUCTION

The variety of lipids found in the brain is large and their amounts differ considerably from other tissues such as liver; cholesterol, plasmalogens and sphingolipids are known to occur in higher concentrations [Norton & Crammer, 1984; Ramsey & Nicholas, 1972]. There are a few rare lipid related genetic disorders which are associated with genetic defects in sphingolipid metabolism; such as Neiman-Pick and Tay Sachs storage diseases, [Stanbury *et al*, 1978] for example. Recently inositol lipids [Fisher *et al*, 1984] and choline lipids [Taylor *et al*, 1990] have been implicated in second messenger events and ion-channel conductance. Anaesthetics have been proposed to act by controlling lipid metabolizing enzymes of the plasma membrane [Memeses & Glonek, 1989; Sotiris *et al*, 1986] and hence to modulate ion-channel conductance.

The growing conviction that lipids not only have structural importance but also play active roles in membrane biology and hence in medicine has necessitated the development of assay methods for all lipids in a given tissue. Enzyme and immunosorbent methods give sensitive and accurate assays for single enzymes and their substrates and products, whilst significant advances in lipid assays have been achieved by thin layer chromatography (TLC) [Jardetski & Roberts, 1981] and high performance liquid chromatography (HPLC) [May *et al*, 1986; Yu *et al*, 1986]. For these methods however, only a limited number of lipids or lipid classes can be differentiated with a single set of chromatographic conditions or parameters. Mass spectroscopy [Toshiyuki *et al*, 1984] has been used in conjunction with chromatography and, although this has been proved to be a significant advance, it still possesses many drawbacks of the chromatographic procedure to which it is coupled.

Phosphorous NMR provided an assay for lipids that contain phosphorous atoms [Sparling *et al*, 1989; Iida *et al*, 1977; Mahadevan *et al*, 1968] and the application of one and two dimensional proton NMR on analysis of lipid extract and synthetic lipid have appeared [Casu *et al*, 1991; Sparling, 1990; Bandi & Ansara, 1985; Bax & Davies, 1985; Drabkowski *et al*, 1980; Sawan *et al*, 1979; Touchstone, 1973]. Application of ^1H -NMR to the characterization and quantitation of lipids in liver has been discussed in

previous chapter, including a comparison with TLC and HPLC assay methods [Casu *et al.*, 1991]. The same technique was used to obtain an NMR lipid profile of a rat brain lipid extract which will be discussed in this chapter. This method has already proved to be more rapid and comprehensive than most conventional chromatographic assays, and permits the simultaneous detection and quantitation of most glycerol and sphingosine phospholipids, glycerides, ceramides and cerebrosides, cholesterol and plasmalogen lipids on 10.0 mg (or less) of brain lipid extract in a period of < 30 min. As in the previous chapter, freeze clamp and Folch procedures were used for brain lipid extractions.

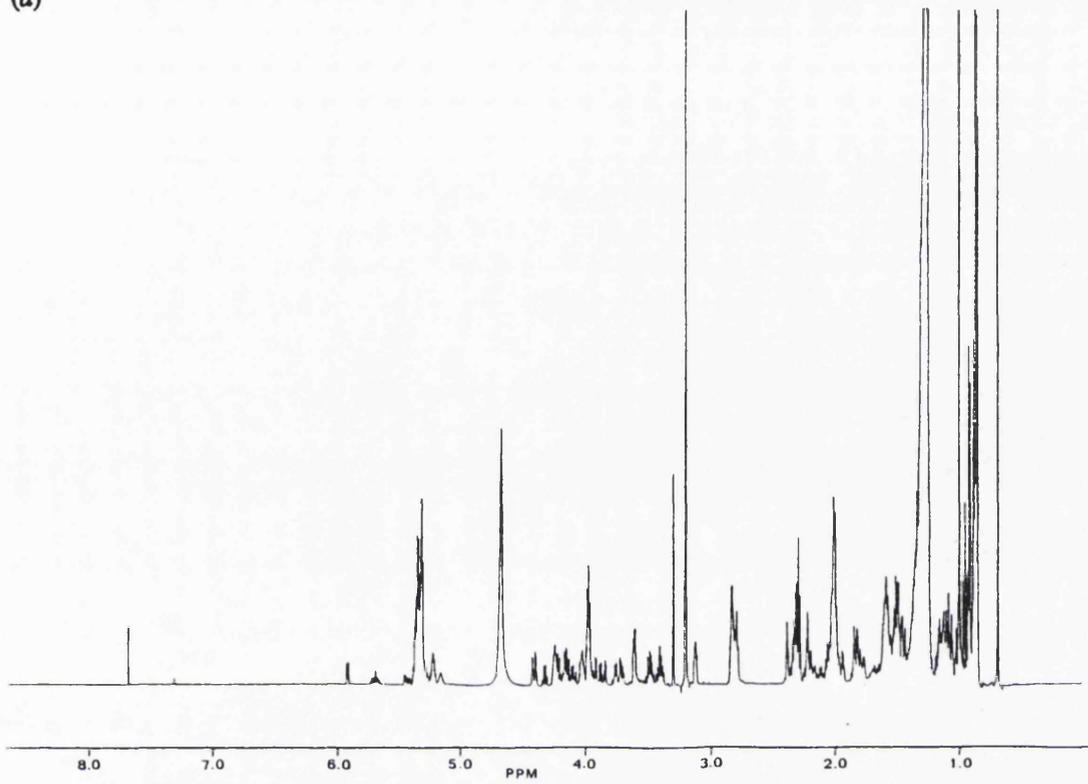
4.2 RESULTS AND DISCUSSION

4.2.1 *In vitro* ¹H-NMR lipid analysis on rat brain

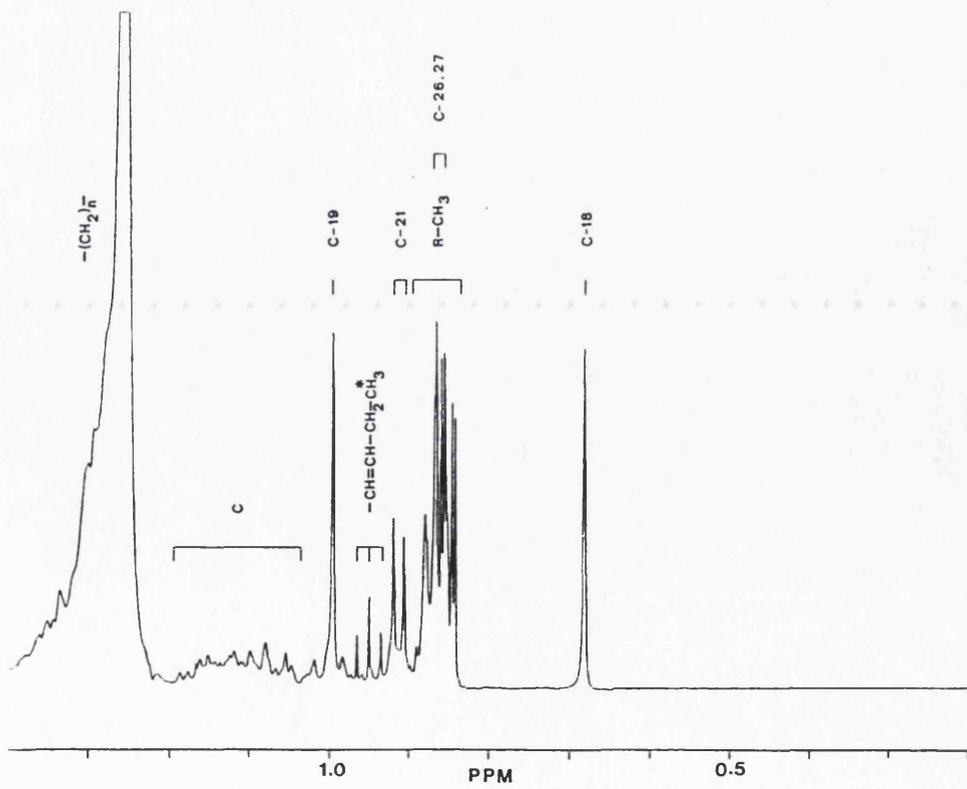
The one and two dimensional proton NMR spectra of the rat brain lipid extract are shown in figure 4.1 and 4.2, respectively. All assignments are accomplished according to the structural-specific moieties of individual lipid species, by comparison with the ¹H-NMR spectra of lipid or fatty acid standards, "spiking" the one or two dimensional spectra with the compound which was suspected to be present, and by analyzing the 2D cross-peaks in the brain lipid mixture. Multiple quantum filtered 2D-COSY (COSYMQF) and 2D homonuclear Hartmann-Hann experiment (HOHAHA) were the 2D pulse sequences most commonly employed to yield the structural assignments, and the assignments of the latter was presented in this report.

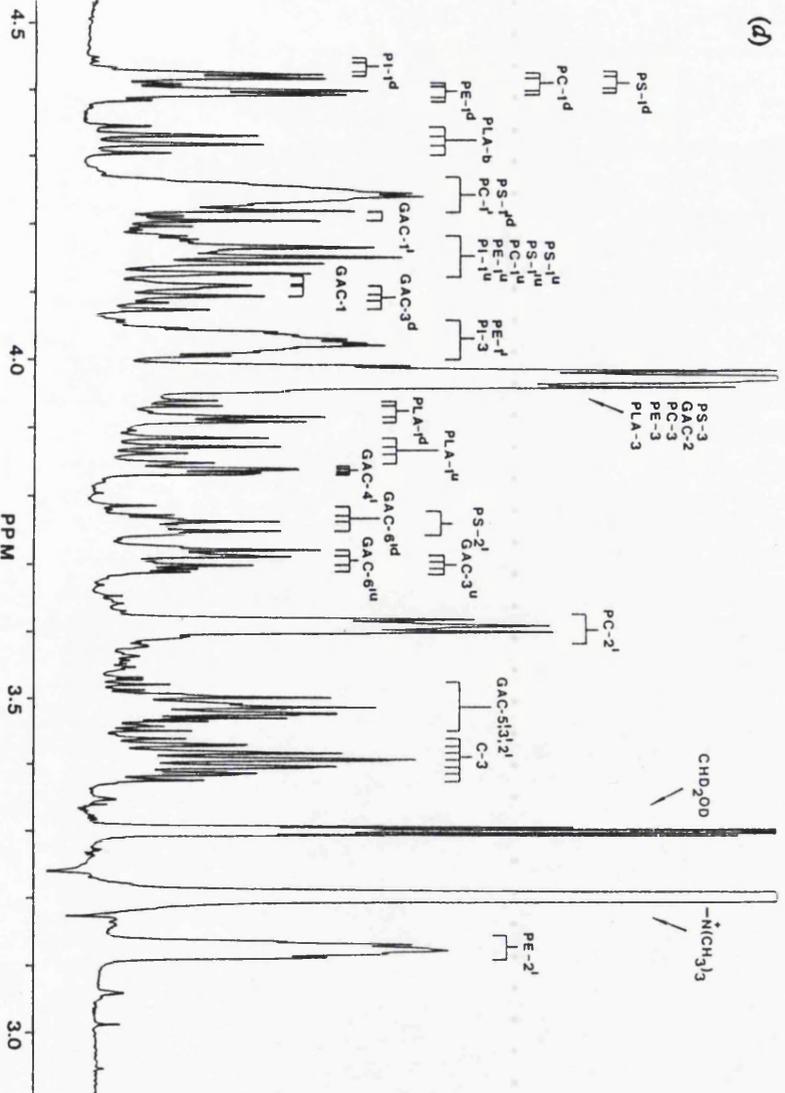
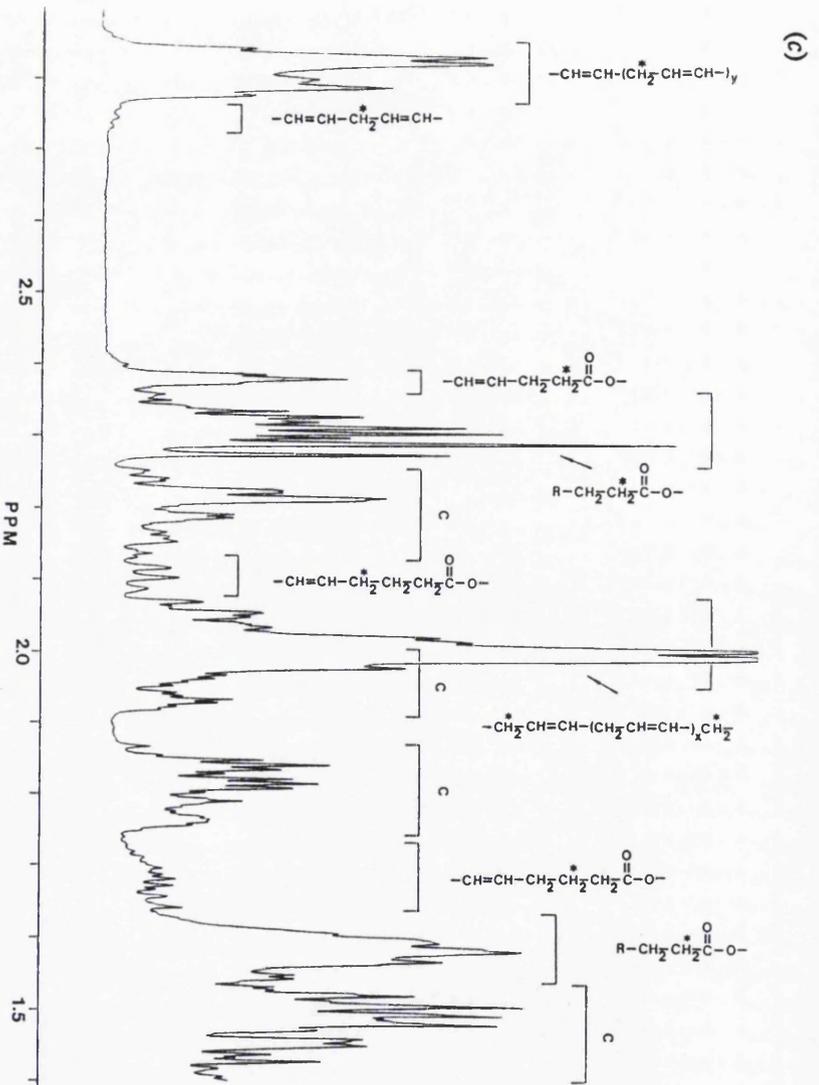
The ultimate development of comprehensive assays for brain lipids and those from other tissues will rely upon finding an extraction procedure or combination of lipid extraction procedures which will quantitatively extract every lipid. Whether or not HPLC, TLC, GC, MS or NMR methods are utilized for assays, they all rely upon the fidelity of the extraction procedures. At this juncture the classical "freeze-clamp" tissue removal procedure and a modified Folch extraction (1957) has been utilized but other lipid extraction procedures have been developed [Klarovic & Fournier, 1986]. Ideally, all will have to be evaluated eventually via the NMR lipid profiles analysis.

(a)



(b)





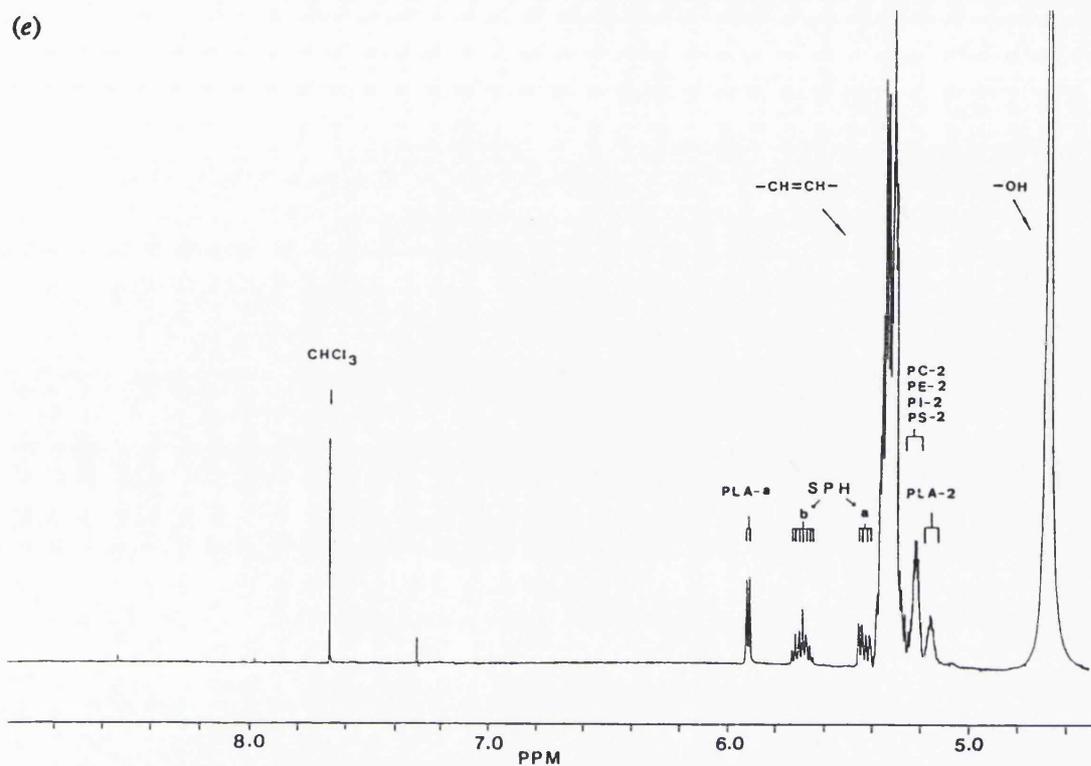
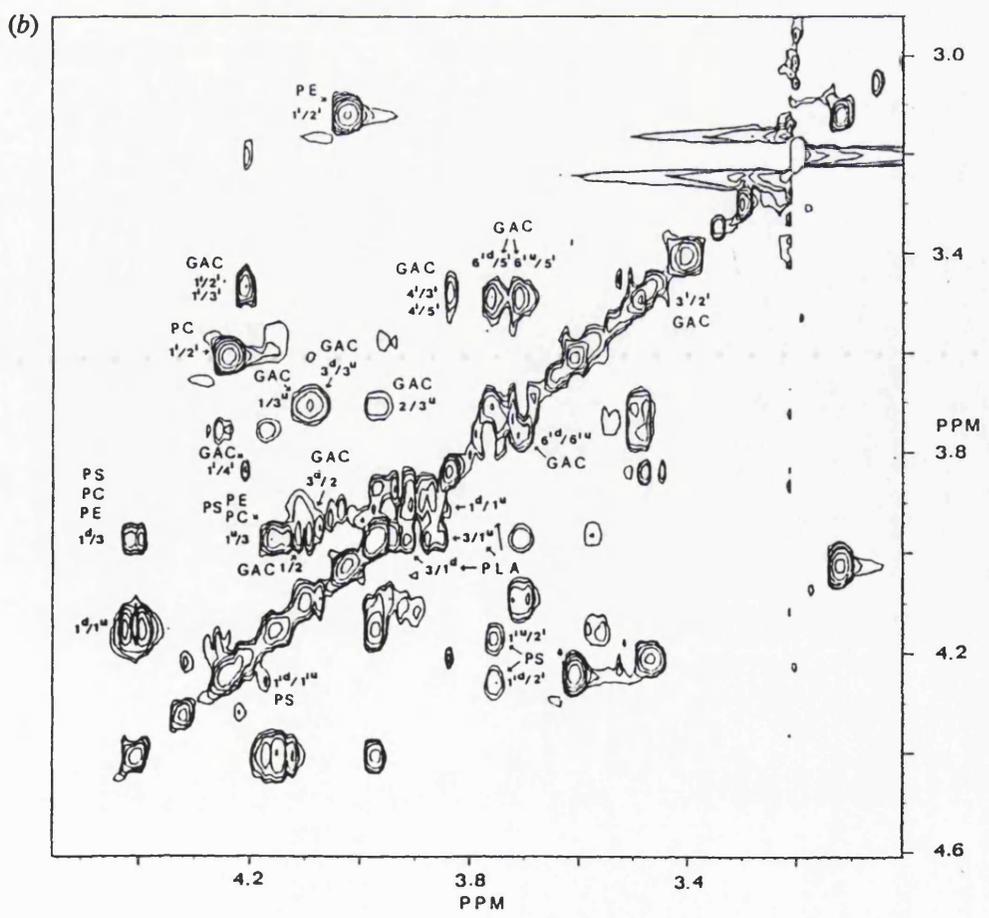
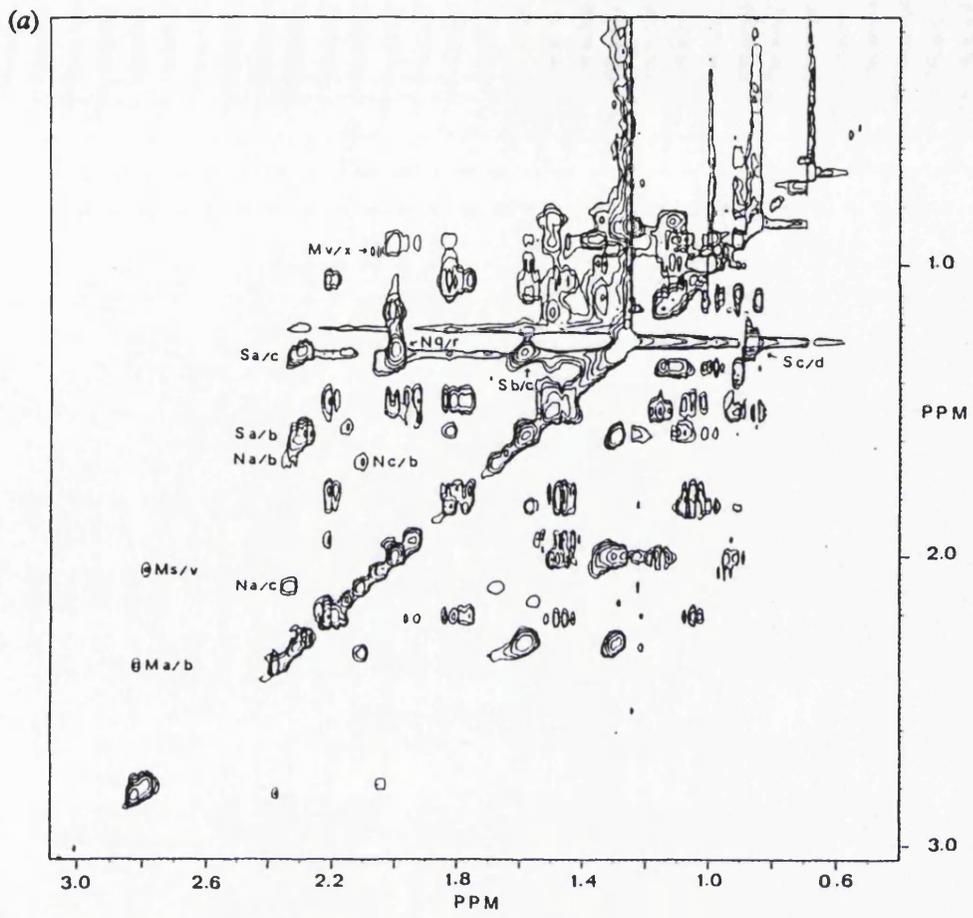


Figure 4.1 (a) One-dimensional proton NMR spectrum of lipids from rat brain [0.50 g of lipid extract in 0.80 ml of $\text{CDCl}_3\text{-CD}_3\text{OD}$ (1:2)]. (b) to (e) Expansions from spectrum (a) showing resonance assignments. The designations \underline{u} or \underline{d} refer to the upfield and downfield protons of a CH_2 group; asterisk indicates the specific resonance in the fatty acid; \underline{x} and \underline{y} are used to indicate the number of repeated chemical group, where $\underline{y} \geq 2$ and $\underline{x} > 1$; only the CH_3 resonances of cholesterol were used for quantitation; nomenclature such as PI-1' and PC-2' are used to designate those protons attached to the specific phospholipid head groups; other designations are explained in the text.



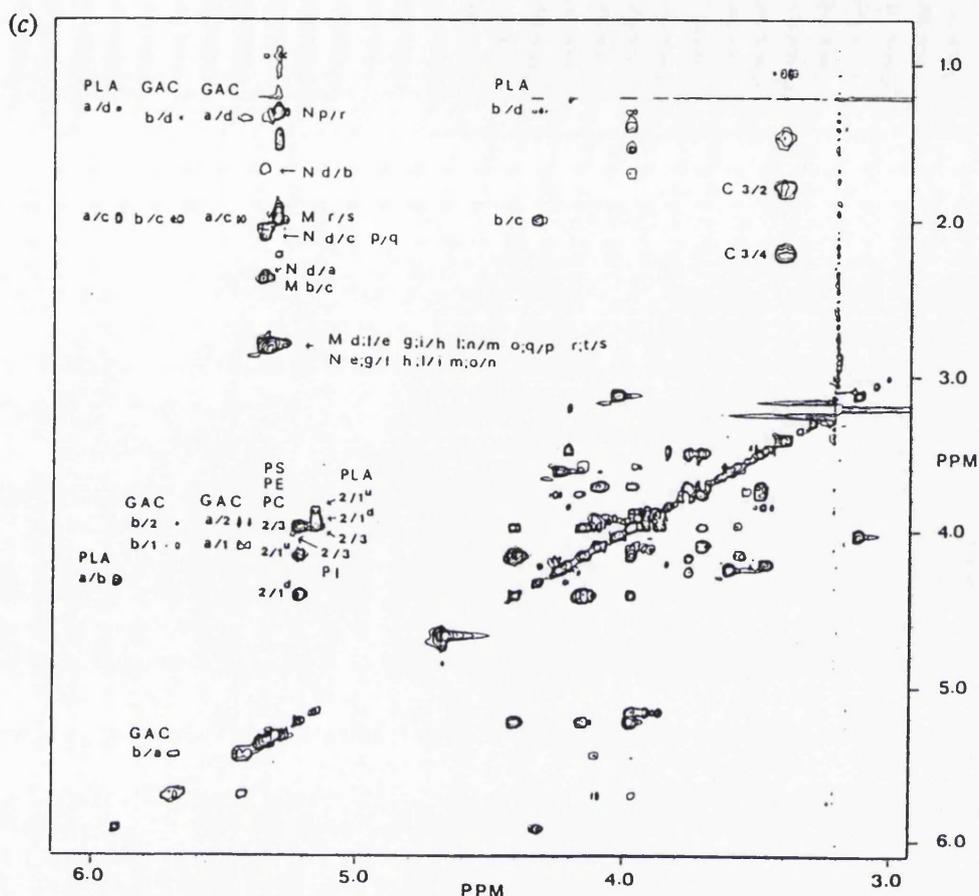
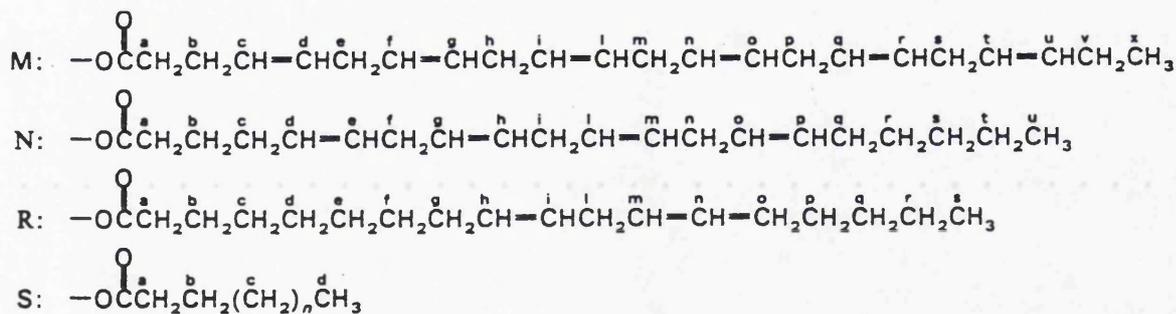


Figure 4.2 (a) Expansion of the 2D-HOHAHA spectrum of lipid extracts from rat brain showing the structural-specific resonances and cross-peaks from the fatty acids of intact lipids. Proton cross-peaks were labelled according to the formulae illustrated below:



For instance, Ma/b and Nc/b indicated respectively the cross-peaks between downfield proton **a** and upfield proton **b** in fatty acid chain N. (b) Expansion of the 2D-HOHAHA spectrum contains the major head group resonances of serine, ethanolamine, choline, inositol and galactose with the backbone resonances of glycerophospholipids, ether glycerophospholipids, cerebroside and sphingolipids. (c) Expansion of the 2D-HOHAHA spectrum of rat brain lipid extracts containing resonances from the olefinic groups of unsaturated fatty acids, the olefinic groups from unsaturated ether lipids plus the C-2 resonances of glycerol and sphingosine moieties.

4.2.2 Lipid quantitation by proton NMR

Quantitation of lipids and esterified fatty acids featured in this report are given in terms of weight percentage (wt. %). The integrated areas were measured from the diagnostic structural resonances, and compared with the integrated area of residual CD₃Cl used as an external standard. This gave the relative concentration of every identified lipid species. In the few cases where no structure-specific information was obtained due to extensive spectral overlap, the quantities were calculated by subtracting other resolved components from the extensively overlapped signal complex. The brain lipid profile analysis was performed on seven individual samples and proved highly reproducible, as indicated by the \pm S.D. values in table 4.1. In general, good agreements were obtained, both qualitatively and quantitatively, between NMR lipid profile and existing chromatographic data, as summarized in table 4.1. Absolute amount of lipids can be estimated by referring to the wet weight of brain tissue used for lipid extractions and the dried weight of lipid extracts.

Table 4.1

Comparison of weight percentages (wt. %) of individual lipids determined by NMR, GC and TLC.

Lipid	NMR ^a	GC ^b	TLC ^c
PC	25.40 \pm 1.1	22.00	22.50 \pm 0.6
PE	9.50 \pm 0.3	8.20	20.40 \pm 0.9
PI	2.70 \pm 0.3	2.40	1.40 \pm 0.2
PS	-----	7.20	5.50 \pm 0.5
GAC	7.80 \pm 0.8	14.60	-----
Total unsat. ether lipids ^d	11.30 \pm 0.5	-----	-----
Total unsat. sphingolipids ^e	4.10 \pm 0.3 ^f	3.80	2.90 \pm 0.1
Cholesterol	23.60 \pm 0.6	23.00	-----

^aErrors represented the \pm S.D. from the analysis of seven different brains.

^bData obtained from reference [Norton & Crammer, 1984].

^cData obtained from reference [Jardetski & Roberts, 1981].

^dPlasmalogen being the principal component.

^eSphingomyelin being the principal component.

^fTotal sphingolipids, not measured here, can be estimated from the C-2 amide resonance if CD₃OH-CDCl₃ is used as solvent.

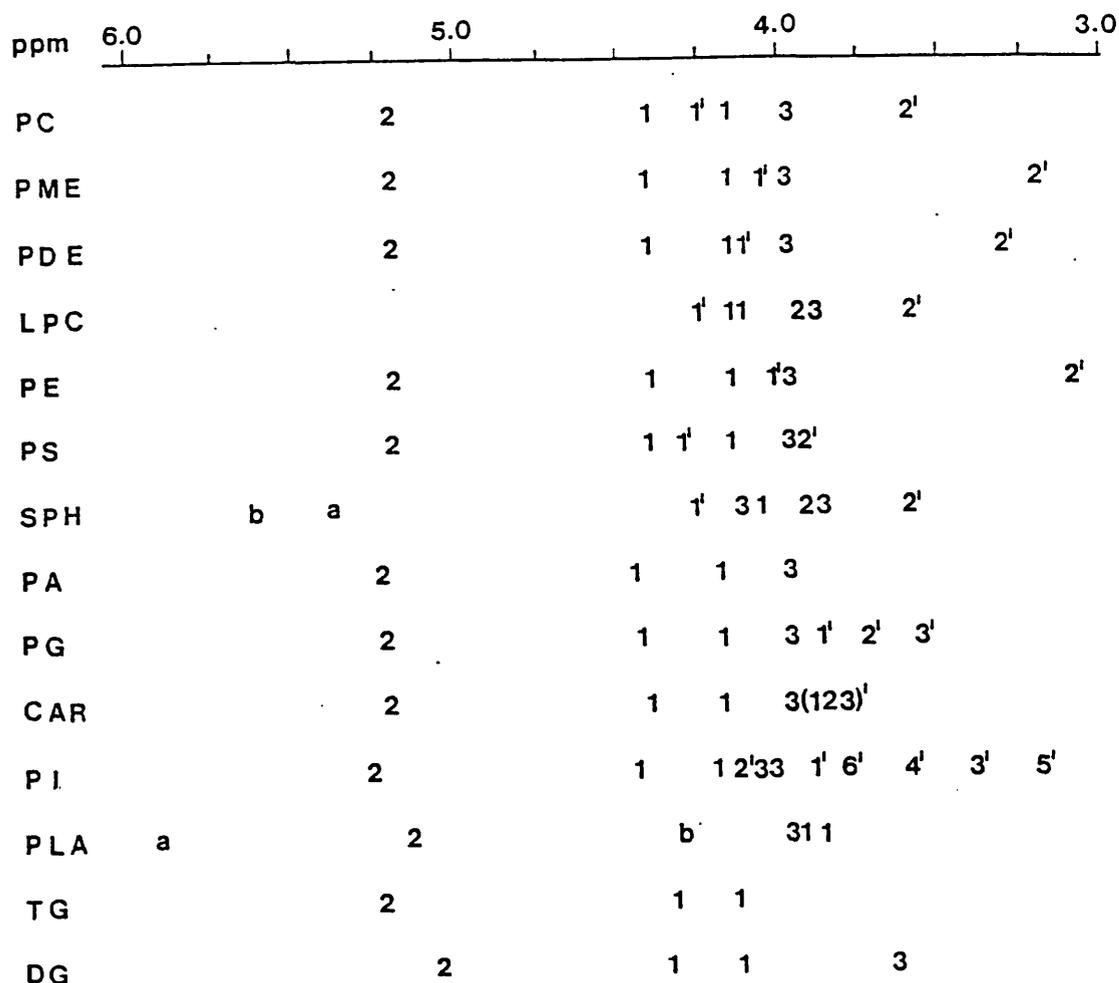


Figure 4.3 A diagrammatic comparison of proton chemical shifts from the glycerol moieties and the head groups of lipids.

4.2.3 Proton NMR spectral assignments: Glycerophospholipids

All compounds of this class possess diacylglycerol moieties covalently linked to a variety of head groups by a phosphodiester bridge. Hence the identification of individual glycerophospholipid is particularly based upon the differences between their head group moieties. The head groups which existed in brain lipids include serine (PS), ethanolamine (PE), choline (PC), inositol (PI) and phosphorylated inositols (PIP_x), glycerol (PG) and proton atom (PA). Cardiolipin (DAG- P_1 -glycerol- P_1 -DAG) is also a member of this class. The lipids can be further sub-classified, in terms of the structures of fatty acids which form acylester groups with the *sn*-1 and *sn*-2 oxygen atoms of the glycerol backbone.

The glycerol moiety carbon atoms are numbered conventionally, with the head groups numbered with primes as illustrated in formulae [i] and [ii]:



In principle each lipid has its structure-specific resonances which distinguish it from the others, these structure-specific resonances were initially identified in the spectra of lipid standards, and subsequently compared to the brain lipid spectrum. The lipid standard assignments are shown schematically in figure 4.3. The 1D and 2D $^1\text{H-NMR}$ spectral assignments of rat brain lipid extract are shown in figure 4.1 and 4.2. As can be seen, all the chemical shifts of lipids assigned in the extracts corresponding to the standard, except for phosphatidylserine (PS). The head group methylene protons of PS, attached to C-1' and C-2', shifted to higher field in the brain lipid spectrum (C-1'^d at 4.26 ppm, C-1'^u at 4.17 and C-2' at 3.75 ppm). This phenomenon could be caused by the presence of paramagnetic metal ions (Fe^{2+} from the accidentally extracted hem group, for instance) or some other causes. For they shifted back to the expected chemical shifts (4.28 and 3.93 ppm) on the addition ("spiking") of standard PS, as revealed in figure 4.4.

All other major lipids belong to this class were successfully identified from their head group signals, which included phosphatidylcholine (PC) (C-1' at 4.22 and C-2' at 3.59 ppm), phosphatidylethanolamine (PE) (C-1' at 4.02 and C-2' at 3.11 ppm) and phosphatidylinositol (PI) (diagnostic resonances from the inositol ring: C-1' at 3.90; C-2' at 4.20; C-3' at 3.40; C-4' at 3.60; C-5' at 3.20 and C-6' at 3.80 ppm). Existence of these lipids was additionally confirmed by their glycerol backbone moiety signals, as labelled C-1^{u/d} (upfield/ downfield), C-2 and C-3 in figure 4.2c. The weight percentage (wt. %) of these lipids in the brain extract derived from the 1D spectra are shown in table 4.1. The wt. % obtained via $^1\text{H-NMR}$ were compared with, and showed a remarkable consistency, with those data obtained via conventional chromatographic (HPLC and GC) methods [Norton & Crammer, 1984].

In this particular study, chemical shifts of lipids such as phosphatidylglycerol (PG), phosphatidic acid (PA) and cardiolipin (CAR) were neither observed in the 1D nor 2D

$^1\text{H-NMR}$ spectra. This indicated that the amount of these lipids in rat brain were below the detection limit of $^1\text{H-NMR}$ (0.10 wt. %). In contrast, a limited quantity of CAR and PA were detected in rat liver extract by $^1\text{H-NMR}$, as described in the previous chapter. As with liver extracts the highly phosphorylated forms of PI (i.e. PIP, PIP₂, PIP₃ etc.) were not identified owing to their low natural abundance.

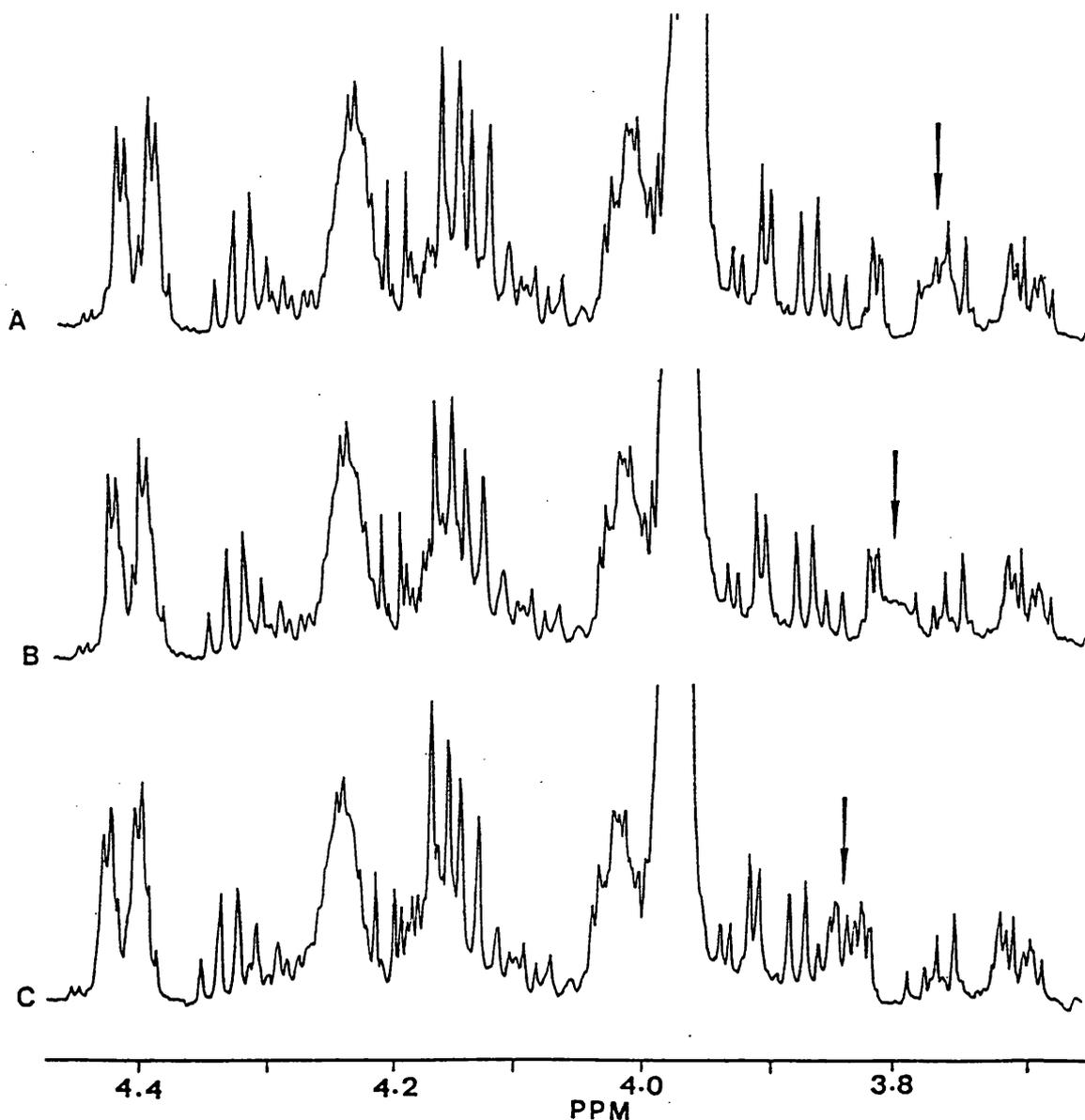


Figure 4.4 (a) Expanded region of a proton NMR spectrum of the brain lipid extract, note the suspected C-2' proton resonance occurred at *ca.* 3.75 ppm. (b) to (c) the same spectrum after gradual addition of standard PS. The arrows indicate the shift of C-2' signal.

4.2.4 Proton NMR spectral assignments: Glycerolipids

Triacylglycerol (TG) is the predominant species of this class, and serves as a major energy reserve. Its metabolites diacylglycerol (DG) and monoacylglycerol (MG) are present in minor quantities in animal cells [Vance & Jacobson, 1988b]. Glycerolipids can be distinguished from each other as well as from their phospholipid counterparts by ^1H -NMR, through the differences between their glycerol backbone moieties resonances. For instance, the glycerol backbone resonances of TG occurred at 4.32, 4.15 and 5.25 ppm, while the corresponding DG signal appeared at 3.65 and 5.10 ppm respectively (fig. 4.3). Thus TG and DG were readily detected in rat liver as discussed in the previous chapter. However, there was no sign of TG, DG or MG in the brain extract spectra as seen in figure 4.1 and 4.2 and hence suggested < 0.10 wt. % (weight percentage) of these lipids are present in the rat brain extracts. Such finding are coincided with existing data [DeWille & Horrocks, 1992].

4.2.5 Proton NMR spectral assignments: Ether phospholipids

Unlike the majority of glycerophospholipids, this particular class of lipids formed an alkenyl or alkyl ether-linkage instead of an acyl-linkage between the hydrocarbon chain and the *sn*-1 glycerol moiety, as depicted by the structural formulae [iii] & [iv]. In contrast with the liver lipid profiles from the previous chapter, significant quantities of ether lipids were detected in brain as expected [Jacobson & Saier, 1988]. Plasmalogen, the principal lipid of this class, was easily identified by its distinctive olefinic resonance (namely protons **a** as shown in formula [iii]) at 5.91 ppm (fig. 4.1b), and the cross-peaks of the 2D HOHAHA spectrum (fig. 4.2) at 5.91/ 4.33 ppm (**a**/ **b** cross-peak), 5.91/ 2.00 ppm (**b**/ **c** cross-peak) and 4.33/ 2.00 ppm (**a**/ **c** cross-peak). The two major plasmalogens, namely, plasmalogen-choline (PLA-C) and plasmalogen-ethanolamine (PLA-E), were not distinguished directly by ^1H -NMR and thus the quantitation of plasmalogen in this report represented a total value of the mixture (table 4.2).





By observing the $^1\text{H-NMR}$ spectra of the lipid standards, discrimination of the saturated ether lipid (as shown in formula [iv]) from its unsaturated analogous (i.e. plasmalogen) should be possible via the 2D cross-peak of methylene protons a/b at 3.44/1.59 ppm. In the 2D spectra of brain lipid extract however, this characteristic signal from the saturated ether lipid was not detected, implying the existence of this particular lipid species is small (< 0.10 wt. %).

4.2.6 Proton NMR spectral assignments: Sphingolipids and Glycosphingolipids

Sphingolipids are lipids which feature a sphingosine (4-sphingenine) or (to a lesser extend) 4-sphinganine, rather than a glycerol, as the backbone moiety. As with their glycerophospholipid counterparts, sphingolipids exhibit a diversity of head groups including phosphocholine (which gives sphingomyelin), phosphoethanolamine (which gives ceramidephosphoethanolamine), and carbohydrates to give a sub-class namely glycosphingolipids, better known as cerebrosides. The carbohydrates most often associated with the cerebrosides are galactose, glucose, *N*-acetylgalactosamine and *N*-acetylglucosamine. Additionally, there is a sub-division of glycosphingolipids called gangliosides, which contain one or more molecules of *N*-acetylneuraminic acid (sialic acid).

The unsaturated sphingolipids in brain can be detected by $^1\text{H-NMR}$, from the structure-specific olefinic resonances at 5.43 ppm and 5.68 ppm, as labelled SPH a and b respectively in figure 4.1b. These two resonances in principle, arise collectively from unsaturated sphingomyelin, ceramidephosphoethanolamine and the unsaturated glycosphingolipids. In practice however, based upon previous investigations [DeWille & Horrocks, 1992], the natural abundance of ceramidephosphoethanolamine in rat brain was found to be low. Furthermore, galactocerebroside, the predominant glycosphingolipid, can be determined separately via the distinctive doublet of the

galactose anomeric C-1' proton at 4.21 ppm. Thus quantitative measurement of sphingomyelin was possible by subtracting the wt. % of galactocerebroside from the wt. % obtained by resonance SPH a or b. The quantitation of the rat brain lipid extract by $^1\text{H-NMR}$ is summarized in table 4.1. Significantly, galactocerebroside was found to be the most abundant lipid in this class, which is in agreement with the conventional chromatographic determinations [Norton & Crammer, 1984; Jardetski & Roberts, 1981]. The total sphingolipids (i.e. both saturated and unsaturated sphingolipids), not measured here, can be estimated from the C-2 amide resonance if $\text{CD}_3\text{OH-CDCl}_3$ is used as solvent.

No trace of gangliosides were detected neither in 1D nor 2D $^1\text{H-NMR}$ spectra. Whether this is attributed to its low natural abundance or due to inefficient extraction is unclear at this stage. Such doubts can potentially be clarified by comparing different extraction methodologies via the $^1\text{H-NMR}$ analysis.

4.2.7 Proton NMR spectral assignment: Steroidal lipids

The brain contains a significant amount of cholesterol which was readily detected in the 1D spectrum by its C-18 methyl resonance at 0.68 ppm (fig. 4.1e). But more specifically, cholesterol was confirmed by the 2D HOHAHA spectrum through the C-3/ C-4 cross peak at 3.40/ 2.22 ppm and the C-3/ C-2 cross peak at 4.55/ 1.82 ppm (fig. 4.2). The weight percentage (wt. %) of cholesterol was calculated from the 1D integrated area of C-18 as 23.60 wt. %.

4.2.8 Analysis of fatty acid composition by proton NMR

A major advantage of the $^1\text{H-NMR}$ lipid profile approach is that considerable amount of information concerning the structure of fatty acid components of brain lipids can be obtained without involving lipid hydrolysis or degradation. The relative quantitation (in wt. %) was determined by assuming the 1D integrated areas of the total CH_3 methyl group and the CH_2COOR resonances at *ca.* 0.87 ppm and 2.30 ppm respectively, as 100.0 wt. %. The quantitation of total unsaturated fatty acids, 44.90 wt. %, was

determined from the intensity of the allylic resonance that begin and end a run of one or more double bonds (as underlined in formula [v]), between 1.98 and 2.10 ppm (fig. 4.1d).



The C-3 methylene group underlined in formula [vi] provided the structural specific resonance for arachidonic acid (20:4) and eicosapentaenoic acid (20:5) at *ca.* 1.65 ppm. Docosahexaenoic acid (22:6) was determined as 6.50 wt. % from its characteristic C-2 methylene (underlined in formula [vii]) resonance at 2.38 ppm. The collective amount of (*n*-3) polyunsaturated fatty acids (i.e. 22:5, 20:5, 22:6 & 18:3) was obtained from their ω (omega)-CH₃ resonance at 0.95 ppm (formula [viii]), which yielded 11.80 wt. %, thus signified that after the elimination of docosahexaenoic acid (22:6) the combined amount of linolenic acid (18:3), eicosapentaenoic acid (20:5) and docosapentaenoic acid (22:5) *ca.* 5.30 wt. %. Linoleic acid (18:2) was determined as 1.90 wt. % from its specific biallylic resonance at 2.74 ppm.

In addition, unsaturated fatty acids where diagnostic resonances were difficult to observe, principally monosaturated, could not be specifically identified and thus were quantified indirectly, by subtracting all detectable fatty acids values from the of total unsaturated fatty acid (44.90 %) and yielded 25.30 wt. %. The quantity of free fatty acids was small, as indicated by the absence of their resonances at 3.48 and 1.16 ppm. The fatty acid composition data are summarized in table 4.2.

4.3 CONCLUSION

Freeze-clamped procedures and a modified Folch extraction were used to extract intact lipids from the homogenized brains of rat: 100.0 mg of brain tissue yield *ca.* 14.0 mg of dried lipids and 70.0 mg of dried lipids dissolved in 0.80 ml of CD₃OD-CDCl₃ (2:1 v/v) was used for the 1D and 2D proton NMR experiments. The weight percentage (wt. %) of different lipids present in the extract was also calculated from the 1D spectra by the mean of integrated area in the 1D spectra.

Among the glycerophospholipid class, PC, PE, PI and PS were unequivocally assigned and quantitatively determined, but CAR, PG, PA and the highly phosphorylated inositols were remained to be individually identified, and estimated as below 0.10 weight percent in the extracted lipid mixture. The structural specific resonances of the individual mono, di and triacylglycerols were also not resolved or observed and their concentrations were similarly estimated as below 0.10 weight percent in the extract.

Table 4.2

Composition of esterified fatty acids from rat brain lipid extract obtained by proton NMR.

Systematic name	Trivial name	Shorthand designation	Content (wt. %)
9,12-Octadecadienoic	Linoleic	18:2 (<i>n</i> - 6)	1.90
5,8,11,14-Eicosatetraenoic	Arachidonic	20:4 (<i>n</i> - 6)	5.90 ^a
5,8,11,14,17-Eicosapentaenoic	-----	20:5 (<i>n</i> - 3)	0.40 ^a
9,12,15-Octadecatrienoic	Linolenic	18:3 (<i>n</i> - 3)	5.30 ^b
7,10,13,16,19-Docosapentaenoic	-----	22:5 (<i>n</i> - 3)	
4,7,10,13,16,19-Docosahexaenoic	-----	22:6 (<i>n</i> - 3)	6.50
Total Unsat. fatty acids	-----	-----	44.90
Monounsatur. fatty acids	-----	-----	25.30 ^a

^aDiagnostic signal was superimposed with signal from other fatty acid classes, thus quantitation was achieved indirectly by subtraction.

^bThe wt. % was obtained from a mixture of two fatty acids.

Significantly, galactocerebroside was distinguished from sphingomyelin and hence was specifically quantitated. However, at this stage of the NMR lipid assay development, cerebroside containing head groups other than galactose, and gangliosides, were not identified or estimated. Whether this is attributed to their low natural abundance or due to inefficient extraction is unclear. Additional experiments such as comparing different extraction methodologies via the $^1\text{H-NMR}$ analysis should prove beneficial.

It has been traditionally complicated to identify and distinguish ether lipids from the corresponding PE, PC, cerebroside and sphingomyelin. This assay did identify each of these structurally-related lipids by their diagnostic resonances and/ or 2D cross peaks that arose from either their *sn*-1 linked moieties, their phospho headgroups or their sphingosine/ glycerol backbone moieties. Sometimes a combination of two or three moieties per molecule was required for unambiguous assignment.

The NMR lipid analysis approach has one other advantage over the conventional lipid profiles based upon solely chromatographic assay procedures — in the intact non-chemically degraded lipid mixtures from the brain, it was possible to obtain significant information on the ratios of saturated to unsaturated fatty acid chains. In addition, different classes of unsaturated chains were identified and quantified from their structure specific methylene, allylic and olefinic groups.

Although many aspects and problems of the extraction procedure and the NMR lipid analysis remain to be explored and solved, it is clear from the data presented in this report that the method is complementary to and has many advantages over other methods of lipid analysis. The lipid profiles via NMR are more comprehensive, more rapidly obtained and yield the relative concentrations of individual lipids. It should be possible to utilize this procedure, or modifications of it, to study the role of lipids in brain biology, neurodegenerating diseases and many branches of medicine.

Chapter 5
1D and 2D proton NMR of lipids from human erythrocytes and blood plasma

5.1 INTRODUCTION

Blood is the most abundant body fluid that plays a central role in body transportation system — it carries nutrients, minerals, oxygen to cells and organs, and removes wastes and toxic materials. Erythrocytes, the oxygen carrying cells, together with white blood cells and hemoglobin-rich cytoplasm contribute about 50 % to the total blood volume. Blood plasma, the liquid portion of blood contains numerous proteins, including albumin and apoproteins. The latter are the main vehicles for body lipid transportation, as they associate with lipids to form lipoproteins. There are five major types of lipoproteins, with various sizes, densities and lipid compositions [Vance, 1988b]. The lipoproteins of lowest density, the chylomicrons, are the largest in size and contain the most lipid and the smallest percentage of protein. At the other extreme are the high-density lipoproteins (HDL). Between these two classes, are the low-density lipoprotein (LDL), the intermediate-density lipoprotein (IDL) and high-density lipoprotein (HDL). Each type of lipoprotein contains different proportions of triglycerides, phospholipids, cholesterol and cholesterol esters.

Lipid and cholesterol levels in blood can provide crucial information concerning one's physiological condition. For instance, disturbed lipid metabolism is recognized as an indicator of atherosclerosis risk [Weisweiler *et al*, 1985; Berenson *et al*, 1980; Connor, 1979] and cardiac diseases [Talmud & Humphries, 1991; Kim & Clapham, 1989; Chin *et al*, 1984]. Moreover, alteration of the lipid compositions in erythrocyte ghosts and blood plasma of diabetic patients have been reported [Watala & Kordacka, 1987; Watala *et al*, 1987; Weisweiler *et al*, 1985; Connor, 1979]. Defects in the lipid organization of erythrocyte membrane leads to diseases such as hereditary haemolytic anaemia [Watala & Józwiak, 1990]. Other lipid related pathological disorders include Niemann-Pick disease [Lehninger, 1981], hepatocellular liver disease [Cooper *et al*, 1974] and high phosphatidylcholine haemolytic anaemia (HPCHA) [Shohet *et al*, 1971].

Although cholesterol and triglyceride levels in blood are routinely measured in clinical chemistry [Grubits, 1992; Gamble *et al*, 1978; Heider & Boyett, 1978; Trinder, 1969], the information provided is limited. Since the levels of other lipid components are found

to have pathological importance, there are obvious reasons for clinical biochemists and physicians to demand a more comprehensive, accurate, yet rapid lipid assay. Techniques such as thin layer chromatography (TLC) [Gurr & James, 1980], high performance liquid chromatography (HPLC) [Samet *et al*, 1989; Christie, 1987a], gas chromatography (GC) [Kuksis *et al*, 1990] and mass spectroscopy (MS) [Harvey, 1991] have been extensively explored for lipid analysis and refined assays based upon these systems have been established. Nevertheless, none of these single approaches can analyze biological lipid mixtures comprehensively and thus a combination of these technique are necessary in order to obtain a more complete lipids profile. Furthermore, lipids which are analyzed via chromatographic approaches are often subjected to irreversible chemical degradations such as lipid hydrolysis.

In contrast, the non-destructive approach of one and two dimensional proton NMR provides an appealing alternative to the labour-intensive, time-consuming traditional chromatographic lipid assays. This has been successfully demonstrated on the total lipid extracts from rat liver and brain, as described in the previous chapters. The analysis of human erythrocytes and blood plasma lipid extracts, based upon the same technique, will be discussed in this chapter.

5.2 RESULTS

5.2.1 *In vitro* $^1\text{H-NMR}$ lipid analysis of human erythrocytes and blood plasma

By applying 1D and 2D proton NMR experiments to the lipid extracts from human erythrocytes and blood plasma, the lipid compositions were determined more comprehensively. In addition, structural information about the esterified fatty acids from intact lipids was simultaneously obtained, without the need for extra experiments or lipid hydrolysis. Significantly, quantitative information about all major lipids and fatty acids was also extracted by measurement of spectral intensities, and compared favorably with the corresponding data obtained by conventional chromatographic assays.

The one dimensional proton NMR and two dimensional COSY spectra of lipid extracted from human erythrocytes are shown in figure 5.2 and 5.3, whereas the same

spectra obtained from blood plasma are shown in figure 5.4 and 5.5 respectively. All assignments were achieved by: (a) comparison with the spectra from individual classes of lipid standard, (b) comparison with the spectra from previous assignments (i.e. lipid extracts of rat liver and rat brain) and (c) the analysis of the two dimensional cross peaks in the erythrocytes and plasma lipid extracts.

5.2.2 Lipid quantitation by proton NMR

The relative concentration of all spectrally assigned lipids and esterified fatty acids were determined and expressed in weight percentage (wt. %). The integrated areas of the diagnostic resonances, which in principle are directly proportional to the lipid concentrations, were measured and subsequently divided by the integrated area of residual CDCl_3 for normalization.

In the cases where no specific diagnostic signals were observable due to excessive overlapping, the quantities were determined alternatively by eliminating the integrated areas of all known components from the overlapped signal-complex, until the stage that only the species of interest remained. Both the human erythrocytes and blood plasma lipid profile analysis were performed on ten individual samples and proved highly reproducible, as indicated by the \pm S.D. values in table 5.1 and 5.2. In general, good agreement was obtained, both qualitatively and quantitatively, between NMR lipid profiles and existing chromatographic data, as again summarized in table 5.1 and 5.2. Absolute amount of lipids was estimated by referring to the wet weight of tissues used for lipid extractions and the dried weight of lipid extracts.

Table 5.1

Comparison of weight percentages* (wt. %) of individual lipids extracted from human erythrocytes determined by HPLC and NMR.

Lipid	NMR ^a	HPLC ^b
PC	28.70 ± 1.2	27.0
PE	17.10 ± 1.0	22.2
PS	3.80 ± 0.8	10.7
LPC	4.40 ± 0.3	3.2
Total unsat. ether lipids ^c	14.50 ± 1.0	-----
Total unsat. sphingolipids ^d	31.00 ± 1.5 ^e	22.7
PC/ CHOL.	0.33 ± 0.0(2)	0.4

*Percentage weight based upon total phospholipids.

^aErrors represented the ± S.D. from the analysis of ten different samples.

^bData obtained from reference [Watala & Józwiak, 1990].

^cPlasmalogen being the principal component.

^dSphingomyelin being the principal component.

^eTotal sphingolipids, not measured here, can be estimated from the C-2 amide resonance if CD₃OH-CDCl₃ is used as solvent.

5.2.3 Proton NMR spectral assignment: Glycerophospholipids

This class of lipids is defined by the presence of their glycerol backbone moiety, with acyl linkages to hydrocarbons at *sn*-1 and *sn*-2 positions, and a phosphodiester head group at *sn*-3 position. Characterization of different lipids within this class is based upon the diversity of their phospho-head groups. Glycerophospholipids commonly observed in animal cells include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidylinositol phosphates (PIP_x), phosphatidylglycerol (PG), phosphatidic acid (PA) and cardiolipin (DAG-P_i-glycerol-P_i-DAG). Lysophospholipids, the glycerophospholipids with one hydrocarbon chain missing at *sn*-2, is also a member of this class. Lipids can be further sub-classified via the variation of the structures and the degree of unsaturation among hydrocarbons (esterified fatty acids).

Since the chemical shifts of *sn*-1 and *sn*-2 backbone protons are very similar between glycerophospholipids, individual species were diagnosed from their specific head group proton resonances. Typical diagnostic signals are head group methylene protons of PC, PE and PS, labelled as 1' and 2'; and inositol ring of PI labelled 1' - 6'. The precise chemical shifts of all diagnostic resonances are summarized in figure 5.1. The quantitation of all detected lipids, calculated on the basis of the peak intensities from diagnostic signals, are listed and compared with corresponding chromatographic data in table 5.1 and 5.2. With a few exceptions, lipid concentrations determined by ¹H-NMR exhibited close resemblance to the published data.

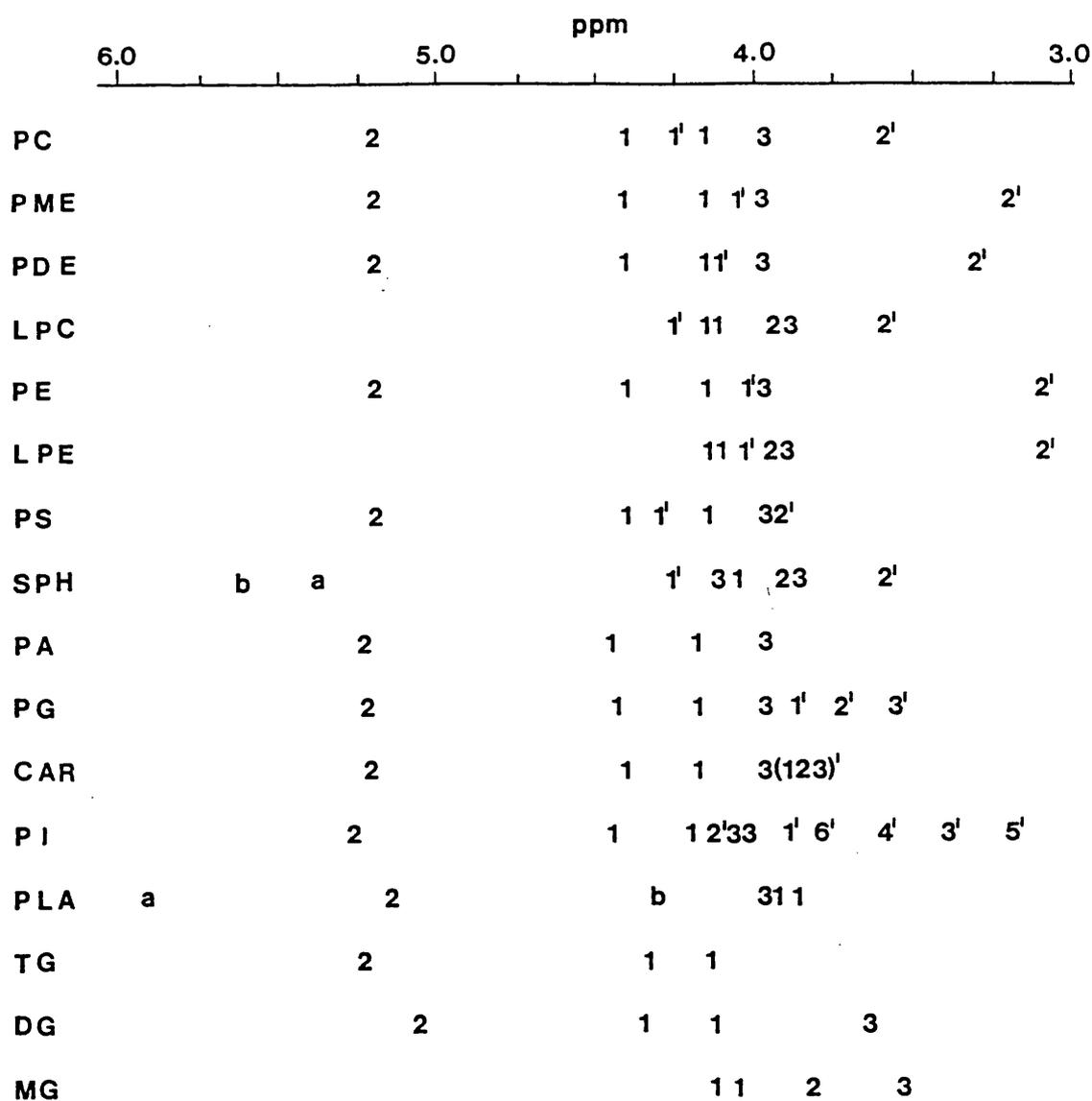


Figure 5.1 Schematic comparison of the resonances from the backbone moieties and the head groups of lipids.

Table 5.2

Comparison of weight percentages* (wt. %) of individual lipids extracted from plasma determined by $^1\text{H-NMR}$, $^{31}\text{P-NMR}$ and HPLC.

Lipid	$^1\text{H-NMR}^a$	$^{31}\text{P-NMR}^b$	HPLC ^c
PC	70.00 ± 3.1	67.00	73.10
PE	-----	2.60	3.90
PI+PS	-----	1.00	3.70
LPC	5.50 ± 0.4	6.80	4.30
Total unsat. ether lipids ^d	5.10 ± 0.3	3.4	-----
Total unsat. sphingolipids ^e	20.90 ± 1.9 ^f	14.20	18.00
PC/ TG	2.3 ± 0.1	-----	-----
PC/(CHOL+CHOL E.)	0.42 ± 0.0(3)	-----	0.50
CHOL. E/ CHOL.	1.72 ± 0.4	-----	-----

*Weight percentage based upon total phospholipids.

^aErrors represented the ± S.D. from the analysis of ten different samples.

^bData obtained from reference [Bradamante *et al*, 1990].

^cData obtained from reference [Watala & Józwiak, 1990].

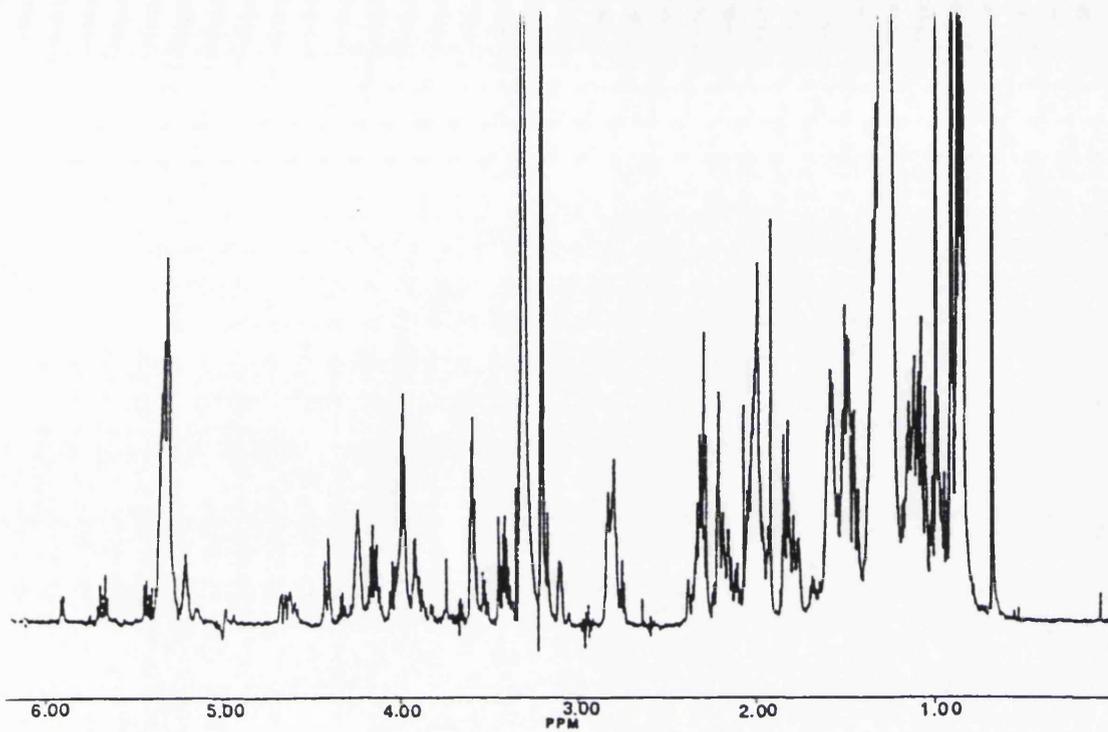
^dPlasmalogen being the principal component.

^eSphingomyelin being the principal component.

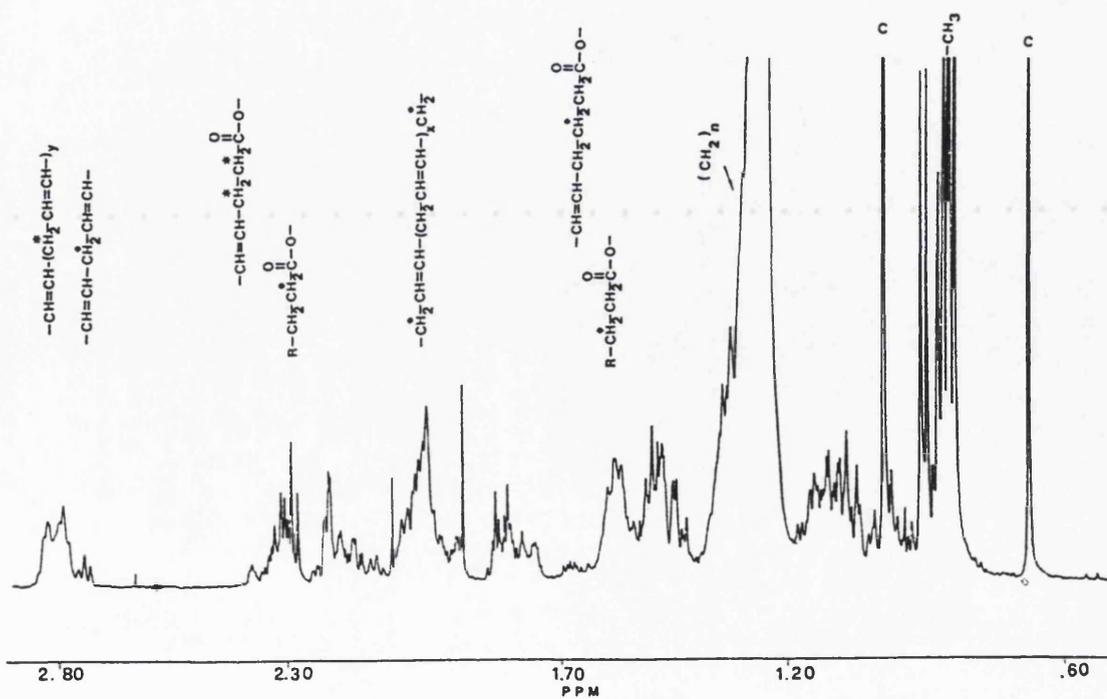
^fTotal sphingolipids, not measured here, can be estimated from the C-2 amide resonance if $\text{CD}_3\text{OH-CDCl}_3$ is used as solvent.

In the proton NMR spectra obtained from human erythrocytes (fig. 5.2 and 5.3), most major phospholipids including PC, PE and PS were unambiguously assigned. Nevertheless, characteristic resonances from PI were not observed in this particular case. Apart from PI, the list of undetected phospholipid species included PG, PA and CAR. Thus the amount of these lipids in human erythrocytes extract was considered to be below the detection limited of $^1\text{H-NMR}$, which is < 0.10 weight percent (wt. %). Such findings are consistent with published chromatographic data [Watala & Józwiak, 1990]. One unusual feature of erythrocyte lipid extracts was the relatively high concentration of lysophosphatidylcholine (LPC), as readily detected in the $^1\text{H-NMR}$ spectrum by its characteristic C-1 resonance at *ca.* 3.95 ppm. In the previous discussions on rat liver and rat brain lipid extracts, evidence of PS and its diagnostic resonances were obscured either

(a)



(b)



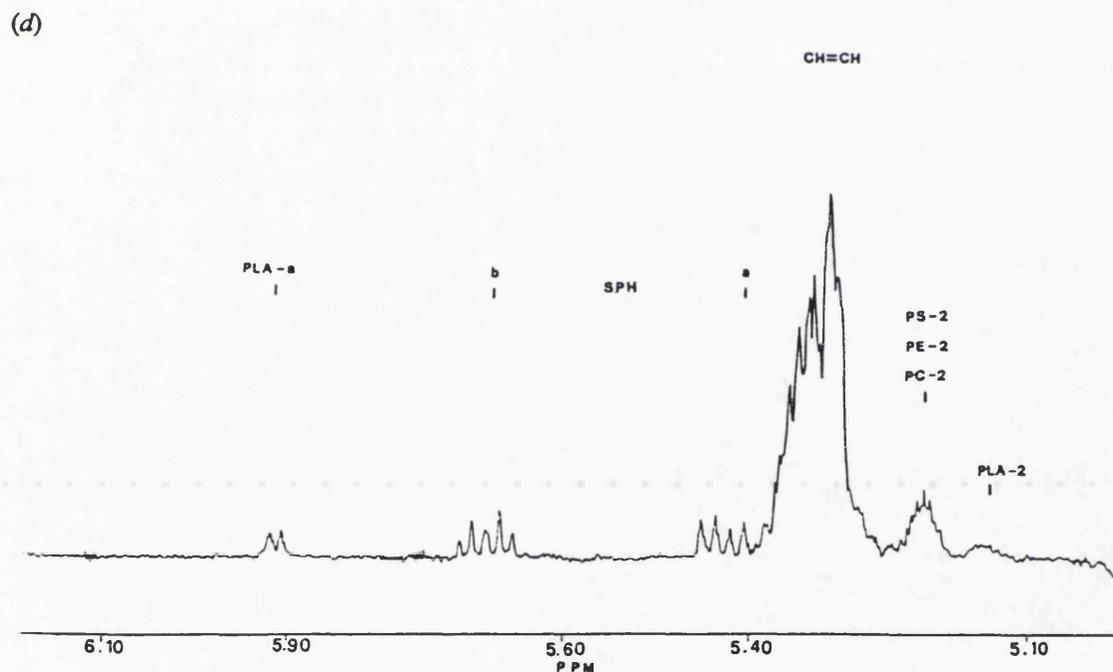
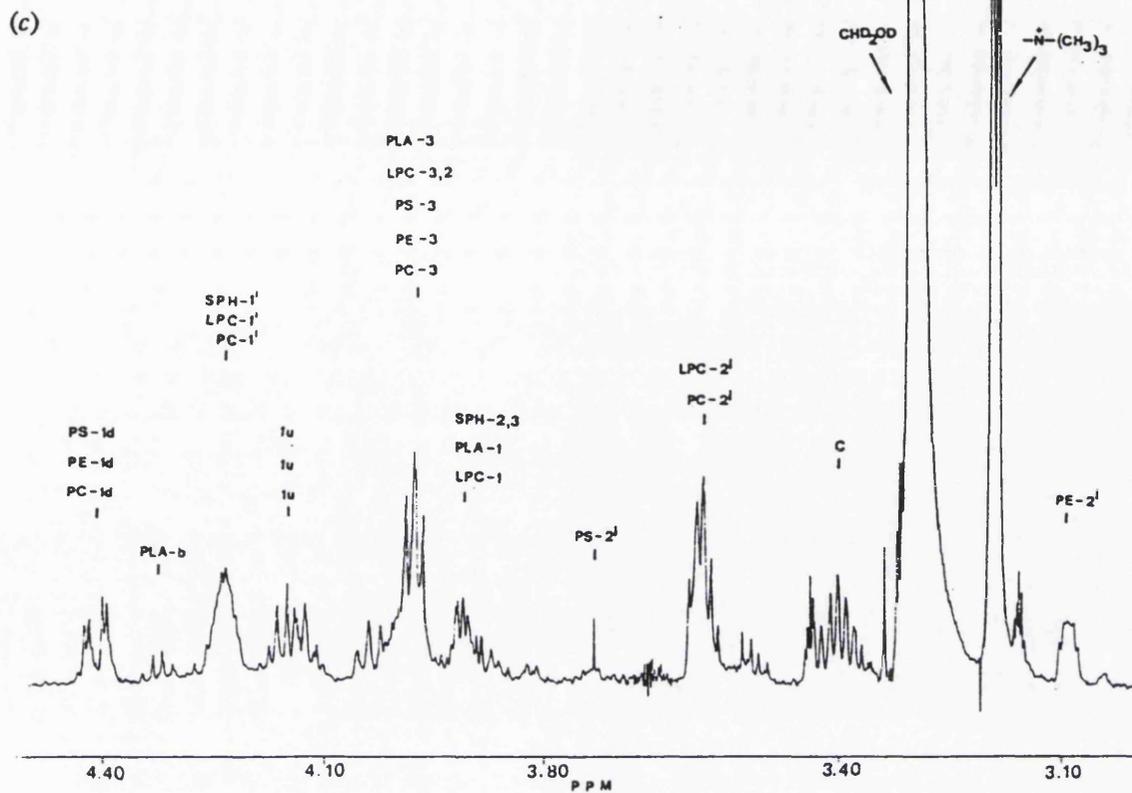


Figure 5.2 (a) One-dimensional proton NMR spectrum of lipids from human erythrocytes extracts [0.50 g of lipid extract in 0.80 ml of $\text{CDCl}_3\text{-CD}_3\text{OD}$ (1:2)]. (b) to (d) Expansions from spectrum (a) showing resonance assignments. The designations μ or \underline{d} refer to the upfield and downfield protons of a CH_2 group; asterisk indicates the specific resonance in the fatty acid; \underline{x} and \underline{y} are used to indicate the number of repeated chemical group, where $\underline{y} \geq 2$ and $\underline{x} > 1$; nomenclature such as PI-1' and PC-2' are used to designate those protons attached to the specific phospholipid head groups; other designations are explained in the text.

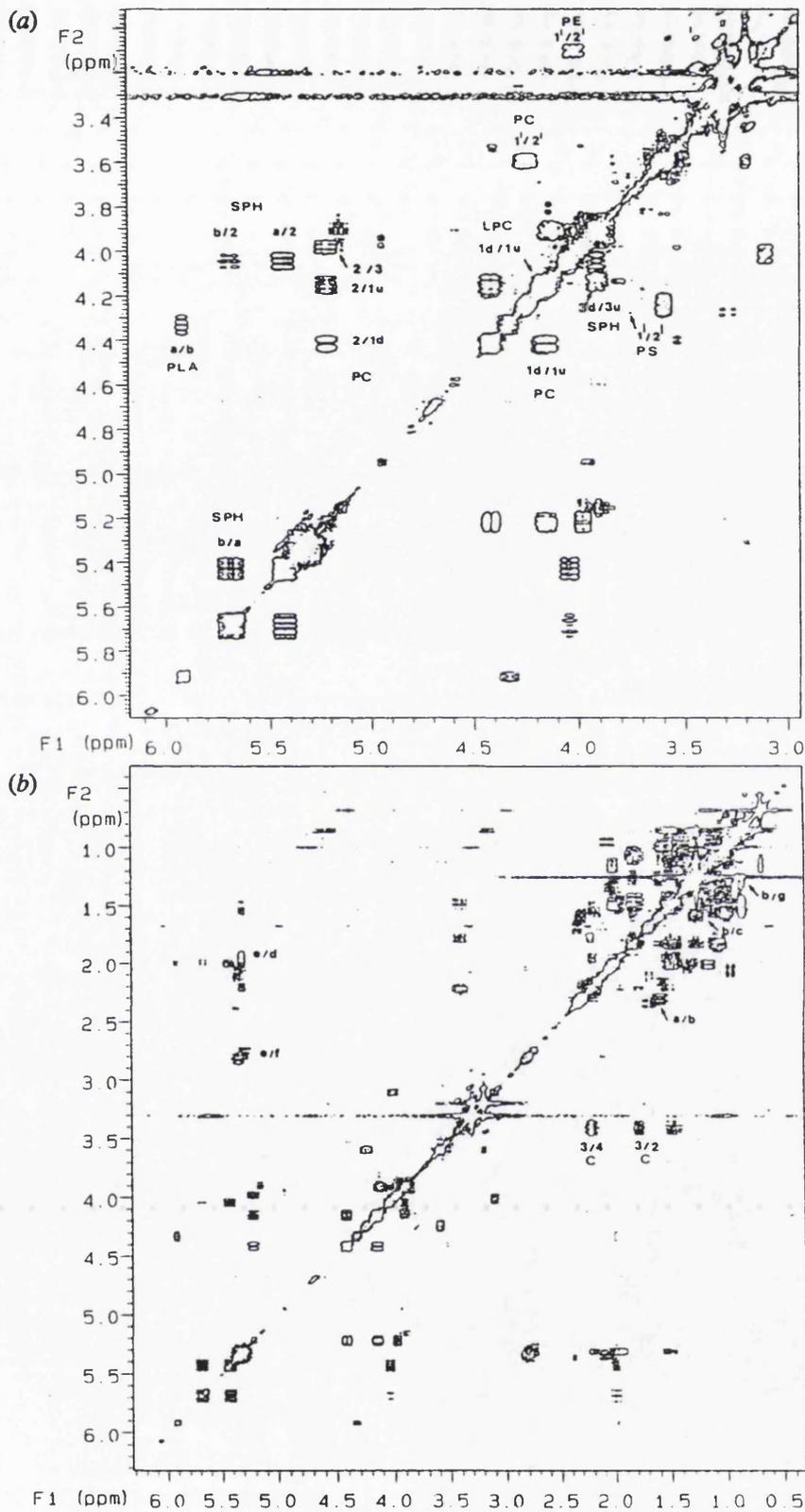


Figure 5.3 (a) Expansion of the 2D-COSY spectrum of lipid extracts from human erythrocytes showing the major head group resonances of glycerol, serine, ethanolamine, choline, with the backbone resonances of LPC, glycerophospholipids, ether glycerophospholipids and sphingolipids. (b) Expansion of the 2D-COSY spectrum of human erythrocytes lipid extract showing diagnostic cross peaks of cholesterol.

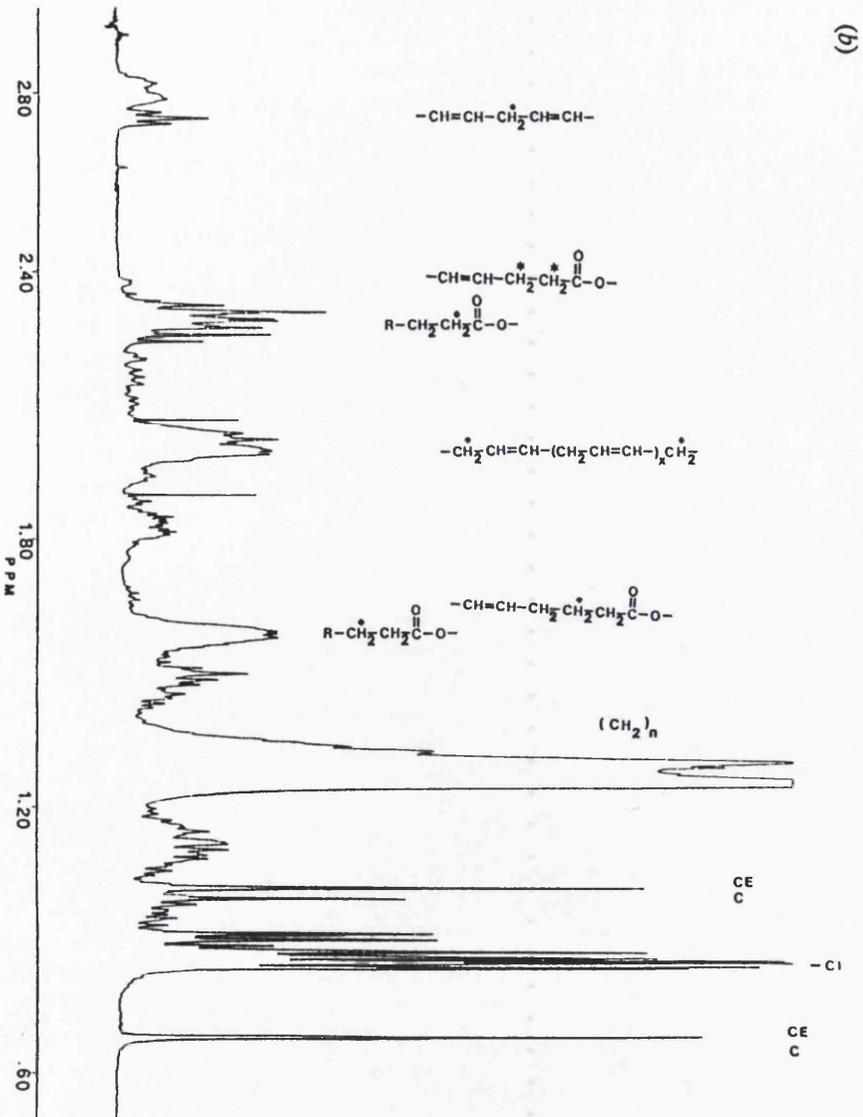
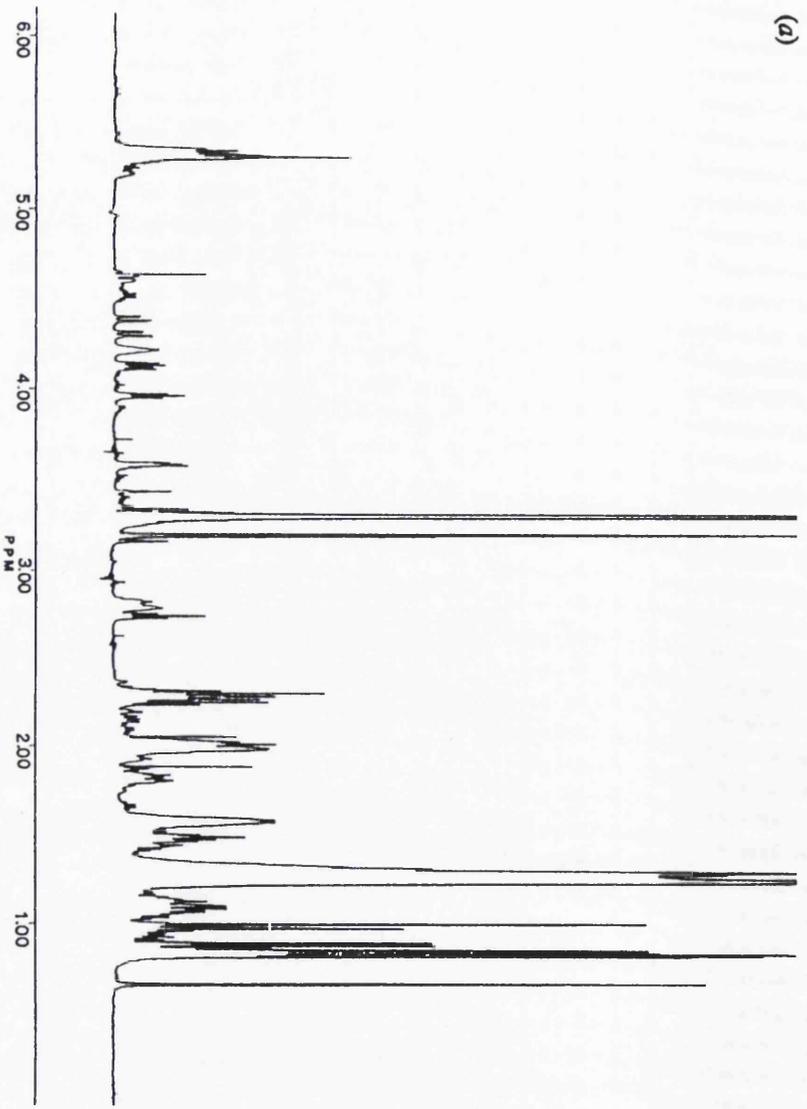
due to the lack of abundance or extensive overlap with other lipid resonances. In the erythrocyte lipid spectra however, the resonance signal generated from C-2' methylene protons at 3.75 ppm was unequivocally assigned (fig. 5.2c).

The proton NMR spectra of blood plasma showed a relatively simple spectral patterns of the phospholipid head group and backbone regions (fig. 5.4 and 5.5). The only principal phospholipid detected in the plasma lipid extract was PC, which contributed *ca.* 70.0 wt. % to the total phospholipid content, with a minor amount of LPC also detected (*ca.* 5.50 % wt. %). The lipid composition of blood plasma determined by ¹H-NMR is summarized and compared with established data in table 5.2.

5.2.4 Proton NMR spectral assignments: Glycerolipids

Glycerolipids are glycerol based lipids with acyl hydrocarbon chains attached at all three *sn*-positions. Therefore unlike their phospholipids counterparts, glycerolipids have no phospho-head groups hence diagnosis were accomplished by distinguishing their glycerol backbone moieties resonances. For instance, the glycerol backbone resonances of TG occurred at 4.32, 4.15 and 5.25 ppm, while the corresponding DG signal appeared at 3.65 and 5.10 ppm respectively (figure 5.1).

As expected, neither TG nor its metabolites (i.e. DG and MG) were observed in human erythrocytes lipid extract. Such findings are confirmed by existing data [Watala & Józwiak, 1990]. In comparison, a significant amount of TG was detected in blood plasma, as clearly shown by its characteristic C-1^d(downfield) resonance at *ca.* 4.30 ppm. The ratio of PC to TG in this particular study was 2.30 with \pm S.D. of 0.10, within the normal range of human TG blood levels [Vance, 1988b]. Again, no DG or MG was detected in blood plasma, thus suggesting the quantities of these lipids were below 0.10 wt. %.



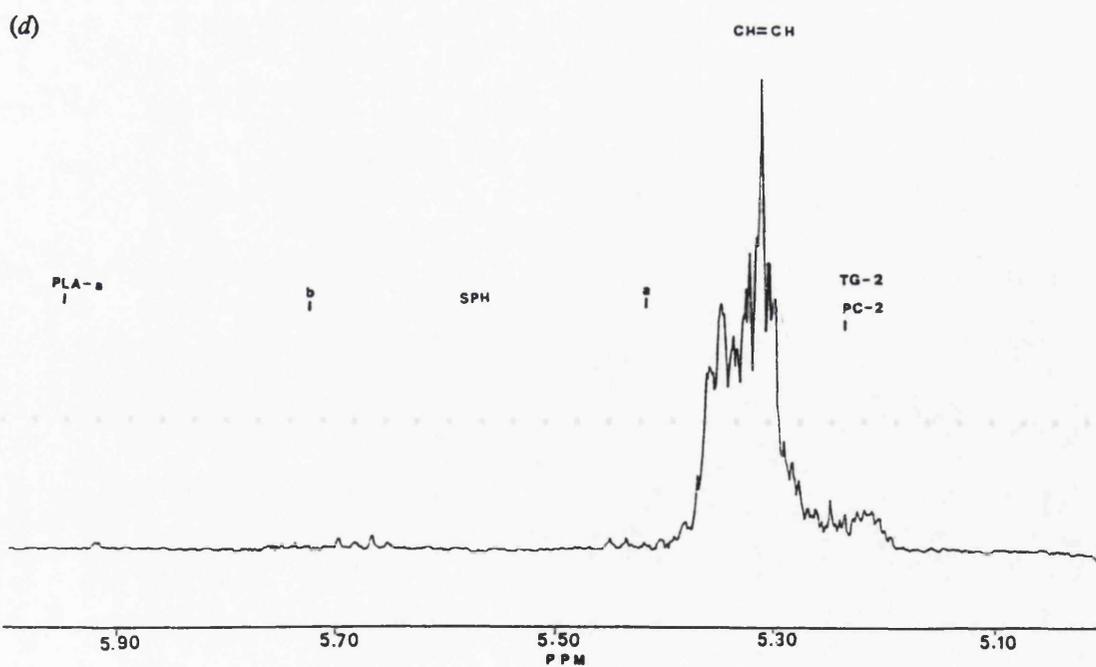
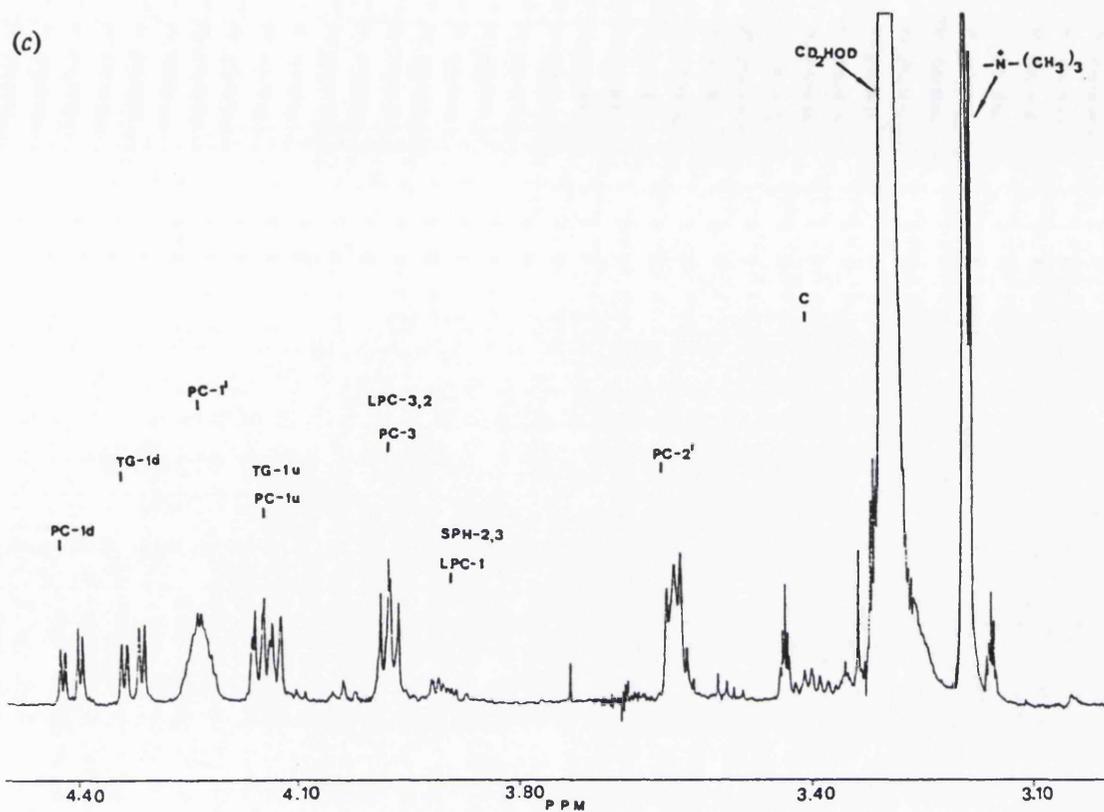


Figure 5.4 (a) One-dimensional proton NMR spectrum of lipids from human blood plasma extracts [0.50 g of lipid extract in 0.80 ml of $\text{CDCl}_3\text{-CD}_3\text{OD}$ (1:2)]. (b) to (d) Expansions from spectrum (a) showing resonance assignments. The designations u or d refer to the upfield and downfield protons of a CH_2 group; asterisk indicates the specific resonance in the fatty acid; χ and γ are used to indicate the number of repeated chemical group, where $\gamma \geq 2$ and $\chi > 1$.

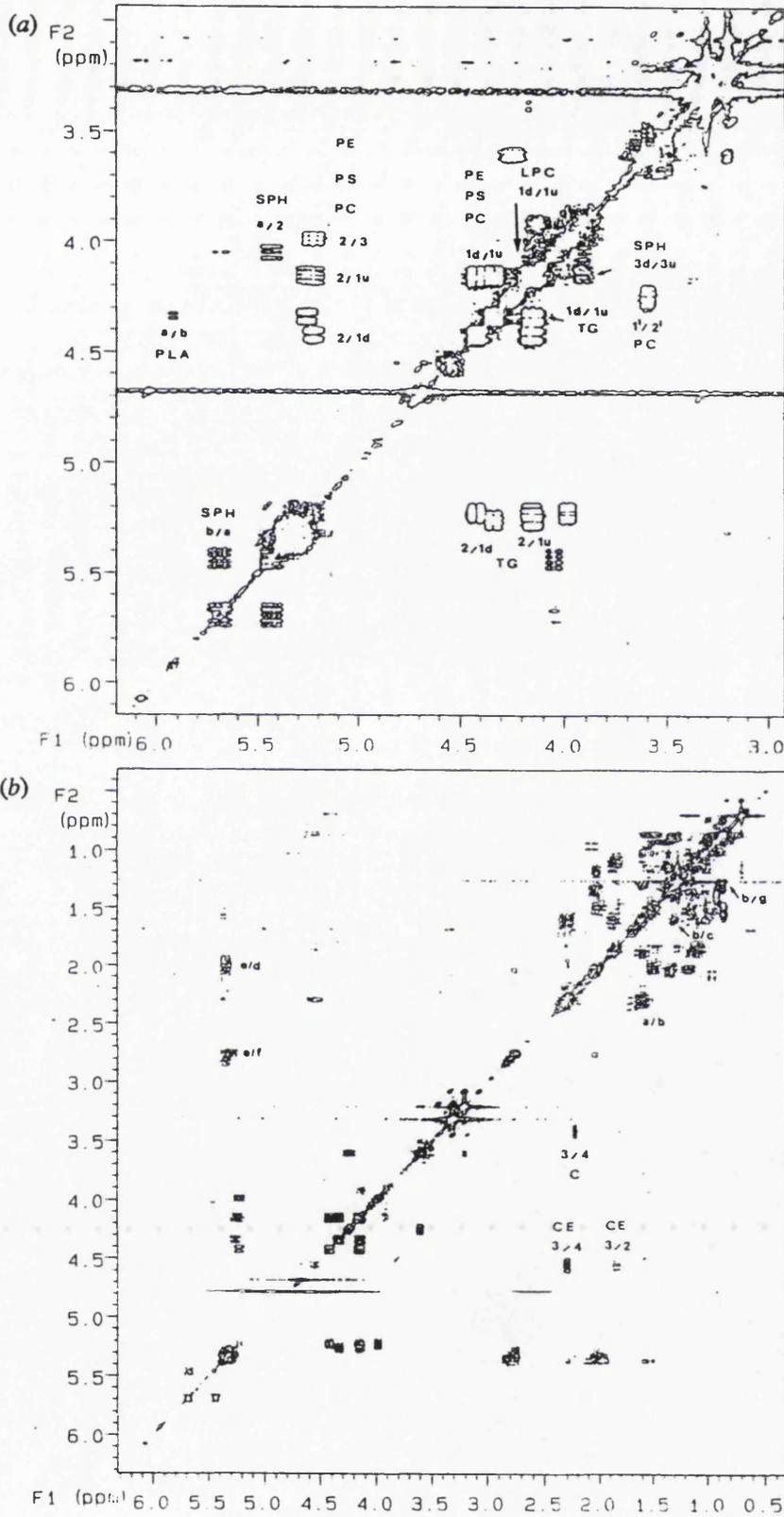


Figure 5.5 (a) Expansion of the 2D-COSY spectrum of lipid extracts from human blood plasma showing the major head group resonances of choline, with the backbone resonances of TG, LPC, glycerophospholipids, ether glycerophospholipids and sphingolipids. (b) Expansion of the 2D-COSY spectrum of human erythrocytes lipid extract showing diagnostic cross peaks of cholesterol and cholesterol ester.

5.2.5 Proton NMR spectral assignments: Ether lipids

These are glycerophospholipids in which the hydrocarbon chains (fatty acids) are connected to the glycerol backbone via an alkenyl or alkyl ether linkage. These two classes of ether lipids can be readily identified via their diagnostic resonances **a** and **b**, as illustrated in formulae [i] and [ii] below:



The 2D COSY **a**/**b** cross peak of alkenyl ether lipids occurred at 5.90/ 4.30 ppm, while the corresponding cross peak from alkyl ether lipids occurred at 3.44/ 1.60 ppm. The majority of ether lipids exist in the alkenyl form, with plasmalogen being the principal lipid of this class. This was confirmed by the distinctive resonances generated by the plasmalogen and the absence of diagnostic cross peak from alkyl ether lipids in both erythrocytes and plasma samples. The level of plasmalogen in blood was found to be relatively high in comparison with other tissues, particularly in the erythrocytes lipid extract where 14.50 wt. % was observed via ¹H-NMR.

There are two ether lipid classes in term of phospho-head group, namely phosphocholine and phosphoethanolamine. Unfortunately, both species were not discriminated by ¹H-NMR at this stage of development. However, the slight splitting of the choline N⁺(CH₃)₃ chemical shift, observed at 3.20 ppm, suggested choline-contained phospholipids (including plasmalogen) can be characterized individually if the lipid sample was placed in a stronger magnetic field.

5.2.6 Proton NMR spectral assignments: Sphingolipids

Instead of having glycerol as the backbone structure, sphingolipids contain a 4-sphinganine or more commonly, a sphingosine (4-sphingenine) backbone moiety. The labelling of the two sphingolipid moieties are summarized in formulae [iii] and [iv]:



Sphingomyelin, the principal compound in this class, was detected in both erythrocytes and blood plasma extracts. The characteristic resonances from the 1D spectrum included proton **b** at *ca.* 5.70 ppm and proton **a** *ca.* 5.42 ppm, and the cross peak resonances from 2D COSY at 5.38/ 5.63 ppm (**a**/ **b**), 5.63/ 1.96 ppm (**b**/ **c**) and 5.38/ 3.97 ppm (**a**/ C-1). As with plasmalogens, the level of sphingolipids in human blood was found to be higher than in other tissues. The sphingomyelin content in erythrocytes was determined to be *ca.* 31.0 wt. % within total phospholipids, and *ca.* 20.90 wt. % in blood plasma by ¹H-NMR.

There are other classes of sphingolipids, namely cerebroside and gangliosides, which have carbohydrate rather than phosphodiester moieties as the head group. Low concentration of these sphingolipids are found in most animal cells (except in brain tissue), and blood plasma and erythrocytes are no exception. Thus the quantities of these lipids, undetected by ¹H-NMR, are estimated < 0.10 wt. %.

5.2.7 Proton NMR spectral assignments: Steroidal lipids

The levels of blood cholesterol and cholesterol esters are probably two of the most commonly measured parameters from the clinical point of view; such tests are performed routinely in clinical biochemistry [Grubits, 1992; Gamble et al, 1978; Heider & Boyett, 1978; Trinder, 1969]. Significantly, ¹H-NMR allowed the identification and quantitation of these two compounds, with the bonus of yielding corresponding information on all other major lipids and fatty acids simultaneously.

Cholesterol and cholesterol esters were detected by ¹H-NMR, from their characteristic C-18 methyl resonance at 0.68 ppm, and cholesterol ester specifically at *ca.* 4.60 ppm in the 1D spectrum. The 2D COSY spectrum provides a complementary

confirmation on cholesterol by the cross peaks at 3.40/ 2.22 ppm (C-3/ C-4) and 1.76/ 3.40 ppm (C-2/ C-3), and cholesterol ester by the cross peaks at 4.55/ 4.25 ppm (C-3/ C-4) and 1.82/ 4.55 ppm (C-2/ C-3). The ratio of PC to cholesterol in the erythrocyte lipid extracts was determined as 0.33 by $^1\text{H-NMR}$, versus the ratio of 0.4 obtained by HPLC assay [Watała & Józwiak, 1990]. In the case of blood plasma, the ratio between cholesterol ester and cholesterol was found 1.72 by $^1\text{H-NMR}$, and the ratio of PC to cholesterol plus cholesterol ester being 0.42, which is comparable with the corresponding HPLC value of 0.50.

5.2.8 Analysis of fatty acid composition by proton NMR

Identification and quantitation of fatty acids from biological lipid mixtures has traditionally been complicated and time-consuming, since lipid extracts are subjected to chemical hydrolysis in order to release fatty acids for the chromatographic determination. In contrast lipid analysis via proton NMR can readily provide the structural information and relative concentration of fatty acids from intact lipids, without the need for extra experimental procedures such as chemical hydrolysis. Thus proton NMR lipid profiles are an appealing alternative for *in vitro* lipid analysis.

The overall quantity of fatty acid chains was determined from the 1D spectrum using the areas of the total CH_3 resonance at 0.87 ppm. The C-2 methylene (immediately after the acyl group) resonance at 2.30 ppm provided the total amount of esterified fatty acids. The weight percent of total unsaturated fatty acid chains was obtained as 46.90 % in erythrocytes and 50.90 % in plasma by integration of the terminal allylic CH_2 groups (see formula [v]) at *ca.* 2.00 ppm. Free fatty acids present in blood plasma were obtained by the difference between the total ($-\text{CH}_3$) and esterified fatty acids (C-2 methylene), which yielded 23.80 wt. %. Various specific fatty acids structures are illustrated in the following formulae:





Several highly unsaturated fatty acids were also identified and quantified specifically by $^1\text{H-NMR}$. For instance, arachidonic acid (20:4) and unsaturated fatty acids with the structure of formula [vi] were quantitatively measured from the resonance at *ca.* 1.65 ppm, as 16.20 wt. % was found in the erythrocytes and 11.90 wt. % in the blood plasma respectively. Linoleic acid (18:2) was detected by its structure-specific allylic resonances at 2.74 ppm, 7.70 wt. % of this particular fatty acid was found in erythrocytes and 19.80 wt. % was found in blood plasma. The *n*-3 fatty acids were measured collectively via the structure-specific C-2 methylene protons at 2.38 ppm, and gave 3.10 wt. % for the erythrocytes and 1.40 wt. % for the blood plasma.

5.3 DISCUSSION

Human erythrocytes and blood plasma were taken from 10 healthy individuals and the lipid extractions were carried out according to the Folch *et al* (1957) methodology. All 1D and 2D experiments described in this chapter were performed with *ca.* 50.0 mg of dried lipid extract dissolved in 0.80 ml of $\text{CD}_3\text{OD-CDCl}_3$ (2:1 v/v) at 30°C. The weight percentages (wt. %) of individual lipid species were measured by the spectral intensities (i.e. integral areas) and subsequently divided by the residual CDCl_3 at 7.70 ppm for normalization. With a few exceptions, the quantitations obtained by $^1\text{H-NMR}$ were compatible with existing literature values, particularly for the major lipids. Moreover, quantitation of lipid was highly reproducible, as indicated by the \pm S.D. values in tables 5.1 and 5.2.

There are striking differences between the erythrocytes and blood plasma in terms of lipid composition, as revealed by their distinctive $^1\text{H-NMR}$ lipid profiles. Whilst erythrocytes exhibited a larger diversity of phospholipids in regard to their higher cell membrane content, blood plasma contained a high concentration of triglyceride and

cholesterol principally from its lipoprotein components. PC, the highest abundant phospholipid was unequivocally assigned in both extracts as expected, while PE and PS were observed only in erythrocyte extracts. Significantly, the erythrocytes spectrum provided convincing evidence for the otherwise obscure PS assignment mentioned previously. PI, PG, PA and CAR were not detected in neither erythrocytes nor blood plasma extracts, and hence suggested the concentrations of these lipids are < 0.10 wt. % of total phospholipids (recalled that *ca.* 50.0 mg were used), which is below the detection limit of $^1\text{H-NMR}$ as used here.

There is one lipid species that exists in erythrocytes and plasma which was undetected in previous chapters, namely lysophosphatidylcholine. This lipid occurred in erythrocyte extracts at high concentration and was assigned in both 1D and 2D spectra. An observable, but considerably smaller amount of LPC was also detected in the blood plasma. In addition, relatively higher levels of sphingomyelin and plasmalogen were observed in these two extracts. Despite the drawback that the choline and ethanolamine derivatives of these lipids were unresolved at this stage of assay development, the potential exists since a minor splitting occurred in the choline $\text{N}^+(\text{CH}_3)_3$ chemical shift (3.20 ppm), presumably from different choline containing lipids. Such splitting will expected to increase when the sample is subjected to a higher applied magnetic field (> 500 MHz), and eventually should be sufficient for specific characterization.

The *in vitro* proton NMR approach of blood lipid analysis not only yielded the traditional triglyceride and cholesterol lipid levels but also demonstrated several additional features. The principal lipids of each class, neutral phospholipids and some acidic phospholipids for instance, were simultaneously determined. Equally important was the detection and measurements of the fatty acid composition in the blood lipids including the ratio of saturated and unsaturated fatty acids, and the concentrations of individual polyunsaturated fatty acids.

More attention has been paid on the development of lipid and related compounds analysis in clinical biochemistry than ever, since recent studies have emphasized the importance of lipids in cell regulation [Edwards & Crumpton, 1991; Nesmeyanova &

Bogdanov, 1989; Neer & Clapham, 1988], signal transduction [Schulam *et al*, 1991; Exton, 1990; Nigam *et al*, 1990; Pacini *et al*, 1990; Radab-Thomas *et al*, 1987], their association with various pathological conditions [Talmud & Humphries, 1991; Watala & Kordacka, 1987; Watala *et al*, 1987; Weisweiler *et al*, 1985; Chin *et al*, 1984; Berenson *et al*, 1980; Connor, 1979] and with major biological events such as ageing [Snyder, 1985; Naccache *et al*, 1985; Hallam *et al*, 1984]. The conventional chromatographic lipid assays available at present, although well established, are often highly labor-intensive and time consuming. In comparison, the NMR lipid profile approach has in its favour the advantages of rapidity, comprehensiveness, and a non-destructive nature. Even at this early stage of development, with the minor drawbacks of sensitivity and a few unresolved lipid sub-classes, $^1\text{H-NMR}$ remains a highly appealing complementary technique for the *in vitro* blood lipid analysis. With refinements or modifications, research on biological lipids and metabolism via $^1\text{H-NMR}$ can be expanded to genetic diseases, physiological process of ageing or to the pathological status such as hypertension, arteriosclerosis, diabetes etc.

Chapter 6

**Neutral lipids, non-acidic and acidic phospholipids
analysis from rat liver by 1D and 2D proton NMR &
Bond Elut ion-exchange chromatography**

6.1 INTRODUCTION

Lipid has traditionally been regarded as merely a structural component of cell and body tissues. Phospholipids being one of the major constituent of the cellular membrane, and the triglycerols serve as efficient reserves for the storage of energy. Sphingolipids are important components of the myelin sheath, a multilayered membranous structure that protects and insulates nerve fibres. However, this point of view has changed radically since the last decade. Many of the vitamins and hormones found in animals are lipids or derivatives of lipids. Recent discoveries such as phosphatidylinositol cycle [Exton, 1990]: activation of protein kinase C by diacylglycerol and intercellular calcium flux by inositol phosphates [May & Calder, 1993; Lameh *et al.*, 1990; Neer & Clapham, 1988], arachidonic cascade [Choi, Gao & Gibbons, 1991; DuBourdieu & Morgan, 1990; Rustenbeck & Sigurd, 1989; Kurachi *et al.*, 1989]: whilst arachidonic acid and its derivatives serve as the cellular signal messengers [Barbour *et al.*, 1989; Williams *et al.*, 1989; Bevan & Wood, 1987]; include the release of prostaglandins by cyclooxygenase as lipid hormones, with leukotrienes and lipoxins derived from arachidonic acid by 5-lipoxygenase as the potential mediators of inflammation and hypersensitivity reactions [Piomelli *et al.*, 1987], and direct/ indirect regulation of ion channels by fatty acids [Ordway *et al.*, 1991; Kurachi *et al.*, 1989] etc., all emphasize the importance of lipid as the potent molecule in biological systems.

In vitro lipid assays are increasingly used as a more rapid and comprehensive technique for monitoring lipid compositions of cells, body fluids and tissues. The commonly used techniques like thin layer chromatography (TLC) [Touchstone, 1973], high performance liquid chromatography (HPLC) [Christie, 1985], ion-exchange chromatography [Bandi & Ansara, 1985] and gas chromatography [McGrath & Elliott, 1990] provide a simultaneous detection and quantitation of several lipids in a given sample or lipid class [Gurr & James, 1989; Christie, 1987a; Christie, 1987b]. However, most lipid assays are time consuming and usually more than one single step is needed for a comprehensive analysis. Recently, nuclear magnetic resonance spectroscopy (NMR) has been demonstrated to provide a rapid and comprehensive lipid assay on various

biological tissues and body fluid. Relative quantification of phospholipids by one-dimensional phosphorus NMR spectra [Meneses & Glonek, 1988; Stoiros *et al*, 1986], and assignments of the 1D and 2D proton NMR spectra of lipids from cell and tissue extracts have been reported [Casu *et al*, 1993; Casu *et al*, 1991; Daniel & Jardetzky, 1990].

Although this NMR approach to lipid analysis is promising there are several drawbacks still to be overcome. For instance, only the fatty acid compositions from total intact lipid mixtures are readily detected by NMR, further discrimination of fatty acid compositions between individual lipid classes is not possible. It would be propitious to distinguish, say, the fatty acid composition of triglycerides from their glycerophospholipids counterparts. Furthermore, parts of the 1D spectrum are excessively overlapped (fatty acid methylene region and phospholipid head group region in particular) even under the high magnetic field of 500 MHz. Such drawbacks can be overcome by applying a simple ion exchange chromatographic procedure using commercial solid phase ion-exchange (Bond Elut) columns [Egberts & Buiskool, 1988; Kates *et al*, 1988; Kaluzny *et al*, 1985] prior to the NMR assay. Lipids are separated into four individual classes according to their polarity. Thus improved and more specific analysis of individual lipids in each class can be achieved. The recovery after column separations was found to be high and reproducible, and the NMR analysis of each fraction gave improved results both quantitatively and qualitatively due to reduced overlap and the separations of low abundance lipids from each other.

6.2 RESULTS AND DISCUSSIONS

6.2.1 Calculations and quantitation of lipids

Linear baseline correction was applied to all proton 1-D spectra prior to integral calculations. The relative concentrations of individual lipids were determined by measuring the integral at their characteristic chemical shift. Integration of CDCl_3 was used as an internal standard for spectral normalization. Hence all quantitation described in this chapter were expressed in weight percentage (wt. %).

6.2.2 Proton NMR of rat liver subjected to Bond Elut Chromatography

The published NMR profiles of liver [Casu *et al.*, 1991], brain [Casu *et al.*, 1993] and macrophages [Daniel & Jardetzky, 1990], carried out by one and two dimensional techniques provided a rapid and quantitative assay for most of the major classes of lipids in cells. The spectra also yielded information on the fatty acid composition of the intact lipids. Eventually 2D NMR analysis should yield even more information. There were however disadvantages to this purely NMR approach to lipid analysis, including a) fatty acid analysis was only of total lipid and not of each class of lipid, b) some low abundance lipids, even in 2D NMR spectra, were difficult to identify and quantitate, c) acidic and highly acidic lipids, such as phosphatidylserine, cardiolipin, PG, PA and the highly phosphorylated inositol lipids were not quantitatively analyzed, d) the majority of glycolipids were not identified, e) saturated and unsaturated sphingolipids and ether lipids were not distinguished and quantitated specifically.

Solid phase ion-exchange chromatography, such as DEAE, has been used for lipid separation [Christie, 1987b]. A commercial available pre-pack column, Bond Elut with NH-aminopropyl silica was chosen for this particularly study. Bond Elut ion exchange chromatography can efficiently fractionate lipids into four classes, according to their differences in polarity: Class I — Neutral lipids especially glycerides and steroids, Class II — Free fatty acids, Class III — Non acidic phospholipids and Class IV — acidic phospholipids. The one dimensional proton NMR spectrum of total liver lipids is shown expanded in figure 6.1. Figure 6.2 shows the comparison of this spectrum and the four chromatographic fractions.

6.2.3 Proton NMR of rat liver: Glycerolipids and Steroidal lipids (Class I)

In the rat liver, the most abundant glycerolipid and steroidal lipid are triacylglycerol and cholesterol respectively, as discussed in the earlier chapters. Thus the efficiency of the first Bond Elut fractionation, also checked by TLC, was readily seen by the presence of marker cholesterol resonances at 0.70 ppm and glycerides resonances 4.30 ppm in figure 6.2b and not figure 6.2c, d & e. Significantly the figure 6.2b and c spectra

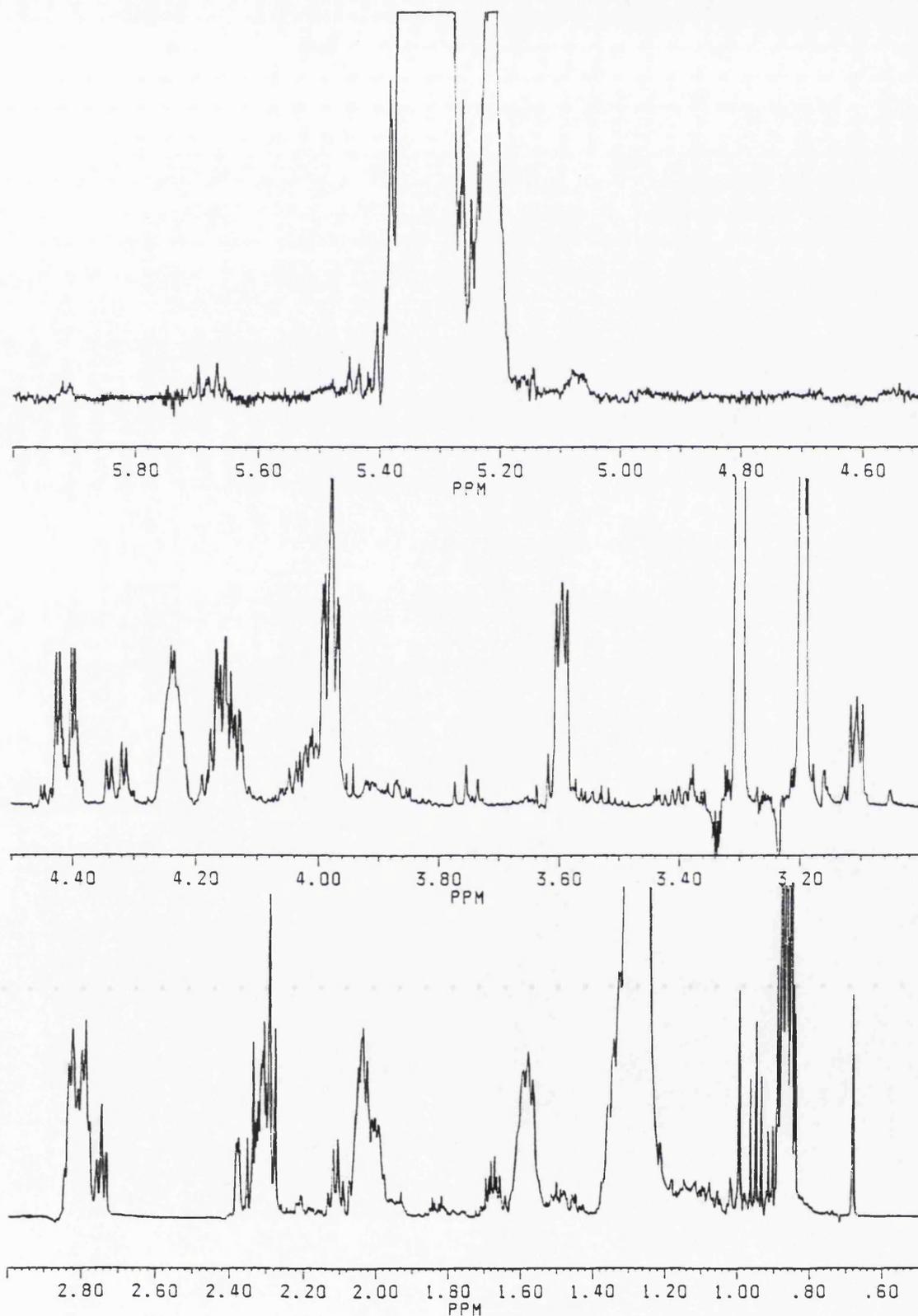


Figure 6.1 Expanded 1D proton NMR spectrum of lipid extract from rat liver. 50.0 mg of lipid extract in 0.80 ml of $\text{CD}_3\text{OH}-\text{CDCl}_3$ (2:1 v/v) was used. Spectrum was recorded at 30°C .

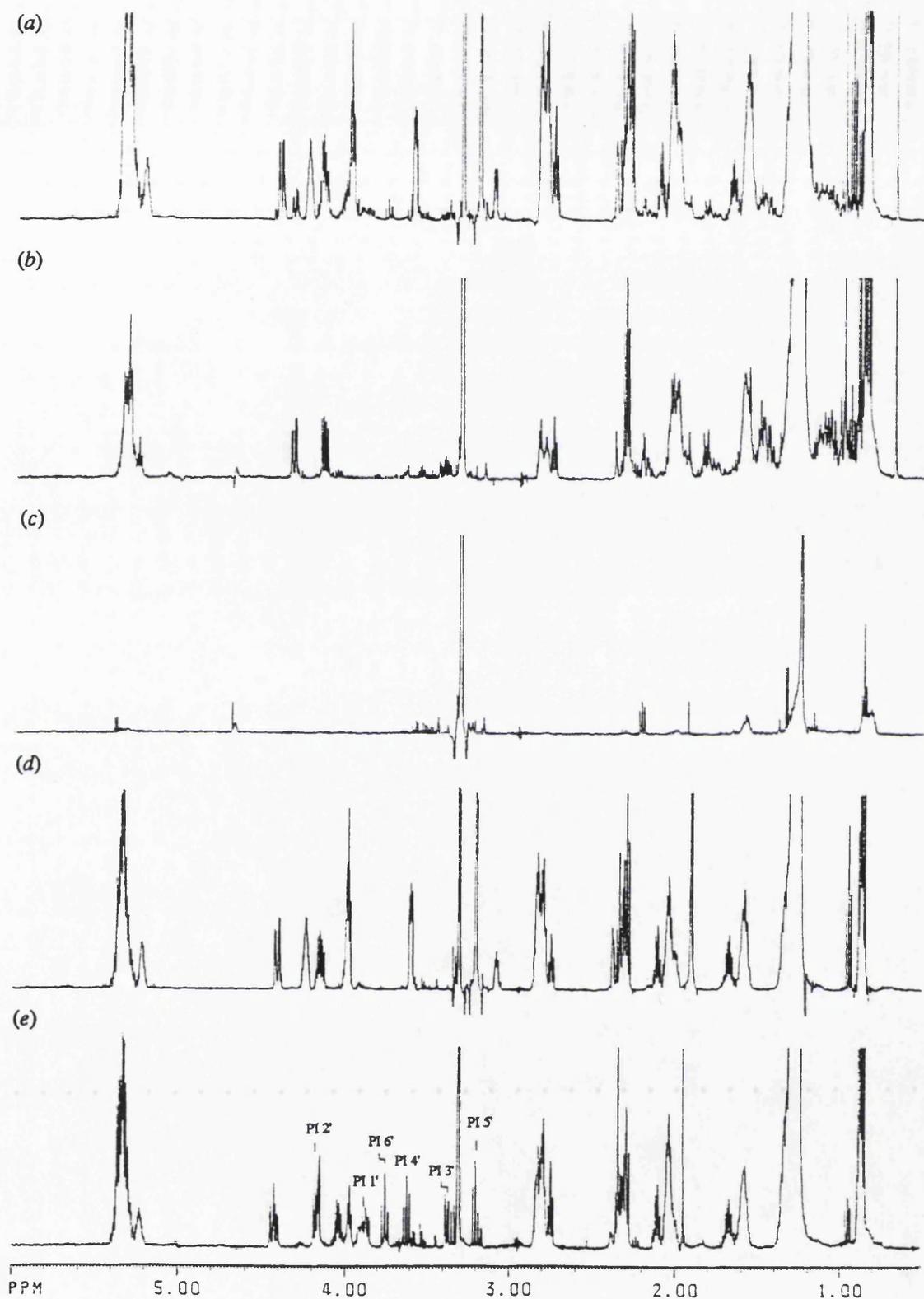


Figure 6.2 1D NMR spectra of rat liver extract with and without Bond Elut separation. (a) Spectrum of lipid extract without separation. (b) Spectrum of fraction I (eluted glycerolipids and cholesterol). (c) Spectrum of fraction II (eluted non-esterified fatty acids). (d) Spectrum of fraction III (eluted non-acidic phospholipids). (e) Spectrum of fraction IV (eluted acidic phospholipids). All spectra were recorded at 30°C with lipid extracts dissolved in 0.80 ml of $\text{CD}_3\text{OD}-\text{CDCl}_3$ (2:1 v/v).

contained no detectable phospholipid or acidic phospholipid signals, as reflected in the phospholipid specific resonance at 4.40 ppm. Cholesterol and glyceride analysis was therefore made straightforward by the absence of overlapping Class III and IV lipid spectra. Tables 6.1 show that the loss of cholesterol and glycerides during Bond Elut fractionation was minimal, with *ca.* 97 % and 89 % recoveries respectively.

6.2.4 Proton NMR of rat liver: Free (non-esterified) fatty acids (Class II)

The extractable free fatty acids, figure 6.2c, were principally saturated fatty acids as reflected in the ratio of the multiple resonances at *ca.* 0.9 ppm, 1.3 ppm and 1.5 ppm, with the lack of resonances at 2.0 ppm, 2.8 ppm and 5.4 ppm. As expected most fatty acids in liver were covalently or strongly bound to glycerides and phospholipids or carrier proteins.

6.2.5 Proton NMR of rat liver: Non-acidic Phospholipids (Class III)

Fraction III, the nonacidic phospholipids, in figure 6.2d were readily analyzed by the absence of resonances from fraction I and IV lipids. Again table 6.1 reflects that the levels of these lipids were relatively unaffected by chromatographic fractionation. As previously published the unsaturated ether and sphingoid lipids, although in low concentrations, were more accurately quantitated by their CH=CH resonances below *ca.* 5.90 ppm and 5.65 ppm respectively, in figure 6.2d. A new feature however was that total ether lipids could now be measured from the *ca.* 4.30 ppm glycerol moiety that was previously hidden by triglycerol resonances. By comparing the total and unsaturated ether lipids resonance intensities, this revealed that most of the ether lipids were of plasmalogen or vinyl ether type. This was confirmed by the low abundance resonances of the saturated ether lipids at 3.44 and 1.59 ppm and by the integrated areas of the C-2 and C-1 proton resonances of ether lipid glycerol moieties.

The ratio of total choline to total ethanolamine lipids was obtained from the areas of the 3.6 ppm to 3.1 ppm head group (CH-2') signals, namely, 1.34 to 0.48. The existence of at least two different choline lipids in the ratio of 6.80 to 1 was obtained from their

separate $N^+(CH_3)_3$ resonances at *ca.* 3.20 ppm. These corresponded to total phosphatidylcholine (3.20 ppm), and ether lipids (3.19 ppm) respectively. The latter value also corresponded to total ether lipids as determined also from the $CH-1^d$ glycerol resonance at 4.30 ppm in the fraction III (class III) spectrum. The absence of a third sphingoid $N^+(CH_3)_3$ resonance at 3.20 ppm indicated that ceramide ethanolamine was the principal sphingoid. Total sphingolipids were quantified by their C-2 sphingosine resonance at 5.07 ppm attributed to the methylene protons adjacent to NHCO group. Thus the two sphingoid resonances at 5.07 ppm (C-2) and at 5.68 ppm (CH=CH) yielded 1.05 as the ratio of sphinganine lipids plus sphingenine lipids to sphingenine lipids. Thus concluded that over 95 % of sphingolipids are sphingenine contain lipids. The N-methyl resonance of lysophosphatidylcholine (LPC), phosphatidylglycerol (PG) and the methylated ethanolamines were too small (< 0.1 wt. %) to measure under these conditions.

6.2.6 Proton NMR of rat liver: Acidic Phospholipids (Class IV)

Analysis of acidic lipids before chromatographic fractionation was quite difficult. Among the common acidic phospholipids the inositols (PI) and serine (PS) lipids have characteristic head group resonances that can distinguish them. Cardiolipin (CAR), phosphatidylglycerol (PG), phosphatidic acid (PA) on the other hand do not have head groups that give diagnostic resonances and there are no significant differences between the glycerol moiety resonances from 4.5 to 3.9 ppm.

In figure 6.2e, the resonances for the PI inositol group permitted an easy quantitation (Table 6.1); these occurred at 3.75, 3.85, 3.60 and 3.35 ppm. Also, the inositol cross-peaks from PI were unambiguously assigned in the 2D COSY spectrum, as shown in figure 6.3.

The integral of the triplet at 3.85 ppm arising from the $CH-1'$ resonance of PI was significantly lower from that of the C-2, C-1 and C-3 glycerol moiety signals in this spectrum thus indicating the presence of the other acidic lipids.

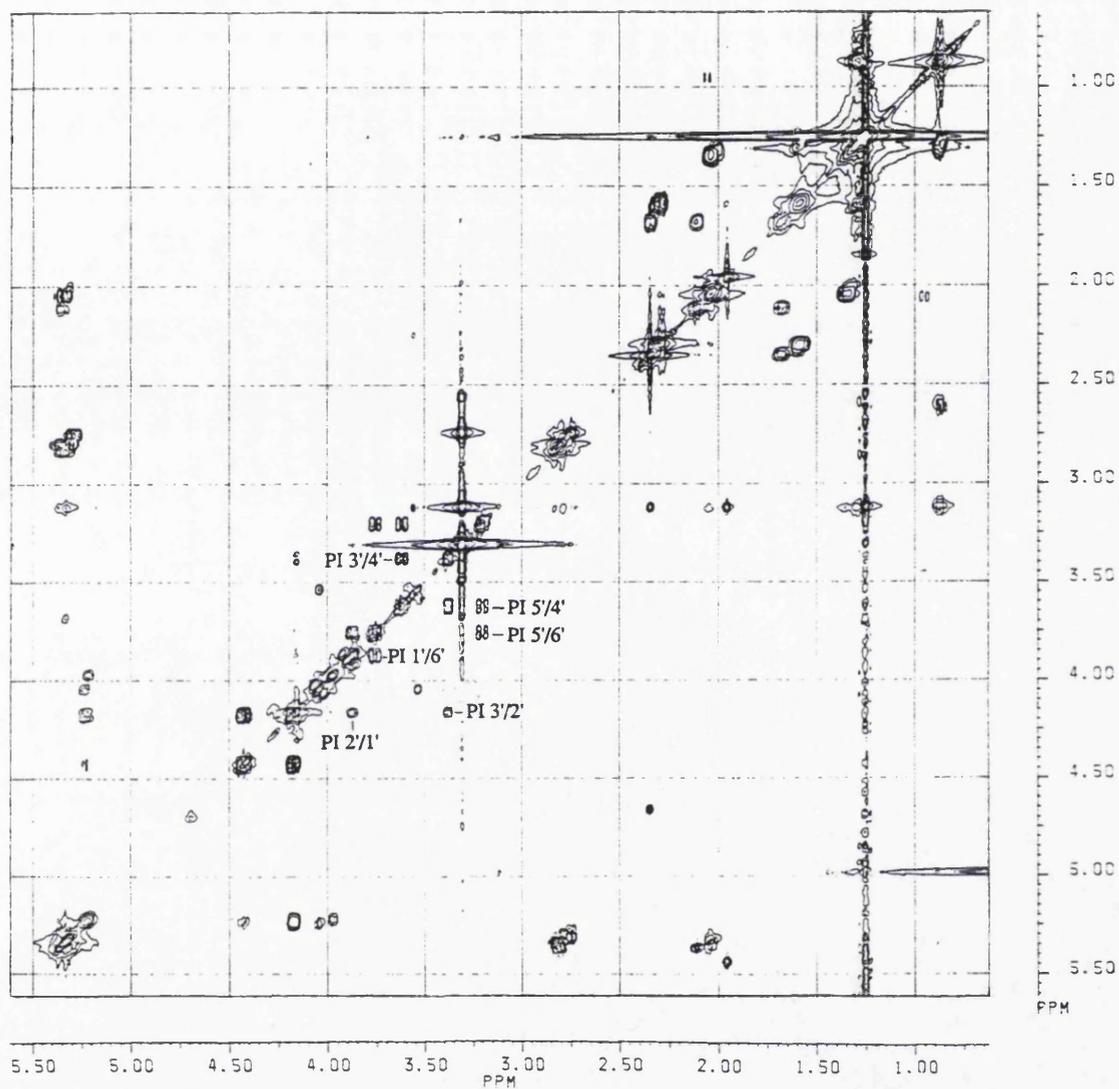


Figure 6.3 2D COSY NMR spectrum of acidic phospholipids separated by Bond Elut chromatography. PI was shown to be the principal component in this particular fraction, as the diagnostic inositol cross peaks were unequivocally assigned. The 2D COSY experiment was performed at 30°C by using standard pulse sequence, with a relaxation delay (D1) of 4.0 s and recorded in 2K data points, obtained from 400 FID's of 32 scans each, with zero-filling in the F₁ dimension. The data were multiplied with a square sine-bell function in both dimensions prior to transformation.

Table 6.1

Recoveries of rat liver lipid extracts after Bond Elut chromatography, in weight percentage (wt. %), as determined by comparative 1D proton NMR spectra.

Chemical species	Chemical shift (ppm)	Area before separation	Area after separation			Recovery (wt. %)
			Fraction I	Fraction III	Fraction IV	
Total P. ^a	4.40	0.91	-----	0.70±0.02	0.17±0.01	95.9
PC	3.20	0.62	-----	0.55±0.01	-----	87.5
PE	3.08	0.25	-----	0.20±0.01	-----	79.4
PI	3.75	0.11	-----	-----	0.09±0.00(1)	86.9
PS	3.75	-----	-----	-----	-----	-----
CHOL.	0.70	0.15	0.15±0.003	-----	-----	97.3
SPH	5.70	0.04	-----	0.03±0.01	-----	91.7
PLA	5.90	0.00(3)	-----	0.00(2)±0.00(1)	-----	66.7
TG/ DG ^b	4.30	0.28	0.25±0.01	-----	-----	89.4

^aTotal P. = Total phospholipid content

^bTG/ DG; ratio of triacylglycerol to diacylglycerol

The 2D COSY had a single cross-peak in the head group region at (4.05, 3.55) which corresponded closely to the β(beta) and α(alpha) proton signals of the serine head group of PS. Analysis of the 1D and 2D spectral components of cardiolipin, phosphatidylglycerol and phosphatidic acid was not achieved in this analysis but the relative integrals of the glycerol proton resonances indicate that one or more is present.

6.2.7 Fatty acid analysis of intact lipids

Previously it was shown that 1D and 2D NMR gave a great deal of information about the overall fatty acid composition of liver lipids but not about the composition of individual classes of lipid [Casu *et al*, 1993; Casu *et al*, 1991]. By separating lipids into four classes, the NMR spectrum of each class could then be analyzed for the class-specific fatty acid composition.

A complete fatty acid analysis of class III lipids by NMR has not yet been achieved

but a great deal of information was easily gained from figure 6.2. For example docosahexaenoic and related acids have a distinctive ω (omega)-methyl triplet at 0.95 ppm, yet docosahexaenoic acid gives another specific C-2 methylene resonance at 2.40 ppm. Arachidonic acids have their characteristic C-2 β (beta) and C-3 γ (gamma) methylene resonances at 2.20 ppm and 1.70 ppm and linoleic acids gave diagnostic biallylic CH_2 resonances at 2.75 ppm, while other polyunsaturated fatty acids give their biallylic resonances at 2.80 ppm. These structure-specific resonances yielded accurate quantitation of these fatty acid components.

The ratio of total fatty acid chains to PUFA chains of *ca.* 2 to 1 was derived from the integrated ratios of $\omega\text{-CH}_3$ resonances (0.9 ppm) as the total fatty acid chains to that of total olefinic $\text{CH}=\text{CH}$ resonances (at 5.4 ppm).

Table 6.2 and footnotes summarize the fatty acid analysis of the intact neutral lipids, nonacidic phospholipids and acidic phospholipids. Analytical information was obtained as follows:

- a) The spectral areas regarding structures 8 and 9 gave the ratio of total unsaturated fatty acid chains to fatty acid chains in control sample as 1.69/ 2.83 equal to 0.60. These values were reasonably constant from fractions I, III and IV and were 0.51, 0.56 and 0.45 compared to the control value of 0.57.
- b) The linoleic acid and di-unsaturated fatty acid composition was higher in nonacidic phospholipids compared to both neutral and acidic phospholipids (Structure 3, Table 6.2).
- c) PUFAs containing the structural features analogous to arachidonic acid were not detected in triglycerides as evidenced by the absence of the structure-specific resonance at 1.7 ppm compared to control (i.e. non-separated sample) and fractions III and IV. The percentage of fatty acid chains containing this structural feature (principally arachidonate) was different in the control, non acidic and acidic

Table 6.2

Fatty acid compositions of rat liver lipid extracts after Bond Elut fractions, in weight percentage (wt. %), via proton NMR.

Chemical structures*	Chemical shift (ppm)	Area before separation	Area			Recovery (wt. %)
			Fraction I	after Fraction III	separation Fraction IV	
1	5.61	4.74	0.85±0.021	2.72±0.030	0.82±0.004	92.6
2	2.80	3.10	0.42±0.011	1.92±0.016	0.46±0.009	90.3
3	2.75	0.52	0.13±0.001	0.23±0.001	0.13±0.001	94.2
4	2.40	0.16	0.03±0.002	0.10±0.001	0.01±0.000	87.5
5	2.00	1.28	0.37±0.008	0.64±0.006	0.07±0.004	84.4
6	1.70	0.50	-----	0.31±0.007	0.05±0.004	72.0
7	0.95	0.33	0.11±0.005	0.15±0.001	0.05±0.004	93.9
8	0.85(total)	2.83	0.76±0.028	1.52±0.014	0.63±0.011	102.6
9	Total UFA	1.69	0.39±0.009	0.85±0.041	0.28±0.009	89.9

***Table 6.2 Footnotes**

Structure 1, 5.61 ppm: includes the vinyl -CH=CH- groups of every unsaturated fatty acid.

Structure 2, 2.80 ppm: includes all alkylic CH₂ groups for PUFAs containing more than two unsaturated fatty acid chains [e.g. -CH=CH-CH₂-CH=CH- (excluding linoleic acid)].

Structure 3, 2.75 ppm: represents the -CH=CH-CH₂-CH=CH- alkylic resonances of linoleic acid and diene fatty acids.

Structure 4, 2.40 ppm: the multiplet includes all alkylic groups in fatty acids containing the following underlined structural features -CH=CH-CH₂CH₂CO₂-.

Structure 5, 2.00 ppm: this multiplet arises from alkylic groups external to a mono or polyunsaturated fatty acid [e.g. structures such as -CH₂CH=CH-(CH₂-CH=CH)_x-CH₂-, in which x≥0].

Structure 6, 1.70 ppm: This resonance arises from beta alkyl groups of fatty acids containing the following underlined structural features -CH=CH-CH₂CH₂CH₂CO₂-. The gamma alkylic group resonance of this type of fatty acid occurs at 2.1 ppm. Arachidonic acid is a primary example of this class of fatty acid.

Structure 7, 0.95 ppm: This methyl triplet arises from fatty acid chains containing a high degree of unsaturation (e.g. docosahexaenoic acid and linolenic acid) and arises from structural features such as CH₃-CH₂-CH=CH-. Information on highly unsaturated fatty acid can also be obtained from the resonances which arise from the -CH=CH-CH₂CH₂CO₂-.

Structure 8, 0.85 ppm: includes the CH₃ groups of fatty acid chains, saturated and unsaturated. In fraction 1 this also includes cholesterol methyl groups.

Structure 9, total UFA, total unsaturated fatty acids.

phospholipids. The values respectively were 30 %, 36 %, and 18 % obtained from the ratio of structures 6 and 9.

- d) PUFAs containing low field shifted methyl groups due to the present of ω -3 olefinic group were analyzed as depicted in structure 7, in table 6.2, as docosahexaenoic acid being the prime example. The percentage of fatty acids with this feature varied from the control to fractions I, III and IV as 12 %, 14 %, 10 % and 8 % respectively.
- e) The 2.4 ppm resonances (Structure 4, Table 6.2) also gave information on highly unsaturated fatty acid chains which have olefinic group start at C-4 position throughout the lipid classes. Thus the composition varied in control and the fractions as follows: control, 6 %, fraction I, 3 %, fraction III, 7 %, fraction IV, 15%.

6.3 CONCLUSIONS

The combination of Bond Elut chromatography plus 1D and 2D proton NMR spectroscopy has been shown to give superior qualitative and quantitative analysis than either technique alone. The neutral lipid fraction was analyzed for steroids and glycerides; the non acidic lipids for the various structural lipids containing choline and ethanolamine — this included ether, diacylglycerol and sphingoid lipids. Specifically two major choline lipids, namely PC and plasmalogen, were detected and the major sphingoid lipid was ceramide ethanolamine as indicated by the lack of a third choline $N^+(CH_3)_3$ resonance. The ratio of alkyl ether lipids to alkenyl ether lipids (plasmalogen) was easily determined by these procedures.

Apart from improved lipid classes analysis, considerable information on the saturated, unsaturated and polyunsaturated fatty acid composition of each class of lipid was obtained rapidly and quantitatively without the need of lipid hydrolysis.

Although the advantages of *in vitro* NMR analysis of cellular lipids have been documented, this purely NMR approach also had drawbacks. Rapid and quantitative separation of total lipids into neutral lipids, free fatty acids, nonacidic phospholipids and

acidic phospholipids using Bond Elut ion exchange columns as demonstrated here permitted a more quantitative and complete NMR analysis of glycerides, cholesterol, saturated and unsaturated sphingolipids and ether lipids as well as diacylphosphocholine and ethanolamine lipids. Acidic lipids were also analyzed. The fatty acid compositions of the intact lipids in each of the four Bond Elut fractions were determined from the NMR spectrum of each fraction.

Chapter 7

**Neutral lipids, non-acidic and acidic phospholipids
analysis from rat heart, kidney and brain by 1D and 2D
proton NMR & Bond Elut ion-exchange
chromatography**

7.1 INTRODUCTION

Nowadays lipids are regarded not simply as structural components of cellular membranes, but have major functions including such roles as: 1) electrical conduction, insulation, membrane fluidity and permeability [Jacobson & Saier, 1988], 2) components of membrane receptors, ion-channels, transport proteins and enzymes [Dinh & Kennerly, 1991; Ordway *et al*, 1991; Habenicht *et al*, 1990; Lamah *et al*, 1990; Kurachi *et al*, 1989], 3) substrates and products of membrane enzymes that modify phospholipids, and precursors of lipidic hormones and intracellular messengers [Schulam *et al*, 1991; Exton, 1990; Nigam *et al*, 1990; Pacini *et al*, 1990; Ragab-Thomas, 1987] and 4) participation in the regulation of membrane proteins [Edwards & Crumpton, 1991; Nesmeyanova & Bogdanov, 1989; Neer & Clapham, 1988]. Recent discoveries such as the phosphatidylinositol cycle [Exton, 1990], arachidonate cascade [Choi, Gao & Gibbons, 1991; DuBourdieu & Morgan, 1990; Rustenbeck & Sigurd, 1989; Kurachi *et al*, 1989], phosphorylation by protein kinases and intracellular calcium flux [May & Calder, 1993] all emphasize the potency and biological importance of lipids.

The study of lipids in biological systems has never been simple. There is a variety of lipid components in cell membranes, the principal lipids being glycerides, steroid lipids, phospholipids, ether-phospholipids and sphingolipids, plus a number of sub-classes depending upon their head groups and fatty acid compositions. Moreover the lipid compositions diverge significantly among internal organs, tissues and body fluids. Hence there has been an ever-increasing demand for rapid and comprehensive lipid assay methodologies to investigate the biological and clinical roles of lipids.

Development of lipid analysis has been carried on for some time, thus there are well established assays available for both lipids and their metabolites, such as thin layer chromatography (TLC) [Pal & Davis, 1991; Jheem & Weigel, 1989], high-performance liquid chromatography (HPLC) [Seta *et al*, 1990, Araki *et al*, 1990; Vercaemst *et al*, 1989], gas chromatography (GC) [McGrath & Elliott, 1990; Beyer *et al*, 1989] and mass spectroscopy (MS) [Chen *et al*, 1990]. However, most of these conventional assays are

non-comprehensive, an extensive lipid profile was often achieved by the combination of various methods [Guido *et al*, 1993; Thomas *et al*, 1992; Chilton, 1991].

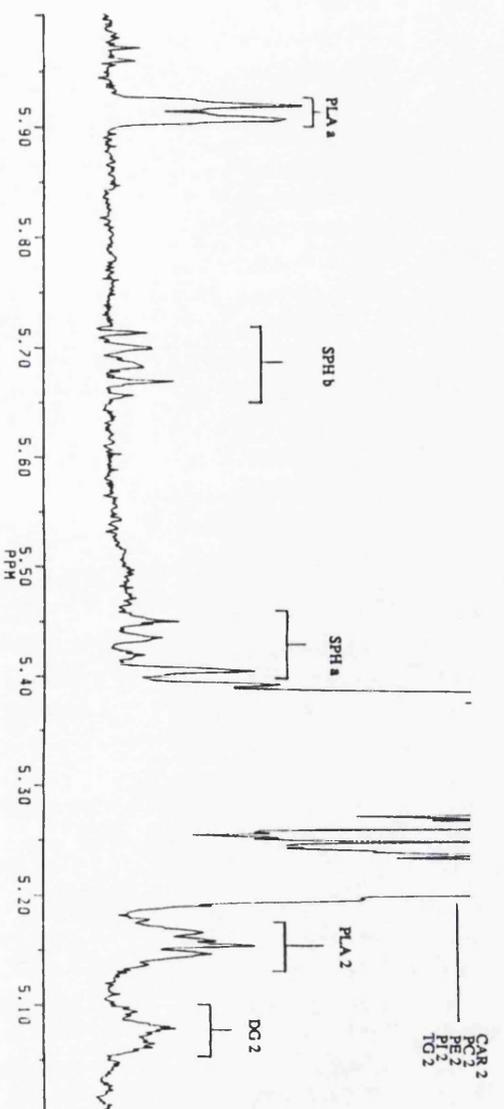
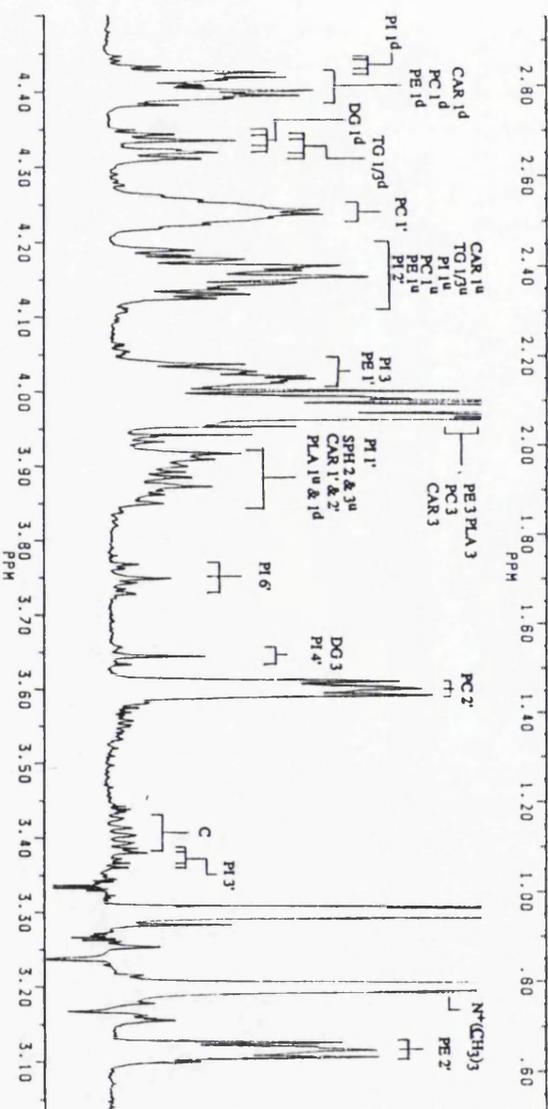
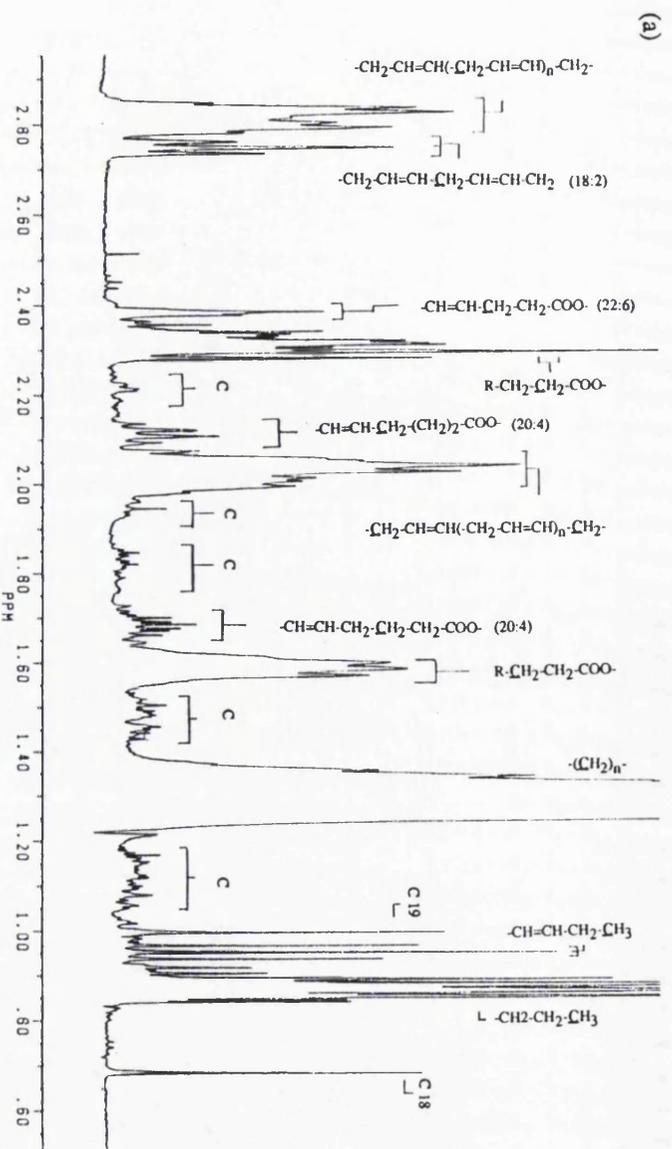
Recently NMR spectroscopy has been explored as a complementary technique for lipid profiling. Proton and heteronuclear NMR spectroscopy provide a rapid, comprehensive and non-destructive method of analyzing the lipids of tissues, cells and body fluids. This has been successfully demonstrated on synthetic lipid mixtures [Sparling, 1990; Sparling *et al*, 1989] and the lipid composition of tissues like liver [Casu *et al*, 1991], body fluids [Casu *et al*, 1992] and macrophages [Sze & Jardetzky, 1990]. Such *in vitro* lipid profiling approaches were further improved with the aid of simple solid-phase ion-exchange chromatography [Choi *et al*, 1993]. In this approach, prior to the NMR experiments, lipids were separated into four or five fractions according to their polarities. This lead to an improvement of resolution, reduced spectral overlap, and significantly, to the comparisons of lipid-bound fatty acids between individual lipid fractions.

In this particular study the combination of 1D and 2D proton NMR and Bond Elut column chromatography was used to obtain the lipid profiles of rat heart, kidney and brain. Once again the recovery after column separation was found to be consistent and reproducible, as previously reported [Choi *et al*, 1993]. The lipid compositions determined by NMR were compared to existing data [DeWillie & Horrocks, 1992; White, 1973], and good agreement was achieved with previously published data in all three tissues.

7.2 RESULTS AND DISCUSSION

7.2.1 *Calculations and quantitation of lipids*

Linear baseline correction was applied to all proton 1D spectra prior to integral calculations. The relative concentrations of individual lipids were determined from the integrated area at their diagnostic chemical shift, as reported previously [Choi *et al*, 1993; Casu *et al*, 1991]. With the integrated area of CDCl₃ as an external standard for spectral normalization.



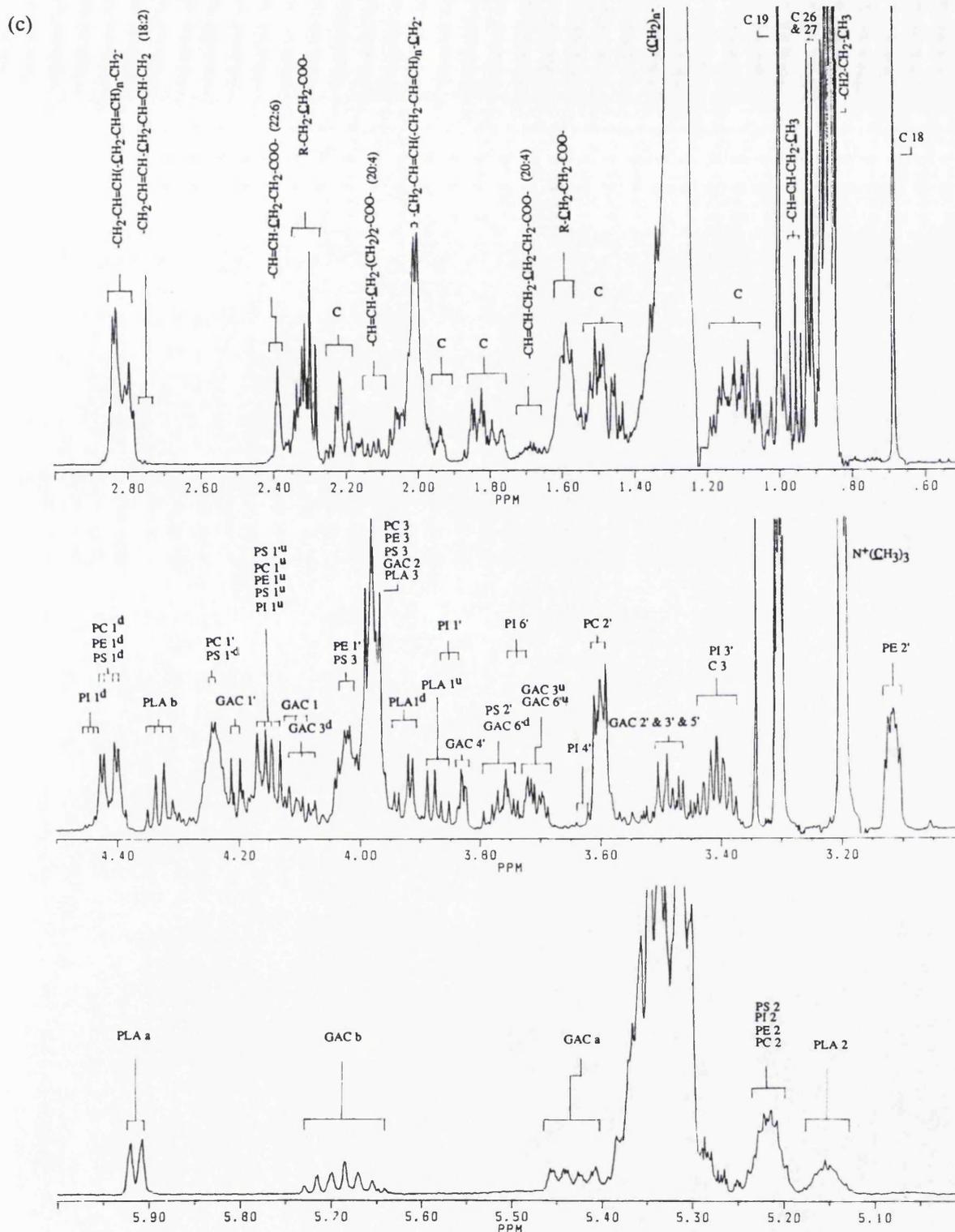


Figure 7.1 Expanded 1D proton NMR spectrum of lipid extract from rat heart (fig. 7.1a), rat kidney (fig. 7.1b) and rat brain (fig. 7.1c). Lipid extracted from 1.0 g of tissue was resuspended in 0.6 ml of $\text{CD}_3\text{OD}-\text{CDCl}_3$ (2:1, v/v) for the experiment. The spectrum was recorded using a Bruker AM 500 NMR spectrometer, at 30°C in the Fourier Transform (FT) mode with 32K data points, using a 45° detection pulse and 2.0 acquisition time. CD_3OD solvent resonance at 3.30 ppm was used as the reference signal.

7.2.2 Lipid profiles of rat heart, kidney and brain

The expanded $^1\text{H-NMR}$ spectra of rat heart, kidney and brain lipid extracts are shown in figure 7.1. Assignments of spectra were based on previous studies [Choi *et al*, 1993; Casu *et al*, 1992; Casu *et al*, 1991], information obtained from 2D NMR experiments and the comparison with standard spectra. Figure 7.2, 7.3 and 7.4 show the spectra of the separated Bond-Elut chromatographic fractions of each tissue respectively. In the cases of heart and kidney, the lipid extracts were separated into four fractions, in the order of: [I] — non-polar (neutral) glyceride lipids and steroidal lipids (Fig. 7.2 & 3b), [II] — non-esterified (free) fatty acids (Fig. 7.3c), [III] — Non-acidic phospholipids (Fig. 2c & 3d) and [IV] — acidic phospholipids (Fig. 7.2d & 7.3e). A five-step fractionation was performed on the brain sample (Fig. 7.4a-f), with the extra fraction eluting phosphatidylserine (Fig. 7.4e). Figure 7.5 shows the expansions of 2D-HOHAHA spectrum from the total brain lipid extract.

The phospholipid compositions of all three tissues, in weight percentage (wt. %), are summarized and compared to published data in table 7.1. Table 7.2 illustrates the recovery of lipids after Bond Elut column fractionations. Table 7.3 lists the fatty acid compositions of the three tissues, in weight percentage, after Bond Elut column fractionations.

7.2.3 Neutral lipids and non-esterified fatty acids.

Neutral lipids (non-polar glycerolipids and steroid lipids); which are principally the mixture of triacylglycerol and cholesterol were eluted in fraction I. The most distinctive feature of this fraction was the existence of cholesterol, which was present in all three tissues (Fig. 7.2, 7.3 & 7.4b). The separation of cholesterol and triglycerides from phospholipids reduced the spectral overlap and hence improved both the neutral lipid quantitations, and the phospholipid quantitations (lipid-bound fatty acids in particular) in later fractions. The chemical shift of the cholesterol C-18 methyl protons at 0.70 ppm was used as a diagnostic signal. Triacylglycerols were also identified in both heart and kidney lipid extracts, from their diagnostic moiety *sn*-1/ 3 proton signal at 4.30 ppm.

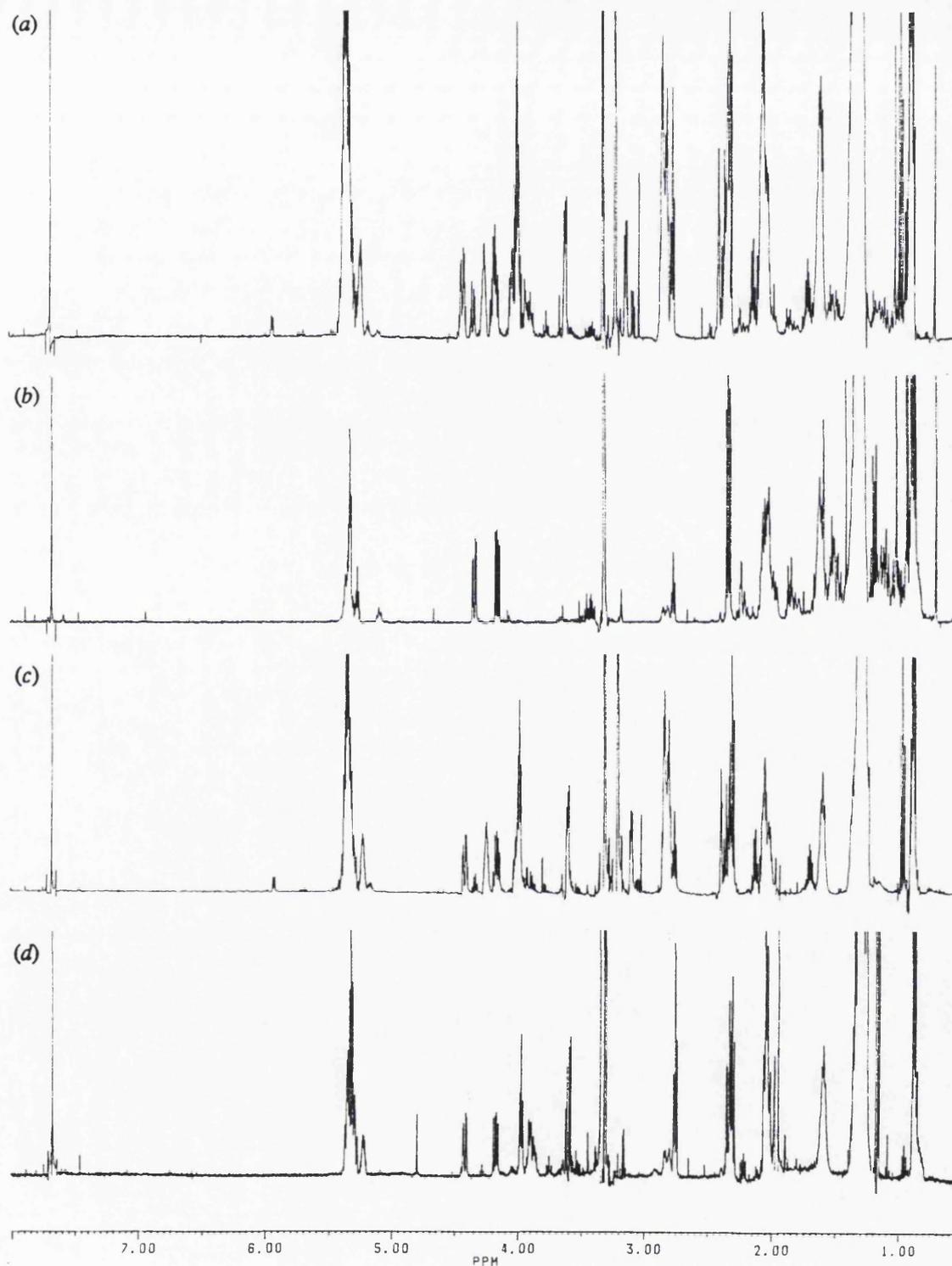


Figure 7.2 Comparison of 1D proton NMR spectra of rat heart extract, prior to and after solid-phase (aminopropyl) ion-exchange column separation. (a) Spectrum of lipid extract without separation, (b) spectrum of fraction I (eluted neutral lipids and cholesterol), (c) spectrum of fraction III (eluted non-acidic phospholipids), (d) spectrum of fraction IV (eluted acidic phospholipids).

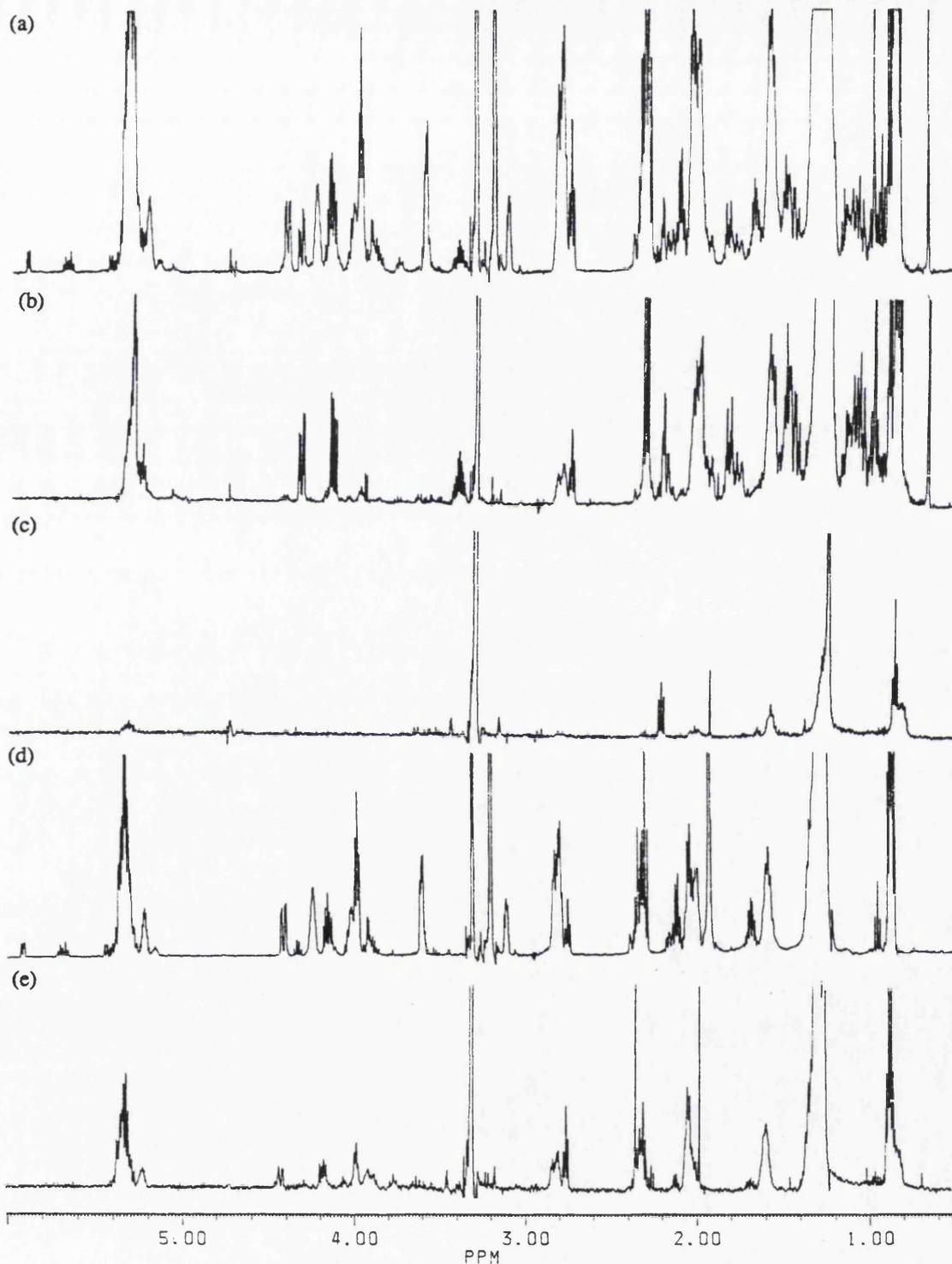
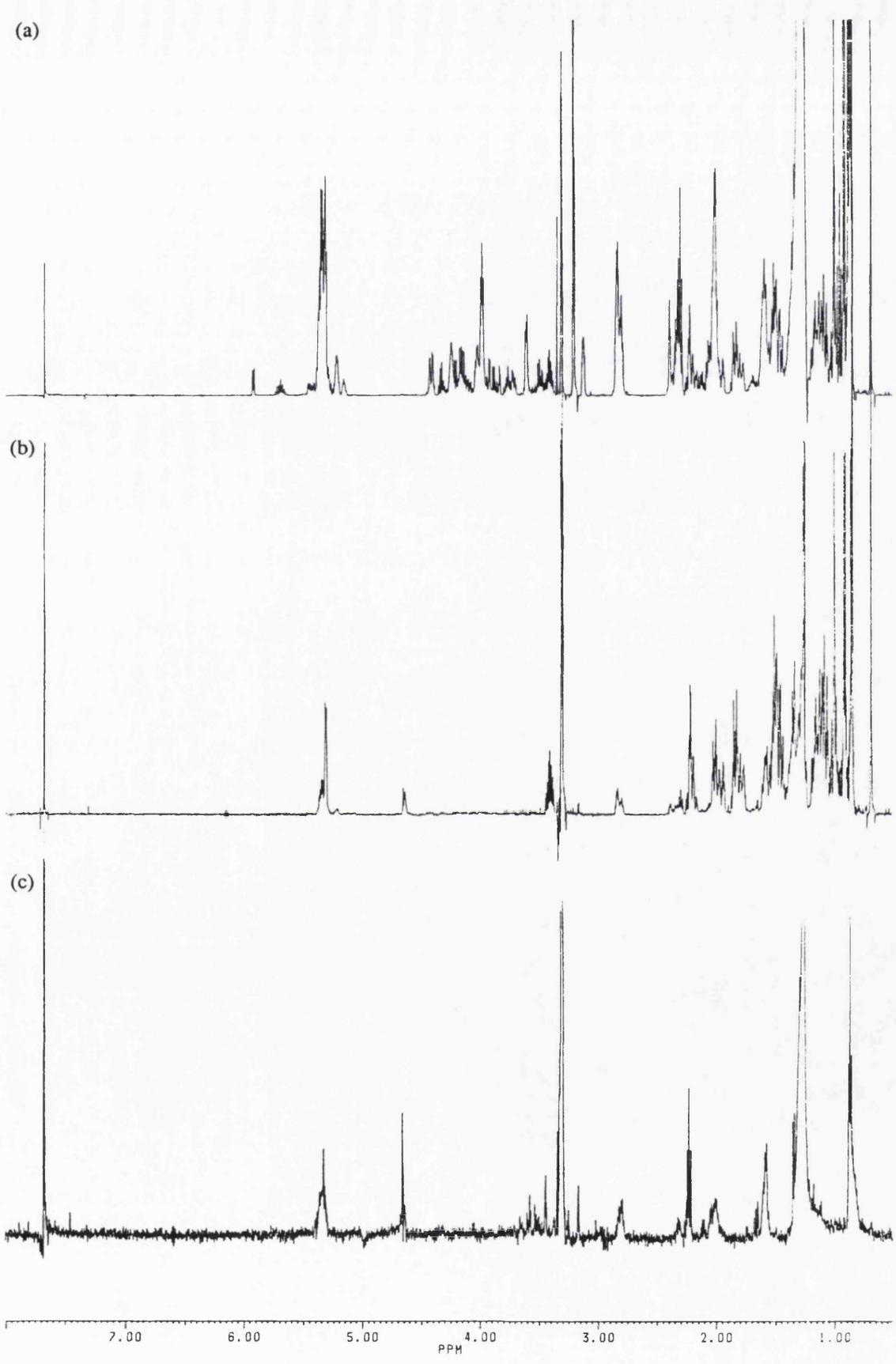


Figure 7.3 Comparison of 1D proton NMR spectra of rat kidney extract, prior to and after solid-phase (aminopropyl) ion-exchange column separation. (a) Spectrum of lipid extract without separation, (b) spectrum of fraction I (eluted neutral lipids and cholesterol), (c) spectrum of fraction II (eluted non-esterified fatty acids), (d) spectrum of fraction III (eluted non-acidic phospholipids), (e) spectrum of fraction IV (eluted acidic phospholipids).



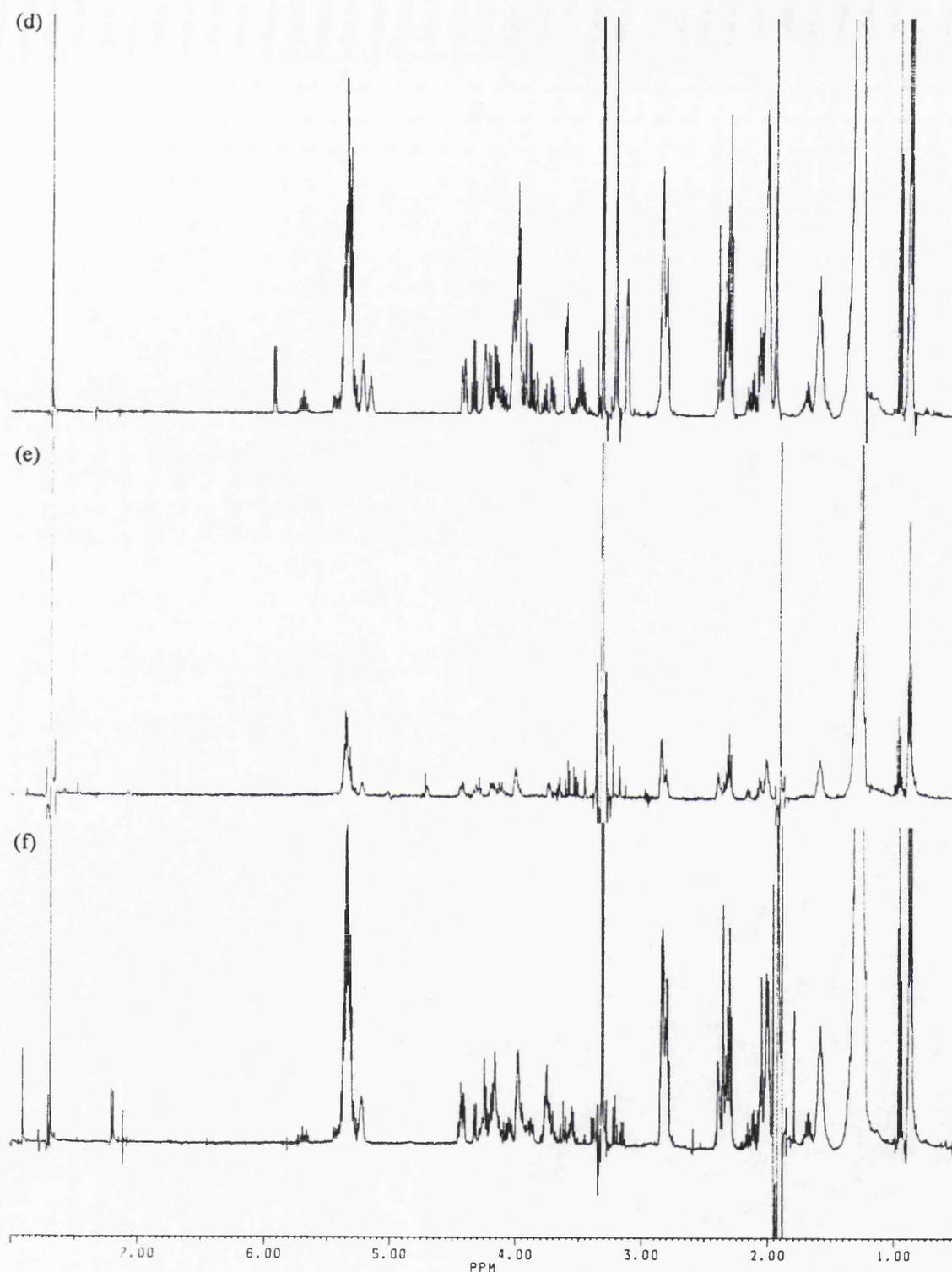


Figure 7.4 Comparison of 1D proton NMR spectra of rat brain extract, prior to and after solid-phase (aminopropyl) ion-exchange column separation. (a) Spectrum of lipid extract without separation, (b) spectrum of fraction I (eluted neutral lipids and cholesterol), (c) spectrum of fraction II (eluted non-esterified fatty acids), (d) spectrum of fraction III (eluted non-acidic phospholipids), (e) spectrum of fraction IV (eluted phosphatidylserine) and (f) spectrum of fraction V (eluted other acidic phospholipids).

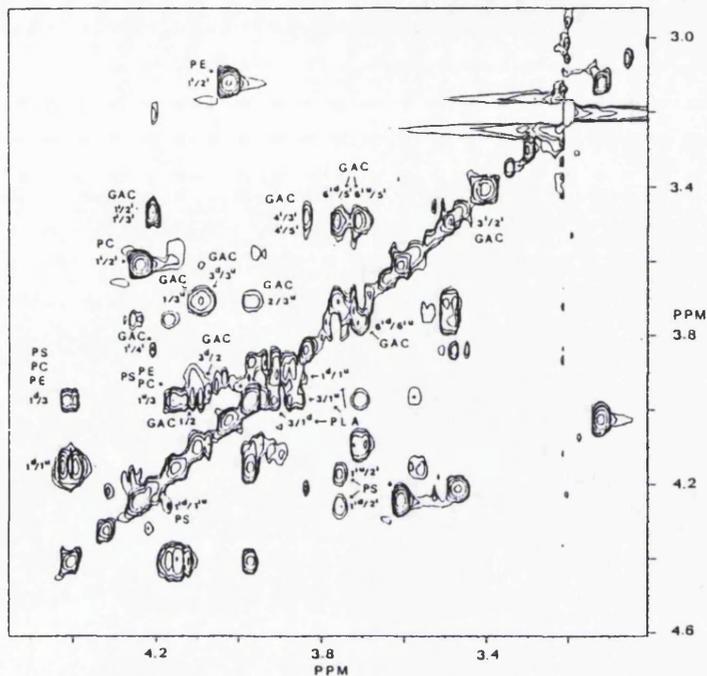


Figure 7.5b Expansion of the 2D-HOHAHA spectrum containing the major head group resonance of glycerol, serine, ethanolamine, choline, inositol, galactose of the glycerophospholipid, ether glycerophospholipid, sphingolipid classes. For instance, PI 2/3 indicates the cross-peak between proton C2 and proton C3 glycerol protons of PI.

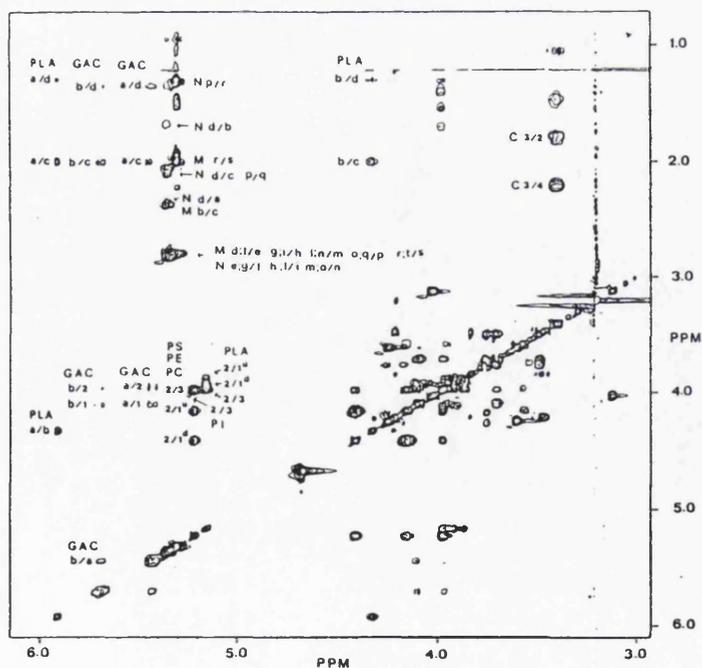


Figure 7.5c Expanded region of the 2D-HOHAHA spectrum from brain lipid extracts, containing resonances from the olefinic group of fatty acid chains, the vinyl group from unsaturated ether lipids, proton C2 resonances of glycerol plus sphingosine moieties of lipids.

However, the *sn*-2 proton which occurred at 5.23 ppm overlapped with other corresponding phospholipid signals. No triacylglycerol was found in the brain lipid extract as predicted [DeWillie & Horrocks, 1992]. The efficiency of this fractionation, was confirmed by the absence of detectable phospholipid signals in figures 7.2, 7.3 and 7.4b, was also checked by TLC. Table 7.2 shows that the loss of cholesterol and triglycerides during column separations was minimal (with recovery being over 90 % on average).

Table 7.1

Lipid compositions of rat heart, kidney and brain extract, in weight percentage (wt. %), obtained by ¹H NMR are compared to the published data.

Lipid	Heart		Kidney		Brain	
	¹ H-NMR	Ref. ^a	¹ H-NMR	Ref. ^a	¹ H-NMR	Ref. ^b
PC	35.29±1.62	36.00 [†]	35.80±1.72	34.30	29.75±2.44	22.00
PE	23.56±0.86	29.70 ^α	22.90±2.96	27.10	17.72±0.88	19.80
PI	6.19±0.31	3.70	9.44±0.63	5.90	7.60±2.25	2.40
PS	5.59*±0.30	3.20	6.91*±0.45	7.30	8.80±0.37	7.20
SPH	3.84±0.18	3.10	12.28±0.36	12.10	1.68*±0.13	3.80
PLA	8.66±0.52	-----	8.09±0.24	-----	13.93±0.98	11.60
CAR+others [‡]	17.97±1.00	12.50	-----	6.50	-----	-----
GAC	-----	-----	-----	-----	16.45±1.32	14.60
GAC(S)	-----	-----	-----	-----	2.69±1.06	4.80

Errors represented ± S.D. from three separate experiments.

^aObtained by reference [White, 1973].

^bObtained by reference [DeWillie & Horrocks, 1992].

*Estimated indirectly by subtraction.

[‡]Others = PA and PG.

[†]Mixture of phosphatidylcholine and plasmalogen-choline.

^αMixture of phosphatidylethanolamine and plasmalogen-ethanolamine.

Non-esterified or 'free' fatty acids were eluted in fraction II. However, no observable free fatty acids were detected in the heart and kidney samples. This was illustrated in figure 7.3c as there was no diagnostic signal from any major fatty acids. As expected, most fatty acids in heart and kidney were covalently or strongly bound to glycerides and phospholipids or carrier proteins. In contrast, a limited amount of free fatty acids was

detected in the brain lipid extract (Fig. 7.4c). Arachidonic acid was identified from its characteristic C-3 and C-4 proton chemical shifts at 1.70 and 2.15 ppm respectively, and contributed about 6.20 % to the total non-esterified (free) fatty acids content. The quantity of total free fatty acid was estimated at about 1.20 % of overall lipid weight (data calculated via the division by terminal-CH₃ areas in table 7.2c, since terminal-CH₃ represent the overall fatty acid content).

7.2.4 Non-acidic diacylglycerophospholipids, Sphingolipids and Ether-phospholipids.

The eluate in fraction III contained principally non-acidic diacylphospholipids, ether-phospholipids and sphingolipids, including: phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SPH) and plasmalogen (PLA) from all three tissue extracts; plus galactocerebroside (GAC) from the brain extract. Again, the data in table 7.2 shows that the levels of these lipids were relatively unaffected by the column separations.

As reported in the previous chapter, more specific and detailed quantitation of ether-phospholipids was achieved after column separation, since the removal of the triacylglycerol resonances unveiled another vinyl resonance from α - β (alpha-beta) unsaturated ether-lipid (principally plasmalogen) at approx. 4.30 ppm; namely PLA **b** as shown in figure 7.1c. The complementary vinyl signal was labelled as PLA **a** and occurred at 5.91 ppm. Another significant improvement for brain lipid extract analysis, was the separation of galactocerebroside (GAC) from phosphatidylinositol (PI) and 3'-sulphate-galactocerebroside, which made the specific quantitation of all three species possible. The chemical shift of galactocerebroside C-4' proton at 3.82 ppm was used for diagnostic purposes. Other proton resonance signals from the galactose head-group were unequivocally resolved in the 2D brain lipid extract spectrum, as shown in figure 5 b. Since the sphingosine moiety of galactocerebroside, 3'-sulphate-galactocerebroside and sphingomyelin are identical, all three species exhibit the same resonance from their backbones and their characteristic α - β unsaturated alkene group which linked to the C-1 of the ceramide moiety; labelled as protons **a** and **b** at 5.43 ppm and 5.70 ppm

Table 7.2a

Recoveries of lipids from rat heart extract after Bond-Elut chromatography as determined by comparative 1D proton NMR spectra.

Lipids	Chemical shift (ppm)	Area before separation	Area after separation			Recovery (%)
			Fraction I	Fraction III	Fraction IV&V	
Total P.	4.40	1.83	-----	0.88±0.05	0.54±0.01	91.01
PC	3.20	0.64	-----	0.55±0.02	-----	84.63
PE	3.08	0.43	-----	0.40±0.03	-----	92.56
PI	3.75	0.11	-----	-----	0.11±0.05	94.69
PS	3.75		-----	-----	0.10*±0.10	
CAR	5.70	0.38*	-----	-----	0.33±0.04	85.64
SPH	5.70	0.07	-----	0.06±0.03	-----	82.86
PLA	5.90	0.16	-----	0.12±0.05	-----	77.85
CHOL	0.70	0.15	0.14±0.01	-----	-----	91.37
TG&DG	4.30	0.32	0.28±0.03	-----	-----	89.62
CH ₃	0.90	3.67	1.15±0.03	1.67±0.05	0.34±0.03	86.66

Table 7.2b

Recoveries of lipids from rat kidney extract after Bond-Elut chromatography as determined by comparative 1D proton NMR spectra.

Lipids	Chemical shift (ppm)	Area before separation	Area after separation			Recovery (%)
			Fraction I	Fraction III	Fraction IV&V	
Total P.	4.40	1.62	-----	1.31±0.10	0.24±0.04	95.68
PC	3.20	0.58	-----	0.57±0.04	-----	97.45
PE	3.08	0.37	-----	0.30±0.01	-----	81.67
PI	3.75	0.15	-----	-----	0.13±0.02	84.31
PS	3.75		-----	-----	0.11*±0.05	
SPH	5.70	0.20	-----	0.19±0.01	-----	95.48
PLA	5.90	0.13	-----	0.13±0.01	-----	97.71
CHOL	0.70	0.42	0.41±0.01	-----	-----	96.45
TG&DG	4.30	0.60	0.55±0.03	-----	-----	91.23
CH ₃	0.90	3.73	0.73±0.03	2.26±0.06	0.35±0.04	89.58

Table 7.2c

Recoveries of lipids from rat brain extract after Bond-Elut chromatography as determined by comparative 1D proton NMR spectra.

Lipids	Chemical shift (ppm)	Area before separation	Area after separation					Recovery (%)
			Fraction I	Fraction II	Fraction III	Fraction IV	Fraction V	
Total P.	4.40	9.29	-----	-----	6.57±0.50	0.82±0.08	0.75±0.02	87.51
PC	3.20	2.77	-----	-----	2.70±0.07	-----	-----	97.65
PE	3.08	1.65	-----	-----	1.56±0.03	-----	-----	94.72
PI	3.75	0.71	-----	-----	-----	-----	0.50±0.01	70.82
PS	3.75	0.95*	-----	-----	-----	0.82±0.04	-----	86.33
GAC	3.82	} 1.53	-----	-----	0.92 ^a ±0.02	-----	0.25 ^b ±0.01	} 86.72
SPH	5.70		-----	-----	0.16*±0.03	-----	-----	
PLA	5.90	1.29	-----	-----	1.23±0.05	-----	-----	95.05
CHOL	0.70	3.94	3.469±0.15	-----	-----	-----	-----	88.05
(20:4)	1.70	-----	-----	0.01±0.02	-----	-----	-----	-----
CH ₃	0.90	18.49	-----	0.23±0.02	10.80±0.50	4.66±0.15	2.68±0.06	99.29

Table 7.2a & b footnotes:

Errors represented ± S.D. from three separate experiments.

*Areas estimated indirectly by subtraction.

Lipid samples were analyzed by proton NMR combined with solid-phase (amino-propyl) ion-exchange chromatography. The column separated the lipid extracts into four fractions with different eluates depending upon the lipid polarity, namely, fraction I: neutral and steroid lipids, fraction II: non-esterified (free) fatty acids, fraction III: non-acidic phospholipids and fraction IV: acidic phospholipids.

Table 7.2c footnotes:

Errors represented ± S.D. from three separate experiments.

*Areas estimated indirectly by subtraction.

^aArea of galactocerebroside.

^bArea of 3'-sulphate-galactocerebroside.

The lipid extracts were separated into five fractions via Bond-Elut chromatography depending upon the lipid polarity, namely, fraction I: neutral and steroid lipids, fraction II: non-esterified (free) fatty acids, fraction III: non-acidic phospholipids, fraction IV: phosphatidylserine and fraction V: acidic phospholipids.

respectively. Other major diagnostic resonances included: 1) The C-2 moiety proton resonance from most glycerolipids occurred at 5.23 ppm, with the exception of plasmalogen and diacylglycerol, which occurred at 5.15 ppm and 5.07 ppm respectively. 2) The C-1 proton resonance of all diacylglycerophospholipids, up-field and down-field, occurred at 4.15 ppm and 4.40 ppm respectively. 3) $N^+(\text{CH}_3)_3$ methyl proton resonance from choline-lipids, contain mainly phosphatidylcholine occurred at 3.20 ppm, and 4) the C-2' methylene proton from phosphatidylethanolamine head-group occurred at 3.08 ppm. The phospholipid compositions of all three tissues, in weight percentage, are summarized and compared to published data in table 7.1. All major phospholipids were successfully detected by $^1\text{H-NMR}$, and their concentrations generally shown to be in good agreement with previous determinations by chromatographic methods [DeWillie & Horrocks, 1992; White, 1973]. Slight variations occurred between phosphatidylethanolamine values in heart and kidney samples, since the combined values of phosphatidylethanolamine and plasmalogen-ethanolamine lipids were presented in the references. The efficiency of Bond Elut chromatography was reflected by the high recovery of total phospholipids (over 90 % on average) and supported by other data listed in table 7.2. The diagnostic chemical shifts used for calculations were also summarized in table 7.2.

7.2.5 Acidic Diacylglycerophospholipids and Galactocerebroside-sulphate

Unlike the separations of heart and kidney lipid extracts, a five-step separation, instead of four, was performed on brain lipid extract (Fig. 7.4 b-f). The additional step, which was applied between the non-acidic and acidic-phospholipid elution, eluted exclusively phosphatidylserine (PS) (Fig. 7.4 e). This step was omitted in heart and kidney samples, since accurate quantitation was difficult in those separated PS spectra, due to the low natural abundance of PS in both tissues. Consequently, the small amounts of PS in heart and kidney samples were eluted with other acidic phospholipids into fraction V, and quantitation of PS was then achieved by subtraction (i.e. total phospholipids minus other specifically identified phospholipids). In fraction IV of the

brain extract, PS was quantified by its characteristic C-2' proton signal at 3.75 ppm. Other lipid species eluted into fraction V included: phosphatidylinositol (PI) from all three samples, plus cardiolipin (CAR) from heart and 3'-sulphate-galactocerebroside from brain extract.

Due to spectral overlap and their relatively low abundance (≤ 5.0 wt. %), analysis of acidic lipids before column fractionation was quite difficult. Thus more specific and accurate analysis could be achieved after column separation. For instance, head group diagnostic resonances for PI and PS became more distinguishable (e.g. PI C-6' proton & PS C-2' proton at 3.75 ppm, PI C-3' proton & 5' proton at 3.40 and 3.20 ppm). The *sn*-3 proton moiety resonance from cardiolipin, occurred at 3.97 ppm, and was distinct from other corresponding phospholipid signals and was used for quantitation (*sn*-3 protons from PI occurred at 4.02 ppm). In the brain sample 3'-sulphate-galactocerebroside was separated from galactocerebroside (eluted into fraction III) thus can be quantified directly. Lipid compositions of all three extracts are listed in table 7.1, and compare favorably with the published values. However, there are higher values of phosphatidylinositol appeared in all three samples, such phenomena was possibly due to the strain or dietary differences [Ulmann *et al*, 1991; Brouard & Pascaud, 1990]. Recovery after column chromatography once again proved to be satisfactory, as summarized in table 7.2.

7.2.6 Fatty acid analysis of intact lipids

One of the strongest features of *in vitro* NMR lipid analysis, is the ability to obtain information about the fatty acid components of lipids rapidly and without additional experiments. Various fatty acid species can be specifically identified, with the degree of unsaturation determined [Choi *et al*, 1993; Casu *et al*, 1991]. Nevertheless, a comparison of the fatty acid compositions of individual classes of lipid or individual lipids could not be achieved without chromatographic separation. By separating lipids into four or five fractions, the specific fatty acid composition of each fraction was analyzed. It was found

to be particularly beneficial to separately determine the fatty acid composition of neutral lipids from their non-acidic and acidic phospholipid counterparts.

Details of the fatty acid analysis via $^1\text{H-NMR}$ has been discussed previously [Choi *et al.*, 1993; Casu *et al.*, 1991]. The major diagnostic assignments are summarized as follows: 1) the C-3 & 4 protons chemical shifts, from (Δ -5) fatty acids, principally arachidonic acid (20:4; Δ -5), appeared at 1.70 and 2.20 ppm respectively, 2) C-3 proton of (Δ -4) fatty acids, principally docosahexaenoic acid (22:6; Δ -4), occurred at 2.40 ppm, 3) signals from the biallylic group of linoleic acid (18:2; Δ -9) occurred at 2.75 ppm, 4) biallylic group from other polyunsaturated fatty acids (PUFA) at 2.80 ppm, 5) methylene signals which are immediately adjacent to the vinyl groups from UFAs, with the exceptions of arachidonic and docosahexaenoic acids occurred at 2.00 ppm, and 6) the terminal methyl resonances of fatty acids occurred approx. from 0.82 to 0.97 ppm.

Quantitation of fatty acids from the three lipid extract fractions are listed in table 7.3. Significantly, direct measurements were achieved on linoleic acid, arachidonic acid and docosahexaenoic acid. The overall unsaturated fatty acid contents, were determined from the integrated area of methylene resonance at 2.00 ppm, plus the integrated areas from arachidonic and docosahexaenoic acid.

In general, the degree of fatty acid unsaturation in neutral glycerol lipids (fraction I) are lower than their phospholipid (fraction III, IV & V) counterparts as reflected in the overall unsaturated fatty acid values, and the lower PUFA (i.e. arachidonic and docosahexaenoic acid) content in the former fraction. The only exception were fractions IV & V from the kidney extract, with 43.0 % of total unsaturated fatty acid versus 51.4 % in fraction I. However, despite the higher overall unsaturation, fraction I from kidney extract still had a significantly lower linoleic and PUFA content compared to fractions IV & V, hence suggesting that the excessive unsaturation was contributed mainly by monounsaturated fatty acid.

Interestingly, the linoleic acid content in the brain lipid extract was relatively low compared with other tissues, and only fraction III (non-acidic phospholipids and galactocerebroside) showed any detectable linoleic acid, fraction IV and V had shown no

trace of linoleic acid. In contrast, the highest linoleic acid level from heart and kidney samples were found in fraction IV & V. However, the brain lipid extract had a noticeably higher PUFA content than the other two tissues, particularly in the acidic phospholipid fractions (fraction IV and V), which contain *ca.* 37.4 % of PUFAs, the highest among all fractions. Thus it is confirmed that PI, PS, GAC and 3'-sulphate-cerebroside in the brain lipid extract each possessed a higher polyunsaturated fatty acid contents than other major tissues. Whether this is related to cellular signal transduction in neuro-systems remains to be explored. In the cases of heart and kidney lipid extracts, PUFAs are principally distributed in the non-acidic phospholipid fraction (> 20.0 %).

Table 7.3

Fatty acid compositions of individual lipid fraction in weight percentage (%) from rat organ extracts, determined by ¹H-NMR after column separation.

Fatty acid / Chemical Shift (ppm)	Fraction I			Fraction III			Fraction IV & V				
	Heart	Kidney	Brain	Heart	Kidney	Brain	Heart [‡]	Kidney [‡]	Brain [‡]	IV [†]	V [†]
18:2 / (2.75)	15.45	9.71	-----	6.72	11.14	1.16	30.12	22.64	-----	-----	
20:4 / (1.70)	-----	2.14	-----	17.27	21.14	11.13	1.81	5.27	2.49	18.69	
22:6 / (2.40)	0.61	0.76	5.68	3.84	3.93	14.37	1.61	0.44	11.62	18.69	
PUFA [⊠]	0.61	2.90	5.68	21.11	25.07	25.50	3.42	5.71	14.11	37.38	
Total Unsat.*	47.97	51.41	29.25	78.89	71.00	73.71	66.26	43.00	63.08	40.16	

[‡]Obtained by combined fraction IV & V.

[†]Obtained by separated fraction IV and V.

[⊠]Obtained by the addition of arachidonic acid (20:4) and docosahexaenoic acid (22:6).

*Obtained by the sum of methylene signal at 2.00 ppm, C-4 methylene (20:4) at 1.70 ppm and C-3 methylene (22:6) at 2.40 ppm.

Footnote: The quantity of individual fatty acids were determined from the integrated area at their diagnostic chemical shift, with the integrated area of CDCl₃ as an external standard for spectral normalization.

7.3 CONCLUSION

Simple ion-exchange column chromatography of total lipid extract was used for lipid separation and classification prior to *in vitro* ¹H-NMR analysis of lipid classes. Lipid

extracts from rat heart, liver and whole brain have been analyzed by proton NMR combined with solid-phase (amino-propyl) ion-exchange chromatography. The latter separated the lipid extracts into four or five fractions depending upon lipid polarity, namely, fraction I: neutral and steroid lipids, fraction II: non-esterified (free) fatty acids, fraction III: non-acidic phospholipids, fraction IV: phosphatidylserine and fraction V: acidic phospholipids. This permitted a more comprehensive comparison of the lipid classes, and the assessment of the fatty acid composition from each fraction without resorting to lipid chemical hydrolysis. Such combination has proved to be superior, both qualitatively and quantitatively, to the purely NMR approach. The advantages included the isolation of non-polar glycerol lipids and cholesterol from phospholipids, plus the sub-classification within the phospholipid species according to their acidity. Consequently such fractionation procedure lead to more specific and precise analysis, particularly of low-abundant lipids. For instance, several previously non-separated lipid species were revealed after fractionation and were thus directly measured by NMR. Additionally, information on both the saturated and unsaturated fatty acid composition of each lipid class was obtained rapidly and quantitatively, for each fraction without resorting to chemical hydrolysis and derivatgation.

The data obtained by $^1\text{H-NMR/ Bond Elut}$ approach were in substantial agreement with similar analysis by other conventional chromatographic techniques, and were obtained rapidly, in a comprehensive and non-destructive manner. Specific lipids that were compared included glycerides, cholesterol, free fatty acids, sphingolipids, ether phospholipids, non-acidic and acidic diacylglycerophospholipids.

Heart, kidney and brain tissues were analyzed in this particular study, to illustrate the diversity of their lipid compositions and the potential interests of NMR lipid analysis in biomedical research. The results agreed with published data, both qualitatively and quantitatively. Thus $^1\text{H-NMR/ Bond Elut}$ assay therefore provides an valuable alternative to purely lipid chromatographic assays in cardiac, renal and brain lipid research and form a basis for total lipid analysis of normal and pathological tissues.

Chapter 8

In vitro analysis of rat liver lipid by 1D ^{13}C -NMR and 2D ^{13}C - ^1H NMR spectroscopy

8.1 INTRODUCTION

Research into the structural and dietary importance of lipids has been carried out for a long period of time among biological systems. Nevertheless, only from relatively recent studies, has the functional significance of lipids been finally realized. Current discoveries such as phosphatidylcholine and phosphatidylinositol cycle [Exton, 1990], arachidonic cascade [Ordway *et al.*, 1991; Choi & Gao, 1991; Kurachi *et al.*, 1989], phosphorylation of protein kinase C and intercellular calcium flux [May & Calder, 1993; Lamah *et al.*, 1990; Neer & Clapham, 1988] all emphasize the importance of lipids as potent molecules in biological systems.

Lipid analysis has traditionally been achieved via chromatographic techniques, such as thin layer chromatography (TLC) [Pal & Davis, 1991; Jheem & Weigel, 1989; Touchstone, 1973], gas chromatography (GC) [McGrath & Elliott, 1990; Beyer *et al.*, 1989] and high performance liquid chromatography (HPLC) [Araki *et al.*, 1990; Seta *et al.*, 1990; Vercaemst *et al.*, 1989] etc. However, most of these assays are relatively time-consuming and labour-intensive. Thus there is an increasing demand for rapid and comprehensive alternatives.

Nuclear magnetic resonance spectroscopy has been known as a powerful technique for chemical structure analysis especially for polypeptides and proteins. Also it has long been employed routinely for structure determination and confirmation in organic chemistry. Although phosphorus (^{31}P) NMR has been developed for lipid analysis [Radda *et al.*, 1991; Meneses & Glonek, 1988; Sotiros *et al.*, 1986], it can only detect phosphorus-containing lipids (i.e. phospholipids). Whereas proton (^1H) and carbon-13 (^{13}C) NMR can theoretically detect all classes of lipid, and with the combination of high-field magnets, modern computing technology and multidimensional experiments, they remain a highly appealing alternative to existing conventional lipid assays. Recently, a few laboratories [Casu *et al.*, 1991; Sze & Jardetski, 1990; Canioni *et al.*, 1983 etc.] have explored the potential of high-field NMR spectroscopy as a complementary technique for lipid profiling. *In vitro* proton NMR has been shown to be a rapid, comprehensive and non-destructive technique for the analysis of complex mixtures of lipid and phospholipid

extracts from cells, tissues and body fluids [Choi *et al*, 1993; Casu *et al*, 1992; Casu *et al*, 1991]. It has also been applied to lipid profiling of lower organisms [Adosraku *et al*, 1993]. The major advantages of using ^1H -NMR for lipid analysis are: all lipid species are in principle detectable simultaneously via ^1H -NMR, and fatty acid profiles of intact-lipids can be determined instantly without additional experiment (e.g. lipid hydrolysis). However, due to practical difficulties such as spectral overlapping or low lipid abundance etc., even 2D ^1H -NMR methods have not yet provided complete analysis of the fatty acid components of intact lipids in these mixtures. Here we report a qualitative and quantitative analysis of the principal lipids and their fatty acid moieties in a total lipid extract from rat liver using 1D carbon-13 and 2D heteronuclear NMR methods.

Although ^{13}C -NMR suffers from an intrinsic sensitivity problem compared to its ^1H -NMR counterpart, its advantages over the purely ^1H -NMR approach included: 1) the lower complexity of spectra particularly in the fatty acid regions, 2) the higher resolution and decreased interference from solvent signals and lower concentration of impurities. Such advantages has been discussed [Jeffrey *et al*, 1991] and have already been demonstrated on the analysis of fatty acids in essential oils [Gunstone, 1992; Gunstone, 1991]. Assignments of the olefinic carbon resonance from unsaturated lipids permitted relative quantitation of the fatty acids components of the intact lipids. This was confirmed by a complementary analysis using the methyl, alkyl and allyl carbon resonance of the fatty acids. The phospholipid head group resonances and triglycerol moiety resonances allowed quantitative analysis of phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylethanolamine (PE), and triacylglycerol (TG) in the extract. These analyse were in reasonable agreement with corresponding chromatographic assays by high-performance liquid chromatography (HPLC), thin layer chromatography (TLC), gas chromatography (GC) and by proton NMR spectroscopy [Choi *et al*, 1993; Guido *et al*, 1993; Casu *et al*, 1991; Jheem & Weigel, 1989; Christie, 1985]. When extended to lower concentration lipids and phospholipids, this ^{13}C or combined ^{13}C - ^1H NMR approach should provide a rapid and complementary comprehensive lipid profiling in biology and medicine.

8.2 RESULTS AND DISCUSSION

8.2.1 Carbon-13 lipid analysis of rat liver

The carbon-13 NMR spectrum of a rat liver lipid extract is shown in figure 8.1. The expansion in figure 8.1a was dominated by cholesterol and alkyl groups from different fatty acids. Figure 8.1b contains signals from the glycerol- and sphingo-moieties, plus the head groups of phospholipids, and the olefinic carbon resonance of the fatty acid components are shown in figure 8.1c. Figure 8.2 shows the expansion of 2D HETCOR spectrum in the lipid backbone and head group regions (^1H ; 0.0 to 6.0 ppm, ^{13}C ; 40.0 to 80.0 ppm) with all major lipid species unambiguously assigned.

8.2.2 Carbon-13 spectral assignments: Glycerophospholipids

These lipids contain glycerol moieties with acyl substitution at both the *sn*-1 and *sn*-2 positions, plus a phosphate diester bond joining the *sn*-3 carbon to the head groups. The head groups expected to be found in liver tissue includes choline, ethanolamine, serine, inositol, phosphatidate and glycerol [White, 1973; Ansell & Hawthorne, 1964]. The distinctive resonances from these head groups can often provide a convenient way for diagnostic purposes. Moreover each individual species can be further divided into subclasses according to the degree of unsaturation and length of their fatty acid chains. The diversity of esterified fatty acids can also be detected using the NMR approach.

On the basis of 1D and 2D carbon-13 NMR, resonances from PC, PE and PI were unequivocally assigned. No signals from the acidic lipids PS, PA or PG were detected, due to their lower abundance in liver tissue extract (1-4 %).

Since the *sn*-1, *sn*-2 and *sn*-3 carbon shifts of the PC and PE glycerol moieties are significantly overlapped, signals of their head group methylene carbons (labelled as 1' and 2') were used for diagnostic purposes. The chemical shifts for PC carbons 1' and 2' occurred at 58.70 and 66.30 ppm respectively and the corresponding PE head group signals were found at 61.20 and 40.30 ppm. The chemical shift of $\text{N}^+(\text{CH}_3)_3$ at 53.70 ppm provided another diagnostic resonance for PC and other choline contain lipids. A characteristic weak set of cross-peaks due to PI was observed by 2D HETCOR (Fig.

(c)

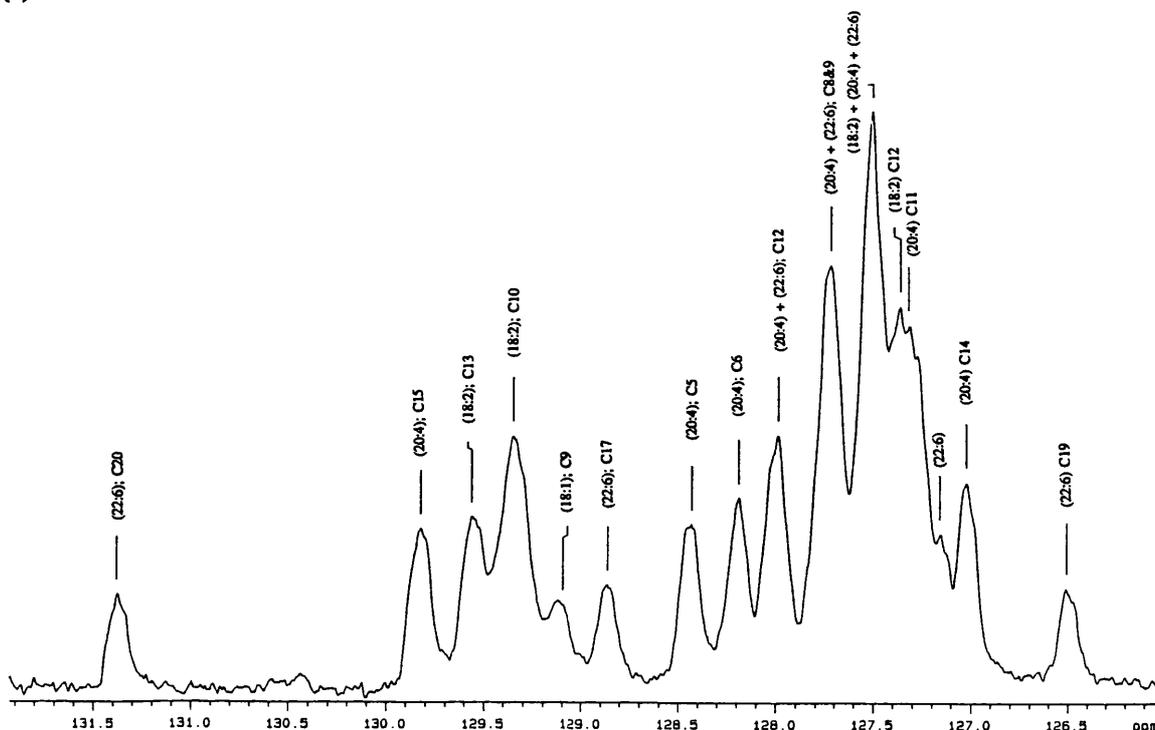


Figure 8.1 (a) Expanded 1D carbon-13 NMR spectrum (10 - 50 ppm) of lipid extract from rat liver. This region consist of chemical shifts from cholesterol and esterified fatty acids. Signals which generated from any particular fatty acid species were indicated by the fatty acid abbreviations. (b) Expanded 1D carbon-13 NMR spectrum (50 - 90 ppm) of lipid extract from rat liver. This region included all major phospholipid head groups, backbone moieties and triglycerol backbone moiety signals. (c) Expanded 1D carbon-13 NMR spectrum (126 - 132 ppm) of lipid extract from rat liver. This region included the olefinic chemical shifts from all major mono, di and polyunsaturated fatty acids. Signals which generated from any particular fatty acid species were indicated by the fatty acid abbreviations. Folch method was used for the lipid extraction. 50.0 mg of lyophilised lipid extract was used for each experiment. Samples were dissolved in a 2.0 ml mixture of deuterated chloroform and methanol with volume ratio 1:2. The CDCl_3 resonance signal at 77.0 ppm was used as reference.

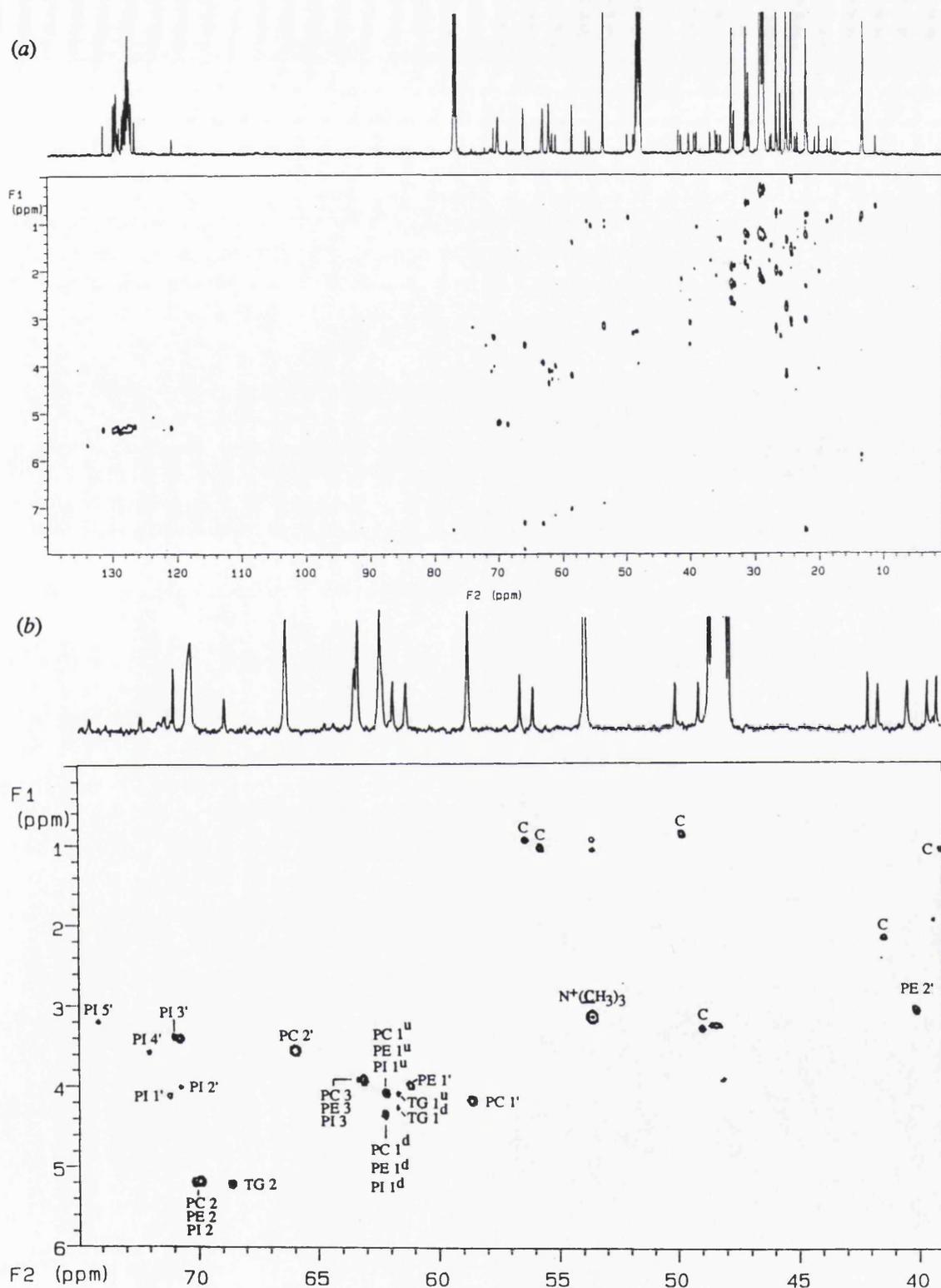


Figure 8.2 (a) 2D HETCOR spectrum of rat liver. (b) Expansion of the spectrum (^1H ; 0.0 - 6.0 ppm, ^{13}C ; 40.0 - 80.0 ppm). All major phospholipid head groups, phospholipid and triglycerol backbone regions are unequivocally assigned. The CDCl_3 resonance signal at 77.0 ppm was used as reference for ^{13}C spectra, and the CD_3OD resonance at 3.30 ppm for a ^1H reference.

8.2) at 3.20/ 69.00 ppm, 3.60/ 71.80 ppm, 3.40/ 70.80 ppm, 4.03/ 70.50 ppm, 4.15/ 71.00 ppm and 4.00/ 63.00 ppm. These signals originated from inositol carbons number 5', 4', 3', 2', 1' and 6' respectively, with 6' overlapped with the *sn*-3 phospholipid moiety signal. The characteristic chemical shifts of different lipids are summarized in table 8.1.

Table 8.1

Summary of diagnostic carbon signals from the major lipids of rat liver extract (ppm). All samples were dissolved in CDCl₃:CD₃OD (1:2 v/v) and experiments were performed at 30°C.

Lipid	C1	C2	C3	C1'	C2'	C3'	C4'	C5'	C6'	N ⁺ (CH ₃) ₃
PC	62.20	70.30	63.30	58.70	66.30	-----	-----	-----	-----	53.70
PE	62.20	70.30	63.30	61.20	40.30	-----	-----	-----	-----	-----
PI	62.20	70.30	63.00	71.00	70.50	70.80	71.80	69.00	63.00	-----
PS	62.30	70.30	63.70	53.40	62.80	-----	-----	-----	-----	-----
TG	61.60	68.70	61.60	-----	-----	-----	-----	-----	-----	-----

8.2.3 Carbon-13 spectral assignments: Alkyl and alkenyl ether phospholipids and sphingolipids

Ether phospholipids are glycerophospholipids in which the hydrocarbon chains at the *sn*-1 position is linked either by an unsaturated or to a lesser extent, a saturated ether linkage, with plasmalogen being the most abundant species in the former class. The diagnostic signals for plasmalogens are the olefinic carbons following the ether bonding at *sn*-1, labelled as a and b. In sphingolipids, the glycerol moiety is replaced by sphingoid, sphingomyelin being the principal sphingoid phospholipid. The diagnostic signals for unsaturated sphingomyelin are the olefinic carbons next to *sn*-1 of the sphingosine moiety, also labelled as a and b. Once again due to the low abundance, neither sphingomyelin nor plasmalogen were detected in the liver lipid extracts. However, all key resonances were readily found in human erythrocyte ghost and plasma extracts, which will be discussed in the following chapter.

8.2.4 Carbon-13 spectral assignments: Glycerolipids and Steroidal lipids

As expected, triacylglycerol was the only major glycerol lipid detected in this particular study. No diacylglycerol signals were observed, due to its lower abundance in liver tissue extract. In TG the diagnostic chemical shift of *sn*-2 carbon was found to be at 68.70 ppm. The *sn*-1 with *sn*-3 TG signals overlapped at 61.60 ppm and were slightly up-field compared to the corresponding signals from phospholipid moieties.

Two varieties of cholesterol, the free and esterified forms, can be found in large concentration in some animal tissues, however, only the former was detected in the rat liver extracts. The ester group of cholesterol ester gives a diagnostic 2D HETCOR cross-peak at 4.55/ 73.50 ppm, which was absent in this particular spectrum. In contrast, this signal was detected in the blood plasma lipid extract and will be discussed in the following chapter. Most cholesterol signals were located within the region from 10 to 70 ppm [Turner & Freeman, 1978], however, the most distinctive signal at 11.30 ppm came from the methyl carbon 18.

8.2.5 Quantitative analysis of lipid extracts via Carbon-13 NMR

To obtain information about lipid components from carbon-13 NMR at a quantitative level, comparison has been restricted to signals of molecular groups exhibiting similar structural and spectroscopic parameters, due to the N.O.E. effect and relaxation behaviour. Despite such reservations, the percentage weight of the three major phospholipids PC, PE and PI, based on the integrated areas of diagnostic head-group resonance, were 57.29 %, 26.25 % and 6.77 % respectively, with a S.D. of $\pm 5\%$. Such values were in close agreement with previously published data (table 8.2a) [Harrocks & Sharma, 1982; White, 1973]. Although direct quantitation of PS and PA was not possible due to their low natural abundance, their concentrations were approximately estimated by subtracting the sum of PC, PE and PI from the total phospholipid concentration which obtained from the chemical shift at 62.20 or 70.30 ppm (table 8.2a).

Table 8.2a

Individual concentrations, in weight percent, of rat liver lipid extract obtained by ^{13}C -NMR, ^1H -NMR and other chromatographic methods.

Lipid	$^{13}\text{C}^{\text{a}}$	$^1\text{H}^{\text{b}}$	HPLC ^b	Ref. ^c
PC	57.29±4.7	60.60±1.8	64.00±0.2	50.80
PE	26.25±6.5	26.40±1.0	23.30±0.1	25.20
PI	6.77±0.34	6.40±1.2	4.70±0.1	7.20
Others*	9.69±0.90	-----	-----	9.20
PC/PE	2.16	2.75	2.30	-----
CHOL/TG	1.03	1.21	1.00	-----

Errors of ± S.D. obtained by three individual values.

*Others = PS, SPH, PA & LPC in column Ref.^c, and PS, SPH & PA in column $^{13}\text{C}^{\text{a}}$.

^aDetermined from ^{13}C NMR from characteristic moiety resonances.

^bBased on data obtained from reference [Exton, 1990; Gunstone, 1992].

^cBased on data from reference [Gunstone, 1991; Gunstone, 1990].

Table 8.2b

The ratio of docosahexaenoic acid (22:6) versus various major fatty acids of rat liver extract obtained by ^{13}C -NMR, comparison with published data.

(22:6)/ fatty acid	$^{13}\text{C}^{\text{d}}$	$^{13}\text{C}^{\text{e}}$	Ref. ^c
Palmitic plus Stearic	-----	0.15	0.17
Oleic (18:1)	0.43	0.39	-----
Oleic plus Palmitoleic	0.83	-----	0.91
Linoleic (18:2)	0.53	0.41	0.65
Arachidonic (20:4)	0.50	0.43	0.46

^cBased on data from reference [Gunstone, 1991; Gunstone, 1990].

^dDetermined from ^{13}C NMR olefinic resonances.

^eDetermined from ^{13}C NMR methyl, methylene or allyl fatty acid resonances.

8.2.6 Determination of fatty acid composition by Carbon-13 NMR.

In carbon-13 NMR the fatty acid composition can be extracted from two possible sources: 1) the structure specific methyl, methylene and allylic carbon resonance from

individual class of fatty acids and 2) their characteristic olefinic carbon signals which depend on the locations within the fatty acid chains. Fatty acid spectra assignments were achieved by the comparison with the spectra of standards, 2D HETCOR and by referring to the known composition in rat liver [White, 1973].

8.2.7 Fatty acid analysis from methyl, methylene and allylic carbon resonance

Alkyl carbon signals were observed from 10 to 40 ppm and several structurally diagnostic resonances were identified, in particular 1) signals from ω (omega)-CH₃ and ω -2 carbon of docosahexaenoic acid (22:6) at 13.50 ppm and 20.10 ppm respectively, 2) specific resonance from arachidonic acid (20:4) including carbon 3 at 26.10 ppm and carbon 2 at 33.30 ppm, and 3) 33.70 ppm provided a diagnostic signal for monosaturated species, mainly oleic acid (18:1) and palmitoleic acid (16:1) [White, 1973].

There are also structural resonance common to some major fatty acids. For instance, the signals of ω -CH₃ and ω -2 carbon from all fatty acids except docosahexaenoic acid occurred at 13.30 and 22.20 ppm respectively. The allylic signal from linoleic acid (18:2) and higher unsaturated fatty acids occurred at 25.20 ppm, which superimposed with the carbon 3 of saturated fatty acids (mainly palmitic acid and stearic acid). The carbon 3 resonance from monosaturated fatty acids, linoleic acid and arachidonic acid are appeared at 24.50 ppm. The complete spectral assignment are shown in figure 8.1a.

From the integrated areas of the pertinent signals, the percentage weights of fatty acids were obtained and compared with the data determined by other methods (table 8.3) [Casu *et al*, 1991; Christie, 1985; White, 1973]. The ratio of different major fatty acids are shown in table 8.2b. Most values were similar to reported data, and the few minor variations that occurred were probably due to dietary differences, which could affect the fatty acid composition [Ulmann *et al*, 1991; Brouard & Pascaud, 1990].

Table 8.3

comparison of the fatty acid levels in rat liver lipid extracts obtained by ^{13}C -NMR, ^1H -NMR and chromatographic methods.

Fatty acids	$^{13}\text{C}_a$	$^{13}\text{C}_d^*$	$^1\text{H}^b$	Ref. ^c
Total Sat.	44.78±2.40	-----	46.90	47.18
Total Unsat.	55.23±2.43	-----	53.10	52.82
Oleic plus Palmitoleic	17.15±0.86	17.94±1.69	-----	-----
Oleic	-----	9.22±0.83	-----	8.64
Linoleic	15.87±0.79	14.38±0.72	14.60	12.13
Arachidonic	16.61±0.80	15.24±0.76	19.20	17.00
Docosahexaenoic	6.80±0.34	7.68±0.69	10.50	7.84

Errors of ± S.D. obtained by three individual values.

*Calculations were based on the value of the weight percentage of total unsaturated fatty acids; obtained from column $^{13}\text{C}_a$.

^aDetermined from ^{13}C NMR of characteristic methyl, methylene or allyl resonances.

^bBased on data obtained from reference [Exton, 1990; Gunstone, 1992].

^cBased on data from reference [Gunstone, 1991; Gunstone, 1990].

^dDetermined from ^{13}C NMR of characteristic olefinic resonances.

Table 8.4

Summary of diagnostic olefinic carbon signals from major fatty acids of rat liver lipid extracts (ppm), samples were dissolved in CDCl_3 - CD_3OD (1:1 v/v) and experiments were performed at 30°C.

Fatty acid	C5	C6	C9	C10	C13	C14	C15	C17	C19	C20
Palmitoleate	-----	-----	-----	129.30	-----	-----	-----	-----	-----	-----
Oleate	-----	-----	129.20	-----	-----	-----	-----	-----	-----	-----
Linoleate	-----	-----	-----	129.40	129.60	-----	-----	-----	-----	-----
Arachidonate	128.30	128.50	-----	-----	-----	127.10	129.80	-----	-----	-----
Docosahexaenoate	-----	-----	-----	-----	-----	-----	-----	128.80	126.60	131.40

8.2.8 Fatty acid analysis using olefinic carbon signals

The olefinic carbon signals of monoenoic to hexaenoic fatty acids were found between

126 and 131 ppm. The detail assignment are shown in figure 8.1c, which included: 1) docosahexaenoate carbons 19, 17, 20 at 126.60, 128.80 and 131.40 ppm respectively, 2) arachidonate carbons 5, 6, 14, 15 at 128.30, 128.50, 127.10 and 129.80 ppm, 3) Linoleate carbons 10 and 13 at 129.40 and 129.60 ppm, 4) oleate carbon 9 at 129.20 ppm and 5) palmitoleate carbon 10 at 129.30 ppm.

Integrated areas of olefinic carbon signals were calculated as described earlier, and the results were found to be similar to their alkyl group counterparts (table 8.2b and 8.3).

8.3 CONCLUSION

Total lipids, extracted from rat liver according to the method of Folch *et al* (1957), were analyzed by 1-dimensional ^{13}C and 2-dimensional heteronuclear correlation (HETCOR) NMR spectroscopy. The fatty acid composition and their relative concentration were also determined. Assignments of all principal lipids and phospholipids in a rat liver lipid extract based upon carbon-13 NMR has been achieved by the comparison with standard 1D and 2D ^{13}C -NMR lipid spectra, 2D HECTOR of the liver extract, and by referring to published ^1H and ^{13}C NMR assignments where available.

Although complete quantitation using every lipid signal was risky due to different relaxation and NOE effects, relative quantitations were obtained by measuring the integrated areas of (a) the head group resonance of phospholipids, (b) the backbone moiety resonance of triacylglycerol, and (c) either the olefinic carbon signals, or the methyl, methylene and allyl resonance of saturated, mono, di, tri, tetra, hexaenoic acids and cholesterol. Significantly, lipid quantitations via ^{13}C -NMR were found to be in close agreement with those obtained by conventional chromatographic techniques.

Further developments such as 2D NMR quantitation should permit analysis of the low concentration lipids in biological fluids and tissues and the NMR technique should eventually provide a more comprehensive, rapid and non-destructive method for characterizing tissues and body fluids in normal and diseased states. With its rapid, non-destructive, comprehensive nature and high resolving power, ^{13}C -NMR should provide a complementary method for lipid analysis of cells, tissues and body lipids in biology and

medicine. Toxicological and pharmaceutical applications are also envisaged, as will be discussed in chapter 10.

Chapter 9
**In vitro analysis of human erythrocytes and blood
plasma lipid by 1D ^{13}C -NMR and 2D ^{13}C - ^1H NMR
spectroscopy**

9.1 INTRODUCTION

Some of the most active research areas nowadays involve lipids and lipid metabolism, such as phosphatidylinositol cycles [Exton, 1990], arachidonate cascade [Choi & Gao, 1991; Kurachi *et al*, 1991; Ordway *et al*, 1991], phosphorylation of protein kinase C and intracellular calcium flux [May & Calder, 1993; Lameh *et al*, 1990; Neer & Clapham, 1988]. Thus there has been an ever-increasing demand for lipid assay methodologies. Established techniques include thin layer chromatography (TLC) [Pal & Davis, 1991; Jheem & Weigel, 1989], high performance liquid chromatography (HPLC) [Araki *et al*, 1990; Seta *et al*, 1990; Vercaemst *et al*, 1989], gas chromatography (GC) [McGrath & Elliott, 1990; Beyer *et al*, 1989], mass spectroscopy (MS) [Chen *et al*, 1990] or the combinations of these [Guido *et al*, 1993; Thomas *et al*, 1992; Chilton, 1991]. Techniques such as HPLC/ MS have been used for the analysis of blood and tissue steroids and their metabolites [Seta *et al*, 1990], cholesterol and triglycerides analysis, lipid analysis in cholesterolemia, hypolipidemias and hyperlipidemias. While these are of cardinal importance it would be advantageous to have a non-destructive, simple and rapid method of simultaneously analyzing blood plasma lipids in general and also erythrocyte and white blood cell lipids. To some extent this has been achieved through 1D and 2D proton NMR [Casu *et al*, 1992; Sze & Jardetzky, 1990].

The potential of NMR spectroscopy has been demonstrated in both natural oils [Gunstone, 1992; Gunstone, 1991; Gunstone, 1990] and *in vitro* biological lipid analysis [Adosraku *et al*, 1993; Choi *et al*, 1993; Casu *et al*, 1992; MacKinnon *et al*, 1992; Ling & Brauer, 1991; Sze & Jardetzky, 1990; Canioni *et al*, 1983; Avila *et al*, 1978; Stoffel *et al*, 1972]. Most of the major classes of lipids and their components were detected and successfully quantified by the combination of 1- and 2-dimensional methods. These techniques also offer the possibility to access information about the fatty acid compositions of intact glycerides and phospholipids, without additional experimental procedures such as chromatography. However, despite the use of proton 1D and 2D NMR at 500 MHz, a complete analysis has not been achieved due to overlapping of spectra and the difficulty of relating fatty acid compositions to individual lipids without

prior separation. Potentially, fatty acid composition and quantitation can be further improved by carbon-13 1D and 2D NMR techniques. Although carbon-13 NMR suffers from an intrinsic sensitivity problem compared to proton NMR, its advantages over the purely proton NMR approach included: i) the lower complexity of spectra particularly in the fatty acid regions, ii) the higher resolution and decreased interference from solvent signals and lower concentration of impurities. Thus by combining the proton and carbon-13 NMR information, a more comprehensive and informative lipid analysis can be possible. This approach has been used here to study the lipid profiles of human erythrocyte and blood plasma extracts.

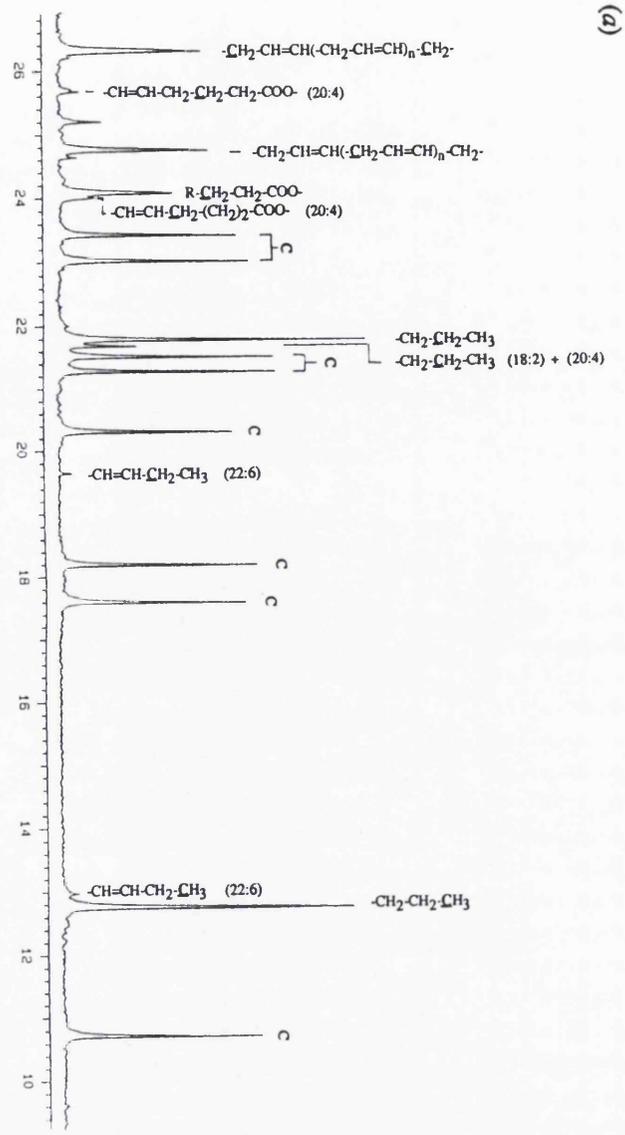
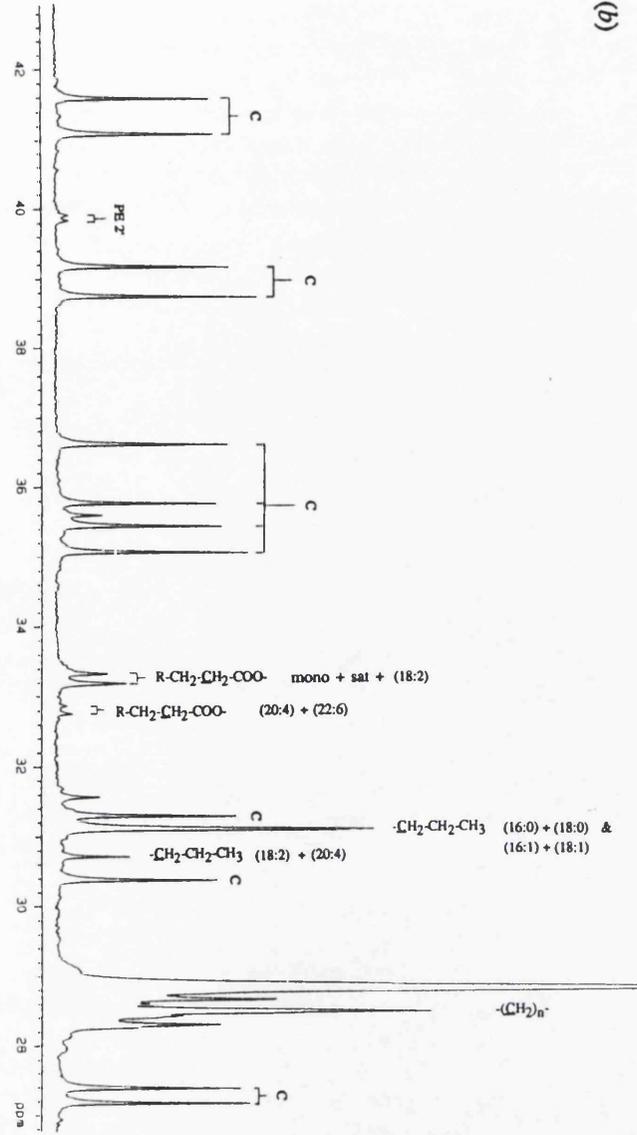
9.2 RESULTS AND DISCUSSION

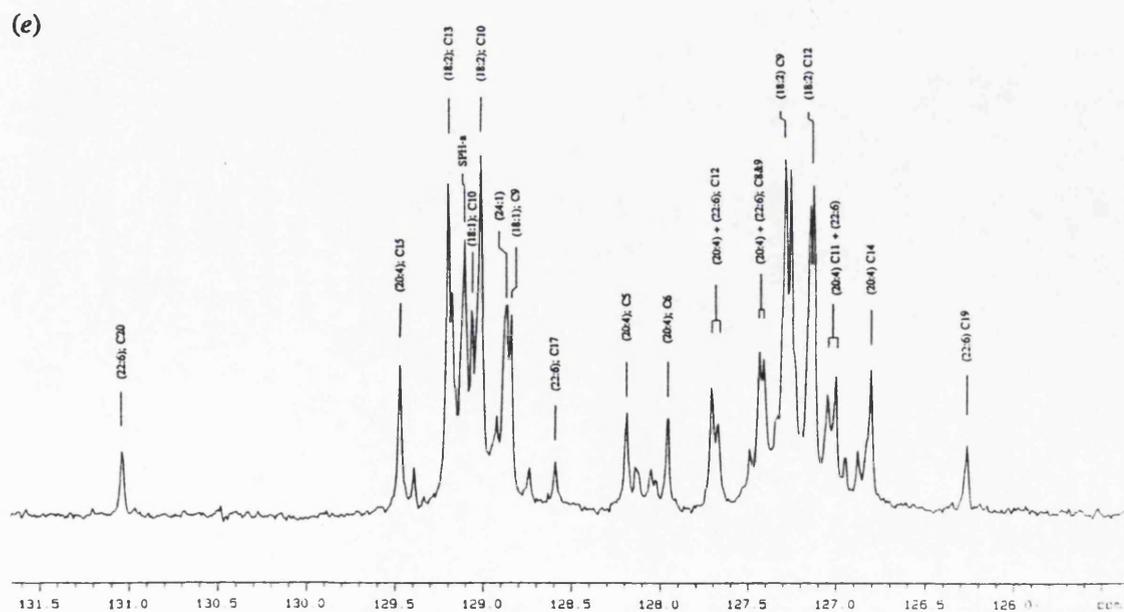
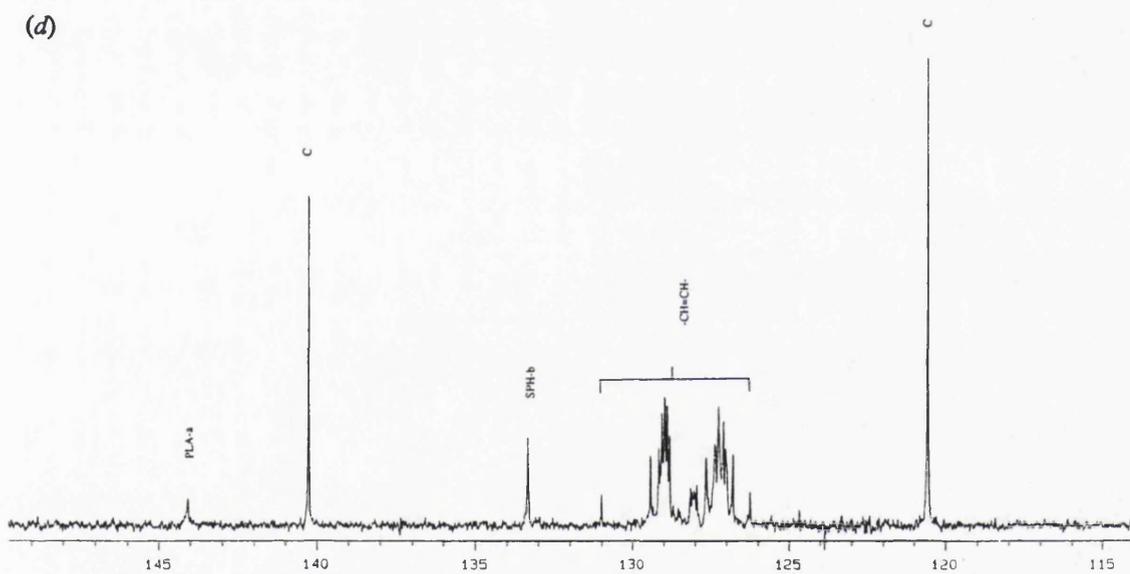
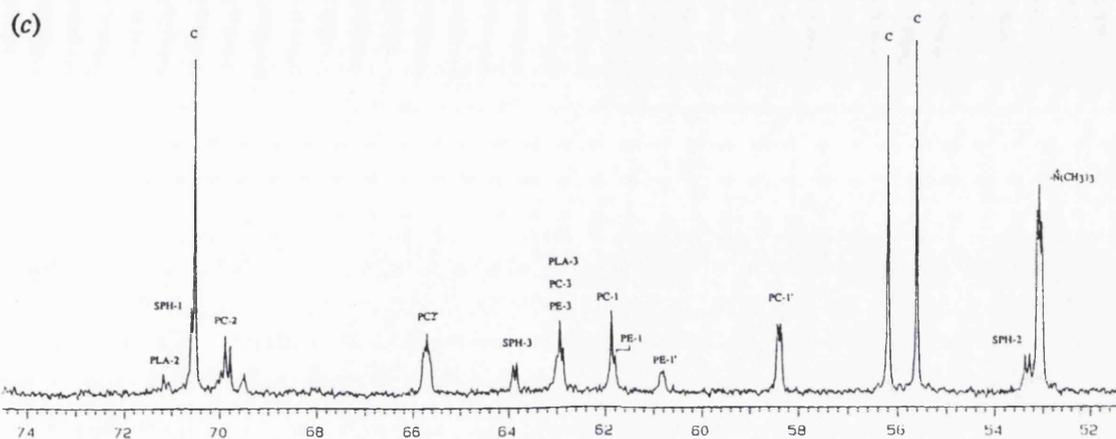
9.2.1 Carbon-13 NMR of human erythrocyte and plasma lipid extracts

The advantages of carbon-13 NMR spectra for in vitro lipid analysis of erythrocytes and blood plasma are clear from the 1D spectra in figure 9.1 and 9.2; single carbon resolution was obtained for most carbon atoms of the intact lipids. This was particularly useful for determining the fatty acid compositions of the phospholipids and glycerides in the total extract although without prior chromatographic fractionation or perhaps the use of very advanced NMR methods, assignments of the specific fatty acid components of individual lipids was not possible. Nevertheless in the areas of clinical chemistry and biochemistry such assignments of simple carbon-13 spectra of total lipids extracts should prove very useful. Carbon-13 and 2D HETCOR NMR spectra of human erythrocyte and plasma lipid extracts are shown in figures 9.1, 9.2 and 9.3. The spectral assignments were achieved by comparison with carbon-13 spectra of lipid standards, analysis of cross peaks in HETCOR spectra plus references to earlier studies [Choi *et al*, 1993; Casu *et al*, 1992; Casu *et al*, 1991; Gunstone, 1992; Gunstone, 1991; Gunstone 1990; Doddrell, 1984; Turner & Freeman, 1978].

9.2.2 Neutral lipid assignments and analysis; cholesterol and glycerides

Although cholesterol and triglyceride levels are routinely measured in clinical chemistry [Grubits, 1992; Gamble *et al*, 1978; Heider & Boyett, 1978; Trinder, 1969],





(f)

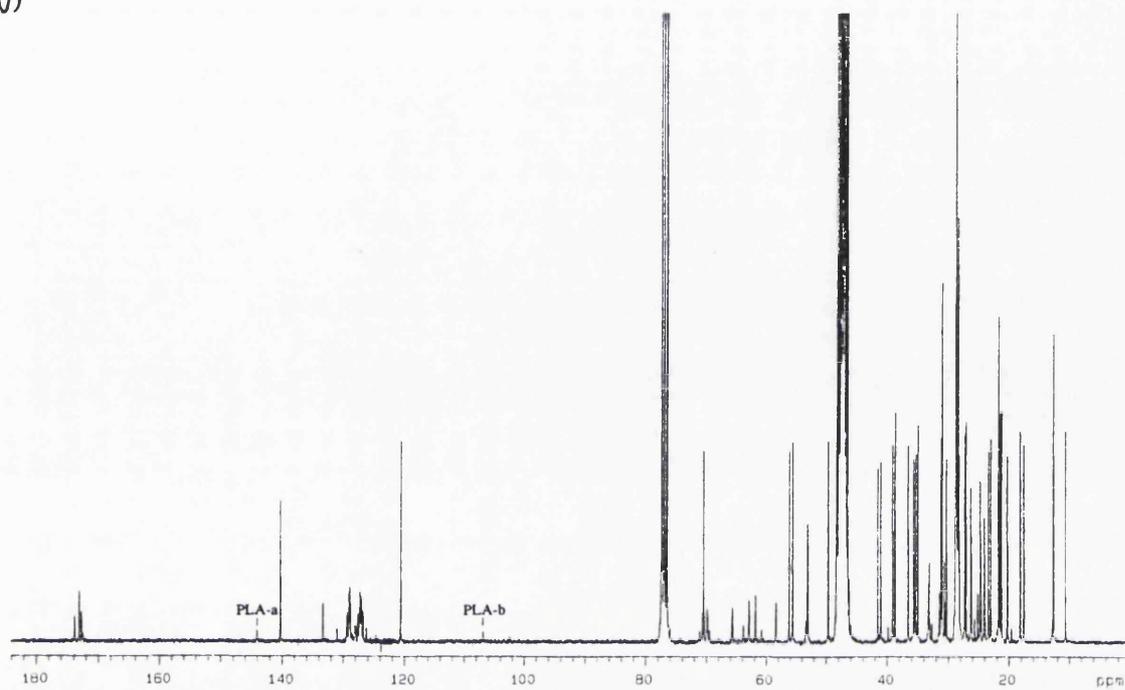
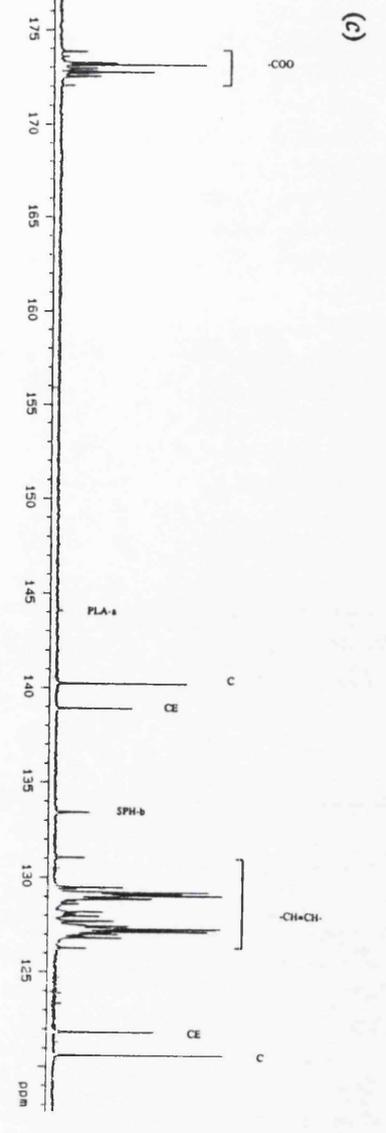
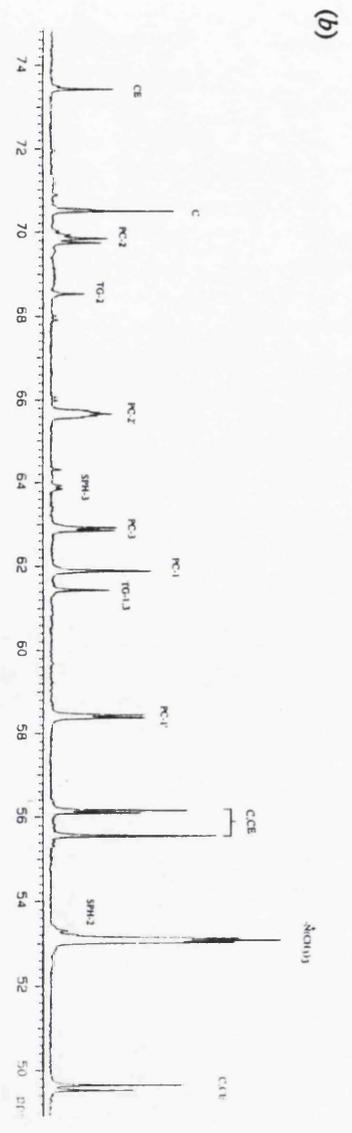
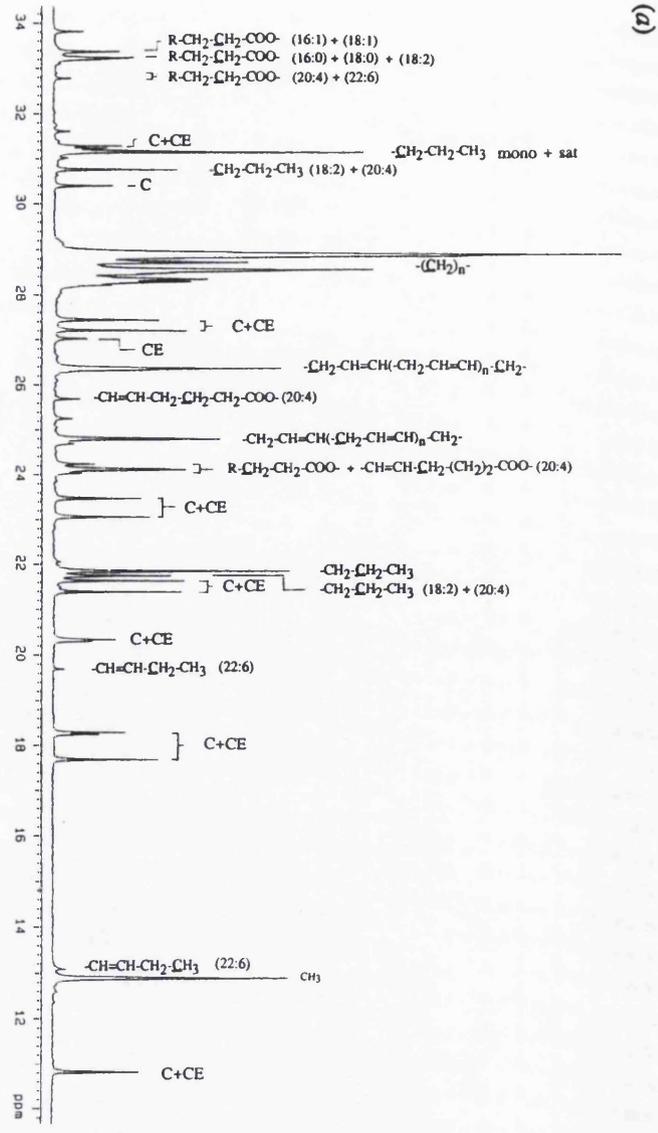


Figure 9.1 (a) Expanded 1D carbon-13 NMR spectrum (10 - 27 ppm) of lipid extract from human erythrocytes. (b) Expansion of the same spectrum from 27 to 43 ppm. These two expansions consist of chemical shifts mainly from cholesterol and esterified fatty acids. Signals which generated from any particular fatty acid species were indicated by the fatty acid abbreviations. (c) Expansion of the same spectrum from 52 to 74 ppm. This region included all major phospholipid head groups and backbone moiety signals. (d) Expansion of the same spectrum from 110 to 150 ppm. This region included the diagnostic olefinic carbon signals from cholesterol, sphingomyelin and plasmalogen, plus olefinic chemical shifts from all major mono, di and poly-unsaturated fatty acids. (e) Expansion from 125.5 to 131.5 ppm, which shows in detail the olefinic carbon chemical shifts from all major unsaturated fatty acids. Signals which generated from any particular fatty acid species were indicated by the fatty acid abbreviations. (f) The full expansion of the 1D carbon-13 NMR spectrum (0 - 200 ppm) from the lipid extract of human erythrocytes. Both diagnostic olefinic chemical shifts from plasmalogen, labelled **a** and **b** are unequivocally assigned. Samples were dissolved in a 2.0 ml of $\text{CDCl}_3\text{-CD}_3\text{OD}$ (1:1 v/v). The CDCl_3 resonance signal at 77.0 ppm was used as reference.



(d)

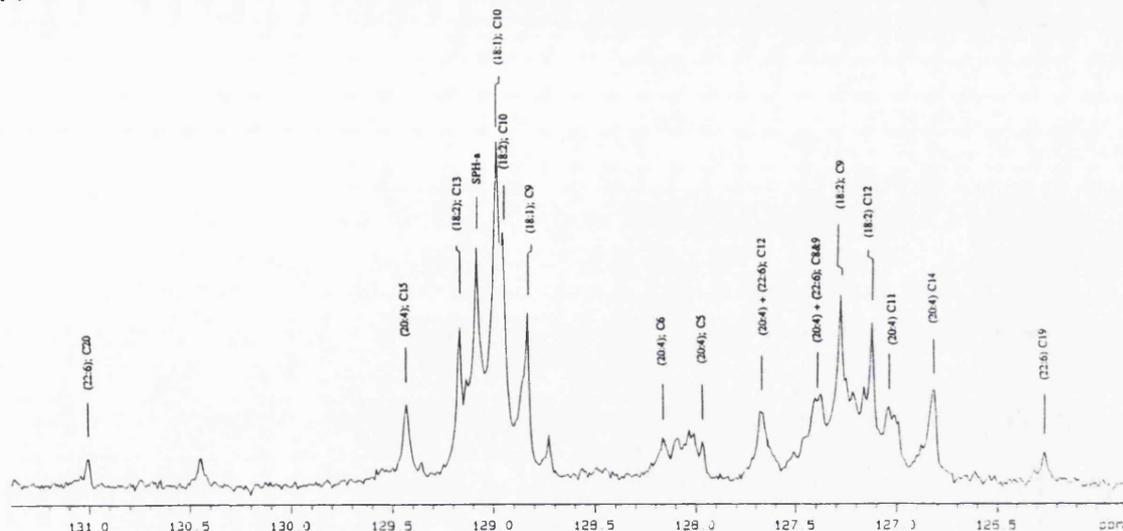


Figure 9.2 (a) Expanded 1D carbon-13 NMR spectrum (10 - 34 ppm) of lipid extract from human blood plasma. This region consist of chemical shifts mainly from cholesterol and esterified fatty acids. The chemical shifts of cholesterol-ester are uniquely exhibited in this sample. Signals which generated from any particular fatty acid species were indicated by the fatty acid abbreviations. (b) Expansion of the same spectrum from 50 to 74 ppm. This region included all major phospholipid head groups and backbone moiety signals. In addition, the chemical shifts of triglycerol moieties, which are absent in erythrocyte extract, are also showed in this particular spectrum. (c) Expansion of the same spectrum from 120 to 175 ppm. This region included the diagnostic olefinic carbon signals from sphingomyelin and plasmalogen, the olefinic chemical shifts from cholesterol and cholesterol-ester, plus olefinic chemical shifts from all major mono, di and poly-unsaturated fatty acids. The chemical shifts of carboxyl groups are located between 172 and 174 ppm. (d) Expansion from 126 to 131.5 ppm. The in detail assignment of the olefinic carbon signals from major unsaturated fatty acids. Signals which generated from any particular fatty acid species were indicated by the fatty acid abbreviations. Experimental conditions were the same as described in figure 9.1.

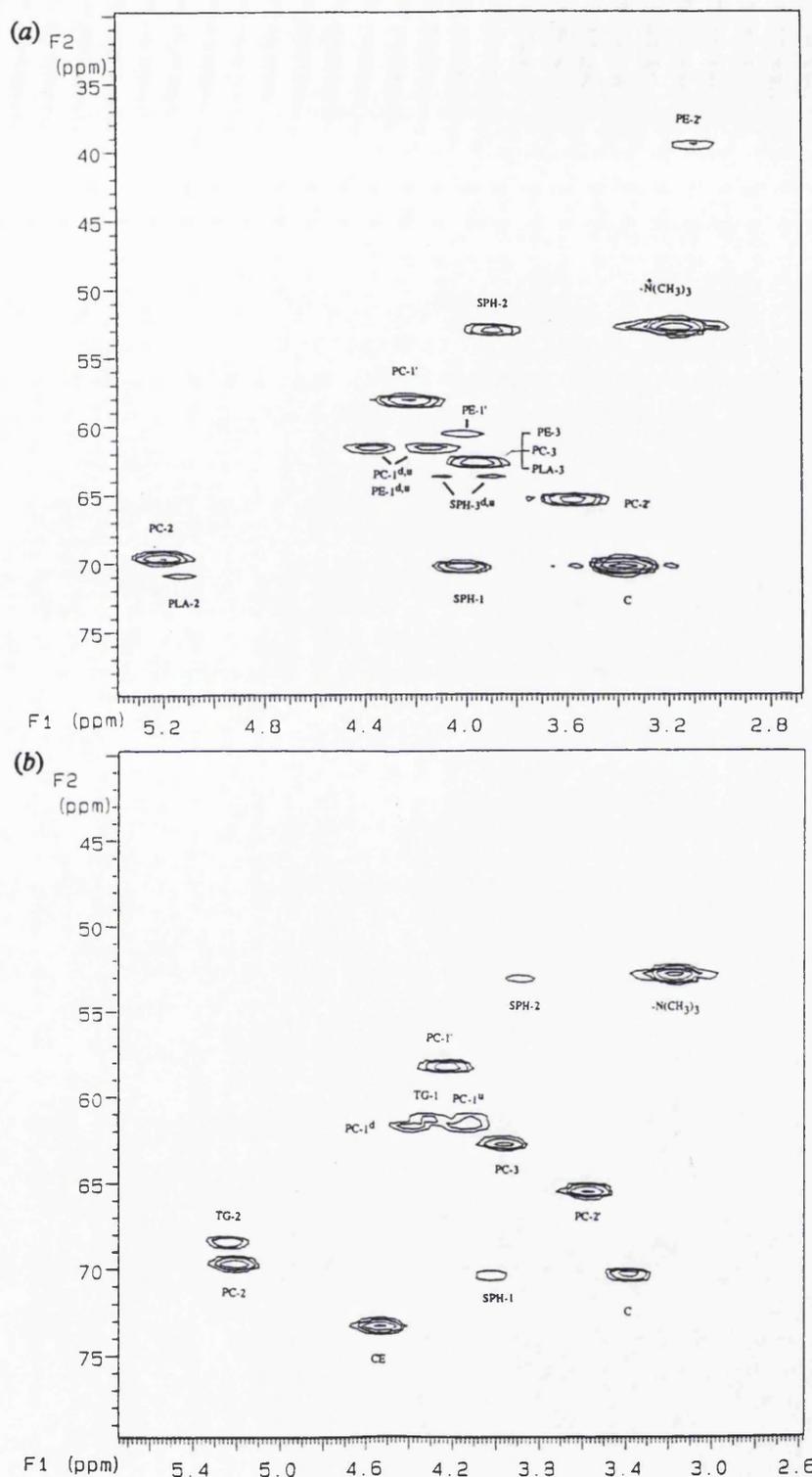


Figure 9.3 (a) Expansion of 2D HETCOR spectrum (^1H ; 2.7 - 5.4 ppm, ^{13}C ; 30.0 - 80.0 ppm) from the lipid extract of human erythrocytes. (b) Expansion of 2D HETCOR spectrum (^1H ; 2.6 - 5.7 ppm, ^{13}C ; 40.0 - 80.0 ppm) from the lipid extract of human blood plasma. All major phospholipid head groups, phospholipid and triglycerol backbone regions are unambiguously assigned. 40.0 mg of lyophilised lipid extract was used for each experiment. Samples were dissolved in a 2.0 ml of $\text{CDCl}_3\text{-CD}_3\text{OD}$ (1:1 v/v). The CDCl_3 resonance signal at 77.0 ppm was used as reference for ^{13}C spectra, and the CD_3OD resonance at 3.30 ppm for a ^1H reference.

each was analyzed via carbon-13 and proton NMR spectra which simultaneously also yielded information on many other lipids, such as phospholipids. In addition, the total fatty acid compositions of intact lipids can be determined without extra experimental procedures, and specific analysis of individual lipid classes can be achieved by combining NMR with simple column chromatography prior to NMR experiments [Choi *et al.*, 1993]. The triglycerol *sn*-2 carbon signal at 68.60 ppm in the plasma lipid extract was distinctive as were the *sn*-1 and *sn*-3 chemical shifts at 61.40 ppm (Fig. 9.2b). Several cholesterol signals in figure 9.1a and 9.2a at 11.70 ppm, 17.60 ppm, 18.20 ppm and 20.30 ppm proved particularly useful for diagnostic purposes. A further advantage of the carbon-13 NMR method was that it readily yielded the ratio of cholesterol ester to cholesterol in blood plasma, from their distinctive C3 and C20 signals at 70.50 ppm and 73.40 ppm respectively (Fig. 9.2b). In this particular study, by comparing the integrated areas between these two chemical shifts yielded a ratio of 1.82, a result that compared favorably with the ratio of 1.72 obtained from a previous proton NMR analysis (Table 9.2c). The absence of triglyceride and cholesterol ester signals in the erythrocyte spectra is consistent with their low abundance or absence from these cells.

9.2.3 Carbon-13 spectral assignment: Sphingolipids and ether phospholipids

The unsaturated sphingosine phospholipids can be identified by their characteristic olefinic carbon signals of the fatty acid moiety adjacent to the sphingosine *sn*-1, labelled a and b, which occurred at 129.10 ppm and 133.20 ppm (Fig. 9.1d & e, 9.2c & d), Sphingomyelin is the principal sphingosine phospholipid in both blood plasma and erythrocytes. The corresponding olefinic carbon signals from plasmalogen, the principal ether phospholipid in this particular study, occurred at 144.20 ppm and 106.50 ppm respectively (Fig. 9.1f) and also labelled as a and b. The sphingosine *sn*-1, *sn*-2 and *sn*-3 carbon signals occurred at 70.5 ppm, 53.3 ppm and 63.8 ppm respectively. Both erythrocyte and plasma lipid extracts exhibited all the characteristic resonances of sphingomyelin and plasmalogen, in 1D and 2D spectra (Fig. 9.1c, 9.2b & fig. 9.3). The characteristic chemical shifts of different lipids are summarized in table 9.1.

Table 9.1

Summary of diagnostic carbon signals from major lipids of human blood plasma and erythrocyte extracts (ppm), experiments performed at 30°C and samples were dissolved in CDCl₃:CD₃OD (1:1 v/v).

Lipid	C1	C2	C3	C1'/Ca*	C2'/Cb*	C3'	C4'	C5'	C6'	N ⁺ (CH ₃) ₃
PC	61.80	69.90	62.90	58.40	55.70	-----	-----	-----	-----	53.10
PE	61.80	69.90	62.90	60.80	39.90	-----	-----	-----	-----	-----
PI	61.80	69.90	62.70	70.60	70.10	70.40	71.40	68.60	70.60	-----
PS	61.90	69.80	63.20	52.90	62.30	-----	-----	-----	-----	-----
SPH	70.50	53.50	63.80	129.00	133.20	-----	-----	-----	-----	-----
PLA	53.00	71.10	62.90	144.20	106.50	-----	-----	-----	-----	-----
TG	61.40	68.60	61.40	-----	-----	-----	-----	-----	-----	-----

* C1' & C2' represent resonance signals from PC, PE & PI, and Ca & Cb from SPH & PLA respectively.

9.2.4 Carbon-13 spectral assignments: Diacylglycerophospholipids

These assignments were based principally upon previous proton assignments [Choi *et al*, 1993; Casu *et al*, 1992; Casu *et al*, 1991] plus the information obtained by 2D HETCOR spectra (Fig. 9.3), and the comparison with spectra of lipid standards and literature values [Choi *et al*, 1993; Casu *et al*, 1992; Casu *et al*, 1991; Gunstone, 1992; Gunstone, 1991; Gunstone 1990; Doddrell, 1984; Turner & Freeman, 1978] where available. They are depicted in figures 9.1 & 2 and their characteristic chemical shifts in ppm listed in table 9.1.

Signals from the head group of PC and PE, namely methylene carbon 1' and 2', occurred at 58.40, 65.70 and 60.80, 39.90 ppm respectively. Other diagnostic signals, as shown in figure 9.1c and 9.2b, include the characteristic choline N⁺(CH₃)₃ shift at 53.10 ppm. The glycerol *sn*-1, *sn*-2 and *sn*-3 resonances of PE occurred at 61.80 ppm, 69.90 ppm and 63.00 ppm respectively, and overlapped significantly with the corresponding PC resonances. Thus the ratio of choline to ethanolamine phospholipids was measured from their methylene carbon 1' resonances as 2.90/ 1.10 ≈ 2.80, a ratio comparable to that from chromatographic as well as proton NMR methods (table 9.2a) [Casu *et al*, 1992]. As expected, no PE was found in the blood plasma extract (Fig. 9.2b) due to its low natural abundance. In contrast, signals from both PC and PE were unambiguously

assigned in erythrocytes spectra (Fig. 9.1b & c). Moreover, the corresponding signals from PS were not observed at 53.40 and 62.80 ppm also due to its lower natural abundance.

Table 9.2a

Individual concentrations, weight percent of extract of human erythrocyte lipids obtained by ^{13}C NMR, ^1H NMR and chromatographic assays.

Lipid	$^{13}\text{C}^a$	$^1\text{H}^b$	HPLC ^b	Ref. ^c
PC	17.51±0.90	15.39	17.61	17.00
PE	10.89±0.50	9.17	14.48	15.00
PLA	4.67±0.30	7.70	-----	-----
SPH	13.23±0.50	16.62	14.81	13.00
CHOL	45.64±2.80	46.64	44.03	42.00
PS + PI	2.49*±0.12	2.04	6.98	8.00
PC/PE	1.61	1.68	1.22	1.45
PC/CHOL	0.38	0.33	0.40	0.40

Table 9.2b

The ratio of docosahexaenoic acid (22:6) versus various major fatty acids of human erythrocytes extract obtained from ^{13}C -NMR are compared to published data.

(22:6)/ Fatty acid	$^{13}\text{C}^d$	$^{13}\text{C}^e$	Ref. ^c
Palmitic plus stearic	-----	0.07	0.08
Oleic (18:1)	-----	0.14	0.16
Oleic plus Palmitoleic	0.25	-----	0.21
Nervonic (24:1)	0.56	-----	0.59
Linoleic (18:2)	0.22	0.25	0.25
Arachidonic (20:4)	0.39	0.38	0.30

Errors of ± S.D. obtained by three individual values.

*Quantity too low to be specifically detected, value was obtained indirectly by subtraction.

^aDetermined by ^{13}C -NMR from diagnostic moiety resonances.

^bBased on data obtained from references [Casu *et al*, 1991; Christie, 1985].

^cBased on data from references [Barenholz & Gatt, 1982; Shohet, 1977; White, 1973; Florkin & Stotz, 1970; Ansell & Hawthorne, 1964].

^dDetermined by ^{13}C -NMR olefinic resonances.

^eDetermined from ^{13}C -NMR fatty acid alkyl resonances.

9.2.5 Fatty acid composition from carbon-13 NMR

Fatty acid compositions were determined by carbon-13 NMR in two ways. One involved analyzing the structure-specific methyl, methylene and allylic resonances of the fatty acid components (Fig. 9.1a, 9.1b, & 9.2a), and the other by assigning the olefinic carbon signals from unsaturated fatty acids (Fig. 9.1e & 9.2d). Both approaches yielded similar results. The fatty acid assignments of both erythrocyte and plasma lipid extracts were again achieved by comparison with the spectra of standard fatty acids, 2D HETCOR and by comparing the spectra with the published lipid and fatty acid compositions in erythrocyte and blood plasma [Christie, 1985; Barenholz & Gatt, 1982; Shohet, 1977; White, 1973; Florkin & Stotz, 1970; Ansell & Hawthorne, 1964]. Substantial agreement between NMR compositions reported in this article and the published data was observed.

9.2.6 Fatty acids analysis from methyl and allylic carbon resonances

The methyl, methylene and allylic carbon resonances that occurred from 10 to 40 ppm provided significant structural information for the majority of fatty acids moieties. For instance docosahexaenoic acid was identified by its ω (omega)-CH₃ and ω -2 carbon resonances at 13.00 and 19.60 ppm respectively; arachidonic acid specific resonances included carbon 4 at 25.70 ppm and carbon 2 at 32.80 ppm; also observed is the diagnostic resonance for monosaturated fatty acids at 33.70 ppm, arise principally from oleic acid and palmitoleic acid [White, 1973; Ansell & Hawthorne, 1964].

There were other structural resonances which are common among major fatty acids. The resonance signals of ω -CH₃ and ω -2 carbons from all species except docosahexaenoic acid occurred at 12.80 and 21.80 ppm respectively. Signals of biallylic carbons given by dienoic and higher unsaturated fatty acid species occurred at 24.80 ppm. The comprehensive fatty acid structural assignments are shown in figure 9.1a, 9.1b and 9.2a.

Quantitative analysis of the major fatty acids was achieved by using their integrated diagnostic peak areas. Despite the potential complication arising from NOE effects and

relaxation differences caused by the diversity of chemical environments, the results compared favorably with other published data listed in table 9.2b and 9.3.

Table 9.3

Composition in weight percent of fatty acids of human erythrocyte lipid extract obtained by ^{13}C -NMR, ^1H -NMR and other chromatographic methods.

Fatty acids	$^{13}\text{C}^{\text{a}}$	$^1\text{H}^{\text{b}}$	Ref. ^c
Total Sat.	50.26	53.10	57.51
Total Unsat.	49.74	46.90	42.49
Oleic plus Palmitoleic	25.64	19.90	18.53
Linoleic (18:2)	14.35	7.70	11.46
Arachidonic (20:4)	6.15	16.20	9.61
Docosahexaenoic (22:6)	3.59	3.10	2.89

^aDetermined by ^{13}C -NMR from characteristic moiety resonances.

^bBased on data obtained from references [Casu *et al*, 1991; Christie, 1985].

^cBased on data from references [Shohet, 1977; Barenholz & Gatt, 1982; White, 1973; Florkin & Stotz, 1970; Ansell & Hawthorne, 1964].

Table 9.4

Summary of diagnostic olefinic carbon signals from major fatty acids of human erythrocyte and blood plasma lipid extracts (ppm).

Fatty acid	C5	C6	C9	C10	C13	C14	C15	C17	C19	C20
Palmitoleate	-----	-----	-----	128.80	-----	-----	-----	-----	-----	-----
Oleate	-----	-----	128.90	-----	-----	-----	-----	-----	-----	-----
Linoleate	-----	-----	-----	128.95	129.10	-----	-----	-----	-----	-----
Arachidonate	128.90	129.10	-----	-----	-----	126.80	129.40	-----	-----	-----
Docosahexaenoate	-----	-----	-----	-----	-----	-----	-----	128.50	126.30	131.00

9.2.7 Fatty acid analysis using olefinic carbon signals

The olefinic carbon signals of fatty acids between 126 to 131 ppm provided additional confirmatory information about the unsaturated fatty acid compositions (Fig. 9.1e & 9.2d). The most distinctive signals included : i) docosahexaenoate carbons 19, 17, 20 at 126.30, 128.50, and 131.00 ppm respectively, ii) arachidonate carbons 5, 6, 14, 15 at 128.90, 129.10, 126.80 and 129.40 ppm, iii) linoleate carbon 10 and 13 at 128.95 and

129.10 ppm, iv) oleate carbon 9 at 128.90 ppm and v) palmitoleate carbon 10 at 128.80 ppm. The list of diagnostic olefinic chemical shifts is shown in table 9.4.

The ratio of docosahexaenoic acid to that of the other detected fatty acids in the erythrocyte lipid extract is shown in table 9.2b. Methylene, allylic and olefinic carbon quantitation show similar results, which are remarkably close to the reference values.

9.3 CONCLUSION

Total lipids, extracted from human erythrocytes and blood plasma based upon the method of Folch *et al* (1957), were analyzed by 1-dimensional ^{13}C and 2-dimensional heteronuclear correlation (HETCOR) NMR spectroscopy and by comparison of these spectra with lipid standards and published data. The assignments of the ^{13}C -NMR spectra from human erythrocyte and blood plasma lipid extracts were achieved by comparison with standard lipid spectra, 2D HETCOR, references to ^1H -NMR assignments and previous publications. The principal neutral lipids and phospholipids were identified and then quantified from the integrated areas of 1D spectra. The fatty acid composition and their relative concentration were also determined. Despite the potential complication arising from NOE effects and relaxation differences caused by the diversity of chemical environments, the quantitations were achieved by measuring the relative integrated areas of (a) phospholipids head group resonance, (b) backbone glycerol resonances for neutral glycerolipids and (c) major fatty acids using their olefinic carbon signals and/or their methyl and allylic resonances. Both the qualitative and quantitative data on the major lipids and fatty acids, obtained rapidly and without prior chromatography, agreed substantially with similar data obtained by traditional chromatographic techniques (HPLC, TLC etc.) (Table 9.2 and 9.3). With its rapid, non-destructive and comprehensive nature, such assignments of simple carbon-13 spectra from lipid extracts should provide a complementary method for lipid analysis, particularly in the areas of clinical chemistry and biochemistry.

Further developments including quantitation via 2D NMR and the combination of ^1H - & ^{13}C -NMR, should permit such analysis for much lower concentrations of lipids in

body fluids and tissues, hence provide a more comprehensive, non-destructive and rapid method for lipid analysis. Such lipid profile analysis should proved particularly valuable as a diagnostic tool in fields such as clinical chemistry medicine and nutritional study.

Chapter 10
**Investigation of the effect of carbon tetrachloride
hepatotoxicity on lipid composition by ^1H -NMR**

10.1 INTRODUCTION

Hepatotoxic effects of CCl₄ (carbon tetrachloride) have been widely studied [Aragno *et al*, 1992; Recknagel, 1983; IARC, 1979]. Information on the toxicity of CCl₄ is extensive, and CCl₄ has become a reference and comparative substance in studies of hepatotoxicity. There is well-established evidence that CCl₄ hepatotoxicity causes various pathological events, including cytochrome P-450 activation and destruction [Castro *et al*, 1989; Slater, 1982], fading of the capacity of the liver endoplasmic reticulum (ER) to sequester Ca²⁺ ions [Dolak *et al*, 1988; Long & Moore, 1986; Moore, 1982; Lowrey *et al*, 1981; Moore, 1980], alternation of membrane permeability [James *et al*, 1986; Kakis *et al*, 1980; Chien *et al*, 1978; Boyer *et al*, 1977], lipid peroxidation [Benedetti *et al*, 1986; Poli & Gravela, 1982; Benedetti *et al*, 1982; Recknagel *et al*, 1977] and fatty liver [Aragno *et al*, 1992; Hoyumba *et al*, 1975; Jeanrenaud, 1977]. Such pathological events can potentially trigger further destructive reactions [Anders & Pohl, 1985; Glende & Pushpendran, 1986; Lamb *et al*, 1984; Hewish & Burgoyne, 1973; Burgoyne *et al*, 1970]. However, the major contributor to final cell death is still unclear [Chiarpotto *et al*, 1990; Albano *et al*, 1989; Tsokos-Kuhn, 1988].

The advantages of rapid, non-destructive and comprehensive lipid analysis by NMR has been successfully demonstrated for cells, body fluids and tissues [Choi *et al*, 1993; Casu *et al*, 1991; Sze & Jardetski, 1990; May *et al*, 1986; Yu *et al*, 1986; Toshiyuki *et al*, 1984; Canioni *et al*, 1983; Jardetski & Roberts, 1981; Drabkowski *et al*, 1980; Sawan *et al*, 1979; Llida *et al*, 1977; Mahadevan *et al*, 1968]. Intact lipid and their fatty acid compositions can be analyzed simultaneously, without further chromatographic procedures such as HPLC or GC, and such NMR lipid assays are a valuable complementary method for studying CCl₄ hepatotoxicity mechanisms in liver. However, more precise and specific information can be obtained, by the addition of simple column chromatography prior to NMR and to divide the liver lipid extract into four or five different classes according to their polarity. Qualitative and quantitative information of intact lipids and fatty acids from each class can be obtained individually. In this particular study the liver lipid extract was separated into 1) neutral lipid classes, included

triacylglycerol (TG), diacylglycerol (DG) and steroidal lipids, 2) non-acidic phospholipid classes, included phosphatidylcholine (PC), phosphatidylethanolamine (PE), plasmalogen (PLA) and sphingomyelin (SPH), 3) acidic phospholipid classes, which included principally phosphatidylinositol (PI) and phosphatidylserine (PS). The lipid profile of rat liver obtained from normal and CCl₄ treated rats via 1D ¹H-NMR were compared. The data obtained from ¹H-NMR technique in this report were found to be highly consistent and reproducible, and correlated with other recent studies [Aragno *et al*, 1992; Chiarotto *et al*, 1990; Albro *et al*, 1988].

10.2 RESULTS AND DISCUSSION

10.2.1 Hepatotoxic effects of CCl₄ on rat liver lipid composition determined by ¹H-NMR

Recent studies have suggested that the fatal damage from hepatotoxic substances is probably due to secondary evoked mechanisms, rather than the initial hepatotoxic events [Recknagel *et al*, 1989]. For instance, the disturbance in hepatocellular calcium homeostasis, caused mainly by the early depression of calcium sequestration by the liver ER, can lead to an abnormally high intracellular calcium level, thus trigger various Ca²⁺ activated enzymes such as phospholipase A₂ (PLA₂) in an uncontrollable manner [Glende & Recknagel, 1992; Glende & Pushpendran, 1986]. Other studies suggested major cell injury involved lipid peroxidation induced by free-radical mechanisms [Castro *et al*, 1989; Slater, 1982]. Regardless of their origin, such lipid degeneration processes should be able to be monitored by the change of lipid and fatty acid compositions in liver. In this report ¹H-NMR was applied to determine the effects of CCl₄ on total liver lipid composition and upon the composition of each class of lipid separated by Bond-Elut chromatography, as illustrated in figure. 10.1.

All lipids and fatty acids were identified and quantified by the 1D ¹H-NMR technique developed previously [Choi *et al*, 1993; Casu *et al*, 1991]. Both control and CCl₄ treated experiments were performed in triplicate, and the error-bar in all figures represents the

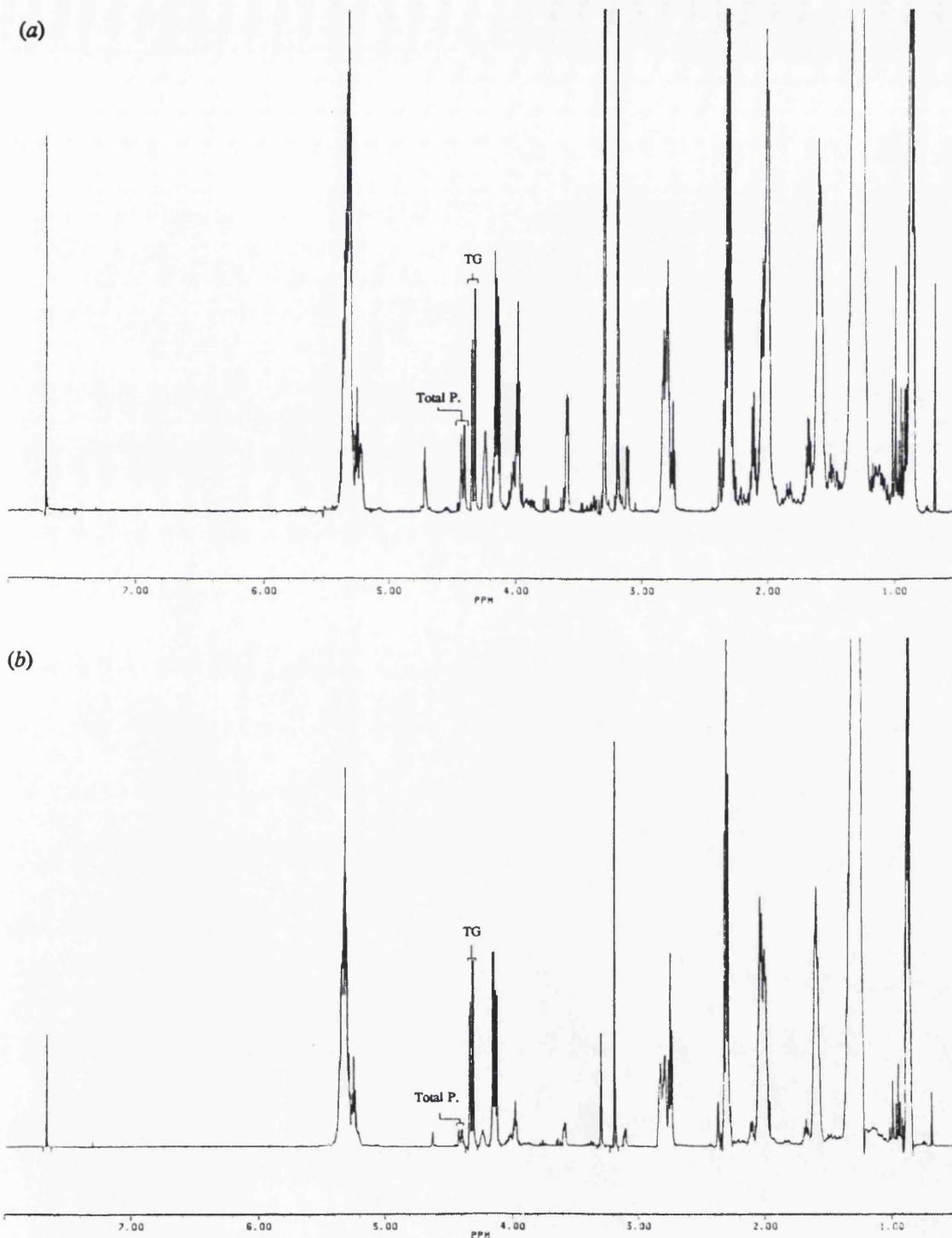


Figure 10.1 One-dimensional proton NMR spectra of lipids extract from (a) normal (control) rat liver and (b) CCl_4 treated rat liver. The effect of CCl_4 on the lipid composition was readily detectable, as emphasized by the difference of phospholipid /TG ratio in the two spectra.

Note: Two spectra are presented in different scale, due to the overwhelming size of TG peak in spectrum (b). The spectral scale of (a) is five times larger than of (b).

standard-deviation of three measurements. All chemical shifts used for diagnostic purposes are summarized in table 10.1.

Table 10.1

Diagnostic proton chemical shifts for major lipids and fatty acids.

Chemical species or structures	Chemical shift (ppm)	General remarks
Total P.	4.40	Total phospholipids content
PC	3.20	-----
PE	3.08	-----
PI	3.75	-----
PS	3.75	-----
CHOL.	0.70	-----
SPH	5.70	-----
PLA	5.90	-----
TG	4.30	-----
DG	3.60	-----
-CH=CH-	5.61	Fatty acid olefinic groups
-CH=CH-(CH ₂ -CH=CH) _x -	2.80	[x>1]; PUFAs except linoleic acid
-CH=CH-CH ₂ -CH=CH-	2.75	Principally linoleic acid
-CH=CH-CH ₂ -CH ₂ -COO-	2.40	Δ ⁴ fatty acids, mainly docosahexaenoate
-CH ₂ -CH=CH-(CH ₂ -CH=CH) _y -CH ₂ -	2.00	[y≥0]; fatty acid allylic groups
-CH=CH-CH ₂ -CH ₂ -CH ₂ -COO-	1.70	Δ ⁵ fatty acids, principally arachidonate
CH ₃ -CH ₂ -CH=CH-	0.95	n-3 fatty acids
CH ₃ -CH ₂ -CH ₂ -R	0.85(total)	R = long-chain alkyl groups

10.2.2 Diacylglycerolipid, steroidal lipid and total phospholipid compositions

The comparison of liver lipid extract contents (in mg per kg of animal wt.) between control and CCl₄ treated rat is shown in figure 10.2. As expected, a substantial increase in triglycerol was observed in CCl₄ treated liver sample. This fatty liver phenomenon has been attributed to the depression of VLDL (very low density lipoprotein) secretion from liver by CCl₄ [Aragno *et al*, 1992; Jeanrenaud, 1977; Hoyumba *et al*, 1975]. There is also a detectable but smaller decrease in total phospholipid content possibly caused by the combination of lipid peroxidation, and the action of PLA₂ [Glende & Recknagel,

1992; Glende & Pushpendran, 1986]. Since phospholipids are known as the principal source of PUFAs (polyunsaturated fatty acids, such as arachidonic acid) and PUFAs are particularly vulnerable to peroxidation process [Thomas *et al.*, 1992], decrease in phospholipid levels could have been caused by the excessive breakdown of PUFAs. Additionally, the rapid fading of the capacity of liver ER to sequester Ca^{2+} ions caused by CCl_4 [Dolak *et al.*, 1988; Long & Moore, 1986; Moore, 1982; Lowrey *et al.*, 1981; Moore, 1980] could lead to a unphysiological rise in concentration of Ca^{2+} ions in the cytosol, which in turn caused the activation of PLA_2 [Glende & Recknagel, 1992; Glende & Pushpendran, 1986] and hence lead to the degeneration of phospholipids. In contrast there is no observable difference in cholesterol concentrations.

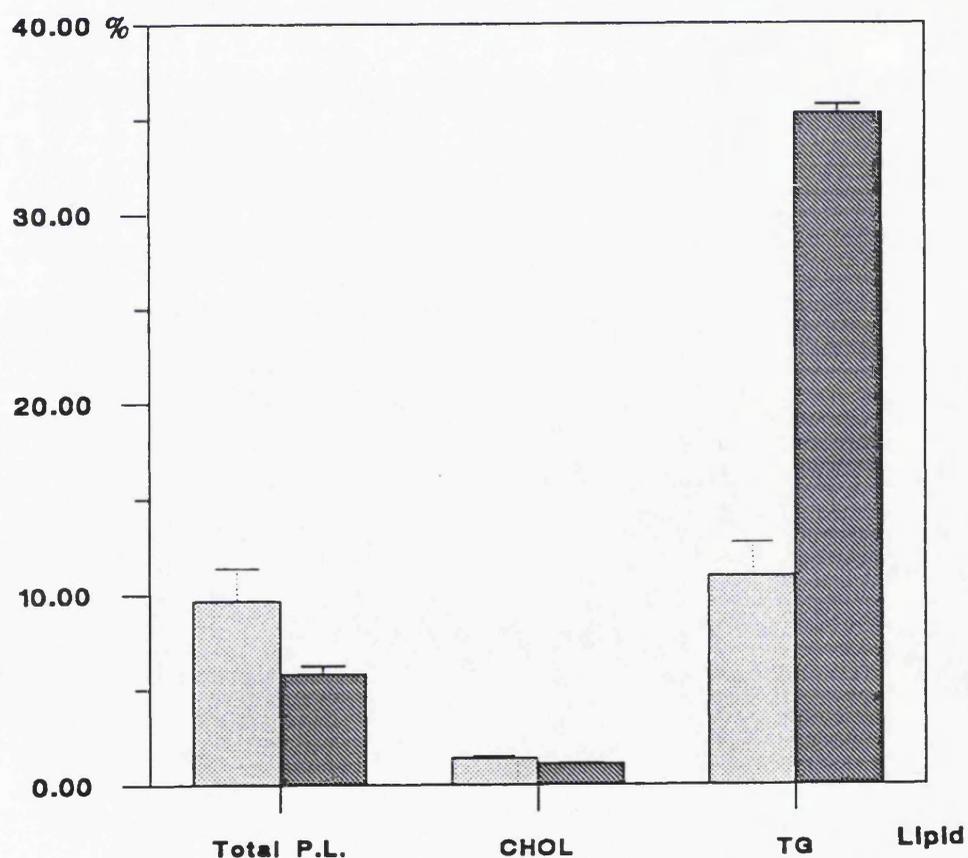


Figure 10.2 The comparison of lipid compositions, between control (□) and CCl_4 treated (▨) rat liver lipid extract (in mg per kg of animal wt.). Error bars represent \pm S.D. from three separate experiments.

10.2.3 Phospholipids and acidic-phospholipids composition

The comparison of liver phospholipid levels (in mg per kg of animal wt.) in control and CCl₄ treated rat is shown in figure 10.3. The decrease in phospholipid levels among CCl₄ treated liver in contrast to the controls as described in previous section, is further confirmed by this data. With the exception of sphingomyelin (SPH) and plasmalogen (PLA), all other major phospholipids were considerably reduced compared with their control counterparts. The lack of a statistically significant reduction in SPH and PLA levels could be due to the low natural abundance hence higher integral errors of the two species. The degree of reduction in each major phospholipid was consistent with the model involving PLA₂ activation and its contribution to phospholipid degradation.

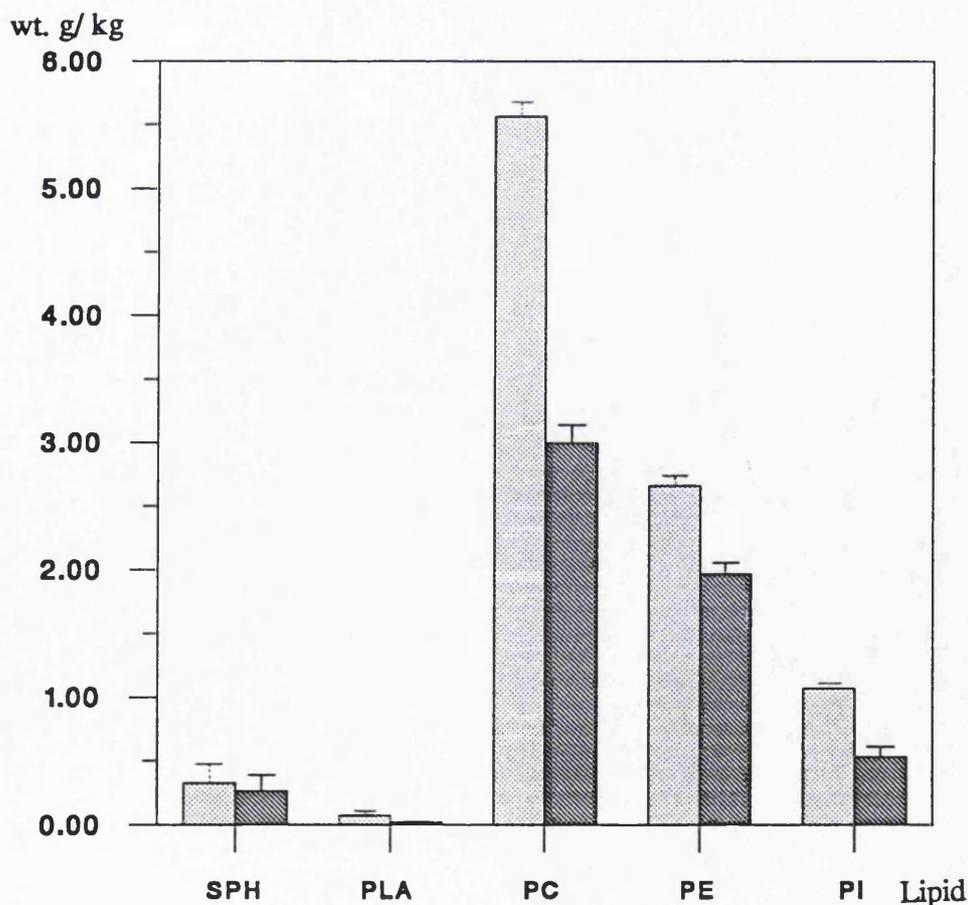


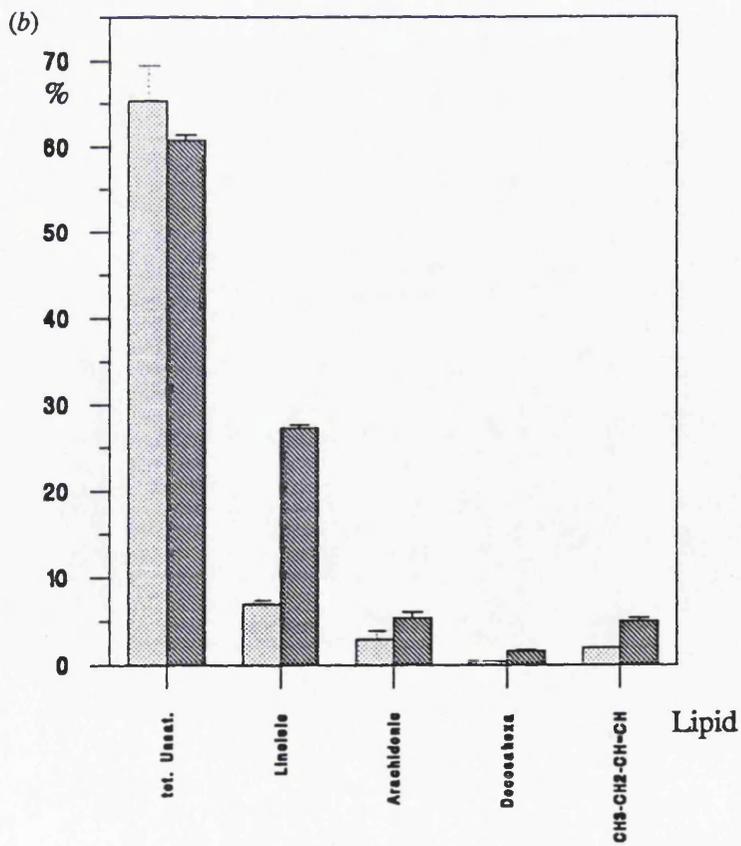
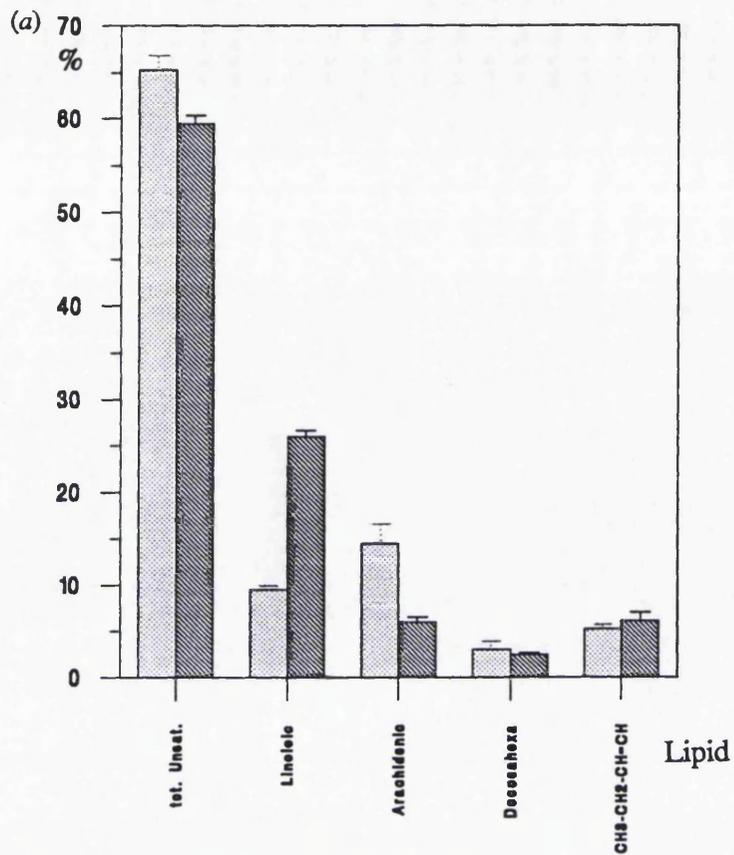
Figure 10.3 The comparison of liver phospholipid levels (in mg per kg of animal wt.) between control (□) and CCl₄ treated (▨) rat. Error bars represent ± S.D. from three separate experiments.

However, among the principal phospholipids, PC is also known to be the major donor of PUFA, thus the larger reduction of PC can also be explained by the higher rate of destruction by peroxidation processes.

10.2.4 Fatty acid compositions from intact lipids

Ion-exchange column chromatography [Christie, 1987b] separated the lipid extract into three individual sub-classes, according to their polarity. Such separation prior to the $^1\text{H-NMR}$ experiment is particularly beneficial for interpreting fatty acid compositions of intact lipids, since fatty acid compositions of the individual lipid classes can then be specifically assigned. The comparisons of major liver fatty acid compositions (in mol %) between control and CCl_4 -treated rats, prior and after column separation, are shown in figure 10.4.

One interesting feature common to all four sub-figures (thus all three lipid sub-classes) is that, despite the general increases of linoleic acid content in CCl_4 treated liver samples, which coincided with previous findings [Aragno *et al*, 1992; Chiarpotto *et al*, 1990; Albro *et al*, 1988], there is no significant difference in terms of total unsaturated fatty acid levels between the two groups. In the case of non-acidic and acidic phospholipids (i.e. figures 10.4c and d), this phenomenon could be explained by the corresponding reduction in arachidonic acid. However, the same argument cannot be applied to the neutral lipid levels in figure 10.4b. In this particular case, which reflected principally triacylglycerol, there were substantial increases in all $^1\text{H-NMR}$ detected fatty acids in CCl_4 treated livers, despite the fact that total unsaturated fatty acid levels remained relatively unchanged compared to the controls. To rationalize the two total unsaturated fatty acid levels, one possible explanation could be a considerable reduction of those fatty acids which could not be specifically detected by $^1\text{H-NMR}$ in CCl_4 treated livers. Since the only major fatty acid species which could not be identified specifically by $^1\text{H-NMR}$ is mono-unsaturated fatty acid, we suggest there is a possibility of significant reduction in the production of mono-unsaturated fatty acid after CCl_4 intoxication. Thus the increase of linoleic acid and PUFA contents within triglycerol after CCl_4 exposure could be



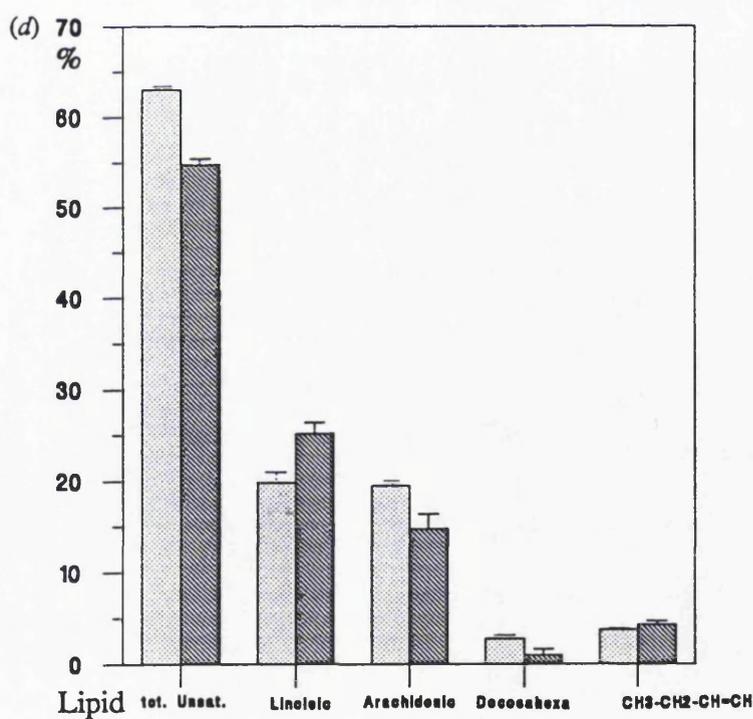
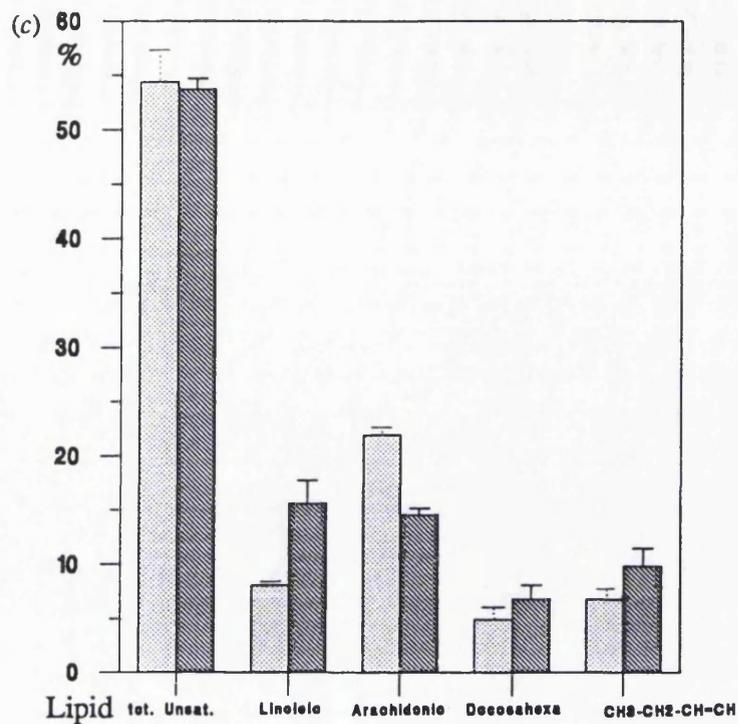


Figure 10.4 The comparisons of major liver fatty acid compositions (in mol %) between control (dark) and CCl₄-treated (striped) rat, prior and after column separation. (a) The comparison prior column separation. (b) Included the comparison of neutral lipids classes (mainly TG), (c) non-acidic phospholipids classes (PC, PE, SPH and PLA) and (d) acidic phospholipids classes (mainly PI) respectively. Error bars represent \pm S.D. from three separate experiments.

explained by the lack of mono-unsaturated fatty acids in the liver for triacylglycerol production. While animals are not able to desaturate fatty acid acyl-chain beyond the Δ^9 position, the raised of both linoleic acid and PUFA indicated the increased up-take of the essential fatty acids into the liver after CCl_4 treatment.

The reduction of arachidonic acid levels in both acidic and non-acidic phospholipids, can be explained by the activation of PLA_2 and lipid peroxidation, as described earlier. In addition, since linoleic acid is the precursor of PUFA synthesis, the increased concentration of such acid after CCl_4 treatment can be either caused by 1) the increased up-take for PUFA production, to compensate the excessive losses of arachidonic acid, or 2) the inhibition of arachidonic acid production, hence causing accumulation of linoleic acid.

10.3 CONCLUSION

In vitro lipid NMR profiling was utilized in this particular study, to illustrate the potential of this novel assay in biochemical and toxicological research. All major lipid classes in the rat liver were unequivocally identified by NMR and with the aid of column chromatography fractionation prior to NMR, analysis became more precise and specific, particularly for acidic phospholipid. The compositions of the esterified fatty acids within each class of lipid was also achieved by NMR. There were considerable changes in both fatty acid and lipid compositions after CCl_4 intoxication. Apart from the expected increase in triacylglycerol, other variations observed include the drop of phospholipid levels and the increase of PUFA content among all lipids. In addition, although not revealed directly, there is a strong implication that monounsaturated fatty acid levels were reduced substantially in triacylglycerol after CCl_4 intoxication. Significantly, such finding of the alteration of lipid compositions in rat liver correspond with published data. In general, the information from NMR analysis are consistent with the models of lipid peroxidation and the failure of calcium flux control. Other speculated mechanisms that could contribute to cell death include the disruption of DNA metabolism and the stimulation of hepatocyte growth factor (HGF) [Asami *et al*, 1990; Nath *et al*, 1990].

This study demonstrated the applicability of *in vitro* NMR lipid profiling to biological lipid metabolic studies. With the appealing advantages such as its rapidity, comprehensiveness and non-destructive nature over other conventional chromatographic approaches, NMR alone or in combination with Bond Elut column chromatography is a complementary alternative for use in clinical, biology, toxicology and medical research. With further developments or modifications, NMR lipid profiling can possibly be expanded to wider applications such as lipid related genetic diseases, physiological process of ageing or in pathological status such as hypertension, arteriosclerosis, diabetes etc.

Chapter 11
Summary and Discussion

11.1 METHODOLOGIES FOR LIPID ANALYSIS

11.1.1 An overview of the conventional lipid assays

It is becoming clear that lipids do not only have structural roles but also participate in major biological events such as cellular signalling. Lipid assays are therefore essential for understanding their role in biochemical, pharmaceutical and medical research. Nevertheless, comprehensive determination of lipid levels in membranes, cells, tissues and body fluids has not been easy, mainly due to the difficulty of separating the variety of lipid species (yet chemically and structurally very similar) before specific analyses could take place. Enzyme and immunosorbent methods use specifically labelled lipids, and although they give sensitive and accurate assays, are restricted to monitoring one single enzyme via its substrate and products only [Chilton, 1991; Gibbons *et al*, 1989]. Whilst significant advances in lipid assays have been achieved by thin layer chromatography (TLC) [Pal & Davis, 1991; Jheem & Weigel, 1989; Jardetski & Roberts, 1981; Gurr & James, 1980; Touchstone, 1973], gas chromatography (GC) [Kuksis *et al*, 1990; McGrath & Elliott, 1990; Beyer *et al*, 1989] and high performance liquid chromatography (HPLC) [Araki *et al*, 1990; Seta *et al*, 1990; Samet *et al*, 1989; Vercaemst *et al*, 1989; Christie, 1987; May *et al*, 1986; Yu *et al*, 1986], for these methods only a limited number of lipids or lipid classes can be differentiated within a single set of chromatographic conditions or parameters. Combination of various techniques or adjustment of chromatographic conditions are necessary for revealing a complete lipid profile. Consequently comprehensive lipid analysis has become a labour-intensive and time-consuming procedure. Mass spectroscopy (MS) [Harvey, 1991; Toshiyuki *et al*, 1984] has also been used in conjunction with chromatography (with HPLC in particular) and, although this has proved to be a significant advance, it still possesses many drawbacks of the chromatographic procedure to which it is coupled. For instance, lipids which are to be analyzed via chromatographic approaches have often to be subjected to irreversible chemical degradations such as lipid hydrolysis. Additional practical procedures are often required in order to analyze esterified fatty acid compositions of intact lipids. Furthermore, recovery of samples can also be difficult in

certain types of chromatographic assays. Despite all the reservations, the techniques mentioned above are the only method of choice today for routine lipid multiple assays.

11.1.2 Lipid analysis by NMR approach

Soon after the discovery of chemical shift phenomenon, the potential of nuclear magnetic resonance spectroscopy for molecular structure analysis has been realized and extensively explored [Akitt, 1983; Martin *et al*, 1980; Harris, 1983]. Since then NMR has rapidly become an essential tool for structural determination and confirmation in synthetic organic chemistry and biology [Williams & Fleming, 1987; Kemp, 1986]. More recently, with the introduction of two-dimensional experiments [Ernst *et al*, 1986; Benn & Gunther, 1983; Freeman & Morris, 1979], the application of NMR on polypeptides, proteins and many biological macromolecules have also been established, as there are also attempts to apply NMR to lipid analysis.

Although there have been developments of lipid analysis involving phosphorus (^{31}P) NMR [Radda *et al*, 1991; Sparling *et al*, 1989; Meneses & Glonek, 1988; Stoiros *et al*, 1986; Iida *et al*, 1977; Mahadevan *et al*, 1968], the application is limited to phospholipids only. However, with the recent advanced high-field magnets (currently up to 17.6 tesla), modern computing technology and multidimensional experiments, NMR of some other nuclei, proton (^1H) and carbon-13 (^{13}C) in particular, could theoretically detect all the present classes of lipids [Jeffrey *et al*, 1991; Sparling, 1991]. In contrast to the chromatographic methods, this NMR approach has the advantages of rapidity, comprehensiveness, and is non-destructive in nature. NMR thus has promises as an highly appealing alternative to the existing conventional lipid assays.

One major advantage of using NMR for lipid analysis is that all lipid molecules can in principle be detected simultaneously. Yet such comprehensive lipid profile can be obtained rapidly; a typical 1D ^1H -NMR experiment can analyze lipid extracts at the mg level in less than 30 minutes [Choi *et al*, 1993; Casu *et al*, 1991]. The NMR lipid analysis approach has one other advantage over the conventional lipid profiles based upon merely chromatographic assay procedures, that is, the fatty acid profiles of intact-lipids can be

determined instantly without resort to additional experiment (e.g. lipid hydrolysis). The NMR experiments are hence non-destructive and lipid samples are fully recoverable. In addition, by measuring the spectral intensities (i.e. integral areas) of characterized diagnostic peaks, the relative quantities (often expressed in weight percentage) of all major lipids and esterified fatty acids are readily obtainable.

11.2 DEVELOPMENT OF THE LIPID PROFILE ANALYSIS BY NMR

11.2.1 Lipid analysis via proton NMR

Recently, a few laboratories [Sze & Jardetski, 1990; May *et al*, 1986; Yu *et al*, 1986; Toshiyuki *et al*, 1984; Canioni *et al*, 1983; Drabkowski *et al*, 1980; Jardetski & Roberts, 1981; Sawan *et al*, 1979; Llida *et al*, 1977; Mahadevan *et al*, 1968] including ours have explored the potential of high-field proton NMR spectroscopy as a complementary technique for lipid profiling. *In vitro* ^1H -NMR has been shown to be a rapid, comprehensive and non-destructive technique for the analysis of complex mixtures of lipid and phospholipid extracts from synthetic lipids [Sparling, 1990; Sparling *et al*, 1989], mineral oil [Gunstone, 1991, Gunstone, 1990], and various cells, tissues and body fluids [Choi *et al*, 1993; Casu *et al*, 1992; Casu *et al*, 1991; Sze & Jardetski, 1990]. It has also been applied to lipid profiling of lower organisms [Adosraku *et al*, 1993].

Lipid extracts from the rat liver, brain, heart, kidney, human erythrocytes and blood plasma were chosen for our development work of NMR lipid profiling, to illustrate the diversity of their lipid compositions and the potential interests of NMR lipid analysis in biochemical research. Of all the studies described in this report, assignments were accomplished by the combination of (a) comparison with the ^1H -NMR spectra of lipid or fatty acid standards, (b) "spiking" the one or two dimensional spectra by gradually adding the compound which was suspected to be present, (c) by analyzing the cross-peaks of 2D NMR experiments (COSY or HOHAHA) [Bax & Davis, 1985; Bax & Freeman, 1981] from the lipid standard or mixture, thus assigning these to the structural-specific moieties of individual lipid and fatty acid species, (d) cross-references with the spectra of lipid extracts from our previous assignments and, (e) by referring to the

published data of lipid compositions or previous assigned NMR spectra when available.

With the ^1H -NMR approach, all major lipids and fatty acids, with concentration higher than *ca.* 0.10 wt. % (in 50.0 mg of sample) were unambiguously identified. The *in vitro* proton NMR approach of lipid analysis not only yielded the phospholipids, triglyceride and cholesterol lipid levels but also demonstrated several additional features. The principal lipids of each class, neutral phospholipids and acidic phospholipids for instance, were simultaneously determined. It has been traditionally complicated to identify and distinguish ether lipids from the corresponding PE, PC, cerebroside and sphingomyelin. This assay did identify each of these structurally related lipids via their diagnostic structural resonances, although sometimes a combination of two or three moieties per molecule was required for unambiguous assignment. Equally important was the detection and measurement of the fatty acid composition in the extracted lipids including the ratio of saturated and unsaturated fatty acids, and the concentrations of individual polyunsaturated fatty acids.

All the quantitation of lipids and esterified fatty acids reported were obtained by measuring the integrated areas of specific diagnostic resonances. All concentrations are in terms of weight percentage (wt. %). However, their absolute amount can also be determined, by referring wt. % to the wet weight of tissue used for lipid extraction and the total weight of the dried lipid extract.

11.2.2 Combination of NMR lipid profiling with ion-exchange chromatography

Although the ^1H -NMR approach to lipid analysis is promising there are several drawbacks still to be overcome. For instance, in regard of the importance of fatty acids in cellular signalling processes, it would be advantageous to distinguish the fatty acid composition within certain classes of glycerophospholipids from other phospholipid and neutral lipid counterparts. However, without any chromatographic separation, only the fatty acid compositions from total intact lipid mixtures are detected by NMR, and further discrimination of fatty acid compositions between individual lipid classes is not possible. Furthermore, parts of the 1D spectrum are excessively overlapped (fatty acid methylene

region and phospholipid head group region in particular) even at high spectrometer frequencies such as 500 MHz, which made some assignments difficult and ambiguous. Such drawbacks can be partially overcome by applying a simple ion exchange chromatographic procedure prior to the NMR assay. By such chromatographic procedure, lipids were separated into various classes according to their polarity, and thus allowed more specific and detailed analyses.

Solid phase ion-exchange chromatography, such as DEAE, has been developed for lipid separation [Kates *et al*, 1988; Christie, 1987; Kaluzny *et al*, 1985]. In effort to maintain the rapidity nature of NMR lipid assays, a commercial pre-pack and disposable column, Bond Elut with NH-aminopropyl silica sorbent, was chosen for this particular task. Bond Elut ion exchange chromatography can effectively fractionate lipids into five classes, based upon their differences in polarity. The recovery of lipids from Bond Elut columns was found to be high and consistent.

Combination of NMR/ Bond Elut has proved to be superior, both qualitatively and quantitatively, to the purely NMR approach. Significantly, non-polar glycerol lipids and cholesterol are entirely separated from phospholipids, with the phospholipids also being sub-classified according to their acidity. This reduced the complexity of all spectra considerably and permitted a more specific determination of fatty acid compositions. Analysis of the low-abundance lipids, such as PS, PI and glycopospholipids were also improved. For instance, several previously non-separated lipid species were revealed after fractionation and can be directly measured by NMR. The results agreed substantially with published data, both qualitatively and quantitatively.

11.2.3 Lipid analysis via carbon-13 NMR

In the previous sections, various advantages of *in vitro* ^1H -NMR lipid analysis over the traditional chromatographic lipid assays have been demonstrated. Nevertheless, due to several practical difficulties including spectral overlapping and low lipid abundance etc., even 2D ^1H -NMR methods have not yet provided complete analysis of the fatty acid components of intact lipids in extracted mixtures. Since even under the applied

magnetic field of 500 MHz, the fatty acid alkyl/ methylene region in the ^1H spectrum is still too overcrowded to allow comprehensive assignments. Thus an alternative approach of using carbon-13 NMR was performed. A qualitative and quantitative analysis of the principal lipids and their fatty acid moieties in a total lipid extract using 1D broad band decoupled carbon-13 and 2D heteronuclear NMR methods was developed.

Apart from the fact that ^{13}C -NMR suffers from an intrinsic sensitivity problem compared to its ^1H -NMR counterpart, its advantages over the purely ^1H -NMR approach include: 1) the lower complexity of spectra particularly in the fatty acid regions, since single carbon resolution was obtained for virtually all carbon atoms of the intact lipids, 2) the higher resolution and decreased interference from solvent signals and lower concentration of impurities. These advantages for analysis of fatty acids in essential oils has already been demonstrated [Gunstone, 1991; Gunstone, 1990]. Assignments of olefinic carbon resonance permitted the identifications of unsaturated fatty acids components within the intact lipids. The data were confirmed by complementary analyses using the methyl, alkyl and allyl carbon resonance of the fatty acids. Other principal lipid components were identified by their characteristic backbone resonances or their head group signals. 2D HETCOR [Benn & Gunther, 1983] experiments were performed in order to resolve or confirm certain 1D assignments.

Despite the potential complications that arise from NOE effects and relaxation differences due to the diversity of chemical environments, the extracted lipid and fatty acid concentrations were determined via ^{13}C -NMR based upon their spectral intensities in a similar manner as its ^1H analogue. Thus both the qualitative and quantitative data on the major lipids and fatty acids were obtained rapidly without prior chromatography. The findings agreed substantially with the corresponding data obtained by chromatographic techniques and ^1H -NMR.

11.3 CURRENT LIMITATIONS OF THE NMR LIPID ANALYSIS APPROACH

11.3.1 Inferior sensitivity

Whereas *in vitro* lipid analysis via NMR certainly has some intriguing preferences

over the traditional chromatographic approaches, the technique is still far from flawless — one of the major disadvantage being its intrinsic insensitivity. By comparison with the chromatographic methods, NMR suffers from a ten or even hundred fold inferior sensitivity. In principle, this problem can be overcome by the increasing the accumulation of FIDs — hence the experimental time. In practice, however, this implies that the appeal of assay rapidity fades proportionally with the increase of sensitivity. In order to retain the advantage of rapidity, the quantity of sample for ^1H -NMR experiment is restricted to ≥ 0.50 mg, although the limit can be reduced to *ca.* 0.10 mg for samples that contained few components. While the optimum sample concentration for ^{13}C -NMR is *ca.* 30-50 mg, with a lower extent to *ca.* 10 mg.

11.3.2 Incomplete analysis of various lipid components

A minority of lipids such as PA and PG are difficult to analyse, partly due to their universally low abundance among animal cells, but also owing to their lack of specific head group signals. These lipids can be quantified indirectly by subtracting other resolved lipid components from the total phospholipid signal. The discrimination between unsaturated and saturated ether lipids can only be achieved indirectly via the combination of ^1H -NMR and Bond Elut chromatography. Other undetected lipid species include lysophospholipids, gangliosides and highly phosphorylated inositol lipids. This can be due to the inefficient extraction or their low occurrence.

There are two varieties of ether lipids with distinctive phospho head groups, namely phosphocholine (i.e. plasmalogen) and phosphoethanolamine. Neither ^1H -NMR nor ^{13}C -NMR differentiated these two species at this stage of development. However, in the ^1H -NMR spectra, a slight splitting of the choline $\text{N}^+(\text{CH}_3)_3$ chemical shift was observed at 3.20 ppm, presumably attributed to different choline-containing lipids (including plasmalogen). Such splitting is expected to be increased when the sample is subjected to a higher applied magnetic field (> 500 MHz), and should eventually be sufficient for specific characterization. An equivalent head group diversity is expected among the sphingolipids, and it should be possible to resolve these in the same manner. In the ^1H -

NMR assay, esterified monounsaturated fatty acids were unassigned again due to the lack of specific diagnostic resonance, and the quantitation was achieved indirectly by subtraction.

11.3.3 Reliability of the lipid extraction and the solubility of samples

The ultimate development of comprehensive assays for biological lipids will rely upon finding an extraction procedure or combination of lipid extraction procedures which will quantitatively extract every lipid. Whether or not HPLC, TLC, GC, MS or NMR methods are utilized for assays, they all depend upon the fidelity of the extraction procedures. At this juncture the classical "freeze-clamp" extraction procedure developed by Folch *et al* (1957) has been utilized but other lipid extraction procedures have been developed [Klarovic & Fournier, 1986]. Ideally, all will have to be evaluated via the NMR lipid profiling assay eventually.

Biological lipid extracts consist of a huge diversity of compounds with equally diverse solubility properties. Hence to solubilize one lipid extract homogeneously can be tricky, yet homogeneous solution is vital for obtaining high quality spectra (especially for ^1H -NMR). To complicate the matter further, solvent compositions also affect the spectral resolution tremendously. The optimum ratio of deuterated chloroform-methanol was found to be 1:2 v/v. However, triacylglycerol only dissolves in this solvent mixture at low concentration. In the cases where high levels of triacylglycerol exist, an increase of the proportion of chloroform may necessary in order to maintain the solution's homogeneity. Thus the ratio of deuterated chloroform-methanol for the blood plasma ^{13}C -NMR experiments were in 1:1 v/v instead.

11.4 PROSPECT OF NMR LIPID PROFILE ANALYSIS

11.4.1 Combination of NMR with HPLC

An obvious approach to improving NMR lipid assays is to couple NMR with HPLC. Lipid assays via HPLC has been developed extensively and even though not truly comprehensive (by using one single system), established multiple lipid assays are

available [Araki *et al*, 1990; Seta *et al*, 1990; Samet *et al*, 1989; Vercaemst *et al*, 1989; Christie, 1987; May *et al*, 1986; Yu *et al*, 1986]. The lipid extract was hence subjected to HPLC column separation prior to NMR profiling procedure. This yields a more specific lipid analysis profile similar to the Bond Elut approach. However, one major advantage of NMR approach — rapidity, is sacrificed in this application. As one complete HPLC elution usually required 30 to 60 minutes, and numerous elutions will be required in order to accumulate sufficient amount of lipids for a decent ^1H -NMR spectrum. Alternatively an exceedingly lengthy NMR experiment will be necessary to compensate the insufficient lipid concentration. In the case of ^{13}C -NMR, the requirement of lipid concentration is so immense that the whole approach may become impractical. In conclusion, the NMR/ HPLC approach is applicable in developmental work but not suitable to be a routine assay.

11.4.2 Quantitation of lipids via 2D NMR

One possible improvement on the current NMR lipid assay, is to extract quantitative information from 2D NMR rather 1D spectra. The peak volume of cross-peaks will be use for quantitation purpose. With the combination of modern NMR processing software such as FELIX[®], the quantitation can be fully automated (e.g. automatic cross-peak identifications and integral calculations) once established. However, even on the basis of our accomplished methodology, considerable effort will still be necessary to achieve such goal. For example, since cross-peak volumes are often not in direct proportion to the concentrations of the corresponding lipids, a particular formula or may be a set of formulae will have to be derived for normalization. Nevertheless the development of lipid quantitation via 2D NMR should eventually provide a more precise and more specific assay methodology.

11.4.3 Refinement of ^{13}C -NMR lipid assay

In the ^{13}C -NMR lipid profile, more precautions on the NOE and relaxation behaviour may prove beneficial [Abraham *et al*, 1990a]. By increasing the relaxation (pulse) delay

between each acquisition process in order to remove the relaxation time variation, apply gate decoupling technique to eliminate the differential NOE, and by inducing paramagnetic relaxation reagent to reduce the overall relaxation time. On the other hand, such precautions may not be necessary since, in general, the larger the molecule, the shorter the relaxation times, and thus these effects become less severe for large molecules such as cholesterol and complex lipids [Abraham *et al*, 1990b]. For instance, with the shorten relaxation times (\ll acquisition time), the relaxation is overwhelmingly dipole-dipole thus the full NOE may reach not only to methylene and methine carbon, but also to quaternary carbon atoms. In this case the NOE effect is practically even-out. Judging from the quantitative data obtained in our studies, which agreed reasonably with other established methods, indicated the relaxations may indeed rather short.

11.4.4 Current progress on NMR technology

Current progress on NMR technology provides promising options for the improvement of NMR lipid profiling assay. Firstly, advance of NMR processing software is able to perform substantial spectral enhancements and this can be valuable for identifying low abundance lipids which are obscure under normal observation. Secondly, the latest commercial NMR to-date provide up to 17.6 tesla (750 MHz in proton frequency) of magnetic field strength. The increase of applied magnetic field strength can improve both the sensitivity and resolution of NMR spectroscopy, as illustrated by the Boltzmann equation:

$$N_{\beta} / N_{\alpha} = \exp (-\Delta E / kT)$$

where N_{α} is the population of the nuclear at lower energy state

N_{β} is the population of the upper energy state.

Since ΔE is field dependent, the population difference is also dependent on the applied magnetic field. In addition, since the J coupling (spin-spin coupling) interaction is

independent of the applied magnetic field whereas the chemical shift separation (in MHz) is proportional to it, consequently the NMR spectrum becomes simpler on going to higher applied fields. Finally, the recent development of pulsed field gradients as an alternative to the traditional phase cycling in 2D and multidimensional NMR can reduce the experiment time significantly, as the lengthy phase cycle is replaced by a one single-shot field gradient pulse in order to disperse the unwanted transverse components of magnetization [Barker & Freeman, 1985]. By combining the technological enhancements mentioned above, the potential for lipid profiling by NMR is optimistic.

11.5 CONCLUSION

Research of lipids and their metabolites has become a major trend in molecular biochemistry, its importance has been extended from merely structural to significant functional role. The analytical assays of lipid and related compounds in clinical and molecular biochemistry has evolved rapidly. Virtually all the lipid assays available for routinely used to-date are based extensively upon chromatographic techniques. Typical examples include the gas chromatography, high performance liquid chromatography or combination of various techniques such as HPLC/ MS. The development of these methodologies has significant impacts on modern lipid research, as they provide sensitive, and often accurate results. Nevertheless, these conventional chromatographic lipid assays, although well established, are often highly labour-intensive and time consuming. In comparison, NMR lipid profile approach has the potential advantages of rapidity, comprehensiveness, and non-destructive nature. In this project we have explored such potentials, successfully accomplished the fundamental development and attempted to refine this *in vitro* NMR lipid assay. NMR lipid analysis via two nuclei, namely proton and carbon-13, were developed as regarded to their universal occurrence in biological (organic) molecules. A variety of animal tissues and body fluids were used for the developmental work, including rat liver, rat brain, rat heart, rat kidney, human erythrocytes and human blood plasma. Lipid extracts of these tissues provide a diversity of lipid profiles and reveal all major lipid species exist in animal cells. As described

previously, the proton NMR assignments of these lipid extract spectra were achieved by: a) the comparison with standard lipid spectra, b) by analyzing the cross peaks of 2D NMR spectra, c) by adding the lipid which suspected to be present into the lipid extract, also known as "spiking" method, and d) by referring and comparing to the published data where available. The majority of lipid species were identified by solely 1D NMR approach, with the benefit of gaining extra information about esterified fatty acid compositions, without additional experimental procedure. Quantitation of lipids and fatty acids, in terms of wt %, were also readily accessible. Moreover, the NMR lipid assay has been further improved, by introducing Bond Elut chromatography for lipid separation prior to NMR experiment. By dividing the lipid mixture into four or five different classes depending on their polarities, NMR assays became more specific and sensitive, particularly to the low abundant lipids. This has been clearly demonstrated on rat liver, brain, heart and kidney lipid extracts. The development of carbon-13 NMR assay was accomplished in a similar manner as their proton counterpart described above, with the extra aid of 2D heteronuclear correlation experiments (HETCOR). In contrast to the proton NMR lipid assay, the carbon-13 approach gives relatively simple spectra, thus a more unambiguous assignment, but suffers from a considerably lower sensitivity.

In general, the results agreed equitably between the two NMR approaches (^1H and ^{13}C) and significantly, were consistent with the established conventional chromatographic technique, both qualitatively and quantitatively. Even with the minor drawbacks on intrinsic sensitivity and a few unresolved lipid sub-classes, potential for further improvement exist thus ^1H -NMR and ^{13}C -NMR remain a highly appealing alternative or complementary technique on *in vitro* lipid analysis. With continuous refinements or modifications such as enhance magnetic field strength and the establishment of routine 2D quantitation, researches on biological lipids and metabolisms via NMR can be expand to genetic diseases, physiological process of ageing or in pathological status such as hypertension, arteriosclerosis, diabetes etc.

Appendix 1

Identification and quantitation of lipids from tissue extracts via NMR and the list of lipid standard spectra

IDENTIFICATION OF THE INDIVIDUAL LIPID COMPONENT IN TISSUE LIPID EXTRACTS

As mentioned in the previous chapters, individual lipid can be distinguished within a lipid extract mixture by ^1H and ^{13}C NMR, according to their characteristic chemical shift resonances. For phospholipids, these diagnostic resonances are usually come from their head groups, such as the inositol ring from phosphatidylinositol. For neutral glycerol lipids and sphingolipids, the diagnostic resonances came from their glycerol or their sphingosine backbone moieties, which exhibit different chemical shifts compare to their phospholipid counterpart. Most of the esterified fatty acids can also be distinguished by their specific methyl, methylene and allylic resonances. In the case of ^{13}C NMR, apart from the information gained as discribed in its ^1H counterpart, extra diagnostic information about the unsaturated fatty acids can be obtained via the olefinic resonances. The dignostic ^1H chemical shifts for all major lipids and fatty acids are listed in table 10.1. Whereas the diagnostic ^{13}C chemical shifts for all major lipids and fatty acid are summarized in table 8.1, 9.1 and table 8.4, 9.4 respectively.

The major disadvantage of NMR lipid analysis is its inferior sensitvity compared to other established mainstream lipid analytical assays. In principle, this problem can be overcome by increasing the accumulation of FIDs, which effectively increase the spectrum resolution and the signal to noise ratio. From a practical point of view however, this implies that the appeal of assay rapidity fades proportionally with the increase of sensitivity. In order to retain the advantage of rapidity, the quantity of sample for ^1H -NMR experiment is restricted to ≥ 0.50 mg, although the limit can be reduced to *ca.* 0.10 mg for samples that contained few components. While the optimum sample concentration for ^{13}C -NMR is *ca.* 30-50 mg, with a lower extent to *ca.* 10 mg. Below is a summary of the typical 1D NMR conditions used throughtout this research project.

	Amount of lipid used (mg)	Solvent volume (ml)	Pulse angle (°)	Pulse delay (s)	Number of FIDs accumulated	Duration of experiment (min)
^1H	5.0	0.8	45	6.0	64 - 128	15 - 30
^{13}C	30 - 50	2.0	45	15.0	128 - 256	60 - 120

QUANTITATION OF LIPIDS AND FATTY ACIDS IN TISSUE LIPID EXTRACTS BY ^1H NMR

Since the coefficient of NMR signal absorption is a constant for any nucleus, and the NMR signal obtained is directly proportional to the number of nuclei producing it, this particular feature allow the spectral quantitation to take place. In the case of ^1H NMR, lipid quantitations were achieved by simply measuring the intensities of diagnostic NMR signals in term of integrated areas. The diagnostic resonances which were selected for quantitation purpose must be well isolated, with as little interferences from other chemical shifts as possible. The results were then normalized by the integrated area of the residual solvent CD_3Cl , in order to allow direct comparison between different spectra. The relative quantities of esterified fatty acids were determined in a similar manner, and the integrated areas were normalized by the integrated area of the methyl terminal group, which occurred at *ca.* 0.90 ppm in the ^1H NMR spectrum. This particular methyl resonance is considered to represent the grand total of fatty acids. Since all fatty acids actually consisted of a methyl terminal group, hence such normalization resulted in a unit of mole percentage of total fatty acids. In addition, all integrated area values were converted into "single proton intensities" — for example, any integrated areas derived from the chemical shifts of methylene (which contain two protons) and methyl groups (which contain three protons), were being divided by a factor of 2 and 3 respectively. Such treatment thus allow the direct quantitative comparison regardless to the differences in chemical structures. The accuracy of quantitation was illustrated by the calibration curves in figure 1.

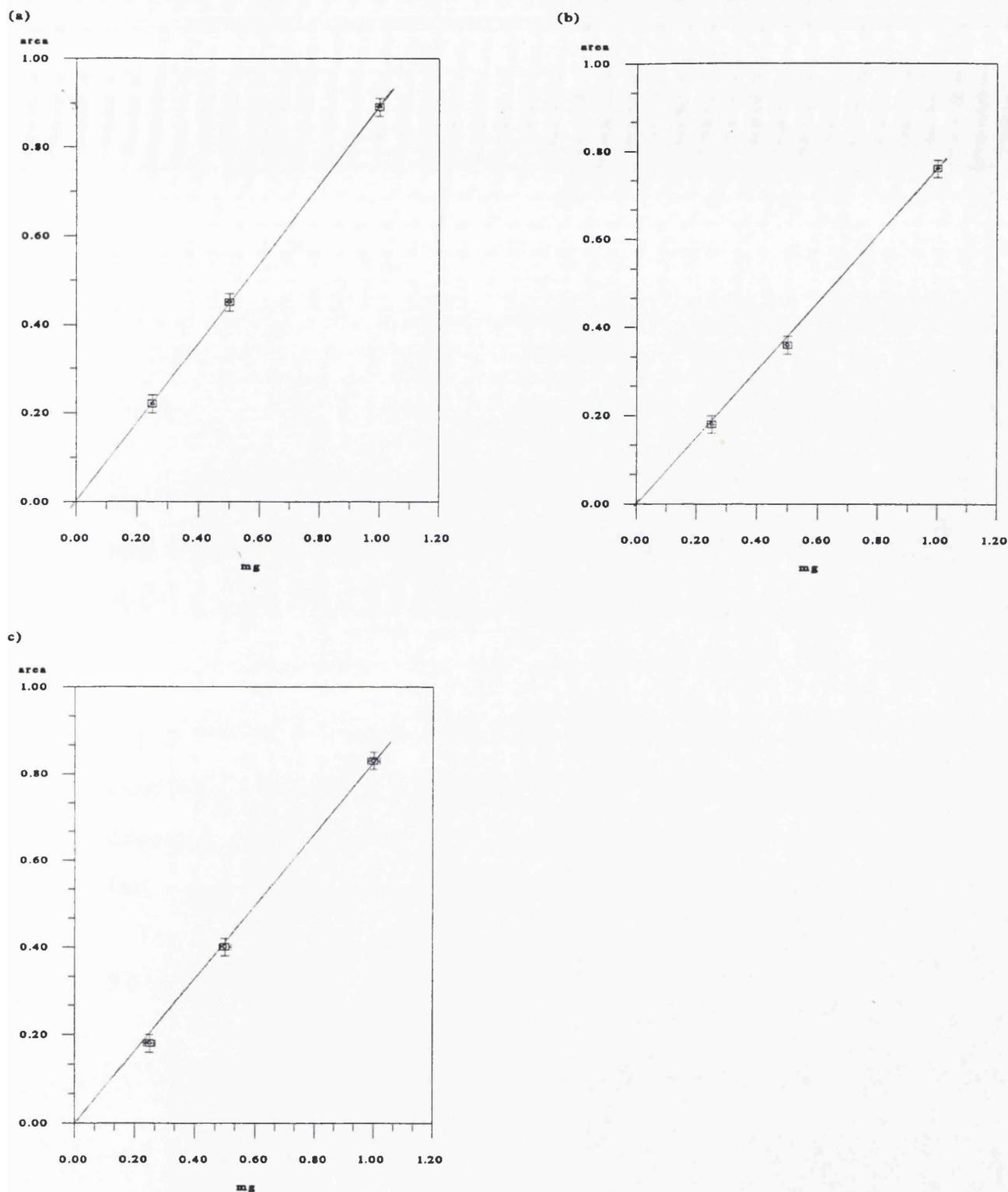


Figure 1 The normalized integrated areas of diagnostic ^1H NMR resonance from (a) Phosphatidylcholine [3.20 ppm], (b) phosphatidylinositol [3.75 ppm] and (c) Triacylglycerol [4.30 ppm] versus different lipid concentrations (from 0.25 to 1.0 mg). The integrated areas of diagnostic ^1H NMR resonances were divided by the integrated area of the residual solvent CD_3Cl for normalization. All lipids were dissolved in 0.80 ml of CDCl_3 : CD_3OD (1:2 v/v) mixture. Measurements were performed in triplicate and the error bars represented \pm S.D. of the three values.

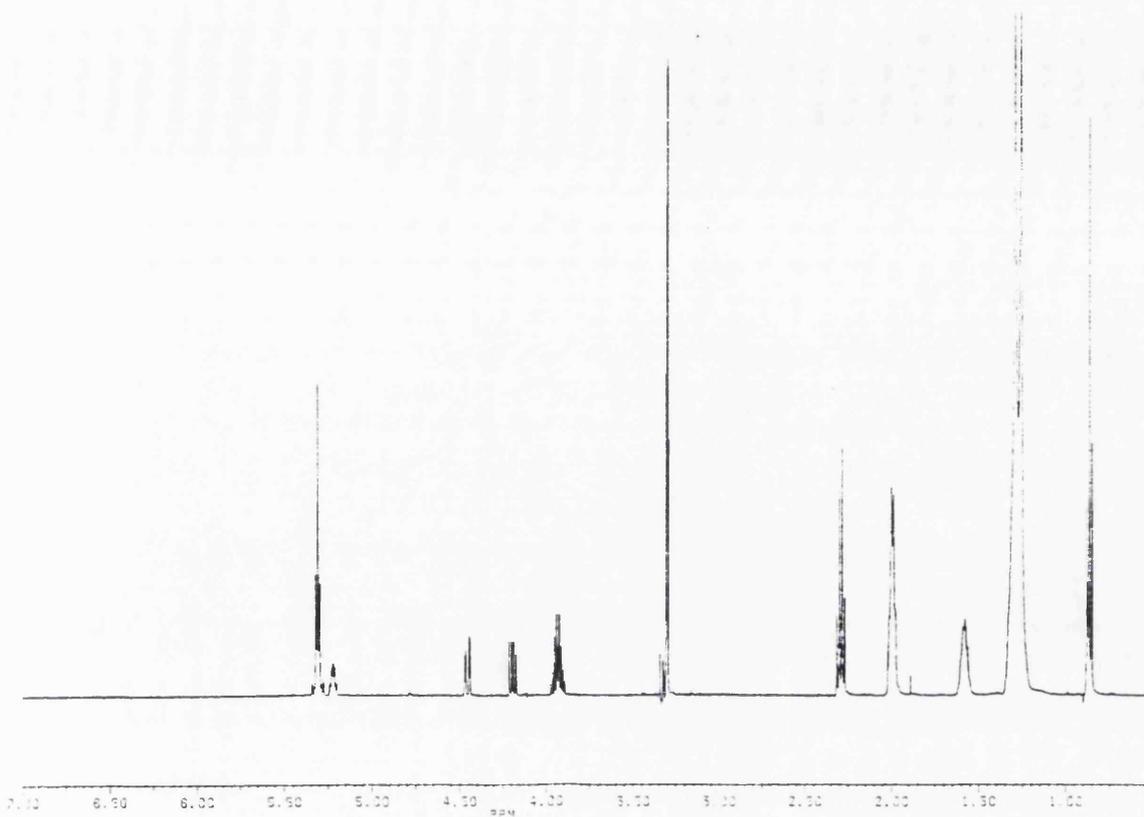
QUANTITATION OF LIPIDS AND FATTY ACIDS IN TISSUE LIPID EXTRACTS BY ^{13}C NMR

Despite the potential limitation arising from NOE effects and the differences of relaxation behaviour, quantitation of lipids and fatty acids were nevertheless carried out in the ^{13}C NMR experiments. Fortunately, the NOE effects were much less severe in these experiments as the relaxation time of large molecules such as complex lipids and cholesterol are considerably shorter. Areas of the well defined and isolated diagnostic signals were measured and normalized. The selection of diagnostic chemical shifts and the procedures for lipids and fatty acids quantitation were identical to the ^1H NMR method summarized in the previous section, with the exclusion of "single proton intensities" normalization. Significantly, the results compared favourably with ^1H NMR and other conventional analytical methods as described in chapter 8 and 9.

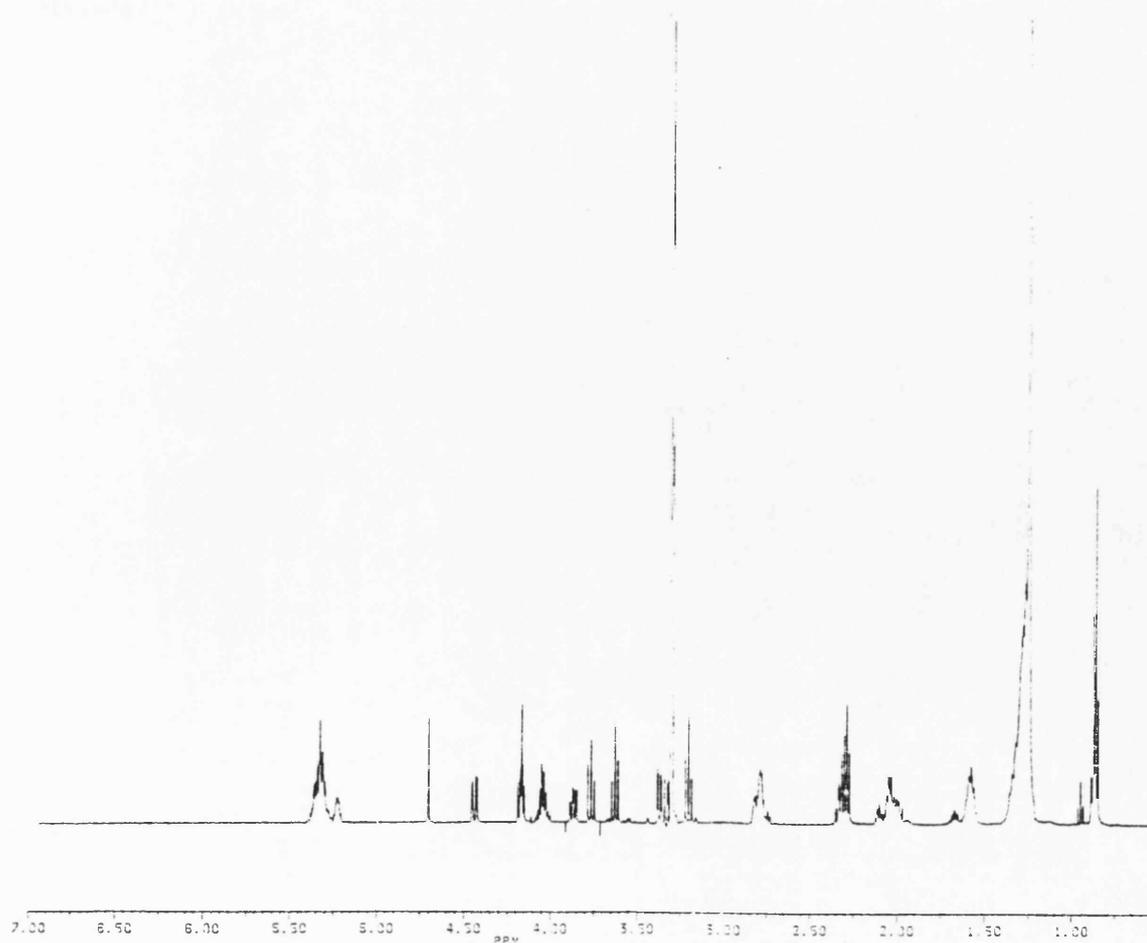
LIST OF ^1H AND ^{13}C NMR SPECTRA

During the development of NMR lipid analysis, spectra of lipid and fatty acids standard were often used to compare with the tissue lipid extract spectra. Many of the diagnostic chemical shifts were assigned in this manner. Thus some of the major lipid and fatty acid standard spectra are listed at the end of this chapter for reference purpose.

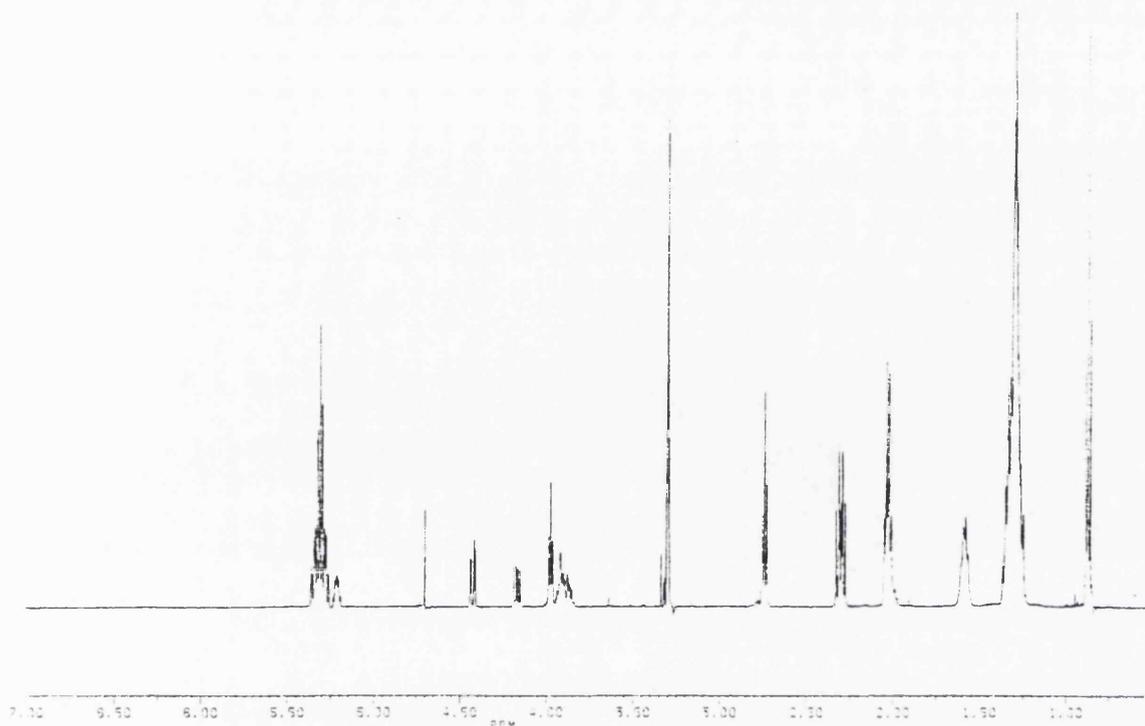
The amount of lipid used for producing each ^1H NMR standard spectrum was about 5.0 mg where available, and 30.0 mg was used for ^{13}C NMR.



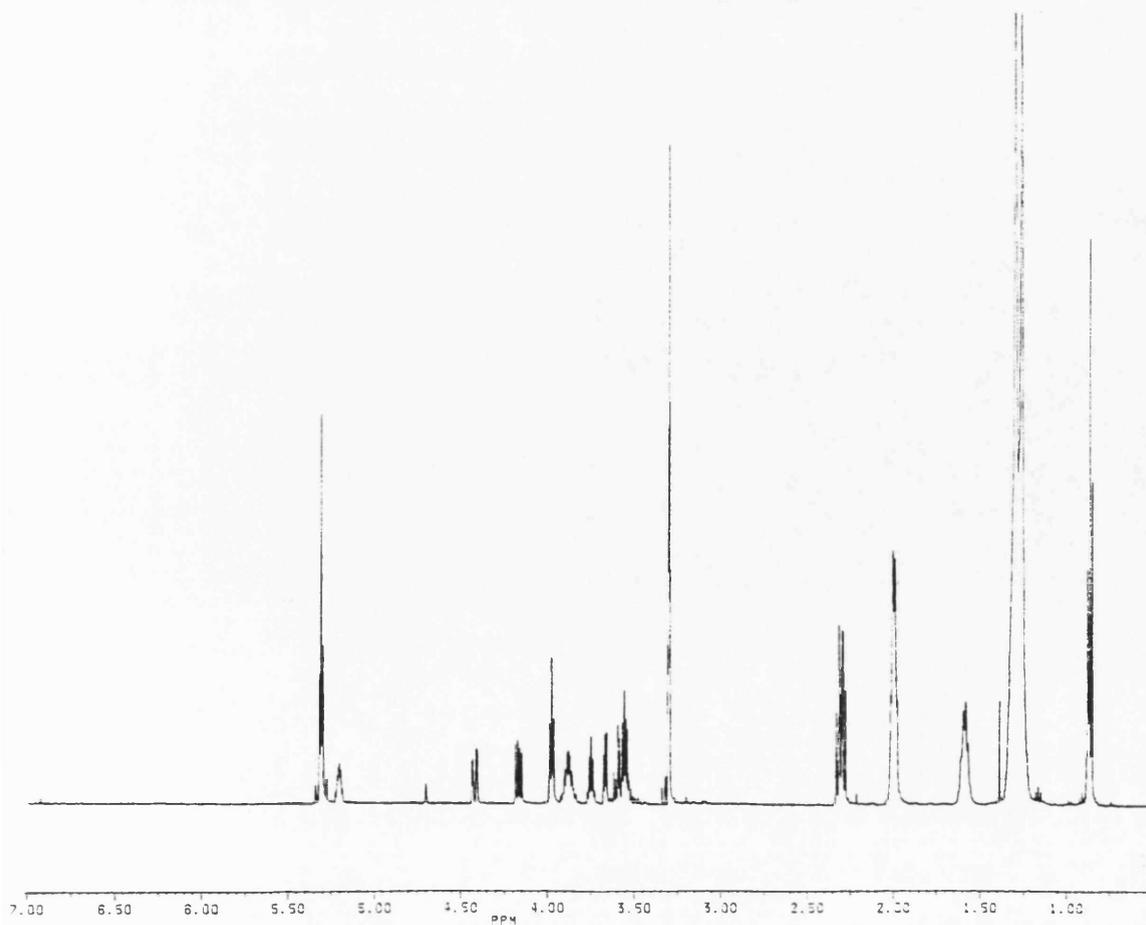
^1H NMR spectrum of phosphatidic acid in 0.80 ml of $\text{CDCl}_3:\text{CD}_3\text{OD}$ (1:2 v/v).



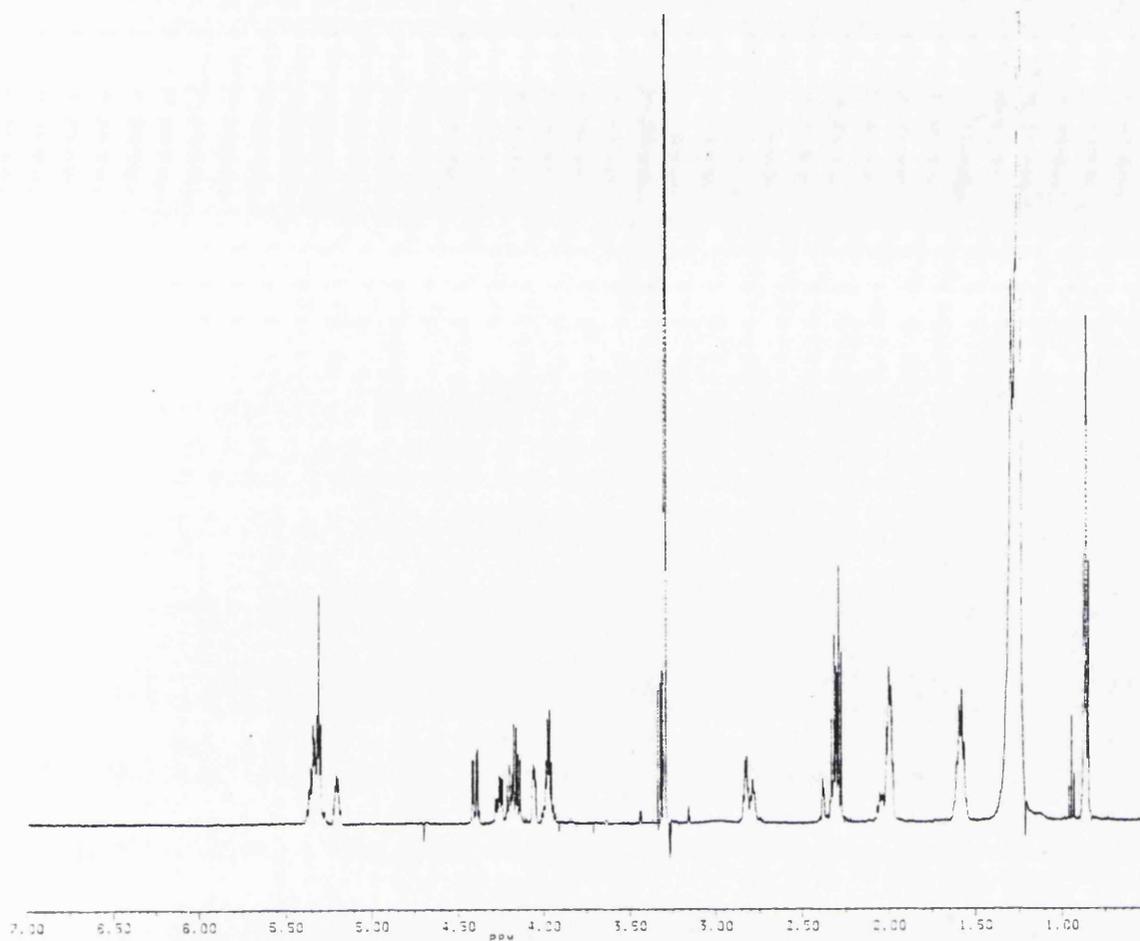
^1H NMR spectrum of phosphatidylinositol in 0.80 ml of $\text{CDCl}_3:\text{CD}_3\text{OD}$ (1:2 v/v).



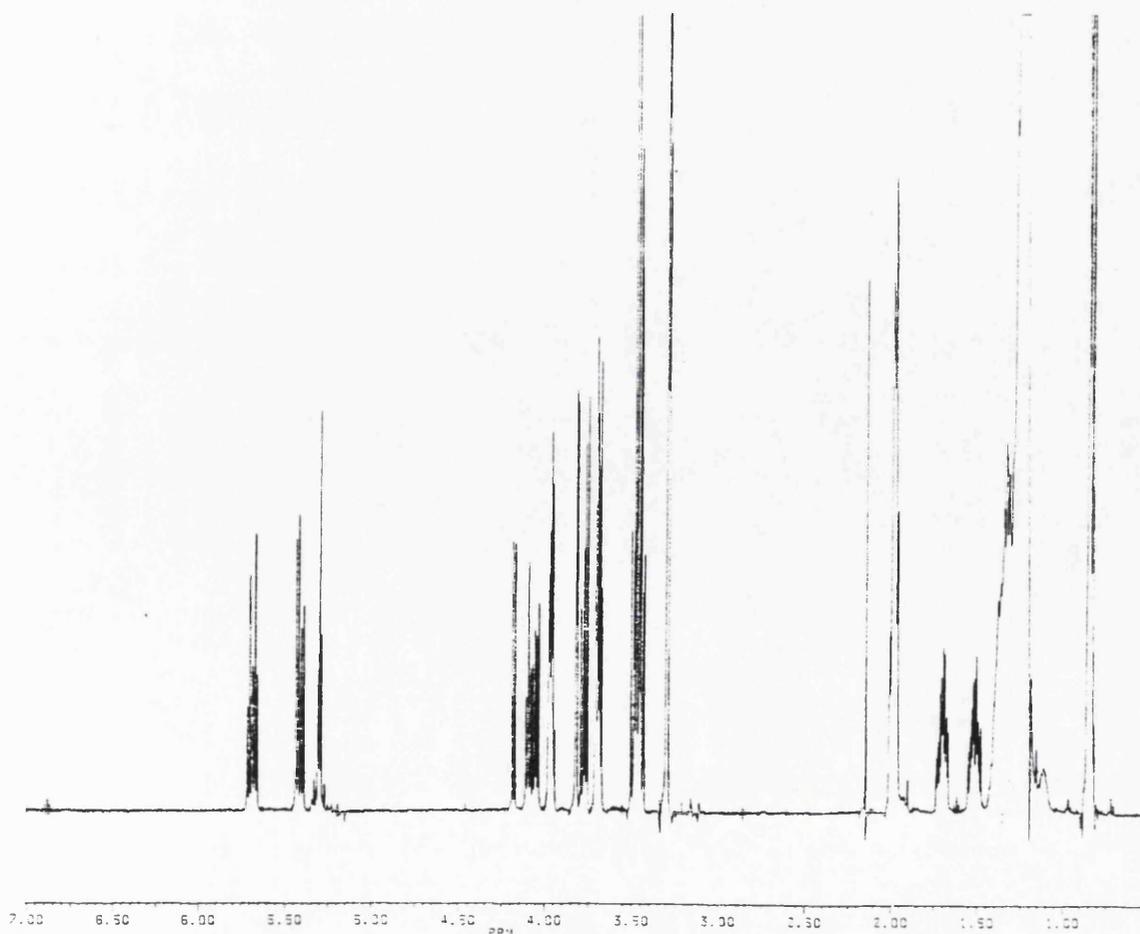
^1H NMR spectrum of cardiolipin in 0.80 ml of $\text{CDCl}_3:\text{CD}_3\text{OD}$ (1:2 v/v).



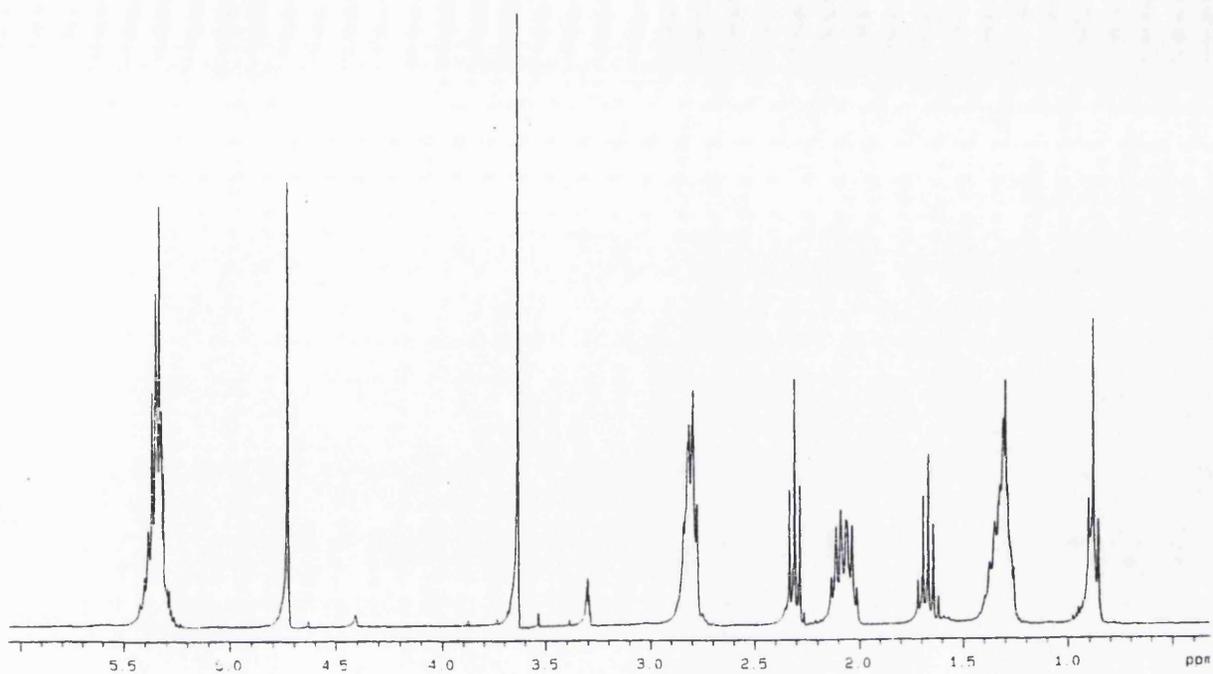
^1H NMR spectrum of phosphatidyl glycerol in 0.80 ml of $\text{CDCl}_3:\text{CD}_3\text{OD}$ (1:2 v/v).



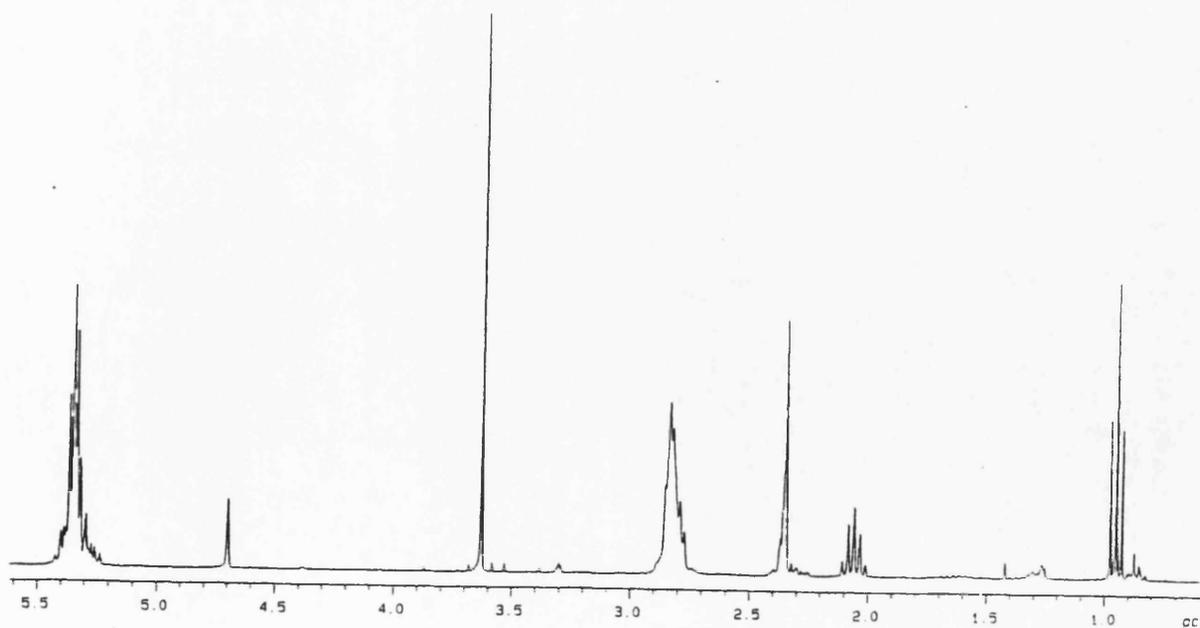
^1H NMR spectrum of phosphatidylserine in 0.80 ml of $\text{CDCl}_3:\text{CD}_3\text{OD}$ (1:2 v/v).



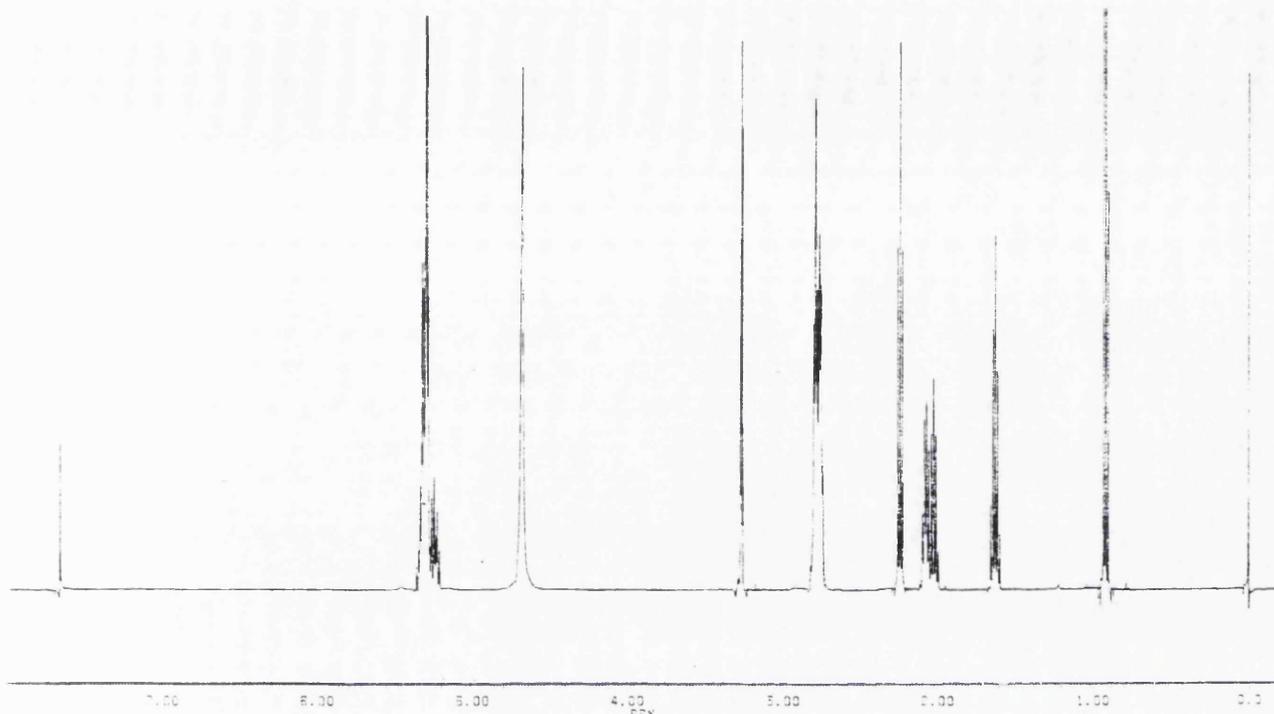
^1H NMR spectrum of galactocerebroside in 0.80 ml of $\text{CDCl}_3:\text{CD}_3\text{OD}$ (1:2 v/v).



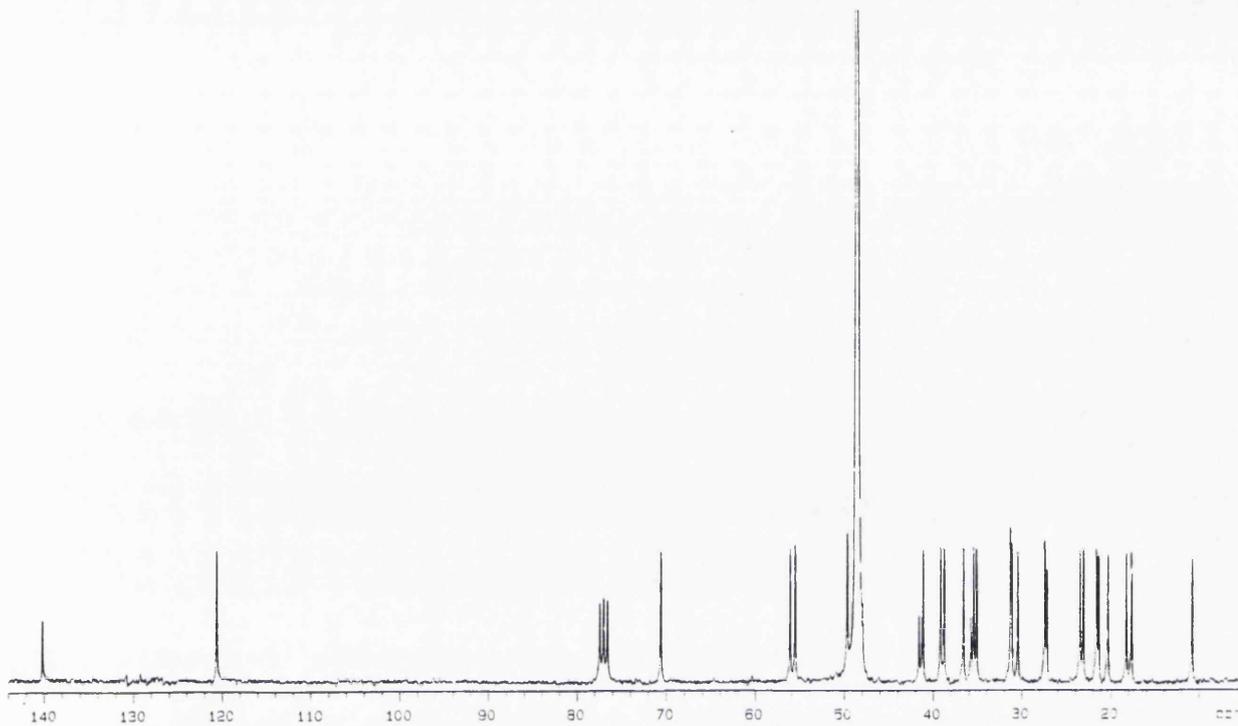
^1H NMR spectrum of arachidonic acid in 0.80 ml of $\text{CDCl}_3:\text{CD}_3\text{OD}$ (1:2 v/v).



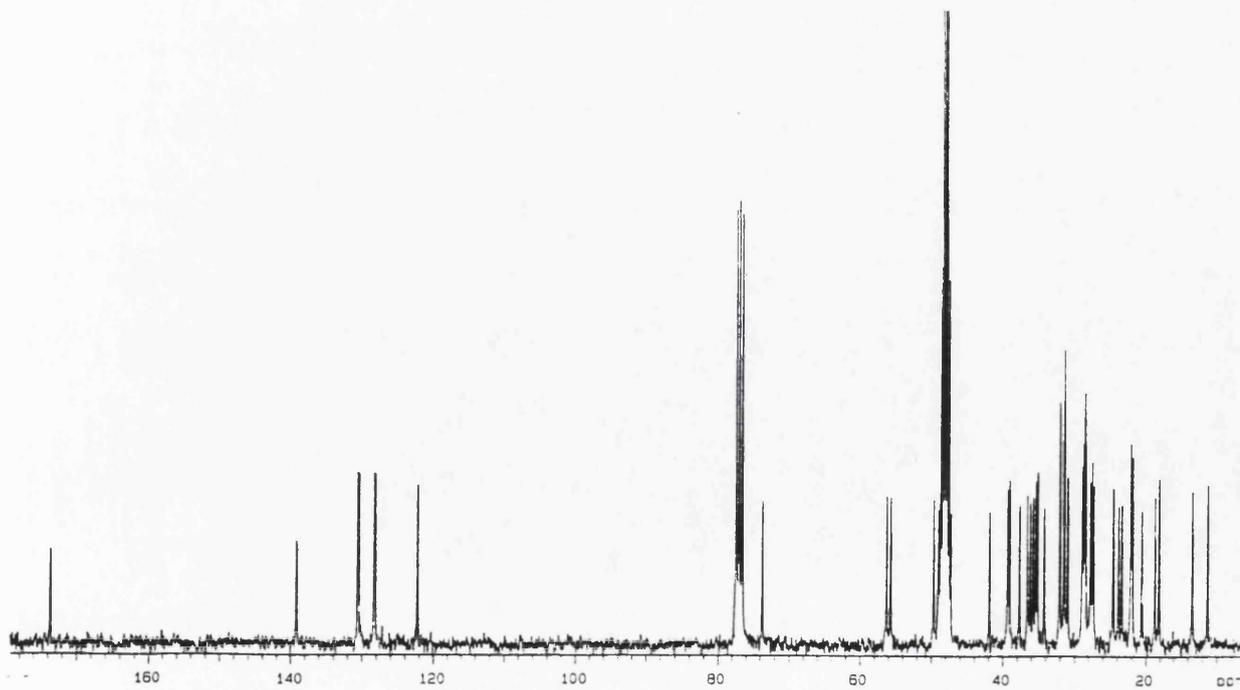
^1H NMR spectrum of docosahexaenoic acid in 0.80 ml of $\text{CDCl}_3:\text{CD}_3\text{OD}$ (1:2 v/v).



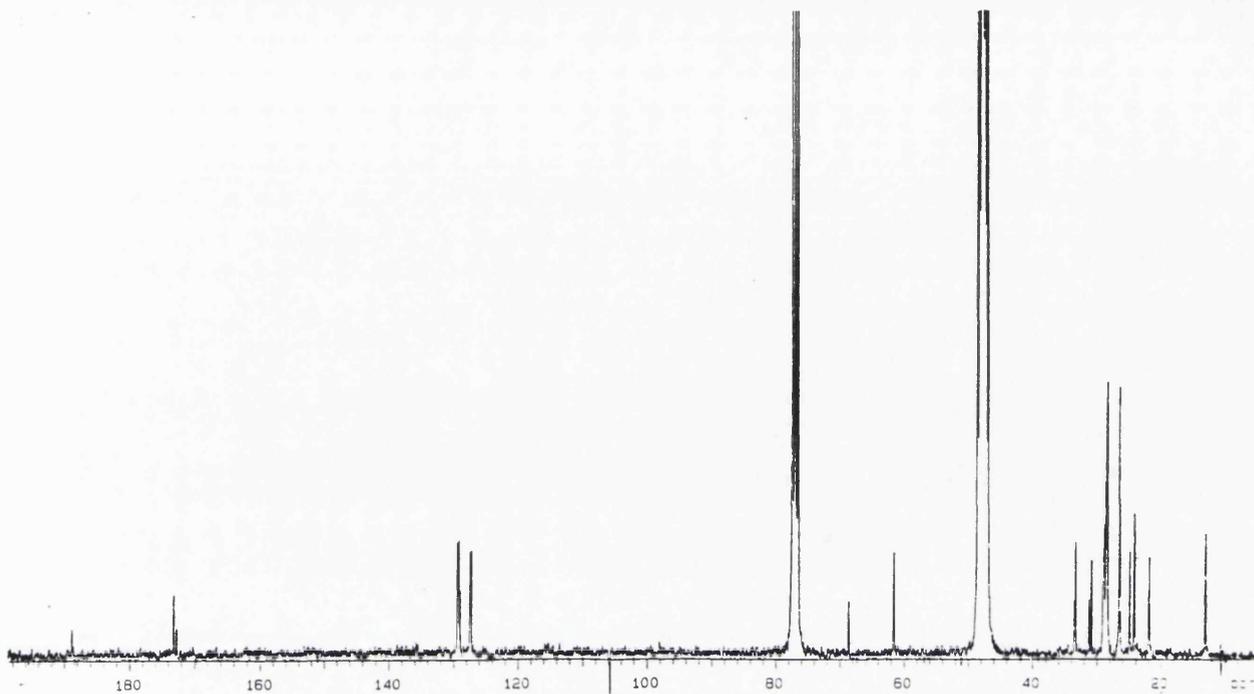
^1H NMR spectrum of eicosapentaenoic acid in 0.80 ml of $\text{CDCl}_3:\text{CD}_3\text{OD}$ (1:2 v/v).



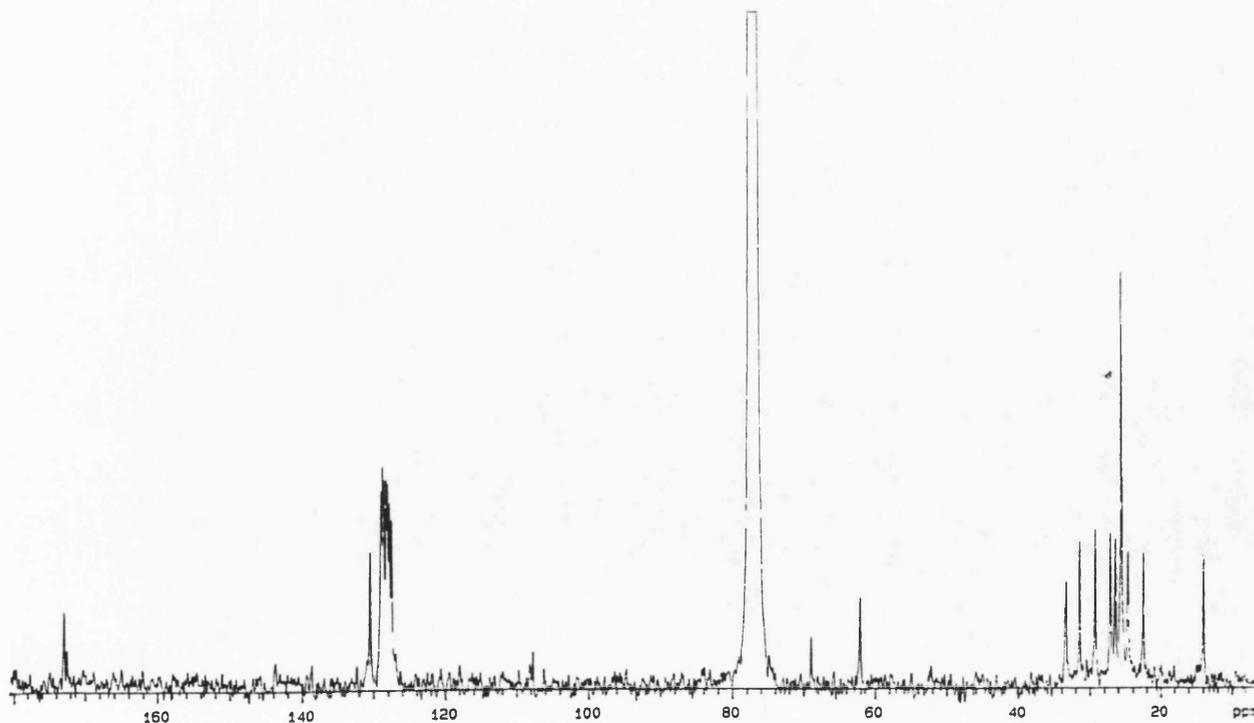
^{13}C NMR spectrum of cholesterol in 2.0 ml of $\text{CDCl}_3:\text{CD}_3\text{OD}$ (1:1 v/v).



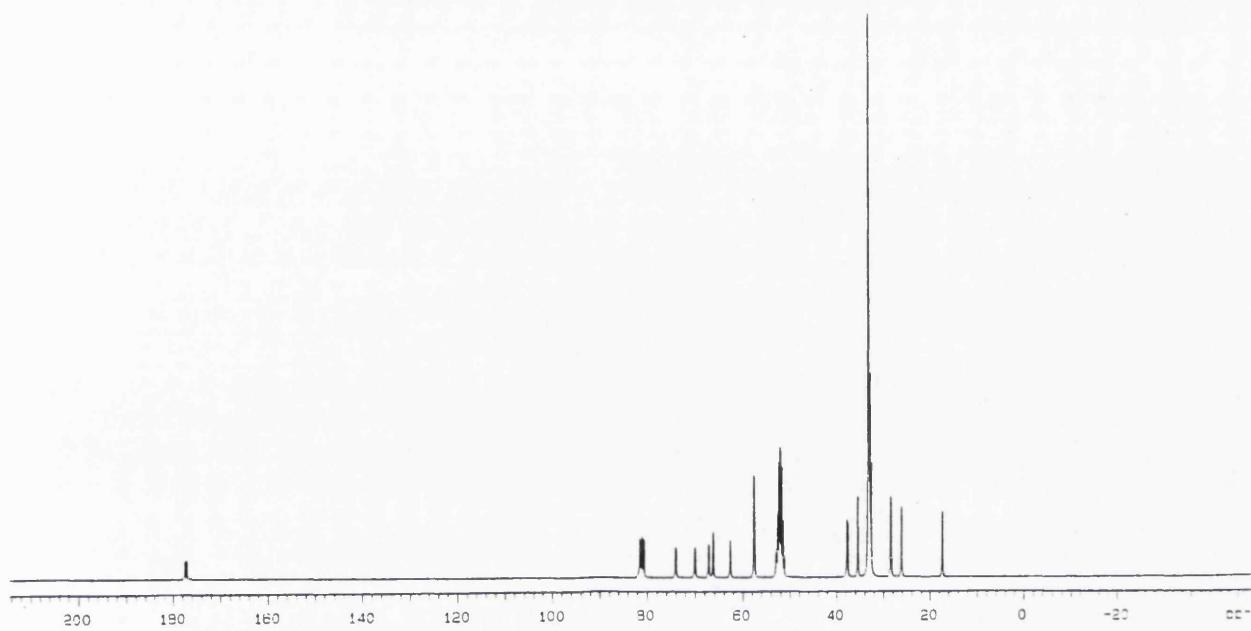
^{13}C NMR spectrum of cholesterol ester linoleioate in 2.0 ml of $\text{CDCl}_3:\text{CD}_3\text{OD}$ (1:1 v/v)



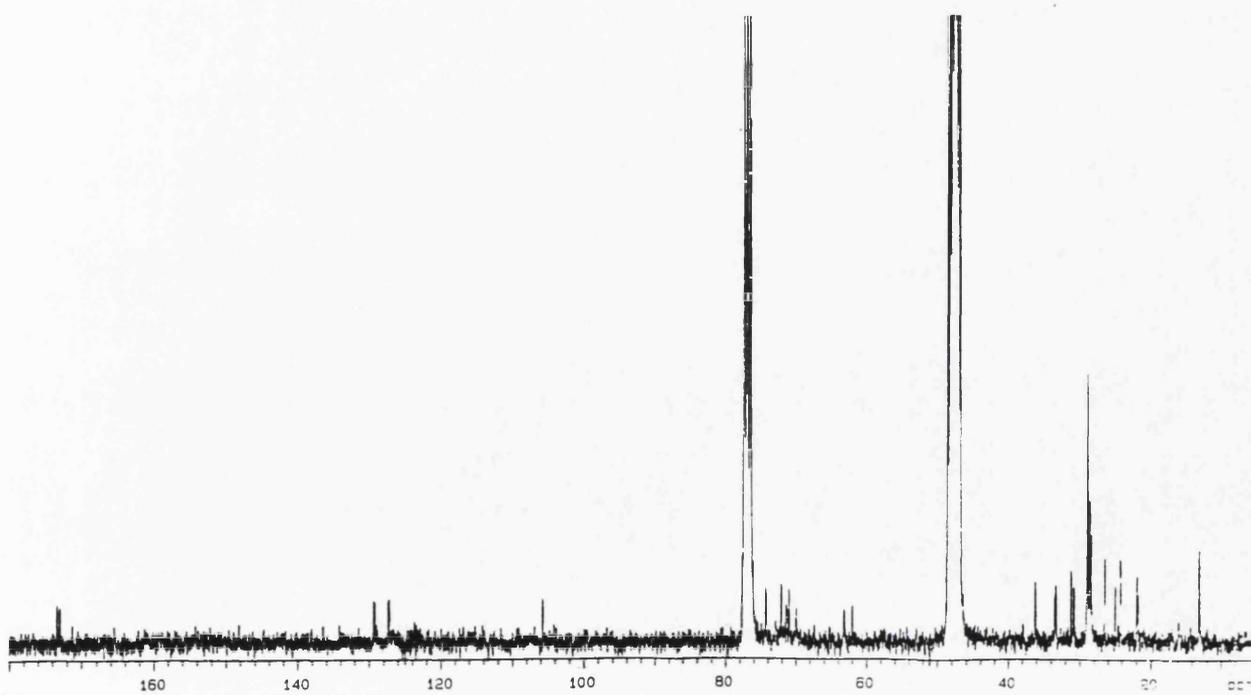
¹³C NMR spectrum of 1,3 dilynoleic, 2 oleic-glycerol in 2.0 ml of CDCl₃:CD₃OD (1:1 v/v).



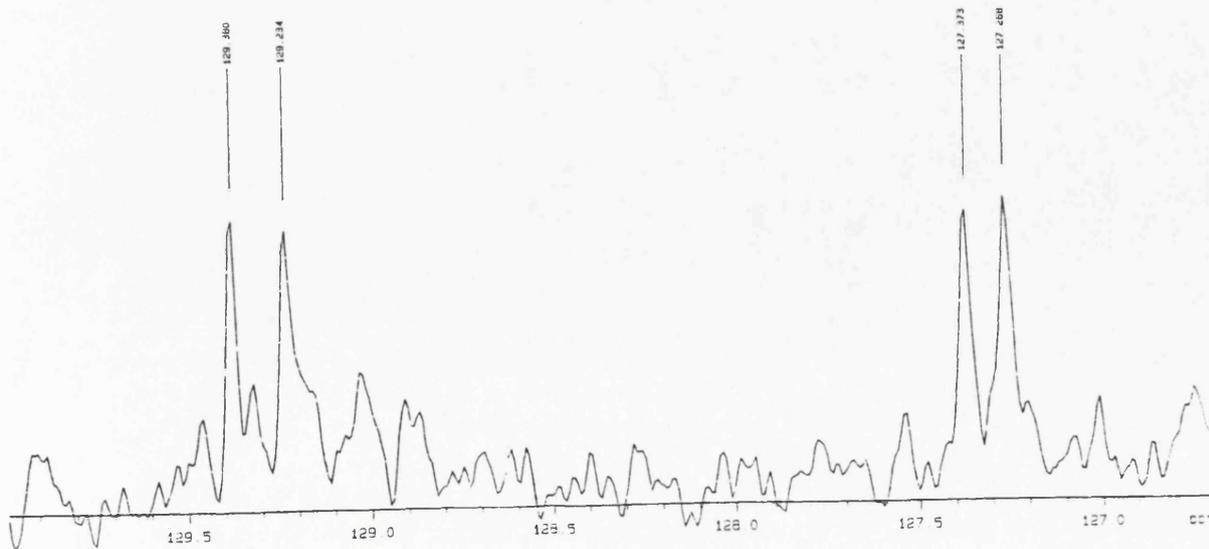
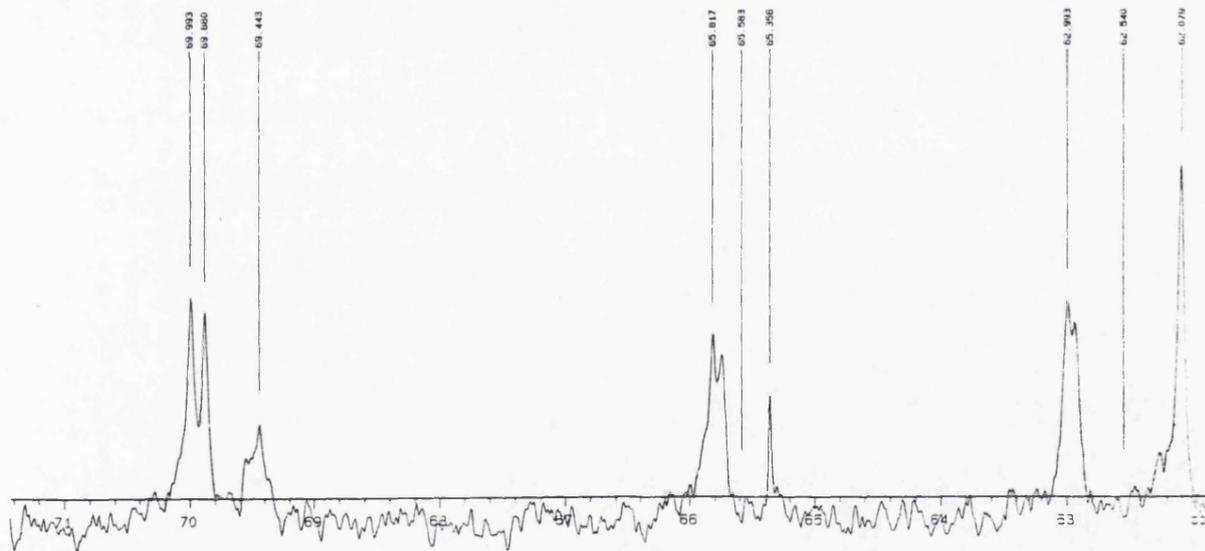
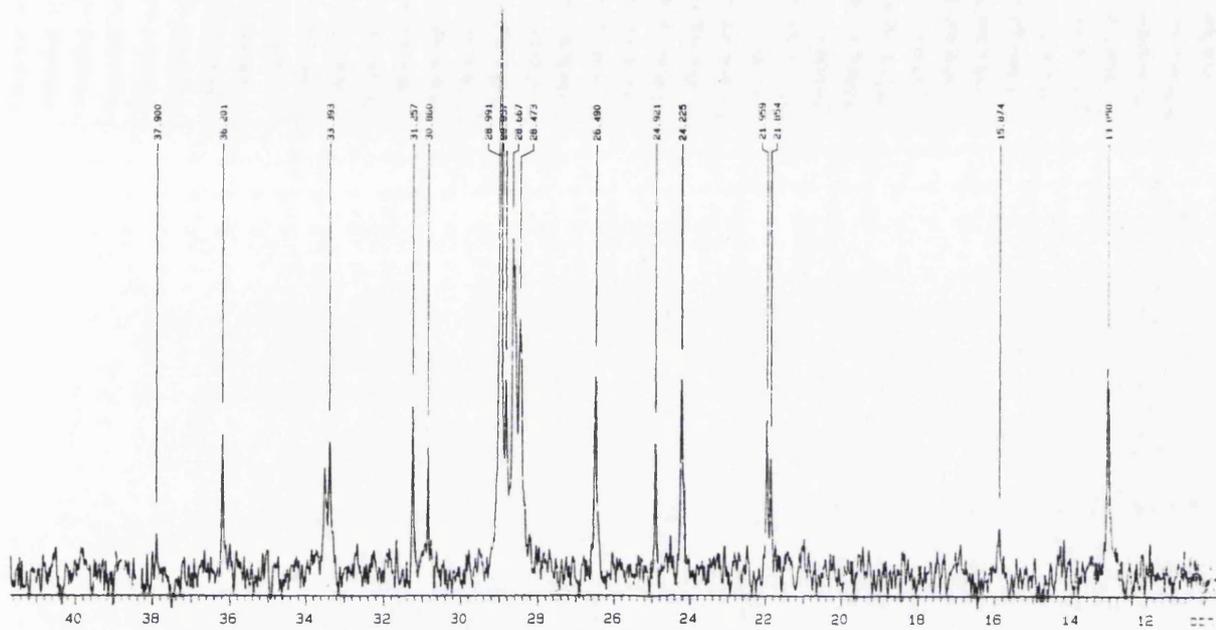
¹³C NMR spectrum of 1,3 arachidonic, 2 oleic-glycerol in 2.0 ml of CDCl₃:CD₃OD (1:1 v/v).



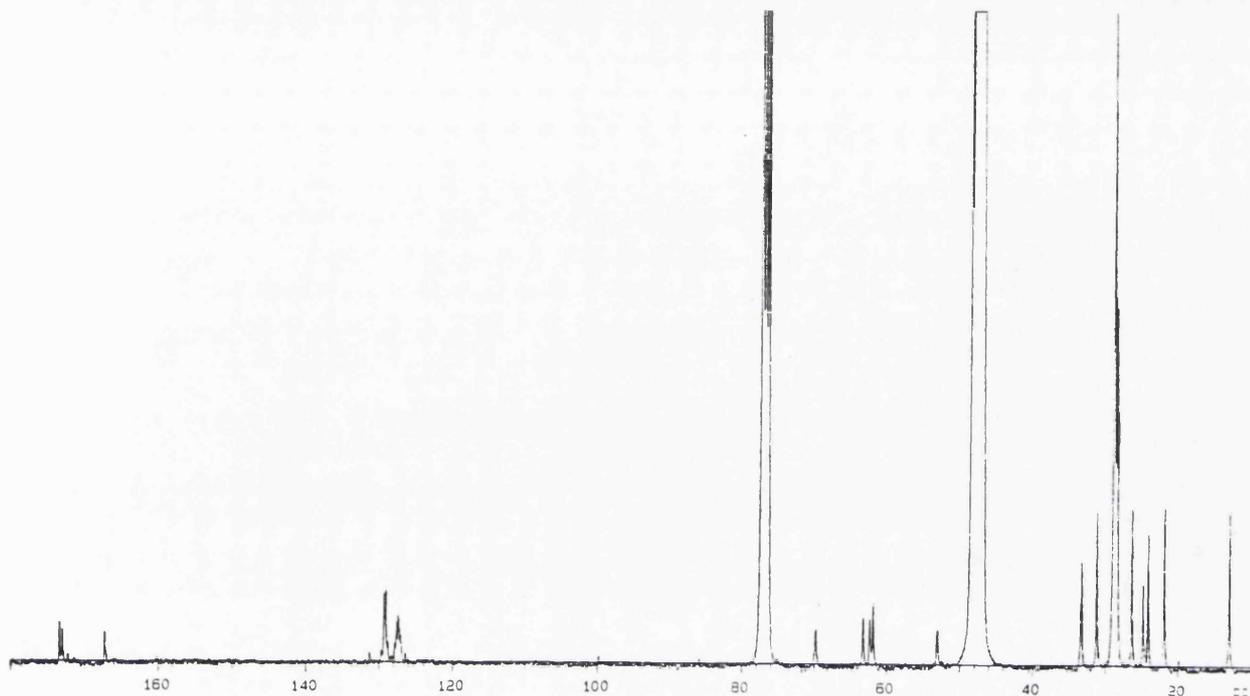
¹³C NMR spectrum of phosphatidylcholine in 2.0 ml of CDCl₃:CD₃OD (1:2 v/v).



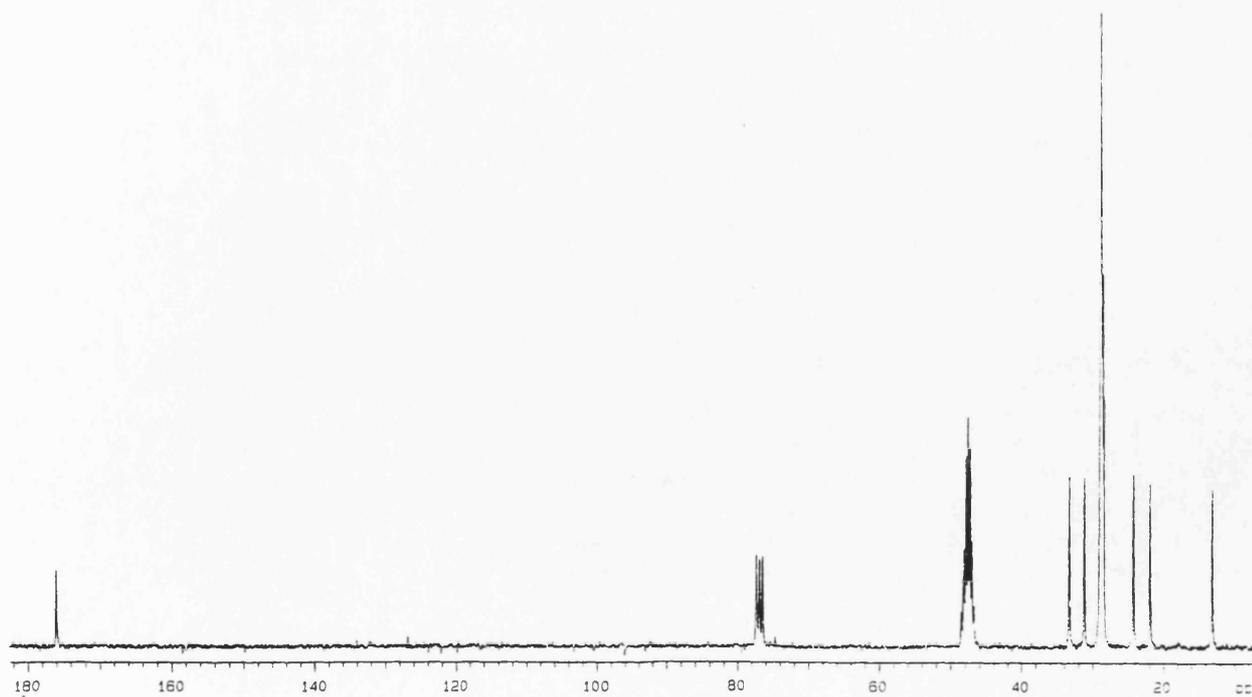
¹³C NMR spectrum of phosphatidylinositol in 2.0 ml of CDCl₃:CD₃OD (1:2 v/v).



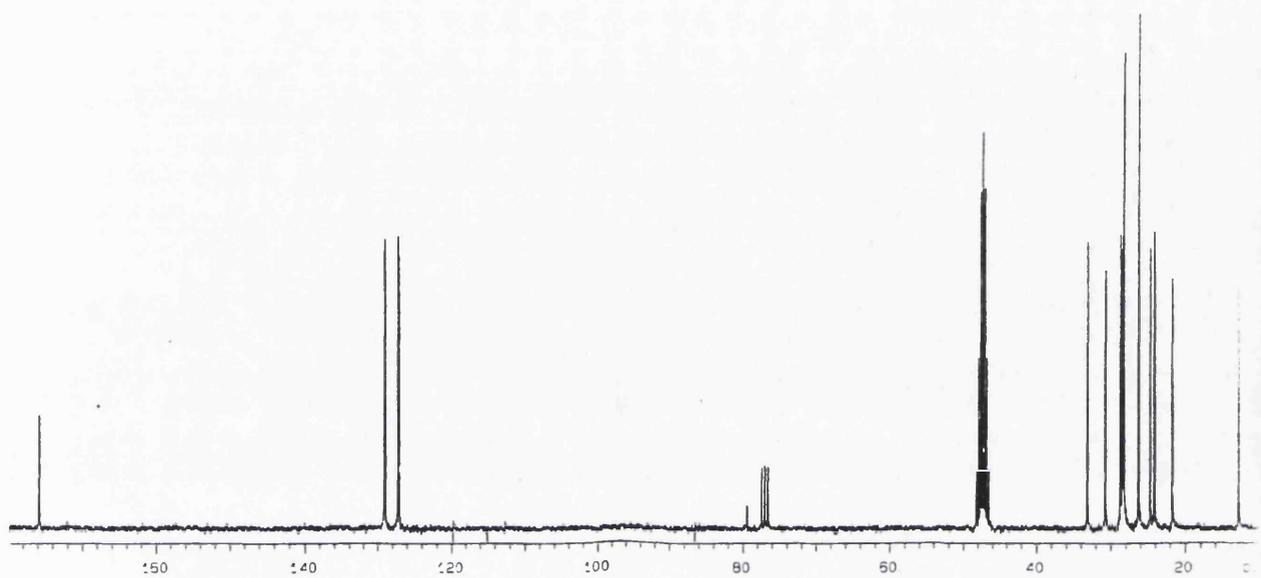
¹³C NMR spectrum of cardiolipin in 2.0 ml of CDCl₃:CD₃OD (1:2 v/v).



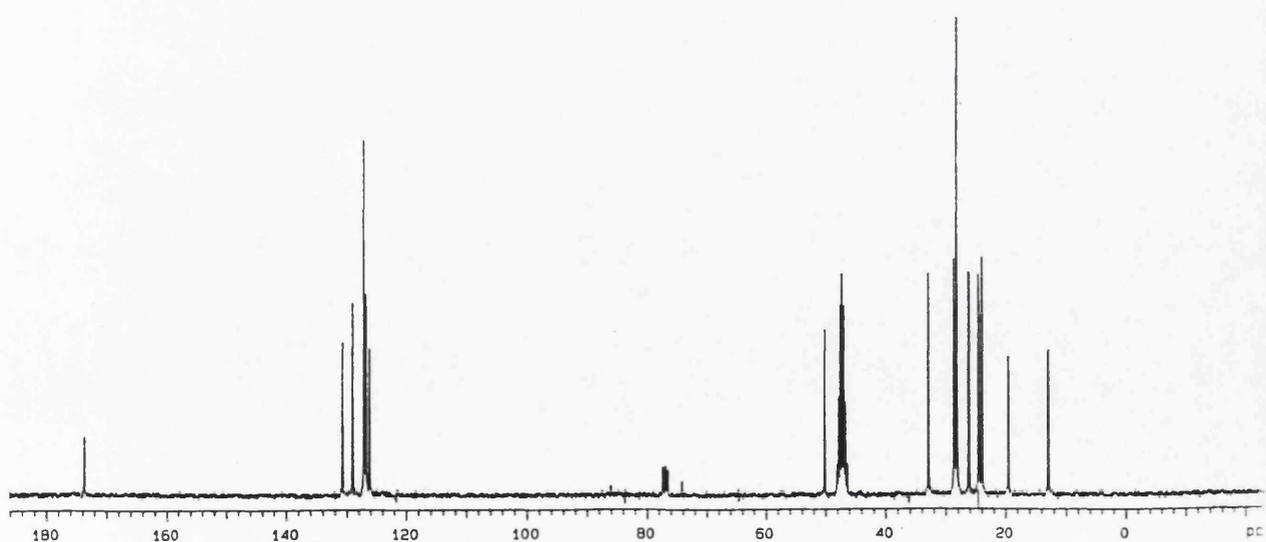
^{13}C NMR spectrum of phosphatidylserine in 2.0 ml of $\text{CDCl}_3:\text{CD}_3\text{OD}$ (1:2 v/v).



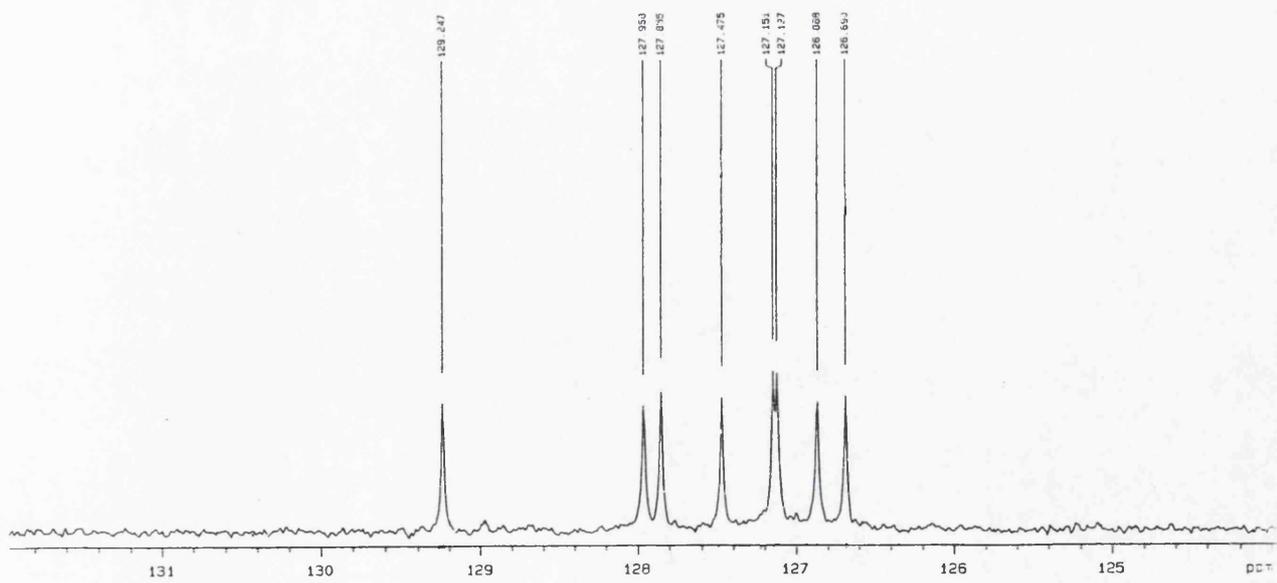
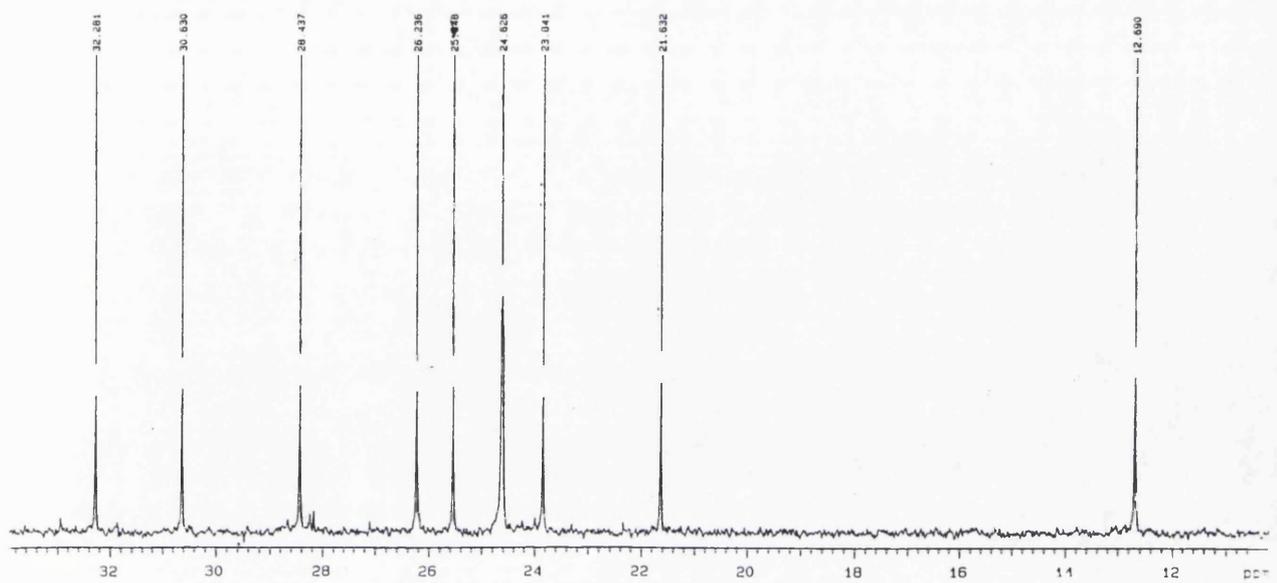
^{13}C NMR spectrum of palmitic acid in 2.0 ml of $\text{CDCl}_3:\text{CD}_3\text{OD}$ (1:2 v/v).



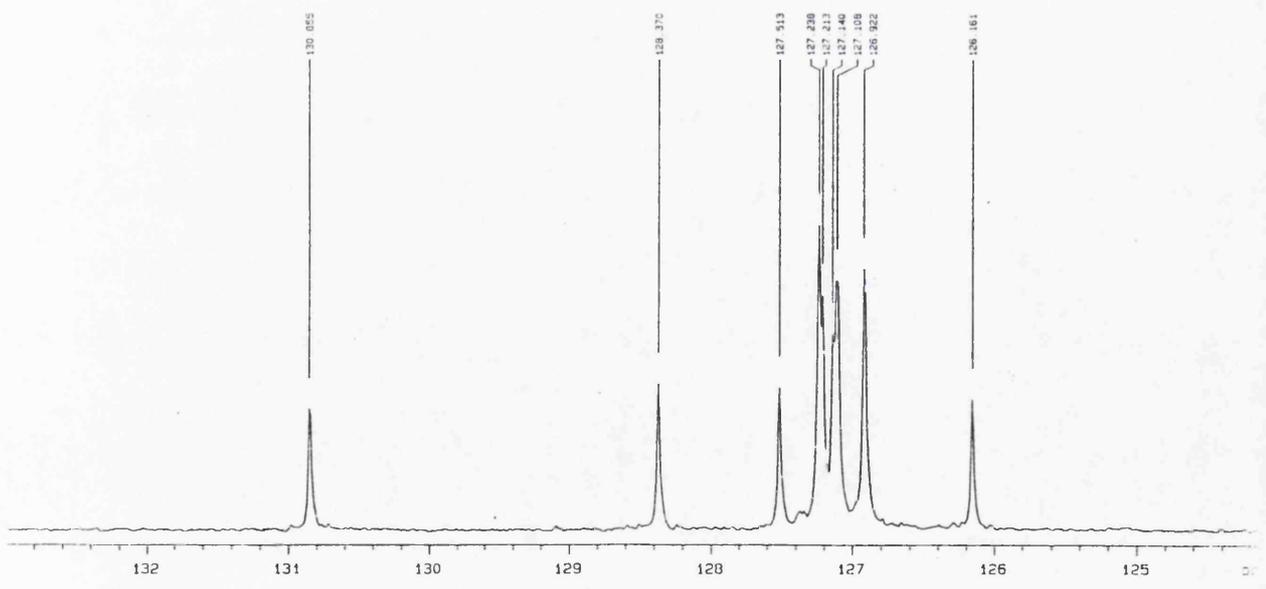
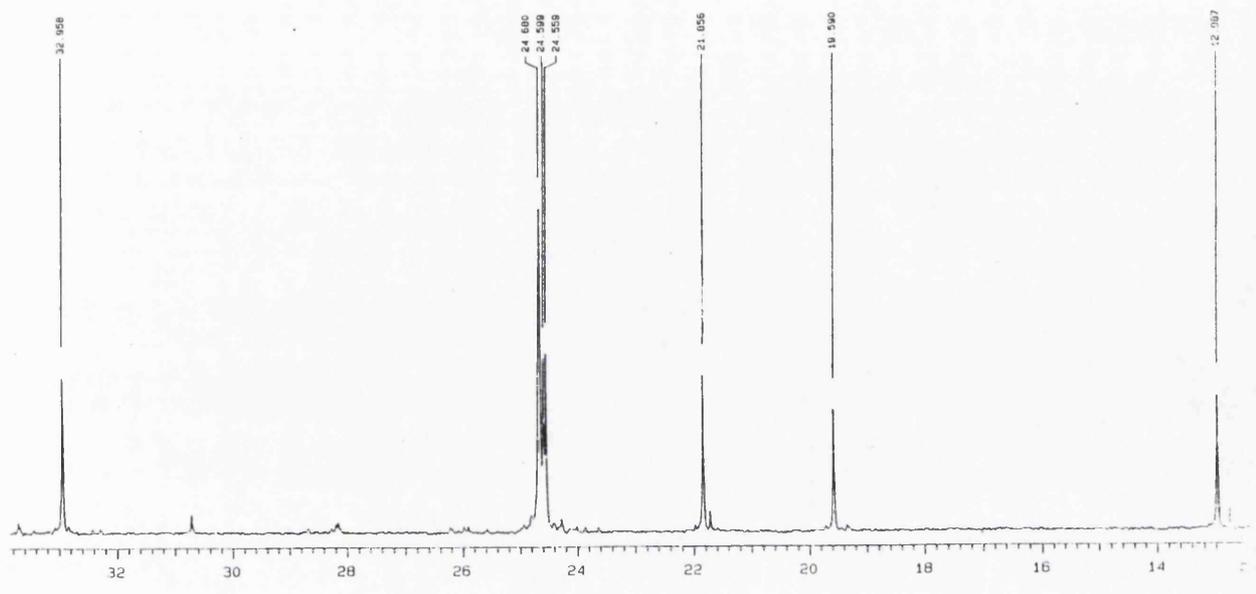
^{13}C NMR spectrum of linoleic acid in 2.0 ml of $\text{CDCl}_3:\text{CD}_3\text{OD}$ (1:2 v/v).



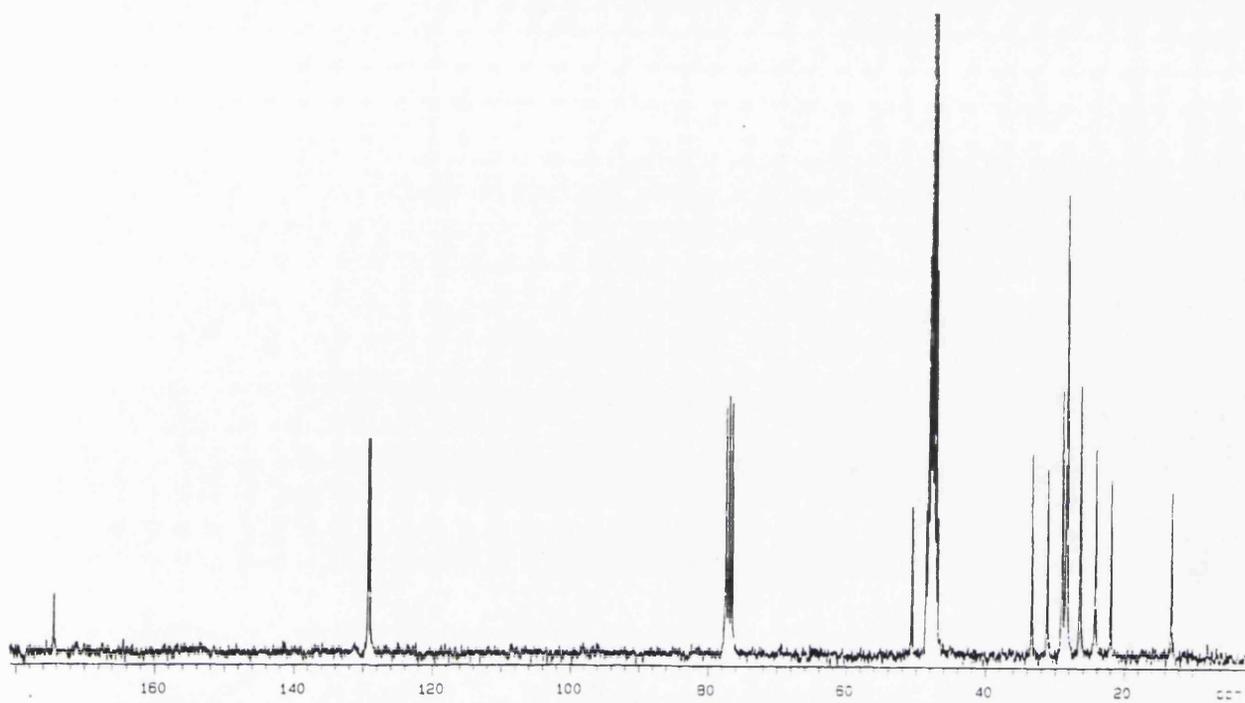
^{13}C NMR spectrum of linolenic acid in 2.0 ml of $\text{CDCl}_3:\text{CD}_3\text{OD}$ (1:2 v/v).



¹³C NMR spectrum of arachidonic acid in 2.0 ml of CDCl₃:CD₃OD (1:2 v/v).



¹³C NMR spectrum of docosahexaenoic acid in 2.0 ml of CDCl₃:CD₃OD (1:2 v/v).



^{13}C NMR spectrum of palmitoleic acid in 2.0 ml of $\text{CDCl}_3:\text{CD}_3\text{OD}$ (1:2 v/v).

Appendix 2
Arachidonic acid as a feedback inhibitor of
phospholipid methylation in rat polymorphonuclear
leukocytes

INTRODUCTION

Phospholipid transmethylation, a biosynthetic step for phosphatidylcholine (PC) formation from phosphatidylethanolamine (PE) is an alternative to the CDP mediated denovo pathway. Although it is a minor synthetic step in most cells the rate of lipid turnover is about 30 times faster than the major CDP pathway, with a half-life of 1 to 10 hours [Sundler & Akesson, 1975]. These features of fast turnover, relatively low productivity, suggest that transmethylation could be localized and involved in cellular signal transduction [Hirata & Axelrod, 1980]. This has already been supported by examples showing changes of PC turnover during cellular activation [Mato & Alemany, 1983]. Additionally a variety of agonists and antagonists affect the turnover of lipids via this process including different classes of anaesthetics, hormones, chemotactic and growth factors etc. [Fonteh *et al*, 1988; Mato, 1986; Hirata & Axelrod, 1980]. Furthermore this step produces PC rich in polyunsaturated chain at the 2-acyl position, which is a substrate for phospholipase A₂ (E.C. 3.1.1.4) production of arachidonic acid, a precursor of various important metabolic mediators [Crews *et al*, 1981; Kannagi *et al*, 1980]. Arachidonic acid itself has also been widely accepted as a second messenger, and a protein kinase C stimulator. Thus transmethylation could be an integral part of an "Arachidonic cascade", and hence the intra/intercellular production and regulation of arachidonic acid. In this report we demonstrated that arachidonic acid inhibits the transmethylation by decreasing of L-[³H] methionine incorporation into phospholipid of rat peritoneal polymorphonuclear leukocytes.

MATERIALS

Wistar strain rats, weight between 150 - 200 g were used in the experiments. L-[³H] methionine was purchased from Amersham Ltd. All radioactivities were counted by a Beckman model LS 5000 CE liquid scintillation counter. 5.0 ml disposable scintillation vials were used in the experiments. All other reagents used in the experiments are reagent grade.

EXPERIMENTAL

Wistar strain rats, each weighed about 200 g were scarified by the treatment of carbon dioxide. 20 ml of sterilized ice-cold Tyrode's buffer was immediately injected into their peritoneal cavities. The peritoneal cavities were then subjected to constant massaged for 3 minutes. Injected buffer was then withdrawn and transferred to a sterilized centrifuge tube. The polymorphonuclear leukocytes suspension was centrifuged for 5 minutes at 1200 rpm. Supernatant was discarded and the cell pellet was washed by blood lysing (ammonium chloride) buffer in order to remove the accidentally extracted erythrocytes. The number of cells were being counted under a light microscope with a neubauer. 5×10^5 cells/ ml of cells were used in this particular study.

A 10^{-2} M arachidonic acid solution was made by adding arachidonic acid (28.01 μ l/ 30.50 mg) into 10.0 ml of Tyrode's buffer. A 10-fold dilution of this solution gave 10^{-3} M arachidonic acid solution. Further dilutions were obtained in a similar manner.

Incubation (Time dependent study): Peritoneal macrophages (5×10^5 cells/ ml) were preincubated in a 37°C shaking water bath for 3 minutes. In the meantime 0.10 ml of 10^{-4} M arachidonic acid was added into individual incubation tube, except for the control which the arachidonic acid was replaced by the same volume of Tyrode's buffer. After the preincubation period radiolabelled L-[Methyl- ^3H] methionine (1.0 μCi / ml) was added into the macrophage suspension. The suspension was then transferred immediately as 1.0 ml portion into each incubation tube. The tubes were incubated at 37°C in a shaking bath for 5.0, 10.0, 20.0 and 30.0 minutes respectively. The reaction was terminated by the addition of 1.0 ml ice-cold Tyrode's buffer containing 3.0 mg/ ml D-L methionine. Tubes were kept at 4°C after the reaction was stopped.

Incubation (Dose dependent study): The incubation procedures were similar as above excepted 0.10 ml of arachidonic acid solution with different concentrations (from 10^{-4} to 10^{-9} M) were added into individual tube. For control the acid were

replaced by 0.10 ml of Tyrode's buffer. All cell reactions were terminated together after 30 minutes.

Incubation (Palmitic acid dose dependent study): The experimental procedures were identical to the arachidonic acid dose dependent study, except the arachidonic acid were replaced by the equivalent quantity of palmitic acid.

lipid extraction: After the reaction were terminated tubes were centrifuged at 1200 rpm for 5 minutes. Supernatant was discarded and pellets were washed twice by cold Tyrode's buffer. After the second wash cell pellets were extracted with chloroform-methanol-2M HCl mixture (6:3:1 v/v). All samples were vortexed for 1 minute, then the aqueous and organic phases were separated by centrifugation (1000 rpm for 3 minutes). The lipid-rich organic phase was retained and washed twice with 2.0 ml of 0.50 M potassium chloride in 50% (v/v) methanol. Aliquots were dried under a stream of nitrogen [Fonteh *et al*, 1899].

Aquaphase scintillant (4.0 ml) was added to the samples and the [Methyl-³H] activity incorporated into phospholipid was detected by liquid scintillation counting.

RESULTS

Our results indicate arachidonic acid is able to produce a 4-fold inhibition of phospholipid methylation, from the concentration of 10^{-5} mol/ L to 5×10^{-8} mol/ L in a dose-dependent manner and with an EC_{50} of 5×10^{-7} mol/ L. The saturated fatty acid, palmitic acid under identical conditions failed to produce such inhibition (data not shown). To eliminate the possibility that arachidonic acid metabolites were responsible for the inhibition, aspirin, known as a lipo-oxygenase inhibitor was added (10^{-5} mol/ L) to prevent further degradation of arachidonic acid. No interference with the inhibition was observed (data not shown).

It is concluded that arachidonic acid inhibits phospholipid transmethylation which could be involved in an "Arachidonate Cascade". As a product of PC possibly from PE, arachidonic acid modulates this pathway via a negative feedback mechanism. Further study about the relationship between products of phospholipid methylation and

"Arachidonate Cascade" will be informative in revealing the function of this inhibition mechanism.

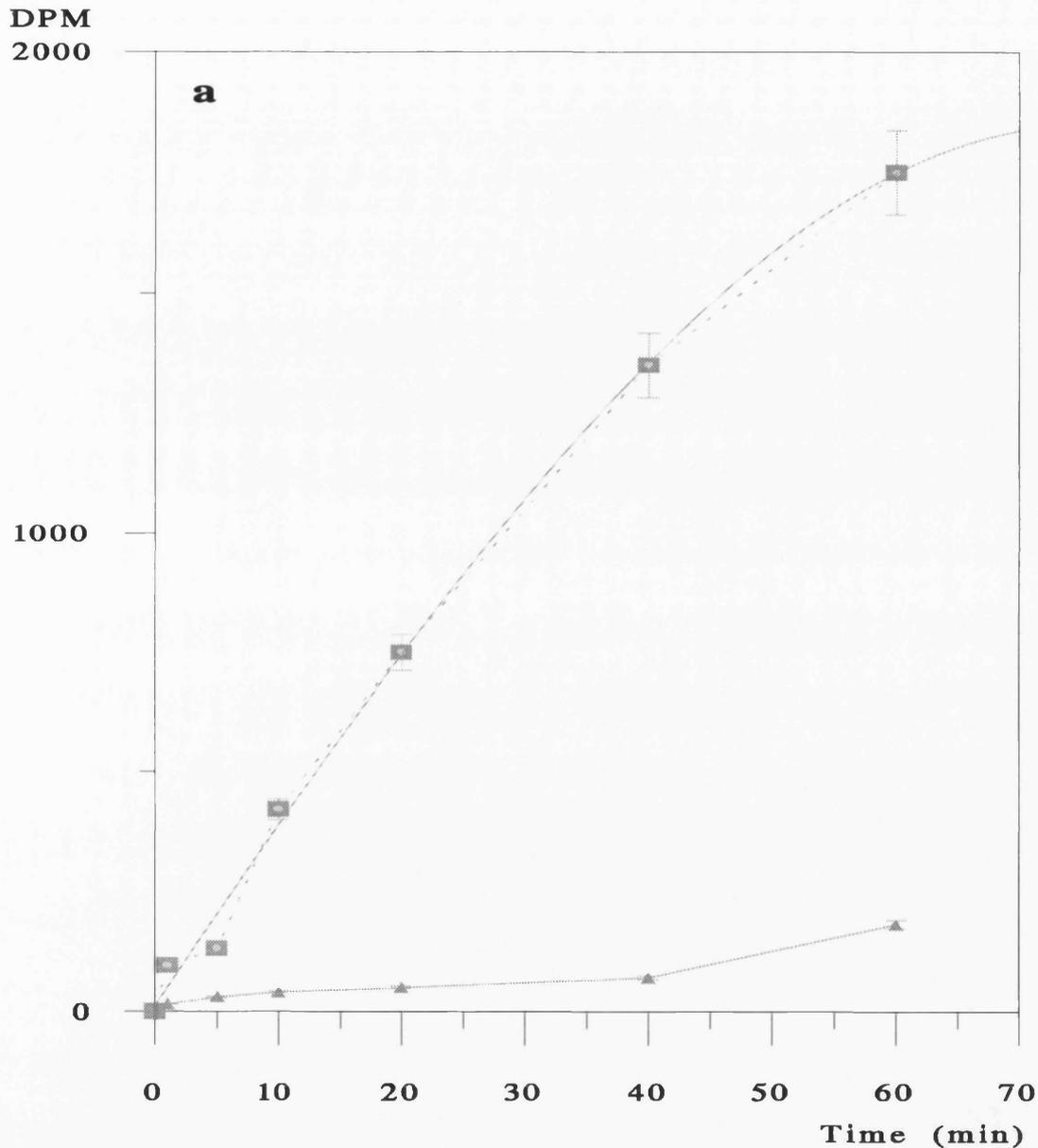


Figure 1a. Time-dependent curve of arachidonic acid inhibition on lipid methylation. Comparison between the control (triangle) and the present of arachidonic acid (square) at different time periods, at 1, 5, 10, 20, 40 and 60 minutes respectively. Cells were suspended in Tyrode's buffer (1.0 ml) with L-[Methyl³H] methionine (1.0 μ Ci/ ml) in each individual assay. Incubation was in a shaking water bath at 37°C. Concentration of arachidonic acid used was 10^{-5} mol/ L. In the control assay arachidonic acid was replaced by the same volume of Tyrode's buffer. A high degree of inhibition was caused by arachidonic acid maintained with increasing time.

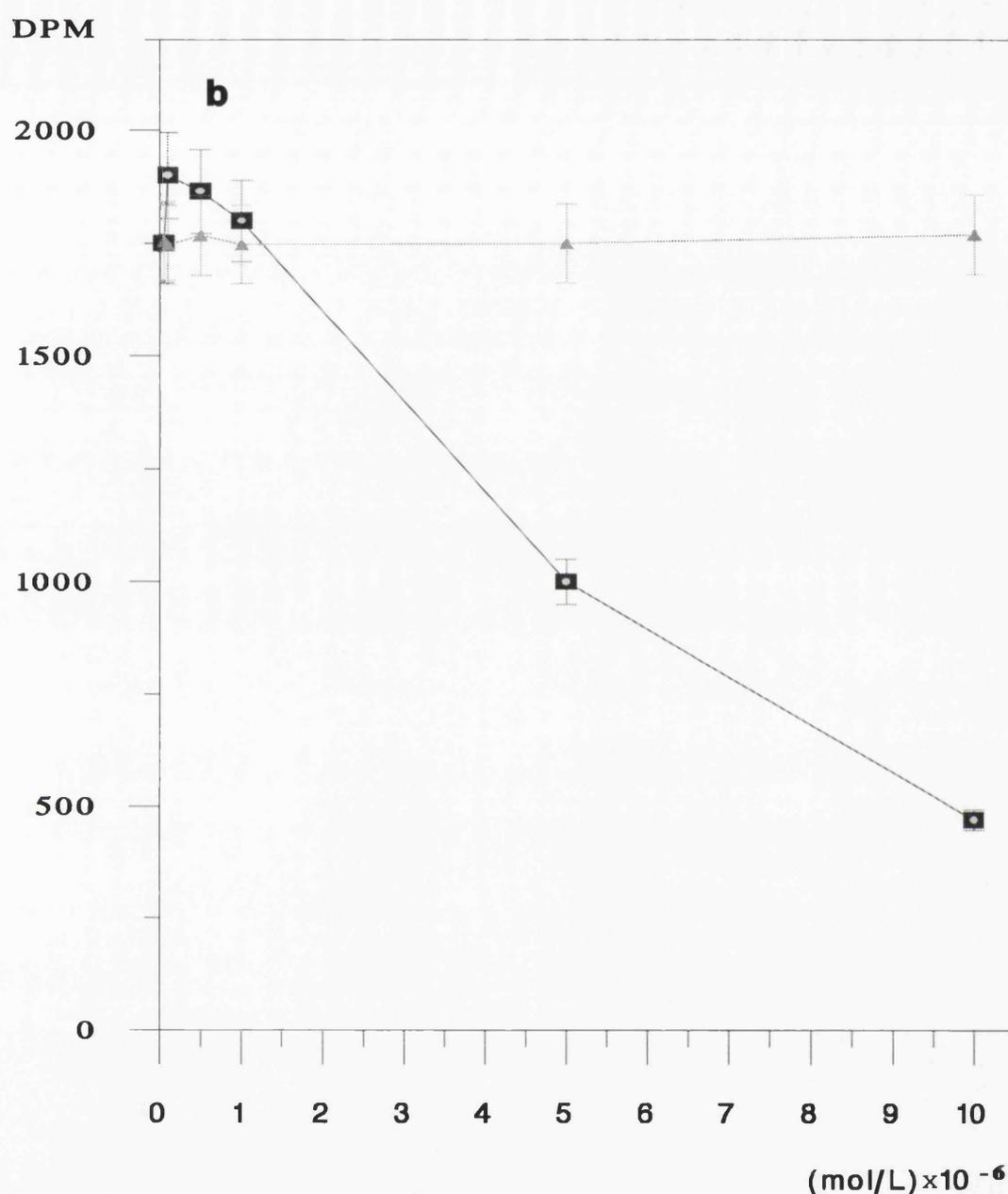


Figure 1b. Dose-dependent curve of arachidonic acid inhibition on lipid methylation. Comparison between the control (triangle) and the present of arachidonic acid (square). Cells were suspended in Tyrode's buffer (1.0 ml) with L-[Methyl³H] methionine (1.0 μ Ci/ ml) in each individual assay. Incubation time was 30 minutes in a shaking water bath at 37°C. Concentrations of arachidonic acid varied from 10^{-5} mol/ L to 5×10^{-8} mol/ L, with an EC_{50} value at 5×10^{-6} mol/ L. In the control assay arachidonic acid was replaced by the same volume of Tyrode's buffer.

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